CARDIOVASCULAR, METABOLIC, ENDOCRINE AND BEHAVIORAL ASPECTS
OF DEVELOPMENT IN POSTNATAL LAMBS IN RELATION TO AGE, SEX,
LAMB NUMBER AND ACUTE FLUOXETINE ADMINISTRATION

by

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Abstract

Human newborns exposed in utero to maternally administered SSRIs such as fluoxetine (FX) have an increased risk of adverse pregnancy outcomes including poor neonatal adaptation. This comprises respiratory difficulty, jitteriness, cyanosis when feeding and persists for several days after birth. Several potential mechanisms underlying these symptoms have been proposed: 1) acute toxicity to the drugs (i.e. serotonin syndrome), 2) withdrawal syndrome due to the sudden discontinuation of maternal-fetal placental drug transfer at birth or 3) an SSRIs-elicited alteration in fetal brain development. However, the actual mechanism has not been elucidated. In addition, the safety of SSRIs for the treatment of depression in children and adolescents is uncertain. Thus, we conducted experiments in which acute i.v FX (1mg/kg) and sterile water were given to FX and control groups in the lambs at ~ 3,10 days, 1,3,6 and 12 months of age. In another cohort, daily 50mg FX was given i.v to 5 pregnant ewes via implanted catheters during late gestation (131-132d, term = 147d) for 2 weeks. Plasma FX concentrations in the newborn acute FX group were within the range measured in human infants at the same age. There was a lack of significant acute FX effects on cardiovascular-respiratory, behavioral and endocrine functions in ~ 4 day old lambs. In the lambs exposed to FX prenatally, plasma FX at birth and postnatal day (PND) 2 were low and undetectable on PND 5,10,14. However, prenatal FX-exposed lambs were hyperactive during PND 4 to14 and their heart rate variability (HRV) was significantly lower than control lambs on PND 2. Results suggested that SSRI toxicity and withdrawal are unlikely to be the mechanism underlying poor neonatal adaptation in human exposed to FX in utero. However, acute FX effects were seen in some, but not all, age groups. No acute FX effects were observed in the young lambs (10d and 3 months of age). However, hypoactivity,
transient hypoxemia, hypertension and increased HRV occurred after acute FX administration in the older lambs (1,6 and 12 months of age). Hypoxemia and hypertension effects were more profound in males. No changes in HPA axis function were observed.
Preface

I wrote this thesis with direction and input from Dr. Dan Rurak and my supervisory committee members: Drs. Tim Oberlander, Ken Lim, Wayne Riggs and Anthony Perks. Surgeries in the ewes and lambs were performed by Dr. Rurak or a clinical veterinarian, Dr. Bev Chua with assistance from myself and Tim Chow, whose doctoral thesis related to the determination of the disposition of FX in the postnatal lambs. (Chow, 2013) Daily catheter flushing and lamb weights were done by myself and Tim. The experiments on the lamb included in Chapters 3 to 5 were conducted by myself and Tim Chow with occasional support from our supervisor, Dr. Dan Rurak. I was responsible for collecting heart rate, blood pressure, heart rate variability data and blood gas samples. Blood gas samples were run by myself and Dr. Rurak. I was also responsible for collecting plasma samples for ACTH and cortisol. Tim Chow was responsible for collecting plasma samples for FX measurement and urine samples as well as developing and running the LC/MS/MS assay. (Chow et al, 2011) Thus the plasma FX concentration versus time curve in figure 4.1 is from Tim’s thesis. However, although Tim analyzed the plasma FX concentrations in Chapter 6, these data are not part of his PhD thesis, I did all the remaining data analysis from actiwatch, Digital Video Recording (DVR), power lab, ECG, blood gas, ACTH and cortisol assay.

All experiments, sample collection and data analysis in the ewes and lambs included in Chapter 6 were done by myself. The catheter implantation procedure in the pregnant ewes was performed by Dr. Bev Chua with assistance from myself, Dr. Rurak and the staff in the Centre of Comparative Medicine (CCM). FX measurement in this chapter was also run by Tim Chow.
Manuscripts will be prepared for future publication based on results from Chapters 3-6. Manuscripts will include relevant sections found in Chapters 1-7 including introduction, methods, results and discussions.

This thesis was conducted under animal care approval from the University of British Columbia Animal Research Ethics (Certificate number: A07-0302)
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<tr>
<td>5-HIAA</td>
<td>5-hydroxyindoleacetic acid</td>
</tr>
<tr>
<td>5-HTP</td>
<td>5-hydroxy-tryptophan</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxy-tryptamine/ Serotonin</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotrophin</td>
</tr>
<tr>
<td>ANS</td>
<td>Autonomic Nervous System</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under the Curve</td>
</tr>
<tr>
<td>BE</td>
<td>Base Excess</td>
</tr>
<tr>
<td>CBT</td>
<td>Cognitive-Behavioral Therapy</td>
</tr>
<tr>
<td>CCM</td>
<td>Centre of Comparative Medicine</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin-Releasing Hormone</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DVR</td>
<td>Digital Video Recording</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FFA</td>
<td>Free Fatty Acid</td>
</tr>
<tr>
<td>FFT</td>
<td>Fast Fourier Transformation</td>
</tr>
<tr>
<td>FX</td>
<td>Fluoxetine</td>
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<tr>
<td>GA</td>
<td>Gestational Age</td>
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<tr>
<td>GFR</td>
<td>Glomerular Filtration Rate</td>
</tr>
<tr>
<td>GH</td>
<td>Growth Hormone</td>
</tr>
<tr>
<td>GLM</td>
<td>General Linear Model</td>
</tr>
<tr>
<td>HF power</td>
<td>High Frequency power</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-Pituitary-Adrenal</td>
</tr>
<tr>
<td>HRV</td>
<td>Heart Rate Variability</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like Growth Factor-1</td>
</tr>
<tr>
<td>LF power</td>
<td>Low Frequency power</td>
</tr>
<tr>
<td>LF/HF ratio</td>
<td>Low Frequency/High Frequency power ratio</td>
</tr>
<tr>
<td>MAOI</td>
<td>Monoamine Oxidase Inhibitors</td>
</tr>
<tr>
<td>MCA</td>
<td>Middle Cerebral Artery</td>
</tr>
<tr>
<td>MDD</td>
<td>Major Depressive Disorder</td>
</tr>
<tr>
<td>msec</td>
<td>Millisecond</td>
</tr>
<tr>
<td>NaSSA</td>
<td>Noradrenergic and Specific Serotonergic Antidepressant</td>
</tr>
<tr>
<td>NDRI</td>
<td>Norepinephrine/Dopamine Reuptake Inhibitor</td>
</tr>
<tr>
<td>NFX</td>
<td>Norfluoxetine</td>
</tr>
<tr>
<td>nu</td>
<td>normalized unit</td>
</tr>
<tr>
<td>OCD</td>
<td>Obsessive Compulsive Disorder</td>
</tr>
<tr>
<td>PMSG</td>
<td>Pregnant Mare Serum Gonadotropin</td>
</tr>
<tr>
<td>PND</td>
<td>Postnatal Day</td>
</tr>
<tr>
<td>PPHN</td>
<td>Persistent Pulmonary Hypertension</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized Controlled Trial</td>
</tr>
<tr>
<td>REM</td>
<td>Rapid Eye Movement</td>
</tr>
<tr>
<td>RMSSD</td>
<td>Square root of the mean of the squared differences between adjacent NN intervals.</td>
</tr>
<tr>
<td>SARI</td>
<td>Serotonin Antagonist Reuptake Inhibitor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>SD Delta NN</td>
<td>Standard Deviation of the differences between adjacent NN intervals</td>
</tr>
<tr>
<td>SDNN</td>
<td>Standard Deviation of the NN intervals</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of Mean</td>
</tr>
<tr>
<td>SNRIIs</td>
<td>Serotonin-Norepinephrine Reuptake Inhibitors</td>
</tr>
<tr>
<td>sO2</td>
<td>Oxygen saturation</td>
</tr>
<tr>
<td>SSRIIs</td>
<td>Selective Serotonin Reuptake Inhibitors</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricyclic Antidepressant/Tricyclic Acids</td>
</tr>
<tr>
<td>VFA</td>
<td>Volatile Fatty Acid</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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</table>
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A final thanks to all the funding sources for the project and my studentship (CIHR, IWRH, CFRI).
Dedication

To the memories of my father

To my beloved mom and brother
1. Introduction

1.1 Postnatal Lamb Development:

1.1.1 Cardiovascular Function:

Birth is an event which involves remarkable changes in the environment surrounding the fetus. Moving from a comfortable warm environment, filled with fluid and maternal soft tissue as a cushion, and readily available nutrients and oxygen supply via the placenta in utero to an extra utero life, the newborn for the first time is exposed to cold challenge, gravity, air, and hard surfaces. In order for a successful transition to be accomplished, it is required that the newborn undergo a series of physiological changes (such as air breathing, shift in cardiovascular patterns, thermoregulation, alteration of oxygen transport (Lister et al., 1984) and behavioral adaptations (e.g: becoming aroused and aware of the surrounding environment, being responsive and interactive in order to start establishing bonding with the mother, and initiating suckling (Mellor & Gregory, 2003; Mellor, 1988).

After birth, the high relative cardiac output observed in the fetus is still maintained in the newborn lambs up to 1 month of age (Minoura & Gilbert, 1987). One of the reasons explained for the high cardiac output in the newborn lambs is that more $O_2$ needs to be transported to the tissues since $O_2$ extraction is limited by the avid binding of $O_2$ to hemoglobin (Lister et al, 1979). As there is an increasing oxygen consumption in the newborn lamb in response to cold exposure after birth (Alexander & Williams, 1968; Sidi et al., 1983), it is vital for them to maintain a high cardiac output. The subsequent decline in heart rate over the first 2 months of life is proportionate to that of cardiac output to keep the stroke volume relatively constant in relation to the body weight (Lister et al., 1979). In
lambs, cardiac sympathetic innervations and parasympathetic influences on the heart are incomplete at birth. However, the sympathetic nervous system appears to be predominant in the lamb less than 1 week of age whereas parasympathetic becomes dominant in lambs older than 3 months. It is believed that the sympathetic activity and circulating catecholamines help to maintain such high cardiac output in 1-3 day old lambs (Minoura & Gilbert, 1987). Siimes et al have reported that the beat-to-beat heart rate variability (HRV) increased in lambs after 3 weeks of age (Siimes et al., 1984). This increasing trend of HRV with advancing age is similar to that observed in human infants (Harper et al., 1976; Wheeler et al., 1979). In addition, HRV varies in relation to sleep state. It is highest during waking and active sleep and lowest during quiet sleep (Siimes et al., 1984).

1.1.2 Homeostasis And Metabolism:

Since nutrients are ingested orally after birth, before the newborns are able to nurse from their mother and before maternal milk production increases, they have to use their own energy reserves to produce heat against the cold stress. In newborn lambs, both shivering (muscle glycogenolysis) and non-shivering metabolism (or brown adipose tissue thermogenesis) are equally important in cold-induced thermogenesis. In brown adipose tissue thermogenesis, the brown adipocyte was stimulated by noradrenaline via β-adrenergic receptors. This gives rise to a series of actions with a final release of free fatty acid (FFA) within the cell. Even though these FFA might be released to the circulation, a significant amount of these FFA remain in the cell and is transferred to the mitochondria where they are oxidized to produce heat via the electron transport change and action of brown adipose specific uncoupling protein (Symonds et al., 2012). With advancing age, the non-shivering contribution declines rapidly while shivering metabolism increases (Alexander & Williams,
1968; Alexander et al., 1968, Symonds et al., 2012). However, lambs exposed to cold showed a much greater increase in free fatty acid concentration in those older than 20 days than those at 1-3 days old (Alexander et al., 1968). As the authors explained, this is because the proportion of brown adipose tissue, which can re-utilize fatty acids from triglyceride lipolysis, decreases while white adipose tissue increases over age, thus during cold exposure in the older lambs, the rate of lipolysis exceeds the rate of FFA recycling within the adipose cell (Alexander et al., 1968).

In the transition from prenatal life, in which nutrient supply and growth are increasingly constrained by the placenta, to postnatal life with major changes in the quantity and composition of nutrient supply, there is a requirement for metabolic adaptations. This includes colonization of the digestive system, which begins soon after birth (Morrison et al., 2009). The composition of nutrients in utero is primarily glucose and amino acids whereas a diet after birth may contain a high level of fat and less carbohydrate (Girard et al., 1997; Greenwood et al., 2002). In sheep, with the dietary switch from milk-fed to solid feed at weaning, the morphology and metabolic aspects of the ruminant digestive system also change accordingly. The rumen increases in size, the intraruminal papillae increase in length and the epithelium lining the rumen becomes keratinized (Lake et al., 1982; Lane, Baldwin, & Jesse, 2000; Lavker, Chalupa, & Opliger, 1969). In the presence of solid feed, ruminal microorganisms produce volatile fatty acids (VFA) via fermentation of the feed. VFA exposure appears to be necessary to stimulate rumen metabolic development. Examining rumen development in lambs with and without solid feed in their diet, Lane et al have concluded that the presence of solid food is needed for rumen metabolic maturation (Lane et al., 2000).
In a study of blood composition in normal and growth retarded lambs over the first 24 hour after birth, Mellor & Pearson (1977) found that plasma glucose and IgG increase a few hours after birth, which is most likely attributed to the absorption of colostrum from the gut. In contrast, lactate concentration decreases sharply after birth. Plasma fructose concentration was decreased progressively in late gestation fetal lambs till birth and had disappeared from the lamb’s plasma by the first day of birth (Comline & Silver, 1970).

A recent study in our lab has found that arterial Po$_2$, pH, BE, So$_2$ decrease and Pco$_2$ and hemoglobin concentration increase in the fetal lambs with advancing gestational age (Rurak & Bessette, 2012). However, to date there is limited information on the changes in arterial blood gas values of newborn and postnatal lambs in response to cardiovascular-respiratory development as well as other physiological processes.

1.1.3 **Behavior: Maternal-Infant Bonding:**

Different from rodents, which are altricial at birth, lambs are precocial and born at an advanced stage of physical development. In addition, all sense organs (tactile, olfactory, auditory and visual) of the lamb are fully-developed and ready to function from birth (Fraser & Broom, 1997). This allows mutual recognition between ewes and lambs, which happens very early following birth, and in which visual appearance of the lamb appears to be an important cue (Walser & Alexander, 1980). After the first two hours of parturition, cross fostering lambs is no longer possible, even when the alien lambs were masked by their foster mother’s amniotic fluid (Walser & Alexander, 1980). Indeed, maternal-infant bonding is first established immediately after delivery by a coordinated expression of behaviors between both the ewe and lambs. Together with the ewe’s behavior at birth such as licking or
grooming, rumbles (frequent low-pitched bleats made by the ewes as she licks the lamb dry) and udder acceptance, it is also vital for the lamb to successfully accomplish a sequence of behaviors directed toward standing, finding the udder and suckling during this immediate postpartum period (Fraser & Broom, 1997). These behaviors from the ewe serve not only for the formation of a strong maternal-infant attachment with her own lamb but also as an expression of nurturing behavior to facilitate the successful transition from the pre to postnatal life for the lambs (Dwyer, 2008a). Normally, the ewe licks to dry the lamb, to clear placental membranes from the lamb’s nose and mouth, to stimulate activity and respiration and to form an olfactory memory for the lamb. In addition, a rumbling sound made by the ewe when she licks the lamb may help to reduce stress in the lamb (Dwyer et al., 1998) and form the auditory memory for later maternal recognition (Walser & Alexander, 1980). These sound cues also strongly influence the lamb’s behavior. It helps the lambs to orientate to the ewe and keep them near to the ewe, especially in the dark or when mingling with other ewes and lambs. At the same time, in response to maternal care, lambs perform a series of behaviors after birth initiated by shaking the head, rolling onto the sternum, bleating, pushing up on the knees and then attempting to stand (Dwyer, 2003; Dwyer, 2008a; Fraser & Broom, 1997). Most newborn lambs are able to stand within the first half hour following birth and sucking occurs within the first 2 hours of birth in normal cases (Dwyer, 2003). Studies have shown that the rate of lamb survival is higher in lambs that stand and suckle quickly after birth (Dwyer et al., 2005). However, the lamb’s first attempts to suck are usually unsuccessful. There are a number of known factors affecting the behavior in the lamb including breed, sex, litter size, prenatal nutrition, placental insufficiency, the ewe’s parity and the birth process (Dwyer, 2003; Dwyer et al., 2005). Male lambs tend to be slower than
females to rise on their knees and to attempt to suck, whereas triplets are slower than twin or single lambs to suck (Dwyer, 2003; Dwyer et al., 2005). Likewise, using an actigraphy method, a previous study in our lab also found that from postnatal day 2 to 4 male lambs seem to be less active than females but the situation was reversed from day 5 to 9 (Rurak et al., 2008). Bonding is also developed between twins or triplet lambs. They seem to stay closer to each other than to any alien lambs. Such attachment is established through visual cues and voice recognition (Fraser & Broom, 1997).

1.1.4 Hypothalamic-Pituitary-Adrenal (HPA) Axis Function:

The fetal HPA axis plays an essential role in fetal development, maturation, homeostasis and even neonatal survival. Studies have shown that the timing of HPA axis maturation is species-specific and depends on the timing of the brain growth spurt. In precocious animals such as sheep, the brain growth spurt, which is associated with a large proportion of neuroendocrine maturation (including corticosteroid receptor development), takes place in utero (Dobbing & Sands, 1979; Matthews et al., 2002).

It had been well established that the plasma concentration of cortisol, a final product of the HPA axis, increases markedly during the last 5-10 days of pregnancy and can actively initiate the onset of parturition by changes in the progesterone/estrogen ratio (Challis et al., 2000; Mellor, Matheson, & Small, 1977; Norman et al., 1985; Thorburn & Challis, 1979). Therefore, fetal hypophysectomy or adrenalectomy prolong pregnancy (Liggins, Holm, & Kennedy, 1966), whereas infusions of adrenocorticotropic (ACTH) or glucocorticoids can cause premature delivery (Liggins, 1968; Thorburn & Challis, 1979). The prepartum surge of
cortisol is considered as a preparation for the fetus to face with stressful events at birth and enable them to adapt to extrauterine life (Mastorakos & Ilias, 2003).

Postnatally, plasma cortisol concentration decreases markedly during the first 1-2 hours after birth and continues to decline for 24h thereafter (Mellor & Pearson, 1977). The circadian rhythm of plasma cortisol does not appear until the third week of age in sheep. This appearance of a 24 h rhythm of cortisol is not influenced by the presence of a light: dark cycle since it appears at the same time in newborn lambs raised both under 12:12 hour light: dark or constant light condition (Parraguez et al., 1989).

Sex differences in the HPA axis function has been observed and attributed to the differences in the levels of gonadal hormones such as estrogens, progesterone, testosterone (Carey et al., 1995; Viau & Meaney, 1991; Young, 1995; Young, 1996). This could contribute, at least in part, to the higher prevalence of depression in females than in adult males (Brummelte & Galea, 2009). In rats, estrogen enhances HPA axis responses (Viau & Meaney, 1991), whereas androgen depresses HPA axis activity (Viau, 2002). In sheep, sex differences in cortisol response only depend on the type of acute stressors but not the gonadal status (Turner et al., 2002).

1.1.5 Renal Function:

Previous studies have shown that renal function changes from the fetal to the newborn period in lambs, which indicates the rapid adjustment of the kidney to the changes in fluid and electrolyte supply. The glomerular filtration rate (GFR) increases from $4.59 \pm 0.27 \text{ml/min}$ to $6.94 \pm 1.0 \text{ml/min}$, whereas urine flow, sodium and osmolar excretion decreased and urinary osmolarity increased (more concentrated urine) in newborn lambs 24 h
following birth compared to fetal lambs (Smith & Lumbers, 1989). Renal adaptations in the newborn lambs appear to be independent of their gestational age (Berry et al., 1995). The mechanisms responsible for the increase in GFR in the postnatal lambs include an elevated net filtration pressure and ultrafiltration coefficient (Turner et al, 2008). In fetal sheep, acute stress due to surgery can reduce urine flow (Gresham et al, 1972), which can also be the case with birth events. Given that urine flow is influenced by water and fluid intake, GFR and sodium reabsorption, dietary changes from milk-fed to solid intake at the time of weaning are hypothesized to cause a decline in urine flow rate.

1.2 Depression: Symptoms And Diagnosis:

Depression is often referred to as being one specific form of a mental health problem; however there are actually several categories of depressive illnesses. The most common form of depression is Major Depression or Major Depressive Disorder. This form of depression comprises a combination of symptoms that interferes with one’s ability to work, study, sleep, eat and enjoy activities that are pleasurable. A less severe form of depression is dysthymia, in which the long-lasting symptoms do not seriously disable one, but keep one from functioning well or feeling good. And another form of depressive illness is Bipolar Disorder, which is characterized by cycling mood changes: high (manic episodes) and low (depressive episodes) (Dubovsky et al., 2003).

The persistence of symptoms is a key to diagnosis. Many of us might feel blue or down several times in our life, especially, for example when we lose a loved one. Sometimes we may even have passing thoughts of suicide. However, in order to differentiate between real depression and these more commonplace situations, we need to consider the time frame
of those feelings or symptoms. Based on the DSM-IV, diagnosis for major depression

disorder is made if symptoms are present for at least 2-week duration (table 1, criteria for
diagnosis of depression, DSM-IV). Conversely, dysthymia is considered as chronic
depression in which the symptoms must persist for at least 2 years to meet the diagnostic
criteria.

Assessing depression in children and youth is different from adults because the

symptoms may be atypical in these populations; for example, depressed mood in children and
adolescents can present as an irritable mood, or the weight loss criteria queried in adults can
be reconsidered as a failure to make the expected weight gain in children. In dysthymic

disorder, the time period for diagnosis is also shorter for children, at least one year (instead of
2 years in adults).
Table 1.1: DSM-IV-TR criteria for major depressive episode

A. Patients must have experienced at least five of the nine symptoms below, for the same 2 weeks or more, for most of the time almost every day. One of the symptoms must be either (a) depressed mood, or (b) loss of interest.

a. Depressed mood

b. A significantly reduced level of interest or pleasure in most or all activities.

c. A considerable loss or gain of weight (5% or more change of weight in a month when not dieting). This may also be an increase or decrease in appetite

d. Difficulty falling or staying asleep (insomnia), or sleeping more than usual (hypersomnia)

e. Behaviour that is agitated or slowed down.

f. Feeling fatigued, or diminished energy

g. Thoughts of worthlessness or extreme guilt (not about being ill)

h. Ability to think, concentrate, or make decisions is reduced

i. Frequent thoughts of death or suicide (with or without a specific plan), or attempt of suicide

Adapted from Quick reference to the Diagnostic criteria from DSM-IV-TR, American Psychiatric Association (Mood disorders. 2000).

1.3 Pathophysiology Of Depression:

Many theories have been proposed for the pathophysiology of affective illness. In 1965, extrapolating the indirect evidence from the pharmacological studies, Schildkraut
proposed the catecholamine hypothesis of affective disorders. This hypothesis states that depression is associated with a decrease in catecholamines, particularly norepinephrine, available at central adrenergic receptor sites (Schildkraut, 1965). On the other hand, the indoleamine theory postulates that it is not only noradrenaline but also a serotonin deficiency that contributes to the disrupted regulation of basic behaviors observed in depressed patients. In addition, studies have shown that an intact serotonin system is necessary for optimal functioning of noradrenergic neurons (Price et al., 1990). Subsequently, the roles of cholinergic-noradrenaline systems in depression were emphasized in the work of David Janowsky et al (Janowsky et al., 1972). Dopamine also appears to play a role in the pathophysiology of depression. Decreased dopamine transmission has been associated with depression in post-mortem and animal studies (Dunlop & Nemeroff, 2007; Nutt et al., 2006). Collectively, these theories are termed the monoamine hypothesis, as they involve deficiencies in norepinephrine, dopamine and serotonin.

Altered brain serotoninergic function has been implicated as an underlying cause of not only depression but also of other psychiatric disorders including anxiety, obsessive-compulsive disorder, eating disorders and substance dependence (Bellivier et al., 1998; Lucki, 1998; Mann et al., 2001). Evidence that supports the serotonin theory includes: (a) significantly lower plasma tryptophan levels in subjects with major depression as compared to healthy controls (Coppen et al., 1973; Cowen et al., 1989), (b) lower cerebrospinal fluid concentrations of 5-hydroxyindoleacetic acid (5-HIAA), a major metabolite of 5-hydroxytryptophan (5-HT), in untreated depressed patients (Owens & Nemeroff, 1994), (c) lower concentrations of 5-HT and 5-HIAA in postmortem brain tissue of depressed and/or suicidal subjects (Owens & Nemeroff, 1994), (d) a decrease in the number of 5-HT transporter or
binding sites in postmortem brain tissues of depressed patients and suicide victims and in platelets of untreated depressed patients (Owens & Nemeroff, 1994). In addition, depriving the brain of tryptophan, which leads to a central serotonin deficiency, can induce depression within hours (Lam et al., 1996) or a profound relapse in remitted depressed patients who previously responded to serotonergic antidepressant therapy (Owens & Nemeroff, 1994).

It is well-known that the essential amino acid tryptophan is a precursor for serotonin (5-HT) synthesis in the human body. Once ingested, tryptophan is converted to 5-HT through a series of reactions. However, the majority of tryptophan is converted to kynurenine following the pathway as described in Fig. 1.1. A portion of serotonin is also converted to melatonin. (Fig. 1.1) (Jonnakuty & Gragnoli, 2008; Kopin et al, 1961; Myint et al., 2007; Watts et al, 2012)

5-HT is found in both the central nervous system, mostly in the brain-stem neurons of the raphe nuclei, and in the peripheral system, mostly in the enterochromaffin cells in the gastrointestinal tract crypts (Furness & Costa, 1982; Maurer-Spurej et al., 2004). After release into the blood circulation from the enterochromaffin cells, 5-HT is rapidly taken up by platelets via the 5-HT transporter and is stored in platelet dense granules. Therefore, peripherally, platelets store almost all the total body circulating serotonin (Maurer-Spurej et al., 2004). Since the blood brain barrier is impermeable to peripheral 5-HT, central 5-HT synthesis depends on the amount of tryptophan or 5-hydroxy-L tryptophan available peripherally to cross the blood brain barrier. While tryptophan has to compete with other amino acids such as valine, leucine and isoleucine for a carrier protein in order to cross the blood brain barrier, 5-hydroxy-L tryptophan can easily cross the blood brain barrier since it does not require a specific transport protein (Rahman et al., 1982; Yuwiler et al., 1977).
Centrally, 5-HT functions at the neuron synapses and influences a variety of behavioral, physiological and cognitive functions such as memory, mood, emotions, wakefulness, sleep, appetite and temperature regulation (Jacobs & Azmitia, 1992).

In the periphery, traditionally as its name suggests, serotonin is a vasoconstrictor causing hypertension (Maurer-Spurej et al., 2004). On the other hand, it also mediates nitric oxide release and functions as an endothelium-derived relaxing factor (Maurer-Spurej et al., 2004). It exerts its action through many subtypes of 5-HT receptors present on vascular smooth muscle cells and endothelial cells (5-HT1B, 5-HT 2A, 5-HT 2B, 5-HT 4 and 5-HT 7) (Nilsson et al., 1999). 5-HT is metabolized primarily in the liver and only a small fraction escapes from this primary metabolism and is metabolized in the endothelium of lung capillaries. Finally, the product of 5-HT metabolism, 5-Hydroxyindoleacetic acid (5-HIAA), from either central or peripheral sources, is excreted in the urine (Green & Curzon, 1968).

In addition to the indolamine theory of depression, collective evidence has suggested a corticosteroid receptor hypothesis in depression (Holsboer et al., 1984; Holsboer, 1999; Holsboer, 2000). Overproduction of corticotrophin releasing hormone which causes an excess activity of the HPA axis with hypercortisolemia is also observed in many depressed patients (Gillespie & Nemeroff, 2005; Vreeburg et al., 2009). This prolonged or excessive secretion of glucocorticoids may lead to suppression of neurogenesis and hippocampal atrophy (Belmaker & Agam, 2008; Heim & Nemeroff, 2001; Sapolsky, 2000).

In parallel, the leptin hypothesis of depression has also been proposed based on supporting evidence from animal and human studies. (Lu et al., 2007) The hypothesis of leptin insufficiency in depression is mainly supported by data from a rodent study. Decreased
plasma leptin is associated with behavioral changes in chronically stressed mice or rats and systemic administration of leptin can reverse the chronic stress-induced behavior changes or depressive-like behaviors (i.e. a decrease in sucrose preference or reduced duration of immobility in force swim test or tail suspension test) together with an increase in c-fos expression in the hippocampus. (Lu et al., 2006; Lu et al., 2007) Thus, leptin acts as an antidepressant in the rat model. However, in the human, leptin insufficiency seems to occur in only a subpopulation of depressed patients and it alone cannot explain the association between obesity and depression. Thus, it raised a complementary hypothesis of leptin resistance, which is similar to insulin resistance in type 2 diabetic patients. In addition, since leptin is also capable of modulating HPA axis function, the leptin hypothesis for depression might be complementary to the aboved mention HPA hypothesis of depression. (Lu et al., 2007)
FIG. 1.1: Biosynthesis and metabolism of serotonin.

(Jonnakuty & Gragnoli, 2008; Kopin et al, 1961; Myint et al., 2007; Watts et al, 2012)
1.4 Depression In Pregnancy, Postpartum And In Children:

1.4.1 Incidence:

Even though depression can occur in both genders, it affects more women than men with a female to male ratio of 1.7-2.7 (Bhatia & Bhatia, 1999; Burt & Stein, 2002; Kessler et al, 1993). It is believed that biologic factors such as endocrine, genetic or social features contribute to this gender difference (Nonacs & Cohen, 2003; Weissman & Olfson, 1995). Studies have shown that acute depletion of tryptophan has a more profound impact on mood in women as compared to men. This implies that women and men are different in terms of the magnitude and functional activity of their central serotonergic system (Nishizawa et al., 1997). Additionally, tryptophan pyrrolase, an enzyme that reduces blood tryptophan levels, which is an amino acid precursor of serotonin, may be stimulated by circulating concentrations of estrogen and progesterone (Khan et al., 2005). This may, in part, account for the greater frequency of depression among women during their childbearing years. Whatever the cause might be, women are much more likely to suffer from depression at any stage of their lifetime, especially during their childbearing and child-rearing years. The incidence of clinical depression during pregnancy is about 10-15% (Bhatia & Bhatia, 1999; Burt & Stein, 2002; Nonacs & Cohen, 2003). Postpartum depression occurs in 10-22% of women (Burt & Stein, 2002), with symptoms beginning two weeks after delivery including tearfulness, despondency, labile mood, feelings of inadequacy and inability to cope, particularly with the baby (Cooper & Murray, 1998; Hopkins et al., 1984; Murray, 1992; O'Hara, 1997).
Depression in children and adolescents is an important issue as depressed adolescents frequently grow up to be depressed adults (Emslie et al., 1997; Harrington et al., 1990; Lewinsohn et al., 1999; Rao et al., 1999; Weissman et al., 1999). They have more social problems later in life at work and with their families, attempt suicide more often and require more medical and psychiatric attention (Birmaher et al., 1996; Brent et al., 1988; Rohde et al., 1994). Suicide is the fourth leading cause of death in 10 to 14 year old children and rises to the third leading cause of death in adolescents (Anderson, 2002). Statistics in Canada reported that the suicide deaths in 1996 have increased three fold in 10-14 age group as compared to the rate in 1971 (Houle & Wilkins, 2010). In addition, there is a subsequent increase in the likelihood of tobacco use, involvement in deviant activities and accidents and impaired relationship with parents and partners, and also impaired academic function in these depressed adolescents (Birmaher et al., 1996; Kandel & Davies, 1986; Rohde et al., 1994).

Childhood depression is more common than it used to be thought. In fact, depression can be diagnosed in children as young as 3 years old (Dubovsky et al., 2003). In general, depression occurs in 2% of prepubertal children and in 3-8% of adolescents (Birmaher et al, 1998; Costello et al., 1996; Lewinsohn et al., 1994).

1.4.2 Risk Factors Of Depression:

Generally, multiple factors may contribute to the occurrence of depression including marital status, socioeconomic factors, residence, seasonal and geographic factors, social stressors, lack of social support and the presence of comorbidity (Kaplan & Sadock's comprehensive textbook of psychiatry2005). Omega-3-fatty acid, which reduces the turnover of membrane phospholipids, is considered as an adjunctive mood stabilizer (Dubovsky et al.,
However, there is evidence that a diet low in omega 3 fatty acids or fish consumption is not associated with depressed mood, major depression or suicide (Hakkarainen et al., 2004).

Early experience of loss or trauma and stress can initiate or exacerbate neurochemical imbalance in vulnerable subjects. This idea is supported by studies in animals in which early separation from peers and inescapable situations profoundly alters the turnover of their biogenic amines and postsynaptic receptor sensitivity (Gilmer & McKinney, 2003). In humans, a polymorphism of the serotonin transporter gene can identify who among traumatized children will develop adult depression (Caspi et al., 2003; Kendler & Karkowski-Shuman, 1997; Lesch et al., 1996; Ogilvie et al., 1996; Ogilvie & Harmar, 1997).

There are many factors that can play a role in the occurrence of postpartum depression. Even though there are no firm causal links, the significant changes in hormone levels of progesterone, estrogen, cortisol, neurosteroids and β-endorphins may lead to depression in this period (McCoy et al., 2003; Weissman & Olfson, 1995). Additional predictive factors are obstetric pregnancy complications, infant irritability and poor motor function in the early neonatal period (Murray, 1993; Murray et al., 1996; Waterstone et al., 2003). Besides these, probably the most attributed risks are psychiatric and psychosocial factors including prior history of mood disorder, low socioeconomic status, lack of partner or family support, an unwanted pregnancy and young maternal age (Burt & Stein, 2002; Hopkins et al., 1984; Murray, 1993; Warner et al., 1996).

When left untreated, depression during pregnancy can lead to changes in maternal behaviours such as decreased appetite, poor pregnancy-related weight gain, increased
likelihood of smoking and/or alcohol and drug consumption and even a risk of suicide in severe cases (Ahluwalia et al., 2004; Bhatia & Bhatia, 1999; Nonacs & Cohen, 2003). There is also evidence that these poor maternal health behaviours or depression per se are associated with an increased risk of ischemic heart disease (Glassman & Shapiro, 1998). These place both the mother and fetus at risk.

1.4.3 Effects Of Maternal Depression On Fetal Neurobehavioral Development:

Studies have shown that depression per se, without poor maternal behavior, can result in pregnancy complications such as preterm birth and/or small for gestational age, and increased rates of maternal obstetric complications (Anderson, 2004; Andersson et al., 2004a; Andersson et al., 2004b; Dole et al., 2003; Hoffman & Hatch, 1996; McCormick et al., 1990; Orr & Miller, 1995; Orr et al., 2002; Peacock et al., 1995; Steer et al., 1992; Weissman & Olfson, 1995). It is not yet clear via which mechanisms depression can lead to preterm birth and fetal growth restriction. One explanation for this relationship is that the elevated plasma concentrations of maternal cortisol and catecholamines associated with depression lead to a decrease in uterine blood flow (Hoffman & Hatch, 1996; Lake et al., 1982; Linkowski, 2003; Weissman & Olfson, 1995; Rurak, 1995), and thus reduce nutrient supply to the fetus and impair fetal growth (Lang et al., 2000). Moreover, the elevated endogenous maternal corticosteroids and catecholamine concentrations can affect the fetal central nervous system causing infant irritability such as excessive crying, difficulty in consoling, which is a predictive factor of postpartum depression (Murray, 1993; Welberg et al., 2000; Zuckerman et al., 1990). As a result, maternal depression during pregnancy can extend into the postpartum period via altered neonatal behaviour (Zuckerman et al., 1990). This, in turn, could further impair neurological development of the offspring. Indeed, some evidence has
indicated that maternal depression can adversely affect temperament and cognitive
development in the infant. Similarly, depressed mothers of preschoolers have more negative
perceptions of and interactions with their children (Beck, 1996; Hay & Kumar, 1995; Lang et
al., 1996).

1.4.4 Treatment Of Depression:

Given the negative impact of maternal depression in pregnancy on both the mother
and offspring, treatment for this condition is essential. Due to the general concern about
exposure of the fetus to drugs, psychotherapy seems to be a preferable choice of treatment.
However, there are few studies that assess the efficacy of psychotherapy during pregnancy.
Drug therapy is a norm and antidepressants are the preferred course of treatment even though
these drugs are not approved for use in pregnancy (Altshuler et al., 2001; Bhatia & Bhatia,
inhibitors (SSRIs) were first introduced into the market in the late of 1980s (DeVane, 1999),
and by 1998 they largely replaced the older classes of antidepressants for the treatment of
depression in pregnancy (Altshuler et al., 2001; Goldstein, 1998; Goodnick & Goldstein,
1998b; Misri et al., 2000; Nonacs & Cohen, 2003; Simon et al., 2002; Skop et al., 1994) due
to their lower incidence of side effects and greater safety in terms of drug overdose
(Goodnick & Goldstein, 1998b; Jones & Blackburn, 2002). One recent study has reported an
increasing use of antidepressants in pregnancy from 2% in 1996 to 7.6% of deliveries in
2004-2005, and in which SSRI use increased 4.9% from 1996 to 2004 (Andrade et al., 2008).
There are similar reports that SSRI accounts for more than 70% of prescribed antidepressant
as a pharmacotherapy in pregnant women (Cooper et al., 2007; Simon et al., 2002). In
addition, these agents have also been used to treat other psychotic disorders in pregnancy,
which are associated with a dysfunctional serotonin system, such as anxiety and obsessive-compulsive disorders (OCD) (Levine et al., 2003; Vaswani et al., 2003).

1.5 Selective Serotonin Reuptake Inhibitors (SSRIs):

1.5.1 Clinical Uses:

SSRIs are considered as a second generation of antidepressant after the first generation of monoamine oxidase inhibitors (MAOI) and tricyclic antidepressants (TCA). In general, SSRIs are widely used in psychiatry. Since SSRIs are relatively safe in terms of overdose and have a more benign side-effect profile than the older generation agents, they offer a greater tolerability and compliance to the treatment. (Lane et al., 1995; Masand & Gupta., 1999) Besides their main indication for the treatment of major depressive disorders, SSRIs are also prescribed as a first-line pharmacological treatment for other disorders such as bipolar disorder, social anxiety disorder, generalized anxiety disorder, obsessive-compulsive disorder (in combination with cognitive-behavioral therapy), panic disorder, eating disorder (bulimia), premenstrual dysphoric disorder, and posttraumatic stress disorder. (Boyer et al., 1992, Masand & Gupta., 1999) In addition, they are also used to control mood disorders in special subsets of the population including post stroke patients, and terminally-ill patients (cancer, HIV/ADIS) (Franco-Bronson et al., 1996; Sauer et al., 2001) A typical response to treatment occurs within four to six weeks of SSRIs treatment.

1.5.2 Mechanism Of Action:

Monoamine transmitter activity may be regulated at many different levels including synthesis, packaging and storage of neurotransmitters in synaptic vesicles, release, reuptake,
metabolism and postsynaptic receptor responsiveness (Fig. 1.3) (Kaplan & Sadock’s comprehensive textbook of psychiatry 2005).

FIG. 1.2: Schematic diagram of monoaminergic synapse and steps involved in synaptic transmission: Monoamine neurotransmitters (1) are synthesized within neurons from common amino acid precursors, which are Tryptophan and 5-Hydroxytryptophan (5-HT) in the case of serotonin, (2) are taken up into synaptic vesicles via a vesicular monoamine transporter, (3) are released into the synaptic cleft upon stimulation, (4) interact with postsynaptic receptors to alter the excitability of postsynaptic cells, (5) interact with presynaptic autoreceptors located on the nerve terminal to suppress further release, (6) Released monoamines may also be taken back up from the synaptic cleft into the nerve terminal by plasma membrane transporter proteins. (7) Once taken up, monoamine may be subject to enzymatic degradation or may be protected from degradation by uptake into vesicles. MAO: monoamine oxidase

Adapted from Kaplan and Sadock’s comprehensive textbook of Psychiatry 2005.
The goal of drug therapy in the treatment of depression is to maintain the circulating neurotransmitter concentration in the synapse at a certain level, either by increasing the amount of neurotransmitter produced for release or the duration of time the neurotransmitter spends in the synapse. Antidepressants are classified based on the type of monoamine neurotransmitter (dopamine, noradrenaline or serotonin), being affected at the synapse and the specific mechanisms being impacted such as synthesis, release, reuptake, and metabolism. There are three major classes of antidepressants. Monoamine oxidase inhibitors (MAOI) prevent the degradation of noradrenaline and serotonin in the presynaptic cell. Tricyclic acids (TCA) block the reuptake of noradrenaline and serotonin to varying degrees at the presynaptic cell. SSRIs selectively inhibit the reuptake of serotonin (5-HT) by presynaptic serotonergic neurons and peripheral serotonin containing cells, particularly platelets, via effects on the serotonin transporter (Anderson, 2004; Goodnick & Goldstein, 1998a; Stahl, 1998; Wright & Angus, 1989). In addition, there are sub-classes of dual inhibitors of the three neurotransmitters mentioned above that have been developed, including norepinephrine/dopamine reuptake inhibitor (NDRI), serotonin/norepinephrine reuptake inhibitor (SNRI), noradrenergic and specific serotonergic antidepressant (NaSSA) and serotonin antagonist reuptake inhibitor (SARI) (Stahl, 1998).

The various SSRIs (sertraline, fluoxetine, paroxetine, citaloprame, fluvoxamine) differ in their potency and inhibitory effects on reuptake of other monoamine neurotransmitters (Kaplan & Sadock's comprehensive textbook of psychiatry 2005). However, comparisons of these agents in acute treatment have shown that they have similar efficacies, probably due to the parallel differences in dose and plasma concentration (DeVane, 2003; Hiemke & Hartter, 2000).
After SSRI treatment, the inhibition of serotonin reuptake usually occurs immediately whereas the onset of the antidepressant effect is delayed for about 2-6 weeks (Goodnick & Goldstein, 1998b). It is generally accepted that the initial rise in extracellular 5-HT in the somatodendritic regions of the dorsal and median raphe nuclei activates somatodendritic 5-HT1A autoreceptors which are responsible for the inhibition of the firing of 5-HT neurons and thus reducing 5-HT release. Yet, when given chronically, SSRI will down-regulate the 5-HT1A autoreceptors and increase serotonergic neurotransmission (Celada et al, 2004; Hensler, 2003; Hjorth, 1996; Stahl, 1998). In addition, other neurotransmitters can also be involved in the control of the firing rate and serotonin release (Kalsner, 2000; Kalsner & Abdali, 2002).

1.5.3 Pharmacokinetics:

The different SSRIs vary in their half-lives and other pharmacokinetic variables. Fluoxetine (FX) has the longest half-life, whereas the values for the other SSRIs are shorter (Hiemke & Hartter, 2000; Vaswani et al., 2003). The wide difference in the half-lives among SSRIs account for the differences not only in the time to reach the steady state, the time to clear the drug from the body after discontinuation but also in the potential for withdrawal symptoms after discontinuing the SSRIs. Concomitant use of SSRIs and other agents metabolized by the cytochrome P450 (CYP) isozymes can lead to drug-drug interactions, because SSRIs inhibit the activity of some of the isozymes. Among SSRIs, FX and paroxetine are strong CYP2D6 inhibitors, and the others are weak CYP2D6 inhibitors. FX is also a moderate CYP2C19 inhibitor (Harvey & Preskorn, 2001). Additionally, FX is also a substrate of CYP2C19 and CYP2D6 (Jeppesen et al., 1996; Ring et al., 2001; Spina et al., 2003, Harvey & Preskorn, 2001)
Genetic polymorphisms have been identified in two CYP isoenzymes, CYP2D6 and CYP2C19, and that divides the population into several groups including ultra extensive, extensive, intermediate and poor metabolizers. The poor metabolizers with nonfunctional allele of CYP2D6 gene have a severely impaired catalytic function of the isoenzymes. The clinical impact of poor metabolizers is that they are unable to metabolize the drugs, which are substrates of these enzymes; therefore the sub-population will have a higher risk of adverse effects, especially when no other metabolic route is available for the drugs or when multiple drugs share the same CYP in their metabolism (Vaswani et al., 2003). This becomes more important when switching medications having a long half live (eg: FX) to other medications metabolized by the same cytochrome since the risk of toxicity still persists long after the drugs were discontinued.

Among the SSRIs, paroxetine, sertraline and fluoxetine are highly bound to plasma proteins and only a small fraction of the drugs stays in the form of free drug. Thus, coadministration of the SSRIs and another highly-protein-bound drug might elevate the free concentrations of the other drug, potentially resulting in adverse events.

All of the SSRIs can readily cross the placenta in humans, rats and sheep because they are highly lipophilic (Hendrick et al., 2003; Kim et al.,2004; Kim et al., 2006; Pohland et al., 1989; Rampono et al., 2009). SSRIs are also excreted in breast milk. Despite this fact, the concentrations of the drugs in an infant are much less than those in a fetus, because of the much lower amount of drug acquired by the infant via breast milk compared to that acquired by the fetus via placental transfer and also because of the progressive development of metabolic capacity in the infant following birth (Kim et al., 2006). Hence, the exposure of a fetus to the drugs during prenatal period is more likely to be the cause of any long-term
effects of the drugs in compared to the exposure of an infant to the drugs during the postnatal period.

1.5.4 Side Effects:

As a class, SSRIs, clearly demonstrate superiority in terms of safety and tolerability compared to the older antidepressant medications. A meta-analysis of 84 double-blind Randomized Controlled Trials (RCT) of the adverse effects of antidepressants, particularly SSRIs and TCA has found that all antidepressants cause adverse effects (Trindade et al., 1998). Typical anticholinergic side effects, including dry mouth, constipation, blurred vision, urinary retention and postural hypotension, which are usually reported in tricyclic antidepressants, occur less frequently with SSRIs. Serotonin has been implicated as playing roles in gastrointestinal motility, appetite control, sexual behavior, sleep regulation, and cerebral vasomotor tone; therefore SSRIs are predicted to cause nausea, appetite loss, sexual dysfunction, and sleep disturbances (Dubovsky et al., 2003). In fact, there is evidence that fluoxetine, fluvoxamine, paroxetine and sertraline have been associated with nausea, diarrhea, insomnia, nervousness, agitation and anxiety. Even though paroxetine was found to induce less nausea than the other SSRIs, the difference was not significant (Trindade et al., 1998). SSRI-associated adverse effects seem to be related to drug dose (Beasley et al., 1991).

Together with the introduction of SSRIs and the increased incidence of depression in children and adolescents, SSRIs is also used to treat children with this condition. SSRIs were found to be associated with a higher rate of agitation and suicidal thoughts, or both, from clinical trials comparing SSRIs treatment and placebo in children and adolescents with major depressive disorder. Thus, a warning for a close observation of signs or symptoms of
worsening depression, including suicidal ideation, early on in therapy with these medications or on titration of dosing, was required to be included in their labels and product inserts (Bridge et al., 2007; Hammad et al., 2006). In the meta-analyses, SSRI treatment had a benefit compared to placebo treatment, but also was associated with significant risks for adverse events, including suicidal behaviour (except for FX) (Jureidini et al., 2004; Whittington et al., 2004)

1.6 Fluoxetine (FX):

FX is an antidepressant that belongs to the SSRI class. It was one of the first SSRIs to be developed. FX (trade name Prozac) was first introduced into the US market in 1988 (Borys et al., 1990). It has been shown to effectively relieve the symptoms of major depression and is better tolerated than the older classes of antidepressant such as TCA (Cohn & Wilcox, 1985). Currently, FX also has received approved indications for OCD, bulimia nervosa and premenstrual dysphoric disorder. In 2003, the FDA approved the combination of FX and olanzapine (trade name Symbyax) for treatment-resistant depression (unresponsive to two separate trials of different antidepressants). Despite the fact that many new SSRI agents have been introduced into the market after FX was, FX remains very popular. In 2008, over 23 million prescriptions of FX were filled in the US, an increase of 4.5% from 2007. Also, it was ranked the second most prescribed antidepressant (Top 200 generic drugs by total prescriptions 2008-2009).
1.6.1 Pharmacokinetics:

1.6.1.1 Absorption:

The bioavailability of FX is relatively high, about 70 to 90 percent. This bioavailability results from an almost complete absorption and a limited hepatic first-pass effect. The time to maximum concentration (tmax) of FX is about 6-8 hours. And the peak plasma concentration (Cmax) of FX is about 15-55 ng/ml (Altamura et al., 1994). Food does not decrease the absorption, but may delay the time to reach the peak concentration by 1-2 hours (Benfield et al., 1986).

1.6.1.2 Distribution:

FX is one of the SSRIs highly bound to plasma proteins, approximately 95%. The volume of distribution (Vd) of FX and its active metabolite, norfluoxetine (NFX), falls in the range from 12 to 45 L/kg (Benfield et al., 1986).

1.6.1.3 Metabolism:

FX is a 1:1 racemic mixture of two optical isomers. Both S- and R- enantiomers have equivalent pharmacologic activity, although the S-enantiomer is eliminated more slowly and therefore presents predominantly in the plasma at steady state (Wong, Fuller, & Robertson, 1990). FX is metabolized by the cytochrome (CYP) P450 isozymes in the liver. FX is demethylated to its major active metabolite, norfluoxetine (NFX) and several inactive metabolites. The two clinically important CYP P450 isozymes involving the metabolism of FX are CYP2C19 and CYP2D6 (Stevens & Wrighton, 1993). CYP2D6 catalyzed the metabolism of R & S FX and S-NFX but not of R-NFX (Fjordside et al., 1999).
1.6.1.4 Elimination:

Hepatic metabolism of FX to its metabolites then renal excretion is the primary route of elimination. The half-life (t1/2) values of fluoxetine following an acute and chronic administration are 1-3 days and 4-6 days respectively. The major active metabolite, NFX, has a longer half-life, which ranges from 4 to 16 days, independent of length of treatment. The relatively slow elimination of FX and NFX ensure that they will remain in the body for weeks after discontinuation. Hepatic impairment will prolong the half-life elimination of FX and NFX.

1.7 FX Effects On Newborns And Infants:

1.7.1 FX Effects On Cardiovascular Functions:

There are a few case reports in adults of adverse cardiac events after starting FX therapy including sinus bradycardia, supraventricular tachycardia, atrial fibrillation, atrial flutter and ST segment depression. (Ahmed et al., 1993; Gardner et al., 1991; Isbister et al., 2004; McAnally et al., 1992; Riddle et al., 1989; Roberge & Martin, 1994). One case report of FX poisoning in which the patient ingested 12g of FX and developed bradycardia, progressing to ventricular fibrillation and asystole (Compton et al., 2005). Another case report of QT prolongation to 600msec and torsades de point in a 74 year-old woman occurred 3 weeks after starting FX therapy (Appleby et al., 1995).

QTc interval prolongation was also reported in a newborn after in utero exposure to FX. To note, a prolonged QTc interval (> 560 msec) predisposes the sufferers to torsade de pointes, an intermittent ventricular dysrhythmia which, in turn, can lead to a potentially life-
threatening monomorphic ventricular tachycardia or fibrillation. A subsequent retrospective cohort study comparing 52 SSRIs exposed newborns with 52 unexposed controls matched by gestational age also confirmed that the exposed newborns had a longer QTc interval than the unexposed controls (409 vs 392 msec, respectively). However, there were no cases of arrhythmia reported. The SSRIs used in the study included paroxetine, citalopram, fluoxetine, fluvoxamine and venlafaxine and there was no drug-specific evaluation or serum electrolytes measurement (Dubnov-Raz et al., 2008). In agreement with these findings, prolongation of QTc interval of no greater than 450 msec existed in 62% of FX overdose patients in one study, but none developed ventricular dysrhythmia (Phillips et al., 1997). Nevertheless, of the SSRIs, FX appears to have minimal cardiotoxicity.

Effects of SSRIs exposure on cardiovascular function are likely to originate in utero. Recently our group has found a reduced fetal middle cerebral artery (MCA) flow resistance and cross sectional MCA area and fetal heart rate variability in the SSRIs-exposed fetus, even after controlling for maternal mood. Importantly, despite a reduced MCA flow resistance, MCA volume flow was not increased, and this was associated with a reduced cross sectional MCA area (Rurak et al., 2011).

Chambers et al., (2006) was one of the first papers that reported an increased risk of persistent pulmonary hypertension (PPHN) development (adjusted OR = 6.1, 95% CI 2.2-16.8) in newborns whose mothers were taking SSRIs during late gestation. Even though there were some methodological limitations in the study such as retrospective design and potential recall bias, her finding was confirmed by a subsequent study by Kallen & Olausson, (2008). Despite the fact that persistent pulmonary hypertension is a rare condition, these findings prompted the FDA to issue a public health advisory to add a warning label of potential risk of
PPHN in 3rd trimester SSRI-exposed infants (Public health advisory: Treatment challenges of depression in pregnancy and possibility of persistent pulmonary hypertension in newborns. 2006). However, two following studies, which involved retrospective examinations of administrative health records, failed to demonstrate this increased risk of PPHN in SSRIs-exposed infants (Andrade et al., 2009; Wichman et al., 2009). However, both studies were underpowered to detect small differences in the incidence of PPHN, which itself is a rare event. In another human study (Wilson et al, 2011), several potential factors, including use of SSRIs after 20 weeks gestation, were examined in 20 case of primary PPHN compared to 120 controls. The only significant association was between cesarean delivery before labor onset and PPHN and the authors noted that the 2 studies reporting an association between antenatal SSRI exposure and PPHN (Chambers et al., 2006; Kallen et al., 2008) did not control for the mode of delivery. A very recent study from our group measuring fetal right pulmonary artery resistance, flow and vessel cross sectional area by Doppler ultrasound also did not support an association between SSRIs exposure and PPHN in the infants even though the authors observed a significantly higher rate of right pulmonary artery flow in the SSRI-exposed fetuses with respiratory difficulties (Lim et al., 2012).

In a study in pregnant rats involving daily administration of fluoxetine by gastric gavage from day 11 to 21 of pregnancy (Fornaro et al., 2007), the offspring from the fluoxetine treated group exhibited an increase in pulmonary arterial medial thickness and in the right/left ventricular weight ratio. In vitro, fluoxetine at lower concentrations increased proliferation of fetal but not adult pulmonary arterial smooth muscle cells, whereas at higher concentrations the drug inhibited proliferation of both the fetal and adult cells.
In sheep, 8 days of late gestational FX infusion in pregnant ewes did not result in any changes in the daily average maternal arterial pressure and heart rate nor in fetal arterial pressure. However, there was a transient decrease of fetal heart rate on both the FX exposed group (infusion d2 and 6) and the control group (infusion day 4 and 8) (Morrison et al., 2002).

1.7.2 FX Effects On The Homeostatic And Respiratory Functions:

Extensive research focused on brainstem mechanisms underlying sudden infant death syndrome has shown the essential role of 5-HT neurons in the modulation of the respiratory response and thus homeostatic functions (Kinney, 2009; Kinney et al., 2009). In vitro studies have indicated that the midbrain 5-HT neurons, which serve as an arousal chemoreceptor, and the medullary 5-HT neurons, which function as respiratory chemoreceptor, increase their firing rate 3-fold on average in response to hypercarnia and a decrease in pH from 7.4 to 7.2 (Buchanan & Richerson, 2010; Richerson, 2004; Wang et al., 1998). In addition, acute hypercapnia can also activate the limbic systems causing dyspnea and panic which are usually observed in panic disorders (Gorman et al., 1994; von Leupoldt & Dahme, 2005). Clearly, given that 5-HT neurons play an important role in homeostatic function, changes in the serotonergic system either by depression itself or pharmacologic intervention may lead to changes in homeostatic systems.

1.7.3 FX Effects On Birth Outcomes:

A majority of research regarding the effects of SSRI on birth and pregnancy outcomes have found that late pregnancy SSRI exposure increases the risk of preterm birth, shorter gestational length, lower birth weight, smaller than gestational age, lower APGAR
score at birth, admission to NICU and reduced postnatal weight gain (Chambers et al., 1996; Einarson et al., 2010; Kallen, 2004; Lewis et al., 2010; Maschi et al., 2008; Oberlander et al. 2006; Oberlander et al. 2008; Pearson et al., 2007; Simon et al., 2002; Suri et al., 2007; Wen et al., 2006; Wisner et al., 2009; Zeskind & Stephens, 2004). However, others have failed to confirm these effects (Casper et al., 2003; Hendrick et al., 2003; Kulin et al., 1998; Lund et al. 2009; Malm et al., 2005; Suri et al., 2004). There are conflicting findings regarding the impact of timing and duration of SSRI exposure during pregnancy on perinatal outcomes. Chambers et al., (1996) found that preterm birth, low birth weight and poor neonatal adaptation were significantly more common in a late gestational exposure group than in an early exposure group. Conversely, Oberlander et al., (2008) reported early and late exposure groups had the same neonatal outcomes while an increase in days of exposure was significantly associated with low birth weight and low gestational age, even after controlling for maternal illness and medication dose. Still, others found no association between late gestation exposure and adverse neonatal outcomes (Cohen et al., 2000; Malm et al., 2005). The potential reason for the lack of consistency in these findings is the differences in doses among studies. Recently, it has been reported that much higher preterm birth rate and lower gestational age were observed in the higher FX dose group, even after controlling for length of exposure (Roca et al., 2011). The mechanisms underlying the association between prenatal SSRI exposure and the shortening of gestational length are not yet been clear. However, it has been proposed that SSRI exposure might promote adverse birth outcome through the dysregulation of the fetal HPA system or through the vasoconstrictor effects of serotonin which could decrease maternal placental blood flow, especially in the 3rd trimister (Morrison et al., 2002; Roca et al., 2011). SSRIs-induced fetal HPA axis disturbances were evidenced
by a previous sheep study in our lab showing an increase in the fetal plasma prepartum
cortisol surge and apparent increased sensitivity of the fetal adrenal gland to ACTH after 8 d
maternal FX administration (Morrison et al., 2004). In addition, a relationship between
elevated placental corticotropin-releasing hormone (CRH) levels, which also regulates
cortisol secretion, and a higher risk of preterm birth and fetal growth restriction has been
established in human pregnancies associated with antenatal SSRI therapy (Wadhwa et al.,
2004). Other potential mechanisms concerning the effect of SSRIs exposure on lower birth
weight or smaller than gestational age include a SSRIs-induced alteration in the fetal IGF-
1/GH axis (Davidson et al., 2009) or a reduction in uterine blood flow by 5-HT, a
vasoconstrictor agent, which limits oxygen and nutrient supply to the fetus (Morrison et al.,
2002) or a direct SSRIs-effect on the growing skeleton by exerting their effect on osteoclast
differentiation and formation (Battaglino et al., 2004; Warden et al., 2005).

1.7.4 FX Effects On Neonatal And Child Behaviors:

Accumulating data from case reports and cohort studies have also indicated that late
gestation exposure to SSRIs increases the risk of a variety of neonatal complications termed
“neonatal behavioral syndrome” or “poor neonatal adaptation” (Chambers et al., 1996;
Cohen et al., 2000; Kwon & Lefkowitz, 2008; Mhanna et al., 1997; Oberlander et al., 2004).
According to some studies, the risk can increase up to 3 times (Chambers et al., 1996; Cohen
et al., 2000; Oberlander et al., 2004). Among SSRIs, most reported cases involved FX and
paroxetine exposure. Behavioral and neurologic symptoms vary from mild central nervous
system, motor, respiratory and gastrointestinal signs and metabolic dysfunction (including
irritability, persistent crying, shivering, tremor, restlessness, feeding difficulties, sleep
disturbances) to severe symptoms (including seizures, dehydration, excessive weight loss,
hyperpyrexia and the need for intubation). These symptoms usually disappear by the second week after birth (Moses-Kolko et al., 2005). These findings led Health Canada and the FDA in 2004 to include a warning in the drug information labeling for SSRI/SNRIs that neonates exposed to these agents in the late third trimester have developed adverse events (Summary minutes of the pediatrics subcommittee of the anti-infective drugs advisory committee, 2004). These symptoms are observed in around 10-30% of the exposed newborns (Koren et al., 2009). Unfortunately, the mechanisms that may account for these complications have yet to be elucidated. They may be multifactorial, with the severity of the maternal mental illness playing a major role.

In fact, one of the big challenges to this type of research in humans is to distinguish between the drug effects and the impact of maternal psychiatric disorders on the occurrence of neonatal behavioral syndrome. Even though some studies have enrolled mothers with untreated depression in their studies to adjust for the possible indication confounders, those are more likely to have less severe psychiatric problems than mothers with treated depression. Using a propensity score matching approach, Oberlander et al., (2006) have found that the incidence of symptoms of behavioral syndrome from SSRI exposed infants is similar to non-exposed infants born to depressed mothers.

Since the symptoms observed in adults with SSRI discontinuation syndrome overlap with those seen in neonates born to mothers treated with SSRIs, one of the mechanisms suggested for poor neonatal adaptation syndrome is a withdrawal syndrome. On the other hand, some symptoms in the neonates are similar to those seen as side effects of SSRIs or SSRI toxicity associated with elevated 5-HT levels (serotonin syndrome) (Birmes et al., 2003; Ener et al., 2003). Therefore, it remains unclear whether neonatal symptoms are due to
SSRI withdrawal or directly due to SSRI toxicity (Koren et al., 2009; Moses-Kolko et al., 2005). However, since theoretically the management for these mechanisms is clearly opposite, there is a compelling need to identify which is the true case. Currently, the management for SSRI/SNRI poor neonatal adaptation syndrome is mainly symptomatic, with supportive care only (Koren et al., 2009).

In parallel with the mechanisms suggested from human studies, previous studies on fetal lambs with chronically 8-day FX-exposure in late gestation in our lab (Morrison et al., 2001; Morrison et al., 2002; Morrison et al., 2004; Morrison et al., 2005) have suggested another mechanism: that late gestational SSRIs exposure could alter fetal brain development, which relates to the importance of serotonin in the maturation of brain pathways. During the period of FX administration, the incidence of fetal rapid-eye-movement (REM) sleep was significantly decreased (Morrison et al., 2001). In addition, a study of human fetal behavioral development also demonstrated an abnormaly high rate of continual bodily activity during non-REM sleep in SSRI-exposed fetuses (Mulder et al., 2011). This mechanism is further supported by a subsequent study from our group which showed additive negative effects in infants of depressed mothers treated with SSRIs as compared to untreated depressed mothers (Oberlander et al., 2006). Our recent findings of a decreased MCA flow resistance without increasing MCA volume flow in the fetus exposed to SSRIs (Rurak et al., 2011) adds more evidence to support this proposed mechanism. Changes in tissue volume in the brain region perfused by the MCA, which is secondary to changes in brain blood flow, could be due to a SSRIs-mediated alteration of serotonergic tone. However, whether the disturbed serotonergic system places any long-term sequelae during postnatal development beyond the exposure period still needs further investigation. To date, only two studies have reported significant
difference in psychomotor development in SSRIs-exposed versus non-exposed infants (Casper et al., 2003; Mortensen et al., 2003).

### 1.7.5 FX Effects On The Hypothalamic-Pituitary-Adrenal (HPA) Axis:

HPA axis activity is proposed to be involved in the pathophysiology of depression since altered levels of corticotropin-releasing factor in the cerebrospinal fluid (Arborelius et al., 1999) and hypercortisolism are observed in depressed patients (Holsboer et al., 1995) and this was normalized with antidepressants (Holsboer & Barden, 1996) possibly by increasing hippocampal glucocorticoid receptor expression (Yau et al., 2002). The function of the HPA axis and serotonergic system are highly interrelated. Hypothalamic CRH might modulate the serotonergic system (Boadle-Biber et al., 1993; Fuller, 1996a). Conversely, serotonergic neurons in the raphe nucleus can directly stimulate HPA axis function via 5-HT1A and 5-HT2A receptors in the paraventricular nucleus of the hypothalamus (Fuller, 1996b). Therefore, increases in extracellular 5-HT levels acutely and in serotonergic neurotransmission chronically by treatment with SSRIs, such as FX, in depressed mothers could have an impact on fetal HPA activity. As mentioned previously, in our sheep studies, 8-day late gestational FX i.v infusion in pregnant ewes resulted in an augmented prepartum rise in fetal ACTH and cortisol levels and reduced low voltage rapid eye movement behavioral state in the fetus (Morrison et al., 2004). Although the mechanisms are not fully understood, alterations in HPA axis activity in the fetus might be one of the possibilities underlying negative perinatal outcomes observed in human studies. In addition, this is particularly important in the context of fetal exposure to excess glucocorticoids either by prenatal stress or maternal illness (e.g: depression, anxiety disorders) or pharmacologic agents effects (eg: SSRIs, synthetic glucocorticoids for lung maturation in preterm labor),
which can program the HPA axis in ways that it will have negative impacts on mental, metabolic and cardiovascular health later in life (Lupien et al., 2009; Seckl, 1997; Tamashiro & Moran, 2010). Interestingly, a recent study from our group, which further examined the long-term effects of prenatal SSRIs (fluoxetine) on the HPA axis in the postnatal period, have shown an association between SSRI exposure and reduced basal cortisol levels as well as an alteration in the HPA stress response pattern (Oberlander et al., 2008). A lower cord blood cortisol level in SSRIs-exposed neonates was also reported in a subsequent study (Davidson et al., 2009). Conflicting findings from the sheep and human studies could be, in part, attributed to the differences in the timing and duration of exposure which, determine the nature of the offspring adaptive responses and the physiological disturbances in later life (Chadio et al., 2007).

Sex differences in the HPA axis normalization in response to antidepressant treatment have been observed (Binder et al., 2009). In rodents, there is a differential HPA axis response between males and females following acute SSRIs (citalopram). Female mice had a greater corticosterone response after citalopram administration than males and testosterone treatment in ovariectomized females eliminated the sex differences (Goel & Bale, 2010). Similar differences were also reported with sertraline treatment in sheep (Broadbear et al., 2004). ACTH and cortisol secretion significantly increased in female but not in male after sertraline s.c injection, independent of circulating steroid hormones. These studies indicate that the HPA axis of the female is more sensitive to the stimulatory effects of serotonin than that of the male.
1.8 Rationale:

The incidence of clinical depression during pregnancy is about 10-15% (Bhatia & Bhatia, 1999; Nonacs & Cohen, 2003). Postpartum depression occurs in 10-22% of women, with symptoms beginning two weeks after delivery (Burt & Stein, 2002). Depression during pregnancy can place both the mother and fetus at risk (Ahluwalia et al., 2004; Orr & Miller, 1995; Zuckerman et al., 1989), and there is abundant human evidence for adverse effects of maternal postpartum depression on the cognitive, motor and emotional development of the offspring (Kurstjens & Wolke, 2001; Murray & Cooper, 1997; Murray et al., 2001). Since their introduction in the late 1980’s, SSRI is remain a first-line pharmacological treatment for both depression in pregnancy and in the postpartum period (Altshuler et al., 2001; Misri et al., 2000). However, numerous studies in humans have shown that infants delivered from women taking SSRIs during pregnancy have a higher risk of a number of adverse pregnancy outcomes, including premature birth, low birth weight, decreased APGAR scores at birth, admission to special-care nurseries, reduced postnatal weight gain, persistent pulmonary hypertension and poor neonatal adaptation (Chambers et al., 1996; Chambers et al., 1999; Lattimore et al., 2005; Oberlander et al., 2006). Human studies have found that prenatal SSRI exposure results in blunted infant heart rate and facial responses to a painful stimulus (heel prick) at 2 days and 2 months after birth (Oberlander et al., 2002; Oberlander et al., 2005) and altered hypothalamic-pituitary-adrenal (HPA) axis function at 3 months of age (Oberlander et al., 2008).

The most commonly reported consequence of prenatal SSRI exposure is poor neonatal adaptation (Laine et al., 2003; Oberlander et al., 2004; Sanz et al., 2005; Simon et al., 2002; Zeskind & Stephens, 2004). This is a self-limited phenomenon usually lasting a
few days and comprising respiratory difficulty, cyanosis on feeding and jitteriness. There are several possible mechanisms for these effects. One is an SSRI-elicited alteration in fetal brain development and/or maturation. This is suggested by the results from our previous studies, which involved 8-day administration of fluoxetine to chronically instrumented pregnant ewes in late gestation. The fetuses of these ewes exhibited acute changes in fetal cardio-respiratory function (Morrison et al., 2002), behavior (Morrison et al., 2001) and in HPA axis function (Morrison et al., 2004). In addition, there was a transient reduction of postnatal weight gain, similar to the human findings. The altered fetal behavior comprised a reduction in REM sleep and increase in quiet sleep, while the altered HPA axis function comprised an increase in the fetal prepartum cortisol surge and an apparent increase in the sensitivity of the adrenal gland to ACTH. These prenatal alterations could thus contribute to the poor neonatal adaptation, as well as some of the other adverse postnatal findings associated with prenatal SSRI exposure in the human. However, other explanations for the poor neonatal adaptation have been proposed. These include an SSRI withdrawal phenomenon in the postnatal period or a persistence of drug in the newborn, leading to drug-related toxicity, i.e., the serotonin syndrome (Andrade et al., 2006; Hendrick et al., 2001; Isbister et al., 2001; Koren, 2004; Ruchkin & Martin, 2005). Moreover, FX and SSRIs are also used in children and adolescents for treatment of depression and there are raising concerns about the safety of this therapy (Sharp & Hellings, 2006; Usala et al., 2008). Therefore, we suggest that fluoxetine injection to the newborn lambs and later in life at different ages is a useful model to test the hypothesis which cannot be implemented in human because of ethical issues. In addition, as the lambs were to be studied from birth to one year of age, longitudinal studies on cardiovascular,
metabolic and behavioral, and endocrine functions could be studied in relation to age, sex and FX exposure.

1.9 Objectives:

To determine the ontogenetic changes in various physiologic, behavioral and endocrine functions in lambs from birth to 1 year of age in relation to sex and acute postnatal fluoxetine administration. The study involved 2 groups of lambs: one group received a bolus FX injection at approximately 2, 10, 30 days and at about 3, 6 and 12 months of age and a control group that was studied at the same time periods. In addition, postnatal outcomes in the prenatally FX-exposed lambs were investigated with 2 groups of lambs: one group maternally exposed to FX for 14 days of late gestation and one control group with no prenatally FX exposure.

Specific objectives:

1. To examine the behavioral functions in newborn and postnatal lambs.

2. To examine cardiovascular function including blood pressure, heart rate and heart rate variability in newborn and postnatal lambs.

3. To determine blood gas, oxygen saturation, pH, glucose, lactate in newborn and postnatal lambs.

4. To investigate HPA axis function by measuring plasma cortisol and ACTH concentrations in newborn and postnatal lambs.
5. To determine the effects of acute fluoxetine administration on the above variables at 2, 10 and 30 days and 3, 6 and 12 months of age.

6. To investigate the postnatal outcomes in the lambs antenatally exposed to FX in late gestation.

1.10 Hypotheses:

1. The major postnatal changes in behavior, cardiovascular and HPA axis functions in postnatal lambs will be temporally related to weaning.

2. FX injection will cause transiently agitation in the newborn and postnatal lambs, disrupt their feeding activities and thus affect their ultradian and circadian rhythms.

3. FX injection in the newborn lambs will transiently decrease their heart rate and increase their arterial pressure, changing their heart rate variability.

4. FX injection will cause transient hypoxemia with the decrease in blood oxygen saturation and Po2.

5. FX injection will alter the activity of HPA axis and lead to an increase in cortisol and ACTH plasma levels.

6. Antenatally FX-exposed lambs will have adverse outcomes: lower birth weight, and poor neonatal adaptation.
2. Materials and Methods

All procedures conducted on the animals were approved by the UBC Committee on Animal Care and conformed to the guidelines of the Canadian Council on Animal Care. This chapter describes the general materials and methods for Chapter 3 to 5

2.1.1 Animals:

Sheep were bred following a protocol in our lab to provide us with lambs for studying all year round. The estrous cycle was synchronized via progestin containing vaginal pessaries (Pharmplex, Milton NSW, Australia) for 14 days. Ovulation was then induced with an intramuscular injection of pregnant mare serum gonadotropin (PMSG) (Wyeth, Ontario, Canada). Ewes were then placed with a ram for natural mating. Subsequently, conception was confirmed by measurement of plasma progesterone by the Gynecologic Endocrinology laboratory in the Division of Reproductive Endocrinology and Infertility, UBC at 19 days after pessary removal and gonadotropin injection. An ultrasound was performed at ~90 days gestation to confirm a successful pregnancy. Pregnant ewes (Dorset/Suffolk) were transported to the Child & Family Research Institute from Three Gates Farm, Gabriola Island, at least 3 weeks before their due date (term = 147 days) to allow them to acclimate with the new environment before their deliveries. They were housed singly in 2.4 x 1.2 m pens or in groups of 2 in 2.4 x 2.4 m pens. Sheep were kept under a 12 hour light: 12 hour dark cycle with scheduled lights on at 6:00 am and off at 6:00pm. The feeding regimen comprised 0.6 kg grain and 0.7 kg local hay at 0730 am and 0.7 kg local and 0.7 kg alfalfa hay at ~1430 pm. The grain ration was increased to 0.8 kg after delivery. Water was available ad lib. Signs of imminent parturition in the ewe (bilateral concave flanks, restlessness,
pawing at the pen floor, moving in circles and looking toward the back) and the lambs’ birth were revealed by digital video recording (DVR) from infrared cameras which will be described in more detail in Section 2.2.5 (DVR and behavioral evaluation). Ewes were allowed to deliver without assistance except in cases of malposition of the fetus. After the lambs were born, they were dried with a towel, sexed, weighed and had an actiwatch placed around their neck (more details about actiwatch in Section 2.2.6: Actiwatch/Rest activity monitoring). Surgeries were conducted on the lambs on the first day after birth. Lambs were housed in the same pen with their mothers until the weaning period. The study involved 2 groups of lambs: one group received a bolus fluoxetine injection at ~ 2, 10, 30 days and at ~ 3, 6 and 12 months of age and a control group that was studied at the same time periods.

2.1.2 Surgical Preparation:

Surgeries were done on the lambs on postnatal day 1.5 ± 0.1 (for those who started the day 3 experiment) or on postnatal day 9 ± 1.1 (for those who started the approximately day 10 experiment). Lamb was anesthetized with 4% isoflurane and 40% NO delivered via a face mask and then intubated with a cuffed endotracheal tube (3.5 to 5.0 mm O.D, depending on the size of the lamb). Anesthesia was maintained with 1.5 to 2% isoflurane, 40% NO in oxygen. The lambs were ventilated at a rate of with a frequency and tidal volume appropriate for their weight and postnatal age (Moss et al., 1995). The eyes were lubricated with Refresh Eye Lube. Bupivacaine hydrochloride (0.5%) was infiltrated subcutaneously at the base of the ear before an ear tag was applied and at the surgical sites. Meloxicam (0.2 mg/kg) was also given subcutaneously for analgesia and 250 mg ampicillin intramuscularly for antibiotic prophylaxis. The lamb was placed on a heating pad during the surgery and a thermistor rectal probe connected to a Yellow Springs Instruments Tele-Thermometer was used to monitor the
body temperature continuously. Heart rate was monitored intermittently via auscultation or later in the study with a pulse oximeter (Nonin Medical Inc, Plymouth, MN, USA). Aseptic surgical techniques were employed. All surgical instruments were autoclaved before surgery. The skin area to be operated on the neck was shaved and washed with antiseptic soap, then sprayed with 70% alcohol and Proviodine solution (USP 10%) with the combination repeated 3 times. A sterile surgical drape was used to cover the lamb with the surgical opening positioned appropriately.

Access to the carotid artery and jugular vein was gained via an oblique transverse skin incision in the neck from the sternomastoid muscle (from ~ 1cm lateral to just above the jugular vein). Heparin-bonded 3.5 or 5 French polyurethane catheters (Instech Solomon, Plymouth Meeting, PA) were filled with sterile heparinized 0.9% NaCl solution (12U heparin/ml) and were implanted into the right carotid artery and jugular vein. The catheters were secured in place with 2-0 silk non-reabsorbable sutures and then the catheters were tunneled subcutaneously and exteriorized through a small skin incision at the dorsal neck. These catheters were available for drug administration and blood sampling in the subsequent series of experiments, while the arterial catheter was employed to measure arterial pressure. The transverse and dorsal neck skin incisions were closed with 2-0 Polysorb reabsorbable (Syneture, Canada) interrupted sutures. The skin and the outer surfaces of the catheters were then cleaned with 70% alcohol. Then the distal ends of the catheters were sealed with sterile, metal pins and the catheters were placed in a small zip-lock bag. The skin incisions were covered with sterile gauze pads, over which was placed in a zip lock bag into which the catheters were placed. An elastic bandage was then placed loosely around the neck and secured with a safety pin.
To prevent the risk of fly strike when the lamb gets older, all newborn lambs also had their tails docked. The section of the tail to be cut was first shaved, disinfected, infiltrated with 0.5% bupivicaine and then cut and cauterized using a Supervet Tail Dock Cutter/Cauterizing Instrument (Syrvet Inc., Iowa, USA). Following this, the skin margins of the tail stump were opposed using a continuous 2-0 Polysorb suture.

Lambs were kept in the operating room until they could breathe spontaneously and were conscious. They were then returned to their mother. The total surgery time from removing the lamb from its mother to returning the lamb post-surgery averaged 2.2±0.01 h. The lambs were weighed daily in the morning. Catheters were flushed daily with sterile heparinized 0.9% NaCl solution (12U heparin/ml) to maintain their functioning until the last experiment (1 year of age). If catheters stopped functioning, another surgery was performed to implant other appropriate catheters at least 2 days before the experiments were started.

2.1.3 Experimental Protocol:

The experiments on the lambs began on the day following surgery. A sterile silicone urinary catheter was placed in the urethra of the female lambs and secured with tissue adhesive and a 2-0 silk suture tied to the wool in the groin area on the day before FX experiments. Since in male lambs, the urethra makes a hairpin turn, it is impossible to insert a urethral catheter. A strap-like urine collection device, which was successfully developed by Tim Chow, another PhD candidate in our lab, was placed on the lamb’s penis, wrapped around the abdomen and tied securely on their back about 30 minutes before the FX experiments. This served for urine collection mainly for Tim Chow’s project which involves developmental fluoxetine pharmacokinetics and metabolism. The urine volume data was also
used in chapter 3 to determine the development of urine flow rate in the lambs with advancing age. For the experiments on the 2, 10 and 30 day old lambs, two experiments were carried out on the same day, starting at 8:30am for the first lamb and 11:30am for the second. The lambs were placed in a cloth sling set up in the pen that contained the lamb’s mother or the biggest pen. The lambs were suspended above the floor in the sling and normally lay quietly or slept. They were only in the sling for the first two and a half hours of the experiment, and then were returned to their mothers, so as not to disrupt the normal suckling regimen. Lambs at 10 days and 1 month of age were bleating and jumping sometimes especially toward the end of the 2.5h duration. For the experiments conducted at 3 months and beyond, the lambs were placed in a small monitoring pen (120 X 65 cm) set up in one corner of the holding pen. They remained in this pen for the duration of each experiment (24h for the control experiment and 72h for the FX experiment) and had access to food and water. Since lambs started having solid food around 2 months old, experiments on lamb at 3 months and above were started at 9:00am (30 min after lambs finished their feeding) to eliminate the affect of eating on changes in heart rate and blood pressure (Jones, Langille, Frise, & Adamson, 1993).

Each experiment involved i.v administration of sterile water (0.1ml/kg, equal to the volume of 10mg/ml FX given in FX experiment) or fluoxetine (1mg/kg) followed by serial collection of arterial blood samples (~ 3 ml) at -30, -15, 5, 15, 30, 45 and 60 minutes and 2, 4, 6, 9, 12, 24, 36, 48, 60 and 72 h (more specifics in table 2). The total volume of blood collected (42ml) represents ~ 8% of the total blood volume in the newborn lamb and much less in the older lambs.
Table 2.1: Time points at which blood samples were collected for arterial blood gases, FX, ACTH and cortisol measurement. SW = sterile water.

The symbols indicate when the samples were taken for the corresponding variables

2.1.4 Sample Collection:

Blood samples for hormone and fluoxetine measurements were collected through the carotid artery catheter and placed in a 4ml EDTA K$_2$ and Lithium Heparin (68USP units) vacutainer tubes (BD Vacutainer, NJ, USA) and centrifuged for 20 min at 4000 rpm in a Centrifuge 5810R (Eppendorf, ON, Canada). Plasma was then transferred to polyvinyl Eppendorf tubes for hormone samples and glass tubes for fluoxetine samples and stored at -20°C until analysis. Arterial pressure was obtained from the arterial catheter using a disposable pressure transducer and Powerlab data acquisition system, which also calculated a heart rate from the pulse pressure. The ECG was recorded using surface neonatal ECG
electrodes and the Powerlab Heart Rate Variability Module was used to assess heart rate variability (HRV).

Blood samples were also collected before the surgery, on postnatal day 2.3 ± 0.2 (n = 11), for ACTH and cortisol measurements. Lambs were taken to the operating room and their wool along the jugular vein was shaved. The skin was disinfected with alcohol 70% and applied with local anesthetic Xylocain 2%. Approximately 2ml of blood was taken to the EDTA K2 and Lithium Heparin (68USP units) vacutainer tubes via venipuncture. Lambs usually slept while blood was collected and were returned to their mother immediately. Samples were centrifuged and plasma was transferred and stored as mentioned above.

2.1.5 Digital Video Recording And Behavioral Evaluation:

One infrared video camera were installed on the wall of each pen in the sheep holding room and connected to a digital video recording system on a computer in the lab, which was directly adjacent to the sheep room. Another mobile infrared camera was put outside the pen or above the monitoring pen to allow for the recording and observations of the lambs from a direct view, as the lambs sometimes could not be observed by the fixed cameras because of the ewe and lamb’s position within the pen. On the day of experiment the mobile camera was also be used to record behavioral changes immediately after FX administration. The infrared function allowed the recording and monitoring the ewe and lamb’s behavior at night without interrupting their circadian rhythm. These cameras could also be accessed remotely from home and the video records could be analyzed off-line.

Lamb’s activities were categorized into lying down sleep, lying down awake, standing (still or moving), walking, suckling attempt, successful suckling and playing.
Playing activities included running, jumping, climbing on their mother’s back, nudging their mother, nosing and playing with hay. These activities were collected for the dominant activity in 15 second epochs. The collected activities from the DVR were then matched with the actiwatch data (see below) to obtain an equivalent score from the actiwatch record for the activity identified from DVR in the same epoch. These scores were used to determine the intensity of each kind of activity. After each type of activity was identified as mentioned above from DVR observations, only standing activity was further analyzed using a cut-off point of 60 on actiwatch score to separate standing still from standing moving. In other words, standing still was defined as standing activity from DVR observation and corresponding actiwatch score lower than 60. Similarly, standing mobile was standing activity from DVR and corresponding actiwatch score equal or greater than 60. The percentage of time per 24 hour lambs spent for each activity was also calculated.

### 2.1.6 Actiwatch Rest/Activity Monitoring:

An Actiwatch Activity Monitor (AW64, Mini Mitter Inc., Bend, OR, USA), which is a small wrist watch sized device, was enclosed in a protective metal case and placed around the neck of the lamb with a dog collar from the first day after birth until at least 90 days after birth to measure rest-activity cycles and sleep patterns. The actiwatch contains an omnidirectional accelerometer which detects and stores the occurrence and intensity of any movement with a force of at least 0.01g. The activity data was stored in 15s epochs and downloaded every 10 days using an Actiwatch reader and analyzed using an Actiware-Sleep version 3.5 software (Mini Mitter). The analyzed variables included total daily activity score (total number of movement counts in each 24h period), active bouts/day (= mean activity score, i.e magnitude of activity per epoch), mean movement score in active (the average
number of movement counts during bouts of activity), episode duration (= moving minutes/mean activity score), % time moving and % time immobile.

The ultradian and circadian rhythms in the lambs were determined using Fast Fourier Transformation (FFT) analysis from the Actiware software. The four most prominent frequencies in the FFT analysis were selected.

2.1.7 Powerlab Data Acquisition System:

The lamb’s carotid artery catheter was connected to a disposable pressure transducer (DTX™ Plus TNF-R; Becton Dickinson, Singapore), which was connected to a polygraph recorder (model TA-4000, Gould Instrument Systems Inc, Valley View, OH) and to a Powerlab data acquisition system (AD instrument, Mountain View, CA, USA) to continuously record heart rate and arterial pressure throughout the first 2h of the experiments in 4, 10 and 30 day old lambs and for the entire 24h or 72h of the experiments at 3, 6 and 12 month old lambs. The transducer was kept on the platform which was adjusted to the lambs’ heart level when they stood or were in the sling. Three ECG snap electrodes were attached to the skin on the back and bilaterally on the chest and secured in place by adhesive bandages. During the experiments these electrodes were connected to the Powerlab system by three snap leads to record the electrocardiogram. The Powerlab Heart Rate Variability Module was used to assess and analyze heart rate variability (HRV) including estimates of parasympathetic and sympathetic tone.
2.1.8 **Arterial Blood Gases Analysis:**

Blood samples were collected at -15, 5, 15, 30, 60 and 120 minutes to measure acid-base balance (pH), base excess (BE), bicarbonate (HCO$_3^-$), arterial blood gases (pO$_2$, pCO$_2$), electrolyte (Na$^+$, K$^+$ and iCa$^{2+}$) using an I-STAT-1 analyzer (Abbott, ON, Canada) and CG8$^+$ cartridges (Abaxis, Union City, CA, USA). Body temperature was measured continuously with the thermistor rectal probe (YSI Instruments, Yellow Springs, OH) over the first 2h of experiment or later by a regular digital thermometer and blood gas results were corrected with the appropriate temperature when the sample was collected. Hemoglobin concentration and oxygen saturation were measured with a Radiometer OSM3 Hemoximeter (Radiometer, Copenhagen, Denmark). Glucose and lactate were determined with a YSI glucose-lactate analyzer (YSI Inc., Yellow Springs, OH).

2.1.9 **Adrenocorticotropin Hormone Assay:**

ACTH level was measured using a commercially available human ACTH ELISA kit (Calbiotech Inc., CA, USA) that detects ACTH$_{1-24}$. The kit has previously been validated for use in sheep (You et al., 2008). 200µl of plasma was added together with 25 µl of biotinylated antibody and 25µl of enzyme labeled antibody into each well of the microplate. The microplate then was covered with aluminum foil to prevent light exposure and was shaken at 170 ±10 rpm for 4.5 hour at room temperature. After the remaining antibodies were washed with wash solution five times, 150 µl of TMB substrate was added into each well and the plates shaken at the above speed for 30 minutes at room temperature. 100 µl of stopping solution was then added to terminate the reaction. ACTH level was determined by an ELISA reader (initially by Dynex Technologies and later by Wallac Victor$^3$V 1420) set at 450nm.
Each sample was measured in duplicate. The sensitivity of the assay was < 1 pg/ml. The intra- and interassay coefficients of variation were 7.27% and 7.08%, respectively.

2.1.10 Cortisol Assay:

Commercially available sheep cortisol ELISA kits (Calbiotech Inc., USA) was used to determine cortisol concentrations. 40µl of sample in duplicate and 200µl of Cortisol enzyme conjugate were added into each well coated with anti-cortisol monoclonal antibody for competitive binding. The microplate was then incubated for 1 hour at room temperature with shaking. The unbound hormone and conjugate was washed off with washing solution 3 times. 100µl of TBM substrate was added and incubated for 15 minutes in the same previous condition. The reaction was brought to end by adding 50 µl of stop solution in each well. The cortisol level was determined by ELISA reader (initially by Dynex Technologies and later by Wallac Victor³V 1420) at 450nm. The sensitivity of the assay was 1.38 ng/ml. The intra- and interassay coefficients of variation for low control (78 ± 3 ng/ml) were 10.7% and 3.2%, and for high control (225 ± 5 ng/ml) were 7.2% and 6.7%, respectively.

2.1.11 FX and NFX assay:

FX and NFX concentrations were measured by Dr. Tim Chow from Dr. Wayne Riggs’ lab, Faculty of Pharmaceutical Science, UBC using a validated liquid chromatography with tandem mass spectrometry method (LC/MS/MS) (Chow et al., 2011). The sample volume used for each enantiomer (R)-, (S)- fluoxetine and (R)-, (S)- norfluoxetine was 250 µl. The limit of quantitation was 1 ng/ml.
2.1.12 Statistical Analysis:

All data were analyzed using the Sigma Plot 11 statistics software (Systat Software, Inc; San Jose, CA, USA). A 2 way General Linear Model (GLM) for repeated measures ANOVA or a three way ANOVA followed by the Bonferroni t-test for multiple comparisons was performed to determine the effects of age, sex and FX treatment on the interested variables. P < 0.05 defines significant differences. Data are presented as mean ± SEM. Other statistical methods that were used in particular results chapters are described in those chapters.
3. Postnatal Lamb Development and Sex Differences

3.1 Introduction:

The geometry of the circulation in the newborn alters remarkably at birth, due to the loss of the umbilical circulation, onset of pulmonary ventilation and increase in pulmonary blood flow. With the onset of air breathing, the pulmonary vascular resistance decreases and the surface area for gas exchange increases. Hemodynamic changes result in the closure of fetal circulatory shunts – formamen ovale and ductus arteriosus. At birth, cardiovascular function also alters significantly with an increase in arterial pressure, heart rate and cardiac output. Much research has been done regarding the cardiovascular and hemodynamic changes immediately following birth. (Klopfenstein & Rudolph, 1978; Lister et al., 1979; Minoura & Gilbert, 1987; Woods et al., 1977) However, little has been known about these physiological developments (heart rate, blood pressure, HRV) in lambs after the initial postnatal period. In lambs, heart rate and blood pressure increase with feeding (Jones et al., 1993) but whether there is any effect of weaning on the development of these variables is unknown. Studies in goats found an elevated cortisol and B-endorphin concentration during suckling in kids but no significant changes in heart rate or blood pressure related to suckling (von Walter et al., 2010).

Heart rate variability (HRV) represents the oscillation of the intervals between consecutive heart beats. It is in part determined by influences of the autonomic nervous system on the heart. Since HRV is a non-invasive method to measure the autonomic impulses, monitoring HRV variables are used clinically as an evaluation of fetal well being in the human fetus (Alfirevic et al., 2006; Graatsma et al., 2009; Maso et al., 2012; Van
Leeuwen et al., 1999) and also in the neonate in neonatal intensive care (Fairchild & O'Shea, 2010). They are also used in a number of conditions in the adult, including coronary artery disease, systemic hypertension and myocardial infarction (Charkoudian & Rabbitts, 2009; Huikuri, 1995; Pagani & Lucini, 2001). However, there is less information about changes in HRV characteristics during development, including the changes which occur around weaning. Even though high HRV usually indicates a good adaptation of an effective autonomic system in a healthy individual while low HRV often indicates the presence of physiological malformation, the threshold is age-dependent. HRV has been shown to be lower in healthy women than in men (Saleem et al., 2012); however, it is not known if the same situation exists in sheep. There are established data on the differences in the prevalence, characteristics and response to treatment of cardiovascular diseases (Dunlay & Roger, 2012). After adjustments for the increased longevity in women, age-specific stroke incidence is generally higher in men than women (Katsiki et al., 2011). Gender differences in cardiac autonomic control might occur early in life and could be one of the potential mechanisms for the observed differences in cardiovascular disease between males and females.

Birth also results in changes in the surrounding environment. The newborn has to adapt with and become entrained to the circadian rhythm of the light dark cycle. Melatonin, which is secreted from the suprachiasmatic nucleus under the influence of light from the environment, is not present in human infants until 9-15 weeks of age (Kennaway et al., 1996) and in lambs at 3-4 weeks after birth (Kennaway et al., 1996; Nowak et al., 1990). A disrupted circadian rhythm may play a role in the pathogenesis of certain illnesses such as coronary heart disease, breast cancer, gastrointestinal disturbances, sudden infant death syndrome even though the mechanisms involved have not yet been established (Mosendane
et al., 2008; Lohr & Siegmund, 1999). In a previous study using actigraphy in newborn lambs, our lab found a short (~ 1 h) activity rhythm not present in adult sheep, in which the circadian rhythm was the most dominant. Only the lactating ewes had a short (~1 h) rhythm as the second, third or fourth most dominant rhythms. Also, the total daily activity level in the lambs over the first ~3 weeks was significantly higher than in adult sheep (Rurak et al., 2008). However, it is still not yet clear when the short term ultradian rhythm in the lamb disappears and when the circadian rhythm becomes dominant as in the adult sheep. In the human, babies who are breast-fed for the first 2 years of life wake up more often at night and have shorter sleep bouts instead of having a long unbroken night sleep (Elias et al., 1986). Thus the short 1 h rhythm observed in the lambs may be the result of suckling activity.

3.2 Methods:

3.2.1 Experimental Protocol:

As described in chapter 2, the lambs in this study were used in a longitudinal study of the disposition of the antidepressant, fluoxetine (FX), from birth to one year of age. All the data used in this chapter was from the values of control experiments with sterile water injection or from the control time point of FX experiments (i.e: 30 mins before FX injection) except for the urine volume data and rest-activity cycles. The animal housing, surgical, experimental and analytical methods are described in Chapter 2.

3.2.2 Statistical Analysis:

A 2 way General Linear Model (GLM) for repeated measures ANOVA followed by the Bonferroni t-test was used to determine the effect of age and sex in cardiovascular,
metabolic and endocrine functions. The relationship between postnatal age and the cardiovascular, metabolic, endocrine and urinary variable was analyzed using piecewise linear regression with 2 elements. The area under the curve (AUC) of the activity variables (total activity score, mean activity score, mean score in active, episode duration, % time mobile, % time immobile, circadian and ultradian rhythms) was calculated for each lamb and analyzed by 2 way ANOVA to determine the effect of age and gender in their rest-activity cycle.

3.3 Results:

For the newborn lambs, the average age of the lambs on the control and FX experiments is 3.2 ± 0.5 and 4 ± 0.4 postnatal days, respectively.

3.3.1 The Development Of Physiological Variables And Sex Differences In Development From The Newborn Period Up To 1 Year Of Age:

The lambs’ heart rate fell dramatically over the first month of life from 220.4 ± 6.3 bpm on postnatal day 3 to 155.2 ± 8 bpm at 1 month of age. A further decrease in heart rate occurred thereafter to 94 ± 3.4 bpm at 1 year of age. There was a significant difference in the values between 10 days, 1 month, 3 months and 6 months. There was also a sex difference in heart rate development. At 6 months, heart rate in female lambs was significantly lower than male lambs (96 ± 7.8 bpm in female vs 115 ± 7.7 bpm in male, (p = 0.016) while the situation seemed to be reversed at 1 month of age (167 ± 10.5 bpm in female and 155 ± 8 bpm in male) even though the difference was not significant at 1 month. However, they both reached a similar heart rate at 1 year of age (97 ± 3.9 bpm in female and 93 ± 5.2 bpm in male). Female lambs tended to reach the adult heart rate value earlier than males, at 6 months.
of age. Arterial pressure development was similar in male and female lambs. Arterial pressure gradually increased from 66 ± 1.5 mmHg on day 3 to 82.9 ± 1.1 mmHg at 1 year of age and the value was significantly different at 1 month and 1 year-old against 3 days of age. (Fig. 3.1)

In terms of heart rate variability, a sex difference was only found in Low Frequency (LF) power. At 3 day-old, LF power in male was significantly higher than that in female (52.4 ± 6.2 nu in male vs 25.7 ± 6.5 nu in female, p = 0.036). In female, LF power increased to peak at 10 day-old (61.5 ± 5 nu) and then decreased to a plateau from 3 months to 1 year of age (range: 38 ± 6.8 – 43.4 ± 4.9nu). In males, LF power decreased from 52.4 ± 6.2 nu on day 4 to a plateau between 4 and 12 weeks (range: 35.8 ± 3.8 – 46.9 ± 5.9 nu ), then increased at 6 months (56.1 ± 4.2 nu), followed by a decrease at 1 year of age (Fig. 3.3A).

The time domain variables of HRV (including SDNN, SD Delta NN, RMSSD) were significantly different between the 3-day, 10 day-old and the remaining ages (1, 3, 6 months and 1 year old) with the lower values at 3 days of age (22 ± 1.7 msec for SDNN, 19.4 ± 3.4 for SD Delta NN and RMSSD) and higher values at 1 year of age (102.8 ± 12.4 for SDNN, 80.5 ± 9.8 for SD Delta NN and RMSSD) (Fig. 3.2). High Frequency (HF) power tended to increase from 18.4 ± 2 nu on day 3 to 28.3 ± 2.2 nu at 1 year of age with the lowest value at 10 days of age (16.4 ± 1 nu). Since HF power did not change much over the time, changes in LF/HF ratio were due mainly to LF power alterations. The LF/ HF ratio peaked at day 10 (3.7 ± 0.48) and was significantly higher than that at 3 months (1.8 ± 0.3) and 1 year of age (1.6 ± 0.2). (Fig. 3.3)

Fig. 3.4 show an inverse relationship between SDNN (msec) and heart rate in the lambs from 3 days to 1 year of age with $r^2 = 0.55$, p < 0.001
FIG. 3.1: Postnatal lamb arterial pressure (A) and heart rate (B) in all (open bar), male (black bar) and female (gray bar) lambs at 3 days (n = 14), 10 days (n = 18), 1 month (n = 15), 3 months (n = 17), 6 months (n = 11) and 1 year (n = 10) of age. Different letters (a, b, c and d) indicate significant differences between ages. Asterisk indicates significant difference between male and female, (p < 0.05).
3d = 3 days; 10d = 10 days; 1M = 1 month; 3M = 3 months; 6M = 6 months; 1Y = 1 year
FIG. 3.2: Time domain of heart rate variability: SDNN (standard deviation of NN interval) (A), SD Delta NN (Standard Deviation of the differences between adjacent NN intervals) (B), RMSSD (Square root of the mean of the squared differences between adjacent NN intervals) (C) in lambs at 3 days (n = 11), 10 days (n = 18), 1 month (n = 15), 3 months (n = 17), 6 months (n = 11) and 1 year (n = 10) of age. Different letters (a,b, and c) indicate significant differences between ages, (p < 0.05). 3d = 3 days; 10d = 10 days, 1M = 1 month, 3M = 3 months, 6M = 6 months, 1Y = 1 year
FIG. 3.3: Frequency domain of heart rate variability: LF (Low Frequency) power (A), HF (High Frequency) power (B), LF/HF ratio (Low Frequency/High Frequency Ratio) (C) in lambs at 3 days (n = 11, male n = 4, female n = 7); 10 days (n = 18, male n = 9, female n = 9), 1 month (n = 15, male n = 8, female n = 7); 3 months (n = 17, male n = 9, female n = 8), 6 months (n = 11, male n = 6, female n = 5), 1 year (n = 10, male n = 6, female n = 4) of age.

Different letters (a,b) indicate significant differences between ages. Asterisk indicates significant difference between male and female, (p < 0.05).

3d = 3 days; 10d = 10 days, 1M = 1 month, 3M = 3 months, 6M = 6 months, 1Y = 1 year
Among the blood chemistry variables collected (glucose, lactate, O$_2$ sat, pO$_2$, pCO$_2$, pH, BE, HCO$_3$, Hb), only the pH value at day 3 was different between male and female (Fig. 3.5), with the value in males (7.379 ± 0.0016) being significantly lower than in females (7.457 ± 0.018), p < 0.0001). Following birth, Po$_2$ and pH increased from 86.6 ± 3.4 mmHg and 7.421 ± 0.016 on postnatal day 3 to 110.7 ± 3.27 mmHg and 7.489 ± 0.009 at 1 year of age, while Pco$_2$ decreased from 41.9 ± 1.01 mmHg on postnatal day 3 to 35.57 ± 0.653 mmHg at 1 year of age, with the main changes occurring over the first 12 weeks. Base excess tended to increase from 3 ± 1.019 mmol/l on postnatal day 3 to peak 5.647 ± 0.342 mmol/l at 3 months of age and slightly decrease thereafter. Both glucose and lactate concentrations decreased from 5.39 ± 0.215 mmol/l and 1.028 ± 0.055 mmol/l on postnatal day 3 to 2.91 ± 0.12 mmol/l and 0.527 ± 0.042 mmol/l at 1 year of age, respectively, again with most of the change occurring in the first 12 weeks, although lactate decreased further at
6 months. Hemoglobin concentration decreased more rapidly, from 12.2 ± 0.34 g/l at 3 days of age to low value 8.729 ± 0.418 g/l at week 4, and was relatively constant thereafter (Fig.3.6-3.8). These suggested that the newborn lambs appeared to be more hypoxemic and acidemic than the adult.

Urine flow rate (ml/min.kg) was based on the mean of urine flow rate at different intervals of the FX experiment and was normalized for the weight of each lamb at the corresponding age. Urine flow rates at 2 and 10 days of age were significantly higher than the values at 3, 6 and 12 months of age. There was no sex difference in urine flow rate at any age. (Fig. 3.9)

The cortisol concentration was not significantly different between day 3 (day of control experiment) and ~ day 2.3 ± 0.2 before the surgery to implant the catheters for the subsequent experimental sampling.

In fact, combining the mean cortisol values in the fetus lamb in late gestation from a previous paper in our lab (Morrison et al., 2004) suggested the lambs’ prior surgery cortisol value was consistent with the cortisol surge which usually occurs right before birth in the sheep. After the day 3 experiment, cortisol concentrations at 10 days and 1 month gradually decreased, but are significantly higher than the values at 3 and 6 months. At 3 months, the cortisol concentration became stable through to 1 year of age. (Fig. 3.10) There were no statistical significant changes in ACTH concentration from before surgery up to 1 year of age. (Fig. 3.11)
FIG. 3.5: Mean pH value in total (open bar), male (black bar) and female (gray bar) lambs at 3 days (n = 13, male n = 6, female n = 7); 10 days (n = 17, male n = 8, female n = 9), 1 month (n = 15, male n = 8, female n = 7); 3 months (n = 17, male n = 9, female n = 8), 6 months (n = 10, male n = 5, female n = 5), 1 year (n = 10, male n = 6, female n = 4) of age. Different letters (a,b,c) indicate significant differences between ages. Asterisk indicates significant difference between male and female, (p < 0.05). The fetal value at 147 days of gestational age (at term) is taken from Rurak & Bessette, (2012) and was not included in the statistical test.

3d = 3 days; 10d = 10 days, 1M = 1 month, 3M = 3 months, 6M = 6 months, 1Y = 1 year
FIG. 3.6: Mean pCO₂ (A), pO₂ (B) and O₂ sat (C) in lambs at 3 days (n = 13); 10 days (n= 17), 1 month (n= 15); 3 months (n= 17); 6 months (n = 10) and 1 year (n = 10) of age. Different letters (a, b, c, d) indicate significant differences between ages, \( p < 0.05 \).

The Fetal values at 147 days of gestational age (at term) are taken from Rurak & Bessette, 2012 and was not included in the statistical test.

3d = 3 days; 10d = 10 days, 1M = 1 month, 3M = 3 months, 6M = 6 months, 1Y = 1 year
FIG. 3.7: Mean HCO$_3$ (A), Base Excess (B) and Hemoglobin (C) in lambs at 3 days (n = 13); 10 days (n = 17), 1 month (n = 15); 3 months (n = 17); 6 months (n = 10) and 1 year (n = 10) of age.

Different letters (a,b) indicate significant differences between ages, (p < 0.05).

The Fetal values at 147 days of gestational age (at term) are taken from Rurak & Bessette, 2012 and was not included in the statistical test.

3d = 3 days; 10d = 10 days, 1M = 1 month, 3M = 3 months, 6M = 6 months, 1Y = 1 year.
FIG. 3.8: Mean Glucose (A) and Lactate (B) in lambs at 3 days (n = 13); 10 days (n = 17), 1 month (n = 15); 3 months (n = 17); 6 months (n = 10) and 1 year (n = 10) of age. Different letters (a ≠ b ≠ c ≠ d) indicate significant differences between ages, (p < 0.05).

The Fetal values at 147 days of gestational age (at term) are taken from Rurak & Bessette, 2012 and was not included in the statistical test.

3d = 3 days; 10d = 10 days, 1M = 1 month, 3M = 3 months, 6M = 6 months, 1Y = 1 year
FIG. 3.9: Mean urine flow rate in lambs at 3 days (n = 10); 10 days (n = 10), 1 month (n = 9); 3 months (n = 12); 6 months (n = 7) and 1 year (n = 10) of age.

Different letters (a ≠ b) indicate significant differences between ages, (p < 0.05).

Fetal value at 130-140 days of gestational age is for reference and was not included in the statistical test. The value is taken from (Turner et al., 2008)

GA = gestational age, 3d = 3 days; 10d = 10 days, 1M = 1 month, 3M = 3 months, 6M = 6 months, 1Y = 1 year
FIG. 3.10: Mean cortisol concentration in fetal lambs from preinfusion to infusion day 2 and from infusion day 5 to 8 adapted from Morrison et al, 2004 and in postnatal lambs prior surgery (n = 11), at 3 days (n = 14); 10 days (n = 12); 1 month (n = 12); 3 months (n = 12); 6 months (n = 8) and 1 year (n = 8) of age.

Different letters (a ≠ b ≠ c ≠ d ≠ e ≠ f) indicate significant differences between ages, (p < 0.05)
FIG. 3.11: Mean ACTH concentration in fetal lambs from preinfusion to infusion day 2 and from infusion day 5 to 8 adapted from Morrison et al, 2004 and in postnatal lambs prior surgery (n = 11), at 3 days (n = 2); 10 days (n = 2); 1 month (n = 2); 3 months (n = 14); 6 months (n = 3) and 1 year (n = 4) of age.
Cortisol, ACTH rhythm in the newborn and postnatal lambs:

At 3 day, 3, 6 month and 1 year–old experiment, the cortisol level at 30min, 1h and 2h tend to be higher than that at other time points during the day but the differences were not statistically significant. However at 10 days and 1 month, respectively the higher cortisol value at 30 min (at 10:00am) (201 ±64.9; 145.6 ± 24.8 ng/ml), 1 h (at 10:30am) (209 ±76.6; 178.1 ± 35.6ng/ml), and 2 h (at 11:30 am) (231.8 ± 72.5; 183.5 ± 46 ng/ml) were significantly different from that at 6 h (at 15:30 pm) (85.7 ± 25.6; 68.2 ± 13 ng/ml) and 12 h (at 21:30 pm) (78.1 ± 17.3; 73.4 ± 19.8 ng/ml), respectively. (Fig. 3.12)

From 3 month onward, lambs were put in the monitoring pen during the whole period of the experiment (4 days). The cortisol level did not change much over the sampling time point during the day with placebo injection at 3 month, 6 month and 1 year-old experiment, but there was a sex difference in cortisol concentration at 30 min (10:00 am), 1 h (10:30 am), 6 h (15:30 pm) and 12 h (21:30 pm) samples when the lambs were 1 year old. Cortisol levels were significantly higher in males than females at 1 year of age (p = <0.001).

3.3.2 Neonatal Behavioral Development:

From the DVR observations on 35 lambs following birth, we found the average times that it took for the lambs to first stand, walk and attempt to suckle were 33 ± 3, 45 ± 4 and 57 ± 5 minutes, respectively. Our results are similar to what have been shown in the literature (Dwyer, 2003; Fraser & Broom, 1997). However, we did not see any effects of sex or lamb number on the time that lambs took to first stand, walk or suckle. Also, we did not see any significant correlation between these first time activities and birth weight.
FIG. 3.12: Cortisol concentration during the day in the lambs at 10 days, 1 month and 1 year of age. Samples were collected at -30, -15 min before sterile water injection and 30 min, 1,2,6 and 12 hour after the injection (10 days group, n= 9; 1 month group, n = 11; 1 year group, n = 8). Different letters (a ≠ b) indicates significant differences between the time points. Asterisk indicates significant differences between male and female.
Activities on postnatal day 2-3 were observed from a smaller group of normal term lambs (n= 11) who were not subjected for any experiments or surgery at this age. The results showed lambs at this age spend most of the time at rest or asleep (~ 74.4%). Ewes of twin and triplets appear more frequently to initiate suckling than their lambs at this age. In general, only 33 ± 7% the number of bouts was initiated by the lamb itself. In terms of infant-maternal bonding, we observed that lambs sleep close to either their mom or with their siblings most of the time. Only one single female lamb showed less attachment to her mother.

In addition, I compared the time spent on suckling on postnatal day 2-3 in single (n=6), twin (n= 14) and triplet (n=3) lambs. The suckling time included both suckling attempts and successful suckling. The triplets spent significantly more time suckling than the singles and the twins. However, they gained less weight than the singleton and twins although the differences were not significant (p = 0.064) (Fig. 3.13)

3.3.3 The Development Of Circadian And Ultradian Rhythms. The Ultradian 1 h Rhythm Is Associated With Suckling:

Since during the period of the circadian rhythm study (i.e from birth to postnatal day 90), the lambs used in this study were also subjected to acute FX exposure intermittently at approximately day 4, 10 and 30, it is important to note that the FX administration did not result in any obvious physiological or behavioral changes in the lambs at day 4 and 10. Since the activity was lower on FX day experiments in lambs at 1 month of age, those who were subjected to the 1 month FX experiment were excluded from the regression analysis of this circadian rhythm study. The results of FX administration are not included here.
The daily total activity score up to postnatal day 90 of the rest activity cycle is shown in Fig. 3.14. There were no differences in the rest activity cycle development in male and female lambs (data not shown). The development of four most dominant FFT rhythms for 21 lambs for 90 days after birth are shown in Fig. 3.15. The results indicate that in the newborn the 1-2 hour ultradian rhythm is the most dominant. About 3 weeks later, the ~ 1 hour rhythm disappears and the circadian rhythm becomes dominant. Subsequently, an 8 hour ultradian rhythm becomes secondarily dominant. A third rhythm, a 6 hour ultradian rhythm also developed at ~ 20 postnatal day, and by about postnatal day 65, this rhythm was consistently the third or fourth most dominant rhythm. No sex differences were found in circadian and ultradian rhythm development (data not shown).

The 1-2 h rhythm in the lambs, which was not observed in the adult sheep, disappeared around 3 weeks after birth. At the same time, the total activity also decreased and became stable (Fig. 3.15). Regression analysis of the day that the 1h rhythm disappeared against the day that the lambs had the lowest activity during the first 90 postnatal days indicated that there was a significant correlation between the ~ 1-2 h rhythm and total activity ($r^2 = 0.5375, p < 0.0001$) (Fig. 3.15). In addition, the correlation between sucking frequency from DVR observations and that from FFT in 21 individual lambs on postnatal day 2 and 3 was also significant ($r^2 = 0.1947, p = 0.045$) (Fig. 3.17). This suggests that the 1-2 h rhythm only seen in the lambs is associated with suckling.

The correlation between the first day the 6h rhythm appeared and the first day lambs ate significant amounts of hay was also statistically significantly ($r^2 = 0.4973, p = 0.00107$) which indicated the link between their rumen development and the 6h rhythm (Fig. 3.16).
FIG. 3.13: Weight gain (A) and total suckling time (B) in singleton (n = 6), twin (n = 14 for weight gain and n = 10 for total suckling time) and triplets (n = 3) in lambs on postnatal day 2-3 (N = 22). Asterisk indicates significant difference between singleton and triplets, (p < 0.05)
FIG. 3.14: Plots of daily total activity score (a), activity bouts/day (b), mean score in active period (c), mean episode duration (d), % time moving (e) and % time immobile (f) against postnatal age in lambs. (n=21)
FIG. 3.15: (A) The average development of 24, 8, 6 and 1-2 hour activity-rest rhythms. The lines with diamond, square, triangle and x symbols indicate the 24h circadian, the 8 hour, the 6 hour and 1 hour ultradian rhythms, respectively. Levels of rhythm dominance of 1 to 4 indicate first to fourth most dominant rhythms. A level of rhythm dominance of greater than 4 indicates that this rhythm is not one of the top four rhythms (n=22). (B) The daily mean total activity score. (n= 21)
FIG. 3.16: (A). Relationship between the day that the 1h rhythm disappears and the day of the lowest total activity (n = 21). (B) Relationship between the day that the 6h rhythm disappears and the first day lamb eating hay (n = 21)
The growth rate was significantly different between the 1st and 2nd of the 25-day intervals and the 3rd, 4th of the 25-day intervals (p< 0.001). The slope of the daily weight gain decreased from 279.0 ± 1.86 g/d for the first 25 days to 209.3 ± 3.03, 142.7 ± 1.95, 143.6 ±7.54 and 122.1 ± 13.2 g/d for the subsequent 25 day intervals.

3.3.4 Weaning And Changes In Physiological Variables:

In our study, the time of weaning was defined as a window between when lambs started to first consume solid food (mostly hay) to when the 1h rhythm due to suckling disappeared (i.e., from 29.6 ± 1.1 to 50.9 ± 4.8 days). We have used piecewise linear regression with 2 elements to determine the break point which is the point where the slope of the regression curve for the variable versus age plot changed abruptly. The break points of the significant changes in heart rate, SDNN, glucose, pO₂ and urine flow rate were at 41.1 ±
5.5 (p < 0.0001); 51.6 ± 13.3 (p = 0.0002); 61.1 ± 7.9 (p < 0.0001); 52.3 ± 22.3 (p = 0.0213) and 38.4 ± 8.7 (p < 0.001) days of age, equivalent to 5.87 ± 0.78; 7.37 ± 1.9; 8.72 ± 1.12; 7.47 ± 3.18 and 5.48 ± 1.2 weeks of age, respectively. However, arterial pressure, LF/HF ratio, Lactate, Hb and cortisol changed significantly with the break points at 16 ± 5 (p = 0.002); 5 ± 2 (p = 0.014); 6.5 ± 2.4 (p = 0.0084); 12 ± 1.7637 (p < 0.001) and 12.2 ± 2.4 (p < 0.0001) days of age, equivalent to 2.28 ± 0.71; 0.71 ± 0.28; 0.92 ± 0.34; 1.71 ± 0.25 and 1.74 ± 0.34 weeks of age, respectively (Table 3.2). Taken together, the changes in heart rate, SDNN, glucose, pO2 and urine flow rate occurred around the window of weaning time, suggesting a relationship between weaning and these physiological changes.
<table>
<thead>
<tr>
<th>Variables</th>
<th>Mean age (days) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1h rhythm lost</td>
<td>50.9 ± 4.8</td>
</tr>
<tr>
<td>Lowest actiwatch total activity</td>
<td>56.6 ± 4</td>
</tr>
<tr>
<td>Solid food was first consumed</td>
<td>29.6 ± 1.1</td>
</tr>
</tbody>
</table>

Table 3.1: The time window of weaning in lambs was defined as when lambs started to first consume solid food to when 1h rhythm due to suckling disappeared.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Breakpoint (days) ± SE (p)</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (bpm)</td>
<td>41.1 ± 5.5 (&lt;0.0001)</td>
<td>0.6914</td>
</tr>
<tr>
<td>Arterial pressure (mmHg)</td>
<td>16 ± 5 (0.002)</td>
<td>0.2345</td>
</tr>
<tr>
<td>SDNN (msec)</td>
<td>51.6 ± 13.3 (0.0002)</td>
<td>0.473</td>
</tr>
<tr>
<td>SD Delta NN (msec)</td>
<td>60 ± 26.2 (0.0247)</td>
<td>0.3296</td>
</tr>
<tr>
<td>RMSSD</td>
<td>60 ± 26.2 (0.0247)</td>
<td>0.3296</td>
</tr>
<tr>
<td>LF power (nu)</td>
<td>3.6 ± 0.54 (&lt;0.0001)</td>
<td>0.1578</td>
</tr>
<tr>
<td>HF power (nu)</td>
<td>2 ± α (1.0)</td>
<td>0.2588</td>
</tr>
<tr>
<td>LF/HF ratio</td>
<td>5 ± 2 (0.014)</td>
<td>0.1417</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>61.1 ± 7.9 (&lt;0.0001)</td>
<td>0.8184</td>
</tr>
<tr>
<td>Lactate (mmol/l)</td>
<td>6.5 ± 2.4 (0.0084)</td>
<td>0.2375</td>
</tr>
<tr>
<td>O₂ sat (%)</td>
<td>99 ± 29.5 (0.0012)</td>
<td>0.3133</td>
</tr>
<tr>
<td>pO₂ (mmHg)</td>
<td>52.3 ± 22.3 (0.0213)</td>
<td>0.2738</td>
</tr>
<tr>
<td>pCO₂ (mmHg)</td>
<td>12 ± 6.4 (0.066)</td>
<td>0.1119</td>
</tr>
<tr>
<td>pH</td>
<td>55.5 ± 80.7 (0.494)</td>
<td>0.0223</td>
</tr>
<tr>
<td>HCO₃ (mmol/l)</td>
<td>99 ± 68.4 (0.1522)</td>
<td>0.0426</td>
</tr>
<tr>
<td>BE (mmol/l)</td>
<td>73.6 ± 38.5 (0.0595)</td>
<td>0.1609</td>
</tr>
<tr>
<td>Hb (g/l)</td>
<td>12 ± 1.7637 (&lt;0.001)</td>
<td>0.2814</td>
</tr>
<tr>
<td>Cortisol (ng/ml)</td>
<td>12.2 ± 2.4 (&lt;0.0001)</td>
<td>0.414</td>
</tr>
<tr>
<td>Urine flow rate (ml/min/kg)</td>
<td>38.4 ± 8.7 (&lt;0.0001)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Table 3.2: Break points of cardiovascular, metabolic, endocrine and renal variables.
Break point is the point (day of age) where the slope of the plots of the variable versus age changes.
3.4 Discussion:

The main findings of this study are that there were sex differences in pH value and LF power at 3 days of age and in heart rate at 6 months of age. Cardiovascular function in the lambs changed over time with a decrease in heart rate and increase in arterial pressure together with the maturation of the autonomic system (i.e., increase in the time domain of HRV and a reduced influence of the sympathetic system on the heart as indicated by a decrease of LF power and therefore LF/HF ratio). The relation between HRV frequency domain variables and autonomic function will be discussed further below.

In addition, we also found the acid base balance shifted toward the alkaline range over age with a decline in pCO₂, and increase in pH. The efficiency of pulmonary oxygen uptake indicated by arterial P₀₂ was progressively increased after birth until 3 months of ages and slowly increased thereafter which suggested that a process of improvements in pulmonary gas exchange still continued at least for the first 3 months of life. Glucose, lactate concentration, urine flow rate and Hb decreased with increasing age, while a slight anemia occurred at 1 month of age. The cortisol concentration was attenuated a few days before birth, decreased rapidly for the first 10 days of age and gradually thereafter.

The 1h rhythm that exists in the lambs but not in the adult sheep is associated with suckling. The disappearance of 1h rhythm was associated with the changes in growth rate and the decline in total activity. The break points of some physiological variables (including changes in heart rate, SDNN, glucose, pO₂ and urine flow rate) occurred around the window of weaning time suggests the effects of weaning and thus the beginning of solid food intake.
Indexes for HRV analysis are usually obtained by linear methods (including time domain and frequency domain analyses) or nonlinear methods. In this study we used the linear method with statistical indexes of the time domain (SDNN, SD Delta NN and RMSSD) and frequency domain indexes. Compared to the time domain, the frequency domain provides better information of the sympathetic-vagal balance on the heart since the SDNN of time domain represents both sympathetic and parasympathetic activity, but does not allow for identification of changes in HRV elicited by autonomic system. Frequency domain analysis, on the other hand, is composed of several oscillatory components reflecting the autonomic system: high frequency (HF), low frequency (LF) and very low frequency (VLF) components. HF covers a frequency from 0.15 to 0.4 Hz and is an indicator of the influence of the vagus nerve on the heart. LF, ranging from 0.04 and 0.15 Hz, represents both vagal and sympathetic components on the heart, with a predominance of the sympathetic system. Therefore, the LF/HF ratio reflects the relative changes between sympathetic and parasympathetic systems, and hence represents the sympathetic-vagal balance acting on the heart. VLF does not have important role in indicating the function of the autonomic system (Vanderlei et al., 2009).

The possibility that the surgery could affect my measurements of cardiovascular function, in particular the postoperative autonomic nervous system hyperactivity (i.e. increased LF power), was considered. However, I do not think that this is the case for several reasons. First, the surgery for catheter implantation in our lambs was a simple procedure performed under both general anesthesia and local analgesia. Second, the cortisol concentration measured on day 3 (i.e day after surgery) tended to be lower than that prior to surgery, suggesting that the surgery was not too stressful event compared to birth itself.
Third, the values of heart rate, arterial pressure and HRV in our study were similar to those reported in other studies which focused on the postnatal changes in cardiovascular function in lambs. Consistent with our results, other studies also found a similar high heart rate after birth, a decrease of heart rate and increase of arterial pressure at rest in lambs from 1 day to 6 months of age (Klopfenstein & Rudolph, 1978; Minoura & Gilbert, 1987). The increasing trend of beat to beat HRV in my study from postnatal day 2-3 to 1 year of age was similar to other findings in lambs from 1 to 10 week-old even though the HRV in lambs in the study of Siimes et al seemed to plateau earlier (after 6 weeks of age) (Siimes et al., 1984). In the human, it has also been shown that HRV increases with gestational age, postnatal age and with maturity (Harper et al., 1976; Katona et al., 1980; Wheeler et al., 1979).

The decline in heart rate values with ages observed from our study may result from an increase of parasympathetic activity or a decrease in sympathetic activity or from simultaneous changes in both regulatory systems. The latter is most likely the case, since the two branches of the autonomic nervous systems do not function on a continuum to regulate cardiac activity, thus the effect of each system on the heart cannot be simply determined by addition or subtraction of the relative components (Hainsworth, 1995). Instead, heart rate is affected by the net interaction between vagal and sympathetic regulation. This can explain the unsynchronized changes among LF power, HF power and heart rate over ages in our lambs. The sympathetic system appears to be a major regulator of vascular function up to 8 weeks of life and by 3 months of age, vagal regulation becomes dominate (Gronlund et al., 1989; Woods et al., 1977). Our results represent the normal postnatal ANS maturation in the term lambs with a progressive decrease in heart rate and increase in HRV which is, in part, consistent with findings in human newborns. (Longin et al., 2005)
Changes in the time domain of HRV was inversely related to changes in heart rate. (Fig. 3.4) When heart rate decreased, the time intervals between the single beats had to become longer, thus, as expected, time domain of HRV increased. However, LF power which mainly indicates the influence of the sympathetic system on the heart altered independently to the changes of heart rate. The increase to peak of LF power at ~ 10 days of age in the lambs was sex specific, due to significantly increased LF power in female from 3 days to 10 days of age but not in male. Also, this peak LF power at ~ 10 days of age occurred around the same time with the peak of total activity score (at ~ 15 days of age) observed from the actiwatch while heart rate slightly decreased at this age. It has been known that physical activity strongly influences cardiac activity and is frequently characterized by decreasing vagal tone and increasing sympathetic influences on the heart (Nakamura et al., 1993; Voss et al., 2002; Warren et al., 1997). This was shown in the work of Voss et al in which heart rate and HRV were evaluated in seven horses at rest and during six incremental levels of exercise from walking on a dry treadmill to trotting in water above the elbow. Heart rate increased together with a decrease in overall variability (SDNN, withdrawal of vagal), LF power increased (higher influence of sympathetic nervous system) and HF power decreased (decrease in parasympathetic activity) during exercise compared to rest. (Voss et al., 2002)

It was also found that LF power in male lambs was significantly higher than in female lambs at 3 days of age, but there was no difference in heart rate. This highlighted the greater sensitivity of HRV in detecting changes in sympathovagal balance in the absence of apparent changes in heart rate or respiratory rate. Indeed, Sleigh and Henderson found HF power, which represented vagal activity, reduced in relation to pre-surgery anxiety, whereas no effect was seen on heart rate. (Sleigh & Henderson, 1995) The sex difference in LF power
could be due to an influence of sex hormones on the ANS. Testosterone is present in the plasma of male lambs by 36h after birth and increases to a peak at 3 days of age (Savoie et al., 1981). The higher LF power in male lambs could also be a very first sign of sympathetic nervous system response to an unfavorable environment. This could be related to the greater degree of acidemia (lower pH) in the males than females at this age. In this case, the high HRV response in males may reflect an appropriate adaptation. On the other hand, it could also reflect a higher innate sympathetic drive in the male which leads to their higher risk of metabolic syndrome, a situation observed in human adults. (Chang et al., 2010; Licht et al., 2010) However, this is not likely to be the case, since insulin resistance, a key phenomenon in the pathogenesis of many metabolic cardiovascular disorders, results from a diminished regional blood flow and tissue glucose delivery as a consequence of vasoconstriction due to an enhanced sympathetic drive (Esler et al., 2001) and we do not see any gender differences in arterial pressure in lambs at this age and even later in life. Another possibility, even though less likely, of high LF power in male lambs is due to the high body fat. In human, obesity is associated with increased sympathetic outflow to the kidneys and skeletal muscle vasculature (Esler et al., 2001) and body fat is the main source of sympathetic discharge. Even though male lambs usually have a higher carcass weight than female lambs at the same age, this difference is attributed to a heavier fat-free weight only (Bennett et al., 1991).

Interestingly, our results showed that male lambs had a lower pH value than females at 3 days of age. Although the extent of acidosis in the males was not great, this could be one of the predisposing factors that lead to a higher mortality rate in male than female neonates (Khoury et al., 1985), especially related to respiratory distress syndrome (RDS) (Ingemarsson et al., 1997; Ingemarsson, 2003; Khoury et al., 1985). Ishak et al (2012) have recently
reported in the fetal lambs delivered by Cesaeren section at 133 days, arterial pH lower in the
male lambs than in female lambs in the initial postnatal period, with the sex difference (~0.15
pH units) being greater than the difference in the 3 day old lambs in my study (~0.08 pH
units). This suggests that an attenuated sex difference in arterial pH is present even in term
lambs. A study in Sweden analyzed acid-base balance in 15,480 human cord arterial blood
samples in term infants and showed a higher rate of pronounced acidaemia (pH < 7) in male
newborns (64%) than female newborns (36%) (Ingemarsson et al., 1997). In our study, the
earliest pH measurement obtained from the lambs was at 3 d, thus the male pH value might
have been even more acidotic at birth. This is especially a serious problem if we consider the
higher rate of preterm birth in humans, which is usually associated with pulmonary
immaturity in male infants compared to females (Ingemarsson, 2003). In addition, there is a
delay of lung maturation in male fetuses (Torday et al., 1981) and antenatal betamethasone
administration is not as effective in preventing RDS in male as in female. (Ballard et al.,
1980; Papageorgiou et al., 1981)

A sex difference in heart rate was also observed in lambs at 6 month of age. Heart
rate was significantly higher in male than female. Experiments of this age group in our study
were conducted on postnatal day 195.2 ± 8.6 and 214.6 ± 4.2 in females and males,
respectively. Female lambs raised under natural light can reach puberty as early as 153 ± 7.1
days in Dorset (Fitzgerald & Butler, 1982) or up to 234 days in Dorset and 245 days in
Suffolk ewe lambs (Quirke et al., 1985). Male lambs (Dorset x Leicester x Suffolk) raised
indoor under natural light conditions, on the other hand, enter puberty a bit earlier between
150 to 200 days of age as defined by an increase in plasma testosterone concentration from
1ng/ml at 150 days of age to 8 ng/ml at 200 days of age (Yarney & Sanford, 1989). Since our
lambs were raised under a 12 hour light/dark cycle, their sexual maturation could be delayed, but in any case male lambs reach puberty earlier than females. Therefore, the differences in their heart rate might be a result of sex steroid hormone influences on the cardiovascular system. With the growing rates of cardiovascular disease and metabolic syndrome around the world, they could become the epidemics of the century (Banos et al., 2011; Iyer et al., 2011, Barouki et al., 2012). There will no doubt be increasing attention paid to the effects of sex hormones on the occurrence of these diseases. It appears that androgen and estrogen exhibit differential effects on the pathophysiology of the vascular beds in male and female. So far, whether testosterone is protective or harmful to the development of those chronic diseases is still a matter of debate (Corona et al., 2011; Traish & Kypreos, 2011). In my study, it is uncertain if a gender difference in heart rate would have any long-term effects on the lambs given that there were no accompanying significant differences in other systemic cardiovascular variables such as blood pressure and heart rate variability between genders at this age.

The rapid increase in arterial $P_{O_2}$ in the postnatal lambs compared to the fetus at term is the result of a transition from gas exchange via the placenta in the fetus to air-breathing in the newborn. As the lung takes over the role of placenta in gas exchange, it is necessary that the lung fluid is cleared from the airway and pulmonary vascular resistance decreases around birth (Hooper & Harding, 2001). However, my results showed that after birth, arterial $P_{O_2}$ continues to increase slowly with age. This increase in $P_{O_2}$ is consistent with a slow increase in $O_2$ saturation and slow decrease in $P_{CO_2}$. These changes could reflect slow changes in alveolar $O_2$ and $CO_2$ concentrations in the lung. If this is the case, the lambs must be hypoventilated initially but increase thereafter due to a slow shift in the set point for
ventilatory control in the carotid body regulating $P_{O_2}$. Alternatively, these changes could reflect slow changes in the alveolar-arterial gas differences. In that case, the lambs are ventilated adequately at first but there is a diffusion limitation that is slowly corrected over time. This is most likely to be the case since it has been shown that in sheep even though the blood air distance becomes quite thin after birth (~0.2µm), it is only at focal points initially and it is not until they reach adulthood that the basement membrane fusion has spread enough to cover more than 50% of the epithelial surface (Hooper & Harding, 2001).

The level of activity in the lambs increased progressively right after birth, reached a peak level at ~13-15 postnatal days and then started to decline, becoming stable around postnatal day 30. The activity results for the first 10 days in these newborn lambs is similar to the activity level that was found in term lambs in a previous study (Rurak et al., 2008). Over the 90 days of monitoring, even when lamb’s activity decreased to a basal level, the lowest average values of their total activity score (212473 ± 23298) which is on postnatal day 60 was still higher than the value reported in adolescent sheep (100927 ± 7189) or in adult ewes (134808 ± 14644) held in the same conditions (Rurak et al., 2008). To determine when the level of activity decreases to the level of adolescent or adult sheep will require a longer monitoring period.

For the first 30 days, the increase in daily total activity score, active bouts per day and the intensity of activity to the peak on postnatal day 13-15 of is probably a sign of the maturation in their bone and skeletal muscle after birth (Rurak et al, 2008). The pattern is reversed for the duration of activity. The shifts in activity pattern may reflect the suckling pattern in lambs which goes from less frequent and intensive for a longer period of time at birth to more frequent and intensive but for a shorter periods of time around postnatal day 15,
and then suckling less and less until eventually the lamb was weaned and eating solid food, which they can do for longer periods of time. The underlying mechanism of this pattern of activity may involve the changes in the hormonal levels, such as insulin-like growth factor I which peaks at around the same time (i.e. postnatal day 13-15) (Greenwood et al., 2002), since from direct observation, lambs start to be interested in grain at around this period. Indeed, a study that was focused on the effects of birth weight and postnatal nutrition on neonatal lambs has shown that the IGF-I concentration is maximum on days 9-11 in high birth weight lambs and on days 11 to 13 in low birth weight lambs (Greenwood et al., 2002). Another explanation for the shifts in lambs’ activity could be due to the changes in milk production from the ewe over time. When the milk production from the ewes is at its peak, the lamb’s activity was also highest. Milk production of 2 year old ewes rearing twin lambs and 1-2 year old ewes rearing singletons peaked at 21 day of lactation and 27 to 30 of lactation, respectively (Cardellino & Benson, 2002). However, the time of the peak of milk production in our ewes could have been shorter, at around 15 day of lactation, since most of them were older than 2 years.

Using FFT we found the initial rhythms present after birth in lambs was dominated by the short 1-2 hour rhythms, which corresponded to suckling patterns from our DVR observations. Over the next four weeks, these rhythms decreased in dominance, matching the decrease in frequency and duration of suckling. When lambs’ nutritional requirement changed from the solely milk regimen to solid food supplement, reflected by the disappearance of the short 1-2 hour rhythm due to suckling, their activity also declined. Interestingly, around the time when the 1-2 hour rhythms disappeared and their activity declined, their growth rate was also reduced.
In human infants, there are ultradian rhythms of 3h, 4h and 6h as sleep, wake and food intake behavioral cycles (Menna-Barreto et al., 1993). The time course of the development of a permanent circadian rhythm for lambs seems to be similar to that found in humans. In humans, Tomioka & Tomioka found that the circadian rhythm of sleep-wake cycle appeared in the first week of life in 3 infants and became dominant in the 3rd and 5th week of life (Tomioka & Tomioka, 1991). However, there was a large interindividual variability of both the ultradian rhythms and the development of a 24 h pattern, whose appearance varied considerably between the 6th and the 16th week of life (Lohr & Siegmund, 1999). In sheep, the dominant circadian rhythm is in synchrony with the development of a postnatal melatonin rhythm in lambs at 3-4 weeks (Nowak et al., 1990). In some lambs in our study, the circadian rhythm existed for 1-2 days after birth and disappeared for almost a month before it became persistently dominant. The existence of the circadian rhythm for the first few days after birth in the lambs could be an influence of the remaining melatonin level from the ewe after birth (McMillen et al., 1989).

Similar to infants, lambs have 2 developmental processes in the development of their circadian rhythm: a decreased ultradian and increased circadian components and the process of synchronization with external time cues or social factors such as light/dark cycle, meal feeding, experiments, etc. The development of circadian rhythm appears to be important, since its existence plays an important role in the well being of both animals and humans, because it guarantees optimal functioning of biological systems with maximum efficiency. Once the circadian rhythm appears, it lasts for a considerable length of time, but begins to break down during illness or old age (Weinert, 2000).
The eight hour ultradian rhythm emerged as a dominant rhythm around 2 weeks after birth and is thought to be due to the daily scheduled feeding times, which occurred twice a day at 7:30-8:00 am and ~ 2:30-3:00 pm, an approximately eight hour interval. Even though the young lambs only fed on their mothers’ milk, they became more active as their mothers were being fed. To further confirm that the 8 hour ultradian rhythm is the result of the feeding time entrainment, further study is needed to determine if this ultradian rhythm exists in the lambs that develop under field conditions where they can graze *ad libitum*.

The 6h rhythm seemed to become the third most dominant rhythm when the lamb was about two months of age. This 6 hour rhythm was most likely a result of maturation of the lambs’ rumen and reticulum, which were stimulated by the intake of solid food, as we found a correlation between the first time lambs eat significant amounts of hay, and the day this 6 h rhythm became dominant. This is supported by research which showed that $\beta$-hydroxybutyrate production from butyrate in isolated sheep ruminal cells increased 10 fold from day 42 to day 56, as compared to the rate at birth; this marks a shift in the ability to synthesize ketones in a mature rumen, and the development of the rumen microbiota (Baldwin & Jesse, 1992). Lane *et al* also observed changes in the morphology of the rumen epithelium (fewer and larger intraluminal papillea per cm$^2$) on day 84 from lambs fed with solid food after postnatal day 49 (weaned lambs), as compared to epithelium of lambs fed solely with milk replacer (non-weaned lambs) (Lane *et al*., 2000).

The suckling frequency was similar in singleton, twins or triplet lambs. However, triplets seemed to have longer feeding bouts which might be secondary to the restriction of the available milk supply in triplet pregnancies. As a result, twins or triplets spent more time in competing with their siblings to obtain milk, which led to an increase in their feeding
bouts, but it did not appear to improve the efficiency of their suckling, as reflected in the
tendency for a lower weight gain in triplets and twins, as compared to the singleton, even
though this difference was not significant. This could be due to my small sample of triplets.
Results arising from a much larger study, comprising approximately 3000 singleton, 1500
twins and 200 multiple lambs have shown that singleton lambs gained significantly more
weight than twins and multiples. As a result, singleton lambs were heavier than those born as
twins and multiples, both at birth and at weaning (Carrillo & Segura, 1993).

The short 1h rhythm was not observed in adult sheep and only the lactating ewes have
the short ~ 1h rhythm, but none of them had this as the first dominant rhythm. This suggested
that the 1h rhythm is the rhythm that the ewes share with their offspring. It has been known
that suckling serves as a mechanical stimulus to inhibit the HPA axis in the ewe to regulate
the estrus cycle. Depending on breed, time of the year and degree of mammary stimulation,
the duration of lactational anestrus varies considerably (Fletcher, 1971; Gordon & Siegmann,
1991; Mallampati et al., 1971). Usually after the termination of lactation, the mother and
lamb relationship does not fade away completely. Indeed, lambs remain associated with their
mothers for several weeks or months (Hinch et al., 1990). Therefore, beside measurement of
maternal prolactin level, the waning of this 1h rhythm in the ewe, which can be non-
invasively obtained by actigraphy, could be an alternative indicator that the ewe was free
from the lactational inhibition of reproduction.

Our results provided a normative data set of cortisol concentration in the normal
lambs at different ages under basal conditions. Cortisol level on postnatal day 2 ± 0.2 (i.e
prior surgery) were higher than that measured in the fetus at 7.1 ± 1.6 days before delivery
(at gestational age = 135.4 ± 0.7 days). This was consistent with the cortisol surge resulting
from activation of the fetal HPA axis at ~ 125-130 of gestation which is required for the initiation of parturition (Wood, 1994, Norman et al., 1985). Cortisol concentration declined significantly thereafter during the first 10 days of life and was returned to the similar level observed in the fetus on gestational day 135.4 ± 0.7, and further decreased after 1 month of age to reach the adult value at 3 months of age. However, plasma ACTH concentration was not well-correlated with the postnatal plasma cortisol changes. ACTH was low on postnatal day 2 ± 0.2, probably due to a refunctioning of cortisol negative feedback after birth, which was desensitized at the end of gestation. In addition, the high cortisol concentration without an adequate increase of ACTH could be a result of increasing cortisol binding protein near term, which exaggerates the total cortisol level but suppresses the efficient negative feedback of cortisol on the pituitary, as well as hypothalamus (Challis et al., 1995). The ACTH level remained lower at other ages as well. It was not clear why there was dissociation between plasma cortisol and ACTH concentration even at these later ages.

Cortisol concentration was significantly higher in the morning (at 10:00, 10:30 and 11:30 am) than in the afternoon (at 3:30 pm) or in the evening (at 9:30 pm) in the lambs at 10 days and 1 month of age which suggested that there was diurnal rhythm of cortisol in the lamb at these ages. Such a rhythm was not found at other ages (3 day, 3, 6 and 12 month-old) which is in agreement with the findings from the work of Bell et al, who reported there were no endogenous circadian or diurnal rhythms in ACTH or cortisol secretion in pregnant, non pregnant cycling, non cycling or ovariectomized adult ewes (Bell et al. 1991). In our study, the samples were collected for only a 12h period; therefore we could not make a firm conclusion about a circadian rhythm of these hormones, which appear to be present in lambs after 3 weeks of age (Parraguez et al., 1989). There were some other factors that might have
contributed to the higher level of cortisol in the morning. First, it has been known that the cortisol level increases after feeding in pregnant ewes (Slater & Mellor, 1981) and in the human (Follenius et al., 1982). Lambs around 10 days to 1 month old had a high activity of suckling. In addition, from direct observations we noticed that after the first week lambs started to participate in nozing the ewe’s daily feed. However, if the higher cortisol level was a response to feeding, the cortisol level measured at the earlier time (around 8:30 am) should have been higher, and also higher at the pm feeding time (at 3:30 pm), which was not the case. Thus, it is more likely that the high cortisol level for the 2 h of experiment after dosing is a result of the lamb’s response to the social cue of the investigators’ close proximity for the 2 h duration when there was frequent sample collection. This social cue disappeared at later ages when lambs become habituated to the investigators.

Although there was no rhythm in the older lambs, we did find a sex difference in the cortisol level in lambs at 1 year of age at 0.5 h (10:00 am), 1 h (10:30 am), 6 h (15:30 pm) and 12 h (21:30 pm) sample. Studies that have examined the influence of sex hormones on the cortisol level have demonstrated estrogen-enhanced HPA axis function in adult animals under both basal and stress conditions (Bell et al., 1991; Carey et al., 1995; Wood et al., 2001). However, my results showed that basal cortisol concentration was lower in the female than male lambs, which suggests a reduced HPA axis activity in the female at this age. Nevertheless, since cortisol levels vary during the estrous in sheep (Bell et al., 1991), it is possible that the cortisol levels in the female were measured in noncycling animals or during anestrus cycles which have no estrogen effects.

In our study the lambs were not weaned abruptly. In studies focused on the effect of diet and weaning on growth, the time of weaning can be as early as 28 days of age (Lane &
Albrecht, 1991) or as late as 42 days, by which time the rumen is believed to be already functional (Holcombe et al., 1992). The earliest our lambs were separated with their mothers was at 47 days of age and in some cases they remained with their mothers for up to 80 days. However, the lambs were gradually weaned by the ewes refusing to let them suckle and walking away from them, even though the lambs still tried to nurse sometimes. In the context of our study, the time of weaning was defined as the time window when lambs started to first consume solid food (hay) to when the 1h rhythm due to suckling disappeared.

The blood glucose concentration, which is related to rumen development (Holcombe et al., 1992) decreased gradually with age, and the value at 1 month was significantly different from that at other ages. This alteration in glucose concentration were consistent with our observations of the time lambs first started to consume solid food (29.6 ± 1.1 days of age) which indicated a functional forestomach already developed. Similar results were also reported in the work of Holcombe et al., (1992). Our results, taken with the data from the Holcombe et al study, suggest that the window of weaning is around 1 month of age. It appears that the greatest changes (i.e break points on piecewise linear regression) of most physiological variables (heart rate, SDNN, Glucose, pO2, urine flow rate) occurred around the window of weaning time (1 to 2 months of age), so that by 3 months of age they are similar to their levels at more mature ages.

A decrease in urine flow rate around the time of weaning was reasonable as the lambs changed from a predominantly milk diet to a solid food diet. In addition, it has been known that feeding activity has effects on the cardiovascular system; thus it was not surprising to observe heart rate and HRV (SDNN) were significantly altered at the time of weaning.
Overall, our results revealed a data set of normal development of physiological, endocrine and behavior characteristics in the lambs in relation to several milestones of the developmental process (birth, weaning and puberty) under basal conditions. This will provide reference information of these variables for future studies related to any relevant disorders.
4. Acute Fluoxetine Effects In The Newborn

4.1 Introduction:

Major depression is probably one of the most burdensome disorders in the world. In the report of World Health Organization (WHO) in 1996, it was estimated that by 2020 depression will rank second only after cardiovascular disease in terms of its disability-associated burden (Murray & Lopez, 1996). Depression affects at least about 15% of women in their lifetime (Burt & Stein, 2002; Kessler et al., 2003). The risk is even higher during the reproductive years. Thus there is a significant risk that one might become depressed during pregnancy or even get pregnant while being depressed or on antidepressant therapy. Recent studies have failed to confirm the previous belief that the state of pregnancy is protective against mood disorders (O'Hara et al., 1990). Moreover, it has been shown that untreated maternal depression leads to long-term neurobehavioral consequences in the offspring, including poor cognitive function, and diminished language and psychomotor development (Deave et al., 2008; DiPietro et al., 2006; Hay et al., 2008; Murray & Cooper, 1997; Murray et al., 2001; Nulman et al., 2002). Therefore, pharmacologic treatment is recommended for pregnant women who have moderate to severe symptoms.

In addition, depression during pregnancy is in turn a risk factor for postpartum depression, affecting 10% to 22% of women (Burt & Stein, 2002). Since the postpartum period is the most vulnerable time for serious symptoms and their short-term and long-term consequences to not only the mother but also the entire family unit, this has resulted in postpartum depression becoming a significant health care problem. Moreover, it has been shown that the risk of relapse increases 5 times in women who discontinued medication.
during their pregnancy compared with those who maintained their medication (Cohen et al., 2006). Thus continuing medication in the third trimester is recommended (Toohey, 2012). Among the classes of antidepressants, SSRIs have been widely used in pregnant women due to their relatively safe side effects profile. In fact, 33% of pregnant women with clinical depression were prescribed an antidepressant (Oberlander et al., 2006), in which SSRIs accounted for 75% of the cases (Simon et al., 2002). Recent studies have reported an increasing use (4.9- 6.2% increase over 9-14 years) of SSRIs during pregnancy (Andrade et al., 2008; Cooper et al., 2007; Simon et al., 2002; Wichman et al., 2008). However, third trimester use of SSRIs, unfortunately, was also reported to increase the risk of a variety of neonatal complications termed “neonatal behavioral syndrome” or “poor neonatal adaptation” after birth (Chambers et al., 1996; Cohen et al., 2000; Kwon & Lefkowitz, 2008; Mhanna et al., 1997; Oberlander et al., 2004; Oberlander et al., 2006), a phenomenon observed in 10% to 30% of infants exposed in utero to SSRIs (Koren et al, 2009). The presenting symptoms include irritability, persistent crying, shivering, tremor, restlessness, feeding difficulties, sleep disturbances or even more severe symptoms, such as seizures, dehydration, excessive weight loss, hyperpyrexia and the need for intubation (Moses-Kolko et al., 2005). Even though this syndrome is self-limiting, it results in longer hospitalization and led to a warning from Health Canada and FDA about the adverse events when using SSRI/SNRIs in pregnant women during the third trimester (Summary minutes of the pediatrics subcommittee of the anti-infective drugs advisory committee.2004).

There are several suggested mechanisms for the poor neonatal adaptation. First is a withdrawal syndrome due to the discontinuation of maternal-fetus placental transfer right after birth. Second is a serotonin toxicity mechanism induced by the high circulating SSRI
concentrations in the newborn at birth, due to their low capability of drug clearance. Third is an SSRIs-elicited alteration in fetal brain development. Findings supporting the latter mechanism arise from the work of our group (Morrison et al, 2001; Morrison et al., 2002; Morrison et al., 2004; Morrison et al., 2005), which reported a decrease of fetal rapid-eye-movement (REM) sleep in lambs whose mother were administered FX i.v. continuously for 8 days in late gestation. Similar findings were also demonstrated in human fetus with SSRI exposure (Mulder et al, 2011). Further supporting evidence comes from the study of Oberlander et al., 2006 and Rurak et al., 2011, who observed altered fetal middle cerebral artery flow characteristics in 36 week human fetuses exposed in utero to SSRIs compared to a control group.

However, which mechanism is responsible for the observed poor neonatal adaptation is not yet clear. Currently, the management for these syndromes is simply symptomatic with supportive care (Koren et al., 2009). Since the strategy of treatment could be completely opposite for the first and second mechanisms mentioned above, it is important to elucidate the underlying mechanism for the syndrome. For this reason, in this study we investigated the effect of acute FX, one of the most prescribed SSRIs on growth, behavior, and cardiovascular-respiratory, and endocrine functions in ~4 d old newborn lambs. The hypothesis that was tested was that acute administration of FX to achieve plasma concentrations similar to those in human newborns exposed to FX in utero would result in effects similar to poor neonatal adaptation in human infants.
4.2 Methods:

4.2.1 Experimental Protocol:

21 lambs were included in the study reported in this chapter, of which 9 lambs were in the control group with sterile water i.v. bolus administration (0.1ml/kg) and 12 lambs were in the FX group with FX (1mg/kg) bolus i.v injection, respectively. The animal housing, surgical, experimental and analytical methods are described in Chapter 2. Heart rate and blood pressure data used in this chapter were averaged at 1 min intervals for 15 mins before dosing and the first 10 mins after dosing and at 5 min intervals for the remaining time while HRV data were averaged over 30 mins intervals and also averaged at 1 min intervals for 2 min before dosing and 5 mins after dosing. Blood gas samples were collected at -15, 5, 15, 30, 60, 120 mins as described above, however, another arterial blood gas sample was taken at 2 mins in 4 lambs in this study to characterize more fully the short term changes in blood gas and acid-base status following water or FX administration. For the rest-activity analysis, beside the control group with sterile water injection and FX group with FX injection, another group of normal term lambs in which no surgeries or experiments occurred were included.

4.2.2 Statistical Analysis:

Activity data were analyzed using a 2 way GLM for repeated measures ANOVA followed by the Bonferroni t-test for multiple comparisons versus the control group to determine the effect of treatment and sex on changes in behavioral activities. Cardiovascular and blood gas data were analyzed using a 3 way ANOVA followed by Bonferroni’s correction for pairwise or control comparisons to determine the effects of treatment, time and sex on the measured variables. Two way GLM for repeated measure ANOVA was used for
not-normally distributed variables (SDNN, SD Delta NN, RMSSD, LF power, HF power, LF/HF ratio, O2 saturation, pCO2, glucose and Hb) to determine the effects of treatment and time. Because plasma cortisol concentrations were not normally distributed, the non-parametric Mann-Whitney U-test was used for the cortisol data to determine the effect of treatment. P < 0.05 defines statistical significance. Data are presented as mean ± SEM.

4.3 Results:

On postnatal day 3.3 ± 0.4 and 4.2 ± 0.4 sterile water (0.1ml/kg, N=9) or FX (1mg/kg, N=12) was acutely injected via the jugular vein catheter, respectively.

4.3.1 Plasma FX Concentration And Weight Gain:

The plasma FX concentration in the ~ 3 day old lambs over 72h of experiment and in 13 individual human infants sampled at postnatal day 2 following in utero exposure to the drug cover a similar range (~ 10-100 ng/ml) (Fig. 4.1A). (Chow, 2013; Kim, 2000) There were no significant differences in postnatal weight gain on postnatal day 4 between the normal term lambs group who were not subjected to any experiments or surgery (0.327 ± 0.048 kg, n = 11), control group (0.411 ± 0.056 kg, n = 9), FX group (0.392 ± 0.045 kg, n = 12), respectively (Fig. 4.1B). This indicated FX does not have any effects on initial postnatal weight gain in the neonatal lambs.
FIG. 4.1: (A) Plasma FX concentration versus time curve in the 3 day-old lambs (closed symbols and solid line) and in 13 individual human infants (open symbols) sampled at postnatal day 2 following in utero exposure to the drug. Human data was taken from the dissertation of Dr. John Kim, 2000 and the lambs data was taken from the dissertation of Dr. Tim Chow, 2013. (B) Weight gain on ~ postnatal day 4 among the normal term lambs (n = 11, open bar), control group (n = 9, black bar), FX group (n = 12, gray bar) and post FX day 1 (n = 12, light gray bar)
4.3.2 Rest Activity Cycles:

The number of active bouts per day, total activity score, the intensity and duration of activity and the percent of time moving and percent immobile time were comparable between the normal term lamb group (n = 10), the control group (n = 6) and the FX group (n = 11) (Fig. 4.2). In addition, from the DVR observation we did not notice any obvious behavioral changes or any symptoms representing serotonin toxicity with FX dosing. During the first 2.5h of experiment when lambs were kept in the sling, they just lay quiet or slept for most of the time, and there were no obvious behavioral changes following FX administration.

4.3.3 Cardiovascular Variables (Heart Rate, Arterial Pressure, Heart Rate Variability):

Heart rate, arterial pressure and heart rate variability (HRV) in the newborn lambs with placebo and FX injection are illustrated in Figures 4.3 and 4.4, respectively. The time points included in the 3 way ANOVA test (treatment x time x sex) were at -15 min, 1, 2, 3, 4, 5 and 10 min only, since the data was not normal distributed with additional time points. Arterial pressure were significantly different between the control and FX group at 1, 2, 3, 4, 5 and 10 minutes post-FX (p<0.001), but not at the control time point (-15 minutes). The maximum increase in arterial pressure in the FX group was 8.5 ± 1.1 mmHg at 4 minutes following FX injection compared to values before FX injection. Heart rate was lower in the FX group than the control group only at 2 minutes post-FX (p = 0.034), the difference was 38 ± 9 bpm. However, these effects were very transient and self-limited since heart rate returned to normal values within 1 min later and by 15 minutes post-FX arterial pressure had also returned to values similar to those observed before FX injection.
FIG. 4.2: Activity variables (active bouts per day, mean score in active, episode duration, total activity score, % time moving, % time immobile) in the normal ~ 4 day-old term lambs (n = 10), control = on day of control experiment (n=6), and FX lambs on the day of FX experiment (n = 11) followed up to post FX day 1 (n = 11), post FX day 2 (n = 11) and post FX day 3 (n = 11). No significant changes were found between activities after FX administration and sterile water injection or normal day without experiments.
FIG. 4.3: Arterial pressure and Heart rate (at 1 min intervals for the first 5 min after dosing and at 5 minute interval for the whole 2.5h of experiment) in the ~4 days old lamb with sterile water injection (control group) and FX injection (FX group).

- a indicates significant differences between variables at -15 min vs 1, 2, 3, 4, 5, 10 min after FX within the FX group.
- b indicates significant differences between control and FX at the corresponding time.
FIG. 4.4: Time domain of HRV (SDNN, SD Delta NN, RMSSD) and frequency domain (LF power, HF power, LF/HF ratio) at 30 min intervals in the newborn lambs at ~ 4 days old in the control group (open diamond and dotted line) and FX group (close circle and solid line)

a indicates significant differences between variables at -2 min vs 1,2,3,4,5 min after FX within the FX group
b indicates significant differences between control and FX at the corresponding time
The time domain of HRV including SDNN, SD Delta NN, RMSSD, which is related to the RR interval variability was significantly higher in the FX group than that in the control group at 1 and 2 min after FX administration but were comparable between the two groups at other time points. Indeed, there were significant relationships between the changes in SDNN, SD Delta NN, RMSSD at 1 min following FX injection and the changes in heart rate at 2 mins following FX with $r^2 = 0.8$, $p = 0.001$ and $r^2 = 0.57$, $p = 0.0017$, respectively (Fig. 4.5). However, the frequency domain of HRV including LF power, HF power and LF/HF ratio, which indicate parasympathetic and sympathetic influences on the heart were not different between the control and FX group and also did not change significantly over time. (Fig. 4.4) Sex differences were observed with SD Delta NN, RMSSD (within the control group, at -30 min) and with LF power and LF/HF ratio (at -30, 60 and 120 min) but the differences were not related to treatment (data not shown).

4.3.4 Arterial Blood Gases:

Figure 4.6 and 4.7 illustrate the changes in arterial blood gas variables (O$_2$ sat, pO$_2$, pCO$_2$, pH, BE, HCO$_3$), and lactate, glucose and Hb concentrations in newborn lambs with placebo and FX injection. There were no consistent changes in these arterial blood gas variables in either control or FX group with time. Arterial pH tended to decline in both groups with treatment but this was not statistically significant. A paired-t test was performed to further compare Po$_2$ and O$_2$ saturation at -15 minute before and 2 minute after FX injection in a smaller group of lambs (n= 4) but no statistical significance was reached. There was a sex difference in pH, HCO$_3$ and lactate but this did not depend on the treatment. Hb was slightly lower, but significantly on the day of FX than the control day. This could due to the blood volume lost for sampling.
FIG 4.5: The relationship between changes in heart rate at 2 mins following FX (delta HR) and changes in time domain of HRV (SDNN, SD Delta NN, RMSSD) (i.e Delta SDNN, Delta SD Delta NN, Delta RMSSD) at 1 min following FX in the lambs at ~ 4 days of age.
FIG. 4.6: Arterial blood gases (pH, O₂ saturation, pO₂, pCO₂, HCO₃, Base Excess) in the newborn lambs (~4 days old) in the control group (open diamond and dotted line, n = 9) and FX group (close circle and solid line, n = 11)
FIG. 4.7: Glucose, Lactate and Hb concentration in the newborn lambs (~ 4 days old) in the control group (open diamond and dotted line, n = 9) and FX group (close circle and solid line, n = 11)

4.3.5 Plasma Cortisol Concentration:

No differences were found between the cortisol concentrations in the control group compared with the FX group as shown in Figure 4.8 (p = 0.925)
FIG. 4.8: Plasma cortisol concentration in the ~ 4 days old lambs in the control group with sterile water injection (n = 8, open bar) and the FX group with FX injection (n = 5, close bar) at 30 and 15 minutes before injection and 0.5, 1, 2, 6, 12 hours after injection.

4.4 Discussion:

We have found that acute FX administration did not cause any significant effects on the feeding behavior, rest-activity cycle, cardiovascular-respiratory functions and the cortisol level in the approximately 4 day old postnatal lambs. Mild and transient increase in arterial blood pressure and decrease in heart rate occurred immediately after FX administration and recovered within 15 minutes without any significant changes in HRV or arterial blood gases.

The plasma FX concentration time curve in our studied lambs over 72h of experiment fell in the range of 10-100ng/ml, which is similar to the plasma FX level found in 13 individual human infants sampled at postnatal day 2 following in utero exposure to the drug (Kim et al., 2006). The wide range of plasma FX concentration in human infants could be due to the differences in maternal FX doses or to cytochrome P450 polymorphisms in the
infants, which could affect their ability to metabolize the drug, a phenomenon that has been reported in adults (Bradford, 2002; Fjordside et al., 1999; Sachse et al, 1997; Scordo et al, 2005)

Acute FX effects on weight gain (feeding behavior):

Studies in humans regarding postnatal weight gain in infants breastfed by mothers taking FX are inconsistent. While Chambers et al., (1999) reported a diminished postnatal weight gain in nursing infants of mother treated with FX, a subsequent study from Hendrick et al, (2003) failed to confirm this finding. In our study, the postnatal weight gain was comparable between the acute FX exposed and the normal term lamb group on postnatal day 4. The activity bouts per day were also not different between the two groups. As was demonstrated in chapter 3, this variable at this postnatal age was always associated with suckling or suckling attempts. Collectively, this suggests that acute FX administration did not affect feeding behavior in the lambs. However, it must be kept in mind that in our study, FX was given acutely single dose, while in the human studies, the infants were exposed to FX chronically from the prenatal to postnatal period. Acute and chronic FX exposure might exert different effects on weight gain and feeding behavior. There are unchanged plasma concentrations of leptin, a feeding-inhibiting hormone, in response to acute administration of the serotoninergic agent, clomipramine, in both normal weight and obese women (Oppert et al., 1997). However, a study in rats showed acute or chronic FX administration resulted in a significant decrease in food intake and plasma leptin levels in lean and obese rats (Dryden et al.,1999). The differences in those findings and ours might be attributed to the differences in animal ages or the dose of SSRIs used (10mg/kg in rat study and 1mg/kg in ours)
In addition, there were also no significant differences in factors that might have influences on postnatal weight gain in sheep such as birth weight, litter, breed, ewe-lamb bonding and neonatal behaviors (Dwyer, 2003; Dwyer et al., 2005) between the normal term lambs and the FX group. In addition, different from human studies, the effect of FX on postnatal weight gain in sheep was not confounded by the severity of maternal depression and other associated risk factors (eg: low socioeconomic status, family conflict, financial stress, ect), which could presumably affect neonatal weight gain (Stewart, 2007; Wright et al, 2006; Zuckerman et al., 1989).

4.4.1 Acute FX Effects On Cardiovascular –Respiratory Functions:

FX and other SSRIs cause an acute increase in plasma serotonin levels through inhibiting the serotonin reuptake by platelets (Maurer-Spurej et al., 2004). The effects of serotonin on the cardiovascular system are contradictory between in vivo and in vitro studies, at least in the rat (Watts & Davis, 2011). Serotonin has long been known as a vasoconstrictor agent, which can lead to hypertension (Maurer-Spurej et al., 2004). However, it appears to have divergent effects on the cardiovascular system. Some studies showed an increase in blood pressure with acute FX injection (Anderson et al, 1996; Cavero et al., 1981; Lazartigues et al., 2000) while others reported a decline in blood pressure (Callera et al, 2005; Fuller et al, 1979). In addition, the cardiovascular response to serotonin (i.v) is species-specific (Watts & Davis, 2011): a decreased blood pressure was observed in broiler chickens (Chapman & Wideman, 2002) while an increased blood pressure occurred in calves (Linden et al., 1999). In conscious sheep, serotonin injection caused a dose-related increase in blood pressure (Nelson et al., 1987). This is in agreement with our findings that arterial pressure increased at 1-10 minutes after FX administration in the newborn lambs. Heart rate was also
declined at 2 minutes post-FX. However, these effects were very transient and self-limited since heart rate returned to normal values within 1 min later and by 15 minutes post-FX arterial pressure was also returned to values similar to those observed before FX injection. Therefore, we think these transient changes did not have any clinical significance in the newborns. Interestingly, we noted that cardiovascular responses to acute and chronic FX were opposite. The previous study from our group with 8 day i.v FX infusion in the pregnant ewes showed an increase in fetal heart rate during the first 6h of FX infusion and no changes in daily maternal arterial pressure and heart rate nor fetal arterial pressure during the period of 8 day infusion (Morrison et al., 2002). The possible explanation for the different cardiovascular response is that serotonin response is different with acute and chronic FX treatment. It was reported in human and mice that acute FX treatment increases plasma serotonin concentration while chronic FX treatment causes a decrease in plasma and platelet serotonin levels (Alvarez et al., 1999; Alvarez et al, 1999). Indeed, in the previous sheep study in our lab with chronic FX i.v infusion, the average maternal plasma serotonin level increased for the first hour (at 15, 30 and 60 minutes) but decreased at 2 and 6 hours compared to values prior to FX infusion (Morrison, 2001).

The transient mild bradycardia could be due to either a direct effect of FX on the cardiomyocytes via 5-HT4 receptors or an indirect effect of FX on blood pressure leading to a baroreceptor reflex in the autonomic nervous system (ANS) to increase vagal tone (Watts & Davis, 2011). This reflex bradycardia could be mediated by 5-HT3 receptors located on afferent vagal and sympathetic neurons (Kaumann & Levy, 2006). Even though it has been reported that FX can inhibit the L-type Ca^{2+} current, which plays a role in reducing cardiac contractility (Pacher & Kecskemeti, 2004), we believe that the bradycardia observed in our
study is due to an ANS response on the heart rather than a negative inotropic effect of FX since we did not notice any significant changes in the electrolyte concentration (i.e: K⁺, Ca²⁺, Na⁺) at the corresponding time points (data in appendix 4).

Given the fact that bradycardia is a result of an ANS response, it is expected that HRV changes accordingly. Indeed, the time domain of HRV including SDNN, SD Delta NN, RMSSD was significantly higher in the FX group compared to the control group at 1 and 2 mins after FX administration. However, the effects appeared to be limited to only two minutes after FX. As it was shown in Fig. 4.5, there was an inverse relationship between the changes in heart rate and the changes of time domain of HRV. The greater the decrease in heart rate at 2 min post-FX, the greater the increase in SDNN, SD Delta NN and RMSSD at 1 min post-FX. In addition, the frequency domain of HRV (LF power, HF power, LF/HF ratio) was comparable between the two groups at all time points. Acute and chronic SSRIs exposure might exhibit different mechanisms. A recent longitudinal observation human study from our group has found a reduced fetal heart rate variability (decreased number of accelerations) in the fetuses exposed to SSRIs throughout most or all of gestation, even after controlling for maternal mood, which might reflect an alteration in prenatal cardiac autonomic control (Rurak et al., 2011).

A FX-induced elevated arterial pressure and mild bradycardia at 2 minutes after FX injection were associated with a very mild and transient hypoxemia reflected by lower arterial Po₂ and O₂ saturation values observed at 2 minutes post-FX in a smaller group of lambs (n = 4), but these changes were not statistically significant. However, there were no significant changes in arterial pH, Pco₂ nor lactate concentration at 2 minutes following FX injection. Moreover, Po₂ and O₂ saturation at 5 minutes post-FX and later time point were
comparable with values observed before FX injection. Thus, transient hypoxemia with acute FX injection was too minor to cause any respiratory acidemia. Collectively, respiratory difficulties, a common feature of poor neonatal adaptation in human infants exposed to SSRIs in utero, did not occur in the lambs with acute FX administration.

4.4.2 Acute FX Effects On HPA Axis Functions:

Similarly, FX did not cause significant changes in plasma cortisol concentrations in the newborn lambs in our study. Also, no sex differences were found. To our knowledge, there is limited information about the acute SSRIs effect on the neonate’s HPA axis as well as the influence of maternal SSRI treatment on the fetal HPA axis. HPA axis function and the central serotonergic system are known to be highly interrelated (Boadle-Biber et al., 1993; Fuller, 1996a; Fuller, 1996b). In our previous sheep studies, 8-day late gestational FX i.v infusion in the pregnant ewes resulted in an augmented prepartum rise in fetal ACTH and cortisol levels and reduced low voltage rapid eye movement behavioral state in the fetus (Morrison et al., 2004). In contrast, another human study from our group, which further examined the long-term effects of prenatal SSRIs (fluoxetine) on the HPA axis in the postnatal period, has shown an association between SSRI exposure and reduced basal cortisol levels as well as an alteration in the HPA stress response pattern in the offspring at 3 months of age (Oberlander et al., 2008). A lower cord blood cortisol level in SSRIs-exposed neonates was also reported in a subsequent study (Davidson et al., 2009). Conflicting findings from the sheep and human studies could be, in part, attributed to the differences in the timing and duration of SSRI exposure. Even though there was no stressor stimulation included in our study, it is unclear why the cortisol level in the lambs did not increase with acute FX treatment as expected. It has been reported that acute administration of SSRIs increased the
secretion of several hormones including cortisol and ACTH (Raap & Van de Kar, 1999) while chronic treatment with SSRI (citalopram) resulted in a decrease (Jongsma et al, 2005). Although the mechanisms are not fully understood, alterations in HPA axis activity in the fetus might be one of the possibilities underlying negative perinatal outcomes observed in human studies. In addition, this is particularly important in the context of fetal exposure to excess glucocorticoids either by prenatal stress or maternal illness (e.g: depression, anxiety disorders) or pharmacologic agents effects (eg: SSRIs, synthetic glucocorticoids for lung maturation in preterm labor), which can program the HPA axis in ways that it will have negative impacts on mental, metabolic and cardiovascular health later in life (Lupien et al., 2009; Seckl, 1997; Tamashiro & Moran, 2010).

4.4.3 Underlying Mechanisms Of Poor Neonatal Adaptation:

There is an ongoing debate in the literature regarding the appropriate mechanism for the observed poor neonatal adaptation, whether the symptoms reflect a true withdrawal syndrome after abrupt discontinuation of transplacental transfer of maternal SSRI at birth or are a result of serotonin toxicity due to SSRI exposure in the newborn (Haddad et al, 2005; Isbister et al., 2001; Stiskal et al, 2001). Clearly, it is difficult to differentiate the syndrome based simply on clinical signs and symptoms since the symptoms appear to be overlapped between an SSRI withdrawal syndrome and serotonin toxicity. Thus, measurement of the plasma concentrations of the administered SSRIs at delivery and at the time the neonate was symptomatic would help in determining whether it is a withdrawal or toxicity. For example, a high level of SSRIs concentration at the time of peak symptoms and a decline thereafter would suggest a toxicity phenomenon. In contrast, if drug concentration is undetectable at the time the symptoms are present, it is more likely a case of withdrawal syndrome. There is
evidence supporting a withdrawal syndrome in the neonates after *in utero* SSRIs exposure (Jaiswa et al., 2005). However, most of the withdrawal syndromes occurred more often with paroxetine use than with other SSRIs (Jaiswal et al., 2003; Sanz et al., 2005), likely due to its short half life and no active metabolite (Hiemke & Hartter, 2000). Since FX has a very long elimination half life, it is unlikely that there is a withdrawal syndrome with FX use. In addition, there is no evidence for an ability to metabolize FX in the fetal lamb and human and most importantly, it has been shown that the plasma FX concentration measured in human infants at day 2 following birth is the same as that in cord blood at delivery (Laine et al., 2003, Kim et al., 2006). Hence, it is less likely, we believe, that a withdrawal syndrome is an underlying mechanism for poor neonatal adaptation after *in utero* FX exposure.

On the other hand, there is also evidence supporting the serotonin toxicity mechanism in the “poor neonatal adaptation” in neonates who were prenatally exposed to SSRIs (Jessel & Stiskal, 2004; Knopper et al., 2006; Kwon & Lefkowitz, 2008; Laine et al., 2003; Rampono et al., 2004). These studies reported measureable concentrations of SSRIs (PX, FX and NFX) in cord blood at birth and/or when the symptoms were present during the neonatal period. In our study, we used a different approach to determine if serotonin toxicity is a mechanism responsible for poor neonatal adaptation in the neonates. The study allowed us to reproduce in the newborn lambs a similar drug level observed in the human neonates and the clinical effects were recorded. As described above, the FX level in the lambs and in human infants cover the same range. However, no symptoms related to poor neonatal adaptation (feeding behavior, rest- activities cycle, irritability, respiratory difficulty) or other serotonergic symptoms (restlessness, myoclonus, tremor, shivering) were observed in our exposed lambs.
Thus, our results do not favor serotonin toxicity as an underlying mechanism for poor neonatal adaptation.

To date, it appears that there is a growing body of evidence supporting the view that SSRI-elicited alterations in fetal brain development may be responsible for poor neonatal adaptation. As were mentioned above, supporting evidence include the initial study in sheep from our group and subsequent findings in human which showed a decrease of fetal REM sleep, an important determinant of brain development, in FX exposed fetuses (Morrison et al., 2001; Mulder et al., 2011; Richardson, 1994). In addition, a recent study demonstrated that the risk of respiratory distress, a common symptom in poor neonatal adaptation, was reduced by stopping SSRI treatment group before the end of pregnancy compared to the group in which SSRI therapy continued until delivery, after controlling for maternal illness severity, suggesting that the mechanism is less likely due to either withdrawal or toxicity syndromes, (Warburton et al., 2010). Furthermore, it is known that serotonin plays an important role in the regulation of early brain development. It autoregulates serotonergic neuron growth in culture and the development of related neural systems (Whitaker-Azmitia, 2001). Thus, alteration the fetal serotonin level in the CNS secondary to prenatal SSRI exposure could potentially influence serotonin signaling pathways in a developing brain, affecting the fetal serotonergic tone and finally lead to changes in fetal/neonatal behavior expressing as poor neonatal adaptation (Oberlander, 2012).

In conclusion, overall our results have shown that there is a lack of significant FX effects on cardiovascular-respiratory, behavioral and endocrine functions in ~ 4-day old postnatal lambs with acute FX administration. Thus SSRI toxicity is unlikely to be the mechanism underlying poor neonatal adaptation in human infants exposed to this drug in
uterine. Further investigation is needed to determine the exact mechanism to provide more sufficient information regarding the safety of FX and similar drugs in pregnancy.
5. Acute Fluoxetine Effects In The Postnatal Lambs (From 10 Days To 1 Year Of Age)

5.1 Introduction:

There is increasing awareness that children and adolescences also suffer from depressive disorders, which were formerly thought only to occur in adults. In fact, the prevalence of depression ranges from 0.4 to 2.5% in children and 0.4-8.3% in adolescents (Birmaher et al., 1996; Fleming & Offord, 1990). And the cumulative incidence of major depressive disorder (MDD) during adolescence is similar to the lifetime prevalence of MDD in the adult population, which ranges from 15-20% (Birmaher et al., 1996). In addition, teenage girls are at higher risk for developing depression than teenage boys, with a male:female ratio of 1:2, similar to adults (Kessler et al., 1993; Cohen et al., 1993; Nolen-Hoeksema & Girgus, 1994). These data support the view that depression in adults might actually originate during the adolescent period (Lewinsohn et al., 1999; Rao et al., 1999). Depression that occurs during such a critical period of development can lead to impaired school functioning, disturbed peer and parent relationships and increased rates of health problems or somatic complaints, as well as long-term serious consequences due to an increase risk of engaging in high-risk behaviors such as smoking, alcohol and other substance abuses and even suicide attempts (Asarnow et al., 2005; Bernstein et al., 1997; Deykin et al., 1992; Egger et al., 1999). Since SSRIs were introduced into the market, due to their safety profile and benign adverse effects compared to the earlier antidepressants, they have also been used in the treatment of depression in children and adolescents. However, SSRI side effects testing has mostly been done in adults; thus their safety in the pediatric population is
not well-established. In their meta-analyses, Whittington et al., (2004) and Jureidini et al., (2004) concluded that the benefits from SSRIs treatment in children and adolescents are offset by the significant risks of serious adverse events (i.e suicidal behavior), except for FX. Thus, cognitive-behavioral therapy (CBT) appeared to be a better approach in regard to efficacy and safety (Harrington et al., 1998). Nevertheless, some subsequent studies have shown that it is more efficacious to treat MDD in adolescent with CBT and FX in combination compared to either therapy alone (Goodyer et al., 2007; J. March et al., 2004; March et al., 2007; Pampallona et al., 2004). However, the availability of CBT is limited in some areas, thus SSRIs can be prescribed in these circumstances. Currently, only FX and escitalopram are approved by the FDA for the treatment of depression in adolescents (Hosenbocus & Chahal, 2011; Ronsley et al., 2010). In Europe, FX is approved by the European Medicine Agency for children and adolescents with moderate to severe symptoms but only after failure to respond to 4-6 sessions of psychotherapy (Eaton, 2006). In Canada, Health Canada has not yet approved any SSRIs for use in children and adolescents because of the concerns about the adverse effects of these agents, particularly suicidal thoughts and behaviors. Hence, any use of SSRIs in this population is off-label (Voysey, 2004). Even though FX and escitalopram were approved for use in the US, the FDA did issue a warning regarding the use of SSRIs in the treatment of depression in adolescents (2007 > FDA proposes new warnings about suicidal thinking, behavior in young adults who take antidepressant medications; Public health advisories (drugs) > suicidality in children and adolescents being treated with antidepressant medications). Besides the potential increased risk of suicidality associated with the use of these agents in children and adolescents, another lethal adverse reaction is the serotonin syndrome. This syndrome is a relatively rare but lethal condition and is a result of
overstimulation of peripheral and central serotonergic systems. It can occur at high SSRI doses, overdose or even at therapeutic dose when combined with other serotonergic medications (eg: TCA, MAOIs, dextromethorphan, lithium) (Nelson et al., 2007). As antidepressants are used in patients at high risk for suicide, the risk of intentional overdosing of the drugs is high. In fact, it was reported that among prescription drug overdoses managed by poison centers in the US, antidepressant overdoses are the most common (Nelson et al., 2007). In addition, children less than 6 years of age accounted for 17% of all cases of inappropriate SSRI ingestion. (Nelson et al., 2007) Since there is still limited information on adverse effects (other than suicide behaviors) of SSRIs use in children and adolescences, especially when the drug is used off-label, there is a compelling need for the study of the safety of SSRIs use in such a special population. Therefore, in this study our aim is to investigate the acute effect of FX on a variety of physiological, behavioral and endocrine variables in the lambs at different ages from 10 days to 12 months old.

5.2 Methods:

Animal housing, surgical procedure, experimental protocol and methods are described in detail in chapter 2.

In this chapter, an additional behavior study was performed in the lambs at 6 months of age in which lambs were not restricted to the monitoring pen for sample collection. Instead, lambs were allowed to move freely in the pen with their penmates (peers or siblings) and have access to food and water ad libitum. Sterile water or FX 1mg/kg were given via their jugular vein catheter or by venous puncture around 10am and their behavior were observed by a continuous DVR system to look for any abnormal behaviors. Through DVR
observations, their activities were divided into lying down asleep, lying down awake, standing moving, standing quiet, walking and headbutting. The activities were collected from the DVR system in 15 second epochs and then matched with the activity score from the lamb’s actiwatch in the same epoch to provide the intensity for each corresponding activity.

In the lambs from 10 days old onward, cortisol and ACTH measurements in the FX group were measured in the 3 month and 1 year old groups only.

Statistical analysis:

Differences in the effects of treatment and sex on behavior activities and weight gain were determined using two-way repeated measure ANOVA. In general, three way ANOVA was used to determine the effects of treatment, time and sex on cardiovascular-respiratory, metabolic, endocrine variables. Two-way repeated measured ANOVA or Mann Whitney U test were used for the non-normally distributed variables to determine the effect of treatment and time. Delta O₂ saturation, which is the difference between O₂ saturation at 5 min after dosing and that at -15 min before sterile water or FX administration, was calculated for each animal in the control and FX group. Then delta O₂ saturation between the control and FX group was compared using the Mann Whitney U test (in the 1 and 6 months old lambs) or 2 way ANOVA (in the 1 year old lambs). For the data presented as percentage, statistical tests were run on the raw data, not on % data, except for O₂ saturation. P < 0.05 defines significant differences. Data are presented as mean ± SEM. All data were analyzed using the Sigma Plot 11 statistics software (Systat Software, Inc; San Jose, CA, USA).
5.3 Results:

Actiwatch activities, arterial pressure, heart rate, heart rate variability, arterial blood gases (O₂ sat, pO₂, pCO₂, pH, BE, HCO₃), lactate, glucose and Hb concentration in FX experiments in the lambs at all age groups are summarized in the table in the appendices.

5.3.1 Acute FX Effects On Cardiovascular-Respiratory Functions:

There were no significant changes and no gender differences in arterial pressure, heart rate and HRV variables with acute FX administration in the lambs at 10 days, 1, 3 and 6 months of age even though arterial pressure tended to increase for the first 5 min post-FX in the lambs at these ages. (data in appendices)

However, in the 1 year old lamb, arterial pressure significantly increased after FX administration and this effect lasted for about 15-30 mins (Fig. 5.1). Mean arterial pressure were significantly different between the control and FX group at 1,2,3,4,5, 15 and 30 minutes post-FX (p<0.001), but not at the control time point (-15 minutes). Pressure increased from 85.1 ± 2.2 mmHg before FX to peak value of 106.6 ± 4.1 mmHg at 2 mins after FX administration, thus the maximum increase was 21.5 ± 4 mmHg. It is noted that the increase in mean arterial pressure was more profound and lasted longer in males than in females. Statistical analysis indicated that the difference in arterial pressure at 2 and 3min after dosing vs -15min before dosing were only significant in male lambs (p = < 0.001 and p = 0.034). At the 2 min sample, the mean arterial pressure increase was significantly higher in males than in females (to 112.6 ± 4.1 mmHg in male vs 99.1 ± 5.2 mmHg in female; p = 0.012), with a maximum increase of 25.8 ± 4.4 mmHg in male compared to 16 ± 6.8 mmHg in female.
FIG 5.1: Arterial pressure and heart rate changes in 1 year old lambs with acute FX administration
a indicates significant differences between variables at -15 min vs 1,2,3,4,5,10,15 min after FX within
the FX group
b indicates significant differences between control and FX at the corresponding time
c indicates significant differences between male and female at the corresponding time within FX
group
There was a transient decrease in heart rate at 2 min after FX (p = 0.026), the difference was 30 ± 4 bpm but it quickly returned to normal within 1 min. No sex difference was found in the heart rate response. (Fig 5.1)

In terms of HRV, we did not find any significant difference between male and female lambs at 1 year of age. Except for the LF/HF ratio, all other variables (SDNN, SD Delta NN, RMSSD, LF power (ms²), HF power (ms²) significantly increased at 30 min after FX dosing in the FX group compared with the control group (SDNN: p = 0.013, SD Delta NN: p = 0.002, RMSSD: p = 0.002; LF power: p = 0.01; HF power: p = 0.002). The difference persisted for 60 min for some variables (SD Delta NN, RMSSD, HF power). (Fig 5.2)

In 4 male lambs at 1 year of age, delta arterial pressure (i.e the difference between arterial pressure value at 2 min post-FX and that at -15 min pre-FX) and plasma log FX concentration at 5 mins followed a linear regression with $r^2 = 0.84$, p = 0.08
FIG 5.2: HRV (SDNN, SD Delta NN, RMSSD, LF power, HF power, LF/HF ratio) changes with acute FX administration in 1 year old lambs.

a indicates significant differences between variables at -30 min vs 30, 60, 90 and 120 min after FX within the FX group.
b indicates significant differences between control and FX at the corresponding time.
5.3.2 Acute FX Effects On Homeostatic And Respiratory Functions:

There was no FX effects on respiratory and metabolic function in the lambs at 10
days and 3 months of age even though Po2 tends to be declined at 5 min post FX in the 3
month-old lambs but this is not significant. (data in appendices)

However, Po2 significantly decreased at 5 min after FX injection in the 1 month-old
lambs (p < 0.001). The FX effect was significantly different between male and female (p =
0.002). In males, Po2 decreased from 92.4 ± 4.4 at 15 min pre-FX to 67.2 ± 4.1 mmHg 5 min
post-FX while in females it fell from 97.6 ± 2.5 at 15 min pre-FX to 92.5 ± 4.5 mmHg 5 min
post-FX. (Fig 5.3) There was also a statistically significant difference in delta O2 sat between
the control group (median = 0.625%) and the FX group (median = -3.35%) (p = 0.031) with
the Mann-Whitney U test. This indicates that FX caused a transient hypoxemia in lambs at 1
month of age and it was more apparent and severe in male than female lambs. Other blood
gas variables (pH, pCO2, HCO3⁻) and glucose and lactate concentrations were similar between
the control and FX groups in this age group.

Similarly, in the 6 month-old lambs, 2 way (treatment x time) repeated measure
ANOVA revealed a significant decrease of pO2 at 5 min post-FX compared to control (p =
0.035). Po2 fell from 111.9 ± 5.4mmHg at 15 min pre-FX to 95 ± 7.7mmHg at 5 min post-
FX. (Fig 5.4) Delta O₂ sat was significantly different between the control group (median =
0.65%) and the FX group (median = -1.05%) (p = 0.004) with Mann-Whitney U test.
However, there were no significant differences in hypoxemia response to acute FX
administration between males and females. Other variables were also not significantly
different between the control and FX groups.
FIG 5.3: pO2 and O2 saturation with sterile water and FX administration in male and female lambs at 1 month of age.

a indicates significant differences between -15 min and 5 min after FX within the FX group
b indicates significant differences between control and FX within the corresponding time
c indicate significant differences between male and female within the corresponding time
Asterisk (*) indicates significant differences in delta O2 sat (i.e differences between values at 5 mins and at -15 mins) between control and FX group using Mann-Whitney u test
FIG 5.4: pO2 and O2 saturation with sterile water and FX administration in male and female lambs at 6 months of age.

a indicates significant differences between -15 min and 5 min after FX within the FX group
b indicates significant differences between control and FX within the corresponding time
Asterisk (*) indicates significant differences in delta O2 sat (i.e differences between values at 5 mins and at -15 mins) between control and FX group using Mann-Whitney u test
In the 1 year-old lambs, a transient decrease in Po2 occurred at 5 min after FX injection and returned to normal by the time of 15 min post-FX. Po2 fell from 110.5 ± 2.5 mmHg at 15 min pre-FX to 94 ± 5.8 mmHg at 5 min post-FX. Delta O2 sat was significantly different between control and FX group and was more severe in male lambs (p = 0.037). (Fig. 5.5) There was a significant inverse relationship between the changes in Po2 at 5 min (delta Po2) and changes in arterial pressure at 2 min (delta AP) following FX injection with $r^2 = 0.54$, $p = 0.037$, $n = 8$. The greater the increase in arterial pressure at 2 min post-FX, the greater the decrease in Po2 at 5 min post-FX. (Fig. 5.6A) The significant relationship between delta Po2 and delta arterial pressure is sex-specific, being significant in males ($r^2 = 0.93$, $p = 0.03$, $n = 4$) but not in females ($r^2 = 0.66$, $p = 0.18$, $n = 4$) (Fig. 5.6B). Similarly, changes in O2 saturation (delta O2) at 5 min and delta AP were significantly correlated in male ($r^2 = 0.98$, $p = 0.01$, $n = 4$) but not in female ($r^2 = 0.27$, $p = 0.48$, $n = 4$) (Fig. 5.6C). Also, there was a significant relationship between delta pO2 (i.e. the difference between pO2 at 5 min post-FX and that at -15 min pre-FX) and log plasma FX concentration at 5 min in lambs at 1 years of age ($r^2 = 0.81$, $p = 0.002$, $n = 8$) (Fig. 5.7A). However, the relationship between delta pO2 and log plasma FX concentration at 5 min was not statistically significant in males ($r^2 = 0.75$, $p = 0.13$, $n = 4$) and in females ($r^2 = 0.69$, $p = 0.16$, $n = 4$) (Fig. 5.7B). Nevertheless, there was a significant relationship between delta O2 saturation (i.e. the difference between O2 saturation at 5 min post-FX and that at -15 min pre-FX) and log plasma FX concentration at 5 min in both males and females with $r^2 = 0.92$, $p = 0.040$, $n = 4$ and $r^2 = 0.97$, $p = 0.016$, $n = 4$, respectively. The higher the plasma FX concentration at 5 min, the greater the decrease in O2 saturation at 5 min following FX injection (Figure 5.7C).
However, pH value did not change significantly over time and was comparable between the control and FX group. $\text{Pco}_2$ (at -15 min), BE and $\text{HCO}_3$ were significantly different between the control and FX experiments and this occurred only in female lambs (Fig. 5.8). Similarly, lactate (at 5 min post FX) and Hb (at 5 and 15 min post-FX) were significantly higher with FX administration compared to placebo only in females (Fig. 5.9). Glucose concentration was significantly different between male and female lambs in both control and FX group (Fig. 5.9). There were no significant changes in temperature after FX administration. (data in appendices)

Arterial blood gas samples were also taken at 2 mins after FX in 5 out of the 10 lambs in this 1 year age group. Except for $\text{pO}_2$ and $\text{O}_2$ sat, which were also decreased as shown at 5 mins after FX (Fig. 5.5), there were no significant differences in other blood gas variables at 2 mins after FX compared with those at -15 mins before FX. However, $\text{pCO}_2$ tended to decrease and pH tended to increase at 2 mins post-FX compared with -15 min pre-FX (32.6 ± 1.6 vs 36.8 ± 2.1 mmHg; 7.531 ± 0.027 vs 7.486 ± 0.027, n = 5, respectively) although they are not statistically significant.
FIG 5.5: pO2 and O2 saturation with sterile water and FX administration in male and female lambs at 1 year of age.

a indicates significant differences between -15 min and 5 min after FX within the FX group
b indicates significant differences between control and FX within the corresponding time
Asterisk (*) indicates significant differences in delta O2 sat (i.e differences between values at 5 mins and at -15 mins) between control and FX group within male, and between male and female within FX group.
FIG 5.6: (A) The relationship between changes in pO2 at 5 mins and changes in arterial pressure (AP) at 2 mins following FX injection in all lambs at 1 year of age (square symbol, solid line, n = 8); (B) The relationship between changes in pO2 at 5 mins and changes in AP at 2 mins following FX injection in male (square symbols, dotted line, n = 4) and female (circle symbols, solid line, n = 4) at 1 year of age and (C) The relationship between changes in O2 saturation at 5 mins and changes in AP at 2 mins following FX injection in male (square symbols, dotted line, n = 4) and female (circle symbols, solid line, n = 4) at 1 year of age.
FIG 5.7: (A) The relationship between changes in pO2 at 5 mins and log FX concentration at 5mins following FX injection in all lambs at 1 year of age (square symbol, solid line, n = 8) (B) The relationship between changes in pO2 at 5 mins and log FX concentration at 5mins following FX injection in male (triangle symbols, dotted line, n = 4) and female (circle symbols, solid line, n = 4) lambs at 1 year of age; and (C) The relationship between changes in O2 saturation at 5 min and log FX concentration at 5mins following FX injection in male (triangle symbols, dotted line, n = 4) and female (circle symbol, solid line, n = 4) lambs at 1 year of age.
FIG 5.8: $P_{CO_2}$, base excess (BE), $HCO_3^-$ concentrations with sterile water and FX administration in male and female lambs at 1 year of age.
FIG 5.9: Glucose, lactate, Hb (Hemoglobulin) concentrations with sterile water and FX administration in male and female lambs at 1 year of age.
5.3.3 Acute FX Effects On Behavior:

There were no differences in activity variables between the average of 3 normal days, control group and the FX group in the ~10 day-old group. In contrast, activities significantly decreased on the FX experimental day compared to both control experimental day and normal day in the 1 month-old lambs. Starting from 3 months old, the lambs were kept in the monitoring pen for 4 days during the control and FX experiments. Thus, their activities decreased on both control and FX experiment day due mainly to the restraint. Summarized data for all age groups are in tables 1, 2 in the appendices. To investigate the actual FX effects on the older lambs (after 3 months old) we conducted a behavior study on the 6 month-old lambs in which they were housed in their regular holding pen with their sibling or peers as usual, as described in the method section. The activity data of this behavior study is presented in figures 5.15-5.17. Note that activity data of the 6 month-old group presented in the appendices are the activities when the lambs were kept in the monitoring pen.

5.3.3.1 Behavioral Activities In The Lambs At 1 Month Of Age:

Active bouts per day, mean score in active and total activity score were significantly lower on FX experimental day as compared to the average of 4 normal days (p<0.001) while they were comparable between the other days (control experiment day, post FX day 1, post FX day 2) and the average of 4 days. In contrast, episode duration was significantly higher on the FX day compared to the average of the 4 normal days. Even though the percentage of moving time was lower and the percentage of time immobile were higher on the FX day compared to the average of 4 normal days, respectively, they were not statistically significant. (Fig 5.10) There were no sex differences in these actiwatch variables.
Results from DVR observations showed that the intensity of suckling attempts determined by actigraphy was significantly higher on the FX day compared to the normal day (FX day: 327 ± 21 counts/15s versus normal day: 247 ± 28 counts/15s). The lambs always attempted to suckle when they were returned to their mothers after every blood sampling but the ewes almost always refused them. Thus, it is reasonable that the intensity of suckling attempts was higher on the FX days as lambs tried harder. Importantly, there were no significant changes in the percentage of time lamb spent on suckling attempts on the FX days compared to normal days, which indicates that blood sampling did not interfere with their feeding behavior. However, the amount of time lambs spent on successful suckling was significantly lower on the day of the FX experiment (FX day: 0.7 ± 0.14 %/24h versus the normal day: 1.1 ± 0.22 %/24h). In addition, the amount of time that lambs spent walking significantly decreased on the FX experiment day (3.6 ± 0.2%/24h) vs the control day (4.9 ± 0.4 %/24h) (Fig 5.11A). Even though the time lambs spent standing was not significantly different between the days, when dividing the standing activity into standing quiet (defined by standing from DVR observation and actiwatch score less than 60) and standing mobile (defined by standing activity from DVR observation and actiwatch score equal or greater than 60), there was a significant decrease in the amount of time lambs spent standing moving on the FX experiment day (8.9 ± 0.9 %/24h) compared to the control experiment day (13.7 ±1.5 %/24h) or normal day (13.7 ± 1.3 %/24h), (p = 0.013). Moreover, the intensity of standing quiet was also significantly lower on the day of FX experiment (28 ± 2.8 counts/15s) versus control day (34 ± 2.3 counts/15s) (Fig 5.12). Although the time lambs spent for experiments was significantly higher on the control and FX day compared to normal day, it was negated by the significantly lower intensity of activity during the
experiment on experiment days compared to normal days (Fig 5.11). In fact, lambs slept or lay awake most of the time during the experiments. Collectively, the results suggest that 1 month-old lambs were less successful at suckling and less active (walking less, moving less and more still while standing) with FX administration. However, their weight gain was comparable between the average of normal days, control, FX experiment and post FX days. (Fig 5.13)

Regardless of the day, there were a sex difference in the intensity of standing and playing activity. Female lambs had a higher average score of standing activity (normal day: 102 ± 2.2, control day: 110 ± 11, FX day: 93 ± 16 counts/15s) than the male (normal day: 74 ± 6.6, control day: 75 ± 8.2, FX day: 63 ± 3 counts/15s). Similarly, there was higher playing activity in females than in males (normal day: 272 ± 33 vs 178 ± 44; control day: 232 ± 43 vs 164 ± 18, FX day: 265 ± 15 vs 174 ± 31 counts/day) (Fig 5.14). In general, around 1 month of age female lambs were more active than male lambs but this was independent of FX administration.
FIG. 5.10: Activity variables (active bouts per day, mean score in active, episode duration, total activity score, % time moving, % time immobile) in 1 month-old lambs with the average of the 4 normal days in the pen before the experiments (n = 12, open bar), on control experiment day (n = 9, black bar), and on FX experiment day (n = 12, dark gray), post FX day 1 (n = 11, light gray), post FX day 2 (n = 11) and post FX day 3 (n = 11).

(*) indicates statistically significant between FX experiment day and average of 4 days, p<0.05.
FIG. 5.11: (A) Percentage of time lambs spent for each activity from DVR observation and (B) the intensity of each activity when matching DVR observation with activewatch score data in lambs at 1 month of age. Normal day was a day lambs were in the pen without either control or FX experiment (n = 6, gray bar). Control experiment (n=6, open bar), FX experiment (n=6, black bar).

(^) indicates significant differences between control experiment day and normal day
(+) indicates significant differences between FX experiment day and control experiment day
(*) indicates significant differences between FX experiment day and normal day, p < 0.05.
FIG. 5.12: (A) Percentage of time lambs spent for standing activity from DVR observation and (B) the intensity of standing quiet and standing moving when matching DVR observation with actiwatch score data in lambs at 1 month of age. Normal day was a day lambs were in the pen without either control or FX experiment (n = 6, gray bar). Control experiment (n=6, open bar), FX experiment (n=6, black bar). (^) indicates significant differences between control experiment day and normal day (+) indicates significant differences between FX experiment day and control experiment day (*) indicates significant differences between FX experiment day and normal day, p < 0.05.
FIG 5.13: Weight gain (mean ± SE) in the lambs during their normal days, control experiment with distilled water injection, FX experiment with FX injection and 3 continuous days after the FX experiment day around 1 month of age.

FIG. 5.14: Intensity of standing and playing activity from DVR observation in the 1 month-old male and female lambs on normal day (day lambs in the pen without any experiments, gray bar, n = 3), on Control experiment day (n=6, open bar) and FX experiment day (n=6, black bar).

(a) indicates significant differences in standing activity on each corresponding day between male and female.

(b) indicates significant differences in playing activity on each corresponding day between male and female.

p < 0.05.
5.3.3.2 Behavioral Activities In The Lambs At 6 Months Of Age:

Active bouts per day, total activity score and % time moving were significantly lower on the FX experimental day and post FX day 1 as compared to the average of 4 normal days (p = 0.012, p = 0.007, respectively) while they were comparable between the other days (control experiment day and post FX day 2) and the average of 4 days (Fig. 5.15). In contrast, % time immobile was higher on the FX day and post-FX day 1 compared to the average of 4 normal days. Mean score in active tends to be lower and episode duration higher on the day of FX experiment but these changes were not statistically significant. (Fig 5.15) There were no sex differences in these activity variables from the actiwatch measurements.

The results from the DVR observations showed that there was a small but significant increase in the amount of time lambs spent on headbutting on the day of the FX experiment (0.8 ± 0.17 %/24h) compared to the day of control experiment (0.39 ± 0.09 %/24h) (p = 0.036) but not significant to the normal day (0.47 ± 0.09 %/24h) (Fig 5.16A). Even though the amount of time spent for standing was not significantly different between the days, when dividing the standing activity into standing quiet (defined by standing from DVR observation and actiwatch score less than 60) and standing mobile (defined by standing activity from DVR observation and actiwatch score equal or greater than 60), there was a significant decrease in the amount of time lambs spent for standing moving (FX: 14.4 ± 1.51%/24h, control: 21.2 ± 2.9 %/24h; normal: 21.2 ± 3.06 %/24h) and an increase in the time standing quiet (FX: 18.2 ± 1.53 %/24h, control: 12.2 ± 1.44 %/24h, normal: 13.6 ± 2.02 %/24h) on the FX experiment day compared to the control experiment day or normal day (p = 0.027 and p = 0.005, respectively) (Fig. 5.17). In addition, the intensity of standing activity was significantly lower on the day of FX experiment (68 ± 7.2 counts/15s) compare to the normal
day (102 ± 15 counts/15s) \((p = 0.042)\) (Fig. 5.17B) and this difference was due to the
significant differences in the intensity of standing quiet (FX day: 12 ± 1.4 counts/15s, control
day: 18 ± 1.9 counts/15s, normal day: 18.9 ± 1.9 counts/15s) (Fig 5.17). These results
suggested that after FX administration the lambs’ behavior were shifted toward two extremes
of activity: they were less active, stood very still for longer time, but were also more
aggressive indicated by an increase in headbutting activity. It was noted from DVR
observations that two lambs (one male and one female) presented with jerky movements
from around 20.5h after FX administration. These jerky movements occurred intermittently
in the lambs for ~10h during the day. However, no differences in the intensity and the % time
of each activity were found between male and female lambs. Unfortunately, no blood
samples were available for the measurement of FX concentration in these lambs.

Interestingly, at 6 months of age, acute FX administration resulted in a significant
weight loss in the lambs (-0.7 ± 0.19 kg, \(n = 8\)) as compared to an average of daily weight
gain of 0.1 ± 0.05 kg at their age \((p = 0.011)\). (Fig 5.18) This FX effect on bodyweight gain
was not observed in the younger lambs.
FIG. 5.15: Activity variables (active bouts per day, mean score in active, episode duration, total activity score, % time moving, % time immobile) in 6 month-old lambs with average of 4 normal days in the pen before the experiments (n = 8, open bar), on control experiment day (n=8, black bar), and on FX experiment day (n = 8, dark gray bar), post FX day 1 (n = 8), post FX day 2 (n = 8) and post FX day 3 (n = 8).

Asterisk (*) indicates statistically significant between FX day or post FX day 1 and average of 4 normal days, p< 0.05.
FIG. 5.16: (A) Percentage of time lambs spent for each activity from DVR observation and (B) the intensity of each activity when matching DVR observation with actiwatch score data in lambs at 6 month of age. The normal day was a day lambs were in the pen without either control or FX experiment (n = 6, gray bar). Control experiment (n=6, open bar), FX experiment (n=6, black bar).

(+) indicates significant differences between FX experiment day and control experiment day (***) indicates significant differences between FX experiment day and normal day, p < 0.05.
FIG. 5.17: (A) Percentage of time lambs spent on standing (quiet and moving) activity from DVR observation and (B) the intensity of standing quiet and standing moving when matching DVR observation with actiwatch score data in lambs at 6 month of age. The normal day was a day lambs were in the pen without either control or FX experiment (n = 6, gray bar). Control experiment (n=6, open bar), FX experiment (n=6, black bar).

(+) indicates significant differences between FX experiment day and control experiment day (*^) indicates significant differences between FX experiment day and normal day, p < 0.05.
FIG 5.18: Weight gain (mean ± SE) in the lambs during their normal days, control experiment with distilled water injection, FX experiment with FX injection and 2 continuous days after the FX experiment day at around 6 months of age. (*') indicates significant differences between FX experiment day and the normal average of 4 days, p < 0.05.

5.3.3.3 Acute FX Effects On Cortisol Level:

No differences were found between the cortisol concentrations in the control group compared with the FX group at 3 month and 1 year old, nor ACTH concentration. (Figs 5.19 & 5.20)

There were also no differences in cortisol level between sexes.
FIG. 5.19: Cortisol and ACTH concentration in the lambs at 3 months of age at 15, 30 minutes before and 0.5, 1, 2, 6 and 12 h after sterile water injection (control group) and FX injection (FX group)
FIG. 5.20: Cortisol and ACTH concentration in the lambs at 1 year of age at 15, 30 minutes before and 0.5, 1, 2, 6 and 12 h after sterile water injection (control group) and FX injection (FX group)
5.4 Discussion:

In this study which included lambs at 10 days, 1, 3, 6 month and 1 year of age, we found that acute i.v. FX administration did not have any significant effects in the lambs at 10 days and 3 months of age. However, transient hypoxemia occurred in the 1 month old lambs with acute FX administration and it was more profound in males. Lambs were also less active and altered their feeding behavior on the day after FX administration. These FX effects on respiration and behavior were also observed in the lambs at 6 months of age but there were no sex differences. At 1 year of age, acute FX administration resulted in an increase in arterial pressure, to a greater extent in the male than the female, and a transient decrease in heart rate. HRV was also altered with FX administration for about 30-60 minutes in both sexes. No changes in cortisol level occurred with acute FX administration.

The daily therapeutic FX dosing for children from 6 to 18 years old ranges from 10 to 20 mg but no mg/kg dose has been provided (Eaton, 2006). However, according to a reference used by poison control centers in the US, a minimum toxic dose of FX in children less than 6 years of age is 60 mg or 5mg/kg (Nelson et al., 2007). In our study 1mg/kg FX dose was used, which is at the high margin of the therapeutic range but does not reach a minimal toxic dose. Moreover, the plasma FX concentrations achieved in the lamb experiments were in the therapeutic FX concentration range in humans (Chow, 2013).

In our study, increase in arterial pressure and decrease in heart rate after acute FX administration were observed in the older lambs. As the mechanism of SSRI group, plasma 5-HT concentration is expected to increase after FX bolus by the inhibition of 5-HT reuptake by platelets, the main reservoir of peripheral 5-HT (Maurer-Spurej et al., 2004; Ortiz &
Artigas, 1992). 5-HT has been known as a vasoconstrictor agent, its increase would lead to an elevated blood pressure (Maurer-Spurej et al., 2004). Even though FX seems to have divergent effects on blood pressure, (Lazartiges et al., 2000; Tsai & Lin, 1986) did report that central acute administration of FX caused hypertension in rats in a dose-dependent fashion. In humans FX is usually administered for longer time durations (several weeks) per os and an increase in blood pressure was also observed in those subjects who had a normal pretreatment mean diastolic pressure (Amsterdam et al., 1999). The diversity of 5-HT effects on blood pressure in the literature could be attributed to many factors such as differences in species, route of administration, doses, and 5-HT receptor subtypes. However, elevated arterial pressure after acute FX injection in the older lambs in our study was consistent with a report by (Nelson et al., 1987) in which increase in blood pressure was observed with 5-HT injection in conscious sheep.

However, our study is probably one of the first to examine blood pressure responses to FX administration over age in relation to gender. The effect of FX on arterial pressure elevation only became statistically significant in lambs at 1 year of age and it also increased higher in males compared to females. It is not yet clear why the FX effects on blood pressure was only significant at 1 year of age. However, one explanation could be due to either a lower level of platelets, which are the main reservoir of peripheral 5-HT, in the younger lambs or a lesser extent of a functioning 5-HT receptor subtypes that mediate 5-HT effects. Platelet concentrations are lower in human newborns and children compared to adults (Merritt & Davidson, 1933) and in addition, the platelet 5-HT concentration in newborns is only ~42% of the value in children and adults (Flachaire et al., 1990). If a similar situation occurs in sheep, the transient increase in blood 5-HT following SSRI administration may
have been less in the younger lambs and not sufficient to elicit effects on arterial pressure and blood oxygenation. Measurement of platelet concentrations, platelet 5-HT concentration and the magnitude of the transient increase in blood 5-HT concentration following SSRI administration in sheep as a function of age could confirm this hypothesis.

In terms of the sex differences in the FX effects on arterial pressure in the year old lambs, the lung is a major site of clearance of plasma 5-HT, with ~95% of 5-HT being removed from plasma by the lung via a first-pass effect (Gillis & Pitt, 1982). However, a small change in the 5-HT clearance rate by the lung can dramatically affect the systemic levels of 5-HT, for example increasing pulmonary extraction from 95 to 97.5% would reduce plasma 5-HT concentration in the systemic circulation by 50%. (Gillis & Pitt, 1982) Estrogen (17β estradiol) or progesterone appears to increase 5-HT metabolism in lung homogenates prepared from female rats (Bakhle & Ben-Harari, 1979). By one year of age, male and female lambs who are raised indoors under the same condition as ours have undergone puberty (Suttie et al., 1991) and the females have thus initiated estrous cycles (Ryan et al., 1991; see Chapter 3), with pulsatile increases in blood estrogen and progesterone levels. Thus it is possible that an effect of ovarian steroids on increasing 5-HT clearance by the lung is responsible for the observed smaller increase in the arterial pressure response to FX administration in the 1 year old female lambs compared to male lambs, because of a lesser transient increase in blood 5-HT levels following FX injection. It has been reported that the rate of sustained hypertension is higher in adults who underwent 12 weeks of venlafaxine (a serotonin and noradrenaline reuptake inhibitor) treatment for depression compare to placebo group (Feighner, 1995). In general, SSRIs are not considered to be cardiotoxic, in comparison to the classical antidepressants (TCA, MAOI) (Lane et al., 1995). However,
hypertension is one of the symptoms included in serotonin syndrome (Boyer & Shannon, 2005) and the serotonin syndrome has been reported in pediatric and adolescent populations after SSRI administration (Asch & Parker, 1988; Gill et al., 1999). However, we did not observe hyperthermia after FX injection in the lambs in our study (data in appendices), and hyperthermia is also a symptom of the serotonin syndrome (Boyer & Shannon, 2005).

In the 1 year old male lambs the magnitude of increase in arterial pressure at 2 min following FX injection (i.e: peak value) and plasma FX concentration at 5 mins followed a linear regression analysis. Even though the relationship was not statistically significant (p = 0.08) because of the small sample size (n = 4), it to some extent implies a correlation between FX concentration and its effect.

Interestingly, we found HRV variables (SDNN, LF power, SD Delta NN, RMSSD, HF power) increased for the first 30 mins after acute FX administration in the older lambs. In the literature, the effects of SSRIs on HRV are still a matter of debate. It has been established that HRV decreases in depressed patients, which increases the risk of cardiovascular disease and mortality in these patients (Carney et al., 2005; Grippo & Johnson, 2002; Thayer & Lane, 2007). It has also been shown that TCA treatment exacerbated the decreased HRV in depressed patient which could contribute to the adverse cardiovascular side effect of TCA (van Zyl et al., 2008). However, there is more controversy when it comes to SSRIs effect on HRV. Recent publications reported an “unfavorable” decrease (Licht et al., 2008; Licht et al., 2010) or unchanged of HRV (Kemp et al., 2010) with SSRIs treatment in depressed patients. However, in a meta-analysis done by van Zyl et al., (2008), SSRI use was associated with a slight decrease in heart rate and increase in SDNN. Among SSRIs included in this meta-analysis, FX was investigated in only one study by Khaykin et al, which reported
an increase of SDNN (Khaykin et al., 1998; van Zyl et al., 2008). This finding is consistent with our HRV results, at least in terms of the SDNN variable. In addition, we did observe increases in other HRV variables (SD Delta NN, RMSSD, HF power, LF power) after acute FX administration. However, in the present study, it is unclear why both LF power and HF power increase at the same time. One possibility is that FX might increase sympathetic tone but, in parallel, increase vagal tone and thus minimize the heart rate changes.

However, the significant increase in HRV was only observed in the lambs at 1 year of age and not in the younger lambs. Given the developmental aspects of the lambs, they were deemed to be an adult at 1 year old. Therefore our results suggested the HRV response to acute FX is age-dependent in parallel with the maturation of the autonomic system and adolescents might respond differently to the drug as compared to the adult.

In vivo studies have shown an opposite respiratory response to acute and chronic SSRI treatment. While acute SSRIs (FX, PX) administration caused a reduction in respiratory rate and minute volume in alert, freely moving rats; chronic treatment for more than 23 days in those rats led to an increase in respiratory rate (Annerbrink et al., 2010). In our study, we did not record respiratory rate, instead the effect of FX on respiratory system and homeostasis was observed through the changes in the lambs’ arterial blood gas status. We found a decline in pO2 and O2 saturation in the lambs at 1, and 6 months and 1 year of age at 5 mins after FX administration. However, there were no significant accompanying changes in pCO2, pH, HCO3⁻ and lactate concentration after FX. FX and other SSRIs agents when given acutely will lead to an acute increase in synaptic levels of serotonin by blocking serotonin reuptake (Stahl, 1998). There is evidence to suggest that serotonin might exert both stimulatory and inhibitory influences on respiration. (Annerbrink et al., 2003; Mulkey et al.,
Thus the observed reduction in $pO_2$ and $O_2$ sat after FX administration could be a result of respiratory inhibition due to an increase in synaptic serotonin concentrations. Although due to technical difficulties we did not succeed in an attempt to measure plasma serotonin following FX administration in the lambs in our study, a study in rat has shown that after acute SSRI (FX) administration plasma serotonin increased 520% as compared to the control (Ortiz & Artigas, 1992). The increase in serotonin also occurred in previous study in our lab in which plasma serotonin level increase for the first hour after the bolus of loading FX dose in 3 adult non-pregnant ewes (Morrison, 2001). In addition, in the present study, we found that there was a significant relationship between the level of hypoxemia at 5 mins post-FX and the magnitude of increase in plasma FX concentration at 5 mins in both male and female lambs at 1 year of age. The higher the level of FX, the greater the decrease in $O_2$ sat or hypoxemia in the 1 year old lambs. This again confirms the direct effects of FX on physiological changes. As it was mentioned above, a study in rats showed that the effect of acute FX on reduced respiratory rate only occurred at the dose of 10mg/kg to achieve mean plasma FX = 1092.8 ± 315.9mmol/L, NFX = 1203.2 ± 308.2 mmol/L and a more reduced respiratory rate at a higher dose of 30mg/kg while there was no FX effect at the dose of 1 mg/kg. (Annerbrink et al., 2010) Similarly, in a human study, SSRI effects on fetal behavior were only observed in the fetuses exposed in utero to the drug at standard and high doses in a dose-dependent manner but not in the low dose group (Mulder et al., 2011). Roca et al., (2011) also reported an increased risk of preterm birth mainly occurred in women treated with high dose SSRIs during pregnancy.

Another possibility is that hypoxemia occurred as a result of acute increase in serotonin which caused vasoconstriction and sudden increase in blood pressure. This could
result in a decrease in cardiac output due to an afterload effect, which would reduce pulmonary blood flow to result in the decrease in arterial pO\textsubscript{2}. In fact, our data have shown that the magnitude of increase in arterial pressure at 2 min following FX injection was significantly related to the magnitude of hypoxemia reflected by a decrease in Po\textsubscript{2} at 5 min following FX in the 1 year old lambs supporting for this hypothesis. The relationship was stronger in males than in females. In addition, an experimental study in dogs showed that pulmonary arterial pressure increased after 20-30 mins of i.v serotonin infusion (Sackner, Will, & DuBois, 1966), which suggests pulmonary vasoconstriction. The decrease in pO\textsubscript{2} could via chemoreceptor activation lead to hyperventilation leading to a decrease in arterial pCO\textsubscript{2} and increase in pH. This is consistent with the trend of pCO\textsubscript{2} and pH changes observed at the 2 min sample in the 1 year old lambs. However, we did not record respiratory rate and so could not determine if in fact hyperventilation did occur, and thus explain the unchanged pCO\textsubscript{2} and pH at the 5 min sample.

It is unclear why hypoxemia after FX injection only occurred in the lambs at 1, 6, 12 months of age but not at the other ages (3, 10 days and 3 months of age) and why it was more severe in male than female at 1 month of age. However, it appeared that FX-induced hypoxemia was coincident with milestone events in their development (i.e weaning, puberty). Other factor(s) which are associated with these events might contribute to the observed FX effects on respiratory and homeostasis in the lambs.

Behavior studies in which lambs were not restrained to the monitoring pen were performed in lambs at 3, 10 days and 1, 6 months of age. In general, at 1 and 6 months of age, lambs were significantly less active on the day after FX administration as shown from the activwatch data (Figs 5.9 & 5.14). The reduction in their overall activity could be due to
either the fall in the intensity of high score activities (such as standing moving, walking, suckling and playing or headbutting) and/or a reduction in the time they spent for these high score activities over the day. Using a combination of DVR observations and actiwatch score data, we found that the reduction in the 1 month old lambs after FX injection was a result of both the reduction in the intensity of standing quiet and a decline in the time lambs spent for standing moving, walking and successful suckling. One question is whether this reduction in activity following FX administration at 1 month has any relation to the normal reduction in lambs’ activity observed around 1 month of age due to weaning onset. Our developmental study in chapter 3 showed that the 1h rhythm due to suckling is a prominent rhythm in lambs for the first month of life. That means their main state for this first month of life was simply either sleeping or suckling. Our results suggested acute FX administration did not disrupt their feeding activity before 1 month since there were no significant differences in activities in experiments at 4 and 10 days of age. In parallel of the disappearance of the 1h rhythm around 1 month, a circadian rhythm and other ultradian rhythms (8h, 6h) emerged and became dominant (Fig 3.14). Since when this 1h rhythm disappeared, their total activity also decreased, it is tempting to postulate that this led to a decrease in activity in the FX experiments at 1 month. If this is the case, one would expect to see the same level of activity on the days after FX compared to that on the day of FX experiment. However, based on the pattern of decreased activity illustrated in Fig 5.8, this is less likely the case since the activity dropped sharply on the day of FX compared to the normal day and recovered quickly right on the next day after FX back to their previous higher level of activity and remain at that level days after. In addition, given the fact that lambs were disturbed more by blood sample collection on the day of control/FX experiment, their activity might have been expected to be
higher on those days compared to normal days rather than exhibiting a decrease in activity. However, the reduction in the lamb activity did not affect weight gain, probably because lambs at this age have already started to consume solid food and although they were less successful suckling on the day of FX experiment, they did attempt to suckle harder. Taken together, the effect of FX on the lambs’ activity at 1 month of age does not appear to be simply due to weaning although it could be a predisposing factor. Instead, the mechanism for the decreased locomotor activity in the lambs might be located at the neurotransmitter level. However, since FX has an antidepressant effect, the finding that activity decreased with FX administration was unexpected. Nevertheless, in clinical practice, it usually requires at least two weeks of treatment before FX can exert its antidepressant effect (Stahl, 1998). Thus, acute administration could elicit a paradoxical decrease in activity through an activation of 5-HT autoreceptor which causes an initial silencing of serotonergic neuron activity (Stahl, 1998). In addition, in our study FX was administered to healthy lambs, which might result in different effects on serotonergic tone compared to individuals with depressive symptoms.

At 6 months of age, a decrease in the lamb activity was a result of a shift in the time lambs spent standing moving to standing quiet and a reduction in the intensity of standing quiet activity. However, the FX exposed lambs were headbutting more on the day of FX experiment. That suggests that these lambs were quieter but also more aggressive with acute FX administration. In addition, we also observed jerky movements, which could represent for one of the symptoms of serotonin syndrome (eg: tremor or akathisia) (Boyer & Shannon, 2005), in two of the eight lambs in this study. Blood samples for FX measurement were not available for those lambs but it is possible that the effects of FX on the lambs’ behavior was due to an acute increase in 5-HT available at the synapse on serotonergic neurons which
regulate the affective behaviors and motor tone (Boyer & Shannon, 2005). Interestingly, the
decrease in activity following acute FX administration in the lambs at 6 months of age was
associated with a bodyweight loss. As a vast majority of their usual standing moving at this
age is attributed to food intake activity at fixed and ad libitum meals, their weight loss
appeared to be consistent with DVR observations showing a decline in the time they spent
with standing moving after FX injection. However, whether the FX-induced decrease in
activity interfered with eating and led to a loss in their body weight or alternatively whether
FX caused a reduction in pre-meal appetite and food intake which in turn led to a decrease in
activity remains unclear. Evidence from human and rodent studies have shown that FX has
an inhibitory role on feeding behavior. (Clifton et al., 1989; Lawton et al., 1995). Elevated
serotonin at the synapse following FX administration might interact with other neuropeptide
systems (neuropeptide Y, orexin, melanocortin) to alter feeding behaviors (Halford et
al., 2007).

Few studies have examined the HPA axis response following acute FX
administration. In rats, acute FX administration caused an increase in CRH, AVP, ACTH and
corticosterone (Fuller et al., 1976; Fuller et al., 1996; Gibbs & Vale, 1983). Similarly,
cortisol level increased following i.v infusion of citalopram (Bhagwagar et al., 2002) or after
a single dose of FX (von Bardeleben et al., 1989) in healthy humans. In sheep, Broadbear et
al., (2004) found an increase in ACTH and cortisol for 40 mins following acute subcutaneous
sertraline injection in gonadectomized ewes. However, we did not observe any significant
changes in ACTH and cortisol in lambs at 3 months and 1 year of age following acute FX
administration. The differences in our result and the Broadbear et al study could be due to the
route of administration and the sampling period. FX was given intravenously and the earliest
A sample of ACTH and cortisol after dosing was at 30 mins in our study while sertraline was given subcutaneously and blood was sampled every 10 mins in the other study. In addition, sex hormones could be a contributing factor in different results since in the subsequent study, Broadbear et al. (2004) did show that the increase in ACTH and cortisol in gonadectomized sheep was abolished by sex hormone treatment. Moreover, it is believed that the weak response of the HPA axis to acute SSRI administration is due to the activation of the negative feedback on the 5-HT$_{1A}$ autoreceptors (Raap & Van de Kar, 1999). The midbrain serotonergic system and HPA axis are highly interrelated; this is mediated by 5-HT$_{1A}$ receptors. (Porter et al., 2004) An overactive HPA axis which is not suppressed by dexamethasone administration is a potential long-term suicide predictor (Pompili et al., 2010). However, whether the finding that no changes in basal cortisol and ACTH level after acute FX administration in our lambs can indirectly imply that FX does not have effects on suicidal behavior in the adolescent humans is uncertain.

In conclusion, acute FX effects on cardiovascular-respiratory function and behavior were seen in some, but not all age groups. The effect tended to occur at milestones events of development such as weaning, puberty and adulthood. Gender difference in FX effect on the cardiovascular-respiratory function was observed in the older lambs with more profound FX effects in males.
6. Postnatal Outcomes in Lambs Exposed Antenatally to FX

6.1 Introduction:

Up to 20% of pregnant women might experience mood disturbances at any stage of their pregnancy and thus might require antidepressant therapy (Burt & Stein, 2002). SSRIs have been widely used as a pharmacological treatment for depression during pregnancy with moderate to severe symptoms thanks to their relatively benign side effects profile compared to other earlier antidepressants (MAOIs, TCA). Indeed, over the last decade, SSRI use during pregnancy has increased from 1.5% in 1996 to 6.4% in 2004 (Andrade et al., 2008). A similar increase to approximately 7% was also reported by Wichman et al., (2008). As the name suggests, SSRIs exert their actions by inhibiting serotonin recycling through the serotonin transporter, thereby increasing the amount of serotonin available in the synaptic cleft, and altering serotonergic transmission (Oberlander et al., 2009). It has been well-established that in adults serotonin is a neurotransmitter which plays a key role in modulating emotion, cognition, attention, arousal and stress responses. In the developing brain, central serotonin also acts as a growth factor regulating serotonergic neuronal growth and the development of other related neural systems (Whitaker-Azmitia, 2001). Since SSRIs are capable of crossing the placenta (Hendrick et al., 2003; Kim et al., 2004; Kim et al., 2006; Rampono et al., 2009), there is a significant risk for the fetus from exposure to SSRIs during such critical phases of their neurodevelopment. Studies in human and sheep have demonstrated SSRIs effects on the fetuses. Mulder et al reported a disrupted quiet sleep reflected by the abnormal phenomenon of continual bodily activity during non-REM sleep periods in human fetuses in utero exposed to SSRIs (Mulder et al., 2011). Similarly,
Salisbury et al. (2009) demonstrated increased jerky movements and decreased fetal breathing movements in SSRIs-exposed fetuses at 26 and 36 weeks gestational age as compared to the non exposed group. This is in agreement with previous findings from our group which showed an alteration of fetal behavioral state and neuroendocrine function in fetal lambs with chronic FX infusion to the mothers during late gestation (Morrison et al., 2001; Morrison, 2001; Morrison et al., 2004). Moreover, a recent study also reported an alteration of fetal middle cerebral artery flow characteristics and blunted fetal heart rate variability across the day in 36 week human fetuses exposed to SSRIs as compared to the control group (Rurak et al., 2011). These findings raise an important question whether these fetal neurobehavioral effects persist beyond the perinatal period into childhood, forming a concept of behavioral teratology in the neonate resulting from fetal exposure to SSRIs. A blunted pain response in terms of facial characteristics and cardiac responses in prenatally SRIs-exposed neonates has been reported by Oberlander et al., (2002). This alteration in pain reactivity was still apparent at 2 months of age in the fetal SSRIs-exposed infants, even after controlling for drug level and maternal mood (Oberlander et al., 2005). In addition, a lower basal cortisol level in the evening was also observed at 3 month of age in prenatal SSRIs-exposed infants (Oberlander et al., 2008).

Yet, it is still inconclusive regarding the long-term effects of prenatal SSRI exposure on neurobehavioral development in early childhood. Some studies showed a delayed in psychomotor and behavioral development in prenatally SSRIs-exposed children (Casper et al., 2003; Pedersen et al., 2010) while others failed to observe these differences (Heikkinen et al., 2002; Heikkinen et al., 2003; Nulman et al., 1997; Nulman et al., 2002). Nevertheless, a challenge for this type of research in human is that it is impossible to distinguish between the
effects of SSRIs and the effects of maternal psychiatric illness on the outcomes. Moreover, the postnatal social environment of a depressed mother could also have negative influences on child development after SSRI exposure (Oberlander et al., 2008). These issues might be resolved with animal studies. Since lambs are precocious at birth and have a very similar physiological characteristics to the human, it appears to be a good model to control for the confounding factors present in human studies. In this study, we chose to investigate the postnatal outcomes including birth weight, postnatal weight gain, behavioral development (neonatal behavior, sleep-rest cycles/activities, feeding activity) and cardiovascular changes (heart rate, HRV, sP_o2) in lambs exposed to FX during late gestation.

6.2 Methods:

6.2.1 Animal And Catheter Implantation:

Seven time-bred pregnant ewes (Dorset/Suffolk Cross) were bred following the same protocol in our laboratory described previously in Chapter 2. Ewes were moved to Centre of Comparative Medicine (CCM), UBC 2-3 weeks before their due date (term = 147 days) to allow for acclimation.

They were housed in groups of two or three in the holding pens of approximately 10x12 feet and were able to go outdoors through a small door in their pen until the end of their pregnancy. Approaching their due date, ewes were kept indoors under natural light conditions. Ewes were fed following the protocol in the CCM: 300 g grain + 450 g alfalfa cubes at 7:00 to 8:00 am, 2 flakes of hay at 8:00 to 9:00 am and 2 flakes of hay at 2:00 pm. The study involved 2 groups of lambs: one group of 7 lambs who were exposed to FX antenatally and another control group of 2 lambs born in CCM and 10 control lambs
previously born in the CFRI without any perinatal FX exposure for the first 11 days after birth.

A heparin-bonded 5.0 French polyurethane catheter (Instech Solomon, Plymouth Meeting, PA) was percutaneously implanted to the ewe’s jugular vein using a 13G sterile stainless steel needle (Instech Solomon, Plymouth Meeting, PA) under local analgesia with Lidocaine HCl 2% (Bimeda-MTC, Animal Health Inc, Cambridge, Ontario). The catheter was implanted at an average gestational age of 131 ± 1 d in 5 pregnant ewes. The catheter was inserted approximately 20cm and proper functioning of the catheter was ensured before it was secured to the skin by a 2-0 Nylon suture. The external part of the catheter was closed with a sterile metal pin at the end and kept in a small ziplock bag and safety pinned under a bandage around the animal’s neck. The catheters were flushed every day with heparinized saline and served for subsequent daily FX injection and blood samplings at delivery.

6.2.2 Experimental Protocol:

Beginning one day after the catheter insertion, the pregnant ewes were given daily 50 mg of FX (5 ml of 10 mg/ml) via the inserted catheter. FX was injected every day in the morning around 10:00-11:00 am for 14 days or until the ewes gave birth, whichever came earlier. Signs of imminent parturition in the ewe (bilateral concave flanks, restlessness, pawing at the pen floor, moving in circles and looking toward the back) and the lambs’ birth were observed by DVR from infrared cameras which will be described more detail in the section DVR and behavioral evaluation. Every attempt was made to measure the lambs’ weight, head circumference, tissue spO2 and to obtain a jugular vein blood sample (via percutaneous puncture) as soon as they were born. They were dried with a towel, sexed,
weighed and had on actiwatch around their neck right after birth (more details about actiwatch in the section Actiwatch/Rest activity monitoring). Lambs were monitored for 14 days and daily weight, tissue spO2, head circumference and abdominal circumference were measured. They were housed in a smaller pen with their mothers (8x8 feet) to facilitate ewe-lamb bonding until the end of the monitoring period. On postnatal day 2, 5, 10 and 14, ECG measurements and blood samplings for FX, NFX concentration were performed (more details in the sections below).

6.2.3 Digital Video Recording And Behavioral Evaluation:

Two infrared video cameras were installed at two corners of each pen in three pens in the sheep holding room and connected to a digital video recording system on a computer which was located on the hallway just outside the sheep pen. Another mobile infrared camera was used to directly observe and record the lambs’ behavior while they were on the sling for ECG measurement. The infrared function allowed the recording and monitoring of the ewe and lamb’s behavior at night without interrupting their circadian rhythm. These cameras could also be accessed remotely from home and the video records could be analyzed off-line.

Lamb’s activities were monitored continuously at least 14 days to determine any abnormal behavior changes in the lambs. The number of daily sleep and suckling bouts were identified from the DVR observations for each lamb from postnatal day 2 until day 10. The total activity bout was defined as a sum of suckling bout and active bout without suckling during 24h of daily observation.
6.2.4 Actiwatch Rest/Activity Monitoring:

A similar method and analysis were used as described in chapter 2. The lambs’ activities were recorded for at least 14 days after birth.

6.2.5 Powerlab Data Acquisition System:

ECG measurement was conducted on the lambs for 1 hour on postnatal day 2, 5, 10 and 14. Lambs were taken to the procedure room for ~ 20 minutes to glue on surface ECG electrodes. Three ECG snap electrodes were attached to the skin on the back and bilaterally on the chest and secured in place by adhesive bandages. Lambs were put in the cloth sling, which located right in front of the holding pen from where the lamb came in full view of the mother, and the ECG electrodes were connected to a polygraph recorder (model TA-4000, Gould Instrument Systems Inc, Valley View, OH) and to a Powerlab data acquisition system (AD instrument, Mountain View, CA, USA) by three snap leads to record their electrocardiograph. HRV data were analyzed using the same method as described in chapter 2.

6.2.6 Pulse Oximeter: Tissue SpO₂ And Heart Rate:

Daily tissue SpO₂ and heart rate were measured using a pulse oximeter (Nonin Medical Inc, Plymouth, MN, USA), with the oximeter sensor clipped on the ear of the lamb. The lamb’s ear was shaved for a better signal. The value is an average of 5 minutes monitoring.
6.2.7 FX And NFX Concentration:

Blood samples were also collected after birth from the ewes and lambs and on postnatal day 2, 5, 10 and 14 from the lambs for fluoxetine measurements. Samples were taken from the ewes via the inserted jugular vein catheters. Lambs were taken to the procedure room and the wool along the jugular vein was shaved. The skin was disinfected with alcohol 70% and Xylocain 2% gel was applied. The jugular vein was accessed by percutaneous puncture. Approximately 4 ml of blood was collected in Lithium Heparin (68USP units) vacutainer tubes (BD Vacutainer, NJ, USA) and centrifuged for 20 min at the speed of 4000rpm in a Centrifuge 5810R (Eppendorf, ON, Canada). Plasma was then transferred to glass tubes for fluoxetine samples and stored at 0°C until analysis. Lambs usually slept or lay quiet while blood was collected and were returned to their mother immediately.

FX and NFX concentrations were measured using a validated liquid chromatography with tandem mass spectrometry method (LC/MS/MS) (Chow et al., 2011) as described in Chapter 2. The sample volume used for each enantiomer (R)-, (S)- fluoxetine and (R)-, (S)- norfluoxetine was 250 µl. The limit of quantitation was 1 ng/ml.

6.2.8 Statistical Analysis:

The areas under the curve (AUC) were calculated for daily variables (weight, sP$_{O_2}$, heart rate) by applying the formula described by (Altman, 1991). Then the AUC of each variable were compared between the control and FX group using the unpaired t-test. The effect of FX exposure and sex on gestational age, birth weight and the time for the lambs to first stand, walk and suckle were determined using 2 way ANOVA. Two-way ANOVA with
repeated measures was used to determine the effect of FX exposure and sex on activities (actiwatch and DVR observations) for the first 10 days of life and on HRV variables on postnatal day 2, 5, 10 and 14 in the lambs with multiple comparison correction (Bonferoni t-test). All data were analyzed using the Sigma Plot 11 statistics software (Systat Software, Inc; San Jose, CA, USA).

### 6.3 Results:

In the FX group, ewes were given daily 50mg FX i.v injection for approximately 2 weeks before their due date. The average duration of antenatal FX exposure in the lambs was 12 ± 1.3 days.

Table 6.1 describes the gestational age (GA) and birth weight of the lambs included in the study in this chapter. There were no significant differences in GA at delivery between the control and FX-exposed group. However, it was noticed that 2 ewes that delivered singleton lambs in the FX group had a shorter gestational length (141 and 142 days) as compared to the normal GA of 147 days at term. In the control group, two lambs from the triplet also had a shorter gestational length (142 days). There was also no significant difference in birth weight between control and FX exposed lambs.

Figure 6.1 illustrates daily postnatal weight gain for the first two weeks of life in the control and FX groups. No significant differences in postnatal weight gain were found between the two groups.
<table>
<thead>
<tr>
<th>Control lamb</th>
<th>GA at birth (days)</th>
<th>Birth weight (gram)</th>
<th>Lamb number</th>
<th>FX-exposed Lamb</th>
<th>GA at birth (days)</th>
<th>Birth weight (gram)</th>
<th>Lamb number</th>
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<td>146</td>
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<td>L752M1</td>
<td>148</td>
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<tr>
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<td>142</td>
<td>3300</td>
<td>Triplet</td>
<td>L752M2</td>
<td>148</td>
<td>5000</td>
<td>Twin</td>
</tr>
<tr>
<td>L310M3</td>
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</tr>
<tr>
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<td><strong>146</strong></td>
<td><strong>4871</strong></td>
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<td></td>
<td>1.1</td>
<td>388</td>
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<td></td>
</tr>
</tbody>
</table>

Table 6.1: Gestational age and birth weight in the control and antenatally FX-exposed lambs
M = male, F = female,
1,2,3 after M,F indicates the order of delivery in twin and triplet lambs

![FIG. 6.1: (A) Daily weight in the control (n=7) and prenatal FX-exposed lambs (n=7) from postnatal day 1 (day of birth) to postnatal day 14.](image)

Plasma FX, norfluoxetine (NFX) enantiomer concentrations in the ewes (n = 5) at delivery and in the lambs (n = 7) at birth, on postnatal day 2, 5, 10 and 14 are presented in Figure 6.2. At delivery, the mean maternal FX and NFX level were 627.9 ± 249 and 66.6 ± 14.4 ng/ml, respectively. Mean plasma FX level was quite low in the lambs at birth, with an average of 17.3 ± 10.9 ng/ml and decreased progressively thereafter. It became undetectable
after postnatal day 5. NFX was undetectable in the lambs from birth to postnatal day 14, which suggests that lambs of this age are unable to metabolize the drug. The relationship between the lamb: ewe ratio for FX concentration and the duration from the last maternal FX dose is shown in figure 6.3. The ratio was highest in the lambs that had the shortest duration from the last FX dose, within 8 hours and it fell rapidly thereafter.

Figure 6.4 illustrates the daily tissue sP02 and heart rate values obtained from pulse oximeter from postnatal day 1 to 14 in two control lambs and seven FX-exposed lambs. The results were similar between the two groups. sP02 over 14 postnatal days fluctuated within the normal range 90-100% while heart rate decreased progressively in both control and FX lambs from an average of 234 ± 17 bpm and 230 ± 19 bpm on postnatal day 2 to 187± 6 bpm and 200 ± 8 bpm on postnatal day 14, respectively.
FIG. 6.2: FX, (R)-FX, (S)-FX and NFX, (R-)NFX, (S)-NFX concentrations in the prenatally FX-exposed ewes (n= 5) and lambs (n=7). Samples were taken at birth in both ewes and lambs and on postnatal day 2, 5, 10 and 14 only in lambs.
PND = Postnatal day
[NFX] levels in the lambs were lower than the limit of quantitation (1ng/ml)
FIG 6.3: The relationship between the lamb:ewe plasma FX concentration ratio and the time from the last FX dose (hours) in the ewe.

A

B

FIG 6.4: Daily (A) tissue spO2 and (B) heart rate measured by pulse oximeter in the control (n=2) and prenatal FX-exposed lambs (n =7) from postnatal day 1 (day of birth) to postnatal day 14.
The lambs with prenatal FX exposure were more active than the control lambs, which was reflected by faster first time activities (stand-walk-suck) and an increase in subsequent activities obtained from actiwatch and DRV observations. (Figs. 6.5-6.7) It took the FX lambs \( n = 7 \) only half the amount of time as compared to the control lambs \( n = 37 \) to achieve their first time activities. While the control lambs started to stand at 32 ± 2 mins after birth, the FX-exposed lambs did this at 17 ± 3 mins, \( p = 0.017 \). In general, the control lambs attempted to suck within the first one hour of life (56 ± 5 mins) whereas the FX-exposed lambs did this within the first half hour (29 ± 6 mins), \( p = 0.006 \). There was no sex difference in the first time activities.

Figure 6.6 shows the activity variables from actiwatch monitoring from postnatal day 2 (the first full day after birth) to postnatal day 14 in seven control and seven FX-exposed lambs. The lambs’ activity increased over days in both control and FX groups, but it was increasingly greater in the FX group than the control group. Total activity score and active bout per days were significantly higher in the FX-exposed group from postnatal day 4 to 14 as compared to that on each corresponding day in the control group, while they were comparable between the 2 groups on postnatal day 2 to 3. Mean score in active periods were also higher in the FX group vs control group, but not significantly different. In contrast, episode duration was significantly lower in the FX group compare to the control on postnatal day 5, 9, 10 and 12. The percentage of time moving was significantly higher in the FX group compare to control on postnatal day 12. A reverse situation occurred with the percentage of time immobile.

From DVR observations, sleep, suckling and non-suckling activity bouts were determined from postnatal day 2 to day 10 in the same seven control and FX-exposed lambs.
and are shown in figure 6.7. The number of total activity bouts is the sum of suckling bouts and non-suckling activity bouts. Prenatal FX-exposed lambs appeared to have a significantly lower number of suckling bouts than the control lambs on postnatal day 2, 5 and 6. However, the AUC of non-suckling activity bouts for 10 postnatal days was significantly higher in the FX-exposed lambs compared to the control. Therefore, the number of total activity bouts (= suckling bouts + non-suckling active bouts) was comparable between the two groups until postnatal day 9 and so was the number of sleep bouts. On postnatal day 10, the number of total activity bouts was significantly lower in the FX group than the control, and this difference is due to the significantly lower number of sleep bouts in the FX group than the control group. There were no significant differences in the number of suckling bouts between the two groups on postnatal day 10. Collectively, the results suggested that lambs exposed to FX prenatally were more active than the control group, but the increase in their activity did not contribute to their feeding behavior and thus, their postnatal weight gain. In addition, we did not observe any other abnormal behaviors in the FX-exposed lambs for the first 14 days of life.

Interestingly, the high level of activity appeared to exist in the FX-exposed lambs even at a low level of FX and undetectable NFX level and this high level of activity still persisted on postnatal day 10, 14 when the level of FX was no longer detectable. (Figs. 6.2 & 6.6)

HRV was only significantly different on postnatal day 2 and was comparable on postnatal days 5, 10 and 14 between FX and control groups. The time domain variables of HRV (SDNN, SD Delta NN, RMSSD) was significantly lower in the FX-exposed lambs than the control lambs at both 30 and 60 mins of the recording period. Similarly, the total power
was significantly lower in the FX group but no significant differences were found with LF power, HF power and LF/HF ratio. (Fig. 6.8) Total activity score from actiwatch during the 1h period of ECG monitoring also showed a lower activity level in the FX group compared to the control group, however the difference in total activity score between the two groups was not statistically significant. (Fig. 6.8)

**FIG. 6.5: Neonatal behavior (first time to stand, walk, suck immediately after birth) in the control lambs (n = 37) and prenatal FX-exposed lambs (n = 7).**

Asterisk (*) indicates significant differences between the control (open bars) and FX group (black bars), p < 0.05.
FIG. 6.6: Daily activity variables (active bouts per days, total activity score, mean score in active, episode duration, % time mobile, % time immobile) obtained from the actiwatches in the control (opened, diamond symbols) and prenatal FX-exposed lambs (closed, circle symbols) from postnatal day 2 (the first full day) to postnatal day 14.

* indicates significant differences in the corresponding values between the control and FX group,
+ indicates significant differences from the day 2 within the control group,
^ indicates significant differences from the day 2 within the FX group.
Stats: 2 way ANOVA repeated measures with multiple comparison correction Bonferroni t-test.
FIG. 6.7: Daily sleep, suckling and non-suckling activity bouts obtained from DVR observations in the control (open, diamond symbols) and prenatal FX-exposed lambs (closed, circle symbols) from postnatal day 2 (the first full day) to postnatal day 10.

*  indicates significant differences in the corresponding values between the control and FX group,
+  indicates significant differences from the day 2 within the control group,
^  indicates significant differences from the day 2 within the FX group.

AUC non-suckling activity bout were significantly higher in the FX group (p = 0.04)
FIG. 6.8: HRV (time domain: SDNN, SD Delta NN, RMSSD; frequency domain: total power, LF power, HF power, LF/HF) on postnatal day 2 in control group (diamond symbol, dotted line, n = 8) and prenatal FX-exposed lambs (circle symbol, solid line, n = 7).

* indicates significant differences between the corresponding values in the control and FX groups.
+ indicates significant differences between the values at 30 and 60 min within the control group,
^ indicates significant differences between the values at 30 and 60 mins within the FX group,
p < 0.05. Stats: 2 way ANOVA repeated measures with multiple comparison correction Bonferroni t-test
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Table 6.2: Mean ± SEM of HRV values for the first 30 mins of the experiment in the lambs on day 2 and the changes from day 5, 10, 14 compared with the values on day 2

(*) indicates significant differences between FX and control group
6.4 Discussion:

In this study we found that the lambs with prenatal FX exposure were more active than the control lambs. This was indicated by a faster first time activities (stand-walk-suck) which represent important neonatal behaviors in sheep and by an increase in subsequent activities obtained from actiwatch and DRV observations. HRV variables on postnatal day 2 were lower in the FX-exposed lambs than the control lambs. There were no significant differences in gestational age and birth weight between the two groups. Daily postnatal weight gain, tissue sPO2 and heart rate obtained from pulse oximeter were also comparable between the two groups.

Maternal FX level at delivery in our study was higher than that reported in the human (Kim et al., 2006) and in a previous sheep study in our lab (Morrison, 2001; Morrison et al., 2002). As was discussed in previous papers (Alfaro et al, 2000; Kim et al., 2004), since CYP2D6 is a key enzyme involved in FX metabolism, the maternal FX concentration might be a result of FX-mediated CYP2D6 inhibition. From the literature, the level of FX in the neonates varies between studies and depends on the postnatal day on which FX was measured (Kim et al., 2006; Kwon & Lefkowitz, 2008; Rampono et al., 2004; Spencer, 1993). One case report gave a FX concentration at delivery of 26 ng/ml and undetectable levels at 96h thereafter in an infant, which is similar to our current study (Spencer, 1993). The FX level in our lambs at birth (mean = 17.3 ± 10.9ng/ml) was lower than that measured in umbilical cord vein at delivery (mean = 41.3, 95%CI = 16.9, 59.8) from 9 pregnant women on FX therapy in another study (Kim et al., 2006). Interestingly, NFX was also not detectable at birth and the days after, which suggests that fetal and newborn lambs are unable to metabolize the drug. Our result is in agreement with previous study in our lab in which NFX
was also undetectable in the lamb fetuses after fetal FX administration, in contrast with the mother (Kim et al., 2004). Since the lamb was not able to metabolize the drug, one would expect to see a higher level of FX in the lambs than it was. However, we also found that there was a relationship between the ratio of lamb:ewe FX concentration at delivery and the duration from maternal the last FX dose (Fig. 6.3), indicating a link between maternal and fetal FX level. This suggests that following the period of maternal FX administration, the elimination of FX from the fetus was largely the result of transfer of the drug back to the mother and maternal elimination.

In the human newborn, motor activity is one of the indices of biobehavioral assessment. In sheep, neonatal behavior is one of the many factors contributing to lamb survival. Lambs that are slow in standing and suckling after birth have a lower survival chance. (Dwyer et al., 2005) Moreover, first time activities such as standing, walking and suckling are indicators of neonatal adaptation to postnatal life (Dwyer, 2008b). In the present study, we found that FX-exposed lambs were faster to stand, walk and suckle as compared to the control lambs. This suggests that FX-exposed newborn lambs do not appear to have any difficulty in neonatal adaptation. Our result was different from a human study reported by (Pedersen et al., 2010) in which the first time to sit and walk was delayed in infants exposed to antidepressants during second and third trimester of gestation. However, in their study different classes of antidepressants (SSRIs, TCAs) were included.

For the first 2 weeks of life, there was a progressive increase in the level of lambs’ activity in both the FX-exposed and control groups, which is similar to our developmental study presented in chapter 3 and the results reported by Rurak et al., (2008). However, FX-exposed lambs were more active than the control lambs, reflected in significantly higher
values for both the number of active bouts per day and total activity score between FX and control groups from postnatal day 4 to 14. Although the FX-exposed lambs were more active, it appeared that their movement bouts were shorter than the control lambs, which was reflected by significantly lower values for episode duration in the FX group on postnatal day 5, 9, 10 and 12. Similar to our findings, there is one study in human infants that also found prenatally SSRIs exposed infants increase their motor activity during sleep compared to the control (Zeskind & Stephens, 2004), however the difference in activities between the groups in their study was attributed to the shorter gestational age in the SSRIs-exposed infants. In our present study, mean gestational age at delivery was similar between the two groups, suggesting that the increase in the level of activity in the FX-exposed lambs is independent of gestational age. Moreover, in our study changes in activity in FX-exposed lambs were not confounded by additive effects of maternal depressive status or stress during gestation as in human study. Instead, in sheep ewe and lamb bonding after birth is the key factor affecting behavior (Dwyer, 2008b) and it appeared that all the ewe-lamb bonding in our sheep was successfully established soon after delivery. As it was shown in chapter 3, the more vigorous activity of the lambs for the first month of life was due to their feeding activity. However, observation from DVR revealed that the increase in the level of activity in FX-exposed lambs was not due to the increase in suckling activity, which is the major activity in lambs in the first month of life. Instead, the increase in the level of activity in FX-exposed lambs was due to the higher in the number of non-suckling activity bouts in the FX group reflected by significant differences in AUC of non-suckling activity bouts between FX and control groups. This is consistent with our finding that there was no significant difference in postnatal weight gain between the two groups. From DVR observation, on postnatal day 10,
FX-exposed lambs had significantly lower number of sleep bouts as compared to the control, suggesting they sleep for longer duration than the control. However, on this postnatal day 10, FX-exposed lambs had a shorter episode duration from actiwatch measurement. This suggested that even though the FX-exposed lambs slept more, their sleep was more disrupted than the control. However we did not observe abnormal behavior in the prenatal FX-exposed lambs during the first 10 postnatal days.

As was mentioned previously, the mechanisms of poor neonatal adaptation in newborns exposed to SSRIs during gestation are still a matter of debate. From our study it is apparent that the high level of activity in our FX-exposed lambs was not due to FX toxicity since the average of FX level in the lambs at delivery was lower than the minimum range of adult human therapeutic level, which is 40-250 ng/ml (Krogh CME, 1995) and NFX, a pharmacologically active metabolite, was also undetectable. The question is whether this is a result of a withdrawal effect. In adults with opioid-withdrawal syndrome, the symptoms can last for weeks after abrupt discontinuation of the drug involved. Methadone appears to have a longest duration withdrawal symptoms among opioids after acute discontinuation with the severity of symptoms and can last up to 16 days after the last dose. (Kosten & O’Connor, 2003) The argument is that in my study, the increased activity in the lambs in the FX groups was observed up to 2 weeks after birth, which can be similar the duration of opioid-withdrawal syndrome in the human. Longer follow-up of the increased activity in the antenatally FX-exposed lambs will help to rule out the mechanism. That is, if the increased activity in the lambs still persisted after 2 weeks, it is less likely due to the withdrawal syndrome.
However, I believe that the increased in activity in the FX-exposed lambs is less likely due to withdrawal syndrome. Rather, it is due to a change in the fetal brain development since evidence from human and sheep studies have shown that the alteration in activity resulting from antenatal SSRI exposure seems to appear in the fetuses before the drug exposure ended and continue until the postnatal period. Mulder et al., (2011) reported an abnormal phenomenon of continual bodily activity during non-REM sleep in SSRIs-exposed fetuses. Similarly, another study found SSRIs-exposed fetuses at 26 weeks of gestation had more jerky movements than the control and MDD/no SSRIs exposure groups (Salisbury et al., 2006). In sheep, there were also increased eye movement and fetal breathing movement with 8 day FX i.v infusion. (Morrison et al, 2001) Collectively, these findings from our study and others support the hypothesis that a possible underlying mechanism for the high level of activity in the FX-exposed lambs is SSRIs-induced changes in fetal brain development. Further evidence for this mechanism comes from studies showing a lower cord blood level of calcium-binding protein S100B, a biomarker of early brain development and central serotonergic function, in SSRI-exposed neonates (Pawluski et al., 2009). It is known that the astroglial 5-HT₁A receptor is activated by an increase of synaptic 5-HT after SSRI administration, and this will stimulate a release of S100B protein, which in turn mediates the growth and survival of neurons (Whitaker-Azmitia et al., 1990; Whitaker-Azmitia, 2001).

In our study we also found that the time domain of HRV (SDNN, SD Delta NN, RMSSSD) and total power were significantly lower in the prenatally FX-exposed lambs on postnatal day 2 compared to the control lambs. It is known that changes in HRV are usually associated with physical activity (Nakamura et al, 1993; Voss et al, 2002; Warren et al, 1997). On postnatal day 2, the 24 h total activity score were comparable between control and
Further analysis of the total activity score for during the 1h of HRV measurement on postnatal day 2 did not show significant differences in activity score between the two groups even though the activity score during this 1h of HRV measurement was lower in the FX-exposed group (Fig. 6.8). Thus, it is uncertain whether activity plays a role in the lower level of HRV in the FX-exposed group. There are limited data on HRV changes in the newborns after prenatal SSRIs exposure in the literature, although two studies did report a similar trend of lower HRV rhythms in the newborns or a blunted facial and HRV response to pain in 2 month-old infants after prenatal SSRI exposure (Oberlander et al., 2005; Zeskind & Stephens, 2004). Since changes in HRV in our study only occurred in the time domain but not in frequency domain indices, it is uncertain if the changes come from sympathetic or parasympathetic systems. Changes in HRV also occurred in SSRI-exposed human fetuses. A recent study has reported that short and long-term variation of fetal heart rate, accelerations and duration of high variability episodes did not change during the day and were lower in the SSRIs-exposed fetuses compared to a significant increase during the day in the control group. (Rurak et al., 2011) Taken together, our results and the other findings showed a similar low HRV or blunted HRV response in the SSRIs-exposed infants which originate from the fetal period with SSRI exposure.

The major effects of prenatal FX exposure on the postnatal lambs in the present study consisting of an increase in activity at birth and from postnatal day 4 to 14 and low HRV on postnatal day 2. There was a lack of FX effects on gestational length, birth weight, postnatal weight gain. Heart rate and tissue SpO2 were also not different between the control and FX groups. Our results are different from other previous human studies which reported an increase in preterm delivery, shorter gestational length, lower birth weight and postnatal
weight gain in third trimester FX exposure fetuses. (Chambers et al., 1996; Goldstein, 1995; Hendrick et al., 2003; Lewis et al., 2010; Oberlander et al., 2006; Suri et al., 2007). However, our results were consistent with those found in previous sheep study in our lab (Morrison, 2001; Morrison et al., 2002) except for postnatal weight gain which was not obtained in Morrison et al study. As was discussed before, different results between human studies and ours are probably due to the presence of confounding factors in human studies but not in animal studies, for example the maternal depressive illness and other associated risk factor such as smoking and alcohol abuse. Even though maternal illness severity was accounted for in the Oberlander et al and Suri et al studies, it is possible that the cumulative effect of FX exposure over most or all of pregnancy in the human studies, compared to the short-term late gestation sheep studies, resulted in the observed lower birth weight and gestation length.

In summary, from this study we found that the newborn lambs were not able to metabolize FX, thus FX metabolise and clearance in the fetal lambs were likely achieved via the mother. FX-exposed lambs were more active than the control lambs at birth and from postnatal day 4 to 14 and had a lower HRV on postnatal day 2. Together with the FX measurement after birth and on postnatal day 2, 5, 10, 14, the increase in activity in the postnatal lambs exposed to the drug in utero suggests the most likely mechanism for these effects is alteration in fetal brain development, due to the importance of serotonin in brain maturation. However, DVR observations showed the increase in activity score was not due to suckling activity but due to a decrease and disrupted sleep activity. Consistently, there was no difference in postnatal weight gain in the two groups. And there was also a lack of FX effect in gestational age, birth weight, heart rate and tissue SpO2.
7. Summary and Conclusions

Depression has been recognized as one of the most burdensome disorders during pregnancy, affecting at least 10-15% of pregnant women (Bhatia & Bhatia, 1999; Nonacs & Cohen, 2003). Pharmacological treatment is a norm. And one third of pregnant women suffering from depression take SSRIs as antidepressants (Oberlander et al., 2006). There is an increasing use of FX and other SSRIs during pregnancy (Andrade et al., 2008). A considerable amount of attention has been paid in the literature to the assessment of birth outcomes following in utero FX exposure during late gestation. However, studies that focus on the mechanisms underlying the observed outcomes are relatively sparse. Several mechanisms have been proposed but there is a lack of supporting evidence. Previous experiments in our lab were conducted with 8 day maternal FX i.v infusion to expose late gestation sheep fetuses to FX and primarily focused on the fetal responses and the underlying mechanisms. (Morrison et al., 2001; Morrison, 2001; Morrison et al., 2002; Morrison et al., 2004; Morrison et al., 2005). The current study is in part a continuation of the previous studies, focusing on the effects of maternal FX treatment on postnatal lambs and the underlying mechanisms of poor neonatal adaptation. As noted elsewhere in this thesis, this syndrome occurs in 30% of newborns prenatally exposed to SSRIs (Koren et al., 2009). In an attempt to elucidate the underlying mechanisms for poor neonatal adaptation, I set out to determine if SSRI toxicity is the responsible mechanism, FX was given to the newborn lambs acutely on approximately postnatal day 4 in an attempt to reach the plasma concentration levels observed in human infants at the same age. This was successful in that the level of FX in the newborn lambs fell within the range of plasma FX concentration measured in human infants sampled on postnatal day 2 following in utero exposure to the drug. However, our
protocol of acute, single dose FX administration differs from the clinical setting since human infants are exposed to FX throughout all or most of pregnancy. Similarly, when FX is prescribed to treat depression in children and adolescents, it is given chronically with multiple, daily doses. However, in my subsequent study to investigate the effects of maternal FX treatment on the postnatal lambs, FX was given to the pregnant ewes for the last ~2 weeks of pregnancy, but again for a relatively short period of gestation. The lambs were exposed in utero to FX at clinically relevant concentrations, followed by evaluation of birth weight, postnatal weight gain, behavioral development and cardiovascular function. In the previous studies in our lab, FX was administered to the ewes using infusion pump and thus required surgical instrumentation of the ewes and fetuses, and the restraint of the ewes in a monitoring pen during the infusion and monitoring period (Morrison et al., 2001; Morrison et al., 2002; Morrison et al., 2004; Morrison et al., 2005). Thanks to the procedure to implant jugular vein catheter subcutaneously in the pregnant ewes, we were able to successfully administer FX allowing the ewes to moving freely in their pen. FX was given i.v to the pregnant ewes once daily to mimic the human dosing with pulsatile FX treatment. Oral administration of the drug, which is the norm in humans, was not attempted in this and previous studies because of the concern about the impact of ruminant nutrition on absorption of orally administered drugs in sheep.

The following conclusions can be made from the results of the current study:

1. The results from the developmental study showed a decrease in heart rate, increase in arterial pressure and maturation of the Autonomic Nervous System (ANS) system as the lambs aged. pH, pO2, O2 saturation increase, while pCO2, glucose, lactate, Hb, urine flow rate decrease with age. The plasma cortisol level is high around the time
of birth and decreases progressively thereafter. The 1h activity rhythm, which only exists and is dominant over the first month of life is associated with suckling. The changes in some of the physiological variables including heart rate, SDNN, glucose, pO₂, urine flow rate occurred around the window of weaning time. Weaning was defined in our study as the time window between when lambs started to first consume solid food to when the 1h rhythm due to suckling disappeared. Alternatively, the weaning time could be determined by measuring the level of plasma ketone bodies (acetoacetic acid and 3-hydroxybutyric acid) in the lambs. Even though the neonatal rumen epithelium is not ketogenic, the ruminal epithelium in a mature ruminant animal is able to produce ketone bodies, which is supplied by the microbial fermentation of feed (Lane et al., 2002). In addition, the developmental data also showed that at 3 days of age male lambs are slightly more acidotic than female lambs. This is similar to the findings of Ishak et al in lambs delivered preterm at 133 d, although in this study male lambs exhibited other differences from female lambs, including a higher pCO₂, blood lactate and glucose concentrations and higher arterial pressure (Ishak et al., 2012). In preterm lambs, this reduced postnatal cardio-respiratory adaptation in male lambs could contribute to a higher mortality rate in male than female neonates, especially related to respiratory distress syndrome (Ingemarsson et al., 1997; Ingemarsson, 2003; Khoury et al., 1985). The current data suggest even with delivery at term, respiratory function or acid-base regulatory mechanisms in male lambs are less effective than in females, although the sex differences are much less than with preterm birth. At 3 days of age, LF power were also higher in male than female lambs, possibly due to an influence of testosterone, which present in the
male lambs by 36h after birth (Savoie et al., 1981), on the ANS or due to an ANS response to an unfavorable environment (i.e. lower pH level).

However, one limitation of the developmental study is that the variables were not measured on a daily basis, and thus cannot provide an exact time when the changes occur as well as the pattern of changes between the experimental intervals. In addition, the lambs included in the developmental study were also used for a longitudinal study of FX effects, although only as control animals.

2. Acute FX administration did not cause any significant effects on the feeding behavior, rest-activity cycles, cardiovascular and respiratory functions and the cortisol level in the ~ 4 day old postnatal lambs. Even though arterial blood pressure increased for the first 10 mins and heart rate decreased at 2 mins after FX injection, the changes were too minor and transient to have any clinical or physiological significance. Therefore, it is less likely that SSRIs toxicity is the mechanism underlying poor neonatal adaptation in human infants exposed to this drug and other SSRIs in utero. This is further confirmed by the subsequent results.

3. Acute FX administration did not cause any significant effects in the lambs at 10 days and 3 months of age. However, at 1 month of age, transient hypoxemia occurred at 5 mins after FX injection. The hypoxemia was more severe in males than females. Lambs were also less active on the day of FX administration at this age. Similarly, at 6 months of age, transient hypoxemia also occurred at 5 mins after FX injection but there was no sex difference. Behavioral observations showed lambs were less active but more irritable following FX administration. At 1 year of age, arterial pressure increased
significantly for 30 mins following FX injection. The increase was more profound and lasted longer in males. HRV increased at 30 mins after FX and hypoxemia also occurred at 5 mins after FX injection. However, no changes in the HPA axis function were observed. FX effects on arterial pressure and blood oxygenation only occurred in the older lambs but not in the younger lambs potentially due to the lower level of platelets, which are the main reservoir of peripheral 5-HT in the younger lambs. In humans, it has been known that the platelet concentrations are lower in newborns and children than in adults (Merritt & Davidson, 1933) and platelet 5-HT concentration in newborn is only ~42% of the value in children and adults (Flachaire et al., 1990). Measurement of platelet concentration, platelet 5-HT concentration and the magnitude of transient increase in blood 5-HT concentration following FX administration in sheep as a function of age could confirm this hypothesis. However, analysis of serotonin from plasma samples is technically difficult. In this regard, I did attempt to measure plasma 5-HT concentration after FX injection in several lambs at 3 months of age but the results were very variable. This was likely due to platelet/aggregation during sample collection, resulting in release of serotonin from the platelets into blood. The sex differences in FX effects on arterial pressure in the older lambs could be due to the effect of ovarian steroids on increasing 5-HT clearance by the lung in the females, leading to a lesser transient increase in blood 5-HT levels following FX injection (Bakhle & Ben-Harari, 1979; Gillis & Pitt, 1982). In addition, our data showed that there was a relationship between FX concentration and the changes in respiratory effects in the 1 year old lambs. The higher the FX concentration, the greater decrease in O₂ saturation. Dose-related effects of SSRIs have also been observed in human and animal studies. In particular, increased risk of preterm birth was
mainly found in women treated with high dose SSRIs during pregnancy (Roca et al., 2011). Increased fetal behaviors were also observed at standard and high SSRIs doses, but not at the low dose and the effect at higher doses was greater than at standard doses. (Mulder et al., 2011) In a rat study, respiratory rate reduced at standard doses and further decreased at the higher doses of FX, while no changes occurred at the low dose of FX. (Annerbrink et al., 2010) Moreover, in our study cardiovascular and respiratory effects of FX were also significantly related. The greater the increased arterial pressure, the greater the decreased pO2 concentration. As the mechanism of action of FX in inhibiting serotonin reuptake by platelets leads to an acute increase in plasma serotonin, and since serotonin is a potent vasoconstrictor, it can cause a sudden increase in blood pressure. This could result in a decrease in cardiac output due to an afterload effect, which would reduce pulmonary blood flow and result in the decrease in arterial pO2.

4. Postnatal lambs who were prenatally exposed to FX showed an increase in activity for the first 2 weeks of life, and a lower HRV (time domain indexes: SDNN, SD Delta NN, RMSSS) on postnatal day 2. Measures of birth and postnatal outcomes such as gestational length, birth weight, postnatal weight gain, tissue sPo2 were not affected by late gestation FX exposure. The increase in activity in the FX-exposed lambs existed in the absence of increase in suckling activity, which is consistent with no difference in postnatal weight gain between FX-exposed and control lambs. Measurement of a low level of plasma FX concentration at birth and on the following postnatal day 2,5,10 and 14 confirmed that FX toxicity is less likely to be the underlying mechanism for poor neonatal adaptation observed in human infants. Since the significant increase in activity in the FX-exposed lambs existed until postnatal day 14, while FX level was low from
postnatal day 2, a withdrawal syndrome is also less likely to be the underlying mechanism. Thus this leaves the mechanism of an SSRI-induced alteration in fetal brain development to be related to the importance of serotonin in brain maturation pathways as the most likely candidate. Indeed, an increase in fetal activity and a lower HRV through the day have been observed in the human fetuses exposed to SSRIs in utero (Mulder et al., 2011; Rurak et al., 2011). Measurement of serotonin concentrations in the cerebrospinal fluid would provide direct evidence for the effects of FX on the lambs’ serotonin brain levels. Alternatively, S100B protein has been used as a biomarker for early brain development and central serotonergic function (Pawluski et al., 2009). An increase in synaptic 5-HT after SSRIs administration activates the astroglial 5-HT1A receptor, which leads to a release of S100B protein which mediates the growth and survival of neurons (Whitaker-Azmitia et al., 1990; Whitaker-Azmitia, 2001). Therefore, in the absence of cerebrospinal fluid samples, determination of S100B protein could provide a marker for changes in extracellular serotonin caused by FX administration. The possibility of measuring plasma S100B concentration in my study arose late in the study, after completion of the developmental studies. Unfortunately, due to the lack of remaining blood samples in the control group, S100B protein analysis was not performed in our studies.

In addition, from our studies it appeared that acute postnatal and long-term prenatal FX exposure exert opposite effects. While acute FX injection caused a reduction in activity, subchronic prenatal FX exposure resulted in hyperactivity in the lambs. Since clinically it usually takes about 2 to 4 weeks for an SSRIs fully work, acute FX administration could elicit a paradoxical antidepressant effect (i.e. sedation rather than
hyperactivity), which is different from the long-term exposure. There was a case report of a paradoxical sedative effects at the onset of FX treatment, but this effect disappeared after a few weeks of continuous FX therapy (Gupta et al., 1994). Moreover, it has been documented that FX treatment is associated with a significant increase in the rate of both activating adverse events including insomnia, agitation, anxiety, nervousness, and sedating events such as somnolence or asthenia, as compared to placebo treatment in patients with different baseline psychomotor activity status (agitated, retarded or neither). Interestingly, 10% of patients that experienced adverse events actually reported both effects, either simultaneously or serially. (Beasley et al., 1991)

Alternatively, it has been shown in a previous study in our lab that an enhanced prepartum cortisol surge is associated with long-term prenatal FX-exposure in the fetal lambs (Morrison et al., 2004). This could result in an early maturation of the musculo-skeletal system, and thus explain the observed hyperactivity in the postnatal lambs exposed to FX prenatally in our current study.

In summary, my study is the first to investigate postnatal outcomes in lambs exposed to FX in utero or postnatally. In this study, we have two study cohorts: one was exposed to FX acutely after birth and the other was exposed prenatally to FX for 2 weeks of late gestation, which is equivalent to the last month of third trimester in human pregnancy. Acute FX administration on postnatal day 4 did not cause any significant effects other than slight and transient changes in heart rate and arterial pressure. In contrast, in the ~ 2 weeks gestational FX exposure lambs, we observed a decreased time required for initial lamb milestones (i.e. first time to stand, to walk and to suckle), a reduced HRV on postnatal day 2, a hyperactivity not associated with suckling. (see Table 7.1; A vs C)
Human studies (Table 7.2 B & C) have shown that antenatal SSRIs exposure results in many adverse birth outcomes in the offspring such as shorter gestational age, increased risk of preterm birth, lower birth weight, small for gestational age, reduced postnatal weight gain, poor neonatal adaptation (respiratory difficulty, cyanosis on feeding, irritability), lower APGAR score, increased admission rate to NICU and reduced endocrine and facial response to pain stimulus (via vaccination) at 1 and 3 months of age (Chambers et al., 1996; Costei et al., 2002; Goldstein et al., 1995; Kallen, 2004; Lattimore et al., 2005; Levinson-Castiel et al., 2006; Lewis et al., 2010; Lund et al., 2009; Maschi et al., 2008; Oberlander et al., 2005; Oberlander et al., 2006; Oberlander et al., 2008; Roca et al., 2011; Simon et al., 2002; Toh et al., 2009; Wen et al., 2006; Wisner et al., 2009; Zeskind & Stephens, 2004). However, we did not observe any differences in the corresponding outcomes reported in human studies such as gestational age, birth weight, postnatal weight gain in the lambs whose mothers were administered FX i.v for ~ 12 days late gestation (see Table 7.1 C vs Table 7.2 B).

Differences observed in my study and human studies could be due to several reasons. In human studies, the infants were exposed to the drugs for all or most of pregnancy, whereas in my study, the exposure period was limited for ~ 12 days late gestation. In addition, women who were treated with antidepressants were also clinically depressed; depression per se, and its associated risk factors such as smoking, alcohol and drug abuse could be major confounders for adverse effects in the offspring. As far as we know, our sheep were not depressed. In the current study, we only investigate one subtype of SSRIs, fluoxetine, while in human studies, many subtype of SSRIs (paroxetine, citalopram, sertraline, fluvoxamine, etc.) and other antidepressants (TCA, SNRIs) were included.
Similar to our results, in one rodent study, there were no differences in body weight at postnatal day 1, 8, 15, 22 between pups exposed to saline or FX during pregnancy and lactation (from GA 0 to PND 21) (Lisboa et al., 2007). Other studies in rodents have also shown changes in postnatal behavior (long-term effects) in mice/rats exposed to SSRIs postnatally over the range from postnatal day 0 to 21 (see Table 7.3B) (Ansorge et al., 2004; Hansen et al., 1997; Lee, 2009; Lisboa et al., 2007; Maciag et al., 2006; Manhaes de Castro et al., 2001; Mirmiran et al., 1981; Popa et al., 2008). For instance, Lisboa et al., 2007 reported that female FX-exposed mice had an increased immobility in forced swimming tests on PND 30 and 70 while male FX-exposed mice decreased their impulsivity on PND 40 and 70 (Lisboa et al., 2007). In the rat, neonatal exposure to citalopram (SSRI) from postnatal day 8 to 21 resulted in a decreased synthesis of tryptophan hydroxylase in the dorsal raphe and 5-HT transporter expression in the cortex, increased locomotor activity and decreased sexual behavior (male) in adult rats. (Maciag et al., 2006). The observed hyperactivity in our prenatally FX-exposed lambs is similar to the increased locomotor activity in the Maciag study but different from the remaining rodent studies which reported an increased immobility following SSRI exposure. (see Table 7.3 B). Despite this, the results of chronic FX or SSRI exposure in my study, human studies (Table 7.2 B &C) and rodent studies (Table 7.3 A &B) showed that there are long-term effects in the offspring exposed to SSRI (FX). These long-term effects following FX exposure has been identified to be mediated by 5-HT transporter blockade in rodent models that were exposed to FX early ex utero (PND 4-21) (Ansorge et al, 2004). Interestingly, the effect of antenatal SSRIs exposure in human and sheep studies actually appears to emerge in the fetal period before the treatment was ended. Indeed, a blunted fetal heart rate response during the day, a reduced MCA pulsatile index, increased
activities in human fetuses on non-REM sleep, increased jerky movement and lower reactivity to the vibroacoustic stimulus were observed (Mulder et al., 2011; Rurak et al., 2011; Salisbury et al., 2006) (see Table 7.2A). In the sheep fetuses, 8 day continuous maternal i.v FX infusion led to a reduced REM sleep, increase quiet sleep, enhanced prepartum ACTH and cortisol surge and a transient decrease in uterine artery blood flow (Morrison et al., 2001; Morrison, 2001; Morrison et al., 2002; Morrison et al., 2004; Morrison et al., 2005). Taken together, the results from this study contribute to elucidating the mechanisms of underlying poor neonatal adaptation in humans exposed to SSRI in utero. So far, data from the current study and other relevant studies have pointed to the mechanism of SSRIs-elicited altered fetal brain development. Particularly, prenatal SSRIs exposure might alter 5-HT signaling in utero. This is important in the context of a “fetal programming” impact on adult health. We hope the data gained from the present study contributes valuable knowledge, and could assist physicians and pregnant women in the assessment of the safety of the drugs, and to make the best informed decisions based on the risks and benefits of antidepressant treatment during pregnancy.

In this study, the postnatal follow-up of prenatal FX exposure was limited only to 2 weeks following birth. Considering the increased activity in the newborn lambs is equivalent to jitteriness, one of the symptoms of poor neonatal adaptation in human exposed in utero to the drug, it would be very interesting to follow up their behaviors at longer time beyond this 2 week period, and at milestones of development, such as weaning (at 2 months), puberty (at 6 months) and adulthood (at 12 months) by actiwatch and DVR observations. A study in human has reported that increased aggressiveness scores in the 4-year old children exposed to SSRIs during gestation is associated with a history of poor neonatal adaptaion, however,
current maternal mood and parental stress appear to be the best predictors for the
externalizing behaviors than a history of prenatal depression or SSRIs exposure. (Oberlander
et al, 2007) The challenge to distinguish between the effects of the drug exposure and a
negative influence of postnatal social environment with depressed mothers on neurocognitive
development in the children in human study might be resolved using our sheep model.

Further studies to confirm the hypothesis of in utero altered 5-HT signaling in
prenatal SSRI exposure could be accomplished by investigating the fetal brain development
in SSRIs exposure subjects. It would be ideal to be able to assess fetal brain structure
development by invasive methods such as Magnetic Resonance Imaging (MRI), Positron
Emission Tomography (PET) and follow-ups evaluating the structure of brain development
and function after birth and up to adulthood. Alternatively, the effect of SSRIs on fetal brain
development could be evaluated by serial measuring plasma S100B protein, a biomarker of
early brain development and central serotonergic function, in the sheep fetuses exposed to
SSRIs (FX) following up to postnatal period into adulthood. In addition, it would be
interesting to investigate the transgenerational effects on fetal programming of altered 5-HT
signaling pathways in utero. Given the time required for a gestation of 145 days and
additional 1-2 years for the female lambs to be able to reproduce, the sheep model is
probably not an appropriate model for these long-term studies. Rodents or guinea pigs might
be better models for this type of study.
Table 7.1: Outcomes of chronic antenatal and acute postnatal FX exposure in fetal and postnatal lambs

<table>
<thead>
<tr>
<th>Sheep studies</th>
<th>Agents</th>
<th>Outcomes</th>
</tr>
</thead>
</table>
| (A) Present study | Acute FX i.v (1mg/kg) on PN day 4 | No differences in feeding behavior, postnatal weight gain, no respiratory difficulties, no differences in cortisol level  
Slight and transient heart rate and arterial pressure changes |
| (A) Present study | Acute FX i.v (1mg/kg) at ~ 10 days, 1,3,6 and 12 months of age | Increased arterial pressure in the 1 year old lambs  
Transient hypoxemia in the older lambs (1,6 and 12 months of age)  
Decrease activities (1 & 6 months of age)  
No changes in HPA axis function |
| (B) Morrison et al, 2001 | Maternal FX i.v infusion x 8 days (~125-140 GA) (98.5µg/kg.d) | Altered fetal behavioral state: Increased quiet sleep (low-voltage ECoG activity), eye movements and fetal breathing movements. Decreased REM sleep (high-voltage ECoG activity)  
Transient decrease in uterine artery blood flow  
Transient fetal hypoxemia and acidosis for the first 24h of infusion |
| (B) Morrison et al, 2002 | Maternal daily FX (50mg) i.v x ~12 days (~131-143 GA) | No differences in gestational age, birth weight  
Decreased time required for initial lamb milestones (to stand, to walk, to suckle)  
Reduced HRV on PN day 2  
PN 2- 14: hyperactivity not associated with suckling, no differences in postnatal weight gain, no respiratory difficulties |
Table 7.2: Outcomes of antenatal SSRI exposure in human (exposure for most or all of pregnancy)

<table>
<thead>
<tr>
<th>(A) Human studies</th>
<th>Agents</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mulder et al, 2011</td>
<td>SSRIs throughout gestation</td>
<td>Fetuses:</td>
</tr>
<tr>
<td>Prospective study</td>
<td></td>
<td>Disrupted non-REM sleep</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increase bodily activity during non-REM sleep</td>
</tr>
<tr>
<td>Rurak et al, 2011</td>
<td>SSRIs throughout gestation</td>
<td>Fetuses (36 weeks gestation)</td>
</tr>
<tr>
<td>Longitudinal observational study</td>
<td></td>
<td>Blunted fetal HRV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lower MCA pulsatility index: MCA mean flow velocity, MCA volume flow, MCA cross-sectional area</td>
</tr>
<tr>
<td>Salisbury et al, 2006</td>
<td>SSRIs + no MDD vs MDD + no SSRIs vs MDD + SSRIs vs control</td>
<td>At 26 weeks GA:</td>
</tr>
<tr>
<td>Prospective study</td>
<td></td>
<td>Less movement, more jerky movements, lower reactivity to vibroacoustic stimulus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>At 36 weeks GA:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SSRI or MDD fetuses: increased reactivity to vibroacoustic stimulus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SSRI + MDD fetuses: decreased reactivity to vibroacoustic stimulus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Newborns:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased tremor, startles, hypotonia and lowest quality of movement scores</td>
</tr>
<tr>
<td>Study Details</td>
<td>Agents</td>
<td>Outcomes (short-term effects)</td>
</tr>
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<tr>
<td>Chambers et al, 1996</td>
<td>FX</td>
<td>Newborn: Increase risk of poor neonatal adaptation</td>
</tr>
<tr>
<td>Prospective/retrospective reports</td>
<td>Early vs late gestation exposure</td>
<td>Increase risk of special-nursery admission</td>
</tr>
<tr>
<td>Simon et al, 2002</td>
<td>SSRIs</td>
<td>Shorter gestational age in SSRIs exposed infants</td>
</tr>
<tr>
<td>Retrospective records</td>
<td>TCA</td>
<td>Lower birth weight</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lower Apgar scores (3rd trimester SSRI exposure)</td>
</tr>
<tr>
<td>Kallen et al, 2004</td>
<td>SSRIs (Citalopram, FX, PX, Sertraline)</td>
<td>Increased risk of respiratory distress, increased risk of low Apgar score, increased risk of convulsions</td>
</tr>
<tr>
<td>Prospective cohort study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wen et al, 2006</td>
<td>SSRIs</td>
<td>Increased risk of low birth weight, preterm birth, fetal death, and seizures</td>
</tr>
<tr>
<td>Retrospective cohort study</td>
<td></td>
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<tr>
<td>Wisner et al, 2009</td>
<td>SSRIs</td>
<td>Increased risk of preterm birth in continuously SSRIs exposure across gestation</td>
</tr>
<tr>
<td>Study</td>
<td>Agents</td>
<td>Outcomes (short-term effects)</td>
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<tr>
<td>Oberlander et al, 2006</td>
<td>SSRIs (1st trimester to delivery)</td>
<td>Increased risk of low birth weight</td>
</tr>
<tr>
<td>Retrospective record-linkage</td>
<td></td>
<td>Increased risk of respiratory distress</td>
</tr>
<tr>
<td>Roca et al. 2011</td>
<td>SSRIs</td>
<td>Increased risk of preterm birth (mainly in high dose)</td>
</tr>
<tr>
<td>Lund et al, 2009</td>
<td>SSRIs during pregnancy</td>
<td>Increased risk of preterm birth, lower 5-min Apgar score and NICU admission</td>
</tr>
<tr>
<td>Lewis et al, 2010</td>
<td>Antidepressants</td>
<td>Increased rate of preterm birth, lower birth weight, smaller head circumference</td>
</tr>
<tr>
<td>Toh et al. 2009</td>
<td>SSRIs vs non-SSRIs</td>
<td>Increased rate of smaller than gestational age in late gestational SSRIs exposure</td>
</tr>
<tr>
<td>Levinson-Castiel et al, 2006</td>
<td>SSRIs (PX, FX, Citalopram, Sertraline, Venlafaxine)</td>
<td>Neonatal abstinence syndrome in 30% SSRIs exposed neonates (Finnegan score)</td>
</tr>
<tr>
<td>(B) Human studies</td>
<td>Agents</td>
<td>Outcomes (short-term effects)</td>
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<tr>
<td>Goldstein et al, 1995</td>
<td>FX (3rd trimester)</td>
<td>Incidence of poor neonatal adaptation to FX exposure: 13.4%</td>
</tr>
<tr>
<td></td>
<td>Prospective &amp; retrospective study</td>
<td>Increased risk of respiratory signs</td>
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<tr>
<td></td>
<td></td>
<td>Increased risk of sleep problems</td>
</tr>
<tr>
<td>Maschi et al, 2008</td>
<td>PX, FX, other antidepressants</td>
<td>Increased incidence of poor neonatal adaptation but not statistical significant</td>
</tr>
<tr>
<td>Costei et al, 2002</td>
<td>Paroxetine (early vs late gestational vs no exposure)</td>
<td>Increased risk of neonatal complications (respiratory distress, hypoglycemia, bradycardia, suckling problems, prolonged hospitalization)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased risk of respiratory difficulties</td>
</tr>
<tr>
<td>Zeskind et al. 2004</td>
<td>SSRIs (citalopram, FX, PX, sertraline) During pregnancy</td>
<td>Shorter gestational age</td>
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<tr>
<td></td>
<td></td>
<td>Increase motor activity (*) and tremulous</td>
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<tr>
<td></td>
<td></td>
<td>Fewer rhythms in HRV (*)</td>
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<td></td>
<td></td>
<td>Fewer changes in behavioral state, fewer different behavioral states and lower peak behavioral state</td>
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<tr>
<td></td>
<td></td>
<td>Increased REM sleep</td>
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<td></td>
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<td>(*) no longer significant after adjusted for gestational age</td>
</tr>
<tr>
<td>(C) Human studies</td>
<td>Agents</td>
<td>Outcomes (long-term effects)</td>
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<tr>
<td>Lewis et al, 2010</td>
<td>Antidepressants</td>
<td>At 1 month, lower body weight in exposed infants</td>
</tr>
<tr>
<td>Prospective case-control study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oberlander et al, 2005</td>
<td>SSRIs (FX, PX, sertraline)</td>
<td>At 2 months of age:</td>
</tr>
<tr>
<td>Prospective study</td>
<td>(prenatal and postnatal</td>
<td>Blunted facial response to heel lance in prenatal SSRI exposure infants</td>
</tr>
<tr>
<td></td>
<td>exposure)</td>
<td>Reduced parasympathetic withdrawal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased parasympathetic cardiac modulation during recovery in pre and postnatal SSRI exposure infants</td>
</tr>
<tr>
<td>Oberlander et al, 2008</td>
<td>SSRIs</td>
<td>Reduced early evening basal cortisol level</td>
</tr>
<tr>
<td>Prospective study</td>
<td></td>
<td>At 3 months of age:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Higher post-stress cortisol levels in SSRIs exposed infants.</td>
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<tr>
<td>(A) Rodent studies</td>
<td>Agents</td>
<td>Outcomes</td>
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<tr>
<td>Forcelli et al, 2008</td>
<td>FX</td>
<td>PN 30: decreased conflict-exploratory behavior</td>
</tr>
<tr>
<td>Prenatal infusion for 14 days</td>
<td>PN 60: increased place preference</td>
<td></td>
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<tr>
<td></td>
<td>PN 90: increased extinction response rate</td>
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<tr>
<td></td>
<td>PN 120: decreased nucleus accubens cell count &amp; serotonin transporter-like in raphe nucleus</td>
<td></td>
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</tbody>
</table>

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<thead>
<tr>
<th>(B) Rodent studies</th>
<th>Agents</th>
<th>Outcomes</th>
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</thead>
<tbody>
<tr>
<td>Lisboa et al, 2007</td>
<td>FX</td>
<td>No differences in body weight @ PN day 1,8,15,22</td>
</tr>
<tr>
<td>GA 0-PN 21 to dams</td>
<td>PN 30, 70: increased immobility (female)</td>
<td></td>
</tr>
<tr>
<td>7.5mg/kg gavage</td>
<td>PN 40, 70: decreased impulsivity (male)</td>
<td></td>
</tr>
</tbody>
</table>

<p>| Lee et al, 2009 | FX | PN day 7-35: |
| PN 0-6 | Neuronal structure deformation in the somatosensory cortex |
| 10mg/kg s.c | PN day 30-35 |
| | Higher thermal threshold (hotplate test) |
| | Impaired whisker-specific tactile function (gap-crossing test) |
| | Altered exploratory behavior (open field test) |</p>
<table>
<thead>
<tr>
<th>Study</th>
<th>Agent</th>
<th>Dosage/Route</th>
<th>Outcomes</th>
</tr>
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<tbody>
<tr>
<td>Ansorge et al, 2004</td>
<td>FX</td>
<td>PN 4-21</td>
<td>Decreased exploratory behavior in open field and elevated plus maze test</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10mg/kg i.p</td>
<td>Prolonged latency to begin feeding in novelty-suppressed feeding test</td>
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<td></td>
<td></td>
<td></td>
<td>Prolonged latency to escape a foot-shock in shock-escape paradigm</td>
</tr>
<tr>
<td>Mashaes de Castro et al, 2001</td>
<td>Citalopram</td>
<td>PN 1-19</td>
<td>Decreased aggressive behavior in footshock test</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20mg/kg s.c every 3 days</td>
<td></td>
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<tr>
<td>Hansen et al, 1997</td>
<td>SSRIs (LU)</td>
<td>PN 8-21</td>
<td>No difference in open field test &amp; social interaction test</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5-15mg/kg bid</td>
<td>Increased immobile time in forced swim test</td>
</tr>
<tr>
<td>Maciag et al, 2006</td>
<td>Citalopram</td>
<td>PN 8-21</td>
<td>Decreased synthesis of tryptophan hydroxylase in dorsal raphe &amp; 5-HTT expression in cortex</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5mg/kg x 2 s.c</td>
<td>Increased locomotor activity</td>
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<td></td>
<td>Decreased sexual behavior</td>
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<tr>
<td>(B) Rodent studies</td>
<td>Agents</td>
<td>Outcomes</td>
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<tr>
<td>Popa et al, 2008</td>
<td>Escitalopram</td>
<td>Sleep anomalies</td>
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<tr>
<td></td>
<td>PN 5</td>
<td>Anhedonia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10mg/kg s.c x 2 weeks daily</td>
<td>Increased helplessness</td>
<td></td>
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<td></td>
<td></td>
<td>Increased response to acute stress</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Increased serotonergic autoinhibitory feedback</td>
<td></td>
</tr>
<tr>
<td>Mirmiran et al, 1981</td>
<td>Chlorimipramine</td>
<td>Decreased active sleep, increased quiet sleep</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PN 8-21</td>
<td>Decreased exploratory behavior in open field test</td>
<td></td>
</tr>
<tr>
<td></td>
<td>x 2 daily</td>
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REFERENCES


Chow, T. W. (2013). The disposition of fluoxetine in newborn lambs up to one year of age. (Doctor of Philosophy, University of British Columbia).


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*Top 200 generic drugs by total prescriptions 2008*(2009).


## Appendices

### Appendix 1: Actiwatch Variables Data In The Lambs At ~ 4, 10 Days, 1, 3, 6 And 12 Months Of Age:

1M = 1 month, 3M = 3 months, 6M = 6 months, 1Y = 1 year.

<table>
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<th>3M</th>
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|                          | Mean   | SEM    | Mean   | SEM    | Mean   | SEM    | Mean   | SEM    |
| N = 6-11 Mean            | 126.6  | 5.76   | 84.5   | 5.82   | 62.5   | 4.70   |
| N = 9-10 Mean            | 116.5  | 8.39   | 85.0   | 4.61   | 66.7   | 3.37   |
| N = 9-12 Mean            | 95.2   | 8.51   | 118.7  | 8.84   | 84.0   | 6.85   |
| N = 12 Mean              | 92.8   | 6.96   | 109.8  | 8.19   | 72.3   | 4.00   |
| N = 15 Mean              | 108.2  | 5.26   | 117.0  | 8.09   | 83.6   | 4.95   |
| N = 24 Mean              | 109.1  | 6.78   | 114.1  | 8.40   | 83.8   | 4.29   |
| N = 30 Mean              | 109.1  | 8.84   | 118.4  | 7.12   | 82.0   | 4.48   |
| N = 35 Mean              | 109.1  | 8.84   | 118.4  | 7.12   | 82.0   | 4.48   |
| N = 40 Mean              | 109.1  | 8.84   | 118.4  | 7.12   | 82.0   | 4.48   |

### Active bout per day

- Normal day in pen
- Average of 3 or 4 days
- Control exp
- FX exp
- Post FX d1
- Post FX day 2
- Post FX day 3

### Mean score in active

- Normal day in pen
- Average of 3 or 4 days
- Control exp
- FX exp
- Post FX d1
- Post FX day 2
- Post FX day 3
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Appendix 2: Heart Rate, Arterial Pressure And HRV In The Lambs At ~ 4, 10 Days, 1, 3, 6 And 12 Months Of Age With FX Injection:
1M = 1 month, 3M = 3 months, 6M = 6 months, 1Y = 1 year.

2.1: Heart Rate And Arterial Pressure:

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<th>6M</th>
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2.2: Time Domain Of HRV (SDNN, SD Delta NN, RMSSD):

1M = 1 month, 3M = 3 months, 6M = 6 months, 1Y = 1 year.

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2.3: Frequency Domain Of HRV (LF Power, HF Power, LF/HF Ratio):

1M = 1 month, 3M = 3 months, 6M = 6 months, 1Y = 1 year.

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Appendix 3: Arterial Blood Gases And Body Temperature In The Lambs At ~ 4, 10 Days, 1, 3, 6 And 12 Months Of Age With FX Injection:

1M = 1 month, 3M = 3 months, 6M = 6 months, 1Y = 1 year.

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1M = 1 month, 3M = 3 months, 6M = 6 months, 1Y = 1 year.

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1M = 1 month, 3M = 3 months, 6M = 6 months, 1Y = 1 year.

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Appendix 4: Glucose, Lactate Concentration And Electrolite In The Lambs At~ 4, 10 Days, 1, 3, 6 And 12 Months Of Age With FX Injection:

1M = 1 month, 3M = 3 months, 6M = 6 months, 1Y = 1 year.

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<th>3M</th>
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1M = 1 month, 3M = 3 months, 6M = 6 months, 1Y = 1 year.

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