Uncovering the relationship underlying postpartum depression and alcohol use

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Abstract

Postpartum depression (PPD) is an onset of a major depressive episode during the period around childbirth, affecting 5-30% of women. Epidemiological data suggests that women with a history of drug and alcohol use are more vulnerable to being diagnosed with PPD and women with PPD are more likely to relapse. In addition, evidence suggests that binge drinking by the rodent dams may negatively impact the fetus. However, determining whether there is a direct relationship between postpartum depression and alcohol abuse is difficult to assess in the clinical population. Thus, the aim of this study is to investigate the interrelationship between postpartum alcohol use and PPD and the potential impact on offspring behavior. Given previous findings from the lab which suggested the role of HPA axis dysregulation in the development of maternal care deficits, we hypothesized that HPA axis dysregulation drives this reversible relationship. To study this, we used 2 novel genetic models of PPD that show dysregulated HPA axis function during the peripartum period. We subjected these models to a heavy binge drinking paradigm to determine whether mice exhibiting PPD-like behaviors exhibited an altered preference for alcohol (20%v/v) and how short-term alcohol exposure altered PPD-like behavior in control mice. Finally, we investigated whether binge drinking during pregnancy altered anxiety-like behavior in their offspring. Short term binge drinking altered maternal behavior in control animals at baseline and following stress, but did not disrupt normal anxiety- or depressive-like behaviors. Interestingly, all PPD and wildtype mice reduced their preference for alcohol after parturition and anxiety-like behaviors were not affected in the offspring. These findings suggest that alcohol exposure

results in neural adaptation in the wildtype dams causing them to display abnormal maternal behavior.

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ACTH	Adrenocorticotropic Hormone
ADH	Alcohol dehydrogenase
AVP	Vasopressin
BAC	Blood Alcohol Concentration
β-EP	β-Endorphins
cAMP	cyclic adenosine monophosphate
CORT	Corticosterone
CRF	Adrenocorticotropic Hormone Releasing Factor
DID	Drinking in dark
ELISA	Enzyme-Linked Immunosorbent Assay
FASD	Fetal Alcohol Spectrum Disorder
FST	Forced swim test
GABA	Gamma aminobutyric acid
$GABA_A\delta$ KOs	$GABA_A$ Receptor δ subunit is knocked out
HPA axis	Hypothalamic-pituitary-adrenal axis
HRV	Heart Rate Variability
KCC2/CRH Kos	Potassium-Calcium-Chloride-2 channels on the Corticotropin Releasing Hormone neurons are knocked out
KM	Michaelis constant
MW	Molecular Weight
NMDA	N-Methyl-D-aspartic acid
PCR	Polymerase Chain Reaction
PKPD	Pharmacokinetics and Pharmacodynamics
PPD	Postpartum depression
PVN	Paraventricular nucleus
SNS	Sympathetic nervous system
THDOC	Tetrahydrodecorticosterone
WT	Wildtype
ZT	Zeitgeber time

Chapter 1: Introduction

1.1. Post-partum depression

1.1.1. Definition:

Childbirth is considered to be one of the most fulfilling, happiest and life changing experiences in a mother's life. However, it is becoming increasingly evident that this period of life may also bring with it a negative shift in mood, which can present as postpartum blues, postpartum depression (PPD), postpartum anxiety, or postpartum psychosis (Martinez, Johnston-Robledo, Ulsh, & Chrisler, 2000). PPD is a diagnosis of major depression within the first few months following delivery. It is estimated that nearly 20% of new mothers suffer from PPD(Gavin et al., 2005) (Steiner, 1998) and around 75% from postpartum blues (ECNaSDE, 2008). Along with pain and anhedonia that follow PPD, additionally seen symptoms are deficits in maternal care including infant neglect.

1.1.2. Risk factors:

Stressful life events (Brett, Barfield, & Williams, 2008) (Boury, Larkin, & Krummel, 2004) (Homish, Cornelius, Richardson, & Day, 2004) including violence by the partner (Brett et al., 2008) lack of social support, early conception (Homish et al., 2004) , and low income status (Jesse, Walcott-McQuigg, Mariella, & Swanson, 2005) are risk factors for PPD.

Previous diagnosis of depression during the course of pregnancy and a previous history of PPD positively predict vulnerability to develop PPD. Anxiety during pregnancy and stressful life events are even stronger predictors of PPD (O'hara & Swain,

1996) (Pfost, Stevens, & Lum, 1990) (Rich-Edwards et al., 2006) (Stowe & Nemeroff, 1995). This raises the potential that mechanisms underlying stress may also contribute to the vulnerability to develop PPD.

Another prominent risk factor contributing to PPD is substance use (Chapman & Wu, 2013). Alcohol for instance, is an easily accessible legal substance and is commonly used for recreational purposes. Several studies have shown the effects of alcohol on the systems involved in the stress signaling cascade, such as the HPA axis. Acute alcohol consumption leads to activation of the HPA axis which results in elevated corticosterone (CORT) levels (Workman, Raineki, Weinberg, & Galea, 2015). Interestingly, elevated CORT levels are also associated with PPD. Fifty three percent of the women use alcohol, with 24% of these women indulging in a dangerous pattern of alcohol use termed binge drinking (Pooler, Perry, & Ghandour, 2013). The National Institute on Alcohol Abuse and Alcoholism defines binge drinking as "a pattern of drinking that brings a person's blood alcohol concentration (BAC) to 0.08grams percent or above." This will typically occur when men consume 5 or more drinks and women consume 4 or more drinks in a span of 2 hours (NIAAA). Although 8.5% of these women do continue to use alcohol during pregnancy, the great majority cease for the gestational period (Forray, 2016). Yet, post-delivery women's consumption levels match the pre-gestational use.

1.1.3. Stress and CORT relationship:

Selye defined stress as a state of a non-specific response (revealed after subtraction of the specific components from the total response) of the body to any demand, emphasizing that the pathological triad (adrenal enlargement, gastrointestinal ulceration and thymicolymphatic involution)- "stress syndrome"- would occur in

response to any stressor (Selye, 1936). The adaptive response to the stressor involves neuroendocrine activation, which includes 2 main response systems: cardiovascular responses (in the form of blood pressure and heart rate), driven by sympathetic nervous system (SNS) activity, and release of the glucocorticoid hormone cortisol from the adrenal cortex, driven by the HPA axis activation (Schlotz, 2013). Though stress activates different pathways, it will eventually result in a series of neural and endocrine adaptations known as the stress cascade (Miller & O'Callaghan, 2002). The main site involved in this activation of the neuroendocrine response is the hypothalamus (Barrett, 2005). Studies from our laboratory and others have previously suggested that the KCC2 co-transporter channels (K+/Cl- co-transporter), responsible for maintaining the chloride homeostasis, play an important role in regulating the response due stress-induced HPA axis activation (Hewitt, Wamsteeker, Kurz, & Bains, 2009) (Sarkar, Wakefield, MacKenzie, Moss, & Maguire, 2011) (Gunn et al., 2013). In particular our laboratory has demonstrated that stress causes dephosphorylation of KCC2 at residue Ser940 and results in downregulation of KCC2. This plays a role in the HPA axis activation (Sarkar et al., 2011). Further studies in our laboratory have also supported the role for KCC2 in regulating the HPA axis function in the peripartum and postpartum period (Melón, Hooper, Yang, Moss, & Maguire, 2017). Studies have also shown that tonic inhibition exhibited by the $GABA_A$ receptor δ-subunits also exert control over the HPA axis activity by regulating the CRH neurons (V. Lee, Sarkar, & Maguire, 2014). The inhibitory actions of GABA through these receptors also maintained through chloride homeostasis. Therefore, their activity is also dependent on KCC2 co-transporters (Deeb, Lee, Walker, Davies, & Moss, 2011).

1.1.4. HPA axis and pregnancy:

It is important to note that during pregnancy, mothers experience stress due to various factors such as immunological, metabolic, environmental, psychological, social and physiological. Depending on the duration of exposure the stressors are classified as acute and chronic, which have varying degrees of implications.

In response to a stressor, the HPA axis gets activated which results, ultimately, in the release of glucocorticoids (cortisol in humans and corticosterone in rodents). However, beginning late in pregnancy and continuing during the early postpartum period (CORT levels normalize at 12 weeks postpartum) (Mastorakos & Ilias, 2000) , there is a suppression of stress induced activation of the HPA axis (Paula J. Brunton & Russell, 2008; P. J. Brunton, Russell, & Douglas, 2008) (P. J. Brunton & Russell, 2011). The exact mechanism underlying the activity of the HPA axis during peripartum is still unknown. The Maguire lab has recently shown that the suppression of the stress-induced activation of the HPA axis is also observed in mice. Virgin mice experience stress induced activation of the HPA axis which leads to elevated CORT levels. However, during pregnancy and postpartum period, there is a suppression in the stress induced activation of the HPA axis along with a decrease in CORT levels. But in mouse models of postpartum depression, there is a failure in suppression of the elevated CORT levels and activation of the HPA axis (J. Maguire & Mody, 2016) (Melón et al., 2017).

1.1.5. Role of HPA axis in postpartum depression:

When a healthy individual is stressed, the adrenocorticotropic hormone releasing factor (CRF) and vasopressin (AVP) are released by the hypothalamus, CRF stimulated

the pituitary to secrete adrenocoticotropic hormone (ACTH) This will in turn stimulate the adrenal cortex to secrete glucocorticoids that is cortisol in humans and corticosterone in rodents. These glucocorticoids will bind to the glucocorticoid receptors and mineralocorticoid receptors, inducing the ones on the HPA axis. This results in a feedback inhibition on both CRF and AVP secretion and eventually affects the whole cascade (Pariante & Lightman, 2008). Patients suffering from PPD display a faulty feedback inhibition. As a result, they exhibit elevated CORT levels and cannot suppress the HPA axis (Bloch, Daly, & Rubinow, 2003) (Chrousos, Torpy, & Gold, 1998).

In order to study postpartum depression, we used 2 mouse models: $GABA_A$ receptor δ-subunit knockouts and KCC2/CRH knockouts. Work done previously has suggested the use of $GABA_A$ receptor knockouts as models that exhibit depression-like behavior which is restricted to postpartum period and displays abnormal maternal behavior (Jamie Maguire & Mody, 2008). Further dwelling into the possible underlying reasons contributing to this behavior, it was discovered that hyper excitability of the HPA axis during the postpartum period could play a role (J. Maguire & Mody, 2016). Also, previous studies from our laboratory have demonstrated a role of KCC2 channels on CRH neurons in regulating the stress response and HPA axis activity (Sarkar et al., 2011) (Hewitt et al., 2009). Further studies on KCC2/CRH knockout mice have indicated its part in the inability to suppress HPA axis activation implicated in maternal deficits during the postpartum period (Melón et al., 2017).

1.2. Alcohol

1.2.1. History of alcohol research:

In the early 1900's research related to the effects of alcohol and its actions on the brain was suffering due to lack of understanding of the neurobiology of the brain and advanced techniques. Earlier, it was thought that alcohol is a mere "prodrug" and its breakdown product, acetaldehyde, is responsible for inducing fundamental brain changes. It was later proposed by a couple of labs that acetaldehyde leads to a condensation reaction (Davis & Walsh, 1970) (Yamanaka, Walsh, & Davis, 1970) with biogenic amines like dopamine, norepinephrine and serotonin, to form a psychoactive alkaloid salsolinol. This claim was refuted in the following years but recently a study has confirmed it. Scientists (J. Lee et al., 2010) argued that there is evidence for salsolinol production. However, another pharmacologically active alkaloid, tetrahydropapavroline may be formed (Sullivan, Harris, & Pfefferbaum, 2010). As far as the actions of ethanol on the neurotransmitters and ion channels is considered, it had been established in 1970 that the release of acetylcholine (Phillis & Jhamandas, 1971) and voltage gated ion channels (Harris & Hood, 1980) was inhibited. Later a study (Davidoff, 1973) found that ethanol acts on gamma aminobutyric acid (GABA) receptors. Its actions on GABA as a major target site have been widely studied (Kumar et al., 2009). It was also identified that ethanol has inhibitory actions on N-Methyl-D-aspartic acid (NMDA) receptors (D. M. Lovinger, White, & Weight, 1989) and affects calcium activated potassium channels (Treistman & Martin, 2009) (Yamamoto & Harris, 1983). The role of ethanol on dopamine release and functioning is also studied. Acute alcohol intake activates reward pathways via dopaminergic system. Whereas, chronic alcohol exposure and withdrawal leads to a hypodopaminergic state resulting in dysphoria and ultimately relapse (George F. Koob & Volkow, 2010) (Black, Hoffman, & Tabakoff, 1980).

1.2.2. Pharmacokinetics and Pharmacodynamics (PKPD) of Ethanol:

The rate of alcohol absorption depends upon the food intake, site of absorption, nature/content of alcohol in the beverage as well as the person's body weight, sex, rate of metabolism (Dubowski, 1985). These factors also determine the time taken to achieve a peak in blood ethanol concentration. In a fasted state, alcohol is mainly absorbed through the duodenum and jejunum since the transit time through the stomach is decreased (Eckardt et al., 1998). Whereas in a fed state, there is delay in gastric emptying and 70% of alcohol consumed is absorbed in the stomach (Eckardt et al., 1998). Absorption through the duodenum and jejunum is faster than that in the stomach (Eckardt et al., 1998). The amount of ethanol in the blood and CNS determines the rate of absorption in the stomach and small intestine (Eckardt et al., 1998). Slower the absorption, faster is the 1^{st} pass metabolism. This metabolism is carried out by the enzymes found in the stomach, intestinal mucosa, and liver as alcohol is ingested, absorbed and is finally carried through the portal circulation into the liver. It finally enters into the blood stream (Eckardt et al., 1998). During absorption, the concentration of alcohol is higher in the portal vein and liver than the blood circulation (Eckardt et al., 1998).

On ingestion, ethanol majorly undergoes oxidative metabolism in the liver. It is thought that the brain also metabolizes ethanol. Finally, the rate of oxidative metabolism determines the extent of the actions of ethanol on the brain. After ingestion, ethanol gets distributed in body water. Ethanol is not generally accumulated in any specific organ or preferentially bound to proteins (Eckardt et al., 1998).Total body water remains fairly constant across the whole population i.e average of 72% of lean body mass (Eckardt et al., 1998). However, the body mass varies with sex age, fat content, other coexistant

conditions, etc (Eckardt et al., 1998). All these factors determine the alcohol dose needed to achieve significant blood alcohol levels and ultimately its effects on the brain (Eckardt et al., 1998). When the rate of alcohol absorption and intake exceeds the rate of elimination, it starts accumulating in blood and tissues (Dubowski, 1985). Before the equilibrium is established, the concentration of ethanol in the brain and arterial blood is significantly higher than that in the muscle. Equilibrium is quickly established in highly vascularised organs (like brain, liver, kidney) as compared to poorly vascularised organs (like muscle) (Eckardt et al., 1998). The period before the equilibrium is reached can last for 2 hrs after ethanol ingestion (Eckardt et al., 1998).

Elimination mainly occurs in the liver through oxidation. It also gets excreted in minor quantities through urine, breath and perspiration in unaltered form (Dubowski, 1985). Alcohol does not get eliminated quickly as compared to its absorption. This allows reaching a peak or plateau in the blood alcohol level. At this point, the peak and plateau represents the equilibrium phase wherein rate of absorption is equal to the rate of elimination. After the plateau/peak, is the period of pure elimination, which is mainly carried out by the Class I oxidative enzyme ADH (alcohol dehydrogenase) (A. W. Jones, 2010). Ethanol follows the Michaelis- Menton model at Km (Michaelis constant) for ADHs is at a BAC of 2-10mg/100ml (A. W. Jones, 2010). This model implies that after the enzymes get saturated with substrate, the elimination follows zero order kinetics (BAC>20mg/100ml) (A. W. Jones, 2010). In a fasted condition, the elimination rate of ethanol is 10-15mg/100ml/hr whereas in nonfasted conditions, it is 15-20mg/100ml/hr (A. W. Jones, 2010).

1.2.3. Pharmacology of Ethanol:

Alcohol is one of the legal drugs that is widely abused. The degree of its effects and the behavioral changes of these interactions depend on the dose of ethanol ingested and the level of tolerance of the drinker. At a certain dose, a particular type of receptor complex maybe responsible to contribute to a phenotypical behavioral demonstration (Eckardt et al., 1998). Particularly considering the effects of ethanol on CNS, it has recently been suggested that are certain neuronal proteins called 'receptive element' that are highly sensitive to ethanol (Eckardt et al., 1998) (B Tabakoff & Hoffman, 1987) (Mihic et al., 1997). These proteins include ligand-dated ion channels and proteins in transduction processes (Eckardt et al., 1998). Similarly, it is known that ethanol interferes in the activity of the HPA axis. It interacts with various neurotransmitter systems that are essential for the normal functioning of the stress axis. It is a possibility that the effects of ethanol on the HPA axis are mediated through these systems.

1.2.3.1. Effects of ethanol on $GABA_A$ receptor complex-

$GABA_A$ receptors are a type of ligand-gated ion channel receptors. It is made up of multiple subunits that form a transmembrane chloride ion permeable channel. This receptor has multiple recognition sites such as those for GABA, barbiturates, benzodiazepines and neurosteroids (R. L. Macdonald & Olsen, 1994). Stimulation of GABA results in increased chloride permeability and causes hyperpolarization of the neuronal membrane (Eckardt et al., 1998). This event produces sedation, anaesthesia and an anxiolytic effect (Eckardt et al., 1998). Binding of ligands to the other sites on GABA receptor is called allosteric modulation. Ethanol does not directly bind to any recognition sites on the $GABA_A$ receptor (Robert L Macdonald, 1995), however, it has been found that it stimulates GABA-stimulated chloride flux in isolated brain membrane vesicles as

well as in cultured mammalian cells (Nestoros, 1980) (Suzdak, Crawley, Schwartz, Skolnick, & Paul, 1986) (Ticku, Lowrimore, & Lehoullier, 1986) (Mehta & Ticku, 1988) (Allan & Harris, 1987) (Celentano, Gibbs, & Farb, 1988) (Aguayo, 1990) (Nishio & Narahashi, 1990) (Reynolds & Prasad, 1991) (Eckardt et al., 1998). It is important to note that this chloride flux is seen only in certain regions of the brain depending on the subunit composition (Givens & Breese, 1990). Studies have shown that $GABA_A$ receptors are made up of multiple subunits and are sensitive to low concentrations of ethanol. Ethanol potentiates tonic inhibition in mice expressing $GABA_A$ receptor δ subunits however, do not exhibit the same in mice that lack these subunits (Glykys et al., 2007). However, previous work has also shown that δ subunit deficient mice exhibit reduced alcohol consumption, develop acute and chronic tolerance and demonstrate normal anxiolytic and hypothermic response to ethanol (Mihalek et al., 2001).

1.2.3.2. Effects of ethanol on glutamate receptor complex-

There are different types of glutamate receptor subtypes. Out of those, the NMDA receptors are considered to be most sensitive to the effect of ethanol (Hoffman, Rabe, Moses, & Tabakoff, 1989) (D. M. Lovinger et al., 1989). NMDA receptors are macromolecular complexes that are permeable to Ca^{2+},Na^{+} and K^{+}. In addition it also has other binding sites such as (a) Glutamate and NMDA recognition site (b) a strychnine-insensitive glycine binding site. (c) a binding site for phencyclidine-like compounds (d) voltage- dependent Mg^{2+}binding site and (e) modulatory site for Zn^{2+}binding (Eckardt et al., 1998). It is also found that it has a modulatory binding site for polyamines. It has been largely observed that acute exposure to low concentrations of ethanol (5-10mM) antagonizes the cation flux and inhibits the action of NMDA receptors. However, it is

also very important to note that a few studies also suggest that on exposure to ethanol in the concentration range of 2-9mM in the hippocampus, enhances the response to the NMDA receptors (Lima-Landman & Albuquerque, 1989). It results in the formation of a U-shaped or J-shaped dose response curve. Having said that, it is widely proven that the degree of sensitivity of the NMDA receptors to ethanol varies according to different brain regions and their subunit complex composition (Boris Tabakoff & Hoffman, 1996) . The NR2B subunit is particularly sensitive to ethanol inhibition (David M Lovinger, 1995) (Yang, Criswell, Simson, Moy, & Breese, 1996). Recent studies have shown that other factors such as post-translational modifications, presence of other modulations can also contribute to the sensitivity (Rabe & Tabakoff, 1990) (Snell, Lorio, Tabakoff, & Hoffman, 1994) (Snell, Tabakoff, & Hoffman, 1994) (Bhave, Snell, Tabakoff, & Hoffman, 1999). Depending on the degree of sensitivity of these receptors to ethanol will result in varying behavioral or physiological outcomes.

Some recent studies have also shown that other subtypes of glutamate such as kainate glutamate receptors are also sensitive to ethanol at lower ethanol concentrations (Snell, Tabakoff, et al., 1994) (Dildy-Mayfield & Harris, 1994) (Dildy-Mayfield & Harris, 1994).

1.2.3.3. Effects of ethanol on 5-HT3 subtype of serotonin receptors-

There have been conflicting studies showing varying effects of interactions between ethanol and serotonin. With respect to the interaction of ethanol directly with serotonin receptors, low to moderate ethanol concentrations cause very little effects on 5-HT1 and 5-HT2 subtypes (Buckholtz, Zhou, & Tabakoff, 1989). At low ethanol concentrations i.e

10-25mM, there is an enhanced serotonin-stimulated ion channel current in different cell lines (David M Lovinger, 1991) (DAVID M Lovinger & White, 1991) (D. Lovinger & Zhou, 1994). However, the effects of ethanol on 5-HT3 receptor depend on serotonin concentration (David M Lovinger, 1991) (DAVID M Lovinger & White, 1991). But the exact mechanism of ethanol's interaction with 5-HT3 is still unclear.

1.2.3.4. Effects of ethanol on Dopamine-

It is widely known that ethanol stimulates dopaminergic neurons in the mesolimbic regions (firing rate) as well as the substantia nigra (Mereu, Fadda, & Gessa, 1984) (Gessa, Muntoni, Collu, Vargiu, & Mereu, 1985). The mechanism proposed for these phenomena are that in the mesolimbic system, ethanol acts through the serotonergic and opioid systems, whereas, in the substantia nigra, ethanol inhibits the GABAergic interneurons located in the pars reticulata (Mereu & Gessa, 1985). This action on the mesolimbic dopaminergic system is responsible for the reinforcing properties of ethanol (George F Koob, 1992) (Kiyatkin, 1995). Furthermore, it is seen that the result of the increased firing rate of the mesolimbic dopaminergic neurons leads to the release of ethanol- stimulated dopamine in the nucleus accumbens (Gessa et al., 1985). There is also evidence of dopamine release in other areas of the brain such as caudate nucleus, medial prefrontal cortex and olfactory tubercle (Fadda, Mosca, Colombo, & Gessa, 1989).

1.2.3.5. Effects of ethanol on endogenous opioid neurotransmission-

The reinstatement seeking of alcohol behavior is due to effects of alcohol on the dopaminergic system in the nucleus accumbens involves opioid receptors. This voluntary

intake of ethanol can be reduced by using opioid antagonists that block dopamine/ethanol interactions in the accumbens (Weiss, Lorang, Bloom, & Koob, 1993) (Froehlich, Harts, Lumeng, & Li, 1990) (Sinclair, 1990) (O'Malley et al., 1992) (Volpicelli, Alterman, Hayashida, & O'Brien, 1992) (De Witte, 1984) (Myers, Borg, & Mossberg, 1986) (Kornet, Goosen, & Van Ree, 1991) (Krishnan-Sarin et al., 1995). Evidence suggests that blocking μ and δ opioid receptors reversed ethanol stimulated dopamine release in the accumbens (Benjamin, Grant, & Pohorecky, 1993).

1.2.3.6. Effects of ethanol on nicotinic acetylcholine receptor-

Ethanol directly and indirectly acts on the nicotinic-cholinergic receptors. It increases acetylcholine in the neuromuscular junction and activates the nicotinic cholinergic receptors leading to increased miniature end plate potentials (Bradley, Peper, & Sterz, 1980). On the other hand, it also increases the frequency of the cation channels that are gated by these receptors (Aracava, FRÓES-FERRÃO, Pereira, & Albuquerque, 1991). These receptors are also involved in the reinforcement of ethanol. In the presence of nicotinic receptor antagonists, the ethanol-stimulated release of dopamine in the accumbens and locomotor activity is blocked (Blomqvist, Ericson, Johnson, Engel, & Söderpalm, 1996). The antagonist also reduces voluntary ethanol intake in rats (Blomqvist et al., 1996) (Potthoff, Ellison, & Nelson, 1983).

1.2.3.7. Effects of ethanol on adenylyl cyclase-

The different types of receptor systems such as dopamine (D1 and D2), opioid (μ and δ) and metabotropic glutamate are G-protein coupled receptors. The activity of these receptors depends on K^+ and Ca^{2+} channels and adenylyl cyclase (Eckardt et al., 1998).

This adenylyl cyclase is responsible for cyclic adenosine monophosphate (cAMP) production. cAMP is a second messenger system.

Ethanol results in an agonist mediated increased levels of adenylyl cyclase activity. This involves the action of ethanol on Gs proteins (Boris Tabakoff & Hoffman, 1998). This results in elevated cAMP levels in the brain. However, recent studies have also shown that the degree of sensitivity of adenylyl cyclase to ethanol also depends on their isoforms, Type VII adenylyl cyclase being the most sensitive (Yoshimura & Tabakoff, 1995). Therefore, ethanol stimulates adenylyl cyclase in different regions of the brain depending on the presence of the isoforms. This in turn has implications on the amount of cAMP production. The interaction of ethanol and cAMP producing systems is the underlying cause of ethanol tolerance (Boris Tabakoff & Hoffman, 1998) (Szabo, Hoffman, & Tabakoff, 1988).

1.2.4. Overall effects of ethanol on HPA axis function:

Several studies have shown that both chronic and acute alcohol consumption leads to HPA axis dysregulation (Bryon Adinoff, Iranmanesh, Veldhuis, & Fisher, 1998) (Hundt, Zimmermann, Pöttig, Spring, & Holsboer, 2001). HPA axis activity can be monitored by checking the cortisol levels in humans and corticosterone levels in rodents. Acute alcohol exposure and withdrawal leads to activation of HPA axis which ultimately results in elevated cortisol and adrenocorticotropin hormone levels (Bernardy, King, Parsons, & Lovallo, 1996) (B. Adinoff et al., 1990). Also, intoxication and withdrawal leading to hyperactive sympathetic system could be the underlying cause of dysregulation (Bryon Adinoff & Ravitz, 1991) (Burov, Treskov, Vedernikova, & Shevelyova, 1986). However, for chronic alcohol exposure, mixed results are observed. It may or may not be

able to sustain the elevated cortisol levels over the period of time. This could be a result of variabilities in hormonal tolerance depending upon the exposure time and amount. It could lead to a mild loss of HPA axis activation induced by alcohol or downregulation of receptors (Wand, 1993). The exact mechanism underlying alcohol-induced activation of the HPA axis is unknown. However, it is believed that alcohol mediates its effects by acting on the CRF neurons (Wand, 1993) (Vargas, Bissette, Owens, Ehlers, & Nemeroff, 1992). The third mechanism that has been suggested to be involved in alcohol induced HPA axis dysregulation is reduced HRV activity (Heart rate variability) and parasympathetic nervous system (Henry, 2002) (Ruiz-Padial, Sollers, Vila, & Thayer, 2003) (Julian F Thayer & Brosschot, 2005). These changes induced by alcohol on the functioning of the neural circuitry do not diminish once it is out of the system. In fact, HPA axis dysregulation is observed in abstinent alcoholics and children of the alcoholics (Bryon Adinoff & Ravitz, 1991) (Bernardy et al., 1996) (Croissant & Olbrich, 2004) (Errico, King, Lovallo, & Parsons, 2002) (Gerra et al., 1999) (J. F. Thayer, Hall, Sollers, & Fischer, 2006) (Lovallo, Dickensheets, Myers, Thomas, & Nixon, 2000)

1.3. Effects on Fetus

During a classic case of PPD, the mothers exhibit abnormalities in their reactivity to stress which is regulated by the HPA axis (Stowe & Nemeroff, 1995) (Melón et al., 2017). Activation of maternal HPA axis, results in the adrenal cortex to release cortisol in humans and corticosterone in rodents as a neuroendocrine response to stress. In the offspring, the basal activity of the HPA axis is higher than normal situations and it results in increased secretion of corticosterone. Inspite of this elevated response, the neurons in the adrenal zona fasciculata seem to have degenerated (Fameli, Kitraki, &

Stylianopoulou, 1994). Because the adrenal system is continuously active, it reaches a level of exhaustion. Thus, it cannot respond effectively to stress and shows underdeveloped HPA axis function (adaptation mediated by stress induced rise of CORT) (Fameli et al., 1994). These individuals cannot effectively reduce CORT levels on stress which is a classic feature seen in depressive illnesses. Thus, such offsprings are predisposed to stress. This could possibly show its effects on the anxiety and depression-like behavior (Sellers et al., 2013).

Implications of maternal drinking can be as serious as FAS (Fetal alcohol syndrome) with nearly 30% of patients suffering from it (Chudley, 2017). Alcohol indulgence by mothers can interfere with the neuronal development of the fetus and can lead to mental retardation and cognitive deficits (Willford, Richardson, Leech, & Day, 2004). The majority of the studies are focused on prenatal drinking and its impact on the fetus. The offspring who had moderate as well as heavy alcohol exposure exhibited an increased risk for psychiatric disorders (Streissguth, Barr, Kogan, & Bookstein, 1996) and were likely to behave impulsive, hyperactive and disruptive (Roebuck, Mattson, & Riley, 1999). The same effects of prenatal alcohol exposure on the animals were shown through several other studies (Savage, Becher, Torre, & Sutherland, 2002) (Driscoll, Streissguth, & Riley, 1990). The mechanism underlying this behavior is suggested as the alcohol effects in utero in animals on the functioning of the HPA axis (Schneider, Moore, Kraemer, Roberts, & DeJesus, 2002). However, it is important to consider the varying effects of alcohol on the offspring taking into consideration the time of exposure, the "pattern" of exposure and dose of alcohol (Huizink & Mulder, 2006).

1.4. Animal models

Hormone withdrawal post gestation has shown to contribute to the development of PPD like symptoms (Green, Barr, & Galea, 2009). In humans, the estrogen and progesterone levels decline concurrently post-delivery. However, in rodents, overall progesterone levels decline before giving birth (Schiller, Meltzer-Brody, & Rubinow, 2015). Thyroid hormone models, lower oxytocin level mouse models are also suggested to display PPD like characters since PPD is usually associated with thyroid dysfunction and lower oxytocin levels. HPA axis dysregulation is also implicated in to be a causative factor in PPD. This is usually observed in cases of stress during pregnancy (Schiller et al., 2015).

We used KCC2/CRH Knockouts (K+/Cl- channels on the Corticotropin Releasing Hormone neurons are knocked out) (Melón et al., 2017) and $GABA_A$ δ Knockouts (δ-Subunit on the $GABA_A$ Receptor are knocked out) (Jamie Maguire & Mody, 2008) which display HPA axis dysregulation.

The goal of this study was to assess the effects of alcohol binge drinking during the postpartum period in animals that do not display HPA axis dysregulation and those that do. It is important to note that acute alcohol consumption and PPD have overlapping effects on the CORT release system. We also wanted to evaluate the alcohol preference of these animals and its behavioral implications. Our aim was also to uncover the postnatal effects of binge drinking by these dams.

Chapter 2: Material and Methods

2.1. Mouse models

The mouse models used throughout the experiment are wildtype, global delta knockouts (the δ subunit of the $GABA_A$ receptor is knocked out) and KCC2/CRH knockouts (KCC2 is knocked out specifically in the CRH neurons)

All animals selected for mating. The vaginal plugs were used to time the pregnancy and the females were paired with males until visibly pregnant. Females were separated about 2 weeks into pregnancy and singly housed until delivery. Postpartum dams and virgins were randomly assigned to different treatment groups (water only or ethanol access) (Figure 2.1).

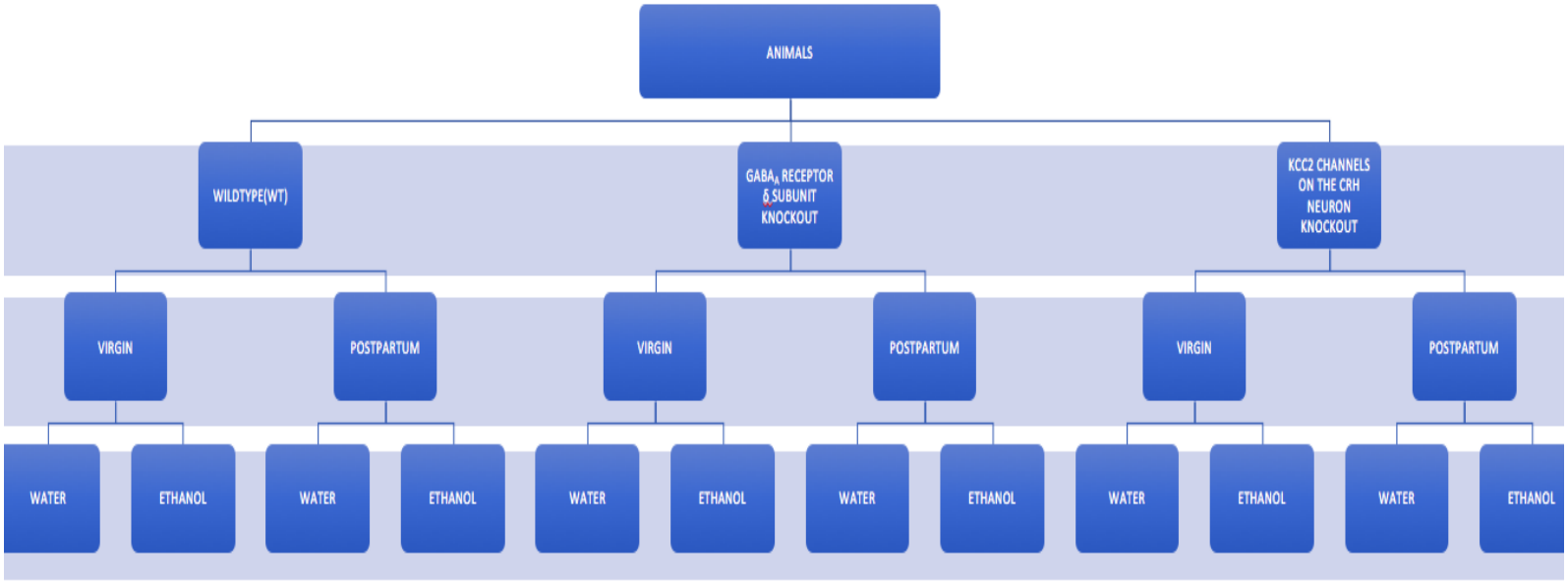

Figure 2. 1- Pictorial Representation of the experimental design for binge drinking and two-bottle choice protocol. The animals are randomly assigned in different treatment groups (water Vs ethanol).

2.2. Drinking models

2.2.1. Ethanol preparation:

20% (v/v) ethanol solution was prepared from a stock solution of '190 proof ethanol'. The 20% ethanol solution was freshly made each week.

2.2.2. Binge drinking protocol:

The animals were placed on a reverse light/dark cycle at least 3 days prior to the beginning of the binge drinking protocol. Mice were randomly assigned to 2 treatment groups (20% v/v of ethanol or water). Beginning at lights out [Zeitgeber time (ZT 12)] the animals had limited access to modified tubes for three 1 hour periods. Each hour was separated by 2 hour break where regular water bottles were returned. Mice had limited access to ethanol in this manner for 7 days. The animals were allowed to rest on the 8^{th} day. On the 9^{th} day the animals underwent behavioral analysis tests.

2.2.3. Alcohol Preference protocol (2-bottle choice protocol):

The animals were placed on a reverse light/dark cycle at least 2 days prior to the beginning of the alcohol preference protocol. 1 day prior to the drinking, the animals were transferred to the locomotor testing room. The binge drinking protocol involves measuring the ethanol intake by the virgin and postpartum mothers of all the 3 genotypes. The animals had access to 2 tubes-ethanol and water, for 24 hours a day. Their preference for alcohol over water is checked for a duration of 7 days. The animals were allowed to rest on the 8^{th} day. On the 9^{th} day, the animals underwent behavioral analysis tests. The animals were in a reverse light/dark cycle during the course of alcohol preference protocol.

2.3. Enzyme-Linked Immunosorbent Assay (ELISA)

Animals were allowed to rest for 30 minutes after the depressive-like forced swim test. Immediately after the completion of the post stress maternal approach test, blood from the submandibular region of the animals was taken and collected into clot activator blood collection tube (Terumo). Serum was isolated from this submandibular blood by centrifugation (14,000 rpm for 5 mins). Corticosterone (CORT) levels were measured by enzyme linked immunoassay in accordance to the manufacturer's instructions. Samples were prepared as duplicates and compared to a standard curve of absorbance at 415nm wavelength using a spectrophotometer. The kits used to measure the corticosterone were by Enzo Life Sciences.

2.4. Polymerase Chain Reaction (PCR)

PCR was performed on mice from the KCC2/CRH KO line using an ear punch sample of the animal. IDT's CRH-Cre Forward (5'- CTG TCT TGT CTG TGG GTG TCC GAT- 3'; M.W= 7,372.8 g/mol) and CRH-Cre Reverse (5'- CGG CAA ACG GAC AGA AGC ATT- 3'; M.W= 6,473.3 g/mol) primers were used. The animals were checked for their Cre status. Animals positive for the expression of Cre recombinase had a knockdown of the target gene.

2.5. Behavior paradigms

Behavioral tests were performed in the Behavioral Core at the Center for Neuroscience Research (P30 NS047243 (PI: Jackson)) at the Tufts University, Boston. The animals were placed in the testing facility 1 hour prior in order to acclimatize the

animals and minimize the confounding effects of stress. All the behavioral experiments were conducted during the reverse light/dark cycle.

2.5.1. Tests for anxiety:

Anxiety can be studied by various tests and the validity of such tests would depend on its ability to detect a change in response or behavior to a stimulus.

2.5.1.1. Elevated Plus Maze-

Rodents prefer to stay in enclosed spaces particularly the edges. This is an unconditioned test that uses a rodent's preference to this behavior called thigmotaxis. The apparatus is elevated and is in the shape of a plus sign. It has 2 arms that are enclosed by the wall and 2 arms that are open. The animals were placed in the center of the maze and their activity was recorded for 10 minutes test period. The number of entries, total distance travelled, amount of time spent in the open arms and the total number of beam breaks (basic movements) were measured using the Motor Monitor software (Hamilton-Kinder). The more time a mouse spends in the closed arm is a measure of its anxiety levels. Similarly, the less anxious it is increases the time and entries in the open arms.

2.5.1.2. Light/Dark Box-

The mice are individually tested using a 22 cm x 43 cm light/dark box apparatus (Hamilton-Kinder). The animal is placed in the dark zone before the test is started. This apparatus has 4 x 8 equally spaced photocells and the activity of the mice is recorded for 10 minutes test period. The latency to enter light compartment, time spent in the dark zone and light zone, the total number of beam breaks (basic movements), number of entries, distance travelled were measured using the Motor Monitor software (Hamilton-

Kinder). The more time it spends in the dark zone is a measure of its anxiety levels. Similarly, the less anxious it is increases the time spent in the light zone. First "emergence" into the light zone with all 4 paws outside is also a measurable parameter for anxiety related behavior.

2.5.2. Pup retrieval-

Maternal behavior is an important factor influencing the growth and development of the pups. Maternal behavior is influenced by a variable number of factors including mental status, diet, environmental stimuli, hormonal factors, etc. This is a baseline maternal test that is done before the animal is exposed to a stressor. In this test, the dams and pups are separated from each other for 20 minutes. Then 2 pups that are found to effectively vocalize are separated from the litter. The dam is then placed close to the litter. She uses auditory cues (ultrasonic cries) to retrieve the 2 separated pups back into the litter which is a normal maternal instinctive behavior. This test is done for 5 mins

2.5.3. Test for Depressive-like behavior-

Forced swim test is performed to detect depressive like behavior in the animals. In this test, the animal is put in a tub of water. Duration of the test is 6 mins. When the animal is put in, the timer is started. Latency to immobility and total immobile time is recorded. Increased latency to immobility is reflective of a decreased depressive like behavior.

2.5.4. Post stress Maternal Behavior-

Duration of the maternal approach test is 30 mins. It is ensured that all the pups in the litter are placed together. This test is done after the stressor and maternal behavior is noted. The dam is placed in the home cage immediately after the forced swim test. The maternal instinct of the dam is to immediately approach, interact and protectively sit over the litter. Latency to approach the litter is recorded which is a measure of abnormal maternal behavior. Also, the total interaction time of the dam with the litter is recorded. Decreased interaction time of the dam with her pups is an indication of abnormal maternal behavior.

2.6. Cannibalization Rate

The litter size is recorded on the day it is dropped. On the test day (Day 9) the litter size is again recorded. A decrease in litter size either due to neglect (malnourishment) or cannibalization is indicative of abnormal maternal behavior.

2.7. Statistical analysis

All data were analyzed using Graphpad Prism 7.0. Comparison between reproductive status (Virgins Vs postpartum) and genotype (WT, $GABA_A$ receptor δ subunit KO, KCC2/CRH KO), reproductive status (Virgins Vs postpartum) and treatment group (water and ethanol), treatment group (water and ethanol) and genotype (WT, $GABA_A$ receptor δ subunit KO, KCC2/CRH KO), treatment group (water and ethanol) and reproductive status (virgin and postpartum), pup gender (Male and female) and treatment group (ethanol and water) was conducted using two-way ANOVA. For post-hoc analyses, the Bonferroni test is used for pairwise comparisons. Comparison between genotype (WT, $GABA_A$ receptor δ subunit KO, KCC2/CRH KO) was conducted using a

one-way ANOVA. All the data is represented as Mean± SEM. Correlation analysis (two-tailed) was also performed on the data wherein the average ethanol intake was one of the variable and time spent in closed arms, time spent in dark zone, total time immobile and latency of approach were the other variables.

Chapter 3: Results

3.1. Binge drinking

AIM 1- Does binge drinking in the postpartum period induce deficits in maternal care?

3.1.1. Drinking in the Dark(DID):

Does reproductive status and/or vulnerability to postpartum depression alter binge drinking behavior?

Mice had intermittent access to ethanol (20% v/v) for 7 days. Data were analyzed using two-way ANOVA with reproductive status (Virgins vs postpartum) and genotype (WT, $GABA_A$ receptor δ subunit KO, KCC2/CRH KO) as factors (Figure 3.1)**.** There was no significant interaction of these factors [F (2,18) = 0.6468, p=0.54] on the amount of ethanol consumed. Specifically, it was observed that the postpartum KCC2/CRH KO mice (M= 5.72±15.07g/kg/3hours) and postpartum $GABA_A$ receptor δ subunit KO dams (M= 4.91±1.6g/kg/3hours) and WT dams (M= 3.29±1.17g/kg/3hours) consumed similar amounts of ethanol. Relatedly, virgin WT (M= 3.71±0.90g/kg/3hours), virgin $GABA_A$ receptor δ subunit KO (M= 2.92±1.19g/kg/3hours) and virgin KCC2/CRH KO (M= 3.40±1.20g/kg/3hours) females consumed similar amounts of ethanol in this limited access paradigm.

FIGURE 3.1 - BINGE BEHAVIOR (BINGE DRINKING WEEKLY)

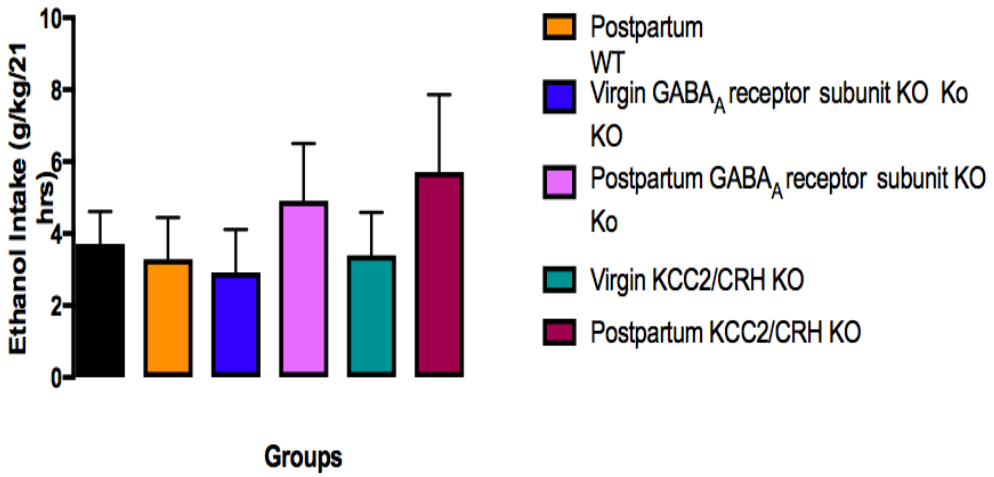

Figure 3. 1- Weekly binge drinking (g/kg/21 hrs) of all the ethanol treatment groups of all the genotypes and reproductive status. The animals had limited access to alcohol/water depending on its treatment group for 7 days. The virgins of all the genotypes consumed an approximate similar amount of ethanol. Same was the case with the postpartum dams of all the genotypes.

3.1.2. Tests for anxiety:

Does binge drinking during the postpartum period alter postpartum anxiety response?

The next question that came up was whether binge drinking during this postpartum depression perturbed the stress signaling pathway to produce significant changes in stress reactivity. Mice were tested on the elevated plus maze and light-dark box in order to assess anxiety-like behavior.

3.1.2.1. Elevated Plus Maze-

Time in the closed arms was assessed using a two-way ANOVA with reproductive status (Virgin vs Postpartum) and treatment (Ethanol vs Water) as factors

(Figure 3.2). There was no significant interaction between these factors [F (1,12) =1.62, P=0.23] on the amount of time spent in closed arms. For water drinking controls, postpartum females (M= 374.2 ±37.20 secs) spent 22.22% more time in the closed arms than virgins (M=306.28 ±42.88secs). Following ethanol, postpartum wildtype dams (M=377.6 ±24.00secs) spent 7.58% less time in the closed arms than the virgin WT females (M=408.83 ±48.70secs). There was also no significant correlation between the average alcohol intake and total time spent in the closed arms for all the WT animals (Postpartum dams p value 0.45, Virgins p value 0.87) (Figures not shown).

FIGURE 3.2- BINGE BEHAVIOR (TOTAL TIME IN CLOSED ARMS)

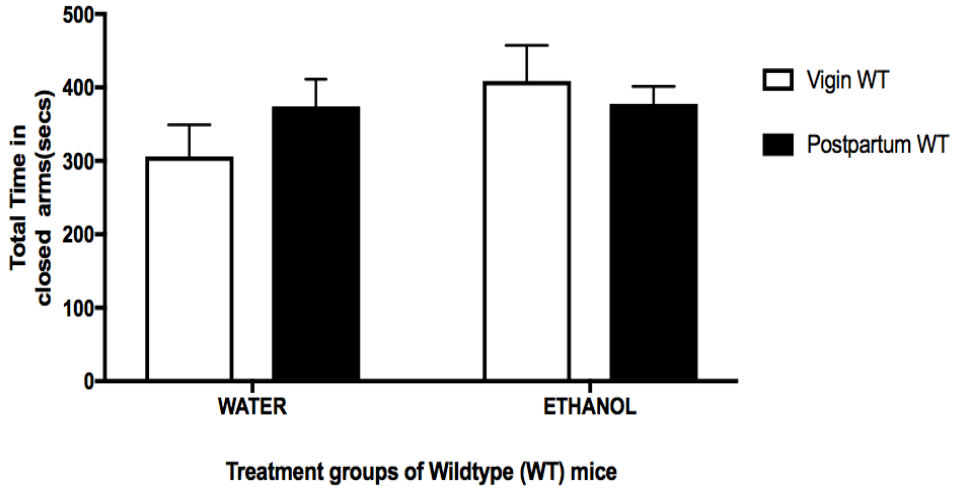

Figure 3. 2- Time spent in closed arms (secs) of the elevated plus maze in wildtype (WT) females with or without a history of binge drinking. This is a 10 minute test for anxiety-like behavior. The amount of time spent in the closed arms is a measure for anxiety response. There was no significant interaction between the factors (reproductive status and treatment group).

3.1.2.2. Light-Dark Box-

Time in the dark side of the light-dark box paradigm and emergence were assessed using a two-way ANOVA with reproductive status (Virgins vs Postpartum) and treatment (water vs ethanol) as factors (Figure 3.3). There was no significant interaction of these factors [F (1,12) = 0.27, p=0.61] on the amount of time spent in the dark zone of the light- dark box. For water drinking controls, postpartum WT dams (M= 269.3667±36.06 secs) spent 19.46% less time in the dark side of the box than virgin females (M= 333.85 ±47.59 secs) however, the difference is not significant. For ethanol, postpartum WT dams (M= 321.14±29.63 secs) spent only 5.87% less time in the dark zone than virgin WT mice (M= 340.73±52.99 secs). There was also no significant correlation between the average alcohol intake and total time spent in the dark zone for all the WT animals (Postpartum dams p value 0.43, Virgins p value 0.76) (Figures not shown).

FIGURE 3.3- BINGE BEHAVIOR (TOTAL TIME IN DARK ZONE)

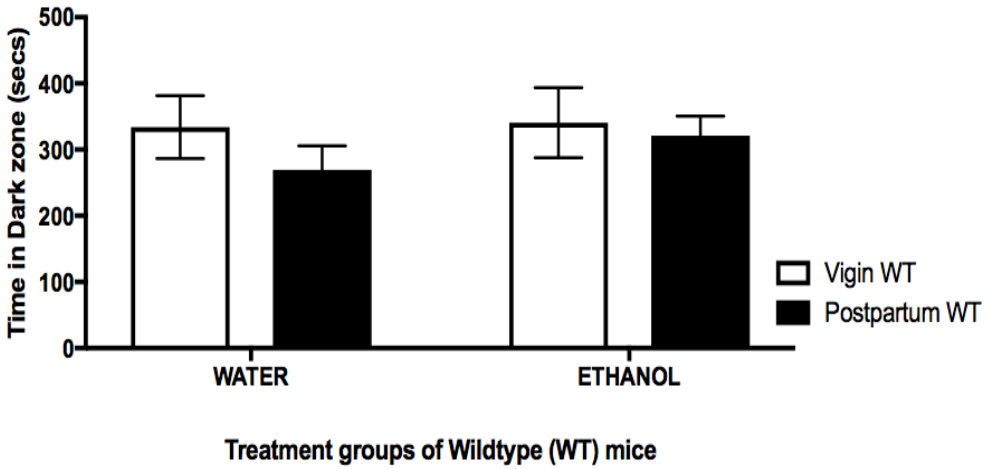

Figure 3. 3- Time spent in dark zone (secs) of the light/dark box in wildtype (WT) females with or without a history of binge drinking. This is a 10 minute test for anxiety-like behavior. The amount of time spent in the dark zone is a measure for anxiety response. There was no significant interaction between the factors (reproductive status and treatment group).

There was no significant interaction of these factors [$F(1,12) = 0.98$, $p=0.34$] on the time to emerge from the dark side of the box (Figure 3.4). However, for water drinking controls, postpartum females (M= 12.67±5.21 secs) take 25.33 fewer seconds to exit the dark box than virgin females (M = 38± 22.39 secs). After ethanol consumption, postpartum females (M= 28 ±7.75secs) only took 1.75 greater seconds to exit the dark box than virgin females (M = 26.25± 10.55 secs).

FIGURE 3.4- BINGE BEHAVIOR (EMERGENCE TIME)

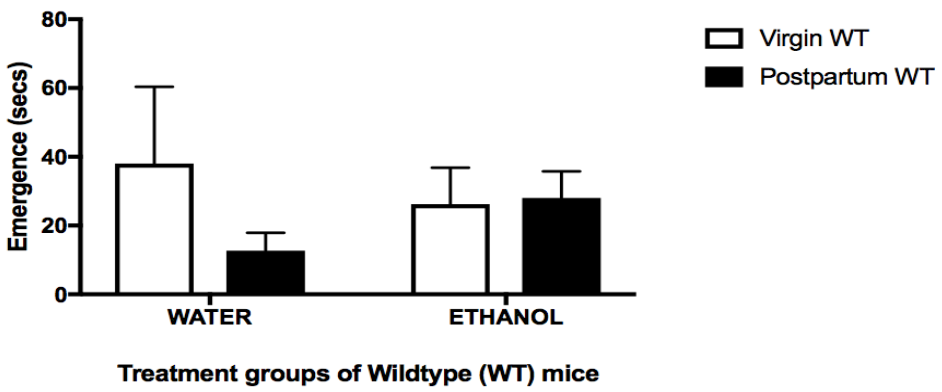

Figure 3. 4- Emergence time (secs) from the dark zone of the light/dark box in wildtype (WT) females with or without a history of binge drinking. This is a 10 minute test for anxiety-like behavior. The amount of time it takes to emerge from the dark zone and enter into the light zone is measure for anxiety response. There was no significant interaction between the factors (reproductive status and treatment group).

Does binge drinking during the Postpartum period alter postpartum anxiety response in genetic models of postpartum depression?

3.1.2.3. Elevated Plus Maze-

Time in the closed arms was assessed using a two-way ANOVA with reproductive status (Virgin vs Postpartum) and treatment (Ethanol vs Water) as factors separately for the two genetic models of PPD (KCC2/CRH KO and $GABA_A$ receptor δ subunit KO). There was no significant interaction of these factors on the amount of time spent in closed arms for either genotype. In the $GABA_A$ receptor δ subunit KO mice [$F(1,12) = 0.21$, $p= 0.65$] (Figure 3.5), there is a significant main effect of the reproductive status [$F(1,12) = 19.96$, $p= 0.0008$]. The water drinking postpartum females (M= 399.43 ±10.91 secs) spent 85.58% more time in the closed arms than virgins (M=215.08±

43.38secs), indicative of more anxiety. And they have a significant difference in the time they spent in the closed arms (Using Bonferroni, p value 0.09). Following ethanol, postpartum dams (M=388.05 ± 39.71 secs) spent 62.94% significantly (Using Bonferroni, p value 0.03) more time in the closed arms than the virgin females (M=238.18 ± 44.94secs), again indicative of increased anxiety. In the KCC2/CRH KO mice [F (1,10) = 1.02, p= 0.34] (Figure 3.6), there is a significant main effect of the reproductive status [F (1,10) = 7.603, p=0.02]. The water drinking postpartum females (M= 366.75 ± 31.58 secs) spent 58.87% significantly (Using Bonferroni, p value 0.048) more time in the closed arms than virgins (M= 231.1± 13.42 secs), indicative of more anxiety. Following ethanol, postpartum dams (M= 375.57 ± 60.63 secs) spent 20.12% more time in the closed arms than the virgin females (M= 312.58 ± 27.95 secs). There was also no significant correlation between the average alcohol intake and total time spent in the closed arms for all the $GABA_A$ receptor δ subunit KO mice and KCC2/CRH KO animals ($GABA_A$ receptor δ subunit KO mice Postpartum dams p value 0.29, Virgins p value 0.078 ; KCC2/CRH KO mice Postpartum dams p value 0.77, Virgins p value 0.80) (Figures not shown).

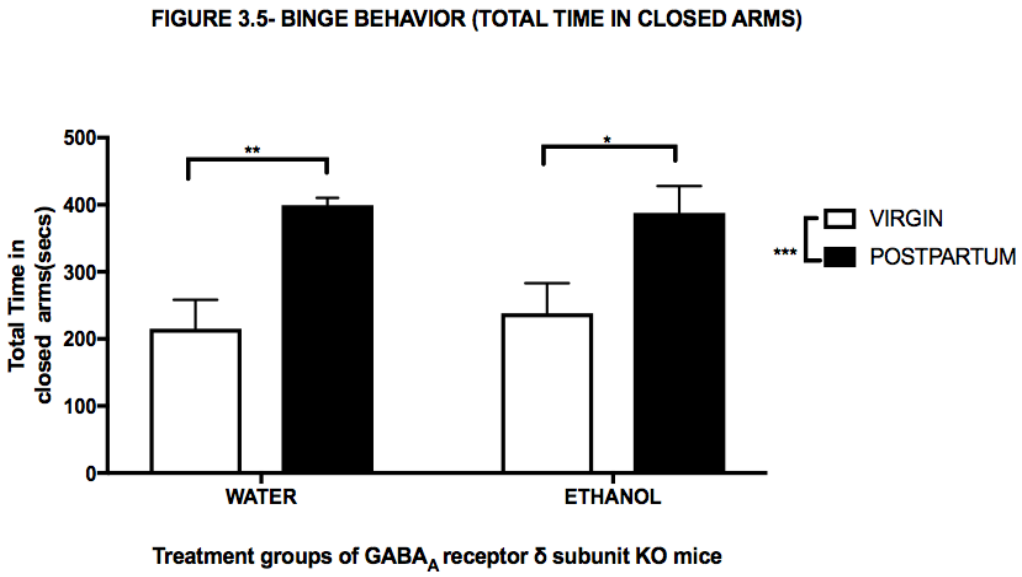

Figure 3. 5- Time spent in closed arms (secs) of the elevated plus maze in $GABA_A$ receptor δ subunit KO females with or without a history of binge drinking. This is a 10 minute test for anxiety-like behavior. The amount of time spent in the closed arms is a measure for anxiety response. There was no significant interaction between the factors (reproductive status and treatment group)

FIGURE 3.6- BINGE BEHAVIOR (TIME IN CLOSED ARMS)

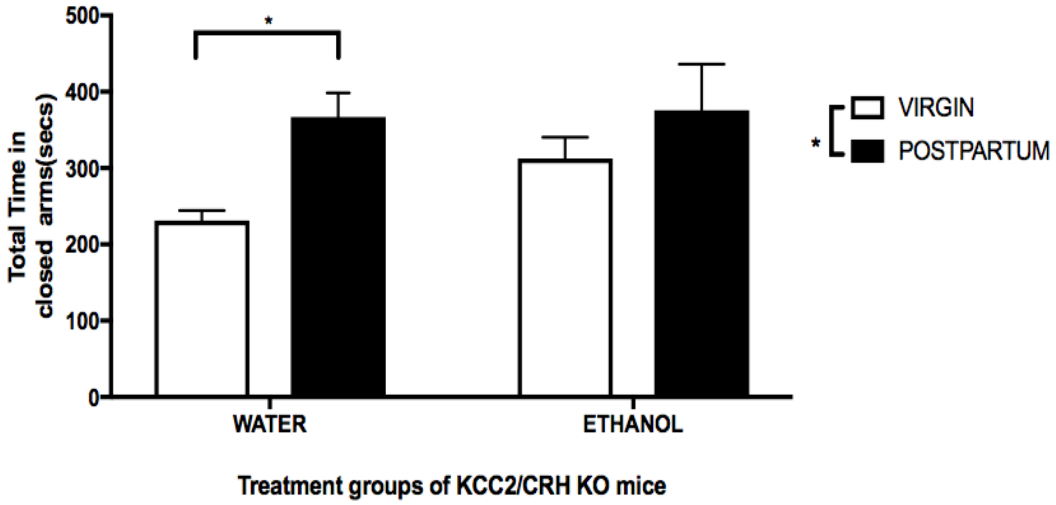

Figure 3. 6- Time closed arms (secs) of the elevated plus maze (secs) in KCC2/CRH KO females with or without a history of binge drinking. This is a 10 minute test for anxiety-like behavior. The amount of time spent in the closed arms is a measure for anxiety response. There was no significant interaction between the factors (reproductive status and treatment group).

3.1.2.4. Light-Dark Box-

Time in the dark side of the light-dark box paradigm and emergence was assessed using a two-way ANOVA with reproductive status (Virgins vs Postpartum) and treatment (water vs ethanol) as factors (Figures not shown). There was no significant interaction of these factors on the amount of time spent in the dark zone of the light- dark box. In the $GABA_A$ receptor δ subunit KO mice [$F(1,12) = 1.69$, $p= 0.22$], for water drinking, postpartum dams (M=317.85 ± 56.40 secs) spent 3.34% less time in the dark side of the box than virgin females (M=329.15± 42.35secs). For ethanol, postpartum dams (M= 378.65± 66.57 secs) spent only 45.21% less time in the dark zone than virgin mice (M= 261.48± 19.65 secs).

In the KCC2/CRH KO mice [F (1,10) = 1.31, p= 0.28], for water drinking, postpartum dams (M=333.9 ± 24.29 secs) spent 36.32% more time in the dark side of the box than virgin females (M=244.87± 51.03 secs). For ethanol, postpartum dams (M= 324.23 ± 29.50 secs) spent only 6.35% less time in the closed arms than virgin mice (M= 345.88 ± 65.88 secs). There was also no significant correlation between the average alcohol intake and total time spent in the dark zone for all the $GABA_A$ receptor δ subunit KO mice and KCC2/CRH KO animals ($GABA_A$ receptor δ subunit KO mice Postpartum dams p value 0.67, Virgins p value 0.79; KCC2/CRH KO mice Postpartum dams p value 0.74, Virgins p value 0.23) (Figures not shown).

3.1.3. Baseline pup retrieval:

Does binge drinking during the postpartum period induce deficits in maternal behavior?

Does binge drinking during the Postpartum period increases the incidence of maternal deficits in genetic models of PPD?

After the anxiety tests which also served as a brief separation period between the dam and her litter, the animals were checked for their baseline maternal behavior. This behavior was assessed using the pup retrieval test whose duration was for 5 minutes (300 secs). From the entire litter, two of the pups were separated in the home cage and the amount of time the dam took to retrieve these two pups back into the litter was noted. These data were analyzed using two-way ANOVA with treatment group (water vs ethanol) and genotype (wildtype, $GABA_A$ receptor δ subunit KO and KCC2/CRH KO) as factors (Figure 3.7). There was significant interaction between these factors on the amount of time they took to retrieve the pups back into its litter [F (2, 11) = 1406,

$p<0.0001$]. There was also a main significant effect of the treatment [$F(1,11) = 1307$, $p<0.0001$] and the genotype [$F(2,11) = 1406$, $p< 0.0001$]of the animals. The postpartum dams of the KCC2/CRH KO (M of the water group= 300 ±0secs, M of ethanol group= 300 ±0secs) and $GABA_A$ receptor δ subunit KO (M of water group= 300 ±0secs, M of ethanol group= 300 ±0secs) genotypes belonging to both the treatment groups did not retrieve the pups within the entire test duration. The animals of these genotypes display HPA axis dysregulation and also exhibit deficits in maternal care. However, it was interesting to find that the postpartum WT dams in the ethanol treatment group (M= 300 ±0secs) displayed abnormal maternal behavior by not retrieving the pups back into its litter until the end of the test duration. On the other hand, the postpartum WT dams in the water treatment group (M= 18.95 ±8.37 secs) retrieved the pups back in its litter. And there was a significant difference (Using Bonferroni, $p<0.0001$) between the retrieval times of the postpartum WT dams in the ethanol vs water groups.

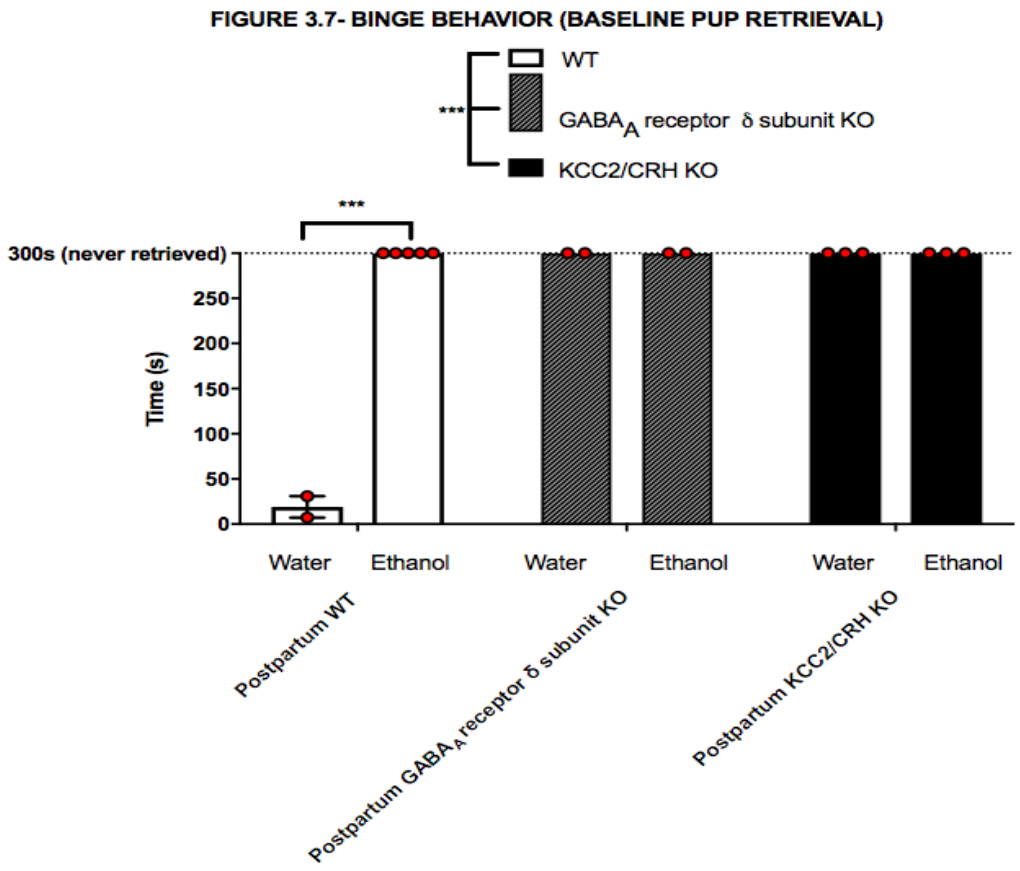

Figure 3. 7- Baseline pup retrieval (secs) of postpartum females of all genotypes with or without a history of binge drinking. The litter is separated from the dam for a brief period. 2 of the vocalizing pups are separated from the litter in the homecage and the amount of time taken by dam to retrieve its pups back into the litter is recorded. There was a significant interaction between the factors (Genotype and treatment group). Postpartum dams (of both the treatment groups) that show HPA axis dysregulation and postpartum WT dams of the ethanol treatment group do not retrieve the pups during the 5 minute test duration. Postpartum WT dams of the water treatment group successfully retrieved its pups back into the litter.

3.1.4. Depressive like Behavior:

Does binge drinking in the postpartum period induce depression like characters?

In order to test the effects of alcohol on HPA axis activity and eventually on the depressive-like behavior, we performed the forced swim test. Data were analyzed using two-way ANOVA with reproductive status (postpartum vs. virgin) and treatment (ethanol vs. water) as factors (Figure 3.8). There was no significant interaction of these factors [F

(1,12) = 0.0065, p=0.94] on the total time immobile. For water drinking controls, postpartum females (M= 289.60 ± 5.589secs) spent 4.34% more time in the closed arms than virgin females (M=277.54 ±25.41secs). Following ethanol, postpartum wildtype dams (M= 288.772 ±11.223secs) spent 5.47% more time in the open arms than virgin wildtype females (274±16.19 secs). There was also no significant correlation between the average alcohol intake and total time immobile for all the WT animals (Postpartum dams p value 0.15, Virgins p value 0.61) (Figures not shown).

FIGURE 3.8- BINGE BEHAVIOR (TOTAL TIME IMMOBILE)

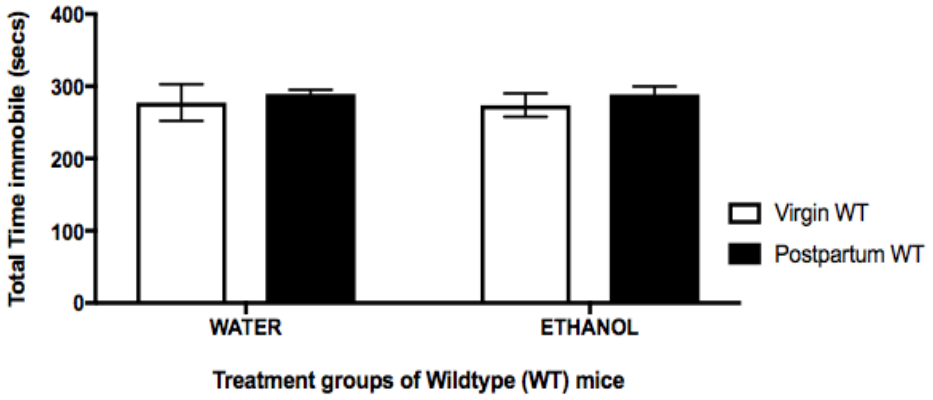

Figure 3. 8- Total time immobile (secs) for wildtype (WT) females with or without a history of binge drinking. The animals were subjected to a 6 minute depressive-like forced swim test. The total time they remain in a state of immobility is recorded as a measure of depressive behavior. There was no significant interaction between the factors (reproductive status and treatment group)

On the other hand, it is seen that binge drinking ethanol in the postpartum period induces helplessness which is measured by the latency to immobility. Data were analyzed

by two-way ANOVA with reproductive status (postpartum vs. virgin) and treatment (ethanol vs. water) as factors (Figure 3.9). There was no significant interaction of these factors [F (1,12) = 0.34, p= 0.57] on the latency to immobility. Therefore, it was observed that the ethanol treated postpartum WT dams (M= 62.9 ±5.23 secs) gave up swimming 23.12 seconds slower than the virgin WT (M=39.78 ± 16.10 secs). For water treated controls, postpartum WT dams (M= 66.666±4.165secs) gave up swimming 35.62 seconds than the virgin WT mice (M= 31.04 ± 11.33 secs). There was also no significant correlation between the average alcohol intake and latency to immobility for all the WT animals (Postpartum dams p value 0.06, Virgins p value 0.36) (Figures not shown).

FIGURE 3.9- BINGE BEHAVIOR (LATENCY TO IMMOBILITY)

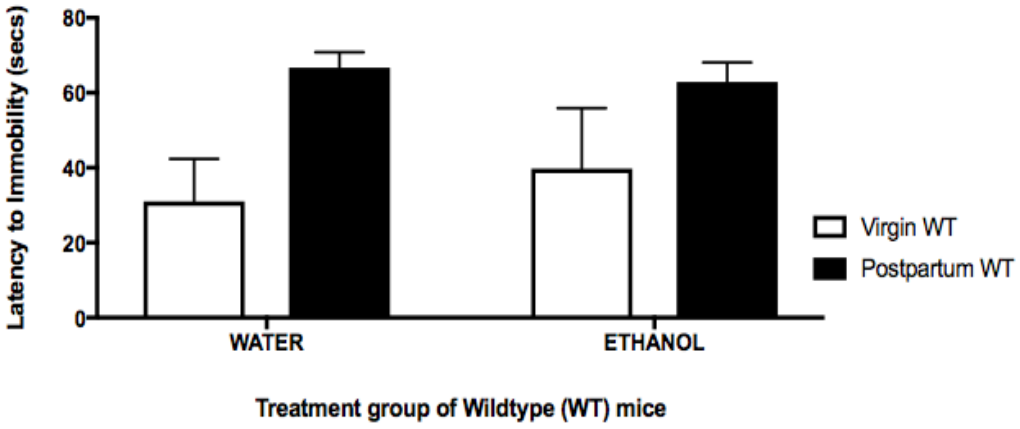

Figure 3. 9- Total time immobile (secs) for wildtype (WT) females with or without a history of binge drinking. The animals were subjected to a 6 minute depressive-like forced swim test. The time taken to go in a state of immobility is recorded as a measure of depressive behavior. There was no significant interaction between the factors (reproductive status and treatment group)

3.1.5. Post Stress Maternal Behavior:

Does binge drinking induce maternal deficits?

In order to assess the impact of binge drinking on the HPA axis function and eventually on maternal behavior, we performed the maternal approach test whose duration of testing was 30 minutes. This study was performed after the depressive–like forced swim test. The data were individually analyzed as genotypes using two-way ANOVA with treatment group (water vs ethanol) and genotype (wildtype, $GABA_A$ receptor δ subunit KO and KCC2/CRH KO) as factors (Figure 3.10). There was no significant interaction between the factors [$F(2,12) = 0.03$, $p=0.97$] for the amount of time it takes to approach the pups after the forced swim test. It was observed that postpartum dams of all the 3 genotypes namely WT (M= 941.8±359.13 secs), KCC2/CRH KO (M= 1222.1±577.87 secs), $GABA_A$ receptor δ subunit KO (M= 937.4±862.57 secs) belonging to the ethanol treatment group showed an increased latency to approach the litter compared to the water treatment dams of WT (M= 478±470.77 secs), KCC2/CRH KO (M= 635.8 ±582.25 secs), $GABA_A$ receptor δ subunit KO (M= 643.6 ±640.29 secs).

FIGURE 3.10- BINGE BEHAVIOR (LATENCY TO APPROACH)

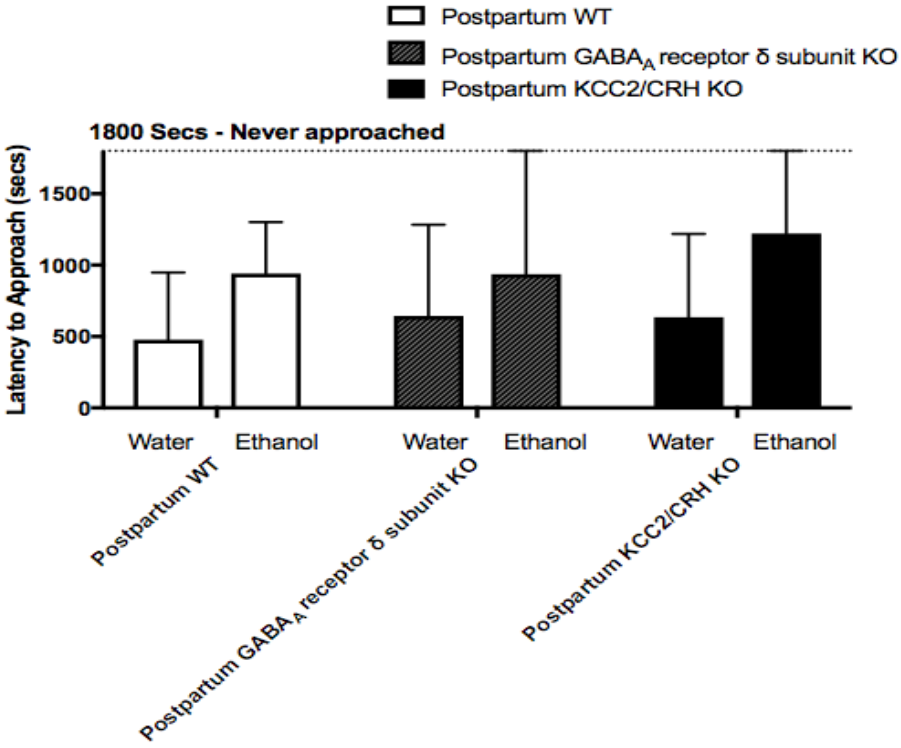

Figure 3. 10- Total time to approach (secs) for postpartum females of all genotypes with or without a history of binge drinking. The animals were immediately put back into the home cage after the forced swim test and their latency to approach the litter was recorded for 30 minutes as a measure for abnormal maternal behavior. There was no significant interaction between the factors (genotype and treatment group). However, it was broadly observed that all the dams (irrespective of the genotypes) showed an increased latency to approach.

The data were also analyzed by two-way ANOVA for the total interaction time between the dams and their litter with treatment group (water vs ethanol) and genotype (wildtype, $GABA_A$ receptor δ subunit KO and KCC2/CRH KO) as factors (Figure 3.11). There was no significant interaction between the factors [$F(2,12) = 0.09$, $p=0.91$] for the amount of time the dam interacts with the litter. Even though not significant, it was observed that postpartum WT dams belonging to the water treatment group (M= 513.19 ± 365.51secs), showed a decrease in the interaction time compared to the ethanol treated dams (M= 744.65 ± 344.05 secs). However, for the other two genotypes namely

KCC2/CRH KO (M= 570.76±471.52 secs) and $GABA_A$ receptor δ subunit KO (M= 988±591.54 secs) belonging to the water treatment group showed an increase in the interaction time of the dam with the litter compared to the ethanol treatment dams of KCC2/CRH KO (M= 478.57±478.58secs) and $GABA_A$ receptor δ subunit KO (M= 863.41±863.41 secs) genotypes.

FIGURE 3.11- BINGE BEHAVIOR (TOTAL INTERACTION TIME)

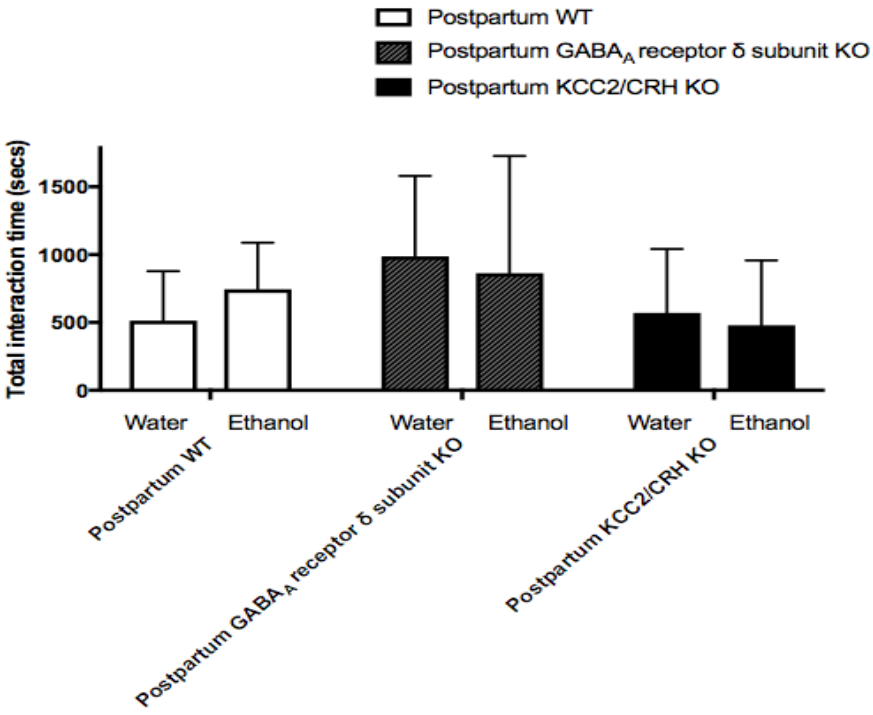

Figure 3. 11- Total interaction time (secs) for postpartum females of all genotypes with or without a history of binge drinking. The animals were immediately put back into the home cage after the forced swim test and their total interaction time with the litter was recorded for 30 minutes. Decreased interaction time is a measure of abnormal maternal behavior. There was no significant interaction between the factors (genotype and treatment group).

3.1.6. CORT Analysis:

What happens to the CORT levels when binge drinking and PPD co-occur?

In order to assess the fluctuations in corticosterone levels due to ethanol binging coinciding with the postpartum period, an ELISA assay was performed. The blood samples from the animals of all genotypes (wildtype, $GABA_A$ receptor δ subunit KO and KCC2/CRH KO) were taken from the submandibular region 30 mins after the FST (immediately after the maternal approach test). Data were analyzed using two- way ANOVA with treatment group (water and ethanol) and reproductive status (virgin and postpartum) as factors.

For WT mice, there was no significant difference between the factors [$F(1,12) = 0.20$, $p = 0.66$] for the CORT levels (Figure 3.12). However, it is important to note that there is a significant difference between the animals that belong to one treatment group depending on their reproductive status [Using Bonferroni- Water group- $p = 0.0101$, Ethanol group- $p=0.015$]. Water treated postpartum dams ($M = 284.76 \pm 34.10$ ng/ml) showed elevated CORT levels compared to the virgins ($M = 102.31 \pm 49.82$ ng/ml). It was also observed that the postpartum animals ($M = 205.22 \pm 31.63$ ng/ml) that belonged in the ethanol treatment group had elevated CORT levels compared to virgins ($M = 54.61 \pm 11.68$ ng/ml). There is also a significant main effect of reproductive status [$F(1,12) = 22.09$, $p = 0.0005$].

FIGURE 3.12- BINGE BEHAVIOR WT (CORT ANALYSIS)

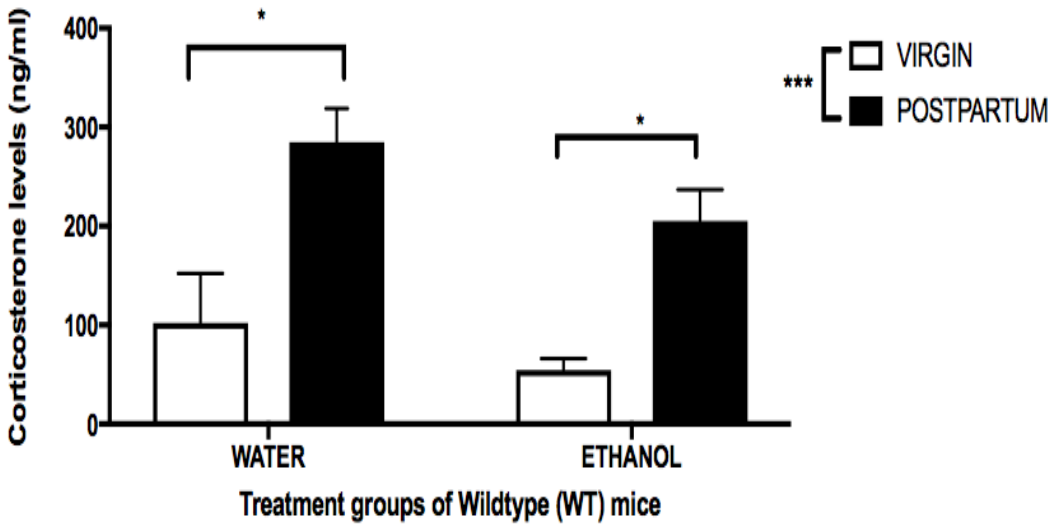

Figure 3. 12- Corticosterone levels (ng/ml) for wildtype (WT) females with or without a history of binge drinking. The average serum corticosterone levels in wildtype (WT) virgins and postpartum dams. Blood samples are taken from the submandibular region following 30 mins of FST and immediately following approach test. There is no significant difference between the factors (Treatment group and reproductive status). However, there is a significant difference between the virgins and postpartum mice within one treatment group.

For $GABA_A$ receptor δ subunit KO mice, there was no significant difference between the factors [$F(1,8) = 0.69$ $p= 0.43$] for the CORT levels (Figure 3.13). In the water treatment group, there is no significant difference between the virgins (M= 52.43± 4.70 ng/ml) and the postpartum dams (M= 45.22± 9.52 ng/ml). Similarly, in the ethanol treatment groups, there is not significant difference in the CORT levels between the virgins (M= 86.69± 35.45 ng/ml) and postpartum dams (M= 49.31±22.18 ng/ml).

FIGURE 3.13- BINGE BEHAVIOR $GABA_A$ receptor δ subunit KO (CORT ANALYSIS)

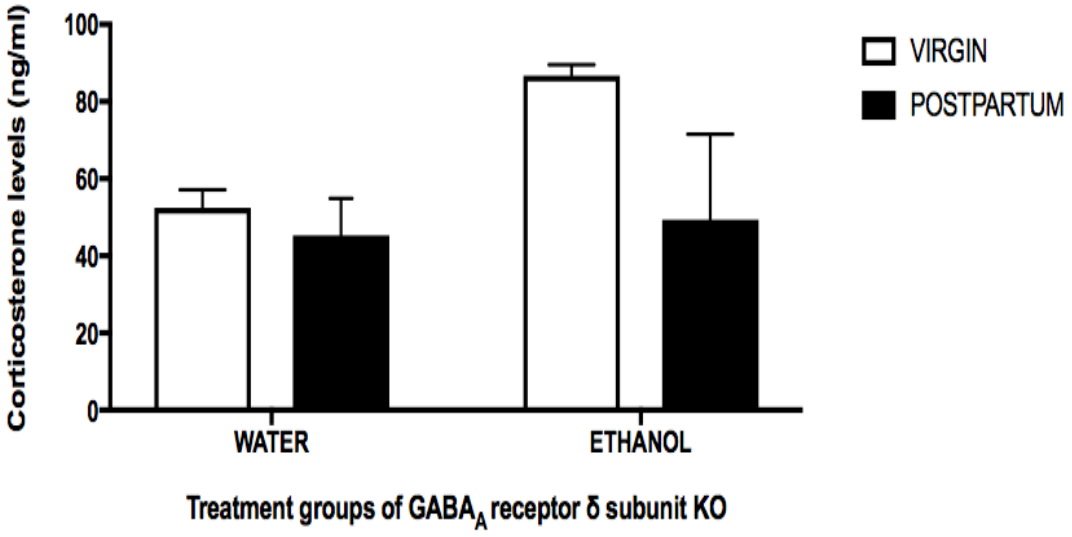

Figure 3. 13- Corticosterone levels (ng/ml) for $GABA_A$ receptor δ subunit KO females with or without a history of binge drinking. The average serum corticosterone levels in $GABA_A$ receptor δ subunit KO virgins and postpartum dams. Blood samples are taken from the submandibular region following 30 mins of the approach test. There is no significant difference between the factors (Treatment group and reproductive status).

For KCC2/CRH KO mice, there was significant difference between the factors [F (1,2) = 33.4, p= 0.029] for the CORT levels (Figure 3.14). It is also important to note that there is a significant difference (Using Bonferroni, p= 0.042) between the animals that belonged to water treatment group depending on their reproductive status, that is virgins (M= 23.29± 8.84 ng/ml) and postpartum dams (M= 152.56 ng/ml). In the ethanol treated group, there was no significant difference observed between the virgins (M= 59.56± 0.99 ng/ml) and the postpartum dams (M= 99.79 ng/ml). Also, it is observed that there is a significant main effect of the reproductive status [F (1,2) = 121, p= 0.0082].

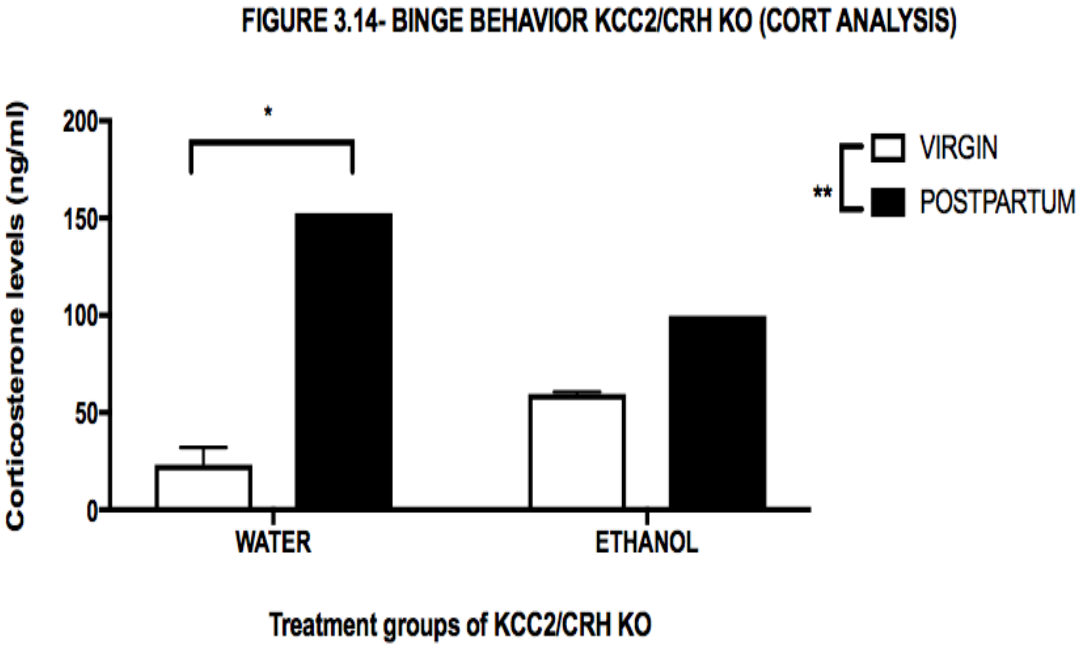

Figure 3. 14- Corticosterone levels (ng/ml) for KCC2/CRH KO females with or without a history of binge drinking. The average serum corticosterone levels in KCC2/CRH KO virgins and postpartum dams. Blood samples are taken from the submandibular region following 30 mins of the approach test. There is no significant difference between the factors (Treatment group and reproductive status). However, there is a significant difference between the virgins and postpartum mice within the water treated group.

3.1.7. Cannibalization rate:

Which models shows sever symptoms of PPD?

Abnormal maternal behavior was displayed by the postpartum WT, KCC2/CRH KO mice and $GABA_A$ receptor δ subunit KO dams belonging to the ethanol treatment group during the baseline maternal retrieval test. The same behavior was displayed by the postpartum KCC2/CRH KO mice and postpartum $GABA_A$ receptor δ subunit KO dams belonging to water treatment group. Cannibalization of the litter is also categorized as abnormal maternal behavior. Data were analyzed using two–way ANOVA with the genotypes (wildtype, $GABA_A$ receptor δ subunit KO and KCC2/CRH KO) and treatment

groups (ethanol and water) as the factors (Figure 3.15). There was no significant difference observed between the factors [$F(2,17) = 0.18$, $p = 0.83$] for cannibalization rate in percent. However, there is a significant main effect of the genotype [$F(2,17) = 5.491$, $p = 0.0145$]. It observed that the survival rate of the pups was decreased in the animals that displayed HPA axis dysregulation such as the $GABA_A$ receptor δ subunit KO (water treatment- M= 62.78±22.28 % and ethanol treatment- M= 64.58±22.14 %) and KCC2/CRH KO (water treatment- M= 25 ± 25% and ethanol treatment- M= 14.29 ±14.29%). WT postpartum dams belong to the ethanol treatment group (M= 12.38± 7.62%) also showed an increased cannibalization rate compared to the water group (M= 0 %)

FIGURE 3.15- BINGE DRINKING DATA- CANNIBALIZATION RATE

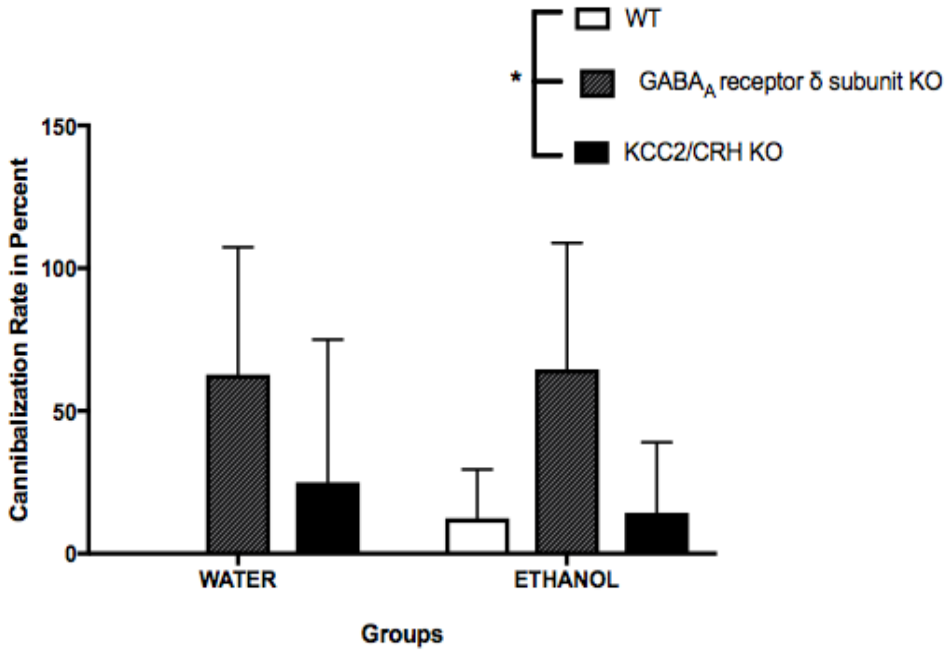

Figure 3. 15- Cannibalization rate in percent of the animals belonging to all genotypes with or without a history of binge drinking. The litter size is recorded on the day it is dropped. On the test day (Day 9) the litter size is again recorded. Models of postpartum depression irrespective of the treatment group and the WT dams in the ethanol treatment group show a decreased litter size.

3.1.8. Pictorial Representation of the results:

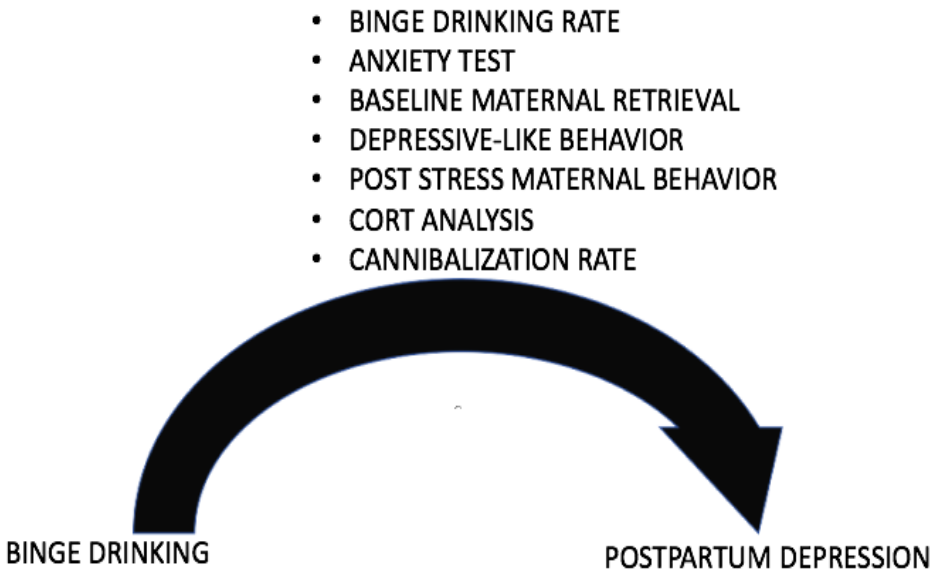

Figure 3. 16- Pictorial representation of the results derived from Aim1.The postpartum WT animals that engaged in binge drinking during the postpartum period exhibited abnormal maternal behavior. Similarly, the same deficits in maternal care were displayed by the water and ethanol treated postpartum KCC2/CRH KO and postpartum $GABA_A$ receptor δ subunit KO females. This same behavior was not shown by postpartum WT dams belonging to the water treated group.

3.2. Alcohol Preference

AIM 2- Under what conditions the alcohol preference changes and are its effects?

3.2.1. Ethanol Preference:

Does reproductive status and/or vulnerability to postpartum depression alter alcohol preference?

The animals of all the genotypes irrespective of their reproductive status (virgins or postpartum) are subjected to a two-bottle choice protocol. In this method, the animals have unlimited access to two bottles of 20% ethanol and water respectively. The preference of the mice to 20% ethanol over water was measured over the course of 7-

days. The data was analyzed using two-way ANOVA with reproductive status (Virgins vs postpartum) and genotype (WT, $GABA_A$ receptor δ subunit KO, KCC2/CRH KO as factors (Figure 3.17)**.** There was no significant interaction between the factors [$F(2,19) = 0.019$, $p=0.98$]. However, there is a main significant effect of the reproductive status [$F(1,19) = 22.04$, $p= 0.0002$]. It was observed that the virgin KCC2/CRH KO mice (M= 38.28 ±14.03) recorded the lowest alcohol preference over the 7-day period. This preference ratio for the amount of ethanol consumed by WT virgins was the highest (M= 39.40± 4.43) followed by virgin $GABA_A$ receptor δ subunit KO (M= 47.49 ±1.00). The alcohol preference of all the postpartum dams including WT (M= 8.98 ±2.93), $GABA_A$ receptor δ subunit KO (M= 15.29 ±3.75) and KCC2/CRH KO (M= 9.28 ±0.52) was lower than virgins. It is important to note that there is a significant difference in the alcohol preference ratio between the virgin vs postpartum dams belonging to WT (Using Bonferroni, $p = 0.025$) and $GABA_A$ receptor δ subunit KO (Using Bonferroni, $p= 0.027$) genotypes. There is no significant in the preference ratio between the postpartum KCC2/CRH KO (M= 9.28 ±0.52) and virgin KCC2/CRH KO (M= 38.28 ±14.03).

FIGURE 3.17- TWO BOTTLE CHOICE PROTOCOL (ETHANOL PREFERENCE RATIOS)

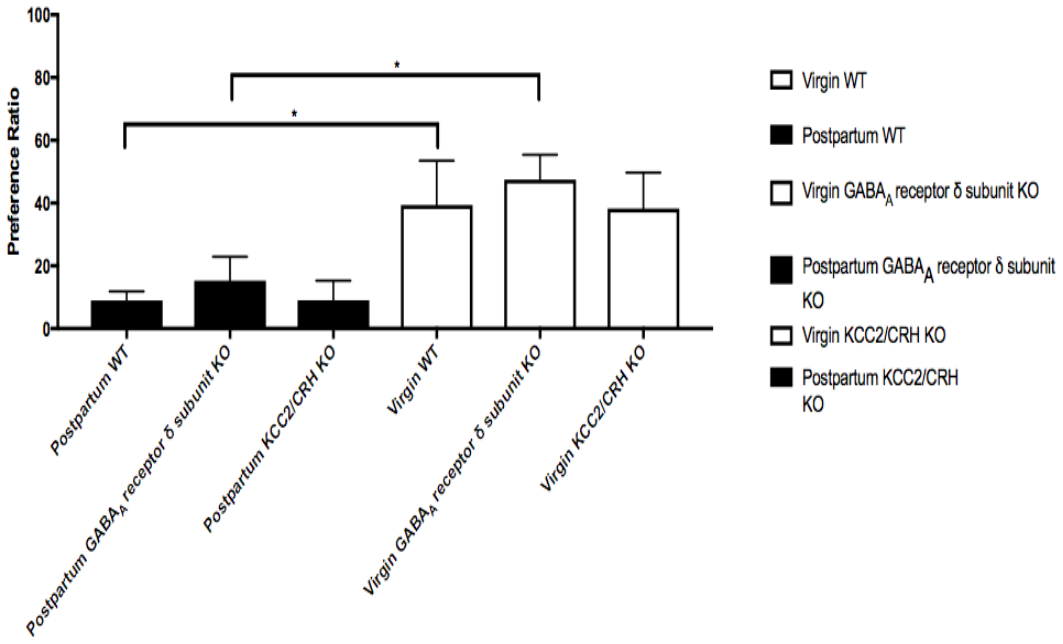

Figure 3. 17- Alcohol preference ratio for females of all genotypes and reproductive status subjected to a two-bottle choice method. The animals had unlimited access to alcohol and water bottles for 7 days. The virgins of all the genotypes consumed an approximate similar amount of ethanol. Same was the case with the postpartum dams of all the genotypes. There was a significant difference between the alcohol preference ratio within a genotype, depending on the reproductive status

3.2.2. Baseline pup retrieval:

Does alcohol preference influence maternal behavior?

Since binge behavior findings provided evidence for the development of maternal deficits in postpartum WT dams that indulged in binge drinking behavior, it was important to observe the effects of alcohol exposed through the two- bottle choice protocol on these animals. These data were analyzed using one-way ANOVA with genotype (wildtype, $GABA_A$ receptor δ subunit KO and KCC2/CRH KO) as a factor (Figure 3.18). There was no significant interaction across the genotypes [$F(2,10) =$

0.64, p= 0.55] for the amount of time taken to retrieve the ups back into its litter. It was observed that the postpartum WT dams (M= 300 ±0secs) and postpartum KCC2/CRH dams KO (M= 300 ±0secs) never retrieved the pups. Thus, they exhibited abnormal maternal behavior. However, the postpartum $GABA_A$ receptor δ subunit KO dams retrieved their pups 66 secs earlier than the other groups (M= 234 ±43.25 secs)

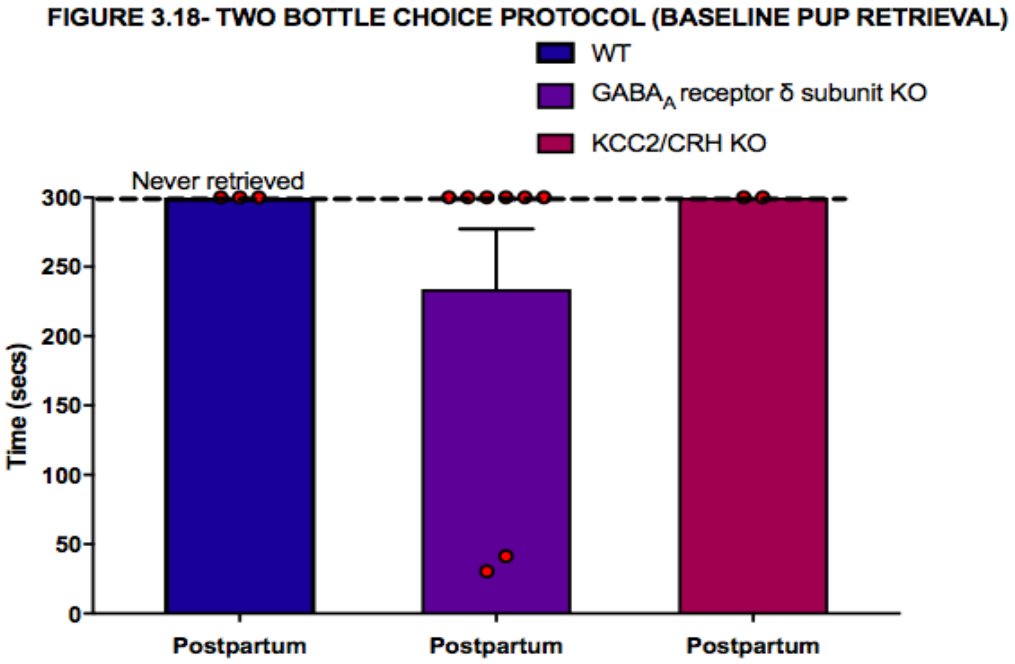

Figure 3. 18- Time to retrieve (secs) the pups by the postpartum females of all genotypes subjected to a two-bottle choice method. The litter is separated from the dam for a brief period. From the entire litter, 2 of the vocalizing pups are separated from the litter in the home cage and the amount of time taken by dam to retrieve its pups back into the litter is recorded. There was no significant interaction between the postpartum dams across the genotypes. The postpartum WT dams never retrieved their pups exhibiting behavioral deficits similar to the other 2 genotypes.

3.2.3. Post Stress Maternal Behavior:

What are the effects of alcohol preference on maternal behavior?

These experiments also were used to assess the impact of the degree of HPA axis dysregulation in the period of postpartum depression depending on the alcohol preference. Data were analyzed using one-way ANOVA with genotype (wildtype, $GABA_A$ receptor δ subunit KO and KCC2/CRH KO) as a factor (Figure 3.19). There was no significant interaction across the genotypes [$F(2,10) = 0.77$, $p = 0.49$] for the amount of time taken to approach the litter post the forced swim test. The postpartum $GABA_A$ receptor δ subunit KO dams showed an increase in latency to approach (M=967.53 ±250.10 secs) followed by postpartum KCC2/CRH KO (M= 879.77 ±868.69 secs) and postpartum WT dams. (M= 775.04 ±372.25 secs).

FIGURE 3.19- TWO BOTTLE CHOICE PROTOCOL (LATENCY TO APPROACH)

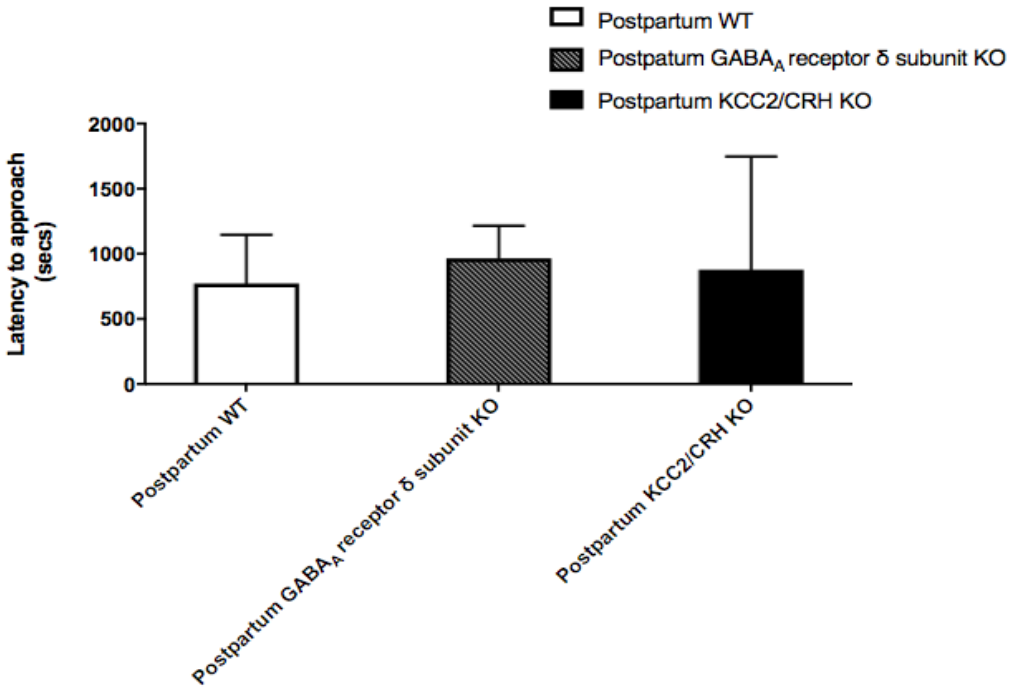

Figure 3. 19- Latency to approach (secs) the pups by the postpartum females of all genotypes subjected to a two-bottle choice method. The animals were immediately put back into the home cage after the forced swim test and their latency to approach the litter was recorded for 30 minutes as a measure for abnormal maternal behavior. There was no significant interaction between the postpartum dams across the genotypes. The postpartum WT dams showed an increased latency to approach their litter, exhibiting behavioral deficits similar to the other 2 genotypes

Data were also analyzed using a one-way ANOVA with as factors. It was observed that genotype (wildtype, $GABA_A$ receptor δ subunit KO and KCC2/CRH KO) as a factor (Figure 3.20). There was no significant interaction across the genotypes [$F(2,10) = 0.026$, $p = 0.97$] for the amount of time the dam interacts with the litter post the forced swim test. The postpartum WT dams showed an increase in total interaction time (M=592.27 ±450.07 secs) followed by postpartum dams KCC2/CRH KO (M= 563.59 ±537.57 secs) and postpartum $GABA_A$ receptor δ subunit KO dams. (M= 536.03 ±208.68 secs)

FIGURE 3.20- TWO BOTTLE CHOICE PROTOCOL (TOTAL INTERACTION TIME)

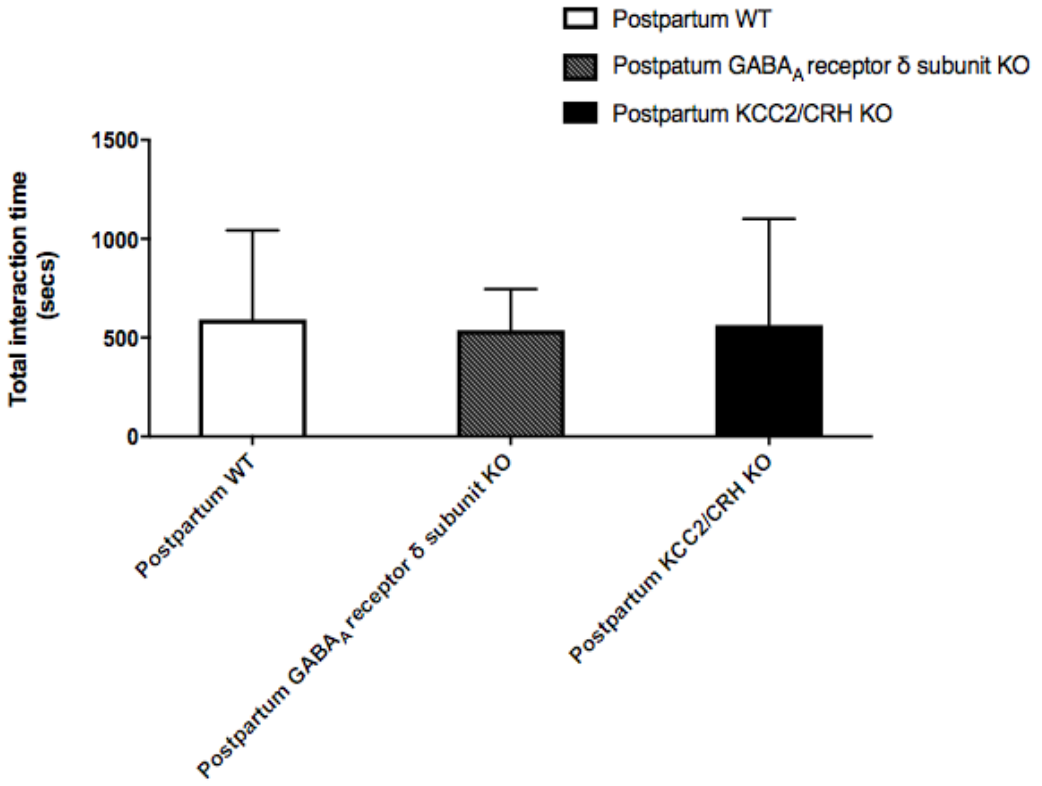

Figure 3. 20- Total interaction time (secs) the pups by the postpartum females of all genotypes subjected to a two-bottle choice method. The animals were immediately put back into the home cage after the forced swim test and their total interaction time with the litter was recorded for 30 minutes as a measure for abnormal maternal behavior. There was no significant interaction between the postpartum dams across the genotypes. Decreased interaction time is an indication of abnormal maternal behavior.

3.2.4. Pictorial Representation of the Results:

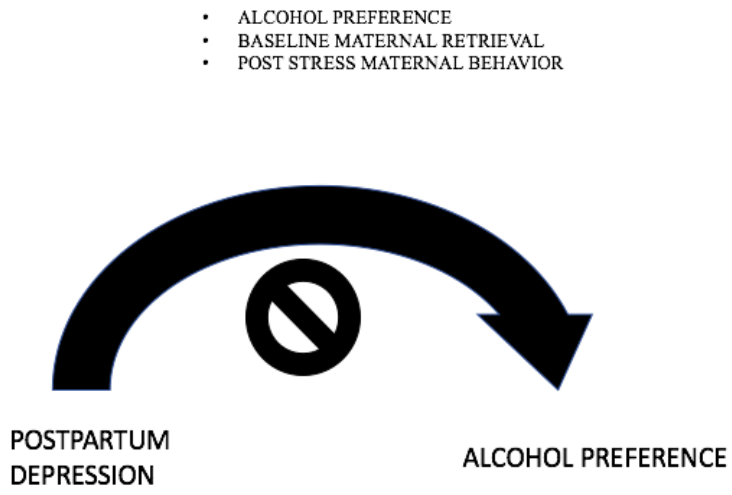

Figure 3. 21- Pictorial representation of all the results derived from Aim2. The postpartum dams that exhibited abnormal maternal behavior ($GABA_A$ receptor δ subunit KO, KCC2/CRH KO) did not have an increased alcohol preference. Similar to them, postpartum WT dams that exhibited deficits in maternal care when exposed to ethanol also show a low alcohol preference. The virgins of all the genotypes had the highest alcohol preference.

3.3. Assessing the Pup behavior

AIM 3- What are the effects of binge drinking on the pups?

3.3.1. Tests for anxiety:

3.3.1.1. Elevated plus maze-

Since binge behavior findings provided evidence for the dysregulation in HPA axis of the postpartum WT dams that indulged in binge drinking behavior, it was important to observe the effects of alcohol on their pups. The pups underwent behavioral analysis on P-21. Data were analyzed using two-way ANOVA with the pup sex (male vs

female) and treatment group (ethanol vs water) as factors (Figure 3.22). There was no significant interaction between these factors [F (1,32) = 0.087, p= 0.77] for the amount of time spent in the closed arms. It was found that the female pups (M= 199.13 ±16.02 secs) and male pups (M= 200.86 ±13.96 secs) of the dams belonging to the ethanol treated groups spent more time in the closed arms than the female pups (M= 167.95 ±15.04 secs) and male pups (M= 165.8 ±6.71 secs) of the dams in the water treated group. However, there is a significant main effect of the treatment [F (1,32) = 9.816, p= 0.0037]

FIGURE 3.22- PUPS BINGE BEHAVIOR (TIME IN CLOSED ARMS)

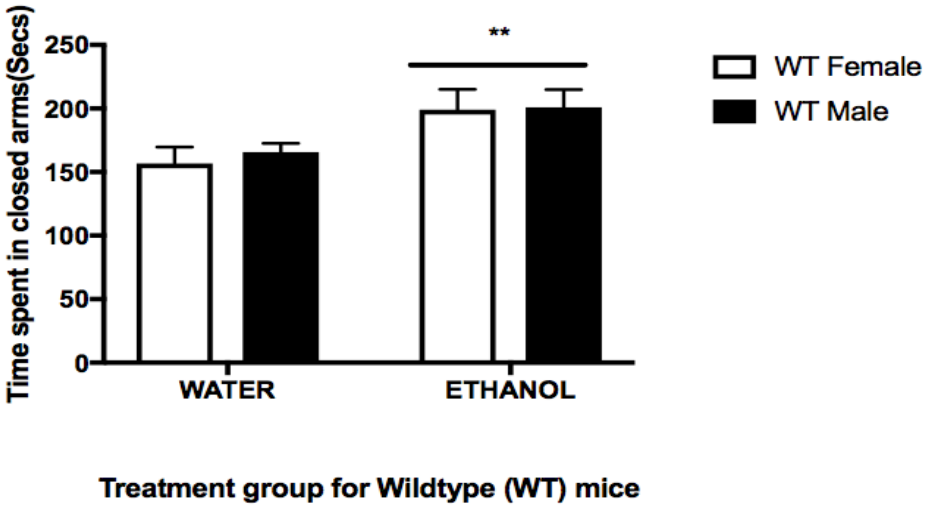

Figure 3. 22- Time spent in closed arms (secs) of the elevated plus maze of male and female pups with or without a history of binge drinking. This is a 5 minute test for anxiety-like behavior. The amount of time spent in the closed arms is a measure for anxiety response. There was no significant interaction between the factors (pup sex and treatment group). However, broadly the WT male and female pups who belonged to the dams in the ethanol treatment group spent longer in the closed arms.

3.3.1.2. Light- Dark Box:

These studies also helped assess the anxiety levels of the WT pups whose dams indulged in binge drinking behavior and displayed HPA axis dysregulation. The anxiety

levels of the pups were tested through the light-dark paradigms. Data were analyzed using two-way ANOVA with the pup sex (male vs female) and treatment group (ethanol vs water) as factors (Figure 3.23) There was no significant interaction between these factors [F (1,32) = 0.808, p= 0.375] for the amount of time spent in the dark zone. The pups of the WT dams that belonged to the ethanol treated group showed an increase in anxiety response. The female pups (M= 184.3 ±12.726 secs) and male pups (M= 186.46 ±15.08 secs) of the dams belonging to the ethanol treated groups spent more time in the dark zone than the female pups (M= 171.74 ±8.65 secs) and male pups (M= 150.18 ±14.22 secs) of the dams in the water treated group.

FIGURE 3.23- PUPS BINGE BEHAVIOR (TIME IN DARK ZONE)

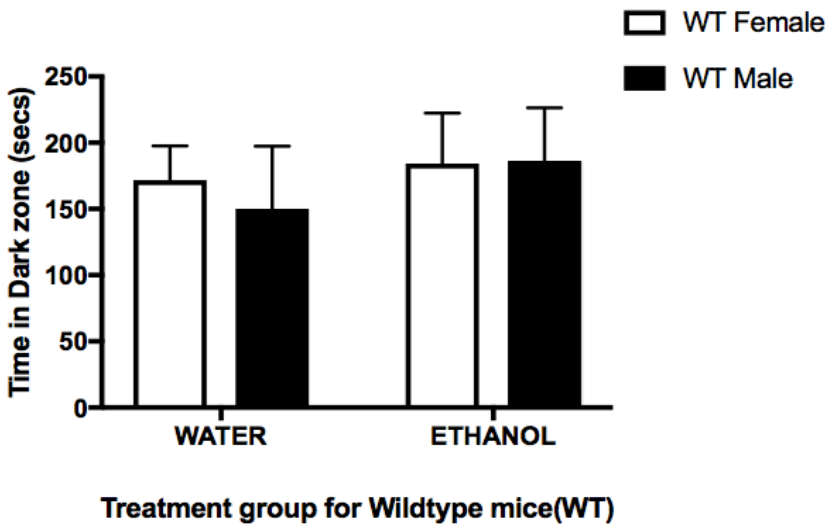

Figure 3. 23- Time spent in dark zone (secs) of the light/dark box in all the male and female pups with or without a history of binge drinking. This is a 5 minute test for anxiety-like behavior. The amount of time spent in the dark zone is a measure for anxiety response. There was no significant interaction between the factors (pup sex and treatment group). However, broadly the WT male and female pups who belonged to the dams in the ethanol treatment group spent longer in the closed arms

Chapter 4: Discussion

The present series of experiments support the role for alcohol binge drinking in the development of maternal deficits and leads to the mimicking of postpartum depression-like symptoms. The results provide an insight on the hypothesis that alcohol possibly causes neural adaptation in the brain and induces abnormal maternal behavior in the postpartum WT mice. Our results also provide evidence that this abnormal maternal behavior of the dams towards the pups is independent of the stress experienced. Moreover, we think that the "pattern" of binge drinking plays a role in this phenomenon. Our results also provide a validation for the use of models displaying HPA axis dysregulation for studies involving postpartum depression. Our findings suggest that alcohol preference varies depending on the reproductive status and that the virgins have the highest preference of alcohol compared to the postpartum mice. We know that the pathology underlying depression and the effects of alcohol leading to activation of the HPA axis overlap. Our results corroborated the hypothesis that fluctuations in CORT on concurrent administration of acute alcohol along with postpartum depression can be the underlying cause producing maternal deficits. Also in the experimental mice (WT) the presence of alcohol prevents the suppression of the HPA axis during the postpartum period. This inability to decrease the CORT levels could possibly play a role in the development of deficits in maternal care observed in the postpartum dams.

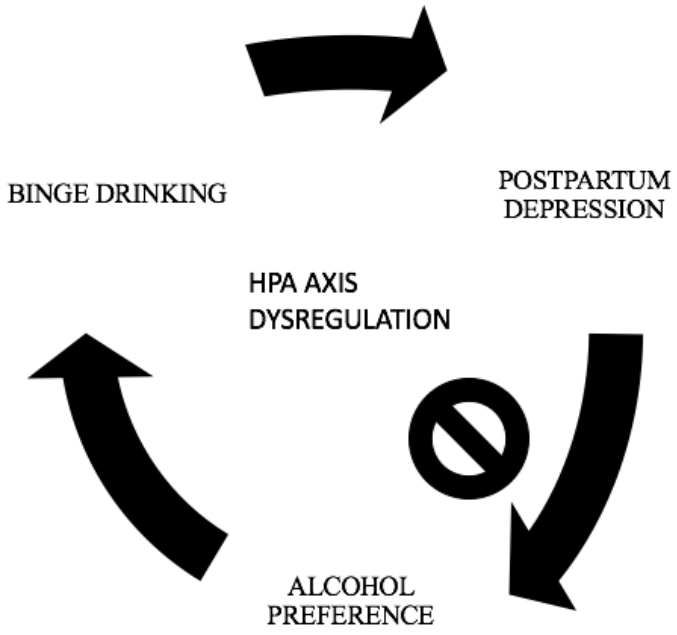

Figure 4. 1- Pictorial representation of the results derived from Aim1 and Aim2.The postpartum WT animals that engaged in binge drinking during the postpartum period exhibited abnormal maternal behavior. The postpartum dams that exhibited abnormal maternal ($GABA_A$ receptor δ subunit KO, KCC2/CRH KO) did not have an increased alcohol preference. The virgins of all the genotypes had the highest alcohol preference.

Corticotropin releasing hormone (CRH) is released from the hypothalamus and acts on the pituitary to release ACTH. It in turn triggers the release of CORT from the adrenal glands. Therefore, CRH controls the HPA axis (Herman et al., 2003) (Ulrich-Lai & Herman, 2009). This control is mediated by the GABA receptor subunits (Decavel & Van den Pol, 1990) (Herman, Mueller, & Figueiredo, 2004). Two types of GABAergic inhibitions are involved in this process: tonic and phasic (Farrant & Nusser, 2005). When the inhibition is produced due to GABA binding to the synaptic $GABA_A$ receptors is called phasic inhibition. Tonic inhibition is referred to the sustained conductance that is produced due to the ambient levels of GABA binding to the $GABA_A$ receptors in the extra synaptic space. The $GABA_A$ receptor- δsubunits particularly play an important role

in regulating the CRH neurons (Sarkar et al., 2011). These subunits are particularly involved in tonic inhibition (V. Lee et al., 2014). The body's homeostasis is maintained by the HPA axis. Stress causes disruption of homeostasis and activity of the HPA axis by signaling through the neural pathways converging on the CRH neurons in the PVN. Under normal conditions (no stress), the stress derived neurosteroid tetrahydrodecorticosterone (THDOC) governs the GABAergic inhibition of the CRH neurons and also decreases the activity of the HPA axis. In particular the δ subunit of the $GABA_A$ receptor regulates this control. However, in case of stress, this THDOC potentiates the activity of the HPA axis and release of the stress hormones like cortisol in humans and corticosterone in mice (Sarkar et al., 2011). The inhibitory actions of GABA are dependent on the maintenance of the chloride gradient through the K+/Cl- co-transporter(KCC2). KCC2 is active when the residue Ser940 in PVN is phosphorylated (Sarkar et al., 2011).

Stress causes dephosphorylation of the Ser940 residue on the KCC2 channel which ultimately leads to downregulation of KCC2 expression (O'Toole, Hooper, Wakefield, & Maguire, 2014). It is already established that KCC2 is required for the maintenance of effective HPA axis function. In virgins, stress causes activation of the HPA axis (increase in CORT levels) through dephosphorylating KCC2 Ser940 residue (Sarkar et al., 2011). During pregnancy and postpartum period stress induced activation of the HPA axis is suppressed and there is a significant decrease in circulating CORT levels (Paula J. Brunton & Russell, 2008) (P. J. Brunton & Russell, 2011) (P. J. Brunton et al., 2008) (de Weerth & Buitelaar, 2005) (Kammerer, Adams, Von Castelberg, & Glover, 2002) (SCHULTE, WEISNER, & ALLOLIO, 1990). This suppression in stress

induced activation of the HPA axis may be due to the inability of dephosphorylating the KCC2 Ser940 residue. Hence, the effects on the HPA axis are dependent on the reproductive status (Melón et al., 2017). Considering the vital role played by the HPA axis in maintaining homeostasis, the inability to regulate its activity plays a role in the development of depression in particular postpartum depression (Bloch et al., 2003) (Chrousos et al., 1998) (Melón et al., 2017). Therefore, in order to mimic postpartum depression-like behavioral model, we used mice lacking the δ subunit of the $GABA_A$ receptor and KCC2 channels on CRH neurons. Hence, these animals displayed HPA axis dysregulation.

Another important factor to be considered throughout this experiment was that the animals were unintentionally being subjected to mild environmental stress for a chronic period of time in the form of changing tubes of alcohol and water. Everyone reacts to these stressors differently. However, in some cases, certain women are more prone to psychosocial distress. Also, their fetuses are subjected to varying intrauterine environments which can affect the fetal brain development (Dipietro, 2012).

We already know that acute alcohol administration leads to activation of the HPA axis (Bryon Adinoff et al., 1998) (Hundt et al., 2001). We also know that alcohol drinking preference increases in cases where there is low dopaminergic activity in the nucleus accumbens (George F Koob et al., 1998). No one has yet studied the concurrent use of alcohol in postpartum depression.

Literature has suggested that different routes of administration can be adopted to expose the rodents to ethanol at varying doses (Costa, Savage, & Valenzuela, 2000) (Cudd, 2005). In order to achieve moderate to high blood alcohol concentrations (BAC),

methods such as intragastric intubation and liquid diets can be used. However, it is observed that it can lead to maternal stress and malnutrition, which can alter the results (Slone & Redei, 2002) (Ward & Wainwright, 1989). Even though it is difficult to obtain moderate to high BAC, voluntary oral consumption is highly effective as it does not induce stress or malnutrition (Finn et al., 2005). However, self-administration rates can be increased by limiting the ethanol exposure time in the voluntary drinking models (Finn et al., 2005) (J. S. Rhodes, Best, Belknap, Finn, & Crabbe, 2005) (J. Rhodes et al., 2007). Therefore, we adopted the binge-drinking paradigm as the pattern of alcohol accessibility to the animals. Also, recently studies have come up with a new binge drinking ethanol model called as 'drinking in the dark' (DID). This procedure takes the advantage of the nocturnal nature of the rodents so that the ethanol exposure occurs during the time of peak of arousal. Also, these studies have shown that pharmacologically relevant blood ethanol concentrations can be achieved by voluntary drinking in the dark phase avoiding any stress that can interfere with the results (Thiele, Crabbe, & Boehm, 2014). Hence, we used the binge-drinking DID paradigm in order to assess the effects of ethanol binging during the postpartum period.

No studies have previously shown the effects of alcohol consumption during the period of postpartum. Our results have shown that the animals drink on an average of 3-5 g/kg/3 hours of ethanol. This amount of ethanol is not enough to show significant effects on anxiety. However, it can be seen that ethanol has broadly nonsignificant anxiogenic effects on postpartum WT dams. These animals spend more time in the closed arms of the elevated plus maze and dark zone of the light/dark box. The baseline pup retrieval test results showed that the postpartum WT dams that belonged to the ethanol treatment

group showed deficits in maternal behavior similar to the models that displayed HPA axis dysregulation. This supported our hypothesis that binge drinking in the postpartum period may induce maternal care deficits and characteristics of postpartum depression. It is also important to note that this abnormal maternal behavior displayed by the postpartum WT dams of the ethanol treatment group was seen before they experienced the stress test. Therefore, this behavior is independent of the effects of the forced swim stress test. In order to test the effects of ethanol on depressive-like behavior, we performed the forced swim test. We observed that ethanol has no effects on the total time immobile and latency to immobility. We think this may be due to the inability of alcohol to reach pharmacologically relevant BAC during the 7-day limited access period. We tested the effects of alcohol again on maternal behavior of the postpartum WT dams. It was observed that they had an increased latency to approach the litter which is a deviation from the normal maternal behavior. These results corroborate our hypothesis that alcohol causes neural adaptation in the brain. It is again important to note that the deficits in maternal care are observed pre- and post- the forced swim test. It was interesting to see that the pup survival rate was also decreased for the WT dams that belonged to the ethanol treated group. An increased cannibalization percent was observed for the $GABA_A$ receptor δ subunit KOs and KCC2/CRH KOs irrespective of the treatment group possibly indicating the role of HPA axis dysregulation in this abnormal behavior. The evidence of this abnormal maternal behavior strengthened our hypothesis that ethanol binging during the postpartum period could possibly play a role in development of abnormal maternal behavior.

One of the drawbacks of this experimental layout was the inability to calculate the BACs throughout the 7-day period when the animals had access to ethanol. Although the average ethanol intake does not reflect the pharmacologically relevant alcohol concentrations, we had to limit to measuring the daily oral intake in order to avoid any stressful response during the behavioral test day period.

Our lab has previously demonstrated that HPA axis dysregulation could play a role in psychiatric disorders like postpartum depression. Thus, in postpartum depression this suppression of the stress induced HPA axis activation is not observed (Melón et al., 2017). The difference in this protocol compared to previous studies is that the animals (all the genotypes and treatment groups) were subjected to continuous stress exposure for 7 days (in the form of changing the bottles 3 times a day) before collecting their blood samples for CORT measurements. The CORT measurements depicted in the figures are indicative of corticosterone levels immediately post the maternal approach test (30 minutes after the depressive-like forced swim test). For the postpartum WT dams belonging to the water treatment group, it was surprising to observe that they were unable to suppress the HPA axis activation. A possible explanation to this could be that an increased duration of environmental stress exposure demands an increased time required to demonstrate a decrease in elevated CORT levels. In this study similar to other experiments the blood samples were taken 30 minutes post the forced swim test and immediately after the maternal approach test. However, there is a difference in the duration, nature and pattern of stress exposure. This would explain why these dams displayed a low interaction time with its litter after the forced swim test. The postpartum WT dams belonging to the ethanol treatment group exhibited deficits in maternal

behavior before the forced swim test (during the pup retrieval) and continued to display abnormal maternal behavior even after the forced swim test (during the maternal approach). Immediately after the maternal approach test, it was seen these animals were not able to suppress the HPA axis activation. For the postpartum $GABA_A$ receptor δ subunit KO dams belonging to the water treatment group, they had shown deficits in maternal behavior during the baseline pup retrieval test. However, 30 mins post the stress inducing forced swim test, the dams show a suppression in the CORT levels. Simulating the decrease in CORT levels, although not significant these animals show a slight increase in the total interaction time with the pups during the maternal approach test. For postpartum $GABA_A$ receptor δ subunit KO dams belonging to the ethanol treatment group, it is observed that the presence of ethanol in the postpartum $GABA_A$ receptor δ subunit KO dams did not affect its ability to suppress the HPA axis activation. For the postpartum KCC2/CRH KO dams belonging to the water treatment group, they also had shown deficits in maternal behavior during the baseline pup retrieval test. However, post the maternal approach test, these animals were unable to demonstrate a suppression in the HPA axis activation. Presence of ethanol on the other hand was able to decrease the CORT levels in the postpartum KCC2/CRH KO dams. Although they are still higher than the virgin CORT levels, they are still decreased compared to the postpartum KCC2/CRH KO water treated dams. Therefore, ethanol seems to have varying effects on the different animal models.

Acute alcohol exposure stimulates the release of CRH (Corticotropin releasing hormone) and arginine vasopressin (Ogilvie & Rivier, 1996) (Rivier, 1996) (Schuckit, Gold, & Risch, 1987). This results in an increase in pro-opiomelanocortin synthesis in the

pituitary gland. Posttranslational cleavage of this pro-opiomelanocortin precursor produces peptides like β-endorphins(β-EP) and Corticotropin. β-EP is responsible for the rewarding and reinforcing effects of alcohol (George F Koob et al., 1998) (Gianoulakis, Krishnan, & Thavundayil, 1996) (Barr et al., 2004). Also, these released corticotropins stimulate the synthesis and release of glucocorticoids from the adrenal cortex. They are proposed to potentiate the positive reinforcing effects of alcohol. Dopamine (Self & Nestler, 1995) (Weiss et al., 1993) (Samson & Hodge, 1996) (Grandy et al., 1989) and serotonin's (Barr et al., 2004) involvement in ethanol self-administration has been suggested due to the overlap with the reward pathway, but it is not fully understood. However, it is known that there is decreased dopaminergic (Meyer et al., 2001) and serotonergic (Malison et al., 1998) activity in conditions like depression. Decreased dopaminergic activity and a D2 receptor deficiency in the nucleus accumbens increases alcohol preference (McBride, Chernet, Dyr, Lumeng, & Li, 1993) (Stefanini et al., 1992) (Phillips et al., 1998) (George et al., 1995). Acute alcohol consumption is thought to stimulate dopamine release (Weiss et al., 1993) and would lead to an aversion to alcohol. Our hypothesis was to check the alcohol preference in dams suffering from postpartum depression who displayed compromised dopaminergic neurotransmission. We wanted to see if the alcohol preference stays the same, increases or switches on concurrent alcohol consumption.

We observed that the alcohol preference ratio is dependent on the reproductive status. It was observed that the virgins have a higher alcohol preference over the 7-day period compared to postpartum dams. We think that it may be due to alternalte-reinforcers and also due to the fact that the presence of pups is rewarding. Since the

postpartum WT mice exhibited deficits in maternal behavior with the limited access to alcohol paradigm, we were curious to check the effects of switched alcohol preference on the baseline maternal retrieval study. Inspite of having a lower alcohol preference, the WT dams exhibited abnormal maternal behavior similar to the models displaying HPA axis dysregulation. It is important to note that these results were observed before the forced swim test was performed (not shown in the results). Therefore, these postpartum depression-like characteristics were independent of the effects of the depressive-like test. Since acute alcohol consumption stimulates the release of dopamine in the mesolimbic regions, we hypothesized that alcohol preference would play a role in determining the effects in depressive like behavior (not shown in the results). Although nonsignificant, increased alcohol preference was broadly seen to induce depressive-like behavior through decreased latency to immobility. Post the depressive-like forced swim test we again wanted to assess the impact of the degree of HPA axis dysregulation due to alcohol preference. The animals continued to display abnormal maternal behavior corroborating our hypothesis that irrespective of the degree of alcohol preference, the animals exhibit deficits in maternal care. It is also important to note that despite subjecting the postpartum WT animals to a two-bottle choice paradigm, they continued to display abnormal maternal behavior. This could possibly indicate that even when given a choice, presence small amounts of ethanol in the system can cause neural adaptations.

It is very crucial to consider that prenatal alcohol exposure does not result in maternal deficits. However, when given alcohol during the postpartum period, it leads to abnormalities in maternal care (Brady, Allan, & Caldwell, 2012) (Boehm et al., 2008). The litter size does not change when the dams are given ethanol prior to and during their

gestation (Boehm et al., 2008) (Brady et al., 2012). However, in our study, the litter size of the dams that consumed ethanol decreased either due to neglect or cannibalization. Studies (Boehm et al., 2008; Brady et al., 2012) have shown that alcohol withdrawal or tapering the nursing dams off ethanol in the postnatal period did not impact maternal care. Hence, abnormal maternal behavior in our study cannot be due to withdrawal. We did not find/ measure the pup weight because it overlapped the period of weight loss experienced by the dam just after parturition. Thus, it would give confounding results.

Previous studies have shown that women who use alcohol during their pregnancy could develop FASD (Fetal alcohol Spectrum disorder) (K. Jones & Smith, 1973). It could lead to prenatal and post-natal growth retardation, CNS alterations, neurological abnormalities, developmental delays, facial dysmorphology, physical malformations etc (Abel & Dintcheff, 1978) (Hellemans, Sliwowska, Verma, & Weinberg, 2010). Studies also indicate that in particular depression and anxiety occur concurrently in adults suffering from FASD (Hellemans et al., 2010) (Chris Famy, Ann P. Streissguth, & Alan S. Unis, 1998). However, it is important to note that the level of alcohol exposure can determine the severity of alcohol induced effects. We hypothesized that the pups' anxiety and depression levels might alter due to the postnatal alcohol exposure.

Besides affecting the mothers as discussed earlier, ethanol can also the placental barrier and blood brain barrier of the fetus. We wanted to check what the effects of binge drinking by the dams would have on their pups. From the behavioral results of the ethanol treated binge drinking WT dams, it was seen that they displayed deficits in maternal behavior using the anxiety test paradigms such as the light/dark box and elevated plus maze to test their anxiety levels. Although the effects on the pups are not

very pronounced due to the low intake of the WT dams, it was seen that ethanol had broadly anxiogenic effects on the WT pups. It is important to note that these tests were performed on the pups atleast 10 days post the ethanol exposure on the dams. What makes these results even more interesting is that these pups are not being nursed by the dams at the time of testing. From the results, there is a significant effect of the treatment group (ethanol) seen on the male and female pups of the WT ethanol treated dams vs male and female pups of the water treated dams. These findings corroborate our hypothesis that the effects are not only seen on the dams but also the pups.

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