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The long-term effects of prenatal stress and/or antidepressant exposure in rats

By

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The long-term effects of prenatal stress and/or antidepressant exposure in rats

By

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B.S., University of Maryland, 2007

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An abstract of
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Abstract

The long-term effects of prenatal stress and/or antidepressant exposure in rats

by

Chase Hanson Bourke

Pregnancy expands a woman's health considerations beyond herself to include her unborn child. Approximately 10-20% of all pregnant women experience depression during pregnancy and pharmacological intervention may be indicated in a substantial proportion of these women. The purpose of this dissertation was to develop a model of clinically relevant prenatal exposure to an antidepressant and maternal depression during pregnancy with the ultimate goal of evaluating the long-term effects of these prenatal exposures on the offspring. Female Sprague-Dawley rats were implanted with osmotic minipumps that were found to deliver clinically relevant exposure to the antidepressant escitalopram compared to daily injections used in most of the extant literature. Subsequently, pregnant females were exposed on gestational days 10-20 to a chronic unpredictable mild stress paradigm that was verified to cause an increase in baseline corticosterone. Maternal behavior was continuously monitored over the first 10 days post parturition but no substantial difference in maternal care (nursing, licking and grooming, or no contact) was observed due to maternal exposure to stress and/or escitalopram. The adult male offspring were analyzed to determine the long-term effects of prenatal exposures. Baseline physiological measurements were largely unaltered by prenatal manipulations. Behavioral characterization of the male offspring, with or without pre-exposure to an acute restraint stressor prior to testing, did not reveal any group differences. Prenatal stress exposure resulted in a faster return of serum corticosterone towards baseline following the peak response to an acute restraint stressor, but not an airpuff startle stressor, in adulthood. Gene expression analysis of select brain regions through microarray and real time PCR revealed no significantly regulated transcripts due to prenatal exposures. This model of maternal depression and its treatment indicate that escitalopram use and/or stress during pregnancy produced no alterations in our measures of male adult behavior or the transcriptome, however prenatal stress exposure resulted in some evidence for increased glucocorticoid negative feedback following an acute restraint stress. The role of stressor and drug dosing or timing in extant studies suggests that study design should be carefully considered before implications for human health are ascribed to prenatal exposure to stress or antidepressant medication.

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5-HT - serotonin; 5HTTLPR - serotonin transporter linked polymorphic region; Ac - acetyl; ACh – acetylcholine; ACTH - adrenocorticotropic hormone; ADHD - attention deficit hyperactivity disorder; Apgar - appearance, pulse, grimace, activity, respiration; AS - antisense; AUC - area under the curve; CGI - CpG island; ChIP - chromatin immunoprecipitation; Chr - chromosome; CMS - chronic unpredictable mild stress; CNS - central nervous system; CpG - cytosine-phosphate-guanine; CRF - corticotropin-releasing factor; EN - embryonic day N; FDA - Food and Drug Administration; FDR - false discovery rate; FLX - fluoxetine; GN - gestational day N; H3K9 - histone 3 lysine 9; HOMA-IR - homeostasis model assessment-estimated insulin resistance; HPA - hypothalamic-pituitary-adrenal; HPLC-UV - high performance liquid chromatography - ultraviolet detection; IPA - Ingenuity pathway analysis; LV - left ventricle; MACS - model-based analysis for ChIP-Seq; MADAM - microarray data manager; MAO - monoamine oxidase; MDD - major depressive disorder; Me3 - trimethyl; NFLX - norfluoxetine; NRI - norepinephrine reuptake inhibitor; NS-Escit - no stress with escitalopram minipump; NS-Sal - no stress with saline minipump; PNDN - postnatal day N; Q-Q - quantile-quantile; RT-PCR - real time polymerase chain reaction; RV - right ventricle; SAM - significance analysis of microarray; SEM - standard error of the mean; SERT - serotonin transporter; S-Escit - prenatal stress with escitalopram minipump; SN - sense; SRI - serotonin reuptake inhibitor; S-Sal - prenatal stress with saline minipump; SSRI - selective serotonin reuptake inhibitor; $t_{1/2}$ - half life (biological); TCA - tricyclic antidepressant; TSS - transcription start site; UPLC-MS/MS - ultra performance liquid

chromatography - tandem mass spectrometry detection; UTR - untranslated region; Emory WMHP - Emory Women's Mental Health Program

CHAPTER 1: INTRODUCTION AND BACKGROUND

Introduction

Historical Perspective

Modern psychopharmacology began in the early 1950's with the introduction of chlorpromazine, other phenothiazines, and a little later, the tricyclic antidepressants that gave clinicians new and effective tools to treat mental health disorders. Regulatory agencies like the FDA were trying to establish standards for the use of drugs during pregnancy when the thalidomide tragedy struck.

Thalidomide was first introduced in 1956 in Germany for morning sickness during pregnancy (Rogers and Kavlock, 2007). As soon as 1961, several physicians began reporting limb deformities in the offspring, including Dr. William McBride (McBride, 1961). Congenital abnormalities usually affect 1.5% of infants, but deformities were observed in nearly 20% of thalidomide-exposed offspring. The drug was withdrawn from the market but doctors and scientists began to re-examine the effects of drugs during the prenatal period.

Tricyclic antidepressants (TCA) were first introduced in 1959 (imipramine), with an active desmethyl metabolite of imipramine, desipramine, introduced in 1964 (Council on Drugs, 1965). It was prescribed during pregnancy and some believed that in the wake of the thalidomide tragedy, the drug could be detrimental to the fetus. In animal studies, it was observed that imipramine may have caused fetal abnormalities in rabbits (Robson and Sullivan, 1963). Dr. McBride, who first reported deformities due to thalidomide, began examining mothers and their infants and reported increased limb deformities associated with this medication (McBride, 1972). The article was reported by several press agencies including *The Times of London* (March 4th, 1972), spurring a reaction

from several physicians reporting the safety of these medications during pregnancy in their patients (Crombie et al., 1972; Kuenssberg and Knox, 1972; Levy, 1972; Sim, 1972). For over 20 years, newer selective-serotonin reuptake inhibitor (SSRI) antidepressants have replaced TCAs such as imipramine and desipramine in North America and much of Europe and modern Asia for the treatment of depression. While these newer antidepressants have been prescribed during pregnancy for several decades, reports of harmful effects, many of which are case reports or of small sample size, are still publicized more frequently than studies reporting their safety.

Defining The Clinical Problem

Pregnancy expands a woman's health considerations beyond herself to include her unborn child. In the case of a woman diagnosed with major depressive disorder, which has been reported to affect 10-20% of pregnant women (Gavin et al., 2005), the effects of untreated depression on the offspring can be profound and long-lasting. Physically, children born to pregnant women diagnosed with active depression have a higher risk of low birth weight and preterm delivery (Steer et al., 1992; Orr and Miller, 1995; Halbreich, 2005; Dunkel Schetter and Tanner, 2012). Behaviorally, neonates (8-72 hours postnatal) with depressed mothers are more inconsolable and cry excessively as assessed by the Neurologic and Adaptive Capacity Scale (Zuckerman et al., 1990). Children also exhibit increased internalizing behavior such as emotional reactivity, depression, anxiety, irritability, and withdrawal (Misri et al., 2006; Tronick and Reck, 2009). Pregnant women who experienced a psychosocial stress exposure during pregnancy are reported to have an increased risk of giving birth to children later diagnosed with schizophrenia or

shortened leukocyte telomere length (Khashan et al., 2008; Entringer et al., 2011). Stress during pregnancy has also been associated with an increased risk of developing an autism spectrum disorder in the offspring (Kinney et al., 2008). The glucocorticoid cortisol is a marker of hypothalamic-pituitary-adrenal (HPA) axis activation and stress-induced salivary cortisol reactivity is elevated in infants born to mothers with a history of depression (Brennan et al., 2008; Field, 2011). Additionally, mothers with a history of an affective disorder have a 2.5-5-fold increased risk of giving birth to a child with attention deficit hyperactivity disorder (Figueroa, 2010). Human studies have shown that maternal depression and anxiety are associated with persistent neuroendocrine and behavioral abnormalities which may influence the development of future psychiatric disorders. Animal studies utilizing prenatal stress exposures to the pregnant dam show disruption of stress responsivity in the offspring when examined at postnatal day 70 (Mueller and Bale, 2008). Chronic stress during pregnancy alters expression of key regulators and mediators of the stress pathway in the offspring including decreased protein expression of the mineralocorticoid and glucocorticoid receptor in the hippocampus and prolonged increases in serum corticosterone concentrations in response to a stressor (Maccari and Morley-Fletcher, 2007). Disruptions due to prenatal stress have been shown to persist via epigenetic miRNA mechanisms to offspring and even subsequent generations (Darnaudery and Maccari, 2008; Dunn et al., 2011; Morgan and Bale, 2011). Pharmacological treatment of maternal depression may help offset these deficits.

Given the fact that these preclinical studies suggest that long term alterations are possible, minimization of stress, or active treatment of mood and anxiety disorders with antidepressants during pregnancy is not uncommon and has been increasing in recent

years. In the Netherlands, based on patient health records, 2% of all pregnant women were prescribed antidepressants (SSRIs or TCAs) during the 1st trimester of pregnancy (Ververs et al., 2006). In the United States of America, the number of women filling antidepressant prescriptions during pregnancy is as high as 8.7% (Cooper et al., 2007). The National Birth Defects Prevention Study in the United States has also observed that antidepressant use during pregnancy has increased 300% from 1998 to 2005 (Alwan et al., 2011). Given the increased use of antidepressants during pregnancy (Figure 1.1), there is a great need for rigorous studies investigating potential adverse effects on the patient and child. However, the lack of clear, long-term adverse effects that coincides in animal and clinical studies should be acknowledged before a treatment decision is made.

Although increased usage during pregnancy is occurring, discontinuation of antidepressant treatment during pregnancy is also increasing and antidepressant use decreases over each trimester (Ververs et al., 2006; Alwan et al., 2011). While many medications are used transiently, affective disorders belong to a class of complex diseases that typically need a continuous treatment (pharmacological or proven psychotherapy techniques) for a response (Kupfer et al., 2012). Pregnancy itself is a major determinant of antidepressant treatment discontinuation and confirmation of pregnancy causes a 1.8-3.5 fold increase in antidepressant discontinuation leading to a steady decline in antidepressant use over the course of pregnancy (Ramos et al., 2007; Bennett et al., 2010; Petersen et al., 2011). Discontinuation should not be attempted without consulting a physician and the physician should be aware of the baseline percentage of untoward outcomes naturally occurring in the human population rather than attribute any untoward effects to prenatal exposure (Kaye and Weinstein, 2005).

The risk of relapse is a significant factor in considering the continuation of treatment during pregnancy and should be carefully considered before any decision is made. Pregnant women with major depression are twice as likely to relapse after discontinuing antidepressant treatment (Figure 1.2) (Cohen et al., 2006). As expected, the risk of relapse is five times higher for women who discontinue antidepressant treatment than for those that continue treatment through pregnancy (Figure 1.2) (Cohen et al., 2006). These results refute the long-held theory that pregnancy protects the mother from the ill effects of psychiatric disorders (Zajicek, 1981; Kendell et al., 1987).

Proper counseling by providing balanced information is vital to making an informed decision about continuation of antidepressant treatment through pregnancy. The FDA safety classification for the majority of antidepressants is currently category C: while there are animal studies indicating an adverse effect on the fetus, there are no adequate and well-controlled human studies and the drug can be used during pregnancy only if the potential benefit justifies the potential risk to the fetus (Figure 1.3). Paroxetine is the only antidepressant medication with a rating of category D: there are adequate and well-controlled human studies indicating a risk to the fetus but the use of the drug in the pregnant woman may be acceptable despite the risk (Food and Drug Administration, 2011). Overall, the FDA recommends antidepressant use in pregnancy based on a risk-benefit decision on a case-by-case basis.

Investigators in the MotheRisk study in Toronto have commented on the absence of evidence-based information in the assessment of treatment decisions (Einarson, 2009). "Positive" studies which have an outcome or association of prenatal antidepressant exposure with some kind of malformation are quickly reported by news outlets and

disseminated. However, "negative" studies which emphasis the safety of antidepressant use during pregnancy are rarely published in science or media outlets. Many clinicians are conflicted about what to prescribe and sometimes do not base clinical decisions on evidence-based information (Einarson and Koren, 2007). Proper counseling is the best approach and each patient should be informed of the benefits and risks (Kalra et al., 2005). For instance, although GI drugs, antibiotics, and antidepressants present similar risks to the fetus (1-3%), 87% of women believe the risk to the fetus is higher than actual for antidepressants (Table 1.1) (Bonari et al., 2005). Patients routinely over-estimate the risk to the fetus when not presented with proper medical information (Einarson et al., 2005).

Routes of Fetal and Infant Exposure

The pharmacokinetics of drug exposure to the infant dictates that there are several steps between direct maternal exposure and direct fetal exposure. These steps consist of maternal metabolism leading to systemic circulation, placental transfer, umbilical cord distribution, and direct *in utero* exposure of the fetus. Pregnancy can affect a psychotropic's metabolism: drug turnover for several SSRIs have been shown to increase over pregnancy (Sit et al., 2008). Postpartum, the mother has also been shown to be in a refractory metabolic state (Sit et al., 2008). After a psychotropic drug is ingested and absorbed into the bloodstream, it first goes through first-pass metabolism by the liver followed by systemic distribution. Metabolism is mediated by drug metabolizing enzymes while plasma binding proteins affects the amount of free drug in systemic circulation. When the drug reaches system circulation, it can transfer across the placenta.

Transfer of the antidepressant across the placenta occurs at different rates, depending on the lipophilic properties of the psychotropic medication. *Ex vivo* studies of isolated human placentas show that tricyclic antidepressants and selective serotonin reuptake inhibitors pass across the placenta barrier. Steady state placental transfer of the parent compound and its metabolites can reach up to 10% (Heikkinen et al., 2001; Heikkinen et al., 2002b). However, *in vivo* studies of umbilical cord distribution have shown much higher transfer of antidepressants.

Distribution of the antidepressant into the umbilical cord varies between the different classes of antidepressants. The TCAs nortriptyline, clomipramine, and their metabolites pass readily into the umbilical cord serum in *in vivo* studies which more closely approximate placental transfer (Loughhead et al., 2006). SSRI exposure results in very high umbilical cord distribution. SSRI cord serum: maternal serum ratio is between 0.52-1.1 depending on the compound, illustrating significant placental transfer. Metabolites, which in some cases are also active at inhibiting the serotonin transporter, readily transfer across the placental barrier (Rampono et al., 2004; Kim et al., 2006; Rampono et al., 2009). Maternal: cord serum ratios have been rated as follows sertraline < paroxetine < fluoxetine < citalopram (Hendrick et al., 2003b). Depending on the compound, the fetus can be exposed to the same amount of drug in the systemic circulation as the mother.

Postpartum TCA exposure through breastfeeding results in a low exposure to the infant compared to *in utero* exposure. TCAs and metabolites pass readily into breast milk but the child does not appear to be exposed to an appreciable dose (Brixen-Rasmussen et al., 1982; Stancer and Reed, 1986; Wisner and Perel, 1991; Breyer-Pfaff et

al., 1995; Yoshida et al., 1997). In many studies of TCAs, the infant serum concentration for drug or metabolite is undetectable. Wisner (1997) performed a controlled study of mother-infant breastfeeding pairs who had not received antidepressants during pregnancy. Nortriptyline and its metabolites were measured in the serum of 6 mother-infant pairs given nortriptyline 24 hours after birth for 4 weeks. While nortriptyline and its hydroxylated metabolites were measured in maternal serum, infant serum concentrations were low or non-detectable (Wisner et al., 1997).

SSRI transfer into breast milk at a much higher rate than TCAs, but exposure to the infant is still minimal. The first step in lactational exposure is transfer from the maternal serum to the breast milk. Newport (2009) has examined the excretion of venlafaxine into breast milk. Infant: maternal plasma ratio was 6.2% for venlafaxine and 58% for the active metabolite desvenlafaxine, indicating some infant exposure through lactation albeit a small fraction compared to the maternal plasma drug concentration (Newport et al., 2009). An examination of other antidepressants has shown that the maternal serum and milk concentrations were high and the ratio of milk: serum was paroxetine (0.7) < sertraline (1.8) < citalopram (2.1) < venlafaxine (2.4). Infant drug concentrations were virtually undetectable and no relationship was associated with the drug metabolizing enzymes CYP2D6 or CYP2C19 genotypes (Berle et al., 2004). After transfer into the breast milk, the question arises if the infant is exposed to an appreciable dose. Several antidepressants are detectable in human breast milk but low or undetectable in infant serum (Altshuler et al., 1995; Epperson et al., 1997; Mammen et al., 1997; Stowe et al., 1997; Wisner et al., 1998; Heikkinen et al., 2002a; Briggs et al., 2009). Infant platelet serotonin uptake, as a functional measure of exposure, is also

unaffected in mother-infant breastfeeding pairs (Epperson et al., 2001). Weissman (2004) has conducted a large scale meta-analysis of 57 studies that examined mother-infant breastfeeding pairs to determine the amount of antidepressant exposure via lactational transfer. Overall, nortriptyline, sertraline, and paroxetine usually had no detectable concentrations in infant serum. Fluoxetine-exposed infants are at risk to have higher serum concentrations and citalopram-exposed infants are marginally at risk to have higher serum concentrations (Weissman et al., 2004). The extant literature indicates that fetal exposure *in utero* is more pronounced than exposure via breast milk.

Atypical antidepressants, which work predominately at the receptor level rather than the transporter level, distribute similarly compared to TCAs and SSRIs. Mother-infant breastfeeding pairs exposed to bupropion had no detectable concentrations of bupropion in the serum of infants (Baab et al., 2002). A patient dosed with mirtazapine had detectable concentrations in milk but no detectable concentrations in the plasma of the infant (Klier et al., 2007). A small cohort of women exposed to oral trazodone was studied to measure trazodone concentrations in breast milk. A very small milk: plasma ratio was observed, indicating that the infant would be exposed to less than 1/100th of the maternal dose (Verbeeck et al., 1986). A similar study administered a dosing regimen of mirtazapine that reached steady state concentrations. The percent of the maternal serum concentration of mirtazapine for infant exposure via breast milk was 1.5% for mirtazapine and 0.4% for desmethylmirtazapine (Kristensen et al., 2007). Although the number of studies investigating exposure to atypical antidepressants is small, there seems to be a similar level of exposure via breast milk among the different classes of antidepressants.

Role of Serotonin in Development

"Teratogenic agents act in specific ways (mechanisms) on developing cells and tissues to initiate sequences of abnormal developmental events (pathogenesis)." - James G. Wilson, Environment and Birth Defects, 1973

The serotonin transporter is a sodium-serotonin symporter that transports serotonin from the extracellular space into the cell (Talvenheimo et al., 1979; Hoffman et al., 1991; Masson et al., 1999). After vesicular release of serotonin into the synaptic cleft, serotonin binds to postsynaptic serotonin receptors to mediate serotonin innervation (Pineyro and Blier, 1999). The most widespread pharmacological treatment of depression currently rely on selective-serotonin reuptake inhibitors that act by binding to the serotonin transporter and preventing uptake of serotonin into the presynaptic neuron (Stafford et al., 2001). Increased serotonin in the synaptic cleft represents the most probable mechanism of action to induce developmental effects on the fetus.

Serotonin has diverse ontogeneological functions *in utero* to guide development. These functions should be considered in the context of laboratory and clinical evidence as a mechanistic approach to investigate the effects of *in utero* exposure to antidepressants. Serotonin increases neurite outgrowth *ex vivo* in mouse embryo thalamic neurons (Lotto et al., 1999). It is likely that the mechanism of this neurite extension is via serotonin-mediated stimulation of S100β release from astrocytes (Whitaker-Azmitia, 2001). Serotonin also plays an important role in axonal guidance: disruption of serotonin in the

forebrain can lead to abnormal thalamocortical axon trajectories (Bonnin et al., 2007; Bonnin et al., 2011). Additionally, a switch from a placental source of serotonin to an endogenous fetal source of serotonin occurs in the second trimester of mice (Bonnin et al., 2011). Therefore, disruption of serotonin signaling during these critical times of development may form the basis of underlying long-term effects on the fetus.

Another possible mechanism of antidepressant-mediated effects *in utero* is through a direct effect on the uterus. The 5-HT_{2B} receptor has been examined in human uterine smooth muscle cells and agonists induce phosphoinositide hydrolysis (Kelly and Sharif, 2006). Serotonin causes relaxation in the porcine oviduct (Inoue et al., 2003) and inhibits myometrial contractility (Kitazawa et al., 1998). These effects are antagonized by mianserin, a 5-HT receptor antagonist. Another group isolated uterine rings of Sprague Dawley rats on G14 and G21 and used these rings for isometric tension recording. No effect was observed for fluoxetine, imipramine, nortriptyline, or 5-HT on contractile activity. Therefore, if there is an effect of these drugs on early gestation, it may not be due to a direct effect on the uterus (Vedernikov et al., 2000). *Ex vivo* experimentation on human placentas showed that a high concentration of doxepin decreases non-neuronal acetylcholine (ACh) release. This may be a possible mechanism of some clinical findings reporting low birth weight due to prenatal antidepressant exposure since ACh may control vascularization and alter energy availability to the fetus (Wessler et al., 2007).

Serotonin also plays a role in the cardiovascular system. Serotonin receptors 5-$HT_{1A,\ 3,}$ and $_{7}$ have been shown to play a physiological role in the parasympathetic regulation of the cardiovascular system (Ramage and Villalón, 2008). Therefore, it has

been posited by some that interference of this system *in utero* may ultimately disrupt normal development of the cardiovascular system. Sloot (2009) has treated rat whole embryos in culture with 12 monoamine reuptake inhibitors. The group found cardiac defects and major malformations when treating these embryos. However, the lowest concentration of drug used was 0.3 μg/mL which is approximately 5-6 times human exposure (Sloot et al., 2009). This article was disputed as not indicative of teratogenicity (Brent, 2010; Scialli and Iannucci, 2010).

Serotonin has diverse roles in development. However, the extant literature has not firmly established a mechanism by which antidepressants *in utero* would disrupt normal serotonin-guided development. James Wilson has identified six principles of teratology and if a xenobiotic is thought to be teratogenic, it should attempt to satisfy these criteria. One of these principles posits that a xenobiotic must act by a defined cellular mechanism to initiate abnormal developmental effects (Wilson, 1973). Current and future studies investigating *in utero* exposure of antidepressants must consider that if there is a teratogenic mechanism, it has not been thoroughly investigated or replicated.

Human Exposure Studies

Monoamine and Hormone Studies

Human studies examining the effects to the fetus or offspring of prenatal antidepressant exposure are the most direct way to determine a possible teratogenic mechanism that can subsequently be tested in controlled laboratory conditions. Examination of 5-HT concentrations in infants reveals some short-term effects of *in utero* antidepressant exposure. SSRI exposure *in utero* yields a cord blood 5-HT concentration

of 25% compared to controls. Whole blood 5-HT in newborns also correlated with mothers. This was a short-term effect and after 1 month, 5-HT concentrations normalized to adult concentrations (Anderson et al., 2004). This effect appears to be isolated to the prenatal period of exposure, as SSRI exposure postpartum in mother-infant breastfeeding pairs resulted in no changes in concentrations of 5-HT in newborns (Epperson et al., 2003). S100β protein concentrations, a regulator of neurite outgrowth modulated by 5-HT_{1A} receptor activation, are decreased in the cord serum following prenatal exposure to SSRIs (Pawluski et al., 2009). While there may be short-term effects to 5-HT whole blood concentrations in the offspring, 5-HT normalizes early in infant development and this transient effect is unlikely to disrupt long-term health.

Studies investigating the effects of prenatal antidepressant exposure on hormone systems are currently limited. Davidson and colleagues in Israel investigated 21 infants exposed to SSRIs throughout pregnancy compared to 20 unexposed controls. SSRI exposure *in utero* decreased cord blood cortisol concentrations while thyroid stimulating hormone was increased compared to unexposed controls. Placental insulin-like growth factor-I receptor expression, which participates in fetal growth and has significant cross-talk with the HPA axis, was higher in SSRI-exposed groups. Finnegan score as a measure of gross CNS and respiratory function correlated with placental insulin-like growth factor-I receptor expression and cord cortisol concentrations. Finnegan scores also correlated with dehydroepiandrosterone and its metabolite dehydroepiandrosterone sulfate, which play an important role in regulating neurotransmitter systems (Pérez-Neri et al., 2008). These correlations were only observed in SSRI-exposed groups and not control groups (Davidson et al., 2009). Another study examined the cortisol response of

second and third trimester SSRI-exposed infants. While mothers had higher anxiety and depressive-like symptoms, infants at 3 months of age had lower evening basal salivary cortisol in SSRI exposed (~2.2 ng/mL) compared to controls (~0.9 ng/mL) (Oberlander et al., 2008b). These studies indicate a role of prenatal SSRI exposure in the regulation of fetal/infant adrenal output and growth-promoting systems.

Genome Association Studies

The advent of shotgun sequencing demonstrated by Venter and colleagues (Venter et al., 2001) caused a rapid rise in genetic studies investigating the susceptibility to disease. The genome era has significantly impacted the field of psychiatry and a large number of genome-wide association studies have dominated the field. However, only a few studies have investigated the prenatal environment in the context of genetics. One study investigated 20 infants (age 2-6 years old) exposed to citalopram or fluoxetine during pregnancy or lactation in patients diagnosed with MDD or panic disorder. This study found significant associations with genes coding for the degradation of monoamines such as monoamine oxidase (*Maoa*) and catechol-*O*-methyltransferase (*Comt*). High activity *Maoa* alleles correlated with high cord blood norepinephrine metabolite dihydroxyphenylglycol and serotonergic symptoms in newborns (see (Hegerl et al., 1998) for description of these symptoms). The high activity *Comt* alleles correlated with high prolactin. Serotonin receptor or transporter gene polymorphism genotypes did not correlate with cord monoamine concentrations or serotonergic symptoms (Hilli et al., 2009). Although this study was part of a larger study with a control group (Laine et al., 2003), no control group was used so it is unclear whether *in*

utero SSRI exposure perturbed these outcomes or the gene association would have occurred despite *in utero* SSRI exposure.

Oberlander and colleagues in Canada have performed extensive work associating maternal conditions, genetics, and endpoints in the child. These studies are in the minority that control for maternal mood or diagnosis of an affective disorder in relation to prenatal antidepressant exposure. Oberlander's group has investigated the behavior of the infant as it relates to prenatal antidepressant exposure, but also genotypes the offspring to examine the serotonin transporter 5' promoter region. The serotonin-transporter-linked polymorphic region (5HTTLPR) in the 5' promoter region modulates expression of the serotonin transporter. A 43 base pair insertion/deletion polymorphism in this region that has been previously reported to affect serotonin transporter expression and treatment response (Heils et al., 1996; Kim et al., 2000; Pollock et al., 2000). The short variant (*ss*) in this region has been reported to reduce expression of the serotonin transporter *in vitro* and the short variant or heterozygous genotypes (*ss, ls*) correlate with anxiety-related traits (Lesch et al., 1996). However, association of the 5HTTLPR genotype and anxiety-like behavior has not been replicated in subsequent studies . Oberlander's group examined infants prenatally exposed to antidepressant and correlated infant behavior with the 5HTTLPR variants. In a model controlling for maternal depressed mood, higher internalizing behaviors in the infant were only associated with maternal mood and not SSRI exposure. Externalizing behaviors in the infant were associated with anxious or depressed mothers and interacted with anxiety and the *ll* 5HTTLPR variant. Child anxiety and depression increased due to maternal anxiety and the *ss* 5HTTLPR variant. Internalizing behaviors were classified as anxious/depressed,

emotionally reactive, somatic complaints, sleep problems, and withdrawn. Externalizing behaviors were classified as attention and aggression (Oberlander et al., 2010). Further studies replicating these findings and expanding on the interaction of the 5HTTLPR and behavioral outcomes will be paramount to understanding the etiology of infant behavior and the relationship with the serotonin system.

An additional study by Oberlander's group examined respiratory symptoms as they relate to maternal mood, prenatal antidepressant exposure, and the 5HTTLPR variants. This study examined women using the SRIs paroxetine, fluoxetine, sertraline, venlafaxine, and citalopram for an average of 220 days during pregnancy. When controlling for maternal mood, Apgar scores (Appearance Pulse Grimace Activity Respiration (Apgar, 1953)) were reduced in 5HTTLPR genotype *ss*. Exposed infants with heterozygous *ls* genotypes had lower birth weight. Disrupted respiration was observed in exposed *ll* genotypes while *ss* neonates had an increased risk of neuromotor symptoms when exposed to SRIs *in utero* (Oberlander et al., 2008a). Oberlander postulates that the combination of prenatal SRI exposure and presumably low expression of the serotonin transporter leads to an overabundance of serotonin in the presynaptic cleft as well as hypersensitivity of postsynaptic serotonin receptors and thus produces neuromotor irritability. Future studies examining this system more comprehensively may aid this interpretation and understand the influence of serotonin on the developing fetus.

Behavioral Studies in Neonates and Children

The concept of behavioral teratogenicity denotes that prenatal exposure to xenobiotics may manifest subtle changes in infant behavior that may point to more

extensive neurotoxic effects or novel systems to explore. Although several animal studies have explored behavioral teratogenicity in the context of prenatal antidepressant exposure, this is an area that should be explored more fully in human studies of prenatal psychotropic exposure. Heal lances are typically performed in neonates to diagnose phenylketoneuria and thus an excellent way to test the behavioral response to pain. Oberlander's group has assessed prenatal exposure to fluoxetine, paroxetine, or sertraline (n = 22) and SSRI + clonazepam (n = 16) compared to 23 unexposed controls and reported a decreased facial action to the pain of a heal lance in the recovery period. Facial action was assessed by the Neonatal Facial Coding System (Grunau and Craig, 1987; Grunau et al., 1990). Mean heart rate after heal lance displayed a blunted increase in the SRI-exposed offspring (Oberlander et al., 2002). The same group replicated this study, again finding a decreased facial action to the pain of a heal lance in the recovery period but also following up on their heart rate findings. Prenatal and postnatal (breast milk) exposure to these medications showed greater parasympathetic control of heart rate variability in the recovery period after heal lance (Oberlander et al., 2005). These findings point to a role of prenatal antidepressant exposure on the pain response in neonates.

Diagnosing and assessing a patient's mental state is primarily based on their behavioral state. Only a few studies have examined the long-term mental development in relation to prenatal SRI exposure. Misri's 2006 study evaluating both prenatal exposure to fluoxetine, paroxetine or sertraline and maternal mood showed no changes in internalizing behavior compared to controls for prenatal SRI exposure. However, maternal anxiety and depressive symptoms did correlate with increased internalizing

behavior in their offspring (Misri et al., 2006). A separate study evaluating fluoxetine or TCA-exposed infants (ages 1-7 years old at follow up, n = 135 exposed infants) showed no differences in perinatal complications, gestation age, cognition, IQ, or behavior compared to unexposed controls (Nulman et al., 1997). A 4 year follow up study of infants exposed prenatally to sertraline, paroxetine, or fluoxetine (N = 22) was conducted to examine internalizing and externalizing behaviors. Umbilical cord serum concentrations of antidepressants correlated with increased externalizing behaviors but this effect was lost when controlling for maternal mood. Increased aggressive behavior was correlated with maternal stress levels and poor neonatal adaptation. Controls were not exposed to prenatal antidepressants and the maternal mood was neither depressed nor anxious (Oberlander et al., 2007). These studies illustrate an important role of maternal mood in discriminating the effects of prenatal antidepressant exposure from prenatal stress/maternal affective disorders.

Increased awareness of childhood neurodevelopmental disorders in recent years has prompted further investigation in relation to prenatal antidepressant exposure. Using the MarketScan database, exposure to bupropion during second trimester has been shown to be correlated with increased risk of ADHD in children but SRI exposure as a whole group was not correlated. Psychiatric disorders in mother were also positively correlated with ADHD risk in the child (Figueroa, 2010). A population-based case control study found an increased risk of developing childhood autism spectrum disorder following prenatal antidepressant exposure (Croen et al., 2011). Further studies of these behavioral endpoints will be valuable as more attention is given to this disorder.

Rating scales of infant development have also been used to assess the behavioral effects of prenatal SRI exposure. Children examined at 12-40 months of age for Bayley Scales of Infant Development showed that length of exposure, by trimester of SRI used, increased the risk for lower Psychomotor Developmental Index and Behavioral Rating Scale scores (Casper et al., 2003). The group also found that prenatal exposure to SRIs (sertraline, fluoxetine, paroxetine, or fluvoxamine) caused lower psychomotor development at age 6-40 months. This effect was still significant when controlling for maternal mood and comparing antidepressant-exposed children to children born to women with MDD (Casper et al., 2003). Another study using the Brazelton Neonatal Behavioral Assessment Scale (BNBAS scoring described in (Als et al., 1977)) showed that exposed neonates had lower habituation, social-interactive, motor, and autonomic behavior although maternal mood was not controlled for (Rampono et al., 2009). Controlling for maternal mood in these studies point to an important role in the relationship between the diagnosis of the pregnant woman and effects on neonatal development.

Sleep has also been considered to be modulated by prenatal SRI exposure since a known side effect of SSRI use is disruption of sleep (Sharpley and Cowen, 1995; Trivedi et al., 1999). A study using a median and high dose of SRIs showed increased fetal motor activity in first and second trimesters compared to control or unmedicated, depressed mothers. Additionally, SRI exposure increased fetal movements in the 3rd trimester, purported to be indicative of disrupted non-REM sleep. Co-varying for mental state resulted in an association with the observed results and may potentially confound the association with SRI exposure (Mulder et al., 2011). Examination of neonates

prenatally exposed to SRIs showed disrupted REM sleep, slightly disrupted autonomic homeostasis, and increased motor activity in 1-2 day old neonates (Zeskind and Stephens, 2004). Some animal studies, especially in sheep, have observed a similar disruption in sleep patterns (*vide infra*).

Case Reports

Case reports are useful for elucidating symptoms to further examine in population case-control or prospective studies. However, a percentage of all infants are statistically likely to be affected by certain neonatal outcomes. Therefore, case reports may indicate an coincidental effect with prenatal exposure to antidepressants but are useful to consider new endpoints to study. Some case reports have indicated no negative effects of prenatal antidepressant exposure on the infant (Kesim et al., 2002; Gentile, 2006; Gentile and Vozzi, 2007; Werremeyer, 2009) and one patient was on 15 different psychotropic medications during pregnancy with no ill effects on the infant (Yaris et al., 2004b). Several reports have reported "jitteriness" in prenatally-exposed infants (Spencer, 1993; Mohan and Moore, 2000; Anbu and Theodore, 2006; Alehan et al., 2008; Eyal and Yaeger, 2008; Kwon and Lefkowitz, 2008a; Kwon and Lefkowitz, 2008b). Others have shown that exposed infants have withdrawal symptoms consisting of "irritability" (Boringa et al., 1992; Nordeng et al., 2001). Respiration problems have also been seen in cases (Eggermont et al., 1972; Eggermont, 1973; Mohan and Moore, 2000; Kwon and Lefkowitz, 2008a; Kwon and Lefkowitz, 2008b). These case reports represent several endpoints that have been examined in controlled or population-based studies.

Sporadic cases have documented prenatal antidepressant exposure and necrotizing enterocolitis (Potts et al., 2007), tachyarrhythmia (Prentice and Brown, 1989), hypothermia (Sokolover et al., 2008), long QTC interval (Dubnov et al., 2005) and a craniofacial defect (Farra et al., 2010). While all of these case reports may elucidate important outcomes to be aware of from a clinician's perspective, almost all of these symptoms were shown to normalize at follow-up. Many controlled studies have followed up on these symptoms to determine if there is any definitive association of prenatal antidepressant exposure and postnatal outcomes.

Growth, Developmental, Gross Anatomical and Physiological Outcomes

Birth weight and gestational age have been examined in relation to prenatal SRI exposure since low birth weight has been associated with impaired development (Vohr et al., 2000). Several studies have shown an association with prenatal SRI use (sometimes dependent on trimester of SRI use) and low birth weight, shortened gestational length, or small for gestational age (Ericson et al., 1999; Costei et al., 2002; Källén, 2004; Zeskind and Stephens, 2004; Malm et al., 2005; Oberlander et al., 2006; Lennestål and Källén, 2007; Diav-Citrin et al., 2008; Lund et al., 2009; Toh et al., 2009; Reis and Källén, 2010). One of these studies found a higher risk of shortened gestational length due to prenatal TCA exposure compared to prenatal SRI exposure (Källén, 2004). However, a conflicting study found that shortened gestational length was associated with SRIs but not TCAs, (Simon et al., 2002). Several other studies have shown no association of prenatal SRI use and birth outcomes (Nulman et al., 1997; Kulin et al., 1998b; Cohen et al., 2000; Einarson et al., 2001; Suri et al., 2004; Lund et al., 2009). Only a few of these studies

control for mental health of the mother in their models, with differing results (Suri et al., 2004; Lund et al., 2009). A population-based study of 860,215 women in the Swedish medical birth registry found an increased risk for preterm birth correlating with antidepressant prescription redemption. Preterm birth risk was increased for SNRIs versus SSRIs and late versus early exposure (Lennestål and Källén, 2007). A meta-analysis for gestational length is essential to determine the effect of prenatal antidepressant exposure on gestational length before any conclusions can be made.

Sporadic studies have shown other symptoms of prenatal antidepressant exposure but have not been replicated. These include an association with jaundice, jitteriness (Diav-Citrin et al., 2008), and infantile hypertrophic pyloric stenosis (Bakker et al., 2010). Infant jaundice has also been reported in certain cases of prenatal SRI exposure (Costei et al., 2002) although another study found the opposite effect (Källén, 2004). As birth registry information becomes more available, studies using this endpoint may become more powered and replicated.

Several studies investigating prenatal exposure to SRIs have used Apgar scores since the Apgar test is typically applied to every neonate and is still widely used today (Casey et al., 2001). Studies have shown decreased Apgar scores due to prenatal SRI exposure (Casper et al., 2003) or prenatal SRI and NRI exposure (Lennestål and Källén, 2007; Reis and Källén, 2010) and some studies have found this association even when controlling for maternal psychiatric history (Lund et al., 2009). Prenatal TCA exposure has been shown to increase risk of low Apgar scores compared to prenatal SRI exposure (Källén, 2004). Only one of these studies showed decreased Apgar scores due to prenatal SRI exposure when controlling for maternal mood (Casper et al., 2003) but another study

did not show an association when controlling for maternal mood (Suri et al., 2004). Again, due to conflicting studies, a meta-analysis examining this endpoint will aid in definitive interpretations of the extant literature.

The neonatal intensive care unit in hospitals admits infants that are typically premature or congenitally ill. Admission into the neonatal intensive care unit is therefore an easily identifiable endpoint that has been examined in the context of prenatal antidepressant exposure. One study by Casper and colleagues found that longer exposure to prenatal SSRIs correlated with increased neonatal intensive care unit admission (Casper et al., 2003). Timing of exposure seems to be an important variable as third trimester exposure increased the risk of neonatal intensive care unit admission compared to first trimester exposure (Malm et al., 2005). This association of SSRIs and neonatal intensive care unit admission is still correlated even when controlling for maternal psychiatric condition (Sivojelezova et al., 2005). A conflicting study found that when controlling for maternal mood, prenatal SRI exposure did not increase the risk for neonatal intensive care unit admission (Suri et al., 2004). This line of evidence indicates a neonatal adaptation syndrome that may be caused by respiratory distress or congenital malformations.

Associations with Respiratory Distress and Pulmonary Hypertension

Serotonin plays an important modulating role in respiratory control and much of the extant literature seems to indicate that the serotonin system produces an inhibitory effect on respiratory control (Mueller et al., 1982). Case reports have called attention to respiratory problems in infants prenatally exposed to TCAs (Ostergaard and Pedersen,

1982; Schimmell et al., 1991; Bloem et al., 1999; Frey et al., 1999). Oberlander's group used birth registry data from 120,000 patients and found an association of respiratory distress in newborns with prenatal SRI exposure even when controlling for severity of mental illness (Oberlander et al., 2006). Others have found that an association with respiratory distress is dependent on the antidepressant type: risk of respiratory distress was higher for prenatal TCA exposure compared to prenatal SRI exposure in two studies (Källén, 2004; Lennestål and Källén, 2007), but prenatal exposure to SRIs still has an increased risk for respiratory distress (Costei et al., 2002; Diav-Citrin et al., 2008). While another study has confirmed that SRI-exposed infants had mild respiratory distress, outcomes were normal at 2 and 8 months of age (Oberlander et al., 2004). Prenatal exposure to antidepressants appears to cause a poor neonatal adaptation syndrome that includes respiratory distress. However, this syndrome appears to be transient and resolves during the newborn period.

Serotonin plays a significant role in vasoconstriction and has been hypothesized to play a role in pulmonary arterial hypertension (Maclean and Dempsie, 2010). The role of the serotonin system in hypertension pathology has led to the focus of some studies on this endpoint in infants prenatally exposed to antidepressants. The Chambers study has been frequently cited to illustrate the danger of prenatal antidepressant exposure. In this study, authors found an increased risk for persistent pulmonary hypertension only during late pregnancy prenatal SSRI exposure (Chambers et al., 2006). A second study found a similar association of persistent pulmonary hypertension with SRIs and NRIs (Reis and Källén, 2010). While this study may elucidate an important area to examine in the future, mental state was not controlled for and may alter this association.

Associations with Congenital Malformations

The most established endpoint of teratology are congenital malformations that may be indicative of a disrupted fetal environment. The extant literature indicates that congenital malformations are not associated with prenatal exposure to TCAs or SRIs (McElhatton et al., 1996; Ericson et al., 1999; Djulus et al., 2006; Lennestål and Källén, 2007), SRIs alone (Pastuszak et al., 1993; Goldstein et al., 1997; Kulin et al., 1998b; Simon et al., 2002; Hendrick et al., 2003a; Yaris et al., 2004a; Malm et al., 2005; Sivojelezova et al., 2005; Källén and Otterblad Olausson, 2007; Pedersen et al., 2009), or atypical antidepressants (Einarson et al., 2003; Yaris et al., 2004a; Chun-Fai-Chan et al., 2005). A meta-analysis of first trimester exposure to several classes of antidepressant confirmed this negative association of prenatal antidepressant exposure and congenital malformations (Einarson and Einarson, 2005). Although studies are continually investigating this endpoint, studies to this point do not point to a strong association of prenatal antidepressant exposure and congenital malformations.

GlaxoSmithKline conducted their own study of first trimester paroxetine exposure compared to other antidepressants. An increased risk (adjusted odds ration = 1.89) of congenital malformations was associated with paroxetine exposure (Cole et al., 2007). The observational study was conducted from patient medical records of 791 mothers and any confounding variables such as mental state were not controlled for. Venlafaxine, a dual SRI/NRI antidepressant, was compared in the MotheRisk study to determine any associated risk compared to other SRIs or non-teratogenic drugs. No increased risk of malformations or any other health outcomes was found (Einarson et al., 2001). The Toronto MotheRisk Study also found no association with congenital malformations and

overall prenatal antidepressant exposure (Einarson et al., 2009). A meta-analysis was conducted of first trimester use of fluoxetine. Using the Chambers study and 3 others, authors found no association of malformations with fluoxetine use. Most importantly, based on power calculations, 26 controlled studies are needed to reverse this finding of no association between antidepressants and congenital malformations (Addis and Koren, 2000).

Cardiovascular defects, a subset of congenital malformations, have also been investigated in the context of prenatal antidepressant exposure due to serotonin regulation of cardiovascular function. Birth registry studies have shown an increased risk of cardiovascular defects in infants prenatally exposed to TCAs. This effect was predominately attributed to prenatal exposure to clomipramine (Källén and Otterblad Olausson, 2003). A follow-up study showed that prenatal exposure to paroxetine and clomipramine, but not other SRIs or TCAs, were associated with an increase risk of heart defect such as ventricular/atrial septum defects using birth registries without controlling for mental state (Källén and Otterblad Olausson, 2006; Källén and Otterblad Olausson, 2007). A follow-up study found that this observed effect was primarily due to clomipramine exposure (Reis and Källén, 2010). GlaxoSmithKline's own study of first trimester paroxetine exposure compared to other antidepressants showed an increased risk of cardiovascular malformations associated with paroxetine exposure (Cole et al., 2007; Louik et al., 2007) but another study found the opposite effect: first trimester paroxetine exposure resulted in fewer cardiovascular malformations than unexposed infants (Einarson et al., 2008). Prenatal exposure to the atypical antidepressant bupropion showed an increased risk for cardiovascular defect (Alwan et al., 2010). A

study of Danish birth registries found an association of SSRI prescription redemption and septal heart defects (Pedersen et al., 2009). A meta-analysis of seven studies in the US and Europe found an overall increased risk for cardiovascular malformations (Bar-Oz et al., 2007). More functional studies using echocardiograms have shown a 2 fold increased risk for mild nonsyndromic heart defects due to prenatal exposure to SRIs (Merlob et al., 2009).

There are several mitigating factors that should be considered in the context of the evidence of potential associations of congenital malformations and prenatal antidepressant exposure. Although a Finnish study of congenital malformations found craniofacial malformations due to imipramine/chloropyramine combination, it is interesting to note that the incidence was not above the national average of 1.25% (Idänpään-Heikkilä and Saxén, 1973). A study of the Danish population examining prescription redemption showed an increased risk of congenital malformations and SRI prescription redemption. However, there was a significant number of congenital malformations in women who discontinued SSRI use before pregnancy (4.5% compared to 4.9% in early and 6.8% in mid/late) indicating mental state may be involved (Wogelius et al., 2006).

Summary of Human Exposure Studies

Human studies over the last 20 years have slowly moved to more rigorous study designs to elucidate the safety of antidepressants during pregnancy. Investigational evidence comprised of case reports and cross-sectional studies has slowly been substantiated with case-control studies. The last decade has seen a further improvement

in clinical evidence with rigorous cohort studies designed to more finely control bias and confounding. While these studies have certainly improved our understanding of prenatal antidepressant exposure, further studies to account for several observed confounds are warranted. Problems with current human studies stem from issues with a proper control group of non-exposed depressed pregnant mothers (Tuccori et al., 2010). Since most of these studies are case-control or cohort studies, there is a large amount of confounding factors that may influence the endpoint such as cigarette smoking during pregnancy and malnutrition. Additionally, mental health state is rarely controlled for and may in fact be driving the clinically-observed condition. Rigorous cohort studies that take into account mental health state will be vital to our understanding of this complex mental health problem.

The essential choice in this mental health issue is risk versus benefit. Numerous reviews cite the safety of antidepressants during pregnancy (Koren et al., 1998; Kulin et al., 1998a; Davanzo et al., 2010). Informed consent is advised in all circumstances and antidepressant use should be considered on a case by case basis (Gupta et al., 1998). Others point out that the risk of administering these medications during pregnancy is still uncertain (Tuccori et al., 2009; Ellfolk and Malm, 2010). The issue sparks intense debate and some point out that the risk is too great and psychotherapy should supplant psychotropics (Campagne, 2007) although this is contested by others who conduct large center studies investigating these issues (Campagne, 2008; Einarson and Eberhard-Gran, 2008; Einarson, 2009). Without rigorous animal data to support a mechanism of action, an established endpoint of toxicity, or a dose-response relationship, most of the clinical literature is inconsistent with regard to risk of prenatal antidepressant exposure. The

benefits of continued treatment through pregnancy should also be considered. The risk of relapse is much lower for pregnant women who continue using antidepressant through pregnancy (Cohen et al., 2006). The negative effects of depression during pregnancy have already been discussed. Additionally, the risk of a suicide attempt that would ultimately harm the fetus should be weighed in consideration of treatment. Each clinician and patient should consider this risk-benefit calculation since each case of depression is unique, from mild to severe.

Animal Exposure Studies

Translation of Development Between Animals and Humans

Translating development across mammalian species is problematic due to a human fetus's rapid neural development in gestation compared to other animals (Gottlieb et al., 1977; Clancy et al., 2001). For comparison of rats and humans, different morphological characteristics could be compared. We acknowledge that there is no numeric relationship which could easily compare rat development and human development (30 human years $\neq$ 1 rat year) and to do so would oversimplify the field of embryology. However, brain growth velocity peaks at birth for humans and on PND8 for rats with spurts of growth postnatally. Additionally, markers of neural development in the hippocampus suggest that the last trimester of gestation in humans is roughly equivalent to the first 5 days of postnatal life in rats (Avishai-Eliner et al., 2002). Since no ontogeneological data is available on the development of the prenatal serotonin system in humans, for the purpose of this review I consider the first postnatal week in rodents to most likely correlate to the end of human gestation. Reviewed in (Watson et al., 2006).

However, the *in utero* environment is not present during the first postnatal week of a rodent's life and therefore exposure studies are further complicated. Exposure studies during this early period would result in a bolus effect. Therefore, this review will only focus on *in utero* animal exposure as a translatable approach to present findings from animal studies using these compounds.

Tricyclic/Tetracyclic Antidepressant Animal Studies

1. Exposure studies

The majority of TCA prenatal exposure studies in animals rely on a single, daily administration of the compound or dissolving the compound in drinking water. To extrapolate animal data and apply it to the human population, exposure studies must rely on a clinically-relevant dosing model that approximates human exposure. Pharmacokinetic studies dictate that there is a bolus effect after a single injection that may lead to acute toxicity. However, in many cases, the metabolite of an antidepressant has similar inhibition of monoamine reuptake at discrete transporters. Therefore, an acute dosing model may result in a high bolus effect of the parent drug but in some antidepressants, the persistence of the active metabolite may induce a more persistent and clinically relevant exposure.

Animal exposure studies investigating non-SSRI drugs have largely focused on imipramine, which is metabolized into desipramine. Desipramine and imipramine are norepinephrine-reuptake inhibitors, inhibiting uptake of norepinephrine and epinephrine at the norepinephrine transporter (although imipramine inhibits the serotonin transporter as well). Devane (1985) used a bolus injection of imipramine in pregnant rats to show

imipramine and desipramine at higher concentrations in the fetal brain than the maternal serum, indicating these medications pass readily through the placenta and expose the offspring to a significant dose (DeVane and Simpkins, 1985). When administered to a pregnant rat, a high dose (30 mg/kg) of imipramine on G18-19 resulted in detectable concentrations in the dam plasma, as well as persistent concentration of its active metabolite desipramine (DeVane et al., 1984). A 10 mg/kg dose of imipramine or desipramine administered late pregnancy resulted in significant placental transfer with significant concentration in the fetal serum (Douglas and Hume, 1967; Hume and Douglas, 1968). These studies indicate significant placental transfer in rodents, similar to clinical data.

2. In utero *NRI exposure and effects on monoaminergic function*

Initial radioligand binding and catecholamine detection studies of prenatal antidepressant exposure investigated catecholamine function in the context of receptor binding, affinity, and catecholamine concentrations. Several studies have shown reduced [^{3}H]-imipramine, β-adrenergic and dopamine receptor binding after prenatal NRI exposure (Jason et al., 1981; De Ceballos et al., 1985b; Ali et al., 1986; Montero et al., 1990) but other studies found no changes in D_1 or D_2 receptor binding (Henderson et al., 1991; Stewart et al., 1998), adrenergic receptor binding (Harmon et al., 1986; Henderson et al., 1991), or 5-HT_2 receptor binding (Henderson et al., 1991). These experiments were conducted during the late postnatal period, up to PND25, and therefore may not be long-term alterations persisting into adulthood. Dopamine affinity for dopamine receptors was also increased by prenatal NRI exposure (De Ceballos et al., 1985a).

Norepinephrine and dopamine concentrations in several brain areas in adolescent and adult male rats and norepinephrine turnover were shown to be unaffected by prenatal exposure to imipramine (Tonge, 1972; Tonge, 1973; Ali et al., 1986) although another study showed reduced hypothalamic dopamine concentrations on PND30 (Jason et al., 1981). NRIs typically have weak activity for the serotonin transporter and the serotonin system in general. The 5-HT_{1B} receptors has been shown to be unaffected by prenatal clomipramine exposure (Montero et al., 1991) but 5-HT and its metabolite 5-HIAA were reduced in the striatum on PND60 by prenatal amitriptyline exposure (Henderson and McMillen, 1993). These studies indicate a short-term effect of prenatal NRIs on adrenergic function.

3. Behavioral outcomes

Disrupted behavior may indicate a phenotypic difference due to prenatal NRI exposure. Several groups have investigated exploratory behaviors and social interaction after prenatal NRI exposure. The open field test for rodents is used to measure exploratory behavior in a novel environment and several groups have shown that prenatal NRI exposure decreased rearing behavior in adolescence (Coyle, 1975; File and Tucker, 1983; Drago et al., 1985) and decreased exploration in adolescence and adulthood in male rats (Rodríguez Echandía and Broitman, 1983). Additionally, prenatal and prenatal + lactational NRI exposure produced similar effects while lactation alone did not, indicating that exposure via lactation may not produce any detrimental behavioral effects (Rodríguez Echandía and Broitman, 1983). Measures of social interaction were shown to be increased in adolescence due to prenatal NRI exposure (File and Tucker, 1983; File

and Tucker, 1984) in male and female rats. However, other groups found the opposite: prenatal NRI exposure decreased social interaction in adolescence and adulthood in male rats (Coyle and Singer, 1975b; Rodríguez Echandía and Broitman, 1983). These studies indicate a short-term effect that persists into adolescence on behavioral alterations due to prenatal NRI exposure.

Serotonin syndrome has been documented in newborns after prenatal antidepressant exposure. Clinically, this syndrome consists of jitteriness and twitching. Animal studies can recapitulate this syndrome through postnatal co-administration of clorgyline, a monoamine oxidase inhibitor, followed by administration of 5-hydroxytryptophan, the immediate precursor of serotonin. (De Ceballos et al., 1985b) explored this endpoint in rats as an output to determine the involvement of the developing serotonin system. Prenatal clomipramine, a dual SRI/NRI, prevented drug induced serotonin syndrome-like behaviors such as head twitches and a resting tremor. However, $5HT_2$ antagonists iprindole and mianserin, both potentiated animal serotonin syndrome. Nomifensine, an NRI/dopamine reuptake inhibitor, had no effect on the behavioral characteristics of serotonin syndrome. Although the conflicting effects seen in this study preclude a strong interpretation, there does seem to be a serotonergic influence on postnatal jitteriness observed in infants prenatally exposed to antidepressants.

Learning, startle response, and hyperactivity have also been investigated in the context of prenatal NRI exposure. Only one study has examined prenatal NRI exposure in swim tests used to assess cognitive function but did not find any association between prenatal NRI exposure and the cognitive outcomes (Coyle and Singer, 1975a) although an enriched environment may interact with the prenatal NRI exposure to affect cognitive

behavior. The acoustic startle response, which measures the startle after a loud acoustic tone, has been shown to be mediated in part by the noradrenergic system (Olson et al., 2011). Prenatal NRI exposure has been shown to decrease the acoustic startle response in early postnatal male rats (Ali et al., 1986) and adolescent male rats (File and Tucker, 1984), although a dose-response relationship was not observed in either study. Haloperidol-induced catalepsy was unaltered by prenatal amitriptyline exposure in adolescent or adult males (Henderson and McMillen, 1993). Locomotion has been shown to be increased with prenatal NRI but not prenatal SRI exposure in male and female adolescent and adult rats (Cuomo et al., 1984) and adolescent male rats (Henderson and McMillen, 1990). However, when challenged with amphetamine, imipramine-exposed rats did not display any changes in locomotion compared to unexposed rats and completed negative geotaxis faster than control groups (Ali et al., 1986). Prenatal desipramine also did not cause any differences in quinpirole-induced stereotypy or locomotion (Stewart et al., 1998). Behavioral studies investigating prenatal NRI exposure are sporadic and while some groups have found behavioral differences, several endpoints have a null result or other studies conflict with positive associations with prenatal NRI exposure.

4. Growth, Developmental, Gross Anatomical and Physiological Outcomes

Gross anatomical differences due to prenatal NRI exposure have been investigated in animal models. Changes in birth weight or growth attributed to prenatal NRI exposure are frequently reported in the clinical literature but this outcome has yet to be replicated thoroughly in animal models. Animal studies show conflicting results:

some report no changes in pup weight or litter size (Rodríguez Echandía and Broitman, 1983; De Ceballos et al., 1985b; Harmon et al., 1986; Stewart et al., 1998) while other studies show an increase in fetal and neonatal weights (Cuomo et al., 1984; Swerts et al., 2010) and still others showed a reduced birth weight in pups and reduced litter size (Singer and Coyle, 1973; Jason et al., 1981; Simpkins et al., 1985; Henderson and McMillen, 1990). Congenital malformations have also been investigated in prenatal exposure animal studies, showing a hint of encephalocele in Golden hamsters prenatally exposed to a supraphysiological dose of amitriptyline (Beyer et al., 1984). Another study investigating teratological outcomes found no differences due to prenatal exposure to imipramine in Bonnet and Rhesus primates, even at ten times the clinical dose (Hendrickx, 1975). An important aspect of the thalidomide tragedy was the choice of the test species: rodents were not susceptible to the teratogenic effects while rabbits and non-human primates were susceptible (Delahunt and Lassen, 1964; Fratta et al., 1965; Schumacher et al., 1968). Prenatal NRI exposure studies have investigated several test species without showing any reproducible teratogenicity.

The noradrenergic system has a hypothesized role in thermoregulation (Mills et al., 2004). Adult male rat offspring prenatally exposed to imipramine displayed a baseline hyperthermic body temperature (Fujii and Ohtaki, 1985). Additionally, prenatal exposure resulted in a hyperthermic reaction to chlorpromazine (control rats were hypothermic) and this effect existed on PND57 and persisted to PND90 (Fujii and Ohtaki, 1985; Fujii, 1997). Female control and prenatally treated with imipramine had a hypothermic response similar to controls, indicating a sex-specific difference in thermoregulation.

Cardiovascular function is significantly regulated by catecholamines (Singewald and Philippu, 1996) and some have hypothesized that disturbances in catecholamine homeostasis due to prenatal antidepressant exposure may alter cardiovascular function in the offspring. Prenatal imipramine exposure has been shown to reduce heart weight in neonatal rats (Harmon et al., 1986). Another study examining prenatal doxepin or imipramine exposure found no differences measured in systolic blood pressure but early prenatal exposure to doxepin increased offspring heart rate from PND35-70. Testing of aortas *in vitro* showed that third trimester exposure to doxepin or imipramine increased isoproterenol-induced relaxation of aortic tissue (Simpkins et al., 1985). These studies demonstrate a role of prenatal serotonin in the development of the cardiovascular system whereby changes in this environment may cause long-term cardiovascular disturbances.

Selective Serotonin Reuptake Inhibitor Antidepressant Animal Studies

1. Exposure Studies

Dosing studies in rodents are vital in order to investigate the possible effects of prenatal SSRI exposure. However, translatable exposure concentrations between animals and humans is difficult due to differences in pharmacokinetics. Most SSRI animal studies employ a model of daily injections subcutaneously or intraperitoneally to investigate the effects on the pups. Daily injections in rodents are problematic due to the bolus effect that may lead to transient toxic serum concentrations of the compound. In contrast, human exposure leads to lower and steadier serum concentrations of the compound compared to rodents (Figure 1.5). Similar to TCA studies, some SSRI antidepressants have active metabolites that persist even after the compound has been

metabolized. While this may be the case, animal exposure studies using SSRIs have begun to use more steady exposure models and measure serum concentrations of compounds to determine the level of exposure.

Studies evaluating exposure concentrations in animals are sparse. Continuous infusion of fluoxetine in sheep via maternal femoral vein catheter yielded serum concentrations in maternal [fluoxetine] ~ 150 ng/mL and fetal [fluoxetine] ~ 60 ng/mL. Similar results were found in mice: placental transfer of fluoxetine was 69% whereas human placental transfer was 73% (Noorlander et al., 2008). This similarity in placental transfer between rodents and humans was also observed for fluvoxamine (30% in mice, 35% in humans). Our group has also observed variable placental transfer depending on the antidepressant used (Capello et al., 2011). Current animal studies have shown that placental transfer does occur to a degree but the level of CNS exposure has only recently been quantified (Figure 1.4). While our lab has observed variable placental transfer, fetal CNS exposure is similar to that of the pregnant dam.

Serum concentrations are only an approximation of CNS exposure and direct measurement of monoamine transporter occupancy in human infants is not possible at this time. However, our group has quantified SERT occupancy in rat pups after steady prenatal exposure to SRIs. Minipumps were used to administer steady serum concentrations similar to human exposure and resulted in approximately 80-95% occupancy of the serotonin transporter in E21 pups. This resulted in approximately the same SERT occupancy in E21 pups and their dams. Postnatally, SERT occupancy rapidly dropped and exposure via breast milk resulted in very low SERT occupancy (Capello et al., 2011). This was demonstrated independently for escitalopram, fluoxetine,

paroxetine, sertraline, and venlafaxine (Figure 1.4). This data indicates that exposure *in utero* to SRIs is similar between dams and their fetuses but postnatal exposure does not result in appreciable SERT occupancy. Therefore, postnatal exposure studies in rodents may not be an appropriate model for developmental exposure.

2. Molecular Outcomes

SSRIs may represent a more informative approach to investigate the effects of prenatal antidepressant exposure due to the very selective mechanism of action on the serotonin transporter. Two studies investigating the serotonergic system after prenatal fluoxetine exposure have been conducted by Battaglia and colleagues. Prenatal exposure via maternal fluoxetine injection resulted in increased density of [^{3}H]-citalopram labeled SERT in the CA2 (+47%) and CA3 (+38%) areas of the hippocampus, as well as the basolateral (+32%) and medial (+44%) amygdaloid nuclei in prepubescent progeny. In the diencephalon, the lateral hypothalamus displayed an increased SERT density (+21%) in prepubescent progeny. In contrast, the density of 5-HT transporters was significantly decreased in the dorsomedial nucleus of the hypothalamus (−21%) and in the substantia nigra (−19%) in prepubescent progeny. However, at PND90, there were no significant differences between control and fluoxetine animals (Cabrera-Vera and Battaglia, 1998). This model also resulted in 28% reduction in 5-HT content in the frontal cortex of prepubescent offspring and the midbrain of adult progeny as well as attenuated *p*-chloramphetamine-induced reduction in midbrain 5-HT content (Cabrera-Vera et al., 1997).

Following prenatal fluoxetine exposure, rats showed a lower B_{max} of [^{3}H]-imipramine binding sites in the cerebral cortex up to PND 25 compared to controls, indicative of a reduction in the SERT (Montero et al., 1990). Rats exposed perinatally to a steady concentration of drug from osmotic minipumps resulted in decreased cell count in the nucleus accumbens and decreased SERT-immunoreactivity in the raphe on PND 120 (Forcelli and Heinrichs, 2008). A similar study examining prenatal fluvoxamine exposure found decreased SERT-immunoreactivity in the raphe on PND 20 and 90 (Noorlander et al., 2008). Only one other study has investigated non-serotonergic receptors and found no changes in D1 or D2 binding in the striatum on PND20 after prenatal fluoxetine exposure (Stewart et al., 1998). Other non-CNS studies of monoamine systems have shown that vas deferens PND30 showed decreased affinity for 5-HT, norepinephrine, and phenylephrine (Pereira et al., 2007). Prenatal fluoxetine exposure seems to selectively affect the serotonergic system and not other monoaminergic systems.

The heavy interplay between the serotonergic system and the HPA axis (Owens et al., 1991a; Owens et al., 1991b) has caused Morrison and colleagues to investigate pituitary and adrenal output in the context of prenatal fluoxetine exposure. During continuous infusion of fluoxetine in sheep, at certain days, fluoxetine decreased ACTH serum concentrations compared to preinfusion day but not compared to control. In the fetus, ACTH was increased on G127 compared to preinfusion day. Cortisol was also affected in the fetus but not in the ewes. On G127 and 128, fetal cortisol in fluoxetine exposed animals were significantly elevated compared to controls (Morrison et al., 2004).

Further studies of HPA axis may help elucidate the function of the developing serotonin system on the fetal HPA axis.

Serotonin's role in early development has been explored via prenatal citalopram exposure. Simpson and colleagues have examined prenatal and postnatal exposures to citalopram and effects on cortical network functioning in rats. A comparison of gestational citalopram exposure and postnatal (PND8-21) exposure showed disruptive behavioral effects of postnatal exposure alone. Additionally, abnormal callosal connectivity was observed in postnatally-exposed animals (Simpson et al., 2011). This study describes an exquisitely regulated developmental system with serotonin at the center. However, since the effects were mostly ascribed to the postnatal exposure period, direct translation to prenatal antidepressant exposure is uncertain.

3. Behavioral Outcomes

Studies investigating anxiety-like and depressive-like behavior have been performed in animals to determine any effects due to prenatal SRI exposure. Mice exposed prenatally to fluoxetine had dose-dependent increases in anxiety-like behavior in adolescence and adulthood (Noorlander et al., 2008). Others have shown increased immobility in the forced swim test for females in adolescence and adulthood (Lisboa et al., 2007). However, some have shown no changes in anxiety or depressive-like behavior (Coleman et al., 1999; Hsiao et al., 2005; Lisboa et al., 2007; Favaro et al., 2008). Ultrasonic vocalizations during separation or behavioral test were increased in pups prenatally exposed to fluoxetine or paroxetine (Coleman et al., 1999; Cagiano et al., 2008). Prenatal exposure to the atypical antidepressant bupropion caused a decrease in

rearing and ambulatory activity in the open field test in adult male mice, although in one study the dose used was twice the traditional dose used in animals (Hsiao et al., 2005; Su et al., 2007). Locomotion is a critical variable in behavioral tests and false positives in anxiety-like behavior, for example, could be due to altered locomotion behavior. Studies have shown that prenatal fluoxetine exposure increases locomotion behavior and motor coordination in the rotorod test (Bairy et al., 2007). A similar finding was shown with prenatal bupropion exposure (Su et al., 2007). In contrast, Coleman (1999) and Stewart (1998) have shown no changes in locomotion or quinpirole-induced stereotypy due to prenatal SSRI exposure.

Behavioral tests such as learning and memory have been conducted in animals prenatally exposed to SSRIs. The Morris water maze and passive avoidance tests have been used to assess learning and memory in rodents. Prenatal exposure to fluoxetine resulted in an increase in learning in male and female adolescent rats (Bairy et al., 2007) but others have found no changes due to prenatal SSRI exposure in the same tests (Grimm and Frieder, 1987; Christensen et al., 2000; Cagiano et al., 2008). Therefore, there does not seem to be a role of prenatal SSRI exposure in the development of learning and memory skills.

Indices of social interaction have been investigated in animal studies of prenatal SSRI exposure. Prenatal fluoxetine exposure caused impaired sexual motivation in adult mice. However, this may indicate increased social contact or decreased preference for a female (Gouvêa et al., 2008). Aggressive behavior and foot shock-induced aggressive behavior has been shown to increase with prenatal SSRI exposure (Singh et al., 1998;

Coleman et al., 1999). Several studies show no changes in social interaction or sexual behavior (Lisboa et al., 2007; Cagiano et al., 2008).

Several other indices of behavioral dysfunction have been investigated in the context of prenatal SSRI exposure. Acoustic startle response, a test of the fear-induced startle response (Koch, 1999), and prepulse inhibition, a test of sensorimotor gating typically used to test animal models of schizophrenia (Geyer et al., 2002), were tested by Vartazarmian (2005) in guinea pigs prenatally exposed to fluoxetine. Prepulse inhibition was increased in both males and females after prenatally fluoxetine exposure, but the acoustic startle response was unchanged (Vartazarmian et al., 2005). Pain sensitivity has also been investigated after prenatal SSRI exposure, with one study showing an increase in pain threshold in adult guinea pigs after prenatal fluoxetine exposure (Vartazarmian et al., 2005) while another study showed no changes in pain sensitivity in mice prenatally exposed to fluoxetine (Lisboa et al., 2007). One study has examined drug-seeking behavior after prenatal fluoxetine exposure with a steady exposure model via osmotic minipump implantation in the dam. Prenatal fluoxetine increased cocaine-induced place conditioning on PND60. Prenatal fluoxetine additionally increased the nose-poke response rate during the extinction phase of cocaine self-administration behavior on PND 90 (Forcelli and Heinrichs, 2008) although others have shown no changes in cocaine-induce place conditioning due to prenatal SSRI exposure (Hsiao et al., 2005). Many behavioral studies examining the effects of prenatal SSRI exposure are often conflicting and a reproducible endpoint has not been identified as indicative of behavioral teratogenicity.

4. Cardiovascular Outcomes

Chambers (2006) reported that children exposed prenatally to SSRIs displayed persistent pulmonary hypertension. Only two animal studies have tried to mechanistically determine the effects of prenatal antidepressant exposure on cardiovascular and pulmonary endpoints. Mice prenatally exposed to fluoxetine exhibited pulmonary hypertension as measured by increased right ventricle: left ventricle + septum ratio or pulmonary arterial medial thickness. Functionally, pulmonary arterial smooth muscle response to serotonin was significantly reduced. The lung concentration of serotonin was unaltered by prenatal exposure but placental serotonin content was significantly reduced. Fetal mortality was increased in the first 3 days of life due to fluoxetine. Newborn arterial oxygen saturation was lower in fluoxetine exposed pups, but this normalized by the 3rd day of life, strongly indicative of pulmonary hypertension. This may be due to serotonin reducing but fluoxetine increasing (at low concentrations) pulmonary arterial smooth muscle proliferation rate in fetal versus adult cells (Fornaro et al., 2007). Noorlander (2008) also showed that mice prenatally exposed to fluoxetine manifested decreased left ventricle wall thickness, a measure of dilated cardiomyopathy, on PND20 and 90.

5. Growth, Developmental, Gross Anatomical and Physiological Outcomes

Morrison and colleagues have used Dorset Suffolk sheep and continuous infusion of fluoxetine to examine sleep endpoints. During the last trimester, a bolus injection of fluoxetine was administered followed by eight days of continuous intravenous infusion. The measured plasma concentration of fluoxetine in the ewe was 106 ng/mL while the

fetal fluoxetine concentration was 58 ng/mL, showing substantial and clinically relevant fetal transfer. Fluoxetine infusion caused disrupted fetal sleep measured by low voltage and high voltage electrocortical activity (Morrison et al., 2001). A follow-up study showed no alterations in fetal plasma melatonin or prolactin or disrupted pregnancy during the infusion. No differences were observed in fetal behavioral state, fetal arterial pressure, HR, breathing, or circadian rhythm (Morrison et al., 2005).

Clinical literature focuses on gross indices of development such as weight, growth, or fetal mortality. Several animal studies have examined these properties in the context of prenatal SSRI exposure with somewhat conflicting results. Impaired weight gain, low birth weight, or small litter size after prenatal SSRI exposure has been observed in several studies (da-Silva et al., 1999; Bairy et al., 2007; Pereira et al., 2007; Cagiano et al., 2008; Favaro et al., 2008; Forcelli and Heinrichs, 2008; Noorlander et al., 2008; Van den Hove et al., 2008; Bauer et al., 2010) while other studies have shown no differences in weight gain or birth weight (Byrd and Markham, 1994; Stewart et al., 1998; Vartazarmian et al., 2005; Lisboa et al., 2007). Congenital malformations are also frequently reported in the clinical literature but have only been reported in the animal literature by extracting and exposing mouse embryos to 20 times the clinically observed serum concentration of sertraline (Shuey et al., 1992). Current studies do not support a role of clinically relevant exposure to SSRIs as a cause of congenital malformations or long-term developmental consequences.

The goal of my dissertation is to develop and apply a model of clinically relevant antidepressant and/or stress exposure during pregnancy to determine the long-term effects on the infant. Previously, our lab has shown that five antidepressant drugs administered

during pregnancy show full occupancy of the fetal cortical serotonin transporter, illustrating that these drugs act similarly as a class. Additionally, our lab has shown that these drugs clear from the fetal system postnatally, albeit at different rates. Finally, our lab has shown that *in utero* exposure results in significant serotonin transporter occupancy, while lactational exposure does not have a meaningful effect on serotonin transporter occupancy (Capello et al., 2011). These studies have lead me to use the antidepressant escitalopram to document the long-term effects, if any, on the offspring.

Tables

Table 1.1. Impact of Motherisk counseling on perception of teratogenic risk.

Perception of risk (pre-counseling) *	Perception of risk (post-counseling)	P value
87% of depressed women rated risk of antidepressants as greater than 1–3%	12% of depressed women rated risk of antidepressants as greater than 1–3%	<0.001
56% of women with gastric problems rated risk of medications as greater than 1–3%	4% of women with gastric problems rated risk of medications as greater than 1–3%	<0.001
22% of women with infections rated the risk of medications greater than 1–3%	2% of women with infections rated the risk of medications greater than 1–3%	<0.001

*Actual baseline rate for major malformations in the general population is 1-3 %

Reproduced with permission from (Bonari et al., 2005).

Table 1.2. Summary of endpoints after prenatal tricyclic/tetracyclic antidepressant exposure in animals

Drug	Species	Dose (mg/kg/d)	Route	Exposure	MaternalSerum Monitored for Drug Conc.	5-HT	NE	DA	Behavior	Cardio	Health	Endpoint	Reference
CLO, IPD, MNS	Rat	10	SC	G6-G21	No	+	+		+		ø	P25	De Ceballos, 1985b
AMI	Rat	10	SC	G2-G21	No	+			ø			P60	Henderson, 1993
CLO, DSP, NOM	Rat	10	OG	G6-G21	No	+						P25	Montero , 1990
AMI	Rat	10	SC	G2-G21	No	ø	ø	ø				P30, 60, 180	Henderson, 1991
CLO, IPD, TNP	Rat	10	SC	G6-G21	No	ø						P25	Montero, 1991
IMI	Rat	15	OG	G8-G20	No		+	+	+		+	P14, 30	Jason, 1981
IMI	Rat	0, 5, 10	SC	G8-G20	No		+	ø	+		ø	P1, 7	Ali , 1986
IMI	Rat	Unknown	DW	G0-P21	No		ø	ø				P290	Tonge, 1972, 1973
IMI	Rat	0, 5, 10	SC	G8-G20	No		ø			+	ø	P4, 7	Harmon , 1986
CLO, NOM, IPR, MNS	Rat	10	SC	G6-G21	No			+	+			P25	De Ceballos, 1985a
DSP	Rat	10	SC	G8-G20	No			ø	ø		ø	P20	Stewart , 1998
IMI	Rat	5	OG	G-14-G19	No				+		+	P21	Coyle, 1975
CLO	Rat	3, 10, 30	IP	G8-G21	No				+		+	P35	File, 1983
DSP, MNS, VX	Rat	1.25/5/10	SC	G8-20	No				+		+	P23, 60	Cuomo , 1984
AMI	Rat	10	SC	G2-G21	No				+		+	P30	Henderson, 1990
CLO	Rat	3	DW	G-14-P2	No				+		ø	P50, 110	Rodríguez, 1983
IMI	Rat	5	OG	G-21-P25	No				+			P25, 80	Coyle, 1975a
CLO	Rat	7.5, 15	IP	G8-G21	No				+			P32, P42	File, 1984
IMI	Rat	5	OG	G-14-G19	No				ø		+	P60	Coyle, 1975b
DOX, IMI	Rat	30	IP	Varied	No					+	+	P35-70	Simpkins , 1985
AMI	Hamster	75	IP	G8	No						+	G15	Beyer , 1984
IMI	Rat	5, 15, 30	SC	G-21-G21	No						+	P1	Singer, 1973
IMI	Rat	10	IP	G9-G11	No						+	G21	Swerts , 2010
IMI	Rat	5	SC	G1-21	No						+, ø	P56, 90	Fujii, 1985, 1997
IMI	Primate	20-244	OG	Varied	No						ø	NA	Hendrickx, 1975

Abbreviations used: +, positive association; ø, null association; AMI, amitriptyline; CLO, clomipramine; DSP, deprenyl; DOX, doxepin; DSP, desipramine; DW, drinking water; G, gestation day; IMI, imipramine; IP, intraperitoneal; IPD, iprindole; MNS, mianserin; NOM, nomifensine; OG, oral gavage; P, postnatal day; SC, subcutaneous; TNP, tianeptine; VX, viloxazine

Table 1.3. Summary of endpoints after prenatal SSRI exposure in animals

Drug	Species	Dose (mg/kg)	Admin	Exposure	MaternalSerum Monitored for Drug Conc.	5-HT	DA	Behavior	Cardio	Health	Endpoint	Reference
FLX, FVA	Mouse	0-4.2	IP	G8-G18	Placental transfer	+		+	+	+	P20, P90	Noorlander, 2008
ESC, FLX, PRX, SRT, VEN	Rat	3-80	OM	G12-G21	Yes	+		ø		ø	G21-P7, P70-P100	Capello, 2011
FLX	Rat	10	OM	G14-P7	No	+		+		+	P30-P120	Forcelli, 2008
CIT	Rat	0-20	SC	G11-P21	No	+		+			P22-P25	Simpson, 2011
FLX	Rat	2.5	OG	G6-G21	No	+					P25	Montero , 1990
FLX	Rat	10	SC	G13-G20	No	+					P26, P70	Cabrera-Vera 1997
FLX	Rat	10	SC	G13-G20	No	+					P26	Cabrera-Vera 1998
ZIM	Rat	5	SC	G10-G20	No	ø		+, ø			P20-P90	Grimm, 1987
FLX	Rat	12.5	OG	G8-G20	No		ø	ø		ø	P20	Stewart , 1998
FLX	Rat	8, 12	DW	G6-G20	No			+		+	P18-56	Bairy, 2007
FLX	Mouse	5, 10	SC	G13-G20	No			+		+	P1-P120	Cagiano, 2008
FLX	Guinea Pig	7	OM	G1-G54	No			+		ø	P63	Vartazarmian, 2005
FLX	Mouse	7.5	OG	G0-P21	No			+		ø	P40, P70	Lisboa, 2007
FLX	Mouse	7.5	OG	G0-P20	No			+			P90	Gouvêa, 2008
FLX	Rat	10	IP	G13-G21	No			+			P56	Singh, 1998
FLX	Sheep	0.099	IV	G120-G128	Yes			+			G128	Morrison, 2001
FLX	Mouse	7.5	OG	G0-P21	No			+, ø		+	P40	Favaro, 2008
PRX	Mouse	30	Chow	G-14-G16.5	Yes			+, ø			P13-P90	Coleman, 1999
FLX	Sheep	0.099	IV	G120-G128	Yes			ø		ø	G128	Morrison, 2005
PRX	Mouse	30	Chow	G-14-P0	No			ø			P34-P105	Christensen, 2000
FLX	Rat	10	OG	G11-G21	No				+	+	G21-P3	Fornaro, 2007
FLX, VEN	Rat	8-80	OG	G15-G20	No					+	P1	da-Silva 1999
FLX	Sheep	0.099	IV	G120-G128	Yes					+	G128	Morrison, 2004
FLX	Mouse	10	DW	G0-G21	No					+	G14, G21	Bauer, 2010
FLX	Rat	10	IP	G0-BF	No					+	P3-P30	Periera, 2007
PRX	Rat	10	DW	G14-G21	No					+	P0	Van den Hove, 2008
FLX	Rat	10	IP	G9-G11	No					ø	G21	Swerts , 2010
FLX	Rat/Rabbit	0-15	OG	G6-G15	No					ø	G20/G28	Byrd, 1994

Summary of endpoints evaluated in animal studies after prenatal SSRI exposure. Abbreviations used: +, positive association; ø, null association; BF, breast feeding; CIT, citalopram; DW, drinking water; ESC, escitalopram; FLX, fluoxetine; FVA, fluvoxamine; G, gestation day; IV, intravenous; IP, intraperitoneal; OG, oral gavage; OM, subcutaneous osmotic minipump; P, postnatal day; SC, subcutaneous; SRT, sertraline; VEN, venlafaxine; ZIM, zimeldine

Figures

Figure 1.1. Use of antidepressants during pregnancy: 1996-2005.

The line with *diamonds* indicates any antidepressant use, the line with *squares* indicates SSRI use, the line with *triangles* indicates tricyclic antidepressant use, the line with *crosses* indicates tetracyclic antidepressant use, the line with *asterisks* indicates MAO inhibitor use, and the line with *circles* indicates other miscellaneous antidepressant use. Less than 0.1% of pregnant women were exposed to tetracyclic antidepressants and MAO inhibitors. The pregnancy period is considered to be the period from 1-270 days before delivery, with three 90-day trimesters: first trimester incorporates the period from 181-270 days before delivery; second trimester incorporates the period from 91-180 days before delivery; third trimester incorporates the period from 1-90 days before delivery. Data for 2005 are not available for 1 of the 7 sites included in the analyses. Antidepressant use in the seven health plans for the period 1996-2000 were calculated using data from an earlier CERT study by Andrade et al that evaluated medication use during pregnancy using similar methods (definitions and measures) as the present study. Reprinted with permission from (Andrade et al., 2008).

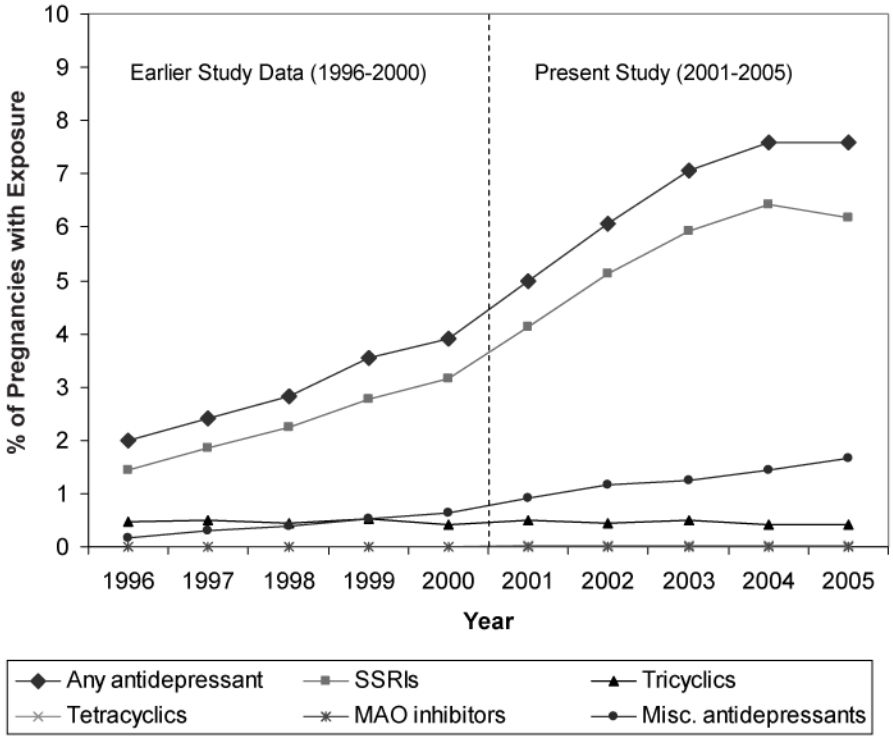

10
9
8
7
6
5
4
3
2
1
0
% of Pregnancies with Exposure
Earlier Study Data (1996-2000)
Present Study (2001-2005)
1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
Year
Any antidepressant
SSRIs
Tricyclics
Tetracyclics
MAO inhibitors
Misc. antidepressants

Figure 1.2. Kaplan-Meier curves illustrating the time to relapse after discontinuation of antidepressants during pregnancy.

Adapted from (Cohen et al., 2006).

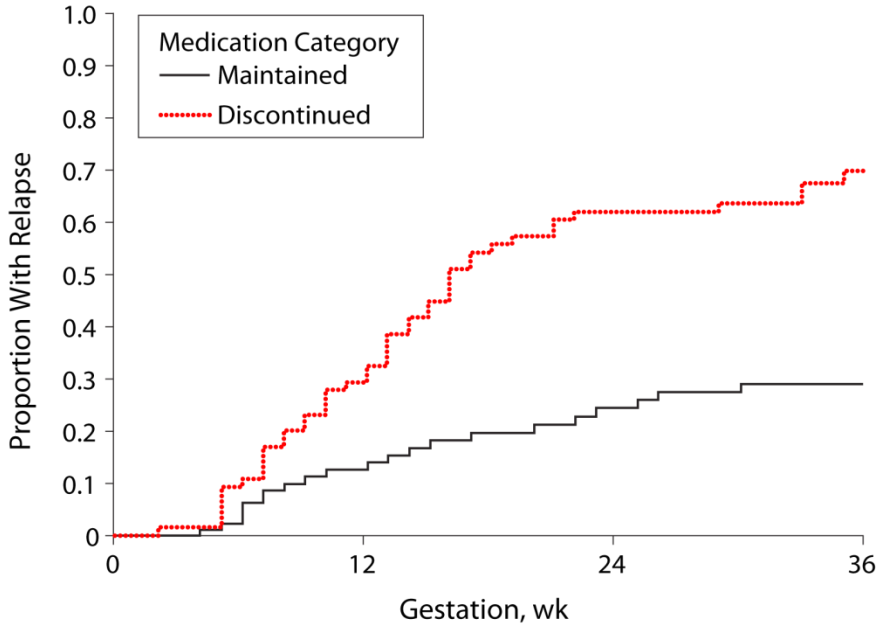


Figure 1.3. Percent of pregnant women taking prescription drugs arranged by the Food and Drug Administration labeling categories between 1996-2000 in the United States. Category A represents drugs that have well-controlled and adequate human and animal studies that show a low risk to the fetus. Category B drugs are classified as having animal studies that do not show a major risk to the fetus and the absence of well-controlled and adequate human studies. In Category C, a drug during pregnancy has an adverse effect in animal studies but there is an absence of well-controlled and adequate human studies. Category D drugs have a clear adverse effect in human and animal studies but the benefit to the pregnant woman may outweigh the risk. Category X drugs have a clear adverse effect in human and animal studies and the benefit to the pregnant woman does not outweigh the risk to the fetus (Food and Drug Administration, 2011). Data adapted from (Andrade et al., 2004).

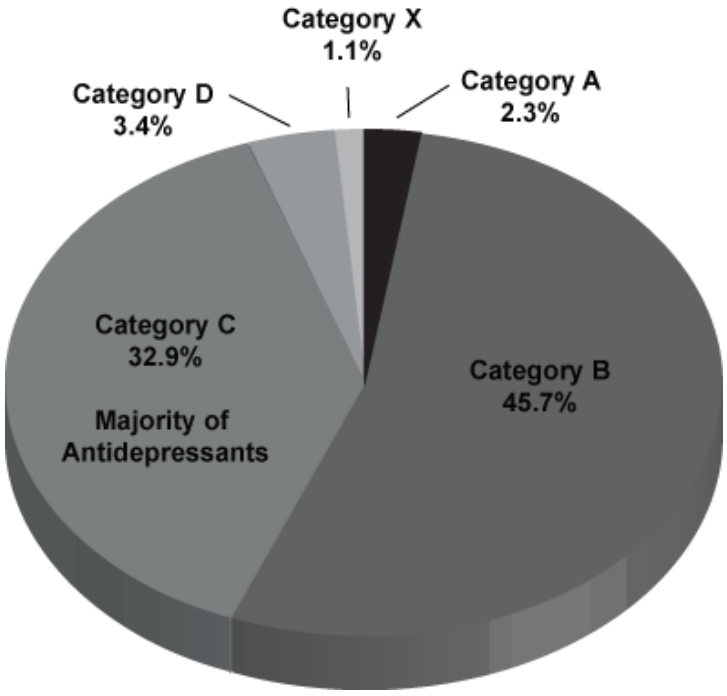


Figure 1.4. Representative autoradiographs of *in utero* exposure to SRIs.

Representative autoradiographs of escitalopram (ESCIT), fluoxetine (fluoxetine), paroxetine (PAROX), sertraline (SRT), and venlafaxine (VEN) including representative pregnant dams exposed to VEN during pregnancy. Each treatment group had its own vehicle run in the same assay but one series is shown for reference. Total binding representative images are displayed for comparison. Dense patches of total binding in the somatosensory cortex are consistent with previous studies investigating SERT binding during the early postnatal period (D'Amato et al., 1987; Kelly et al., 2002). Reprinted with permission from Capello et al., 2011.

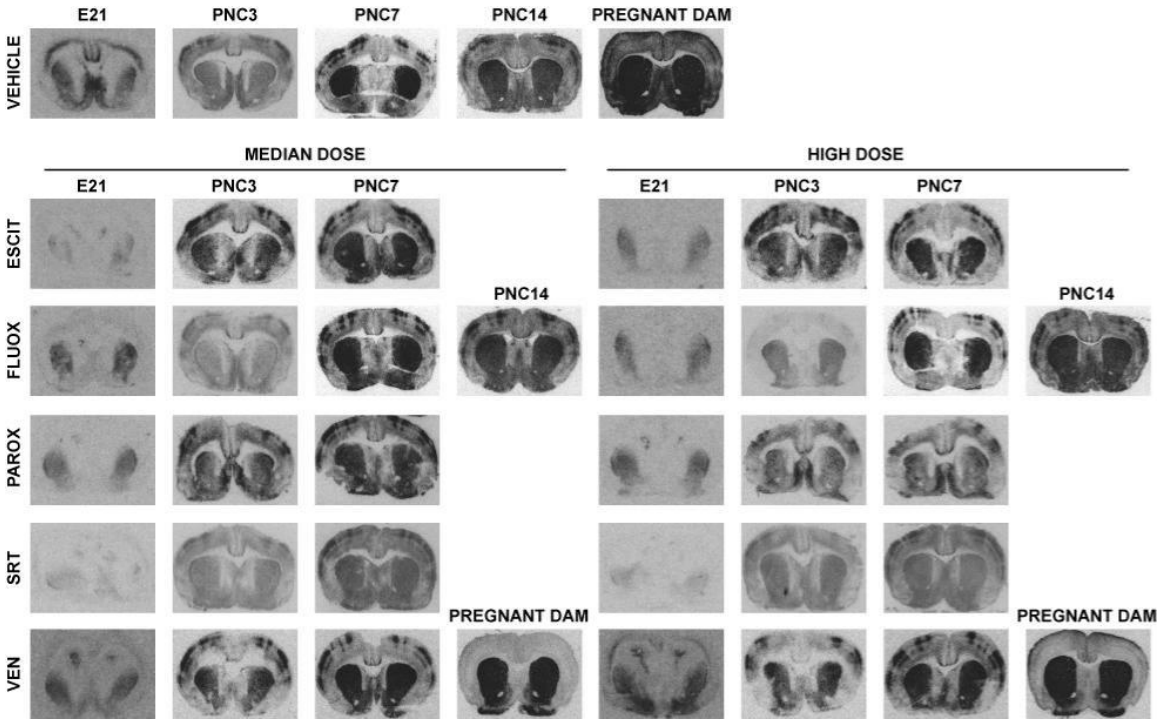
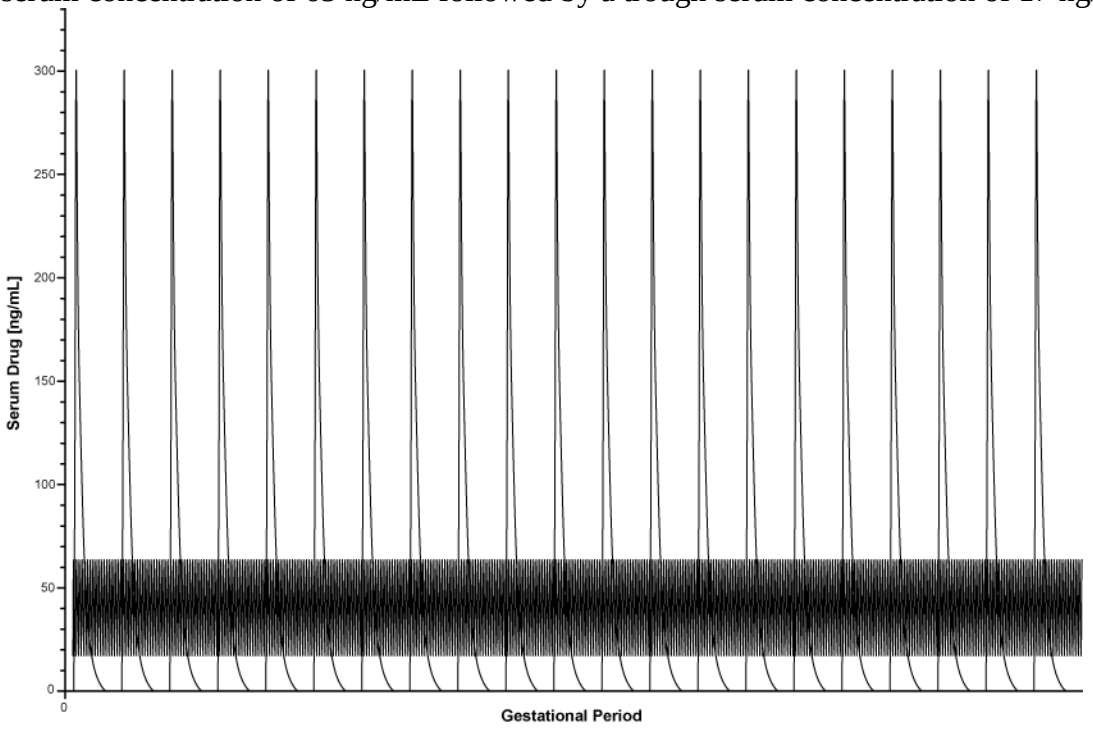


Figure 1.5. Comparison of human and rat pharmacokinetics of the antidepressant escitalopram during pregnancy.

Based on the published half life of escitalopram and observed peak and trough serum concentrations observed in humans and rats. Theoretical serum sampling of escitalopram after single injections during pregnancy in rats would yield a peak bolus concentration of approximately 300 ng/mL followed by rapid clearance. Theoretical serum sampling of escitalopram after daily use during pregnancy in human women would yield a peak serum concentration of 65 ng/mL followed by a trough serum concentration of 17 ng/mL.



CHAPTER 2: MODELING MATERNAL DEPRESSION AND ITS TREATMENT

Abstract

The purpose of this study was to develop a model of clinically relevant prenatal exposure to an antidepressant and maternal depression during pregnancy with the ultimate goal of evaluating the long-term effects of prenatal stress and/or antidepressant exposure on offspring. Acute subcutaneous or intraperitoneal dosing in female Sprague Dawley rats showed that escitalopram was rapidly cleared from the system ($t_{1/2}$ = 1.27 hours) following administration. Female rats implanted with 28 day osmotic minipumps delivering the SSRI escitalopram throughout pregnancy had serum escitalopram concentrations in a clinically observed range (17-65 ng/mL). A separate cohort of pregnant females exposed to a chronic unpredictable mild stress paradigm on gestational days 10-20 showed elevated baseline (305 ng/mL), and acute stress-induced (463 ng/mL), plasma corticosterone concentrations compared to unstressed controls (109 ng/mL). The stressor consisted of exposure to the chronic unpredictable mild stress model with blood draws following the restraint session or damp bedding during the model timeline. A final cohort of pregnant dams were exposed to saline (control), escitalopram, chronic unpredictable mild stress, or escitalopram with simultaneous exposure to chronic unpredictable mild stress to determine the effects on maternal care. Maternal behavior was continuously monitored over the first 10 days post parturition. A reduction of 35% in maternal contact and 11% in nursing behavior was observed due to stress; however these effects were confined to the "lights on" period when the dam and offspring were mostly inactive. Licking and grooming behavior, hallmarks of good maternal care, were unaffected by stress or drug exposure in either the light or dark cycle. These data indicate that: 1) clinically relevant antidepressant treatment during human

pregnancy can be modeled in rats using escitalopram; 2) chronic mild stress can be delivered in a manner that does not compromise fetal viability; and 3) neither of these prenatal treatments substantially altered important indices of maternal care post parturition.

Introduction

Pregnancy expands a woman's health considerations beyond herself to include her unborn child. Approximately 10-20% of pregnant women experience depression (Gavin et al., 2005) and pharmacological intervention may be indicated in a substantial proportion of these women. Women who discontinue antidepressant treatment during pregnancy are twice as likely to relapse (Cohen et al., 2006). Therefore, clinicians are faced with a difficult dilemma: continue treating women to avoid relapse or discontinue treatment and risk a depressive episode. Data exist that shows that untreated depression during pregnancy leads to untoward effects on the infant (*vide infra*). This study aims to develop a clinically-relevant rat model of antidepressant exposure and stress during pregnancy to determine the effects on the offspring and maternal care.

Elucidating the effects of antidepressants or other psychotropic medications on the fetus must rely on a dosing and exposure strategy that approximates clinically relevant human exposure. This study will focus on the selective-serotonin reuptake inhibitor (SSRI) escitalopram that has been purported to have superior efficacy and fewer discontinuations compared to other second-generation antidepressants (Cipriani et al., 2009) and is commonly prescribed to pregnant women at the Emory Women's Mental Health Program (Emory WMHP, http://www.emorywomensprogram.org/). Typical treatment of pregnant women with major depressive disorder results in steady state trough serum concentrations ranging from 17-65 ng/mL of escitalopram, and hereby designated the "Clinically Observed Range" (N = 60, ZN Stowe, unpublished observations). Small laboratory animals in general metabolize xenobiotics at a much faster rate than humans which necessitates consideration of pharmacokinetic differences

between species when developing a dosing paradigm (Martignoni et al., 2006). In rats for example, escitalopram's half-life can be shortened to 15-20% of that in humans due to rapid metabolism and elimination (Bundgaard et al., 2007; Rao, 2007). Consequently, classic single or multiple daily dosing regimens of most antidepressants are unlikely to achieve steady state in a clinically relevant range and, therefore, may not provide the requisite data germane to expanding our understanding of human exposure. I hypothesize that continuous drug delivery with an osmotic minipump will more closely model clinically observed escitalopram concentrations.

Modeling human depression in animals is not possible; however, chronic variable stress or chronic restraint stress have been used as a proxy. Characterizing stress during pregnancy is problematic due to altered endocrine baselines and stress responsivity associated with pregnancy (Williams et al., 1999) as well as changes in the dams morphometry that hinder some behavioral tasks. Although detrimental effects of stress to human infants have been investigated (Cottrell and Seckl, 2009; Davis and Sandman, 2010), animal modeling of stress to the pregnant rat with a definitive measurement of stress-related behaviors has not been well characterized. In humans and animals, depression or stress (animals) during pregnancy can also have effects in the postpartum period by disrupting maternal care of the infant (Lovejoy et al., 2000; Newport et al., 2002; Baker et al., 2008). I hypothesize that exposure to a chronic stress model during pregnancy can be verified by induction of an HPA axis response characterized by elevated basal corticosterone and/or an exaggerated response to a stressor.

The importance of the maternal-fetal serotonin system in development has been recently elucidated (Bonnin et al., 2011) but the concurrent examination of prenatal stress

and antidepressants exposures has yet to be explored. In animal models, there are studies that have examined prenatal stress (Newport et al., 2002; Mueller and Bale, 2006; Mueller and Bale, 2008) and some studies that have examined antidepressant-exposed pregnant rats from the perspective of the offspring (Henderson and McMillen, 1993; Cabrera and Battaglia, 1994; Cabrera-Vera et al., 1997; Cabrera-Vera and Battaglia, 1998; Forcelli and Heinrichs, 2008). To my knowledge, prenatal stress and antidepressant exposure have not been examined concurrently to investigate the effects on maternal care. This study provides a clinically-relevant model of antidepressant exposure and/or prenatal stress as well as investigates the impact of these exposures on maternal care.

Materials and Methods

Animals

Sprague-Dawley male retired breeders and nulliparous females weighing 200-225 grams were purchased from Charles River Laboratories (Charles River, Wilmington, MA). Rats were kept on a 12:12 light: dark cycle (lights on at 7:00 AM) in a humidity (60%) and temperature (20°C-23°C) controlled facility. Rodent diet 5001 chow (Purina Mills, Richmond, IN) and water were available *ad libitum* throughout the study. After two weeks at the Emory University animal facility, female rats were paired with male retired breeders in a breeding cage. Gestational day 0 (G0) was designated by the presence of a sperm plug and pregnant females were single-housed after breeding. Pregnancy was confirmed post-decapitation with a cesarean section for the catheter studies.

Ethics Statement

All animal protocols were approved by the Emory University Institutional Animal Care and Use Committee and carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Resources, 1996) as adopted and promulgated by the U.S. National Institutes of Health. All steps were taken to minimize animal suffering at each stage of the study.

HPLC-UV and UPLC-M/S Detection Methods

High performance liquid chromatography with ultraviolet detection serum analysis of drug concentrations has been previously described in detail (Stowe et al.,

1997; Newport et al., 2012). Briefly, after a solid phase extraction the serum samples were analyzed via isocratic high-performance liquid chromatography separation followed by ultraviolet detection. The limit of detection for each compound is 2 ng/mL.

Ultra performance liquid chromatography with tandem mass spectrometry detection was conducted as follows: sample extraction was accomplished using a standard protein crash (0.1 mL of sample + 10 µL of mobile phase A + 0.2 mL of the internal standard of deuterated citalopram in methanol). The assay was performed on a Waters Inc, Acquity ultra-performance liquid chromatography system with a triple quadrupole detector in the multiple reaction monitoring mode employing electrospray positive ionization (Waters, Milford, MA). The mobile phases were (A) 2 mM ammonium acetate and 0.1% formic acid in water and (B) 2 mM ammonium acetate and 0.1% formic acid in methanol. The flow rate was 0.6 mL/min and the chromatography was developed using a gradient from 25% B to 75% B over 3.5 minutes on an Acquity ultra-performance liquid chromatography, C-18 column (1.7 µM, 2.1 x 50 mm). 5 µL of extract was injected. A seven point standard curve with two levels of quality control were processed in each run. The method is linear from 0.2 to 2000 ng/mL. The limit of detection is 0.05 ng/mL and the limit of quantification is 0.2 ng/mL (10%). The method exhibits no matrix effects by the method of Matuszewski and colleagues (Matuszewski et al., 2003). Absolute recoveries range from 88.9% to 119.6% and inter-assay imprecisions range from 3 to 13% at levels of 75 and 300 ng/mL. The method compares favorably (i.e., greater sensitivity) with the high performance liquid chromatography with ultraviolet detection methods used previously in our laboratory. Extraction and quantification were carried out at the Emory Clinical Translational Research Laboratory.

Acute and Chronic Fluoxetine Administration (Pilot Studies)

For the pharmacokinetic (elimination half-life) experiment, nulliparous females were injected subcutaneously with 11 mg/kg fluoxetine hydrochloride (31.8 μmol/kg) dissolved in a vehicle solution of 40% sterile polyethylene glycol and 60% 0.9% sterile saline (1 mL/kg). For chronic dosing studies, female rats were implanted subcutaneously with Alzet 28-day osmotic minipumps (Alzet, Cupertino, CA) slightly posterior to the scapulae. Osmotic pumps delivered an average dose of 8.8 mg/kg/day or 11.8 mg/kg fluoxetine hydrochloride in a vehicle solution of 40% sterile polyethylene glycol and 60% 0.9% sterile saline based upon the predicted final gestational weight on embryonic day 21 of 353 grams. Three days after pump implantation, female rats were bred. Blood was collected during terminal collections on G20 and blood was spun down at 1,800 x g and the serum fraction was collected. The method to calculate half life is through the following equation:

$$k_{\text{elim}} = \frac{(\ln(C_2) - \ln(C_1)}{t_2 - t_1}$$

$$t_{1/2} = \frac{0.693}{k_{\text{elim}}}$$

Acute and Chronic Escitalopram Administration (Cohort One)

For the pharmacokinetic (elimination half-life) experiment, nulliparous females were injected subcutaneously or intraperitoneally with 12.2 mg/kg escitalopram oxalate (29.44 μmol/kg) dissolved in 0.9% saline (1 mL/kg). For chronic dosing studies, female rats were implanted subcutaneously with Alzet 28-day osmotic minipumps (Alzet,

Cupertino, CA) slightly posterior to the scapulae. Osmotic pumps delivered an average dose of 12.2 mg/kg/day escitalopram oxalate in 0.9% saline based upon the predicted final weight on embryonic day 21 of 440 grams. Three days after pump implantation, female rats were bred. Six days after breeding, jugular catheters were implanted. Blood collected from catheters was spun down at 1,800 x g and the plasma fraction was collected.

Jugular Catheter Implantation

Jugular catheters were implanted according to Thrivikraman et al. (2002). Briefly, animals were anesthetized with a preparation of ketamine:xylazine:acepromazine and assessed for reaction to a painful stimuli prior to surgery. The jugular vein was implanted with a catheter to allow for repeated blood sampling and to prevent a stress response elicited by other sampling methods (i.e. tail nick). Animals were given three days to recover before initiation of stress or dosing models. Blood samples were collected (200 µL) and an equal volume of sterile 0.9% saline was injected to replace the blood volume lost. Catheters were flushed with sterile gentamicin (120 µg/mL, 150 µL) after sampling to prevent infection.

Chronic Unpredictable Mild Stress Model (Cohort Two)

A separate cohort of animals were implanted with jugular catheters and exposed to the chronic unpredictable mild stress model. On gestational day 10 (G10), the chronic unpredictable mild stress (CMS) model of depression began and consisted of restraint three times a day for 45 minutes in a clear acrylic cylinder designed to minimize

movement (2.25 in diameter x 6 in long, Harvard Apparatus, Holliston, MA), a 23.5° cage tilt, damp bedding (450 mL of water), noise (adjusted to 95 dB of intermittent noise), cage changes, or overnight illumination (Table 1). G10 was selected to begin the stress paradigm because the fetal central nervous system begins substantial development at this point (Clancy et al., 2001) and to minimize premature termination of the pregnancy as a result of excessive stress (Mulder et al., 2002). Baseline corticosterone concentrations were assessed by jugular catheter blood sampling which took place at 9:00 AM, the nadir of the circadian cycle of corticosterone. After baseline sampling, animals in the CMS group were exposed to the stressor for that day (45 minutes of restraint on G10, G14, G17, or damp bedding on G12 and G19). At 9:45 AM, a second jugular catheter blood sampling was taken to determine stress-induced increases in plasma corticosterone. The CMS ended following the G20 stress to prevent premature parturition. During the noise and overnight illumination stressors, animals were housed overnight in a separate temperature and humidity controlled room from the unstressed animals. Otherwise, all animals were kept in the same housing room and subjected to identical handling and cage change procedures.

Chronic Restraint Stress Model (Cohort Three)

On G10, the chronic restraint stress model began and consisted of three 45 minute sessions of restraint at 9:00, 13:00 and 17:00 to maximize the number of stress exposures during the light cycle. Animals were restrained in acrylic cylinders designed to minimize movement (Harvard Apparatus, Holliston, MA). For the offspring endpoints, no more than two pups per litter were assigned to a group in order to prevent litter effects (Holson

and Pearce, 1992); in most cases only a single pup from each litter was utilized in any single assay. On PND 63, the offspring were separated from their cage mate and exposed to three 45 minute sessions of restraint at 9:00, 13:00, and 17:00. To test behavioral endpoints in the chronic restraint stress model, pregnant rats and their offspring were tested for anxiety-like and depressive like behaviors. Animals were tested for five minutes in an elevated plus maze during the dark cycle and measured for exploratory behavior. In the defensive withdrawal test, animals were placed in a horizontal cylinder that was placed in a novel arena. Animals were tested for 10 minutes during the dark cycle for the latency to emerge from the cylinder. The forced swim test was used to determine any differences in escape behavior. Animals were placed in a vertical cylinder of warm water for five minutes and tested during the light cycle. For the sucrose preference test, animals were given two bottles in their home cages, one containing tap water and one containing 1% sucrose. Bottles were switched every 12 hours and weighed after 48 hours to measure sucrose and water consumption. No training sessions were conducted for any of the tests. An observer blind to the treatment condition scored the behavior.

Maternal Care Behavior (Cohort Four)

In a separate experiment from the jugular catheter blood sampling experiments, groups were exposed to stress and/or escitalopram in pregnancy. The control group experienced no stress with saline minipump (Saline). Experimental groups consisted of no stress with an escitalopram minipump (Escitalopram), prenatal stress with a saline minipump (Stress-Saline), and prenatal stress with an escitalopram minipump (Stress-

Escitalopram). Maternal care behavior was monitored by video cameras continuously for 10 days postpartum. Our collaborators have reported that a sampling frequency that is equal to one hour of monitoring during the light phase and one during the dark phase accurately characterizes maternal care behavior throughout the entire 24 hour period (Boss-Williams et al., submitted). Light and dark phase behavior was analyzed separately and then combined into a single measure of maternal care behavior and transformed into a percentage of time spent performing the indicated behavior. Three observers blinded to treatment conditions scored three behaviors: licking/grooming, no maternal contact, and nursing. Licking/grooming was defined as the mother's mouth in contact with at least one pup for at least one second. No maternal contact was defined as the mother not engaged in contact with any pup for at least one second. Nursing was defined as at least one pup suckling from the mother for at least one second.

Corticosterone Assays

Plasma corticosterone was assayed using the ImmuChem ^{125}I Corticosterone RIA kit with a sensitivity of 1 ng/mL (MP Biomedicals, Orangeburg, NY).

Statistical Analyses

In all figures, values are expressed as mean ± SEM. GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA) was used to conduct statistical analyses. Experimental differences were analyzed with a 2-way ANOVA followed by a Bonferroni *Post Hoc* Test. Maternal care behavior was analyzed with a 3-way ANOVA (Time x

Drug x Stress) in SPSS 17.0 (IBM, Armonk, NY). Differences were considered significant if $p < 0.05$ except where noted.

Results

Fluoxetine Pharmacokinetics after Acute and Chronic Dosing

Pilot studies determined the serum concentrations of fluoxetine and norfluoxetine after acute or chronic dosing in females. Subcutaneous injection of 11 mg/kg of fluoxetine hydrochloride resulted in rapid rise in serum concentrations of both fluoxetine and the active metabolite norfluoxetine. Fluoxetine and norfluoxetine are inhibitors of many cytochrome P450 enzymes including the enzyme that metabolizes fluoxetine: CYP 2D6 (Sandson et al., 2005). Due to fluoxetine and norfluoxetine's inhibition of CYP 2D6, a disruption of the clearance of fluoxetine is observed at six hours (Fig. 2.1A). The terminal half-life was calculated as 4.6 hours for fluoxetine and 32.54 hours for norfluoxetine in non-pregnant females.

Osmotic minipumps were implanted in pregnant females and designed to deliver fluoxetine hydrochloride at a mean dose of 8.8 mg/kg/day or 11.8 mg/kg//day based on the animal's actual weight of 353 grams on embryonic day 21. The higher 11.8 mg/kg/day dose significantly increased the serum concentrations of fluoxetine and norfluoxetine compared to the lower 8.8 mg/kg/day dose of fluoxetine ($F_{(1,20)} = 20.7$; $p < 0.001$) (Fig. 2.2A). The 8.8 mg/kg/day dose resulted in mean serum concentrations of 93.5 ± 16.0 ng/mL for fluoxetine and 463.7 ± 46.7 ng/mL for norfluoxetine. The 11.8 mg/kg/day dose resulted in mean serum concentrations of 291.5 ± 52.1 ng/mL for fluoxetine and 750.5 ± 78.9 ng/mL for norfluoxetine (Fig. 2.2A). The high dose of fluoxetine decreased the weight gain compared to the low dose ($F_{(1,100)} = 17.9$; $p < 0.001$) (Fig. 2.2B).

Comparison of Escitalopram Delivery and Detection Methods (Cohort One)

Initial studies were conducted to determine the optimal vehicle solution and detection method. After osmotic minipump implantation and breeding, pregnant female rats were killed on gestational day 20 to obtain trunk blood to determine serum concentrations of escitalopram. Both vehicle solutions had no detectable levels of escitalopram (Fig. 2.3A). Either PBS or saline solutions of escitalopram gave comparable serum concentrations of escitalopram (Fig. 2.3A). Both detection methods detected similar serum concentrations of escitalopram (Fig. 2.3B).

Studies were conducted to determine the appropriate clinically relevant dose during pregnancy. To examine daily, acute drug exposure models, 12.2 mg/kg escitalopram oxalate was injected subcutaneously or intraperitoneally. Based on pharmacokinetic data, 11 mg/kg was initially chosen from previous studies showing that an 11 mg/kg dose in a 14-day osmotic minipump results in rat serum escitalopram concentrations in the human therapeutic range (Capello et al., 2011). Pilot studies with the calculated 11 mg/kg dose in a 28 day osmotic minipump were based on a predicted final weight that was found to be lower than initially approximated. Therefore, the actual final dose was 12.2 mg/kg and since this does gave escitalopram serum concentrations in the clinically observed range, 12.2 mg/kg were used for subsequent studies (Fig. 2.3A). Intraperitoneal injection of 12.2 mg/kg of escitalopram oxalate resulted in a lower area under the curve compared to subcutaneous injection (785 ng/mL/24 hours for subcutaneous, 565 ng/mL/24 hours for intraperitoneal) (Fig. 2.4A). The terminal half-life was calculated as 1.27 hours for both routes of administration. The time in the clinically observed range was calculated as 2.46 hours after a single injection and the drug was not

detected in serum after 9 hours (Fig 2.4A). Osmotic minipumps were implanted in pregnant females and designed to deliver escitalopram oxalate at a mean dose of 12.2 mg/kg/day based on the animal's actual weight of 440 grams on embryonic day 21 (mean dose range: 10.76-13.13 ng/mL). A dose of 12.2 mg/kg/day was used for all studies since this dose gave serum concentrations in pregnant rats within the clinically observed range of 17-65 ng/mL observed in human pregnant women. A separate cohort of catheterized, pregnant dams implanted with osmotic minipumps showed a consistent concentration of escitalopram in the serum over the entire course of pregnancy (Fig. 2.4B). The area under the curve was calculated for chronic dosing to yield an average serum concentration of 21 ng/mL/day for the osmotic pump administration (AUC: 510 ng/mL/24 hours for osmotic pump administration). Weight changes due to pregnancy did not affect the serum concentration of escitalopram ($r^2 = 0.003$, $F_{(1,51)} = 0.159$; $p = 0.69$, Fig. 2.5).

Chronic Unpredictable Mild Stress (Cohort Two)

Catheters were implanted in the jugular vein to take plasma samples over the course of pregnancy and measure baseline (9:00 AM) and stress-induced (9:45 AM) changes in plasma corticosterone. Plasma corticosterone samples were analyzed with a 2-way ANOVA (chronic stress x gestational day) Baseline (9:00 AM) measurements of plasma corticosterone concentrations in controls compared to CMS animals showed that the stressed dams displayed an increase in baseline plasma corticosterone due to the stress model ($F_{(1,70)} = 17.0$; $p < 0.001$) (Fig. 2.6A). Baseline increases in plasma corticosterone had the largest difference on gestational day 17: plasma corticosterone

concentrations in control animals were 55.6 ng/mL compared to 305.2 ng/mL in stressed animals ($t_{11} = 3.5$; $p > 0.05$) (Fig. 2.6A).

A second blood draw was taken at 9:45 AM to determine acute stress-induced increases in plasma corticosterone. CMS rats showed an increase in corticosterone due to the daily stressor compared to unstressed animals at 9:45 AM ($F_{(1,58)} = 16.6$; $p < 0.001$) (Fig. 2.5B). Stress-induced increases in plasma corticosterone concentrations were maximal on gestational day 14 (670% difference; $t_9 = 3.7$; $p < 0.01$) and gestational day 17 (296% difference; $t_9 = 3.0$; $p < 0.05$) (Fig. 2.6B). Anesthetized animals were analyzed on G21 but showed no differences between treatment groups for plasma corticosterone (Control = 256.6 ± 60.5, Stress = 310.9 ± 60.15; $t_{15} = 0.6$; $p > 0.05$) or amniotic fluid corticosterone (Control = 173.5 ± 32.4, Stress = 155.5 ± 32.0; $t_{16} = 0.4$; $p > 0.05$).

Chronic Restraint Stress Model (Cohort Three)

In a separate cohort of animals used to compare the effectiveness of stress models, pregnant females exposed to chronic restraint during pregnancy exhibited no alterations compared to controls in weight gain, behavioral endpoints, and post-parturition plasma corticosterone (Table 2.2). Measurement of males and female offspring in adulthood showed no changes in behavior or baseline plasma corticosterone between control and prenatal restraint stress groups (Table 2.3). Additional stress in adulthood produced a decrease in sucrose consumption for prenatal restraint stress in females, but all other behaviors were unaltered (Table 2.3).

Maternal Care Behavior

A combination of stress and escitalopram exposure during pregnancy (Fig. 2.7A) was used to determine alterations in maternal care behavior. The control group experienced no stress and had a saline minipump (Saline). Experimental groups consisted of no stress with an escitalopram minipump (Escitalopram), prenatal stress with a saline minipump (Stress-Saline), and prenatal stress with an escitalopram minipump (Stress-Escitalopram). Maternal care behavior was analyzed with a 3-way ANOVA with repeated measures on one factor to determine changes due to stress or escitalopram exposure over time. Time without maternal contact was increased by 35% due to stress ($F_{(1,32)} = 8.0$; $p < 0.01$) but not due to escitalopram ($F_{(1,32)} = 0.9$; $p > 0.05$). Over the postnatal period, time without maternal contact increased steadily ($F_{(9,288)} = 37.4$; $p < 0.001$) (Fig. 2.7B). Stress increased the time without maternal contact only in the light phase, which is characterized by reduced activity in rats ($F_{(1,32)} = 8.70$; $p < 0.01$). Stress did not alter time without maternal contact in the dark phase, which is the active phase in a 24 hour period ($F_{(1,32)} = 1.3$; $p > 0.05$).

Licking and grooming was not affected due to time ($F_{(9,288)} = 1.5$; $p > 0.05$), stress ($F_{(1,32)} = 0.3$; $p > 0.05$) or escitalopram exposure ($F_{(1,32)} = 0.0$; $p > 0.05$) (Fig. 2.8A). There was an interaction with time x drug ($F_{(9,288)} = 2.2$; $p < 0.05$), but no other interactions reached statistical significance. Licking and grooming was also unaffected by time, stress, or escitalopram in the light cycle ($p > 0.05$) (Fig. 2.8B) and in the dark cycle ($p > 0.05$) (Fig. 2.8C).

There was a significant effect of stress on maternal nursing behavior ($F_{(1,32)} = 5.9$; $p < 0.05$) but no effect due to escitalopram ($F_{(1,32)} = 16.6$; $p < 0.001$). This resulted

in an overall reduction in nursing due to stress by 11%. There was no interaction between these exposures ($F_{(1,32)}$ = 0.6; p > 0.05). Over time, mothers spent less time nursing their pups ($F_{(9,288)}$ = 27.6; p < 0.001). There was a significant interaction between time x stress ($F_{(9,288)}$ = 1.9; p < 0.001) (Fig. 2.8D). The reduction in nursing due to stress was only observed in the light cycle ($F_{(1,32)}$ = 7.3; p < 0.05) (Fig. 2.8E) but not in the dark cycle ($F_{(1,32)}$ = 0.8; p > 0.05) (Fig. 2.8F). Similarly, the interaction of time and stress observed over a 24 hour period was entirely due to the effect in the light cycle ($F_{(9,288)}$ = 2.1; p < 0.05) (Fig. 2.8E) while there was no interaction in the dark cycle ($F_{(9,288)}$ = 1.6; p > 0.05) (Fig. 2.8F).

Maternal Endpoints After Chronic Unpredictable Mild Stress

Measurements from pregnant dams showed that on G15, there was escitalopram exposure that resulted in serum concentrations within the clinically observed range ($F_{(1,32)}$ = 309; p < 0.0001) (Table 2.4, top). Pregnant dams exposed to stress had a trend towards a decreased rate of weight gain ($F_{(1,32)}$ = 4.2; p = 0.050) although this did not reach statistical significance (Table 2.4, middle). Pregnancy duration, litter size, and litter rate of weight gain were unaffected by any exposure (p > 0.05), (Table 2.4, bottom).

Discussion

The purpose of the present study was to develop and characterize a clinically-relevant exposure to an antidepressant, escitalopram, and/or stress during pregnancy as a putative animal model of the treatment of maternal depression during pregnancy. Escitalopram oxalate has excellent aqueous solubility properties (>70 mg escitalopram oxalate/mL 0.9% saline; unpublished observations) and we found that continuous escitalopram administration via an osmotic minipump resulted in an accurate representation of human exposure. Chronic mild and variable stress during pregnancy was found to increase plasma corticosterone concentrations at baseline and in response to the daily stressor. In addition, chronic mild stress slightly altered maternal care in the dam. In contrast, a separate experiment used to model chronic restraint stress in pregnancy did not alter overall dam or offspring behavior (Table 2.2, 2.3). Chronic stress and/or antidepressant exposure during pregnancy did not have any effect on indices of litter viability, indicating that neither maternal stress nor maternal escitalopram treatment overtly interfered with infant vitality

Chronic dosing of many psychotropic drugs in small animals that approximates human exposure is complicated due to issues with rapid metabolism. We have previously examined fluoxetine and norfluoxetine and found rapid clearance of the parent compound from the system, although the persistence of norfluoxetine can allow for clinically relevant exposure (Capello et al., 2011). We have estimated the half-life of the antidepressant paroxetine to be 8.0 hours in the rat (unpublished observations). Daily injections are problematic due to the generally short half-life of fluoxetine and escitalopram in animals which will prevent the compound from achieving and

maintaining clinically relevant drug concentrations throughout the day (Fig. 2.1 and 2.4). This half-life effect was demonstrated in the subcutaneous and intraperitoneal dosing in this study: females demonstrated rapid metabolism of escitalopram that provided less than two hours in the clinically observed range. To approximate human exposure with injections, animals would theoretically have to be dosed approximately once an hour with a lower dose. While this is impractical from a logistic standpoint, much of the extant literature examining chronic antidepressant exposure during pregnancy currently uses a daily injection model to investigate effects to the pregnant animal and fetus.

Daily injections are also problematic due to the bolus effect. A single injection results in a rapid peak of serum escitalopram concentrations. This peak was approximately five times higher than the upper range of clinically observed serum concentrations from human pregnant mothers medicated with escitalopram. These high serum concentrations can logically result in transient toxic effects and produce a false positive effect in the pregnant rat or fetus for studies examining what are thought to be standard or moderate exposure. The majority of the animal literature, which is not limited to the antidepressant area, is based on a daily injection model that may plausibly induce this transient toxic effect.

Current animal dosing paradigms include daily injections, adding the drug in drinking water or food pellets, and implantation of an osmotic minipump for continuous drug delivery. Adding the drug to food and drinking water can be unreliable because the animals eat in a pulsatile manner and the medications may have an undesirable taste leading to a decreased fluid consumption. When the physical properties of the drug are amenable, osmotic minipumps can deliver a steady concentration of drug over a period of

4 weeks and do not subject the animal to repeated stress for an injection. Proper administration in pregnant animals which approximates human exposure is key to investigating any potential long-term effects of antidepressants. Our model utilizing minipumps demonstrated that escitalopram serum concentrations were stable during the entire course of pregnancy. The serum concentrations were consistently within the clinically observed range in the Emory WMHP and as reported elsewhere for trough concentrations associated with therapeutically effective doses in patients with major depressive disorder (6-21 ng/ml; (Sogaard et al., 2005)). SERT occupancy of ~80% has been consistently associated with therapeutic doses of SRIs in patients with major depressive disorder (Meyer et al., 2001; Meyer et al., 2004) and 80% SERT occupancy measured in rodents is obtained at serum concentrations of 18 ng/ml (Kreilgaard et al., 2008; Capello et al., 2011). While humans have some peak to trough fluctuations during dosing intervals that vary according to each drug, they are much more closely reflected by continuous minipump exposure.

Preliminary studies were conducted to first determine the most appropriate model of chronic stress exposure in pregnant female rats. Two prominent models are used in the rodent literature to induce stress in rats: chronic restraint stress and chronic unpredictable mild stress (Maccari and Morley-Fletcher, 2007; Nestler and Hyman, 2010). To my knowledge, a direct comparison of these models during pregnancy has not been reported. Although restraint stress is somewhat easier to administer due to the consistent timing and similar approach between groups, I observed that pregnant females may have habituated to the stressor. I observed after only three days of restraint stress, pregnant females were docile and asleep in the restraints, perhaps indicative of an unstressed

animal. Additionally, since there were no substantial differences in the endpoints measured after prenatal restraint stress, I chose to continue the studies with chronic unpredictable mild stress to negate the observed habituation response.

An animal model of stress exposure in the pregnant rat with a well-established endocrine endpoint has not been established, although it has been investigated. Some groups have characterized the rat's stress response during pregnancy with "high-intensity" stressors typical of experiencing a traumatic event (Barlow et al., 1975). Other groups have examined the HPA response during a rat's pregnancy with a "low-intensity" stressor (Leonhardt et al., 2007) showing that stress-induced corticosterone release can be measured during this period. Linking stress in an animal and clinical depression is very difficult since our group feels that a rodent model of clinical depression has not been conclusively established. Clinical depression is a complex disorder, with a multifaceted pathophysiology. Although animal models can recapitulate some aspects of this pathophysiology (e.g. altered HPA activity, anhedonia), a complete model does not currently exist. Additionally, a subset of patients with major depressive disorder display altered HPA activity (Bourke and Owens, 2010). The current study's model was capable of producing an increase in basal corticosterone and a further increase of corticosterone in response to a stressor. We feel that stress in this study is a suitable proxy for maternal depression due to the quantifiable endpoint (increased baseline of corticosterone concentrations) that is believed to influence the development of the fetal HPA axis (Neigh et al., 2010). While others have shown that classic rises in maternal cortisol are offset by increases in cortisol-binding globulin such that the mother and fetal brains are

protected (Ballard et al., 1982; Coe et al., 1986), the effects of stress on maternal care or possible effects on other pathways in the fetus may cause altered neonatal development.

Chronic stress during pregnancy in our study produced a minor disruption in maternal care by reducing nursing time and increasing the time spent away from the infant during the inactive phase of the light-dark cycle. This did not affect the rate of weight gain of the pups (Table 2.4). Analysis of the light cycle or dark cycle separately showed that the persistent effects in the 24 hour period were confined to the light cycle when the animal is least active. While our scoring system accurately extrapolates the behavior over the 24 hour period, this is an important distinction to note as stress did not affect maternal care during the most active phase of the day. Other groups have found that stress during a rat's pregnancy results in disrupted maternal care, leading to long-term changes in the offspring (Champagne et al., 2003; Champagne and Meaney, 2006). However, these studies also selectively breed for maternal care behavior to determine the interaction of stress and trait-associated licking and grooming behavior. We found that stress itself did not disrupt licking and grooming behavior. Therefore, the limited effects of stress in the face of clear increases in endocrine measures of stress exposure in this study and the extant literature suggest that other factors, such as genetics, play important roles in maternal care behavior. Based on the work of Meaney and colleagues, a gene x environment interaction must be considered when examining maternal care behavior in the rodent (Meaney, 2010), however laboratory animals that have have been commercially bred for decades for research purposes are, arguably, selected for being good at providing maternal care.

Maternal care was altered due to prenatal treatments but licking and grooming behavior was not affected. Previous studies have shown that licking and grooming behavior have profound changes on the offspring's development and produces altered gene expression in adulthood (Weaver et al., 2005; Weaver et al., 2006). These studies of Weaver and colleagues breed the licking and grooming trait selectively in the animals to alter maternal care. Treatment with escitalopram alone did not change nursing behavior compared to saline controls; therefore, we conclude that gestational stress was marginally disruptive to maternal care and that treatment with escitalopram did not offset this deficit nor have independent effects alone.

Our model of stress and antidepressant exposure provides a reproducible endpoint in the pregnant dam. The stress model resulted in alterations in the HPA axis, small but statistically significant decrease in weight gain, and produced modest alterations in maternal care. Minipump administration of escitalopram oxalate resulted in clinically relevant serum concentrations not possible from acute dosing. When these methods were combined, they resulted in no substantial alterations in pup viability at parturition, growth rates between postnatal days 1-21, or maternal care behavior. These experiments provide a model for studies seeking to elucidate detailed investigations of antidepressant and/or stress exposure during pregnancy, as a model of maternal depression and its treatment, on offspring.

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Tables

Table 2.1. Summary of the chronic unpredictable mild stress model.

Gestational Day	Stress
G0	Breeding
G7	Implant catheters
G10	Restraint (3x45 minute sessions)
G11	Cage Tilt (24 hours)
G12	Damp bedding (24 hours)
G13	New cage; Noise (24 hours)
G14	Restraint (3x45 minute sessions)
G15	Overnight illumination
G16	New cage
G17	Restraint (3x45 minute sessions)
G18	Overnight illumination
G19	Damp bedding (24 hours)
G20	New cage; Noise (24 hours)
G21	Amniotic fluid collection

Table 2.2 Behavioral and corticosterone endpoints in pregnant rats exposed to the chronic restraint stress model.

	Time point	Test	Measure	Control	Restraint	p values (N=4-7)
Weight Gain	G5-G19	Rate of Weight Gain	(grams/day)	4.88 ± 0.53	4.84 ± 0.47	$p = 0.96$
Behavior	G19	Elevated Plus Maze	Open Arm Entries	0.429 ± 0.202	1.25 ± 0.620	$p = 0.26$
			Time in Open Arm (s)	20.3 ± 9.17	20.4 ± 14.3	$p = 0.64$
	G20	Defensive Withdrawal	Latency to Emerge (s)	9.43 ± 3.13	89.5 ± 42.8	$p = 0.11$
			Total Time in Tube (s)	419 ± 48.0	396 ± 47.1	$p = 0.74$
	PND30	Forced Swim Test	Struggling (s)	68.4 ± 8.60	56.1 ± 6.32	$p = 0.27$
			Floating (s)	23.0 ± 5.83	24.0 ± 8.43	$p = 0.92$
			Passive Swimming (s)	209 ± 11.0	220 ± 7.57	$p = 0.41$
			Latency to Float (s)	102 ± 12.3	116 ± 22.5	$p = 0.60$
Endocrine	PND30	Radioimmunoassay	Corticosterone (ng/mL)	1340 ± 203	1180 ± 141	$p = 0.53$

Behavior was measured in the elevated plus maze on gestational day 19 the defensive withdrawal test on gestational day 20, and the forced swim test on post-parturition day 30. Immediately after the forced swim test, the mothers were decapitated and trunk blood was collected to assess serum corticosterone in response to a novel stressor. Differences were assessed with an unpaired Student's t test. Data are presented as mean ± SEM, N = 6-8 animals/group.

Table 2.3. Behavioral and corticosterone endpoints in male and female offspring.

	Time point	Test	Measure	Gender	Adult Stress	Control	Prenatal Restraint
Behavior	PND64	Elevated Plus Maze	Time in Open Arm (s)	Male	-	53.3 ± 19.5	26.8 ± 12.2
				Male	+	51.8 ± 14.6	31.0 ± 18.4
				Female	-	34.3 ± 17.7	35.2 ± 12.4
				Female	+	31.3 ± 11.2	29.5 ± 11.4
			Open Arm Entries	Male	-	2.33 ± 0.422	1.83 ± 0.792
				Male	+	2.67 ± 0.803	1.57 ± 0.649
				Female	-	2.00 ± 0.817	2.20 ± 0.735
				Female	+	2.43 ± 0.751	1.67 ± 0.494
	PND65	Defensive Withdrawal	Time in Tube (s)	Male	-	224 ± 77.6	223 ± 37.7
				Male	+	401 ± 63.5	267 ± 48.7
				Female	-	133 ± 4.34	145 ± 45.3
				Female	+	331 ± 74.4	423 ± 57.3
			Latency to Withdraw (s)	Male	-	34.3 ± 19.3	78.8 ± 41.1
				Male	+	69.5 ± 56.9	86.8 ± 52.5
				Female	-	19.8 ± 10.1	11.6 ± 4.76
				Female	+	17.8 ± 8.98	46.8 ± 13.2
	PND65-67	Sucrose Preference Test	% Sucrose Consumed	Male	-	88.9 ± 3.41	90.2 ± 2.46
				Male	+	92.5 ± 0.468	90.9 ± 1.01
				Female	-	86.5 ± 4.29	87.5 ± 2.26
				Female	+	94.0 ± 0.368	80.7 ± 5.90
Endocrine	PND70	Radioimmunoassay	Corticosterone (ng/mL)	Male	-	59.3 ± 18.3	70.1 ± 36.4
				Female	-	82.6 ± 31.0	40.9 ± 25.8

A subset of animals were restrained and singly housed in adulthood. Behavior was measured in the elevated plus maze, the defensive withdrawal test, and the sucrose preference test. Basal corticosterone was measured post-decapitation from trunk blood. Differences were assessed with an unpaired Student's t test. Data are presented as mean ± SEM, N = 3-7 animals/group.

Table 2.4. Maternal and litter endpoints.

	Saline	Escitalopram	Stress-Saline	Stress-Escitalopram
Maternal Drug Conc. (ng/mL)	<0.5 ± 0.07	40.9 ± 3.38	<0.5 ± 0.23	35.1 ± 2.70
G5-19 Maternal Rate of Weight Gain (g/day)	7.01 ± 0.33	7.32 ± 0.74	6.16 ± 0.47 #	6.23 ± 0.39 #
Pregnancy Duration (days)	21.00 ± 0.24	21.00 ± 0.00	20.89 ± 0.11	21.20 ± 0.20
# of Pups Born	12.8 ± 0.7	12.1 ± 1.0	13.3 ± 0.7	12.9 ± 0.5
PND 1-21 Litter Rate of Weight Gain (g/day)	20.1 ± 0.8	19.3 ± 1.0	20.0 ± 1.0	20.7 ± 0.8

Pregnant rats were measured for rate of weight gain, serum escitalopram at G15, and number of pups born (N = 7-11 pregnant rats/group). Maternal rate of weight gain was decreased in dams exposed to stress, although this did not quite reach statistical significant (# $p = 0.05$). Groups were based on treatment to the pregnant dam: no prenatal stress with saline minipump (Saline), no prenatal stress with escitalopram minipump (Escitalopram), prenatal stress with saline minipump (Stress-Saline), prenatal stress with escitalopram minipump (Stress-Escitalopram). The average maternal serum concentrations of escitalopram over the entire course of the study (all cohorts for all chapters) was 31.7 ± 1.4 ng/mL. The culled litter weight normalized to pup number was used to calculate the prepubescent rate of weight gain. Data are mean ± SEM, N = 7-11 litters/group.

Figures

Figure 2.1. Determination of fluoxetine pharmacokinetics after acute subcutaneous dosing in non-pregnant females.

Animals were dosed subcutaneously with 11 mg/kg of fluoxetine hydrochloride in a vehicle solution of 40% sterile polyethylene glycol and 60% 0.9% sterile saline. Serial blood samples was collected from a jugular catheter over 48 hours. Samples were analyzed by HPLC. Serum fluoxetine increased immediately following injection followed by rapid clearance from the system (A). Serum concentrations were semi-log transformed (B). Data are mean ± SEM, N = 6 rats/group.

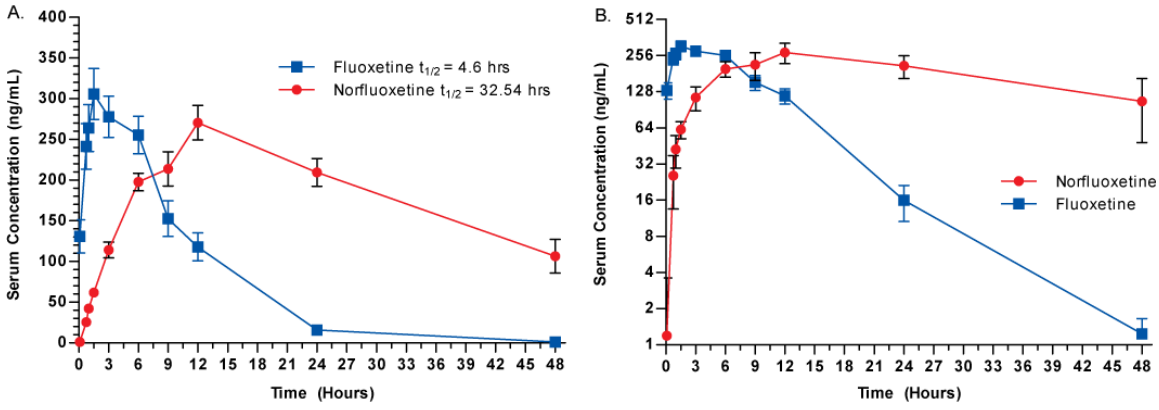


Figure 2.2. Dose-finding study to determine optimal dose of fluoxetine in pregnant rats. Fluoxetine hydrochloride (FLX) was dissolved in a vehicle solution of 40% sterile polyethylene glycol and 60% 0.9% sterile saline (saline), injected into a 28 day osmotic minipump, and implanted seven days before breeding. Blood was collected on gestational day 20. Serum concentrations of fluoxetine and norfluoxetine were determined by high performance liquid chromatography (HPLC) (A). Fluoxetine + norfluoxetine serum concentrations were added together to get FLX + NFLX serum concentrations (TOTAL). Weight was recorded throughout the study to determine if the high dose of fluoxetine significantly impacted rate of weight gain (B). The dose of fluoxetine in saline based on terminal weight was 8.76 ± 1.21 or 11.80 ± 0.88 mg/kg/day. Data are mean ± SEM, N = 6 rats/group.

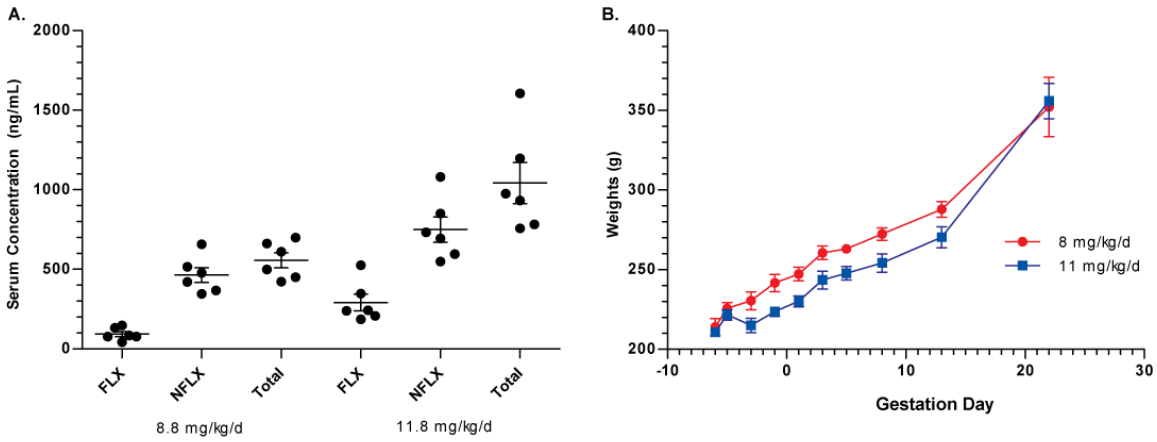


Figure 2.3. Pharmacokinetic study to determine optimal vehicle and detection method for escitalopram in pregnant rats.

Escitalopram oxalate (escit) was dissolved in 0.9% sterile saline (saline) or phosphate-buffered saline (PBS), injected into a 28 day osmotic minipump, and implanted seven days before breeding. Blood was collected on gestational day 20. Serum concentrations of escitalopram were determined by high performance liquid chromatography (HPLC) (A) or ultra performance liquid chromatography with a tandem mass spectrometry-based detection method (LC/MS-MS) (B). The dose of escitalopram in saline based on terminal weight was 12.20 ± 1.03 mg/kg/day. The dose of escitalopram in PBS based on terminal weight was 12.30 ± 1.38 mg/kg/day. Data are mean ± SEM, N = 5-7 rats/group.

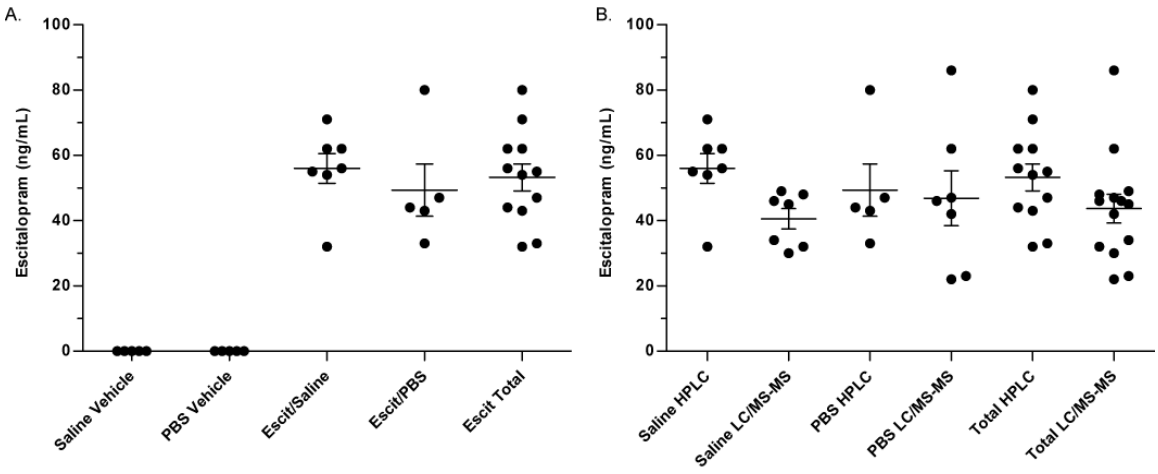


Figure 2.4. Serum drug concentrations following escitalopram administration.

To determine the clearance of escitalopram in acute dosing models, an acute subcutaneous or intraperitoneal dose of 12.2 mg/kg of escitalopram oxalate was injected into catheterized females and serum was collected over 9 hours (A, N = 3-5 rats/group). Pregnant catheterized females were implanted with osmotic minipumps delivering 12.2 mg/kg/day of escitalopram oxalate. Blood was collected on gestational days 6, 10, 13, 16, 18, and 20 via a jugular vein catheter and analyzed for serum escitalopram (B, N = 7-10 rats). Shaded areas reflect the clinically range observed in patients at the Emory Women's Mental Health Program (17-65 ng/mL; N = 60).

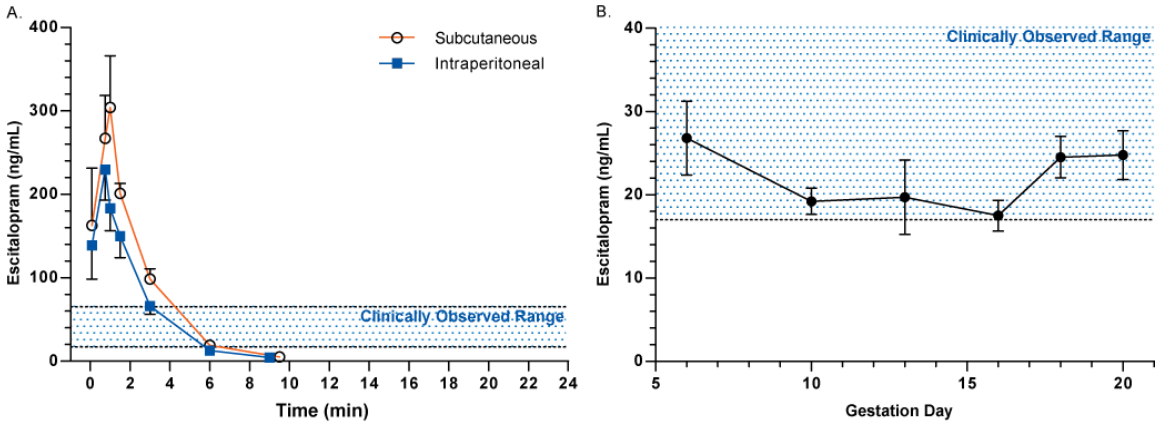


Figure 2.5. Serum escitalopram concentrations compared to weight throughout pregnancy.

Serum escitalopram concentrations were compared to weight gain over the course of pregnancy. There was no effect of weight gain on serum escitalopram concentrations ($p > 0.05$).

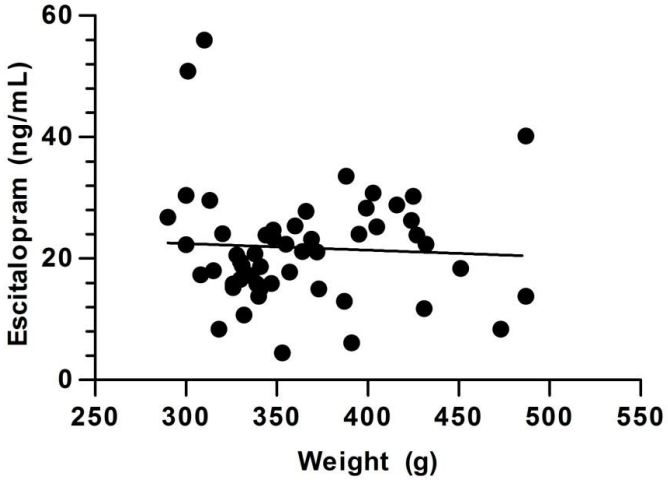


Figure 2.6. Corticosterone measurements during the chronic unpredictable mild stress model.

Pregnant females implanted with jugular catheters were sampled over the course of pregnancy at 9:00 and 9:45. Baseline corticosterone in plasma was measured in control and stress (CMS) animals (A). CMS-exposed animal plasma collected at 9:00 and 9:45 was used to illustrate the daily response to stress in individual animals after the restraint stressor (G10, G14, G17) and the damp bedding stressor (G12, G19) (B). The plasma measurement of corticosterone at 9:45 was compared between control and CMS animals to illustrate the corticosterone response compared to controls at the same time of plasma measurement (C). Data are mean ± SEM, N = 5-11 rats/group. * $p < 0.05$, ** $p < 0.01$ with a Bonferroni *post hoc* Test.

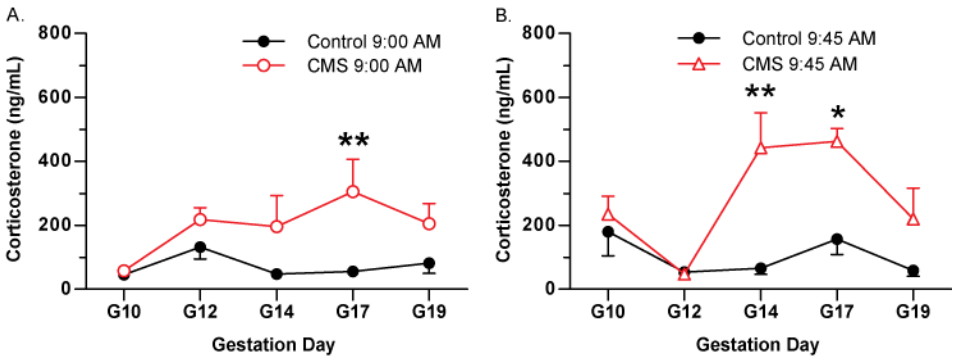


Figure 2.7. Maternal contact behavior following chronic unpredictable mild stress and escitalopram administration during pregnancy.

The timeline of the experiment is summarized including the chronic unpredictable stress model, escitalopram administration, and endpoints (A). Maternal care behavior was videotaped continuously for 10 days and sampled for one hour each day during the light and dark cycles. Time spent without maternal contact was increased due to stress when measured over a 24 hour period for ten days (B). When analyzed separately, the time spent without maternal contact was reduced in the light cycle when the mother is less active (C) but not the dark cycle when the mother is more active (D). Groups were based on treatment to the pregnant dam: no stress with saline minipump (Saline), no stress with escitalopram minipump (Escitalopram), prenatal stress with saline minipump (Stress-Saline), prenatal stress with escitalopram minipump (Stress-Escitalopram). Data are mean ± SEM, N = 7-11 mothers/group. * $p < 0.05$, main effect due to stress in a 3-way ANOVA (Stress x Escitalopram x Time).

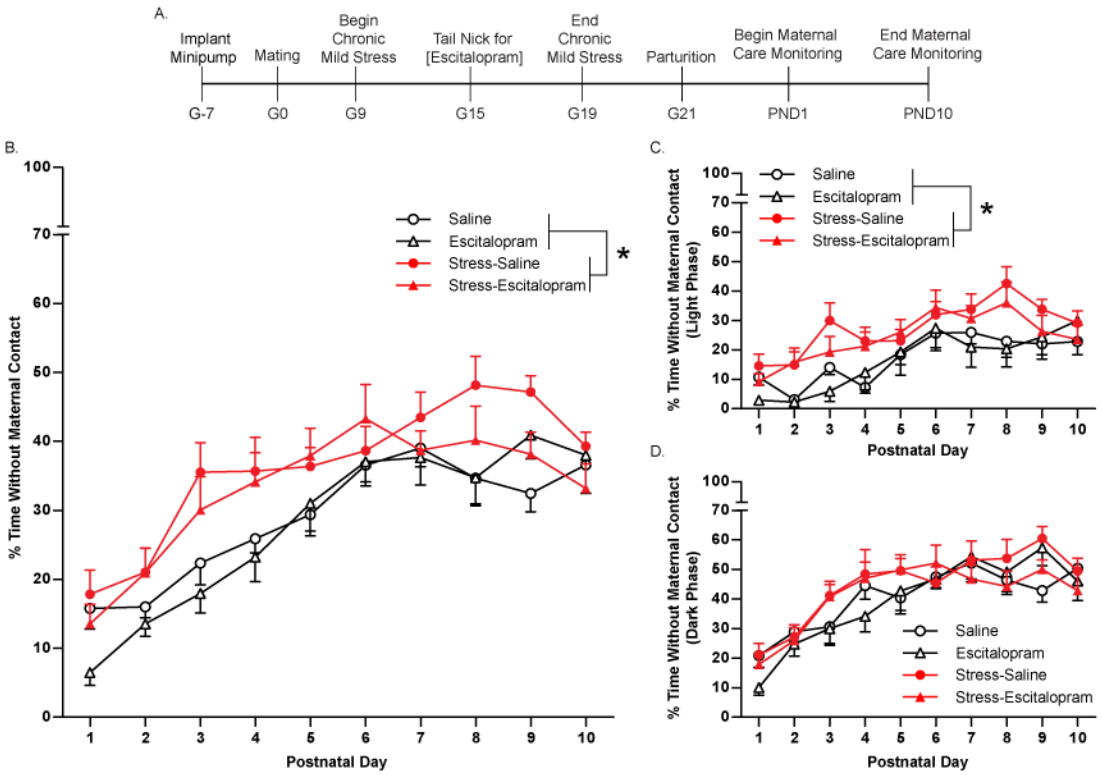
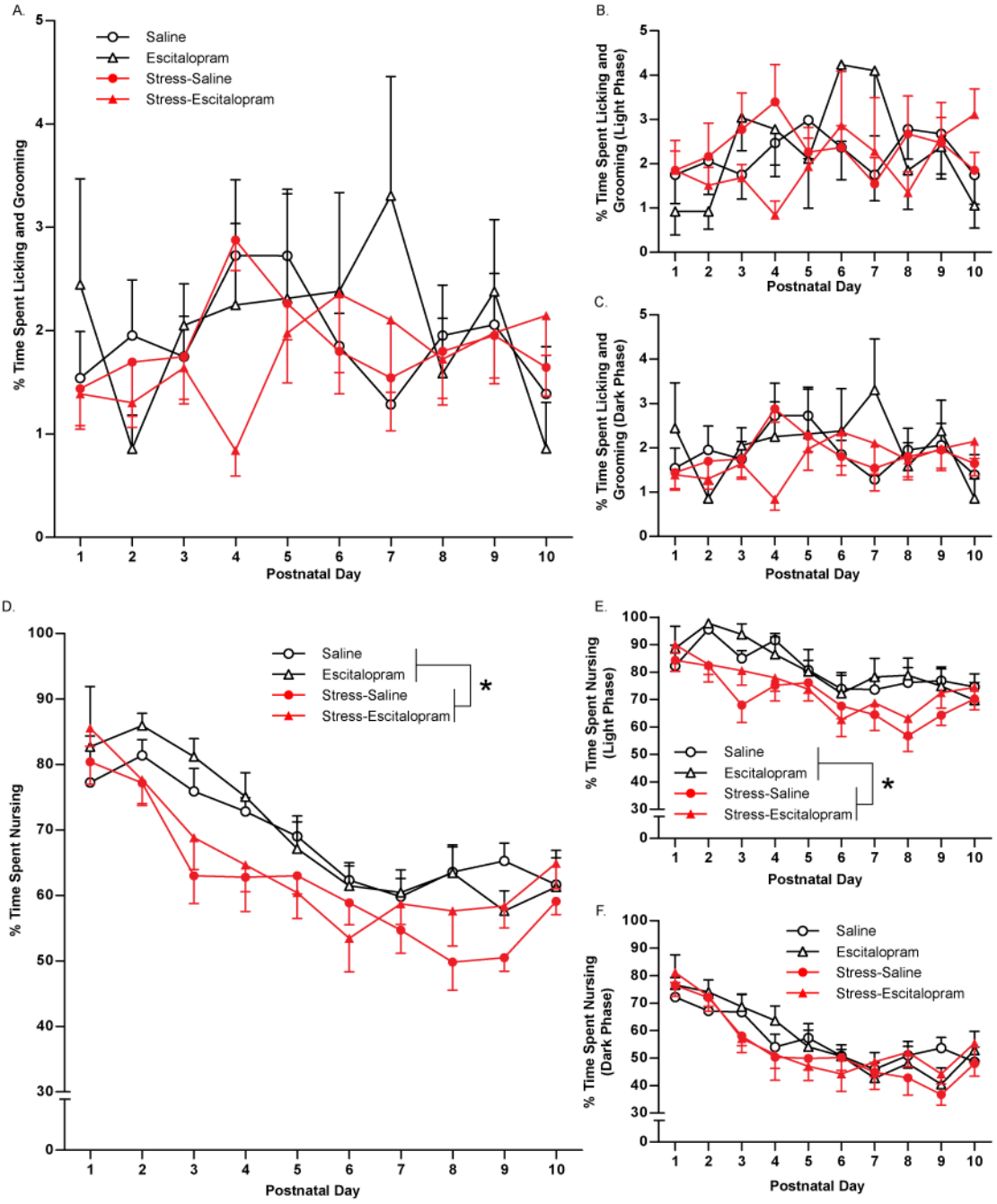

A.
Implant Minipump
Mating
Begin Chronic Mild Stress
Tail Nick for [Escitalopram]
End Chronic Mild Stress
Parturition
Begin Maternal Care Monitoring
End Maternal Care Monitoring
G-7
G0
G9
G15
G19
G21
PND1
PND10
B.
% Time Without Maternal Contact
Postnatal Day
Saline
Escitalopram
Stress-Saline
Stress-Escitalopram
*
C.
% Time Without Maternal Contact (Light Phase)
Postnatal Day
Saline
Escitalopram
Stress-Saline
Stress-Escitalopram
*
D.
% Time Without Maternal Contact (Dark Phase)
Postnatal Day
Saline
Escitalopram
Stress-Saline
Stress-Escitalopram

Figure 2.8. Maternal licking and grooming behavior and nursing behavior.

Maternal care behavior was videotaped continuously for ten days and sampled for one hour each day during the light and dark phases. The percent of time spent licking and grooming was unaffected over a 24 hour period for ten days (A), during the light cycle (B), or during the dark cycle (C). The percent of time spent nursing was reduced due to stress over a 24 hour period for ten days (D). Reduction in nursing due to stress was only observed in the light cycle (E) but not the dark cycle (F). Groups were based on treatment to the pregnant dam: no stress with saline minipump (Saline), no stress with escitalopram minipump (Escitalopram), prenatal stress with saline minipump (Stress-Saline), prenatal stress with escitalopram minipump (Stress-Escitalopram). Data are mean ± SEM, N = 7-11 mothers/group. * $p < 0.05$, main effect due to stress in a 3-way ANOVA (Stress x Escitalopram x Time).


A.
Saline
Escitalopram
Stress-Saline
Stress-Escitalopram
% Time Spent Licking and Grooming
Postnatal Day
B.
% Time Spent Licking and Grooming (Light Phase)
Postnatal Day
C.
% Time Spent Licking and Grooming (Dark Phase)
Postnatal Day
D.
Saline
Escitalopram
Stress-Saline
Stress-Escitalopram
*
% Time Spent Nursing
Postnatal Day
E.
% Time Spent Nursing (Light Phase)
Saline
Escitalopram
Stress-Saline
Stress-Escitalopram
*
Postnatal Day
F.
% Time Spent Nursing (Dark Phase)
Saline
Escitalopram
Stress-Saline
Stress-Escitalopram
Postnatal Day

CHAPTER 3: PRENATAL EXPOSURE TO ESCITALOPRAM AND/OR STRESS IN RATS: LIMITED EFFECTS ON ENDOCRINE OR BEHAVIORAL MEASURES IN ADULT MALE RATS

Abstract

I investigated the long-term effects of prenatal stress and/or clinically relevant antidepressant exposure on male adult offspring in a model of the pharmacotherapy of maternal depression. Female Sprague-Dawley rats were implanted with osmotic minipumps that delivered clinically relevant exposure to the antidepressant escitalopram. Subsequently, pregnant females were exposed on gestational days 10-20 to a chronic unpredictable mild stress paradigm. The male offspring were analyzed in adulthood to determine the long-term effects of prenatal exposures. Baseline physiological measurements were largely unaltered by prenatal manipulations. Behavioral characterization of the male offspring, with or without pre-exposure to an acute stressor, did not reveal any group differences. Prenatal stress exposure resulted in a faster return towards baseline of plasma corticosterone following the peak response to an acute restraint stressor, but not an airpuff startle stressor, in adulthood. This model of maternal depression and its treatment indicate that escitalopram use and/or stress during pregnancy produced no alterations in our measures of male adult behavior, however prenatal stress exposure resulted in some evidence for increased glucocorticoid negative feedback following an acute restraint stress.

Introduction

Depression affects 10-20% of pregnant women (Gavin et al., 2005). Although it is becoming increasingly well-known that untreated maternal depression during pregnancy is detrimental to infant outcome (Dunkel Schetter and Tanner, 2012), the known and unknown risks associated with antidepressant treatment lead to increased discontinuation of antidepressant use during pregnancy (Bonari et al., 2005). Although the short-term effects of prenatal antidepressant exposures have been investigated, the long-term effects, if any, are relatively unknown. This chapter aims to elucidate any long-term behavioral or endocrine alterations due to *in utero* manipulations in order to aid clinicians and their patients in treatment decisions.

Investigation of the long-term effects of prenatal stress, one possible proxy for maternal depression during pregnancy, and antidepressants concurrently has not been rigorously examined. In animal models, there are numerous studies that have examined types of prenatal stress (Newport et al., 2002; Mueller and Bale, 2006; Mueller and Bale, 2008) and several studies that have examined antidepressant-exposed pregnant rats from the perspective of the offspring (Henderson and McMillen, 1993; Cabrera and Battaglia, 1994; Cabrera-Vera et al., 1997; Cabrera-Vera and Battaglia, 1998; Forcelli and Heinrichs, 2008). To my knowledge, prenatal stress and antidepressant exposure have not been examined concurrently and used to investigate the effects on the offspring. However, recent health care data bases indicate that over 6% of pregnant women receive a prescription for an antidepressant (Andrade et al., 2008), therefore it can be argued that investigations of both stress and antidepressant exposure are potentially the most clinically relevant scenario. I hypothesize that, in our rat model, escitalopram

intervention during the stress treatment may attenuate any long-term effects of the prenatal stress exposure on outcome measures in the offspring. Additionally, no published study to date has examined the transcriptome in the context of prenatal antidepressant exposure.

Translation of clinically relevant models of prenatal exposures is necessary to adequately evaluate the risk to the mother and infant. Previously, we have modeled stress and antidepressant exposure to the offspring (Chapter 2; (Capello et al., 2011) with special emphasis on clinically relevant antidepressant exposure using the antidepressant escitalopram as representative of the class. These studies have demonstrated that chronic variable stress during pregnancy produces an increase in basal plasma corticosterone concentrations indicative of an activated hypothalamic-pituitary-adrenal (HPA) axis in response to this chronic stressor. Additionally, we have demonstrated that continuous delivery via osmotic minipumps results in serum escitalopram concentrations maintained within a clinically observed range and providing occupancy of the serotonin transporter in fetal rat brain >80% (Capello et al., 2011). This methodology is utilized in this study to model antidepressant treatment of depression during pregnancy.

Materials and Methods

Animals

The experimental time line is depicted in Figure 3.1. Sprague-Dawley male, retired breeders and nulliparous females weighing 200-225 grams were purchased from Charles River Laboratories (Charles River, Wilmington, MA). Rats were kept on a 12:12 light: dark cycle (lights on at 7:00 AM) in a humidity (60%) and temperature (20°C-23°C) controlled facility. Rodent diet 5001 chow (Purina Mills, Richmond, IN) and water were available *ad libitum* throughout the study. After two weeks at the Emory University animal facility, female rats were paired with male retired breeders in a breeding cage. Gestational day 0 (G0) was designated by the presence of a sperm plug and pregnant females were single-housed after breeding. Three days after birth, rat pups were sexed and litters were culled to six male and two female pups. Animals were weaned on PND21 and kept pair-housed until adulthood. Because pup growth rates and maternal licking and grooming behavior, a hallmark of good maternal care, were unaltered by either prenatal stress and/or escitalopram exposure, maternal care was performed by the biological dam rather than a foster dam (Chapter 2). Escitalopram release from the minipumps ended between 0-2 days post parturition (Alzet model 2ML4 technical information).

For the adult offspring endpoints, in almost all circumstances only a single pup was used from each litter in order to prevent possible litter effects (Holson and Pearce, 1992). When that was not possible, no more than two pups per litter were assigned to a group and are noted at the appropriate point within the manuscript. Each group was assigned between 8 and 12 pups. Rats were killed via rapid decapitation on postnatal day

(PND) 90-95 during the early phase of the light cycle. The brain was removed and immediately dissected on ice. Adrenals were removed and weighed. An additional manipulation of twelve days of chronic immobilization stress in adulthood was used in a separate cohort of animals to determine if a stress-susceptible phenotype existed. Animals were immobilized in DecapiCone® bags (Braintree Scientific, Braintree, MA) for two hours once a day for 12 days (Neigh et al., 2010). One day after the last stress session, control and immobilization stress animals were killed.

Ethics Statement

All animal protocols were approved by the Emory University Institutional Animal Care and Use Committee and carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Resources, 1996) as adopted and promulgated by the U.S. National Institutes of Health. All steps were taken to minimize animal suffering at each stage of the study.

Escitalopram Administration and Chronic Unpredictable Mild Stress Model

Nulliparous female rats were subcutaneously implanted with Alzet 28 day osmotic minipumps (model 2ML4, Alzet, Cupertino, CA) slightly posterior to the scapulae. Osmotic pumps delivered either 0.9% saline or 12.2 mg/kg/day escitalopram oxalate in 0.9% saline based upon a predicted weight of the pregnant dam on G21 of 400 grams (unpublished observations). Three days after minipump implantation, females were bred with retired breeder males. On G10, the chronic unpredictable mild stress model of depression began and consisted of restraint, cage tilt, damp bedding, noise, cage

changes, and overnight illumination (Chapter 2). G10 was selected to begin the stress paradigm because the fetal central nervous system begins substantial development at this point (Clancy et al., 2001) and to minimize premature termination of the pregnancy as a result of excessive stress. On G15, all pregnant dams, regardless of treatment group, were given a tail nick to determine the serum concentration of escitalopram. No pregnant dams with escitalopram minipumps had serum escitalopram concentrations below 17 ng/mL. The stress model ended on G20 to prevent premature parturition.

Physiological Measures

Plasma corticosterone concentrations were assayed using the ImmuChem ^{125}I Corticosterone RIA kit with a sensitivity of 1 ng/mL (MP Biomedicals, Orangeburg, NY). Plasma ACTH concentrations were assayed using the ^{125}I ACTH RIA kit with a sensitivity of 1.5 pg/mL (Diasorin, Stillwater, MN). Non-fasting blood glucose concentrations were measured with the Freestyle Blood Glucose Meter with Freestyle Test Strips (TheraSense, Alameda, CA). Insulin concentrations were measured with a Mercodia Rat Insulin ELISA with a sensitivity of 0.15 µU/mL (ALPCO Diagnostics, Windham, NH). Insulin resistance was calculated by a product of insulin and glucose analogous to the homeostatic model assessment of insulin resistance (HOMA-IR) calculation: [glucose] x [insulin] / 405. Values were normalized to the control group median [glucose] x median [insulin] values such that control mean value would approximate 1. Glucose and insulin measures were not taken after 24 hours of fasting which would have interfered with our main experimental interests and design. However, useful indications of a possible predisposition to a future diabetic phenotype can still be

ascertained from nonfasting glucose and insulin serum concentrations (Wannamethee et al., 1999). Heart tissues were placed in 0.9% sterile saline, dissected on ice, and weighed to determine total heart weight and right ventricle: left ventricle + septum weight as described previously (Fornaro et al., 2007).

Behavioral Testing

Behavioral testing was conducted as previously described (Bourke and Neigh, 2011). Briefly, animals were tested during the beginning of the dark cycle for the defensive withdrawal test (10 min), elevated plus maze (5 min), open field test (15 min), marble burying test (15 min) and acoustic startle response (15 min). The sucrose consumption test was conducted over 48 hours and fluid consumption amounts were adjusted for body weight. The forced swim training (10 min) and test (5 min) periods were both conducted in the light cycle. Although the test was administered, the animals showed extreme difficulty with maintaining an immobile floating position likely due to the heavy mass of the adult animals. While the test was conducted, it was not analyzed due to this confounding variable. Locomotion and exploratory behavior were assessed with the TopScan and ForcedSwimScan systems (Cleversys, Reston, VA). All tests were conducted between PND 90-120 and separated by seven days. A 15 minute restraint using plastic restrainers designed to minimize movement (Braintree Scientific, Braintree, MA) was used in a separate cohort of animals before the open field, marble burying test, acoustic startle response, and the forced swim test session. Other tests (elevated plus maze, defensive withdrawal, and sucrose consumption) did not incorporate the acute restraint in order to minimize habituation to a homotypic stressor.

Jugular Catheter Studies

Jugular catheters were implanted as described previously (Thrivikraman et al., 2002). Briefly, animals were anesthetized with a preparation of ketamine: xylazine: acepromazine and assessed for reaction to a painful stimuli prior to surgery. The jugular vein was implanted with a catheter to allow for repeated blood sampling and to prevent a stress response elicited by other sampling methods (i.e., tail nick). Animals were given four days to recover before initiation of an air puff startle stressor and serial blood sampling (Engelmann et al., 1996). Briefly, a baseline blood sample was collected at 9:00 AM. Animals were then exposed to three trials of three short air puffs (Air Duster, Office Max) on the head and given one minute to recover between trials. Blood samples (200 µL) were collected and an equal volume of sterile, 0.9% saline was injected to replace the blood volume lost. Catheters were flushed with 150 µL of sterile gentamicin (120 µg/mL) solution after sampling to prevent infection. The restraint stress serial blood sampling was conducted four days after air puff serial blood sampling. A baseline blood sample was collected at 9:00 AM and the animals were immobilized for 5 minutes in DecapiCone® bags (Braintree Scientific, Braintree, MA). Blood samples were collected as described above.

CRF Receptor Binding of the Anterior Pituitary

Single point CRF receptor binding assays were performed as previously described (Ladd et al., 1996). Briefly, anterior pituitary samples were homogenized in assay buffer (55 mM HEPES, 10 mM $MgCl_2$, 2.2 mM EGTA, 0.1% BSA, 0.005% TritonX100, 1 µg/mL aprotinin, pH to 7.2). An isotopic dilution of 0.9 nM sauvagine (American

Peptide Company, San Diego, CA) and 0.1 nM ^{125}I-sauvagine (Perkin Elmer, San Jose, CA) was prepared and incubated for two hours at room temperature in assay buffer with homogenized tissue samples. Samples were washed in cold PBS and counted on an LKBγ counter (Perkin Elmer, San Jose, CA; 42% efficiency).

Statistical Analyses

In all figures, values are expressed as mean ± SEM. GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA) and SPSS 17.0 (IBM, Armonk, NY) were used to conduct statistical analyses. Experimental differences were analyzed with a 2-way ANOVA (prenatal stress x prenatal escitalopram) followed by a Dunnett's *post hoc* test where appropriate. A 3-way ANOVA was used to analyze prenatal escitalopram x prenatal stress x adult stress effects. Jugular catheter sampling measures were analyzed with a 3-way ANOVA with repeated measures on time using a Huynh-Feldt correction.

Results

Offspring Endpoints after Chronic Unpredictable Mild Stress and/or Escitalopram Exposure

The experimental time line is depicted in Figure 3.1. Juvenile and adolescent weight gain of the offspring was not adversely affected by either treatment, indicating that maternal stress and/or escitalopram exposure does not interfere with normal growth (Table 3.1, top). Basal measures of pituitary and adrenal output were assessed in adulthood. ACTH concentrations in the prenatal stress alone group were lower than the other groups (Bonferroni *post hoc* test, $p < 0.01$). Plasma corticosterone concentrations were increased due to prenatal escitalopram treatment ($F_{(1,63)} = 7.1$; $p < 0.01$); however, these increases, as well as the observed change in ACTH concentrations (*vide supra*), were well within the ranges routinely observed at trough baseline in nonstressed rats and the physiological significance of these changes are unclear. Adrenal weights were not significantly different.

Previous studies have shown a link between metabolic syndrome and prenatal stress (Tamashiro et al., 2009; Li et al., 2010); therefore, we examined glucose, insulin, and insulin resistance in adulthood. At baseline and after *ad libitum* food availability overnight, blood glucose was not different between groups. Plasma insulin was decreased due to prenatal stress ($F_{(1,77)} = 4.3$; $p < 0.05$) and there was a trend of a decrease in plasma insulin due to prenatal escitalopram ($F_{(1,77)} = 3.4$; $p = 0.07$). Insulin resistance, assessed using the HOMA-IR calculation, was decreased by both prenatal stress ($F_{(1,75)} = 4.1$; $p < 0.05$) or prenatal escitalopram ($F_{(1,75)} = 4.3$; $p < 0.05$) (Table 3.1,

bottom). As with the basal ACTH and corticosterone measures, these were not considered to be physiologically significant after consultation with diabetes specialists.

An increased risk of offspring pulmonary hypertension following antidepressant exposure during pregnancy has been reported in human and animal studies (Chambers et al., 2006; Fornaro et al., 2007). An assessment of pulmonary hypertension was conducted by measuring the right ventricle: left ventricle + septum weight ratio as previously described (Fornaro et al., 2007). Neither total heart weight nor RV:LV + septum ratio was affected by prenatal treatments (Table 3.1, bottom).

Behavioral Characterization of Male Adult Offspring

Behavioral characterization was carried out to determine any phenotypic behavioral differences that arise from prenatal exposure to stress and/or escitalopram. Where denoted, an additional manipulation of chronic adult stress was used to investigate susceptibility or resistance to stress in adulthood. Several behavioral endpoints associated with locomotion, exploratory behavior, and risk-taking behavior were measured and analyzed but none reached statistical significance. The most representative measurement for each test is described in this section and displayed in Figure 3.2. In the elevated plus maze, the defensive withdrawal test, the sucrose consumption test, and the open field test, prenatal treatments had no effect on behavioral measures (Fig. 3.2A-D). In the marble burying test, the number of three quarter or fully buried marbles were unaltered due to prenatal stress ($F_{(1,57)} = 0.2$; $p > 0.05$) or prenatal escitalopram ($F_{(1,57)} = 0.9$; $p > 0.05$), however acute restraint stress challenge before the test decreased marble burying behavior for all treatment groups compared to rats not exposed to acute restraint

($F_{(1,57)}$ = 8.1; $p < 0.01$) (Fig. 3.2E) similar to another report of restraint stress decreasing marble burying behavior (Umathe et al., 2009). The magnitude of the responses to acute restraint stress challenge was not different amongst the various prenatal exposures. Neither prenatal stress, prenatal escitalopram, nor the response to acute restraint stress challenge altered the acoustic startle response in terms of percent habituation or initial startle amplitude (Fig. 3.2F).

Serial Blood Sampling after Acute Stress

An air puff startle was used to assess pituitary (ACTH) and adrenal (corticosterone) responsiveness to a mild stressor in adulthood following the various prenatal exposures. The air puff startle increased plasma ACTH in all groups ($F_{(1.52,36.39)}$ = 25.3; $p < 0.001$) with no significant response differences due to prenatal escitalopram. Prenatal stress had a trend towards increased ACTH responsiveness compared to the nonstressed groups (2-way ANOVA comparison of stress x drug, $F_{(1,24)}$ = 4.1; p = 0.055) (Fig. 3.3A). Cumulative output over 90 minutes was assessed using area under the curve calculations. Prenatal escitalopram did not alter cumulative plasma ACTH ($F_{(1,28)}$ = 0.03; $p > 0.05$), however prenatal stress produced a clear trend of increased total ACTH secretion ($F_{(1,28)}$ = 3.0; p = 0.095).

Acute air puff startle increased plasma corticosterone in all groups ($F_{(4.36,104.64)}$ = 60.2; $p < 0.001$) (Fig. 3.3B). The response was not different between any of the prenatal treatments, however there was a trend over time for prenatal stress to increase plasma corticosterone concentrations via a more prolonged response ($F_{(4.36,104.64)}$ = 2.3; p = 0.062) (Fig. 3.3B). Cumulative adrenal output was assessed by calculating the plasma

corticosterone area under the curve. The corticosterone area under the curve over 90 minutes was unaffected by prenatal stress ($F_{(1,27)}$ = 2.1; $p > 0.05$) or prenatal escitalopram ($F_{(1,27)} = 0.1$; $p > 0.05$). An acute restraint stress was used to evaluate the adrenal response to a heterotypic stressor. Acute restraint increased plasma corticosterone in all groups regardless of a history of prenatal stress ($F_{(4.54,81.78)} = 40.2$; $p < 0.001$) (Fig. 3.3C). There was a trend of prenatal stress exposure to return corticosterone concentrations towards baseline more quickly following acute restraint ($F_{(1,18)} = 4.0$; $p = 0.06$). This trend was also reflected by the area under the curve calculations: prenatal stress exposed rats had decreased cumulative plasma corticosterone over the 90 minute sampling period ($F_{(1,21)} = 7.6$; $p < 0.05$). ACTH sampling was not possible in this experiment.

CRF Receptor Binding in the Anterior Pituitary

To determine if HPA negative feedback was adversely affected at the level of the pituitary, CRF receptor binding in the anterior pituitary was measured with ^{125}I-sauvagine radioligand binding. Neither prenatal stress nor prenatal escitalopram had an effect on CRF receptor binding in the pituitary (stress: $F_{(1,34)} = 0.0$; $p > 0.05$, escitalopram: ($F_{(1,34)} = 0.1$; $p > 0.05$) (Fig. 3.4).

Discussion

The use of psychotropic medications may be clinically necessary in some patients and discontinuation during pregnancy is unrealistic and significantly increases the risk for recurrent depression during pregnancy (Cohen et al., 2006). The majority of epidemiological studies report that the risks to the infant are relatively low (Wisner et al., 1999). These studies have largely focused on birth defects, especially septal heart defects. Others have shown that the risk to the infant is typically overestimated and no higher than antibiotic or gastric medication use during pregnancy: approximately 1-3% (Bonari et al., 2005). In the present study, I found no immediate or long-term gross teratological effects from prenatal exposures.

The justification for the use of male offspring in this study follows primarily from published studies as well as the complications due to using female offspring. A recent study conducted with chronic unpredictable mild stress found that male offspring had a depressive-like behavioral phenotype that was absent in female offspring (Mueller and Bale, 2008). In addition, the choice to use adult offspring would be further complicated with a female group due to the influence of the ovarian hormones on stress responsivity (Bourke et al., 2012). Although these complications could be circumvented through the use of ovariectomy or estrous cycle tracking, the statistical comparisons would be so complex as to be uninterpretable. However, our group is currently exploring the behavioral and endocrine phenotype in females exposed to these prenatal exposures as a separate experiment.

Physiological measures of the male offspring showed almost no changes in growth, development, baseline endocrine measures, or gross histological differences

compared to animals born to dams unexposed to stress or escitalopram (Table 3.1). While measures of ACTH and insulin were statistically significant, I believe these small differences are not physiologically meaningful. Stress-induced plasma levels of ACTH are typically observed above 75 pg/mL (Fig. 3.3A) and elevated plasma insulin levels are usually documented above four ng/mL (Tamashiro et al., 2009).

As shown in Figure 3.2, when assessed in adulthood in males, I observed no changes in behaviors routinely utilized in rodents to study effects on measures of anxiety or affect. Additionally, a stress-challenge in these animals did not reveal any increased susceptibility and/or resiliency associated with any of the prenatal exposures. While I expected an increase in anxiety-like measures from the stress-challenge, these behavioral tests were originally developed for assessing efficacy of antidepressants and anxiolytics. Additionally, the observed decrease in marble burying behavior due to acute restraint is similar to a report in the literature documenting the effects of acute stress in the marble burying test (Umathe et al., 2009). Chronic stress would likely have a robust effect but the present study sought to determine the effect of *in utero* exposures on acute stress reactivity.

Gingrich and colleagues have investigated the use of serotonin reuptake inhibitors during postnatal development and find long-term behavioral alterations in mice (Ansorge et al., 2004; Ansorge et al., 2008). Our results do not replicate their findings, however there are several key differences which may account for this discrepancy. Perhaps most importantly, the studies employ a dosing regimen during the postnatal period (PND4-21) that has no temporal overlap with the present studies. Concise ontogeneological data on human serotonergic function in the CNS is lacking and definitive mapping of our *in vivo*

exposure and their postnatal exposure onto human fetal/infant serotonergic development is not possible at present (Avishai-Eliner et al., 2002). Importantly, the distribution of serotonergic neurons in rats is similar on G19 to adult groupings (Lidov and Molliver, 1982). While the dosing regimens utilized by Gingrich and colleagues postnatally lead to "therapeutic" levels of SERT transporter occupancy and that drug exposure is maintained throughout their studies, I argue that this may not be fully relevant to humans because, following birth, exposure via lactation is low and infants are unlikely to receive any type of therapeutic dose of antidepressants for medical reasons for the first few years of life. Additionally, I observe that peak escitalopram concentrations following subcutaneous injection are up to 12-fold higher than that observed during continuous exposure (Chapter 2). Similar peaks, of unknown magnitude, should also be expected following subcutaneous or intraperitoneal fluoxetine administration. These transient peaks would clearly be suprapharmacological, possibly toxic and may be responsible for their findings but there is no data to support this possibility at present.

Maternal depression poses significant risks to the developing fetus and can produce dysregulation of the child's stress response measured as behavioral and salivary cortisol alterations (Brennan et al., 2008; Oberlander et al., 2008a). Previously, I have shown that chronic unpredictable mild stress produces an increase in baseline and peak corticosterone in pregnant animals (Chapter 2). In the adult male offspring, I observed no major differences in baseline plasma ACTH or corticosterone concentrations. An air-puff startle stress challenge revealed a nonsignificant trend towards increased ACTH and corticosterone responses in animals exposed to prenatal stress with no protective effects of escitalopram co exposure (Fig. 3.3A, B). In a similar cohort of rats, a restraint stress

challenge did not reveal any increased plasma corticosterone responsivity in animals exposed to prenatal stress (Fig. 3.3C). Indeed, prenatal stress was associated with decreased total corticosterone responses (area under the curve) over the 90 minute time period with the curves showing similar peak responses but a faster return towards baseline in the prenatally stressed rats possibly indicative of more efficient negative feedback (no changes were observed in hippocampal glucocorticoid receptor mRNA expression, CRF receptor binding in the pituitary, or chaperone protein mRNA that might explain this effect; Fig. 3.4 and 4.7A). The failure of this study to replicate other studies investigating the long-term effects of prenatal stress may be due to developmental timing of the stress exposure. Although the brain in rodents does not begin to robustly develop until mid gestation, data suggests that early gestational stress may be the most detrimental to the infant (Mueller and Bale, 2008).

The goal of the present study was to evaluate the long-term effects of prenatal stress and antidepressant exposure on adult male offspring. It should be noted that these results only apply to our particular developmental time frame for both exposure and testing. Testing of the exposed offspring in adulthood was chosen based on the fact that mammals, both humans and laboratory rodents, spend the majority of their lifespan as adults and I may have missed changes that may have occurred earlier in life and dissipate by adulthood or changes that do not occur until late life. Alterations due to prenatal exposures may be detected during these time frames but testing in adulthood allows determination of the persistent changes that may be present at earlier time points but do not normalize by adulthood. The present study also focused on male offspring. A study

of sex differences after prenatal exposures using the model described in this study may reveal sex-specific differences based on the *in utero* environment.

In conclusion, I employed a novel and clinically-relevant model of maternal depression and its treatment with the antidepressant escitalopram to examine the long-term effects in the offspring. Prenatal exposures produced no alterations in behavior, transcriptome-wide gene expression, or gene expression of several neuropsychiatric targets in the hippocampus of male rats. Subtle differences were observed in the pituitary and adrenal function of prenatally-stressed rats, indicating possible changes in HPA axis function. For the endpoints examined here, prenatal escitalopram exposure produced no physiologically-relevant alterations in the adult male offspring.

Acknowledgements

Escitalopram oxalate was generously provided by Lundbeck Research USA (Paramus, NJ). I would like to thank Catherine Capello and Faketa Zeljenovik for their assistance in breeding, treating and raising the animals.

Tables

Table 3.1. Physiologic measures in adulthood.

	SAL	ESCIT	S-SAL	S-ESCIT
PND 25-81 Rate of Weight Gain (g/day)	6.9 ± 0.1	7.0 ± 0.1	7.1 ± 0.1	6.7 ± 0.3
ACTH (pg/mL)	28.2 ± 1.9	24.5 ± 1.1	20.4 ± 1.1 *	29.2 ± 2.1
Corticosterone (ng/mL)	11.6 ± 1.1	19.8 ± 4.6 #	10.8 ± 1.4	15.0 ± 2.0 #
Adrenal Weight (mg)	57.4 ± 3.0	61.4 ± 3.1	60.8 ± 2.4	61.7 ± 2.6
Glucose (mg/dL)	102 ± 1.4	101 ± 2.0	101 ± 2.0	102 ± 2.4
Insulin (μU/mL)	3.4 ± 0.3	2.8 ± 0.2	2.7 ± 0.2	2.4 ± 0.2 **
Insulin Resistance (IR)	0.99 ± 0.08	0.80 ± 0.05	0.80 ± 0.06	0.69 ± 0.060 **
Heart Weight (g)	1.83 ± 0.05	1.80 ± 0.08	1.70 ± 0.03	1.75 ± 0.04
RV:LV + Septum	0.25 ± 0.01	0.25 ± 0.01	0.26 ± 0.01	0.25 ± 0.01

Groups were based on treatment to the pregnant dam throughout gestation: SAL (saline treatment during gestation), ESCIT (escitalopram exposure during gestation), S-SAL (chronic unpredictable mild stress + saline during gestation), S-ESCIT (chronic unpredictable mild stress + escitalopram during gestation). Individual males were analyzed for all other endpoints on PND90-95. All endpoints in Table 1 utilized two pups per litter. Data are mean ± SEM, (N = 7-26). # main effect due to prenatal stress, * $p < 0.05$, ** $p < 0.01$ with a Dunnett's *post hoc* Test.

Figures

Figure 3.1. Timeline of chronic unpredictable mild stress and/or escitalopram administration during pregnancy and measurements in adulthood.

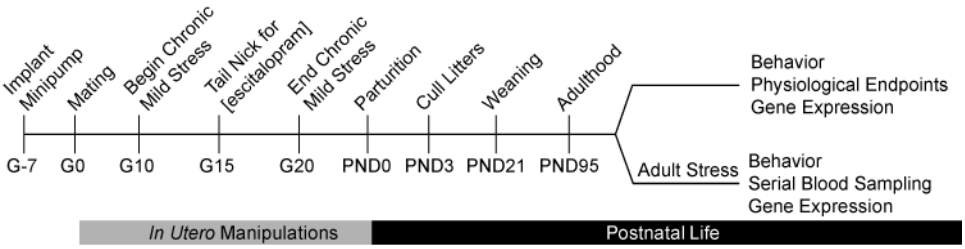


Figure 3.2. Behavioral characterization of adult male offspring prenatally exposed to stress and/or escitalopram.

Males were tested in adulthood in several tests for anxiety-like and depressive-like behavior (figures 2A-2C). A separate cohort was challenged by exposure to 15 minutes of restraint before the test (white bars; figures 2D-2F). Data is presented for defensive withdrawal (A), elevated plus maze (B), sucrose consumption test (C), open field test (D), marble burying test (E), and acoustic startle response (F). Data are mean ± SEM, N = 6-12 per group. SAL (saline treatment during gestation), ESCIT (escitalopram exposure during gestation), S-SAL (chronic unpredictable mild stress + saline during gestation), S-ESCIT (chronic unpredictable mild stress + escitalopram during gestation).

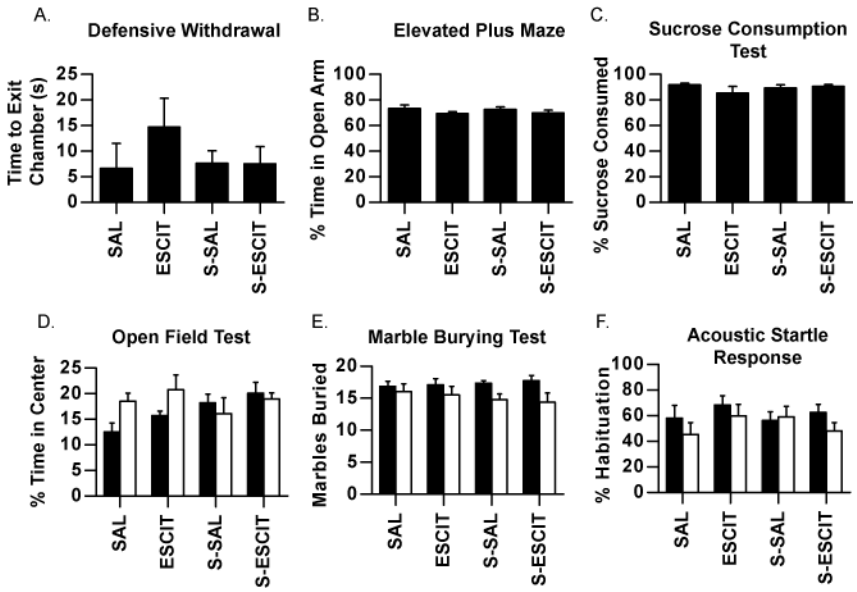


Figure 3.3. HPA axis endocrine response following acute stress in adult male offspring prenatally exposed to stress and/or escitalopram.

Jugular catheters were implanted in adult males and the HPA response to an acute air puff startle (3A-3B) or restraint stress (3C-3E) was assessed. Data are mean ± SEM, N = 9-16 per group for 3A-3C, SAL (saline treatment during gestation), ESCIT (escitalopram exposure during gestation), S-SAL (chronic, unpredictable, mild stress + saline during gestation), S-ESCIT (chronic, unpredictable, mild stress + escitalopram during gestation).

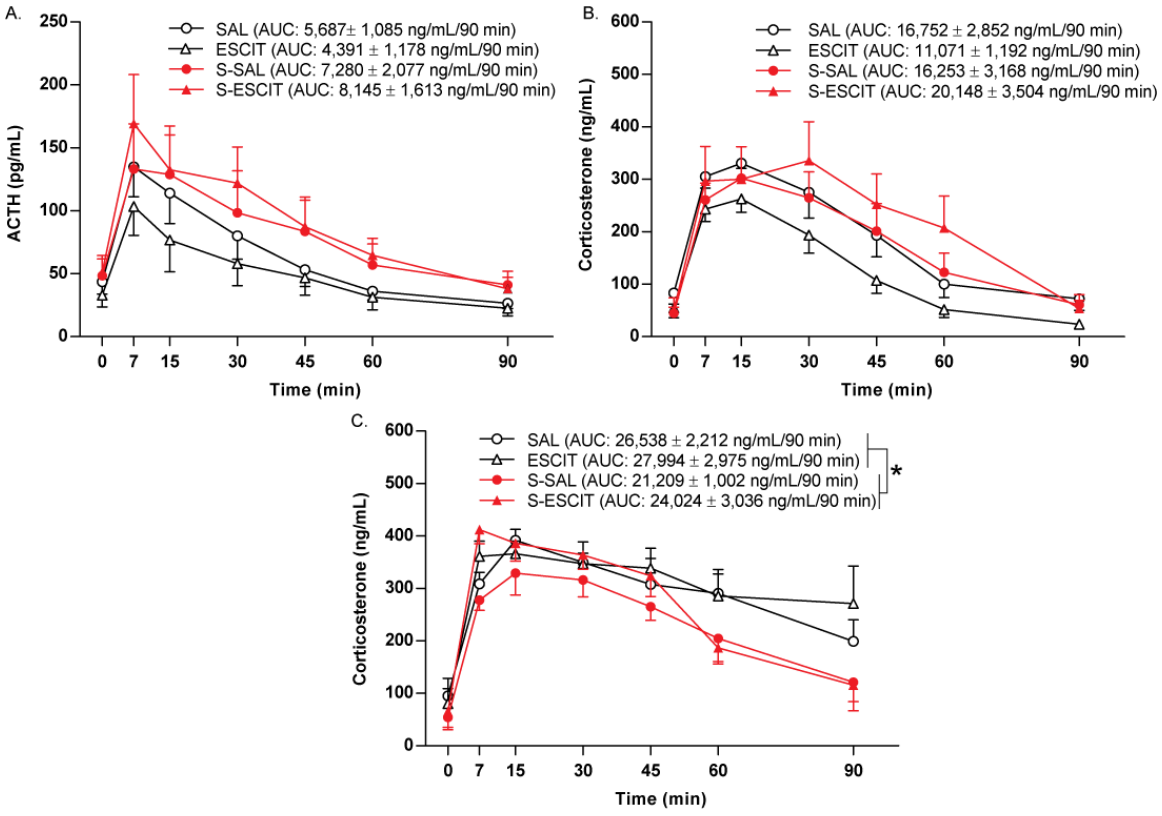
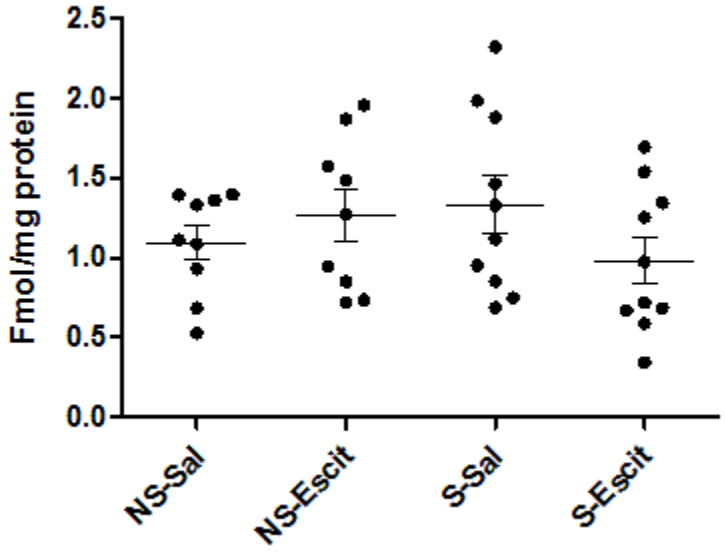


Figure 3.4. CRF receptor binding in the anterior pituitary.

CRF receptor binding was assessed in the anterior pituitary in adult male rats. Data are mean ± SEM, N = 9-10 per group, NS-Sal (saline treatment during gestation), NS-Escit (escitalopram exposure during gestation), S-Sal (chronic, unpredictable, mild stress + saline during gestation), S-Escit (chronic, unpredictable, mild stress + escitalopram during gestation).



CHAPTER 4: PRENATAL EXPOSURE TO ESCITALOPRAM AND/OR STRESS IN RATS: NO EFFECTS ON GENE EXPRESSION MEASURES IN ADULT MALE RATS

Abstract

I concluded my investigation of the long-term effects of prenatal stress and/or clinically relevant antidepressant exposure on male adult offspring with a focus on the transcriptome. Female Sprague-Dawley rats were implanted with osmotic minipumps that delivered clinically relevant exposure to the antidepressant escitalopram. Subsequently, pregnant females were exposed on gestational days 10-20 to a chronic unpredictable mild stress paradigm. The male offspring were analyzed in adulthood to determine the long-term effects of prenatal exposures. Microarray analysis of the hippocampus and hypothalamus, two areas implicated in the stress and antidepressant response, compared all treatment groups but revealed no significantly-altered transcripts. Real time PCR of the hippocampus confirmed that several transcripts in the CRFergic, serotonergic, and neural plasticity pathways were unaffected by prenatal exposures. This model of maternal depression and its treatment indicate that escitalopram use and/or stress during pregnancy produced no alterations in our measures of the transcriptome. The role of drug and stressor dosing or timing in extant studies suggests that study design should be carefully considered before implications for human health are ascribed to prenatal exposure to stress or antidepressant medication.

Introduction

Depression affects 10-20% of pregnant women (Gavin et al., 2005). Although it is becoming increasingly well-known that untreated maternal depression during pregnancy is detrimental to infant outcome, the known and unknown risks associated with antidepressant treatment lead to increased discontinuation of antidepressant use during pregnancy (Bonari et al., 2005). Although the short-term effects of prenatal antidepressant exposures have been investigated, the long-term effects, if any, are relatively unknown. This chapter aims to elucidate any long-term transcriptome alterations due to *in utero* manipulations in order to aid clinicians and their patients in treatment decisions.

Investigation of the long-term effects of prenatal stress, one possible proxy for maternal depression during pregnancy, and antidepressants concurrently has not been rigorously examined. In animal models, there are numerous studies that have examined types of prenatal stress (Newport et al., 2002; Mueller and Bale, 2006; Mueller and Bale, 2008) and several studies that have examined antidepressant-exposed pregnant rats from the perspective of the offspring (Henderson and McMillen, 1993; Cabrera and Battaglia, 1994; Cabrera-Vera et al., 1997; Cabrera-Vera and Battaglia, 1998; Forcelli and Heinrichs, 2008). To our knowledge, prenatal stress and antidepressant exposure have not been examined concurrently and used to investigate the effects on the offspring. However, recent health care data bases indicate that over 6% of pregnant women receive a prescription for an antidepressant (Andrade et al., 2008), therefore it can be argued that investigations of both stress and antidepressant exposure are potentially the most clinically relevant scenario. I hypothesize that, in this rat model, escitalopram

intervention during the stress treatment may attenuate any long-term effects of the prenatal stress exposure on outcome measures in the offspring. Additionally, no published study to date has examined the transcriptome in the context of prenatal antidepressant exposure.

Gene expression analysis through real time PCR and/or microarray analysis in recent years has allowed the identification of numerous biomarkers in fields such as pharmacology, cancer, and psychiatry. The lack of a rigorously investigated mRNA target endpoint due to prenatal stress and/or antidepressant exposure in the extant literature makes a transcriptome-wide microarray approach highly relevant to the field. The choice of gene expression analysis in specific brain regions of interest was based primarily on studies documenting the influence of stress/antidepressants on the amygdala, hippocampus, or hypothalamus. I chose to examine the hippocampus because recent data highlights the importance of forebrain-specific GR in mediating affective-like behaviors (Wei et al., 2004; Wei et al., 2012) and the established role of the hippocampus in negative feedback on the HPA axis (Sapolsky et al., 1985). The hypothalamus and amygdala have an established role of mediating the activation of the HPA axis (Keen-Rhinehart et al., 2009; Flandreau et al., 2012). These three regions of interest represent areas that are globally affected by stress or antidepressant exposure and are the most likely to be altered by prenatal exposures.

Translation of clinically relevant models of prenatal exposures is necessary to adequately evaluate the risk to the mother and infant. Previously, we have modeled stress and antidepressant exposure to the offspring (Chapter 2; (Capello et al., 2011)) with special emphasis on clinically relevant antidepressant exposure using the antidepressant

escitalopram as representative of the class. These studies have demonstrated that chronic variable stress during pregnancy produces an increase in basal plasma corticosterone concentrations indicative of an activated hypothalamic-pituitary-adrenal (HPA) axis in response to this chronic stressor. Additionally, we have demonstrated that continuous delivery via osmotic minipumps results in serum escitalopram concentrations maintained within a clinically observed range and providing occupancy of the serotonin transporter in fetal rat brain >80% (Capello et al., 2011). This methodology is utilized in this study to model antidepressant treatment of depression during pregnancy.

Materials and Methods

Animals

Sprague-Dawley male, retired breeders and nulliparous females weighing 200-225 grams were purchased from Charles River Laboratories (Charles River, Wilmington, MA). Rats were kept on a 12:12 light: dark cycle (lights on at 7:00 AM) in a humidity (60%) and temperature (20°C-23°C) controlled facility. Rodent diet 5001 chow (Purina Mills, Richmond, IN) and water were available *ad libitum* throughout the study. After two weeks at the Emory University animal facility, female rats were paired with male retired breeders in a breeding cage. Gestational day 0 (G0) was designated by the presence of a sperm plug and pregnant females were single-housed after breeding. Three days after birth, rat pups were sexed and litters were culled to six male and two female pups. Animals were weaned on PND21 and kept pair-housed until adulthood. Because pup growth rates and maternal licking and grooming behavior, a hallmark of good maternal care, were unaltered by either prenatal stress and/or escitalopram exposure, maternal care was performed by the biological dam rather than a foster dam. Escitalopram release from the minipumps ended between 0-2 days post parturition (Alzet model 2ML4 technical information).

For the adult offspring endpoints, in almost all circumstances only a single pup was used from each litter in order to prevent possible litter effects (Holson and Pearce, 1992). When that was not possible, no more than two pups per litter were assigned to a group and are noted at the appropriate point within this document. Each real time-PCR group was assigned between 8 and 12 pups. An additional manipulation of twelve days of chronic immobilization stress in adulthood was used in a separate cohort of animals to

determine if a stress-susceptible phenotype existed. Animals were immobilized in DecapiCone® bags (Braintree Scientific, Braintree, MA) for two hours once a day for 12 days (Neigh et al., 2010). One day after the last stress session, control and immobilization stress animals were killed via rapid decapitation on postnatal day (PND) 100-105 during the early phase of the light cycle. The brain was removed and immediately dissected on ice.

Ethics Statement

All animal protocols were approved by the Emory University Institutional Animal Care and Use Committee and carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Resources, 1996) as adopted and promulgated by the U.S. National Institutes of Health. All steps were taken to minimize animal suffering at each stage of the study.

Escitalopram Administration and Chronic Unpredictable Mild Stress Model

Nulliparous female rats were subcutaneously implanted with Alzet 28 day osmotic minipumps (model 2ML4, Alzet, Cupertino, CA) slightly posterior to the scapulae. Osmotic pumps delivered either 0.9% saline or 12.2 mg/kg/day escitalopram oxalate in 0.9% saline based upon a predicted weight of the pregnant dam on G21 of 400 grams (unpublished observations). Three days after minipump implantation, females were bred with retired breeder males. On G10, the chronic unpredictable mild stress model of depression began and consisted of restraint, cage tilt, damp bedding, noise, cage changes, and overnight illumination. G10 was selected to begin the stress paradigm

because the fetal central nervous system begins substantial development at this point (Clancy et al., 2001) and to minimize premature termination of the pregnancy as a result of excessive stress. On G15, all pregnant dams, regardless of treatment group, were given a tail nick to determine the serum concentration of escitalopram. No pregnant dams with escitalopram minipumps had serum escitalopram concentrations below 17 ng/mL. The stress model ended on G20 to prevent premature parturition.

Gene Expression Studies

Brains were immediately dissected on ice with a Jacobowitz 2 mm Brain Slicer (Zivic Instruments, Pittsburgh, PA). The amygdala (bilateral) was removed with a 2 mm sterile biopsy punch (Miltex Inc, York, PA). The hippocampus and hypothalamus were dissected from the same single slice. RNA extractions were performed the day of the dissections. RNA was extracted with the TRIzol method (Invitrogen, Carlsbad, CA) and RNA integrity was assessed using a BioRad Spectrophotometer and an Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA). Samples with RNA integrity numbers under 5.5 units were excluded. RNA was amplified using the GeneChip® WT cDNA Synthesis and Amplification Kit (Affymetrix, Santa Clara, CA). RNA was labeled using the GeneChip WT Sense Target Labeling Kit (Affymetrix, Santa Clara, CA), hybridized to a GeneChip Rat Exon 1.0 ST Array (Affymetrix, Santa Clara, CA) and analyzed on an Affymetrix GeneChip System (Affymetrix, Santa Clara, CA). Expression Console was used to normalize chip data by the RMA-Sketch method (Affymetrix, Santa Clara, CA). The data was filtered to remove duplicate transcript probe sets and analyzed with hierarchical clustering with TM4 Microarray Suite using a Pearson Correlation with

Average Linkage Clustering (Saeed et al., 2003). To find differentially-regulated genes, Significance Analysis of Microarrays (SAM) was used in R to analyze the normalized data (Tusher et al., 2001; R Development Core Team, 2010). SAM was set to examine each two-class unpaired comparison with 1,000 permutations, a 1.5 fold-change cut-off, and a False Discovery Rate (FDR) cut off of 10%. Power analysis was conducted with SAM. In a parallel analysis, data was filtered after SAM analysis with Ingenuity Pathway Analysis 8.7 (Ingenuity Systems, Redwood City, CA) to remove any transcripts that were not expressed in the regions of interest. Individual p-values from the t-tests performed in SAM were adjusted using a Bonferroni correction. To combine the different rounds of microarrays, a meta-analysis was performed using Fisher's method of combining p-values from the MADAM package in R (Kugler et al., 2010).

For real time PCR studies, RNA from the dorsal hippocampus was extracted with TRIzol (Invitrogen, Carlsbad, CA) and to eliminate genomic DNA contamination, a DNAse step was performed using the Turbo DNA-free kit (Ambion, Austin, TX). Reverse transcription was carried out with the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA). cDNA was quantified with the PicoGreen Assay (Invitrogen, Carlsbad, CA). A rat endogenous control plate (Applied Biosystems, Foster City, CA) was run in order to determine the ideal endogenous control (*Hmbs)*. Primers were designed with Primer3Plus (Untergasser et al., 2007) (Table S1) and designed with Ensembl to overlap exon-exon junctions to eliminate amplification of genomic DNA (Flicek et al., 2010). Primer concentrations were optimized to eliminate primer dimers with a dissociation curve. ABsolute SYBR Green (Fisher Scientific, Pittsburgh, PA) was used to detect double stranded cDNA and real time PCR was run on the Applied

Biosystems 7900HT system (Applied Biosystems, Foster City, CA). Gene expression changes were assessed with the following formula: Fold change in gene expression = ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001).

Statistical Analyses

In all figures, values are expressed as mean ± SEM. GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA) and SPSS 17.0 (IBM, Armonk, NY) were used to conduct real-time PCR statistical analysis. A 3-way ANOVA was used to analyze prenatal escitalopram x prenatal stress x adult stress effects. Differences were considered significant if $p < 0.05$. Data analysis of the Affymetrix GeneChip Rat Exon 1.0 ST Array was conducted with R (R Development Core Team, 2010). After RMA-Sketch normalization, data was imported into R and analyzed with the *samr* package (Tusher et al., 2001). Power analysis was conducted with the *samr* package (Tibshirani, 2006).

Results

Microarray Analysis of the Amygdala, Hippocampus, and Hypothalamus

Microarray analysis was used to examine differentially-regulated transcripts in response to prenatal exposures. Post-normalization analysis revealed that mean intensity of each chip was unaffected by chip number or chip run in the robust multiarray average normalization ($F_{(17,\ 2045062)} = 0.2$; $p = 1.0$) (Fig. 4.1). Hierarchical clustering from hippocampus, hypothalamus and amygdala showed grouping of samples based on brain region as expected (Fig. 4.2). An initial microarray analysis identified several differentially-regulated transcripts due to prenatal treatments through SAM analysis (Fig. 4.3). A post-hoc power analysis was performed to ensure adequate sample size. A SAM power analysis with an effect size of 1.5 fold, a false discovery rate of 10%, and an N equal to three revealed that approximately 100-500 differentially-regulated transcripts could be reliably reported (Fig. 4.4). Because all comparisons revealed less than 500 differentially-regulated transcripts, the initial microarray was under-powered. Subsequent power analysis determined that an N of six or greater could reliably detect at least ten differentially-regulated transcripts while keeping the Type I error below 0.01. To satisfy the power requirements, a second microarray was run using a separate cohort of animals having undergone the same prenatal exposures, but the amygdala was excluded from further iterations (Fig. 4.5A). Hierarchical clustering revealed that the first and second microarrays clustered separately (Fig. 4.5B and 4.5C), precluding direct comparison. Meta-analysis was run with Fisher's Method of combining *p*-values using the *p*-values obtained in the SAM analysis. When multiple comparisons were adjusted for using a Bonferroni correction, no genes reached statistical significance. To relax the

Bonferroni correction, regions of interest were filtered with IPA software to only analyze highly-expressed transcripts in the hippocampus or hypothalamus (6,840 transcripts in the hippocampus, 7,339 transcripts in the hypothalamus). Using this relaxed Bonferroni correction, no transcripts reached statistical significance.

Real-Time PCR Analysis of the Hippocampus

Real time PCR was used to complement the hippocampal microarray data and determine if more subtle differentially-regulated transcripts that would not be detected using the 1.5 fold threshold were present. Hippocampal tissue was analyzed with real time PCR to determine the relative abundance of target transcripts by first examining the raw cycle threshold (Ct). High abundance transcripts amplify at a lower cycle threshold (Fig. 4.6). Target genes were selected based on the corticotropin-releasing factor (CRFergic) pathway (Fig. 4.7A), the serotonergic pathway (Fig. 4.7B), and markers of neural plasticity (Fig. 4.7C), all pathways promulgated to play plausible roles in mediating stress and/or antidepressant medication neurobiology. An additional manipulation of twelve days of chronic immobilization stress in adulthood was used in one half of the animals to determine if a stress-susceptible phenotype existed compared to basal (i.e., no stress) expression. Using a Bonferroni correction to account for multiple hypothesis testing ($p < 0.003$), no genes reached statistical significance for differential expression using a 2-way ANOVA (prenatal exposure x adult stress; $p > 0.003$).

Discussion

Although stress and antidepressants can plausibly target/alter parts of the HPA axis, the serotonergic system and/or neurotrophic factors (especially in adults), downstream targets affected by prenatal exposure cannot adequately be determined. Therefore, microarray analysis was initially performed to examine predictable and unpredictable targets for further study. Microarray analysis of the hippocampus and hypothalamus did not detect any differences in gene expression (Figures 1-5). The amygdala was not repeated so this region may still represent an area to explore with a suitably powered study. Our group has reported the importance of the amygdala in HPA dysregulation and anxiety-associated behavior (Keen-Rhinehart et al., 2009; Flandreau et al., 2012). While this region is an important regulator of the stress response and remains an important target for study, the preponderance of scientific studies examine hypothalamic and hippocampal regions in reference to prenatal stress. The aim of this dissertation was to use prenatal antidepressants with a co-exposure to prenatal stress. Therefore, I chose the hypothalamus and hippocampus in order to contextualize any findings.

It should be noted that our two studies clustered separately and produced a significant "batch effect" as others have observed (Yang et al., 2008). Batch effects are frequently observed in microarray studies and indicate that additional normalization procedures are necessary prior to data analysis. Steps were taken to minimize this effect (concurrent array normalization) but the individual arrays still clustered separately. Fisher's method was used to circumvent this confounding variable and I believe these results are still a reliable measure of the transcriptome. While microarray analysis of the

entire transcriptome is indeed a powerful tool, it is limited by certain constraints. Notably, microarray analyses employ a 1.5- or 2-fold change cut-off as a convention. Gene expression differences that are slightly below this cut-off may still have profound effects on the offspring. While other studies examining prenatal stress exposure using RT-PCR have found changes in gene expression (Mueller and Bale, 2008; Nyirenda et al., 2009; Pankevich et al., 2009), our study using a transcriptome-wide and target-specific approach (Figure 4.7) failed to reproduce these findings. However, some have found that these expression changes are dependent on developmental timing of the stressors (Mueller and Bale, 2008) and our stress model extended from mid to late pregnancy. Bale's group have found that maternal stress during early gestation (G1-7) produced robust behavioral and gene expression differences in adulthood. Therefore, future studies should administer a stress model throughout pregnancy or solely during the early gestational period to determine long-term effects on the offspring.

Serotonin plays a well-established role in neurodevelopment by affecting neuronal migration and recent data is clarifying the underlying mechanism(s) (Riccio et al., 2009). In addition to endogenous fetal serotonin, the delicate balance of maternal-placental-fetal interactions of the serotonin system supports a role of serotonin in fetal development (Bonnin et al., 2011). In animals, postnatal exposure to SSRIs utilizing dosing strategies different from our study alters behavior and cortical development (Ansorge et al., 2008; Simpson et al., 2011). To our knowledge, no studies have examined long-term changes in gene expression due to prenatal antidepressant exposure. Other studies have found differences in monoamine receptor density, transporter binding, monoamine concentrations and turnover (Henderson and McMillen, 1993; Cabrera and

Battaglia, 1994; Cabrera-Vera et al., 1997; Cabrera-Vera and Battaglia, 1998; Forcelli and Heinrichs, 2008). Except in the case of Forcelli 2008, these studies have not carefully controlled for the clinical relevance of the antidepressant exposure as is the case here. These studies have found subtle differences which are mostly below 50% and would not be detectable with a microarray if the change is due to altered transcription. Additionally, these autoradiography and biochemical studies have found variable effects on the serotonergic system but the neurobiological consequences are unclear. Using real time PCR to investigate these discrete pathways and to determine changes that would not be detectable with the fold change threshold used in the microarray, I did not find that prenatal escitalopram exposure had any effect on targets in the hippocampus using our model of clinically relevant exposure to escitalopram during pregnancy.

Tables

Table 4.1. Primer sequences for real time PCR.

Target	Accession	Sense Primer (Forward, 5'-3')	Antisense Primer (Reverse, 5'-3')
Crf	NM_031019.1	GAGAAGAGAGCGCCCCTAAC	CTCCGGTTGCAAGAAATTCA
Crfbp	X58023.1	CCGCTACCTAGAGGTGCAAG	CAGGGAGGCTCAGCATGT
Crfr1	NM_030999.3	TCAACGAAGAGAAGAAGAGCAA	AGAGGACAAAGGCCACCAG
Nr3c1	NM_012576.2	CTTTGTGCTGGAAGAAACGA	CGAGCTTCAAGGTTCATTCC
Nr3c2	NM_013131.1	CGTGTCAAGCTCTACTTTACGAA	ACCCCATAGTGACACCCAGA
Pomc	NM_139326.2	AACCTGCTGGCTTGCATC	GACGTACTTCCGGGGATTTT
Fkbp5	NM_001012174.1	TTCGAAAAGGCCAAAGAATC	TGCCTCCCTTGAAGTACACC
5htr1a	NM_012585.1	TGTTGCTCATGCTGGTTCTC	CCGACGAAGTTCCTAAGCTG
5htr1b	NM_022225.1	CTGGTGTGGGTCTTCTCCAT	GTAGAGGACGTGGTCGGTGT
5htr2a	NM_017254.1	TGTAGGTATATCCATGCCAATCC	AGGCAGCTCCCCTCCTTAAA
S100a10	NM_031114.1	GCCCAGGTTTCAACAGATTC	CTGTGAAATGTAAGCATCATGG
Slc6a4	NM_013034.3	CAGTTCTGCAGCGATGTGA	TGATGAACAGGAGAAACAGAGG
Bdnf	NM_012513.3	CGACGTCCCTGGCTGGACACTTTT	AGTAAGGGCCCGAACATACGATTGG
Ntrk2	NM_012731.1	GAGCATCTCTCGGTCTATGC	ACTTGGAATGTCTCGCCAAC
S100B	NM_013191.1	CCTGGAGGAAATCAAAGAGC	TGGAAGTCACACTCCCCATC
Akt1	NM_033230.1	CTGCACAAACGAGGGGAATA	GCCGTTCCTTGTAGCCAATA
Hmbs	NM_013168.2	GAAATCATTGCTATGTCCACCA	AACAGGCTCTTCTCTCCAATCTT

Table 4.2. Primer abbreviations and optimal primer concentrations for real time PCR.

Target	Abbreviation	Sense Primer (nM)	Antisense Primer (nM)
Crf	Corticotropin-releasing factor	50	50
Crfbp	Crf-binding protein	300	300
Crfr1	Crf receptor 1	300	50
Nr3c1	Glucocorticoid receptor	50	900
Nr3c2	Mineralocorticoid receptor	300	300
Pomc	Proopiomelanocortin (ACTH precursor)	300	50
Fkbp5	FK506 protein binding 5	300	300
5htr1a	Serotonin receptor 1a	300	50
5htr1b	Serotonin receptor 1b	50	50
5htr2a	Serotonin receptor 2a	300	50
S100a10	p11	300	300
Slc6a4	Serotonin transporter	300	300
Bdnf	Brain derived neurotrophic factor	300	300
Ntrk2	TrkB receptor	300	900
S100B	S100B	300	300
Akt1	Protein kinase AKT1	300	300
Hmbs	Hydroxymethylbilane synthase	300	300

Figures

Figure 4.1. Average signal intensity across chip number in microarray data.

Average signal intensity per chip was used to validate the robust mean average-sketch normalization and document any chip effects.

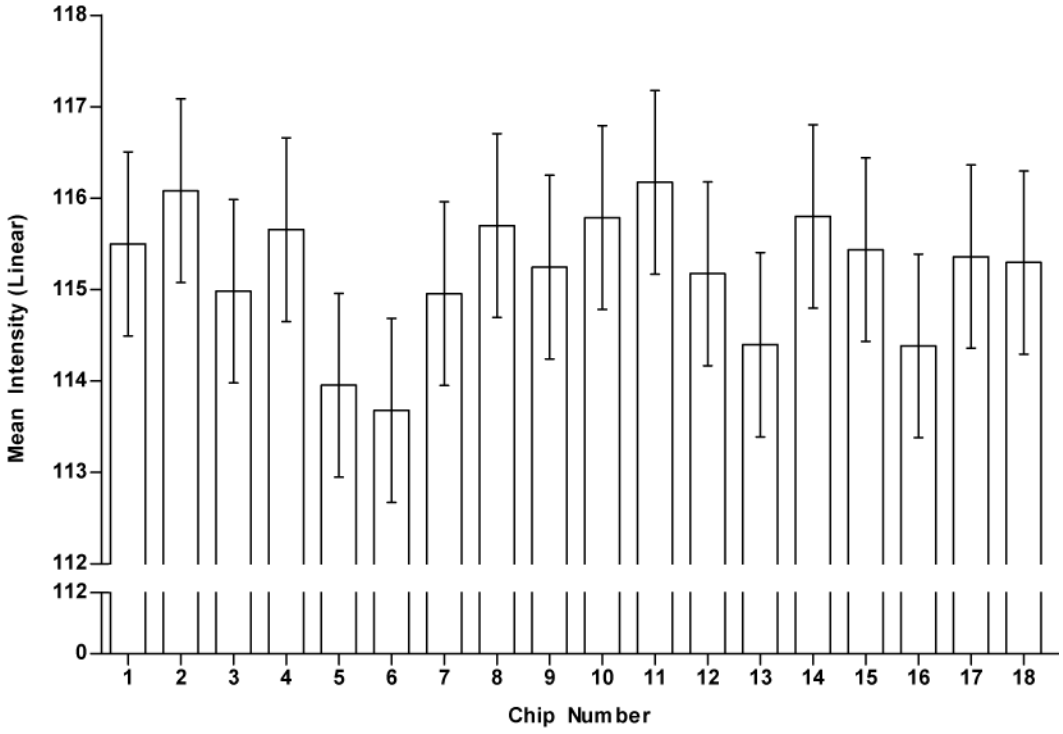


Figure 4.2. Heat map and hierarchical clustering of microarray results clustered by brain region.

Data from the first microarray was used to generate the heat map because the second microarray did not cluster with the first array (Figure 5). Each lane within each brain region represents an individual animal. As expected, analysis showed distinct clustering by brain region, indicating tissue-specific differential transcription.

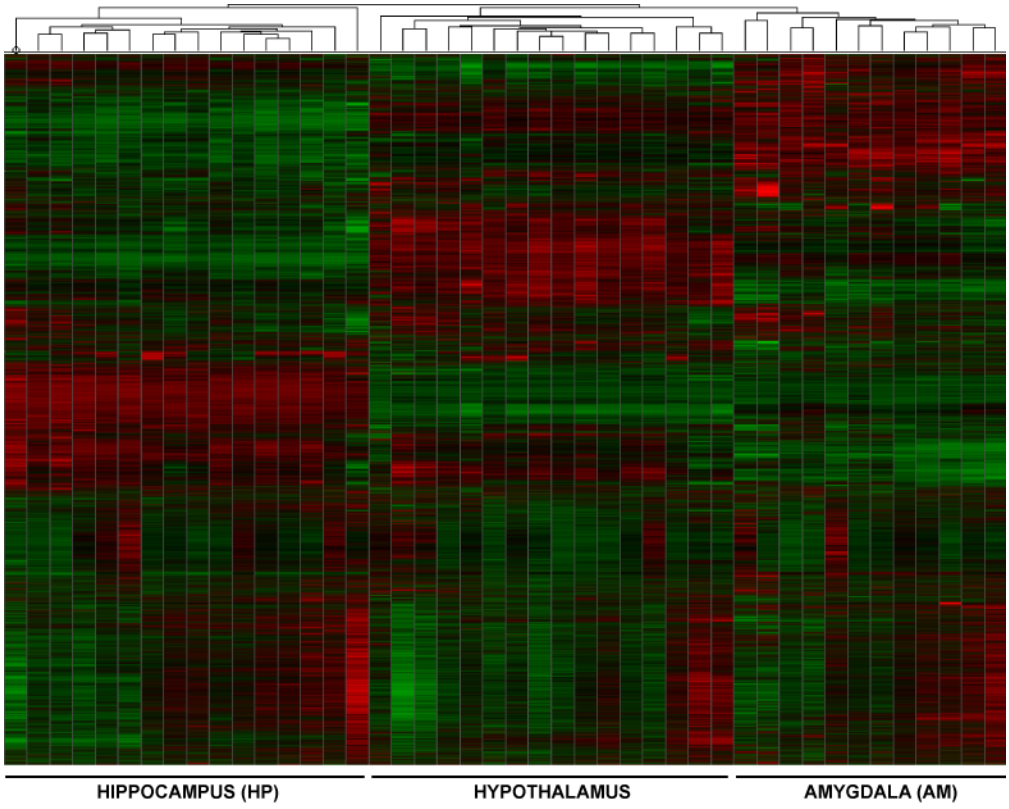


Figure 4.3. Representative SAM analysis Q-Q plot.

Q-Q plot using *samr* package in R. Q-Q (quantile-quantile) plot was used to visualize each transcript of a sample comparison between 2 experimental groups. This analysis was performed for all brain regions and all experimental groups (NS-Sal, NS-ESCIT, S-SAL, S-ESCIT). If each data distribution of transcripts are similar, points lie on the line $y = x$, designated by a line at 45°. As transcripts move farther away from the $y = x$ line, they must cross a specified threshold, related to the False Discovery Rate. This threshold has been designated by the high and low lines parallel to $y = x$. As the threshold becomes larger, fewer transcripts are significant and the FDR remains low. As the threshold becomes smaller, more transcripts are significant but the FDR increases. If a transcript passes this threshold and also satisfies the cut-off criteria where fold change ≥ 1.5, it is considered significant. In our study, no transcripts passed these criteria.

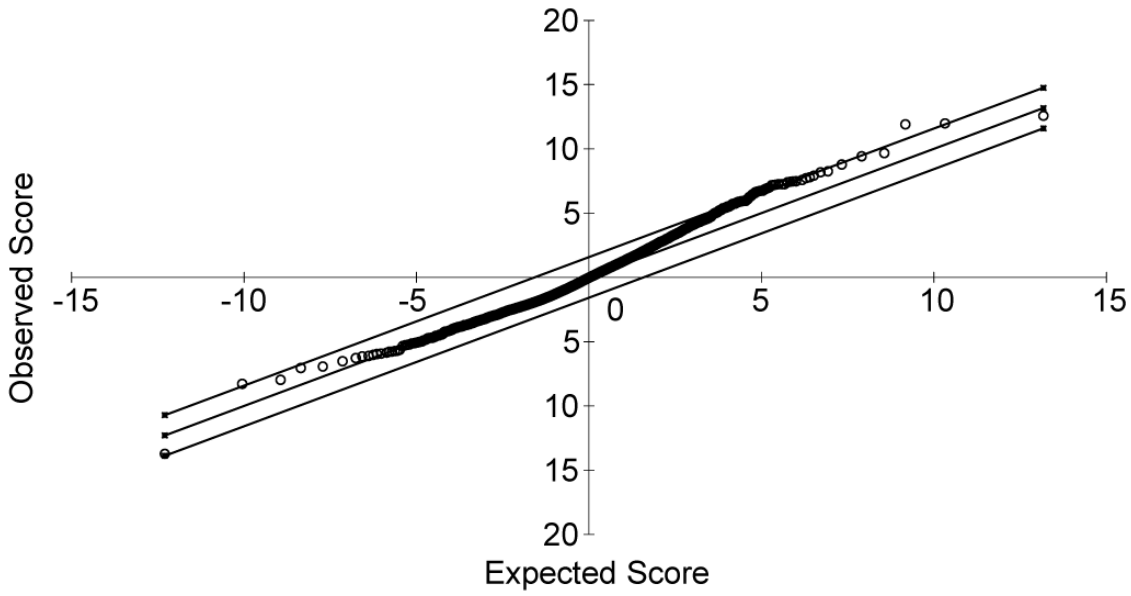


Figure 4.4. Power analysis from SAM.

Power analysis using *samr* package in R. Using a False Discovery Rate (FDR, 1- Power) of 0.1 and a 1.5-fold change cut off, an N = 3 (A) showed that a small sample size would be able to reliably detect over 100-500 genes. Therefore, an analysis finding 90 significant genes would not be reliable from an FDR approach. A study where N = 6 (B) would be able to reliably detect 10 or more genes. Black dots represent the FDR and dotted lines represent the 10th and 90th percentiles of the FDR. The False Negative Rate (FNR, gray dots), analogous to a p value, remained below 0.05 despite the number of transcripts found to be significant.

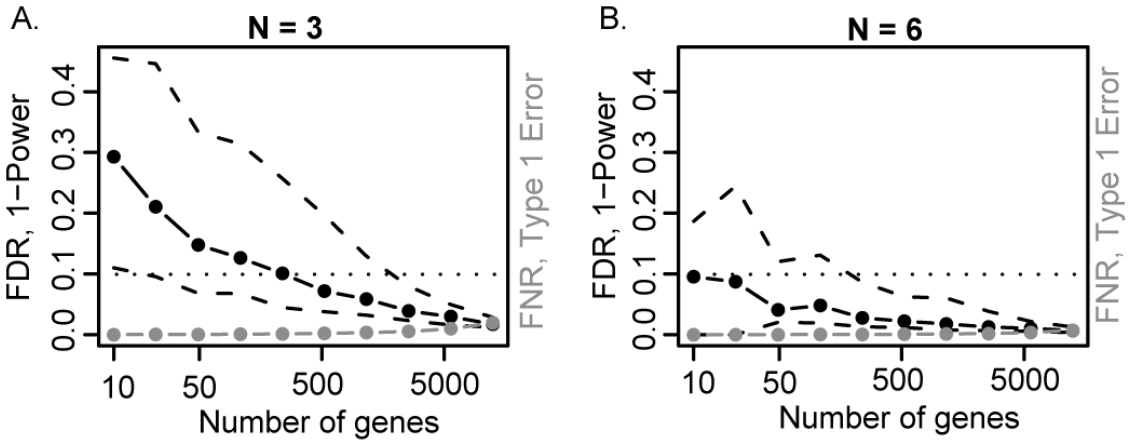


Figure 4.5. Hierarchical clustering of microarray results by specific brain region. Hierarchical clustering in the amygdala (A), hippocampus (B), and hypothalamus (C) was performed to determine clustering of treatment groups and initial versus follow-up microarrays. For the top "Escit" row, a (–) is indicative of a minipump delivering saline during gestation (SAL) while a (+) is indicative of a minipump delivering escitalopram oxalate (ESCIT). For the lower "Stress" row, a (–) is indicative of the offspring's mother exposure to regular handling (S-SAL) while a (+) is indicative of the offspring's mother exposed to chronic, mild stress (S-ESCIT). Clustering analysis showed separate clustering of the initial and follow-up microarray likely due to differing chip lot numbers, time between runs, and/or environmental factors. While these factors precluded direct comparison in a SAM analysis, a meta-analysis was used to determine differential expression that minimizes the influence of extraneous variables.

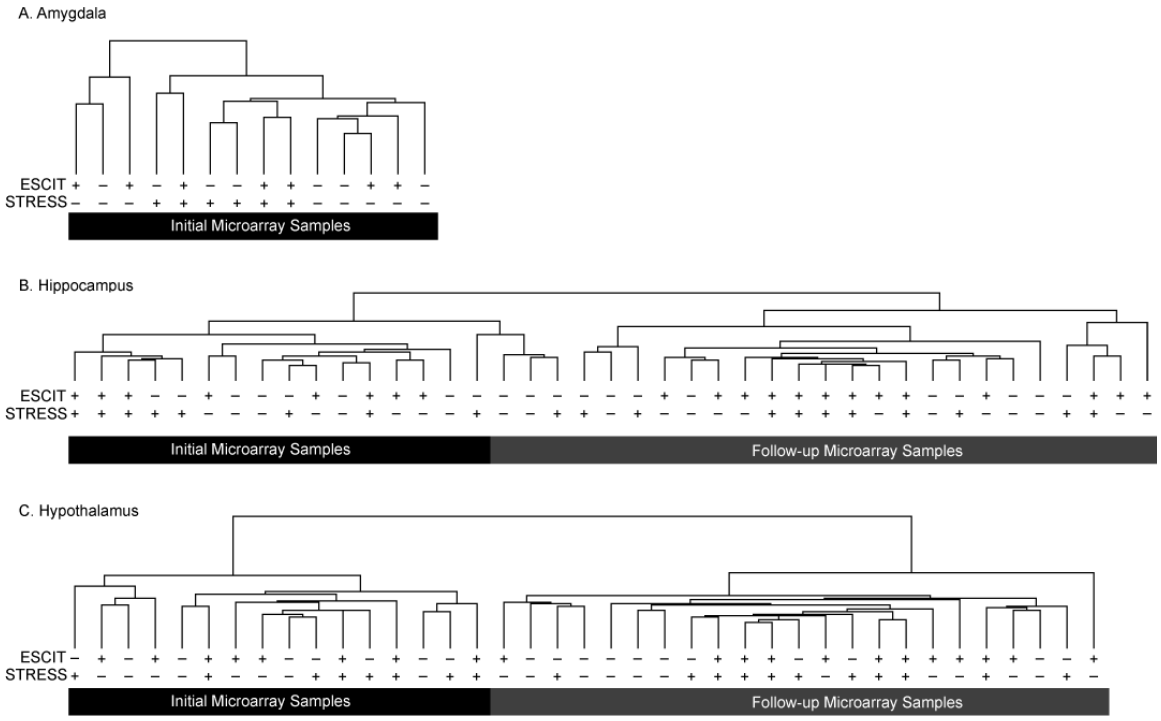


Figure 4.6. Real time PCR of hippocampal tissue from adult males.

Real time PCR confirmed that high abundance transcripts such as the TrkB receptor (*Ntrk2*) was detected at a low cycle threshold, indicating an expectedly high abundance. A detection threshold (Ct) above 35 is considered a low abundance transcript and not detectable. Data are mean ± SEM, N = 82-84.

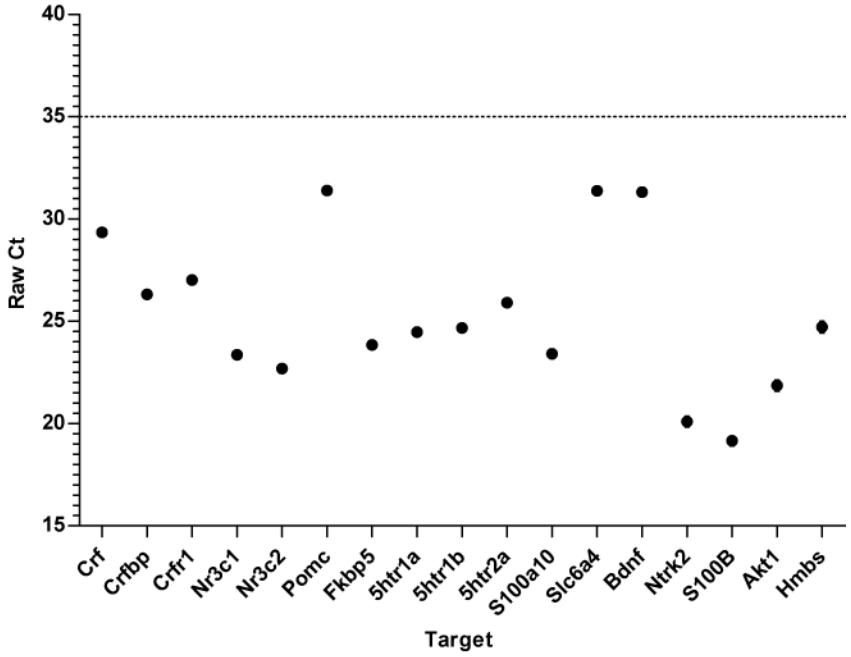
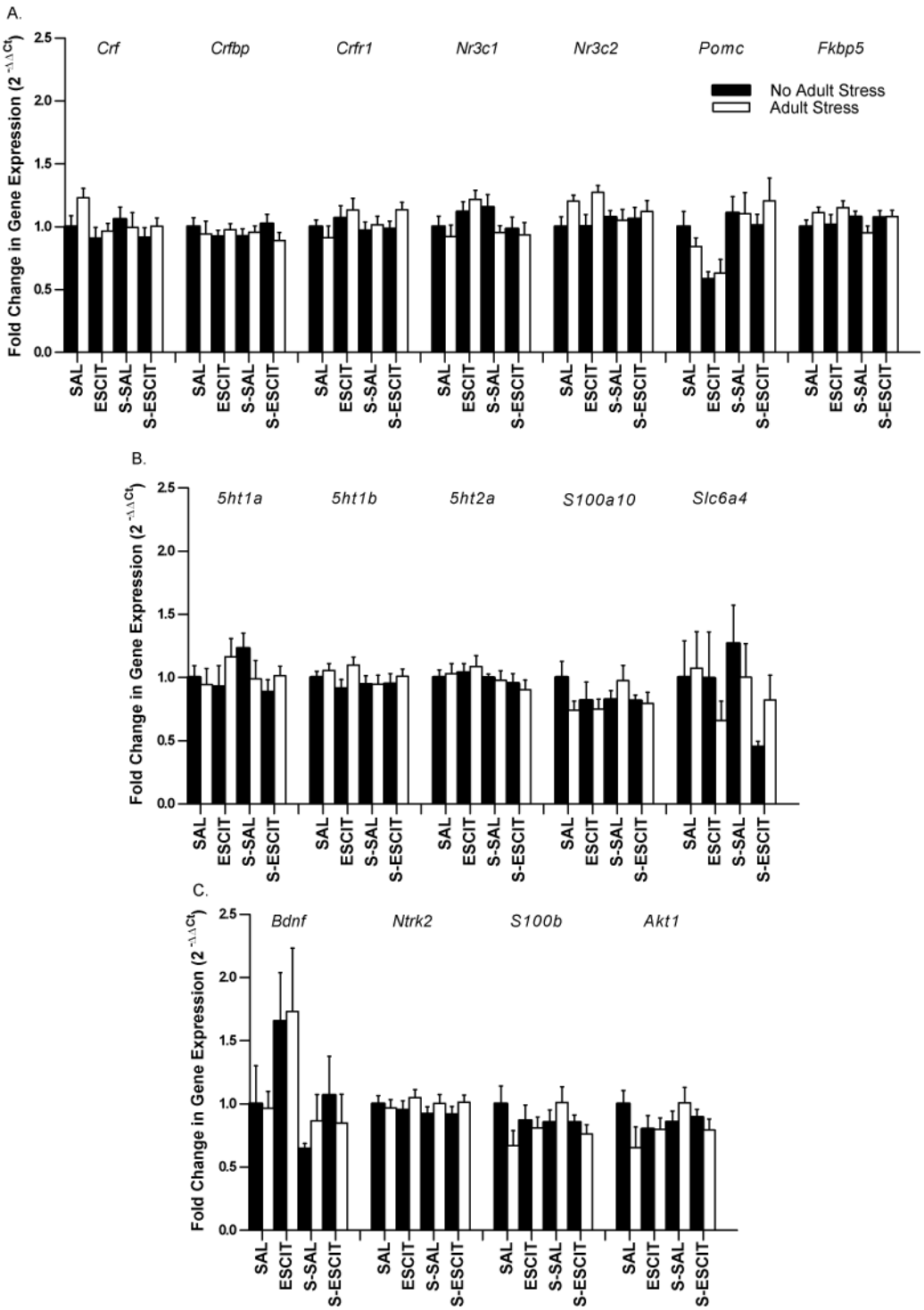


Figure 4.7. Real time PCR of hippocampal tissue from adult male offspring with or without subchronic stress in adulthood.

Following prenatal treatments, half of the male offspring were exposed to twelve days of restraint stress in adulthood in order to investigate stress-activated pathways. Targets related to the CRFergic pathway such as corticotropin-releasing factor (*Crf*), CRF binding protein (*Crfbp*), CRF_1 receptor (*Crfr1*), glucocorticoid receptor (*Nr3c1*), mineralocorticoid receptor (*Nr3c2*), proopiomelanocortin (*Pomc*), and FKBP51 (*Fkbp5*) are depicted in panel (A). Targets related to the serotonergic pathway such as 5-HT_{1A} receptor (*5ht1a*), 5-HT_{1B} receptor (*5ht1b*), 5-HT_{2A} receptor (*5ht2a*), p11 (*S100a10*), and the serotonin transporter (*Slc6a4*) are depicted in panel (B). Markers of neural plasticity such as brain-derived neurotrophic factor (*Bdnf*), Trk_B receptor (*Ntrk2*), S100 calcium binding protein B (*S100b*), and AKT1 (*Akt1*) are depicted in panel (C). No significant effects of prenatal stress, prenatal escitalopram, or the combination were identified ($p > 0.05$). Data are mean ± SEM, N = 8-10 per group.


A.
Crf
Crfbp
Crfr1
Nr3c1
Nr3c2
Pomc
Fkbp5
No Adult Stress
Adult Stress
Fold Change in Gene Expression ($2^{-\Delta\Delta Ct}$)
2.5
2.0
1.5
1.0
0.5
0.0
SAL
ESCIT
S-SAL
S-ESCIT
B.
5ht1a
5ht1b
5ht2a
S100a10
Slc6a4
C.
Bdnf
Ntrk2
S100b
Akt1

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSION

Discussion

Treatment of depression during pregnancy is a growing concern in psychiatry. Pregnant women and clinicians in this situation are faced with a difficult problem: treat the patient and risk effects on the fetus or discontinue treatment and risk a relapse. Indeed, it seems counter-intuitive to prescribe any drugs during pregnancy since pregnant women are discouraged from being exposed to any xenobiotic. However, these recommendations should be put in context as pregnant women are continually prescribed prenatal vitamins containing, among other compounds, folic acid to prevent neural tube defects (Smithells et al., 1980). A risk-benefit assessment is advised before prescribing many drugs such as antihistamines or proton pump inhibitors (Gilbert et al., 2005; Majithia and Johnson, 2012). Nicotine use during pregnancy contributes significantly to long-term diseases and disorders in the offspring (Bruin et al., 2010) and 12.4-21.7% of pregnant women in the United States smoke during pregnancy (Goodwin et al., 2007). The potentially life-threatening mental state of a patient with major depressive disorder in many cases warrants medication throughout life. Discontinuation of pharmacotherapy during pregnancy risks relapse of major depressive disorder and studies examining the negative effects on the child of untreated maternal depression or prenatal stress are well-documented. The current evidence in human patients taking antidepressants during pregnancy and the work presented in this thesis does not indicate any substantial long-term effects in the offspring.

Human exposure studies in the extant literature are mainly composed of case control studies, population observational studies, or retrospective studies. These epidemiological approaches are important to elucidate possible effects that can be

reproduced in controlled prospective cohort studies, but have several limitations. Most of these studies do not quantify exposure concentrations but instead use prescription redemption as an indication of prenatal antidepressant exposure. While this approach does indicate that *in utero* exposure may be present, it does not quantify the level of exposure that is important to determine a dose-response relationship to any observed effects. Additionally, patient compliance becomes a variable in these studies and prescription redemption may not achieve fetal exposure in cases. As mass spectrometry-based techniques become more advanced in the detection of serum drug concentrations, studies correlating serum concentrations of antidepressants with symptoms in the infant will become more common and definitive. Many of these studies are currently underway or only recently reported. Genome wide association studies and studies examining serum drug concentrations in infants are likely to be an important future area of research for psychiatry and obstetrics.

Epidemiological studies investigating prenatal antidepressant exposure also predominately use non-medicated pregnant women as controls without controlling for a history of affective disorders. This becomes a key variable when determining the ill effects from prenatal antidepressant exposure. Any reported ill effects may be incorrectly attributed to prenatal antidepressant exposure when in fact maternal mood is not controlled for. Controlled studies that utilize pregnant women with a concurrent history of mental illness and antidepressant use compared to unmedicated pregnant women with a history of mental illness are necessary to determine possible ill effects (Figure 5.1). The need for this important control group becomes evident when examining the effects of prenatal stress exposure. There are more studies of the effects of stress during pregnancy

than studies examining the effects of prenatal antidepressant exposure that predominately indicate a detrimental effect of prenatal stress. Controlling for this variable will be essential in future clinical studies. One of the strengths of this dissertation resides in using a rational and clinically-relevant control group as well as making comparisons that have both clinical and teratological relevance.

Current laboratory studies investigating prenatal antidepressant exposure also have several limitations that future studies may be able to overcome. The majority of animal studies utilizes acute dosing paradigms to examine adverse effects in the fetus and/or offspring. The clinical relevance of these studies is of limited import and only have some limited application to teratological relevance (Fig. 5.1). Animal pharmacokinetics are fundamentally different from human pharmacokinetics, precipitating a need for a much higher dose in animals than humans. This in turn leads to a pronounced bolus effect in animals that are likely to lead to transient toxic serum concentrations (Fig. 1.6, 2.1, and 2.4). Human dosing regimens and pharmacokinetics lead to much shallower peak and trough serum concentrations of psychotropics. The advent of continuous drug delivery systems such as osmotic minipumps can circumvent these concerns by delivery of a constant concentration of drugs over a long period of time (up to 42 days for a single pump) (Fig. 2.2, 2.3 and 2.4).

Continuous delivery systems via minipump administration are more appropriate to model clinically relevant exposure concentrations. Alternatively, placing medications in food bars or dissolving in drinking water are limited due to the animal feeding schedule. However, the test group would only be exposed to psychotropic compounds during the active cycle, while exposure during the dark cycle would be low to non-

existent due to a lack of feeding behavior. While this would lead to a more constant exposure, the testing group would only be exposed to the pharmaceutical compound during half of the experimental timeline. Osmotic minipumps circumvent this confound by continuously delivery a drug over 24 hours. Therefore, continuous delivery strategies employing minipumps should be favored in the future over the conventional subcutaneous, intraperitoneal, or oral gavage routes of administration.

The decision of a model species to use for testing teratogenicity is central to the investigation of the effects of prenatal antidepressant exposure. Current studies have used sheep, guinea pigs, rabbits, non-human primates, rats, and mice but the majority of animal studies have relied on rats as the model species. Unfortunately, the majority of the endpoints examined in rats have not been examined in a dose-dependent manner and have not been reproduced. In contrast, the sparse number of studies in mice have shown extreme toxicity and are frequently cited to bolster the opinion that prenatal antidepressant exposure causes teratogenicity. The difference between rat and mouse studies should be underscored and balanced from a comparison of each species' maternal behavior: rats are typically "better" mothers than mice. In my dissertation research, maternal care does not appear to be significantly affected by maternal stress and/or antidepressant exposure (Fig. 2.7, 2.8). It remains to be discovered if pregnant mice respond to environmental stressors more adversely than rats but this important piece of evidence may assist in establishing a model test species.

The specific outcome measures evaluated in this study contrast to some studies investigating prenatal stress exposure. While there are numerous groups investigating the effects of prenatal stress, Bale's group performs studies that have significant overlap with

our outcomes. Therefore, these studies represent an excellent comparison. Adult males exposed to prenatal stress displayed increased immobility in the forced swim test, anhedonia, higher corticosterone response to stress, and decreased hippocampal glucocorticoid receptor expression (Mueller and Bale, 2008). These differences were not found in our study. However, it should be noted some significant differences between each study design. For example, mice were used in Bale's study while rats were used in this dissertation. Since many prenatal antidepressant exposure studies appear to find differences in mice, but not rats, mice may be more susceptible to an altered prenatal environment. Additionally, Bale's study found that early prenatal stress during the first seven days of pregnancy were the most disruptive compared to mid or late pregnancy stress. Since I concluded that the fetal serotonergic and CRFergic pathways begin development after this period of time, I reasoned that a stress model from mid to late gestation would be the most disruptive to the fetus. However, early gestational stress appears to be more disruptive, perhaps by altering growth of the dam during this period.

Prenatal antidepressant exposure has been investigated in other animal studies with contrasting results to my outcomes. Battaglia's group performed a series of studies investigating the effects of prenatal fluoxetine exposure on serotonergic CNS function. Similar to this dissertation research, Battaglias' studies found no differences in serotonin transporter density in numerous brain regions including the hippocampus (Cabrera-Vera et al., 1997; Cabrera-Vera and Battaglia, 1998). Although I did not examine serotonin transporter density, I found no differences in hippocampal serotonin transporter gene expression similar to Battaglias' results. Additionally, an adult challenge with DOI (a 5-$HT_{2A/C}$ agonist), resulted in significant HPA activation in control and adult animals

prenatally-exposed to fluoxetine. In Battaglia's study, ACTH plasma concentrations after DOI challenge were blunted in animals prenatally exposed to fluoxetine, opposite to Fig. 3.3 (Cabrera and Battaglia, 1994). The difference between Battaglia's study and my study is likely due to the use of a specific pharmacological manipulation on the serotonin system compared to a psychological stressor that activates numerous monoaminergic and neuropeptidergic systems to activate the HPA axis.

The results presented in this dissertation are a large data set showing an overall null association between prenatal exposures and offspring outcomes. The study itself was carefully designed, well controlled, and conservatively analyzed. While the majority of endpoints presented in this work were not associated with prenatal treatments, parallel studies with collaborators using this model have found some significant differences. For example, gene expression differences due to *in utero* exposures were found in hippocampal vascular factors and behavioral differences were observed in adolescent female rats. Although these aforementioned differences point to an influence of the prenatal environment on the long-term development of the offspring, these studies were tangentially-related to the focus of this dissertation and extended beyond the scope of the dissertation hypothesis. However, these findings illustrate 1) that behavioral and gene expression differences are present in the offspring, 2) these differences can be measured and reliably reported in the future, and 3) although the endpoints in this work did not show any differences in the offspring, other time points or endpoints may yield a difference.

Suitable controls were used throughout the study to ensure that experiments were conducted appropriately. For example, acute stress activated the HPA axis by producing

an increase in plasma ACTH and corticosterone (Fig. 3.3). Gene expression of *Akt1*, a highly expressed transcript in the hippocampus, gave a large signal in both real time PCR and microarray experiments (C_t of 22.5 and microarray signal intensity of 9.72). While intra-experimental controls were numerous, other possible experimental controls could have aided in the interpretation of the results. Since the majority of animal studies investigating prenatal exposure to antidepressants uses a bolus injection (Fig. 1.2 and 1.3), a parallel study could use a bolus injection model to reproduce effects reported in the literature. A small study was conducted to address this experimental control in appendix A. While there were no extreme differences due to the bolus injection of escitalopram, the experiment took place in the neonate and perhaps functional behavioral differences could be observed in this model compared to the continuous delivery model.

In summary, the decision to continue treating a depressed woman who becomes pregnant is a crucial choice that can impact both the pregnant woman and the unborn child. However, evidence-based studies to aid clinicians are sparse. Overall, clinical studies have discovered a neonatal adaptation syndrome that quickly resolves during infant development. Long-term follow up studies of prenatally-exposed infants have only recently been reported and as maternal mood is controlled for, a definitive endpoint may be realized. Animal studies have different obstacles to overcome but the advent of continuous delivery systems will be able to achieve clinically-relevant levels of exposure. This study has found no long-term differences due to prenatal antidepressant exposure. Since the study was carefully designed and modeled to a clinical scenario, these results are highly clinically relevant and indicate no long-term effects of prenatal antidepressant

exposure on the offspring. Antidepressant administration for pregnant women should be considered carefully, weighing all the evidence equally and impartially.

Future Directions

The development and verification in this dissertation research of a continuous model of drug exposure during pregnancy can be applied to a variety of developmental questions. The large number of endpoints and variety of approaches in this dissertation has completed the exhaustive investigation of the long-term effects of prenatal antidepressant exposure. Therefore, future studies could focus on earlier developmental time points (prenatal, neonatal, and adolescent) and/or investigate sex differences with this model of prenatal stress and/or antidepressant exposure. Additionally, this model could easily be applied to different scientific questions in psychiatry and other fields. Experiments within psychiatry could focus on the clinically-relevant problem of developmental exposure to antipsychotics, antiepileptics, and anxiolytics. Experiments outside of psychiatry could examine developmental exposure to persistent toxic compounds such as bisphenol A or pesticides. However, I believe the most important application for this research is surrounding the effects of an altered *in utero* environment on the developing fetus. Therefore, I propose a set of experiments for future studies of the *in utero* environment *vide infra*.

Recent evidence has suggested that autism has an epigenetic mechanism leading to development of this disease (Miyake et al., 2012). Additionally, *in utero* exposure to valproic acid, a histone deacetylase inhibitor, increases the risk of developing autism in humans and causes autistic-like social behaviors in animals (Moore et al., 2000;

Schneider and Przewlocki, 2005; Schneider et al., 2008). Social cognition is impaired in autism spectrum disorders (Meyer-Lindenberg et al., 2011). Theophylline induces histone deacetylase (Ito et al., 2002) and if valproic acid acts through an epigenetic mechanism, theophylline may negate these effects. Therefore, I propose a set of experiments using continuous valproic acid exposure through pregnancy to determine possible neurobiological underpinnings of autism.

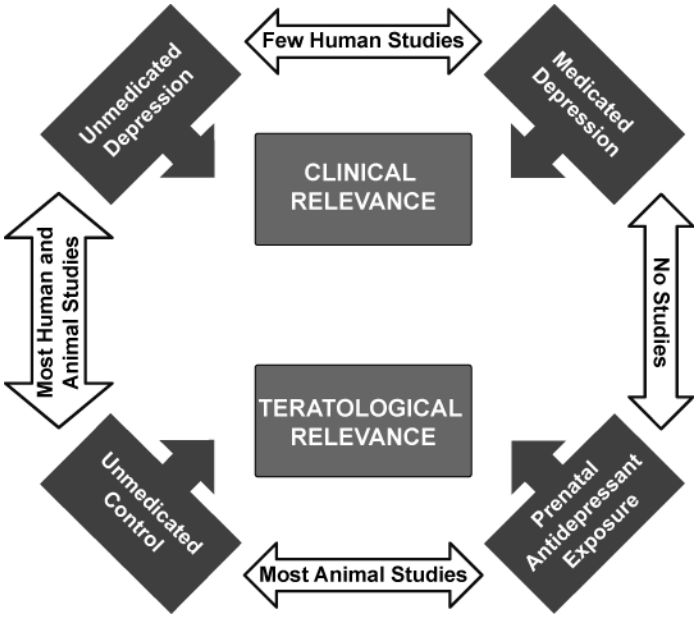
Specific Aim 1. To determine the pharmacokinetics of valproic acid and theophylline in a continuous model of delivery during pregnancy in rats.

Specific Aim 2. To examine the effect of *in utero* valproic acid exposure on social and cognitive behaviors in adolescent animals.

Specific Aim 3. To assess if prenatal theophylline offsets impaired social behavior due to prenatal valproic acid through an epigenetic mechanism.

Figure 5.1. Teratological relevance of animal studies versus clinical relevance of human studies.

The majority of human studies investigating prenatal antidepressant exposure compare medicated and depressed pregnant women to unmedicated and non-depressed control groups to investigated clinical outcomes. The majority of animal studies investigating prenatal antidepressant exposure compare a medicated animal to an unmedicated animal to determine teratological consequences.



APPENDIX A: HYPOTHALAMIC AND HIPPOCAMPAL GENE EXPRESSION IN NEONATAL AND ADULT RATS AFTER PRENATAL ESCITALOPRAM EXPOSURE

Abstract

The long-term effects of prenatal antidepressant exposure in male rats have been extensively characterized from different endocrine, gene expression, and behavioral endpoints in chapters 2-4. However, a more immediate difference may be detectable that normalizes later in life. Experiment one directly compared the neonatal offspring from dams treated through daily injection or continuous delivery of escitalopram. Hippocampal tissue was analyzed by real time PCR for targets in the serotonergic and CRFergic pathways. Daily injections impaired weight gain throughout pregnancy compared to the continuous delivery model. Prenatal escitalopram, delivered through a minipump or daily injections, caused a decrease in *5htr2a* hippocampal gene expression in neonatal rats. Daily injections, regardless of saline or escitalopram, increased mRNA expression of the serotonin transporter in the hippocampus. These studies indicate that daily injections produce stress-associated alterations in maternal rate of weight gain while prenatal escitalopram alters mRNA expression of a serotonergic target in neonatal hippocampal tissue. Experiment two determined if a dose-response relationship existed in adult rats prenatally exposed to escitalopram. Adult male offspring hypothalamic tissue was analyzed with real time PCR for 71 mRNA targets in the monoamine, neuropeptide, neurotrophin, and transcription systems. No significant differences were observed compared to the control group in any of the selected targets, although the sample size was fairly small (N = 4-8). Hypothalamic tissue mRNA studies here indicate that a dose-response relationship for several targets in stress and antidepressant pathways may not exist for prenatal exposure to escitalopram.

Introduction

Previously, I have reported the validation of a clinically-relevant model of escitalopram exposure *in utero* to document the long-term effects on the offspring (Chapters 2-4). While no substantial long-term alterations were observed in the male offspring, the neonatal offspring may represent a cohort who may manifest transient differences in select mRNA targets that may resolve by adulthood. Experiment one determined the immediate effects of prenatal exposure to escitalopram in the previously described clinically-relevant model in neonatal (postnatal day three) rats. An additional cohort was subjected to daily injections of 12.2 mg/kg/d of escitalopram oxalate from gestational day 10-20. Daily injection models currently represent the bulk of animal studies that have found differences in offspring CNS gene expression.

Experiment two determined if a dose-response relationship existed in adult rats prenatally exposed to escitalopram. Currently, animal studies have not replicated a dose-response relationship between prenatal antidepressant exposure and postnatal alterations in physiology, gene expression, or behavior. Vehicle (60% polyethylene glycol (PEG) 400 diluted with isotonic saline) or two doses (4.25 and 10.2 mg/kg/d) of escitalopram in vehicle were administered via 14 day osmotic minipumps from gestational day 11 to 21 implanted in the pregnant dam. While my previous work focused mostly on the hippocampus, I chose to examine if a dose-response relationship existed at the level of the hypothalamus. The hypothalamus is the initial source of HPA activation, with significant modulation by the amygdala and hippocampus. HPA dysregulation is observed in a subset of patients with major depressive disorder, likely though disrupted negative feedback (Budziszewska, 2002). Antidepressants have been shown to regulate

expression of the glucocorticoid receptor, a primary modulator of the negative feedback response, in the hippocampus and the hypothalamus (Budziszewska, 2002). Therefore, I chose the hippocampus and hypothalamus since they have been implicated in both the mechanisms of stress and antidepressant action. I hypothesized that if prenatal escitalopram exposure was affecting gene expression, a dose-response relationship would exist in select targets implicated in the mechanism of action of stress or antidepressant exposure.

Materials and Methods

Experiment One

Animals

Sprague-Dawley male, retired breeders and nulliparous females weighing 200-225 grams were purchased from Charles River Laboratories (Charles River, Wilmington, MA). Rats were kept on a 12:12 light: dark cycle (lights on at 7:00 AM) in a humidity (60%) and temperature (20°C-23°C) controlled facility. Rodent diet 5001 chow (Purina Mills, Richmond, IN) and water were available *ad libitum* throughout the study. After two weeks at the Emory University animal facility, female rats were paired with male retired breeders in a breeding cage. Escitalopram release from the minipumps ended between 0-2 days post parturition (Alzet model 2ML4 technical information). Rats were killed via rapid decapitation on postnatal day (PND) 3 during the early phase of the light cycle. The brain was removed and immediately dissected on ice.

Ethics Statement

All animal protocols were approved by the Emory University Institutional Animal Care and Use Committee and carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Resources, 1996) as adopted and promulgated by the U.S. National Institutes of Health. All steps were taken to minimize animal suffering at each stage of the study.

Escitalopram Administration

For chronic minipump dosing, nulliparous female rats were subcutaneously implanted with Alzet 28 day osmotic minipumps (model 2ML4, Alzet, Cupertino, CA) slightly posterior to the scapulae. Osmotic pumps delivered either 0.9% saline or 12.2 mg/kg/day escitalopram oxalate in 0.9% saline based upon a predicted weight of the pregnant dam on G21 of 400 grams (unpublished observations). Three days after minipump implantation, females were bred with retired breeder males. For daily injection dosing, pregnant females were dosed between G10-20 with 12.2 mg/kg/day escitalopram oxalate in 0.9% saline (1 mL/kg).

Experiment 2

Animals

Female Long Evans rats (Charles River, Cambridge, MA) arrived timed pregnant on embryonic day 10-11 (E10-11). Rats were housed singly and provided water and rat chow *ad libitum* in a temperature controlled facility with a 12 hour light-dark cycle (lights on at 0730 hours). Rats were allowed 24 hours to acclimate before osmotic minipump implantation. All animal protocols were approved by the Emory University Institutional Animal Care and Use Committee and carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Drug Treatment

Drugs were obtained as described in McConathy et al. (2007). Dams were dosed

at rates that mimic the 50th (median) or 85th (high) percentile serum drug concentrations obtained from a sampling of over 750 pregnant and postpartum women from the Emory Women's Mental Health Program (ZN Stowe and DJ Newport, internal data) 60% polyethylene glycol (PEG) 400 diluted with isotonic saline. Escitalopram was administered as the free base at 4.25 or 10.20 mg/kg/day. Escitalopram was first dissolved in ethanol at a concentration of 250 mg/mL and then brought up to the required volume using PEG 400. All drug solutions were sonicated until dissolved. Pumps were loaded with 0.22 μm-filtered drug solution and primed overnight in sterile saline solution at 37°C. Animals were briefly anesthetized with methoxyflurane (Medical Developments, Australia Pty Ltd) and 14 day Alzet osmotic minipumps (model 2ML2, Durect Co, Cupertino, CA) were implanted subcutaneously slightly posterior to the scapulae.

Real Time PCR

For real time PCR studies, RNA from the hypothalamus or hippocampus was extracted with TRIzol (Invitrogen, Carlsbad, CA) and to eliminate genomic DNA contamination, a DNAse step was performed using the Turbo DNA-free kit (Ambion, Austin, TX). Reverse transcription was carried out with the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA). cDNA was quantified with the PicoGreen Assay (Invitrogen, Carlsbad, CA). Primers were designed with Primer3Plus (Untergasser et al., 2007) (Table S1) and designed with Ensembl to overlap exon-exon junctions to eliminate amplification of genomic DNA (Flicek et al., 2010). ABsolute SYBR Green (Fisher Scientific, Pittsburgh, PA) was used to detect double stranded

cDNA and real time PCR was run on the Applied Biosystems 7900HT system (Applied Biosystems, Foster City, CA). Gene expression changes were normalized to *Gapdh* for the hypothalamus and *Hmbs* for the hippocampus and assessed with the following formula: Fold change in gene expression = ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001).

Statistical Analyses

In all figures, values are expressed as mean ± SEM. GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA) was used to conduct real-time PCR statistical analysis.

Results

Experiment 1: Weight Gain of Pregnant Dams

Weight gain for all pregnant dams exposed to a daily injection (saline or escitalopram) was impaired compared to animals implanted with osmotic minipumps ($F_{(1,122)}$ = 23.9, p > 0.001, Fig. A.A.1). Impaired weight gain due to the daily injections was maximal on gestational days 15 (t_{15} = 3.2, p < 0.05) and 16 (t_{15} = 3.2, p < 0.05, Fig. A.A.1). When animals exposed to escitalopram were analyzed separately, the relationship of impaired weight gain in animals exposed to daily injections compared to minipump animals was again observed ($F_{(1,46)}$ = 11.3; p > 0.01). Administration type or drug had no effect on litter size or male-female ration (p > 0.05, Table A.A.1).

Hippocampal Gene Expression in Neonatal Rats Prenatally-Exposed to Escitalopram Via Continuous Exposure or Daily Injections

Prenatal escitalopram exposure produced a significant decrease in *5htr2A* gene expression, regardless of delivery method ($F_{(1,25)}$ = 9.0, p > 0.01, Fig. A.A.2B). Daily injections produced a significant increase in hippocampal serotonin transporter expression ($F_{(1,25)}$ = 5.5, p > 0.05, Fig. A.A.2C). *Bdnf* expression was significantly decreased by daily injections of saline (t_{13} = 2.8, p < 0.05, Fig. A.A.3D). No other targets were altered by delivery method or drug exposure (p > 0.05).

Experiment 2: Hypothalamic Gene Expression in Adult Male Rats Prenatally-Exposed to Escitalopram

Studies were conducted to determine if there was a dose-response effect of increasing escitalopram doses *in utero*. The expression of the gene encoding the nerve growth factor receptor (*Ngfr*) was significantly increased due to a low dose of prenatal escitalopram ($t_{12} = 3.0$, $p < 0.05$, Fig. A.A.6). However, this effect was not replicated for the high dose of escitalopram ($t_8 = 2.0$, $p > 0.05$) and no dose-response relationship was observed. The group exposed to the high dose of escitalopram had a larger amount of variance than the low dose group, likely due to the difference in the group number (8 for the low dose, 4 for the high dose). Without further experiments or replications, it is unknown whether this was a spurious finding or indicative of a phenotypic difference due to *in utero* escitalopram exposure.

No significant differences were measured in any of the other selected targets ($p > 0.05$, *Bonferroni post hoc test*, Fig. A.A.4-7). However, the observed error was very high and follow up experiments would be necessary to verify these results and ensure adequate power of the statistical analysis. These studies indicate that several targets in monoamine, neuropeptide, neurotrophin, and transcription systems were not substantially altered by prenatal escitalopram exposure, complementing the transcriptome-wide approach used in Chapter 4.

Tables

Table A.A.1. Litter weights and male-female ratio after prenatal exposure to saline or escitalopram delivered through a daily injection or minipump administration.

Data are mean ± SEM, N = 3-5 per group.

	Daily Injection		Minipump	
	Saline	Escit	Saline	Escit
PND0 Avg. Pup Weight (g)	7.1 ± 0.4	6.8 ± 0.2		
Total Pups	16.0 ± 0.0	15.4 ± 0.9	14.0 ± 0.6	13.5 ± 2.0
Male Pups	9.0 ± 0.6	9.0 ± 1.0	7.5 ± 1.3	6.5 ± 1.6
Female Pups	7.3 ± 1.1	7.0 ± 1.1	6.0 ± 1.2	6.3 ± 0.5

Table A.A.2. Abbreviations and primer sequences for monoamine receptor, transporter, and neuropeptide targets.

Target	Name	Sense Primer (Forward, 5'-3')	Antisense Primer (Reverse, 5'-3')
5htr1a	Serotonin receptor 1a	TGTTGCTCATGCTGGTTCTC	CCGACGAAGTTCCTAAGCTG
5htr1b	Serotonin receptor 1b	CTGGTGTGGGTCTTCTCCAT	GTAGAGGACGTGGTCGGTGT
5htr1d	Serotonin receptor 1d	TCTTCACTCTGCTCGCTCAA	GCAGAGATCCTCTTGCGTTC
5htr1f	Serotonin receptor 1f	TGGAGGCACCAAGGAAATAG	GTGGGATGTAGAAGGCTCCA
5htr2a	Serotonin receptor 2a	TGTAGGTATATCCATGCCAATCC	AGGCAGCTCCCCTCCTTAAA
5htr2b	Serotonin receptor 2b	TCATGTTTGAGGCTACATGG	CATGATGGAGGCAGTTGAAA
5htr2c	Serotonin receptor 2c	CGCTCCTGATGCACCTAATC	CCACCATCGGAGGAATTAAA
5htr3a	Serotonin receptor 3a	GCATACCATCCAGGACATCA	CCACTCGCCCTGATTTATGA
5htr3b	Serotonin receptor 3b	GTGGTACCGAGAGGTTTGGA	ATGGCACTGAGAGGGAGAGA
5htr4	Serotonin receptor 4	GACAGGCAGCTCAGGAAAAT	AGCACCGAAACCAGCAGAT
5htr5a	Serotonin receptor 5a	AAGCTGTGGAGGTGAAGGAC	CCCTTCTGTCTGGAAGGTGA
5htr5b	Serotonin receptor 5b	ACCACGCAGGCAAAGGAA	GCCAGGAGTCTCCACTTGTC
5htr6	Serotonin receptor 6	TAGCTCAGGCCGTGTGTGAC	CGCATAAAGAGCGGGTAGAT
5htr7	Serotonin receptor 7	ACAGGTACCTTGGGATCACG	AGCCAGACCGACAGAATCAT
5htt	Serotonin transporter	CAGTTCTGCAGCGATGTGA	TGATGAACAGGAGAAACAGAGG
Dat	Dopamine transporter	GTCCTTCCGGGAGAAACTG	TACAGCAACAGCCAGTGACG
Drd1	Dopamine receptor D1	CCTTTGGGTCCCTTTTGTAA	GGGGGTCATCTTCCTCTCAT
Drd2	Dopamine receptor D2	CACCACTCAAGGGCAACTGT	ATCCATTCTCCGCCTGTTC
Drd3	Dopamine receptor D3	GGCTTTCCTCAGCAGTCTTC	TGTGCTCCATTTGTCCTGTG
Drd4	Dopamine receptor D4	GTAGTTGGGGCCTTCCTGAT	CAGGTGACAGCACTGACCA
Drd5	Dopamine receptor D5	AAGGGAGGACGGAGAACTGT	ATGCTCAGCTGCCCTCTCTA
Net	Norepinephrine transporter	TATGGATCGATGCTGCAACC	AGGCATCCCTGTAACAGTTG
Tph2	Tryptophan hydroxylase 2	CCGGAACCAGACACATGC	AGGCTCCCAGAGAGGCTAAG
Vmat2	Vesicular monoamine transporter 2	CTGCAGGCTCCATCTGCTT	CTTTCGGGAACACATGGTCT
Target	**Name**	**Sense Primer (Forward, 5'-3')**	**Antisense Primer (Reverse, 5'-3')**
Cart	Cocaine-amphetamine regulated transcript	GCTGTGTTGCAGATTGAAGC	GTCACACATGGGGACTTGG
Cck	Cholecystokinin	ACTCCGCATCCGAAGATATG	GGGTCCACAGCTTCTACAGG
Clock	Circadian Locomotor Output Cycles Kaput	CTCAATTGTTGACAGAGATGACAG	CTCTTTTTGCTTTGTCCTTGTC
Crf	Corticotropin-releasing factor	GAGAAGAGAGCGCCCCTAAC	CTCCGGTTGCAAGAAATTCA
Crfbp	Crf-binding protein	CCGCTACCTAGAGGTGCAAG	CAGGGAGGCTCAGCATGT
Crfr1	Crf receptor 1	TCAACGAAGAGAAGAAGAGCAA	AGAGGACAAAGGCCACCAG
Crfr2	Crf receptor 2	CCCGAAGGTCCCTACTCCTA	TTCCGGGTCGTGTTGTACTT
Npy	Neuropeptide Y	CACCAGACAGAGATATGGCAAG	GGGTCTTCAAGCCTTGTTCT
Npy1r	Npy receptor 1	GCTACTTCAAGATATACGTTCGC	GATTCGTTTGGTCTCACTGGA
Npy5r	Npy receptor 5	ATCCCGAGCTGGCCATAC	TTGGAACTCAGGTGTGTGGA
Nts	Neurotensin	CTGTGCTCAGATTCAGAAGAAGA	TTTTCCAAGAGGGAGGACTTC
Ntsr1	Neurotensin receptor 1	TCCTCGTCTTACGTGCTGTG	TCGAAGAGGAACGTAGTCCA
Ntsr3	Neurotensin receptor 3	CAGCACTGAAAATGGCCTGT	GATGTTGTTTGGTCCCCATT
Pomc	Proopiomelanocortin (ACTH precursor)	AACCTGCTGGCTTGCATC	GACGTACTTCCGGGGATTTT
Tacr1	Tachykinin receptor 1	AAACGCAAGGTGGTCAAAAT	AGAGATCTGGGTTGATGTAGGG
Ucn1	Urocortin 1	ATAACCTGGCAGGCGGCACCAT	GCTGAACCAGAAGCAGCAAC
Ucn2	Urocortin 2	ACTCCCAGCTCTGTGTCCTC	GACACGGGTGTCCAGGTAAG
Ucn3	Urocortin 3	TCCTCTTACAGGGAGCGATG	GAAGACTGGTCCTGCGTTGT

Table A.A.3. Abbreviations and primer sequences for neurotrophic factor and transcription factor targets.

Target	Name	Sense Primer (Forward, 5'-3')	Antisense Primer (Reverse, 5'-3')
Bdnf I	Brain derived neurotrophic factor I	TTGGACAGTCATTGGTAACCT	ATCCACCTTGGCGATTACAG
Bdnf II b/c	Brain derived neurotrophic factor II	AGTTGGCTTCCTAGCGGTGTAG	CGTGGACGTTTGCTTCTTTC
Bdnf III	Brain derived neurotrophic factor III	TATTTTCCCTCCCCGAGAGT	AACTGGGCTCAATGAAGCAT
Bdnf IV	Brain derived neurotrophic factor IV	GTATTACCTCCGCCATGCAA	CAGCTGCTCTGGGGAAGAC
Mecp2	Methyl-CpG binding protein 2	GATCAATCCCCAGGGAAAAG	CCTCTCCCAGTTACAGTGAAGTC
Ngf	Neurotrophic growth factor	GGCCACTCTGAGGTGCATAG	CTCCCTCTGGGACATTGCTA
Ngfr	Neurotrophic growth factor receptor (LNGFR/p75NTR)	AATGCGAAGAGATCCCTGGT	GGTCTTGCTCTGGAGGAACC
Ngfrap1	Ngfr associated protein 1	GATAGGCCCAGAATAACAACC	GGTCTTCCTGTCCATTCTGC
Nradd	Neurotrophin receptor alike death domain protein	TGTATTCAGATACAGCCCTGAAG	GGGGAAATAGGGAGGAGGTA
Ntf-3	Neurotrophin factor 3	GCGAGACTGAATGACCGAAC	CCTGTAAGATCGTGGCAGAAG
Ntf-5	Neurotrophin factor 5	CTGAGATGTCAGGGAGGAGACT	CACACCTGTCAACAGCACCT
Ntrk1	Neurotrophic tyrosine kinase receptor 1	TGACGGAGCTCTATGTGGAA	CACGATGGTTAGGCTTCTCA
Ntrk2	Neurotrophic tyrosine kinase receptor 2	GAGCATCTCTCGGTCTATGC	ACTTGGAATGTCTCGCCAAC
Ntrk3	Neurotrophic tyrosine kinase receptor 3	GAGTCTGATGCGAGCCCTAC	ACTGCTATGGACACCCCAAA
Target	**Name**	**Sense Primer (Forward, 5'-3')**	**Antisense Primer (Reverse, 5'-3')**
Bcl2	B-cell CLL/lymphoma 2	AGGCTGGGATGCCTTTGT	GCACCCAGAGTGATGCAG
c-Myc		GCGACTCTGAAGAAGAACAAGA	CCTCTTGATGGGGATGACC
Creb1 a/b	cAMP response element binding	CCACATTAGCCCAGGTATCC	CCATGGACCTGGACTGTCTG
Crem 2	cAMP response element binding	TGGAGATGAAACTGATGAGGAG	TTGTGGCAAAGCAGTAGTAGGA
Crem 3	cAMP response element binding	GGTTGTTGTTCAAGATGAGGAG	CACCTTGTGGCAAAGCAGTA
Fkbp5	FK506 protein binding 5	TTCGAAAAGGCCAAAGAATC	TGCCTCCCTTGAAGTACACC
FosB	FBJ murine osteosarcoma viral oncogene homolog B	GCGGAAACGGATCAGCTC	AAACTCCAGGCGTTCCTTCT
Gr	Glucocorticoid receptor	CTTTGTGCTGGAAGAAACGA	CGAGCTTCAAGGTTCATTCC
Hsp70	Heat shock protein 70	GTGCTCTGCAGTGTGCAATC	GCTGGAGAATTCCACCTCAA
Hsp90b	Heat shock protein 90b	TTGGCAGTCAAGCACTTCTC	CACACCACGGATGAAGTTGA
Klf4	Kruppel-like factor 4	ATTAATGAGGCAGCCACCTG	CGCAGTGTCTTCTCCCTTCC
Mr	Mineralocorticoid receptor	CGTGTCAAGCTCTACTTTACGAA	ACCCCATAGTGACACCCAGA
Pax6	Paired box gene 6	CGACAATATACCCAGTGTGTCA	CCTTAGTTTATCATACATGCCGTCT
Pou5f1	POU domain, class 5, transcription factor 1 (Oct4)	GCGTTCTCTTTGGAAAGGTG	AGCTTCCTCCACCCACTTCT
Sox-2	SRY (sex determining region Y)-box 2	CACAACTCGGAGATCAGCAA	CATGAGCGTCTTGGTTTTCC

Figures

Figure A.A.1. Weight gain during pregnancy of animals exposed to minipump delivery or daily injections.

Animals were exposed to 12.2 mg/kg/d of escitalopram oxalate either through osmotic minipump delivery throughout pregnancy or daily subcutaneous injections from G10-20. * $p < 0.05$, *Bonferroni post hoc test*, data are mean ± SEM, N = 4 per group.

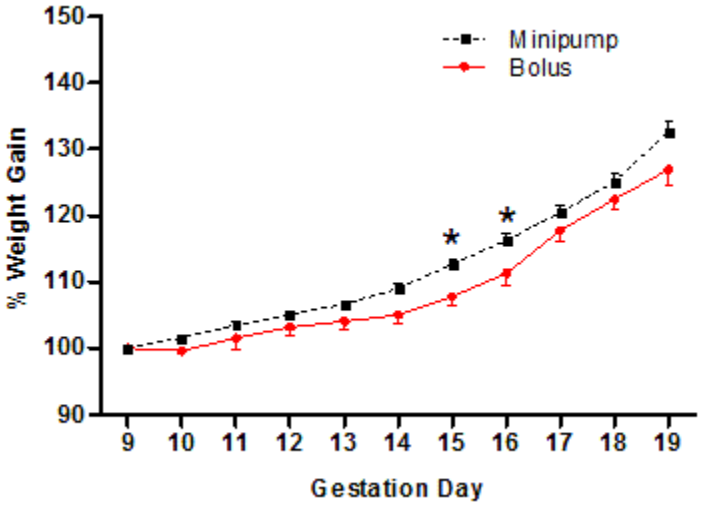


Figure A.A.2. Hippocampal gene expression of serotonergic targets in neonatal rats. Pregnant rats were exposed to escitalopram via minipump delivery or daily injections. Brain tissue was collected from pups on postnatal day three. * $p < 0.05$, main effect due to delivery method, ** $p < 0.01$, main effect due to escitalopram (2-way ANOVA delivery method x escitalopram). Data are mean ± SEM, N = 6-10 per group.

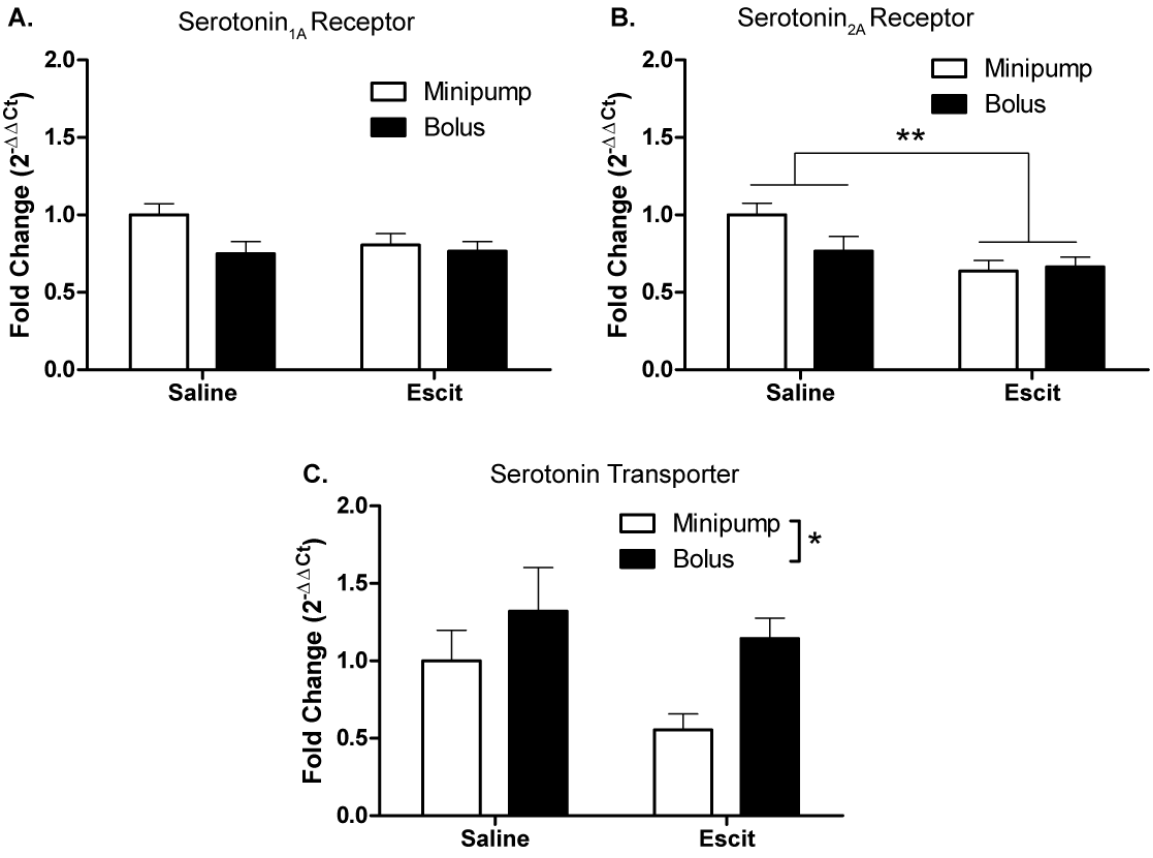


Figure A.A.3. Hippocampal gene expression of neuropeptidergic targets and *Bdnf* in neonatal rats.

Pregnant rats were exposed to escitalopram via minipump delivery or daily injections. Brain tissue was collected from pups on postnatal day three. * $p < 0.05$ *Bonferroni post hoc test*. Data are mean ± SEM, N = 6-10 per group.

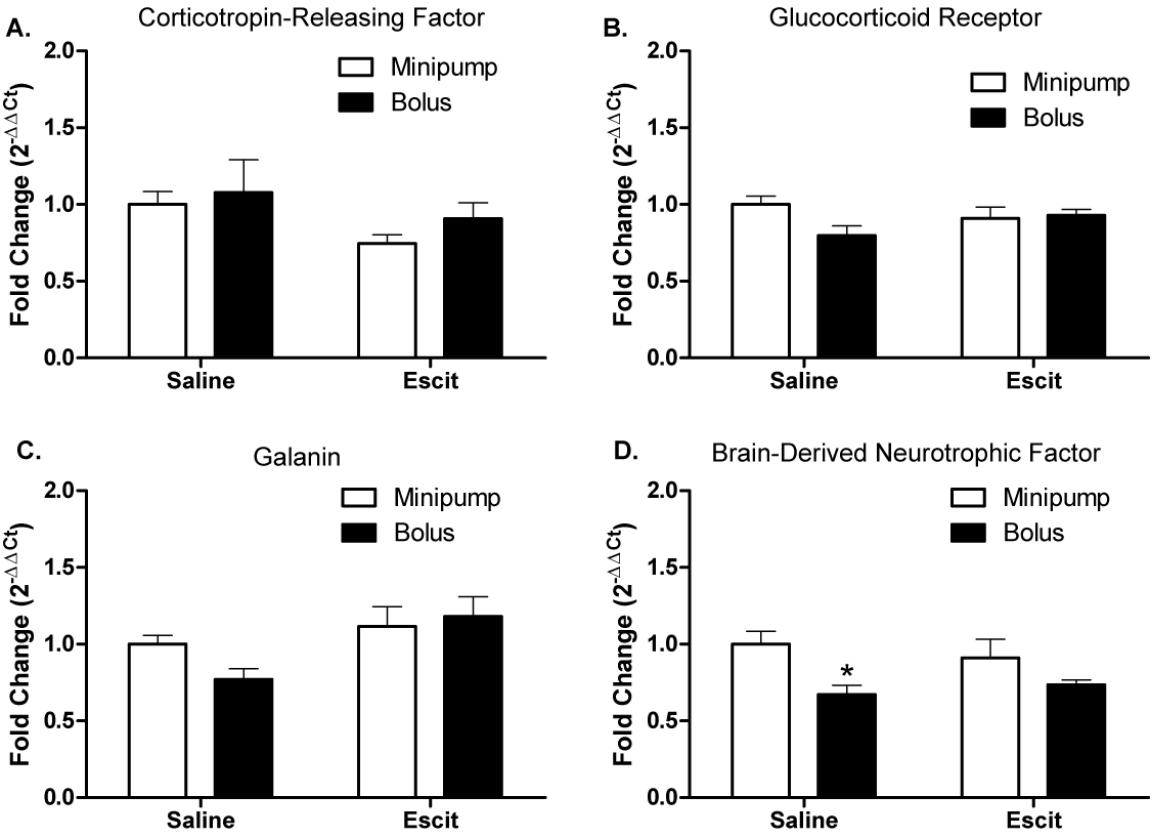


Figure A.A.4. Monoamine receptor and transporter gene expression in the hypothalamus of adult male animals prenatally exposed to escitalopram.

Dams were exposed to vehicle, low dose of escitalopram (5 mg/kg/d) or a high dose of escitalopram (12 mg/kg/d). Data are mean ± SEM, N = 3-8 per group.

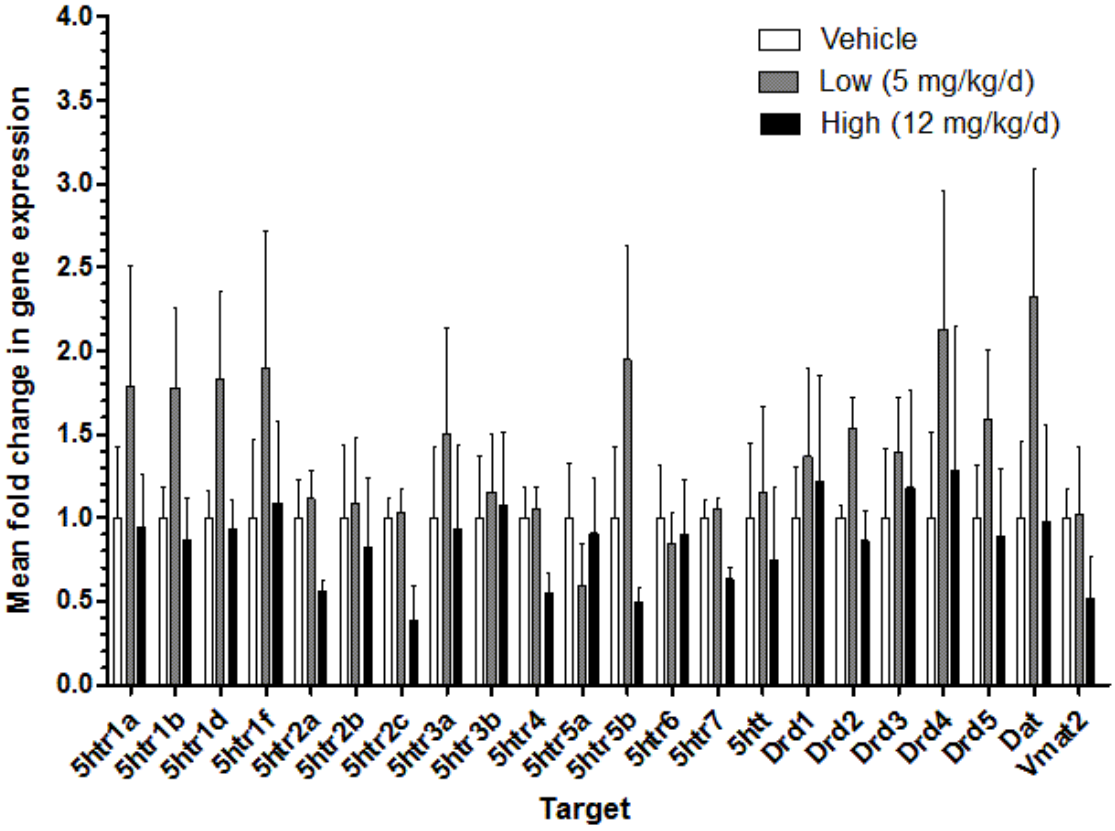


Figure A.A.5. Neuropeptide target gene expression in the hypothalamus of adult male animals prenatally exposed to escitalopram.

Dams were exposed to vehicle, low dose of escitalopram (5 mg/kg/d) or a high dose of escitalopram (12 mg/kg/d). Data are mean ± SEM, N = 4-8 per group.

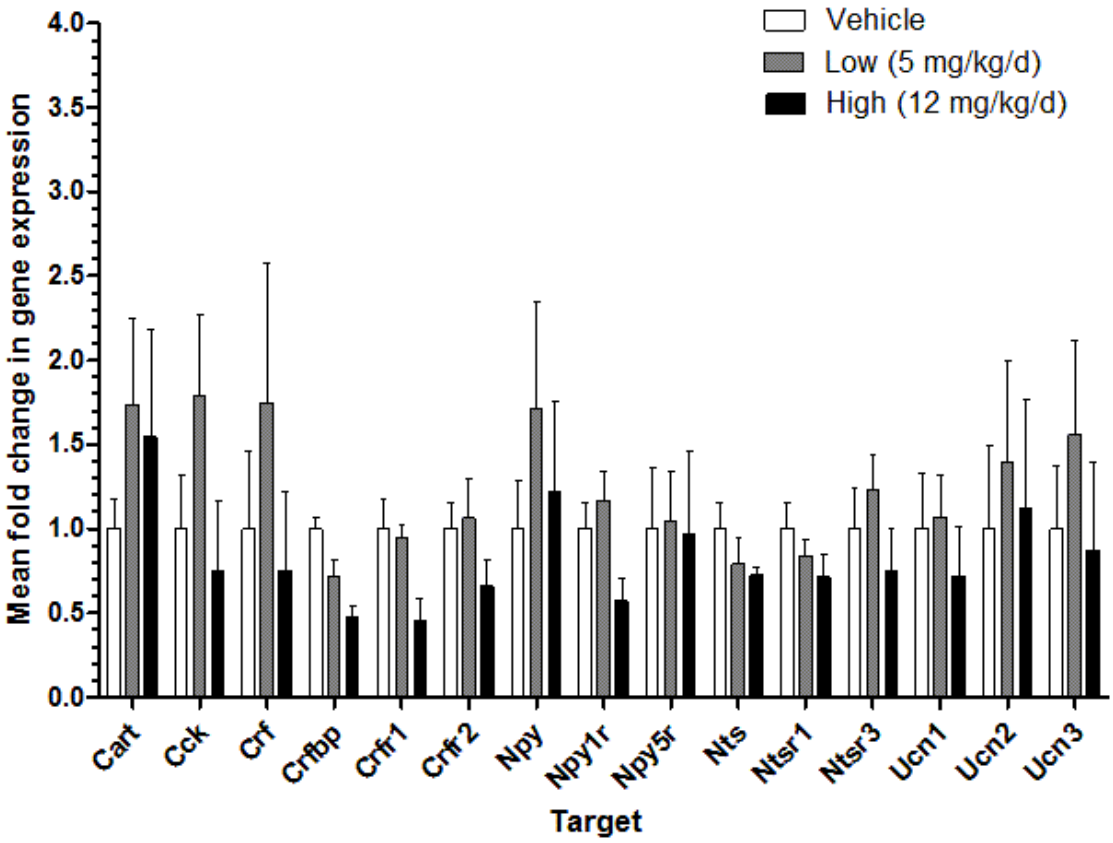


Figure A.A.6. Neurotrophic factor and associated target gene expression in the hypothalamus of adult male animals prenatally exposed to escitalopram.

Dams were exposed to vehicle, low dose of escitalopram (5 mg/kg/d) or a high dose of escitalopram (12 mg/kg/d). * $p < 0.05$, *Bonferroni post hoc test*. Data are mean ± SEM, N = 4-8 per group.

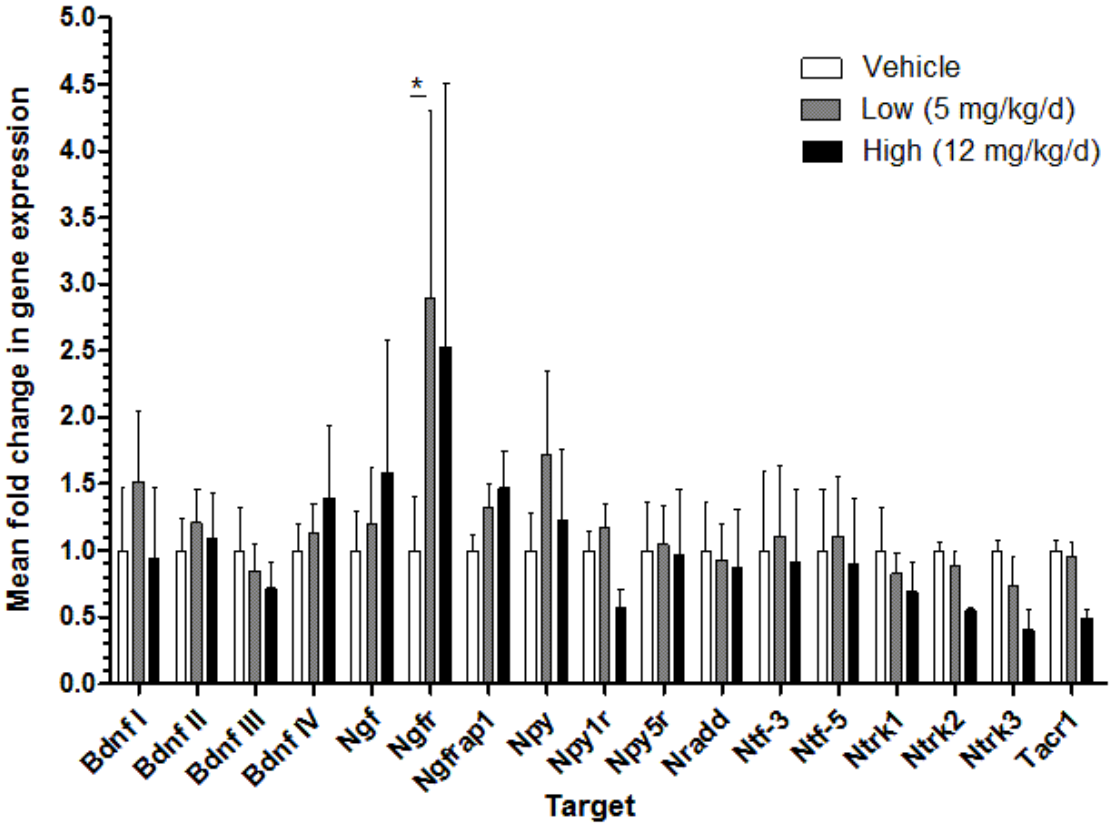
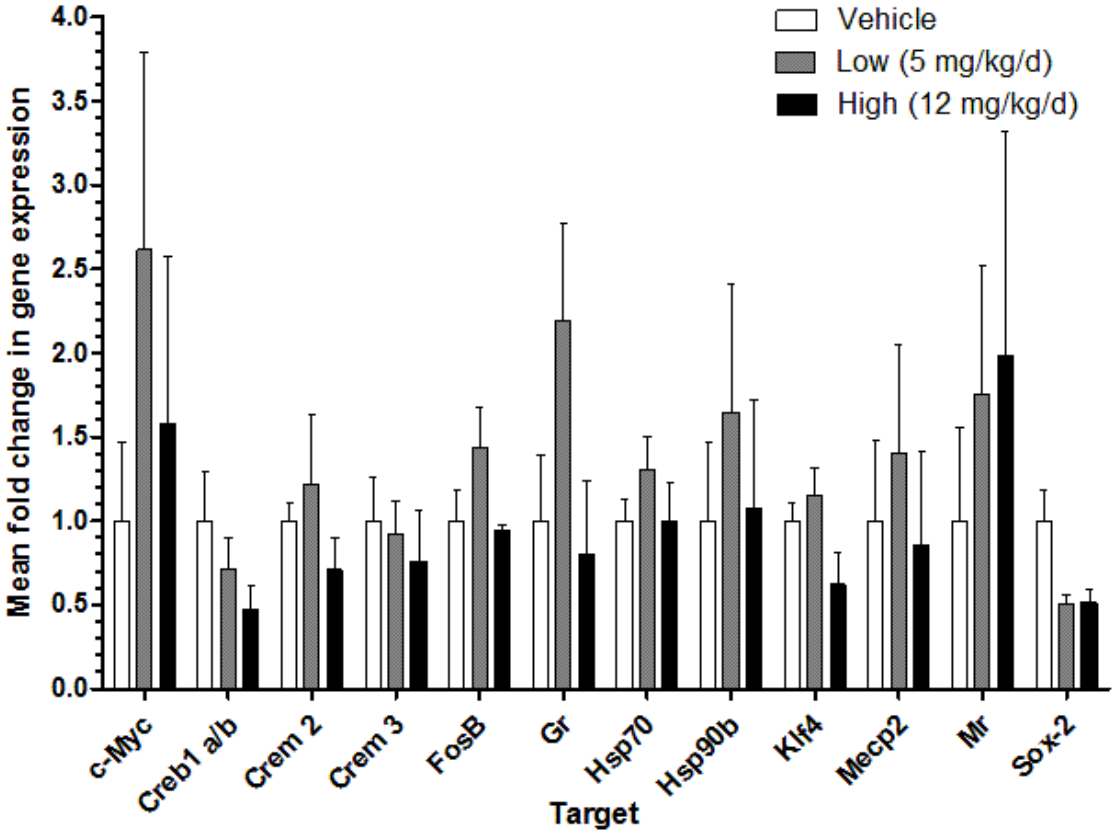


Figure A.A.7. Transcription factor gene expression in the hypothalamus of adult male animals prenatally exposed to escitalopram.

Dams were exposed to vehicle, low dose of escitalopram (5 mg/kg/d) or a high dose of escitalopram (12 mg/kg/d). Data are mean ± SEM, N = 4-8 per group.



APPENDIX B: HIPPOCAMPAL EPIGENETIC MODIFICATIONS IN ADULT RATS AFTER PRENATAL STRESS AND/OR ESCITALOPRAM EXPOSURE

Abstract

Epigenetic mechanisms functionally modify DNA or the dynamics of DNA coiling around histone proteins. Differences in the epigenome caused by prenatal stress are purported to underlie many long-term differences in behavior and HPA activity. Adult males prenatally exposed to stress and escitalopram were killed in adulthood and hypothalamic tissue was used for chromatin studies. Chromatin immunoprecipitation experiments were conducted to determine the binding sites of two histone modifications: H3K9-Ac (marker of transcription activation) and H3K9-Me3 (marker of transcription repression). Primer pairs were designed in the promoter regions of *Crh*, *Nr3c1*, and *Slc6a4*. While significant binding was observed in *Crh* and *Nr3c1* promoter regions, no group differences were found due to prenatal stress or prenatal stress with escitalopram exposure. To determine the global differences in the epigenome due to prenatal stress, 5-hydroxymethylcytosine modifications were measured in hippocampal tissue of adult male rats. Prenatal stress appeared to alter some peaks of 5-hydroxymethylcytosine sites, although these peaks were centered on intergenic regions. Additionally, a small number of animals was used to conduct the studies. Further studies with a larger number of animals are currently underway. However, initial studies do not support a role of prenatal stress in any long-term epigenetic differences.

Introduction

Epigenetics are believed to play a role in the etiology of many disorders and diseases. The two primary parts of the epigenetic code are histone modifications and methylation of genomic DNA (Qiu, 2006). Genomic DNA coils around histone proteins and the tightness of this interaction can prevent transcription factors from reaching the target site and initiate transcription. Histone modification occurs through covalent modification of the histone tails either through methylation or acetylation. Depending on the histone site and the modification, genomic DNA can bind wrap tighter around the histone core proteins or unwind enough to allow transcription factor binding. This process is thought to be dynamic: modification and subsequent transcription can occur in hours. Adverse events early in life, such as poor maternal care, can modify histones in a long-term manner and increase anxiety-like behavior (Weaver et al., 2004; Weaver et al., 2006).

The dynamic process of histone modification is contrasted with the static process of methylation and hydroxymethylation of cytosines on genomic DNA. Methyltransferases can covalently bind a methyl group to cytosine residues. These modifications can in turn alter transcription factor binding or enzymatic cleavage of DNA by preventing protein binding at the DNA response element. Methylation is thought to be static since the methyl signature can persist throughout life. Epigenetic modification can even persist to subsequent generations through RNA-based mechanisms, leading to heritable epigenetic mechanisms of disease or protection (Nelson and Nadeau, 2010).

While my previous work focused mostly on the hippocampus, I chose to examine if prenatal stress caused epigenetic modifications in the hippocampus and the

hypothalamus. The hypothalamus is the initial source of HPA activation, with significant modulation by the amygdala and hippocampus. HPA dysregulation is observed in a subset of patients with major depressive disorder, likely though disrupted negative feedback (Budziszewska, 2002). Stress and glucocorticoids have been shown to regulate expression of the glucocorticoid receptor, a primary modulator of the negative feedback response, in the hippocampus and the hypothalamus (Anacker et al., 2011; Bourke et al., 2012). Therefore, I chose the hippocampus and hypothalamus since they have been implicated in the mechanism of stress.

Materials and Methods

Chromatin Immunoprecipitation Studies

Chromatin Immunoprecipitation (ChIP) assays were performed essentially as described in a procedure obtained from the P. Farnham laboratory (University of Wisconsin). Hippocampal tissue was homogenized in formaldehyde at a final concentration of 1% added directly to the tissue. Homogenate was pelleted after 10 min of formaldehyde exposure, resuspended in phosphate-buffered saline, and repelleted. Homogenate was lysed in lysis buffer (5 mM PIPES [pH 8.0], 85 mM KCl, and 0.5% NP40 containing protease inhibitors [1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin]) and incubated on ice for 5 min. Nuclei were pelleted and lysed in nuclei lysis buffer (50 mM Tris-HCl [pH 8.1], 10 mM EDTA, and 1% SDS), containing protease inhibitors. Lysed nuclei were sonicated using a microtip until the average DNA fragment was approximately 600 base pairs. Chromatin samples were diluted 1:10 with IP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.1], and 167 mM NaCl) containing protease inhibitors and 50 μg/ml of yeast tRNA 20 μg/ml salmon sperm DNA. Immunoprecipitations were performed at 4°C for 2 hr with 5 μg of primary antibody (anti-H3K9-Me3 was purchased from Millipore Corporation, Saint Louis, MO and anti-H3K9-Ac was purchased from Active Motif, Carlsbad, CA). Immune complexes were harvested with secondary antibodies linked to protein G beads (Invitrogen, Carlsbad, CA). Immune complexes were washed three times for a period of 10 min with the following buffers: IP dilution buffer, dialysis buffer (2 mM EDTA, 50 mM Tris-HCl [pH 8.1], and 0.2% sarkosyl), TSE-500 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], and 500 mM NaCl), and IP wash buffer (100 mM

Tris-HCl [pH 8.1], 500 mM NaCl, 1% NP40, and 1% deoxycholic acid), followed by a final wash with TE (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA). Immune complexes were disrupted with elution buffer (50 mM NaHCO3 and 1% SDS) and covalent links reversed by addition of NaCl to a final concentration of 300 mM and heating to 65°C for 6 hr. DNA was ethanol precipitated and further purified by proteinase K digestion, phenol/chloroform extraction, and ethanol precipitated. DNA pellets were dissolved in 30 μL water and 3 μL used as template for PCR reactions. Real time PCR was conducted as described in previous chapters.

Genomic DNA Isolation for 5-hydroxymethylcytosine Sequencing Studies

5-hydroxymethylcytosine studies were conducted as described in (Szulwach et al., 2011). Genomic DNA was isolated by cell lysis in digestion buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl), Proteinase K treatment (0.667 ug/ul, 55°C overnight). The second day, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 Saturated with 10 mM Tris, pH 8.0, 1 mM EDTA) (P-3803, Sigma) was added to samples, mixed completely, and centrifuged for 5 min at 14,000 rpm. The aqueous layer solution was transferred into a new Eppendorf tube and precipitated with 2 volumes 100% ethanol and 1/10 volume 3 M NaOAc. The genomic DNA was recovered and dissolved with 10 mM Tris-HCl, pH 8.0. Genomic DNA samples were further sonicated into ~500 bp by Misonix sonicator 3000 (using microtip, 4 pulses of 27 s each, with 1 min of rest and a power output level of 2; the sonication was performed always on ice). The fragment size of sonicated DNA was verified by agarose gel electrophoresis. The DNA concentration was determined with NANO-DROP 1000 (Thermo Scientific).

5-hmC enrichment was performed using a previously described procedure with an improved selective chemical labeling method (Song et al., 2011). Briefly, the 5-hmC labeling reactions were performed in a 100 μL solution containing 50 mM HEPES buffer (pH 7.9), 25 mM MgCl2, 300 ng/μL sonicated genomic DNA (100–500 bp), 250 μM UDP-6-N3-Glu, and 2.25 μM wild-type β-GT. The reactions were incubated for 1 h at 37°C. After the reaction, the DNA substrates were purified via Qiagen DNA purification kit or by phenol-chloroform precipitation and reconstituted in H_2O. The click chemistry was performed with the addition of 150 μM dibenzocyclooctyne-modified biotin into the DNA solution, and the reaction mixture was incubated for 2 h at 37°C. The DNA samples were then purified by Pierce Monomeric Avidin Kit (Thermo) following the manufacturer's recommendations. After elution, the biotin-5-N3-gmC-containing DNA was concentrated by 10 K Amicon Ultra-0.5 mL Centrifugal Filters (Millipore) and purified by Qiagen DNA purification kit.

Sequencing of 5-hmC–Enriched and Input Genomic DNA

DNA libraries were generated following the Illumina protocol for "Preparing Samples for ChIP Sequencing of DNA" (Part# 111257047 Rev. A). 25 ng of input genomic DNA or 5-hmC-captured DNA was used to initiate the protocol. DNA fragments of ~150–300 bp were gel-purified after the adapter ligation step. PCR-amplified DNA libraries were quantified on an Agilent 2100 Bioanalyzer and diluted to 6-8 pM for cluster generation and sequencing. We performed 38-cycle single-end sequencing using Version 4 Cluster Generation and Sequencing Kits (Part #15002739

and #15005236 respectively) and Version 7.0 recipes. Image processing and sequence extraction were done using the standard Illumina Pipeline.

Sequence Alignment, Binning, and Peak Identification

FASTQ sequence files were aligned to the Rat reference (rn4) using Bowtie 0.12.6, retaining only unique, non-duplicate genomic matches with no more than 2 mismatches within the first 25 bp. Unique, non-duplicate reads from non-enriched input genomic DNA and each 5-hmC-enriched sequence set were counted in 1000-, 5000-, and 10,000-bp bins genome-wide and subsequently normalized to the total number of non-duplicate reads in millions. Our collaborators have found that bins of varying size produce largely similar patterns genome wide and have reported values within a bin size of 10 kb within all figures. Input-normalized values were subtracted from 5-hmC-enriched values per bin to generate normalized 5-hmC signals.

Chromosome-wide densities were determined as reads per chromosome divided by the total number of reads in millions. Expected values were determined by dividing 106 by the total rn4 length, and multiplying by chromosomal length. Expected values were divided by 2 for chromosomes X and Y.

5-hmC peaks were identified using MACS (Zhang et al., 2008) with the following parameters: effective genome size = 2.7e+09; Tag size = 38; Bandwidth = 200; P-value cutoff = 1.00e-08; ranges for calculating regional lambda are: peak_region, 200, 1000.

Association of 5-hmC peaks with genomic features was performed by overlapping peak locations with known genomic features obtained from UCSC Tables for rn4: RefSeq Whole Gene, 5′UTR, Exon, Intron, 3′UTR, +/−500 bp of RefSeq TSS, RefSeq Intergenic

(complement of Whole Gene), CpG Islands (+/−2 kb of CGI, Intergenic/Intragenic/TSS based on RefSeq Whole Gene). Predicted enhancer locations were obtained from (Hawkins et al., 2010). Peaks were assigned to a given genomic feature if overlapping ≥1 bp. Expected values were determined based on the percent base coverage of each defined genomic feature in rn4.

Results

Optimization of Cross-Linking and Sonication Times for Hypothalamic Tissue

Initial experiments were conducted to determine the ideal crosslinking times (incubation time in formaldehyde to bind histone proteins to genomic DNA) and sonication times (sonication time necessary to shear genomic DNA into short fragments for real time PCR). Real time PCR was used after various crosslinking and sonication times were applied to various chromatin immunoprecipitation experiments. Ten minutes of crosslinking and ten minutes of sonication was consistently successful across different genomic regions to give a high percent of input (Fig. A.B.1).

Elucidation of the H3K9-Ac and H3K9-Me3 Binding Sites in Genomic Regions of Target Genes

Chromatin immunoprecipitation of H3K9-Ac or H3K9-Me3 and subsequent real time PCR of genomic regions revealed different amplification of targeted genomic regions. 43 bps upstream of *Crh* and 216 or 8 bps upstream of *Nr3c1* had the highest histone occupancy through H3K9-Ac (Fig. A.B.2). However, other regions had little to no binding and many of these regions were only slightly above background (Fig. A.B.2).

H3K9-Ac and H3K9-Me3 Histone Modifications Due to Prenatal Exposures in Genomic Regions of Target Genes

Chromatin immunoprecipitation of IgG (negative control pull down), H3K9-Ac or H3K9-Me3 and subsequent real time PCR of genomic regions revealed no differential

regulation of histone binding at targeted regions in the *Crh* (Fig. A.B.3), *Nr3c1* (Fig. A.B.4), or *Slc6a4* (Fig. A.B.5) promoter regions (*Bonferroni post hoc tests*, $p > 0.05$).

ChIP-Sequencing of Adult Hippocampal Tissue After Prenatal Stress

ChIP-Seq of 5-hydroxymethylcystosine sites revealed a high number of reads in hypothalamic tissue between control and prenatally-stressed animals (Table A.B.2). The percent of unique, monoclonal reads was very high (>98%), showing the relative purity and high integrity of the sample. However, the percent of reads with at least one reported alignment was lower than expected (~60%), possibly due to differences in the enzymatic reaction to enable affinity purification. Currently, more samples are being sequenced to verify the number of alignments and increase the power of the experiment. Despite the low number of overlapping reads, 5-hydroxymethlcytosine groups were analyzed to determine the genomic sites of hydroxymethylation. Many of the reads were in genomic regions associated with coding sequences, introns, and exons (Fig. A.B.6). Chromosome-wide distribution of sequencing reads were similar between control and prenatally-stressed animals (Fig. A.B.7). Regions with significant 5-hydroxymethlation (MACS peaks) were further analyzed to determine regions of expression. Differences between control and prenatally-stressed animals were found on chromosomes 4 and 14. ChrUn is an unverified region: ChrUn contains clone contigs that can't be confidently placed on a specific chromosome. While these MACS peaks were significantly different between control and prenatally-stressed animals (Chr4: $t_2 = 3.3$, $p < 0.05$; Chr14: $t_2 = 8.8$, $p < 0.001$, *Bonferroni post hoc test,* Fig. A.B.8), the sample size was small and more

samples are needed for the necessary power. Current studies are adding samples to ChIP-sequencing experiments to increase the number of samples per group.

Tables

Table A.B.1. Chromatin immunoprecipitation primer sequences and concentrations. [SN]: sense primer concentration, [AS]: antisense primer concentration in nM.

Target	Accession	Sense Primer (Forward, 5'-3')	Antisense Primer (Reverse, 5'-3')	[SN]	[AS]
GR exon 7	AJ271870.1	TGTGACACACTTCGCGCA	GGAGGGAAACCGAGTTTC	900	900
Crh (-1196->-1046)	NC_005101.2	TTTTCTGGTCTGTATCTGGCCTA	TATTTATCGCCTCCTTGGTGA	50	50
Crh (-698->-544)	NC_005101.2	CCAAGGGAGGAGAAGGTAGG	AAGGCACAGTTAGCGACACA	50	50
Crh (-373->-253)	NC_005101.2	GGAGGAAATGATGTCCGAAA	GGAGGAAATGATGTCCGAAA	900	300
Crh (-42->+50)	NC_005101.2	TCTCCCTCTACCCCAACCTC	ACAGAGCCACCAGCAGCAT	300	900
Nr3c1 (-584->-496)	NC_005117.2	AGCAGCTCAGCACTGCTTTT	CCGATCCCAGCACTTCTAAA	50	50
Nr3c1 (-393->-279)	NC_005117.2	GGGGCTGGAAATGTAAGGAT	TTGGTAACCATGGGGATTGT	50	50
Nr3c1 (-216->-104)	NC_005117.2	TGTCTCTGTGCAAATGAGCTG	AACTCCCAAATCAAAGTCTCCA	50	300
Nr3c1 (-8->-111)	NC_005117.2	ATTTGCCAATGGACTCCAAA	TCCTCCCCTCAGGCTTTTAT	900	300
Slc6a4 (-672->-566)	NC_005109.2	GTCTCTTCTGGCTGGTAGGC	CCCAGAGGAATGTCAGGAGA	50	300
Slc6a4 (-528->-428)	NC_005109.2	TGACACAAGTGCTGGTCCTT	GGCCAAATCACCACCAAGTA	50	300
Slc6a4 (-110->+5)	NC_005109.2	CAGAGCTTTCCGTCTTGTCC	TCCATGCTGCTGGTCAGTAA	300	50

Table A.B.2. Read summary of ChIP-Seq experiment

	Control 1	Control 2	Stress 1	Stress 2	Mean	Standard Dev
# reads processed	5,369,900	5,263,574	4,008,503	3,971,790	4,653,442	767,283
# reads with at least one reported alignment:	3,384,910	3,646,603	2,490,776	2,160,113	2,920,601	708,462
% reads with at least one reported alignment:	63.03%	69.28%	62.14%	54.39%	62.21%	6.11%
# reads that failed to align:	1,090,947	726,596	846,830	1,162,281	956,664	204,373
% reads that failed to align:	20.32%	13.80%	21.13%	29.26%	21.13%	6.34%
# reads with alignments suppressed:	894,043	890,375	670,897	649,396	776,178	134,277
% reads with alignments suppressed:	16.65%	16.92%	16.74%	16.35%	16.67%	0.24%
Reported alignments to 1 output stream(s)	3,384,910	3,646,603	2,490,776	2,160,113	2,920,601	708,462
# monoclonal reads	3,350,649	3,621,415	2,474,063	2,137,351	2,895,870	703,920
% monoclonal reads	98.99%	99.31%	99.33%	98.95%	99.15%	0.20%
# MACS peaks	374	491	182	292	335	131

Figures

Figure A.B.1. Optimization of cross-linking and sonication times for H3K9-Ac hypothalamic tissue.

XN: crosslinking time (minutes), SN: sonication pulses

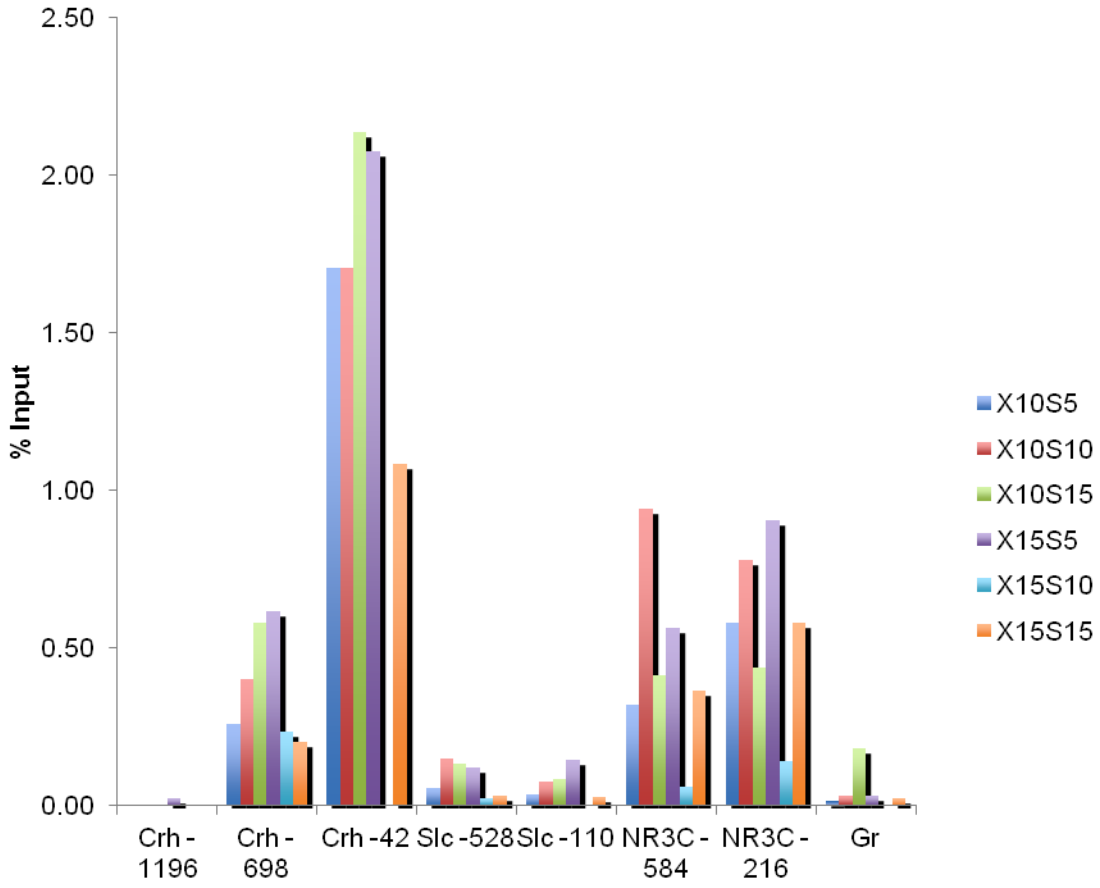


Figure A.B.2. Approximate binding sites of acetylated and trimethylated H3K9 histone. Red represents more than 0.5% of input (relatively high binding), yellow represents 0.1-0.5% of input (relatively modest binding), no shading represents less than 0.1% of input (relatively low to no binding).

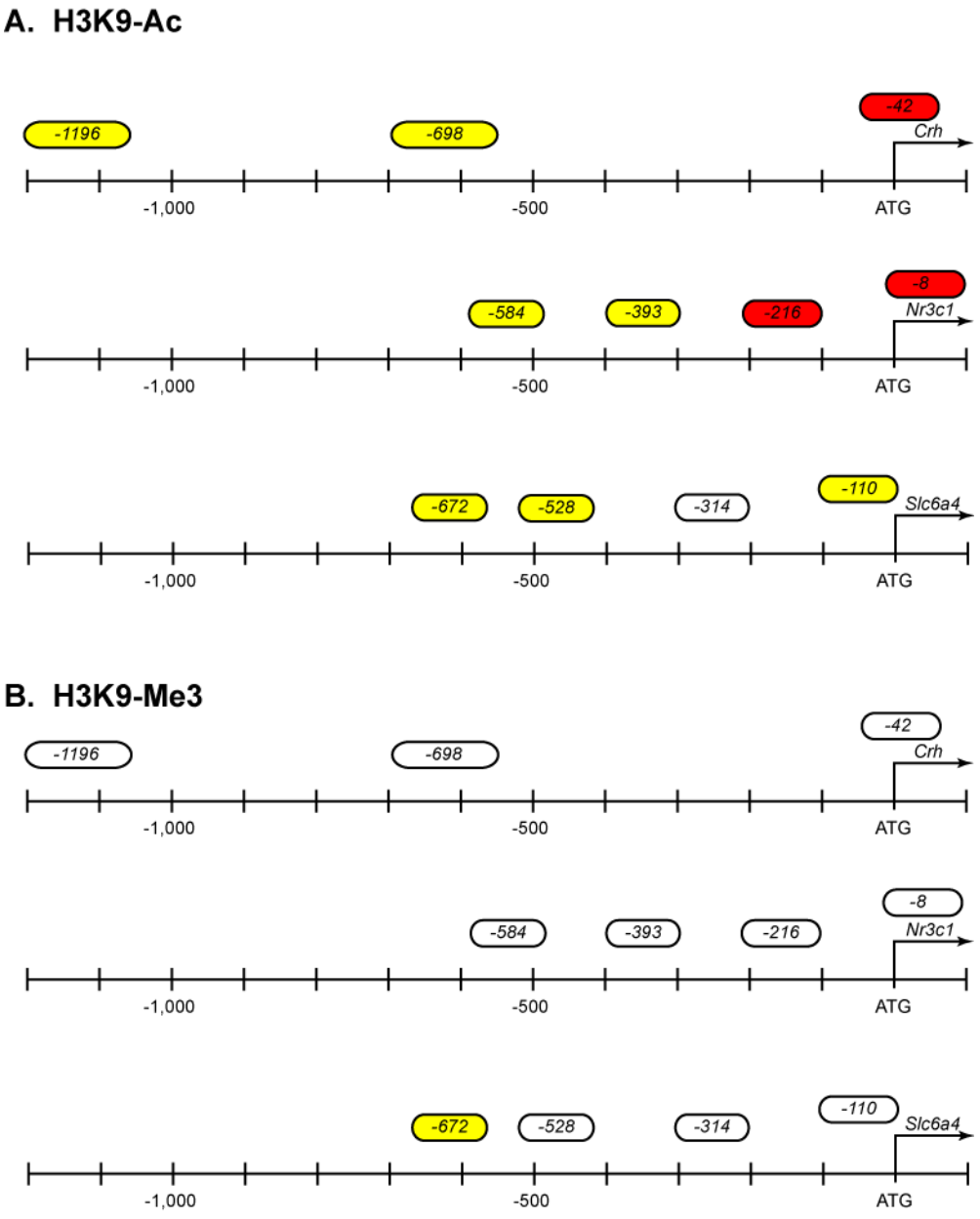


Figure A.B.3. Histone modifications in the *Crh* promoter region.

Primer sequences were made to span upstream regions of the *Crh* promoter region starting 1196, 698, or 42 base pairs upstream of the ATG transcription start site. A negative control (IgG) pull down was included to show a lack of enrichment. Data are mean ± SEM, N = 2 per group.

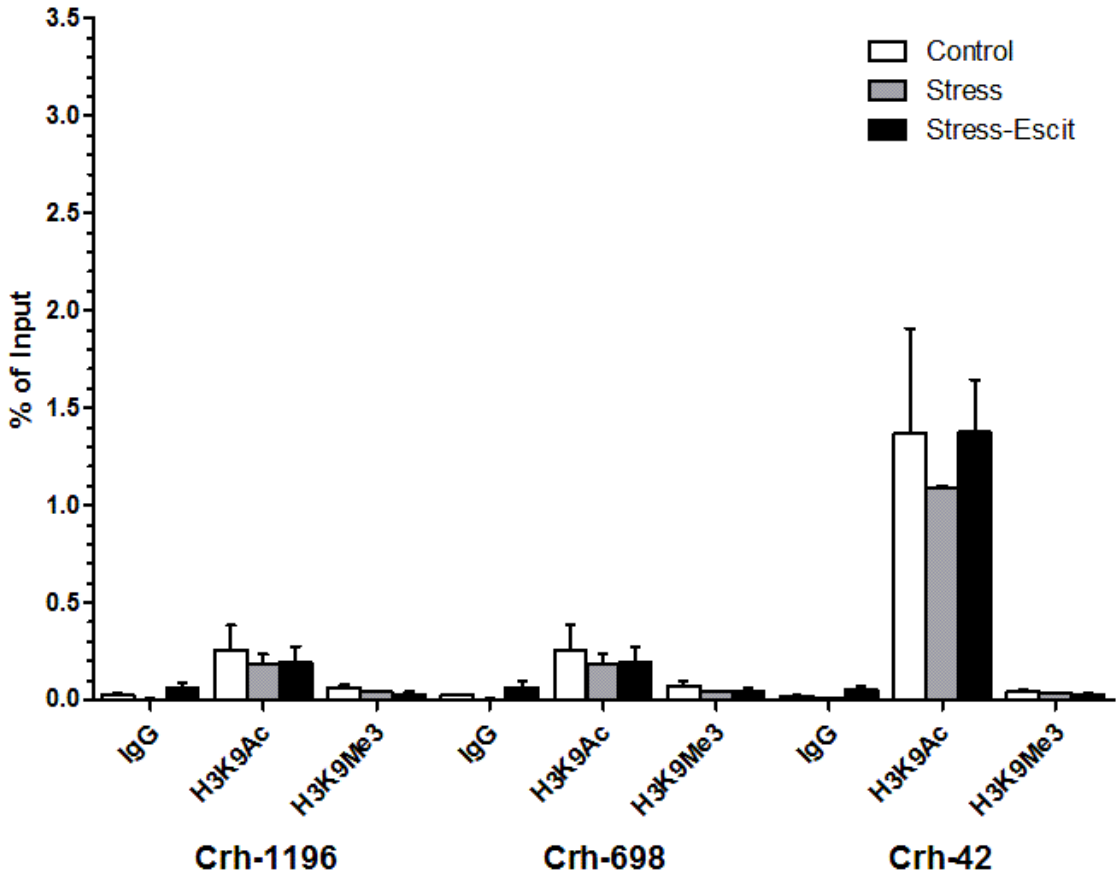


Figure A.B.4. Histone modifications in the *Nr3c1* promoter region.

Primer sequences were made to span upstream regions of the *Nr3c1* promoter region starting 584, 393, 216 or 8 base pairs upstream of the ATG transcription start site. A negative control (IgG) pull down was included to show a lack of enrichment. Data are mean ± SEM, N = 1-2 per group.

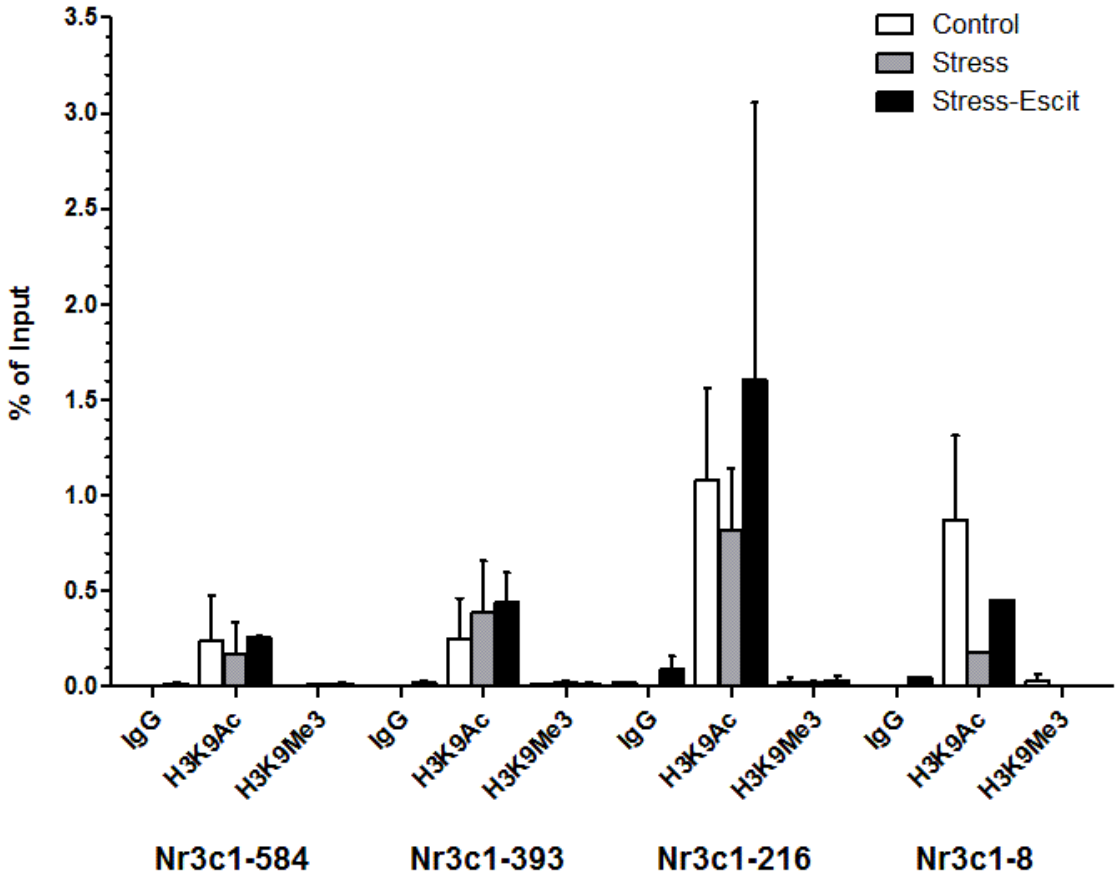


Figure A.B.5. Histone modifications in the *Slc6a4* promoter region.

Primer sequences were made to span upstream regions of the *Slc6a4* promoter region starting 672, 528, 314 or 110 base pairs upstream of the ATG transcription start site. A negative control (IgG) pull down was included to show a lack of enrichment. Data are mean ± SEM, N = 2 per group.

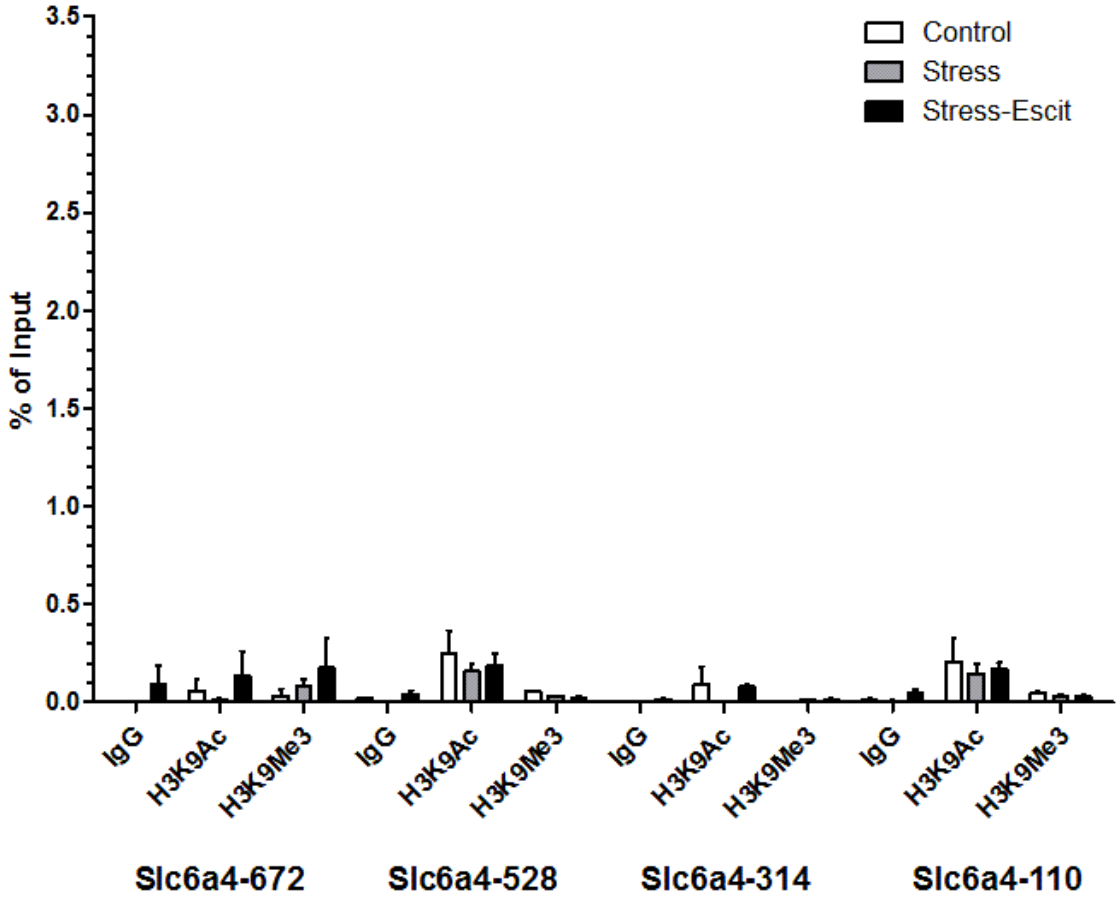


Figure A.B.6. Genomic features of 5-hydroxymethylcytosine DNA in adult male hippocampal tissue.

CpG: cytosine-phosphate-guanine feature; CGI: CpG island; CDS: coding sequence; UTR: untranslated region; Up500: 500 base pairs upstream of a ATG start site.

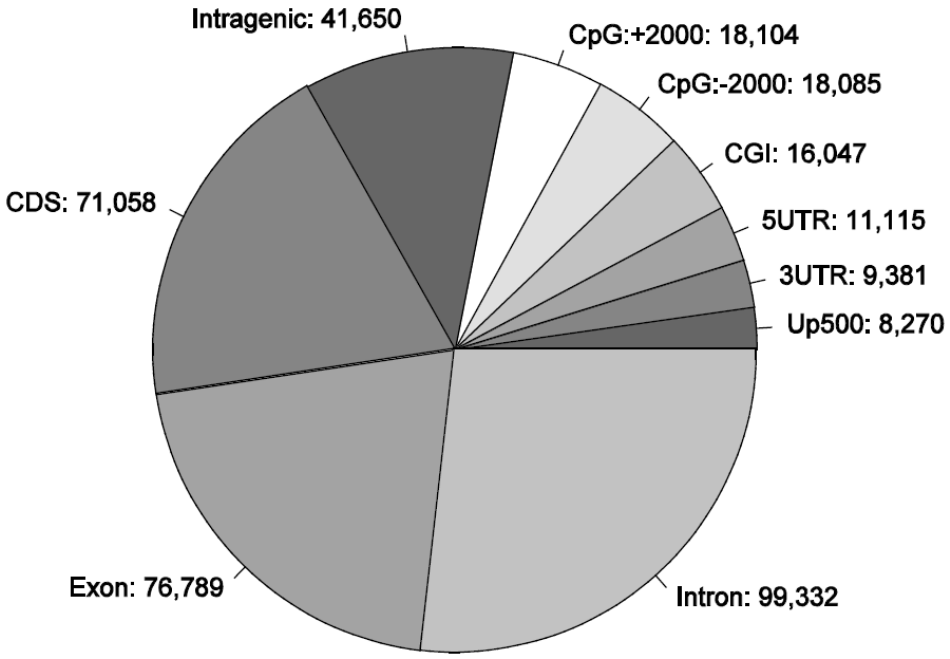


Figure A.B.7. Chromosome-wide distribution of 5-hydroxymethylcytosine sites. Data are mean ± SEM, N = 2 per group.

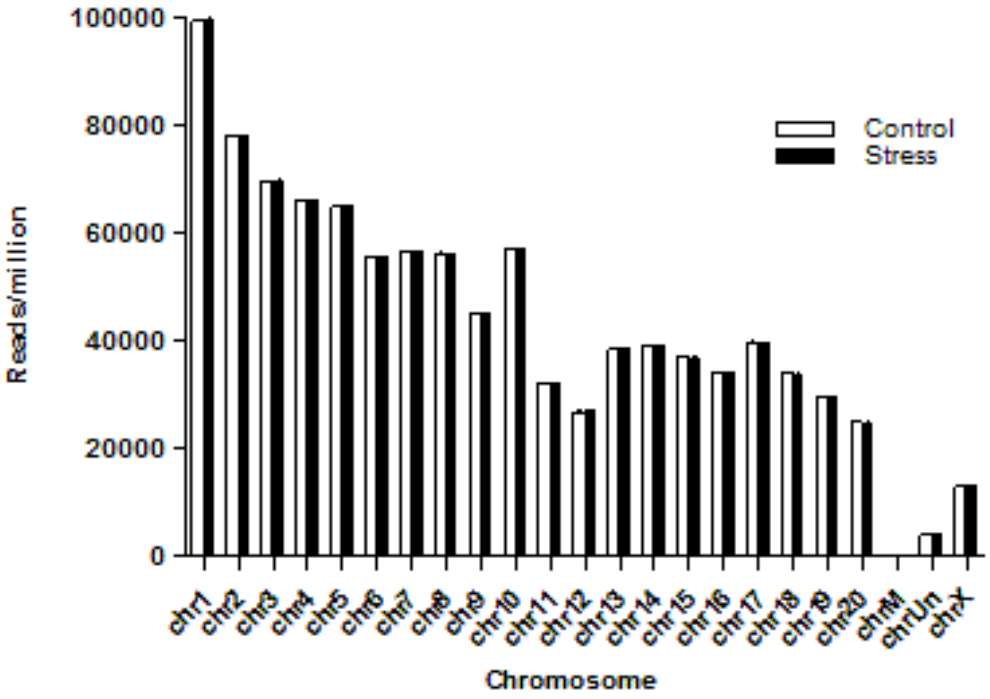
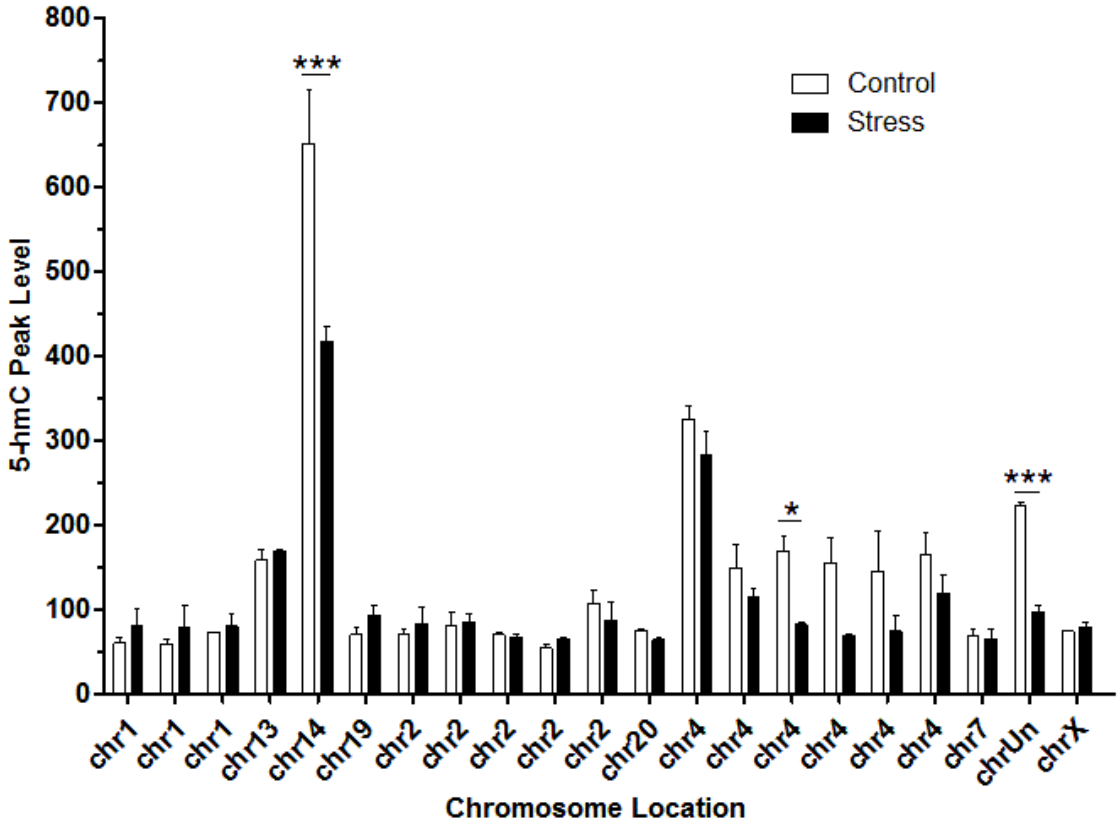


Figure A.B.8. MACS peaks analysis of the adult male hippocampus from controls or animals prenatally exposed to stress.

MACS peaks were identified on several different chromosomes and compared. * $p <$ 0.05, *** $p < 0.001$ *Bonferroni post hoc test*. Data are mean ± SEM, N = 2 per group.



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