NOTE TO USERS

EFFECTS OF FLUOXETINE ADMINISTRATION TO GUINEA PIGS DURING PREGNANCY ON SEROTONIN RECEPTORS AND BEHAVIOR IN RESULTING OFFSPRING

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ABSTRACT

Fluoxetine is a selective serotonin reuptake inhibitor, which is used to treat depression during pregnancy. The aim of this study was to test if treatment of pregnant guinea pigs with fluoxetine affects serotonin (5-HT) receptors or behaviors modulated by 5-HT in the resulting offspring. The first experiment consisted of injecting pregnant guinea pigs with 7 mg/kg/day of fluoxetine or vehicle through gestation, or delivering no treatment. Hippocampal 5-HT_{1A} receptor densities were measured in male offspring at 2 weeks of age, and 5-HT_{2A} receptor densities were measured in the striatum and frontal cortex of male offspring at 9 weeks of age. There were no significant effects of treatment on receptor densities in these offspring. Secondly, pregnant guinea pigs were administered fluoxetine (7mg/kg/day) or vehicle using osmotic mini-pumps through gestation, or no treatment was delivered. In this study, pregnancy characteristics and maternal and pup weights were assessed. Acoustic startle responses, prepulse inhibition of acoustic startle and nociception using a hot-plate test were analyzed in the offspring. No significant treatment effects were found for all outcome measures except in the hot-plate test where the vehicle group had significantly lower nociceptive thresholds compared to both fluoxetine and no treatment controls, when data for 9 week-old males and females were combined. The reduced nociceptive threshold after prenatal vehicle treatment is consistent with previous reports that prenatal stress can reduce pain threshold. Our finding that fluoxetine exposure in the pregnant dam has anti-nociceptive effects in offspring as adults is a novel finding, and is consistent with previous reports that fluoxetine administered to adult animals has antinociceptive properties.

RÉSUMÉ

Le fluoxétine est un inhibiteur sélectif du recaptage de la sérotonine et est utilisé dans le traitement de la dépression durant la grossesse. Le but de cette étude était de vérifier si traitement de cobayes enceintes à la fluoxétine aura un effet sur les recepteurs à la serotonine (5-HT) ou sur le comportement de leurs rejetons. La première expérience incluait des animaux recevant 7 mg/kg/jour de fluoxétine ou le véhicule durant toute la grossesse. Ces animaux étaient compares à des cobayes enceintes ne recevant aucun traitement. La densité des récepteurs 5-HT_{1A} de l'hippocampe a été mesurée dans les rejetons males agés de 2 semaines et la densité des récepteurs 5-HT_{2A} au niveau du striatum et cortex frontal dans les rejetons mâles agés de 9 semaines. Il n'y avait aucun effet due traitement sur les densités de récepteurs de ces rejetons. Dans la deuxième expérience, des cobayes enceintes ont reçu de la fluoxétine (7 mg/kg/jour) ou, véhicule ou aucun traitement, par l'intermédiaire de mini-pompes osmotiques durant la grossesse. Dans cette étude les caractéristiques de la grossesse, le poids maternel et le poids des rejetons étaient analysés. La réponse de sursaut acoustique et l'inhibition du sursaut acoustique ainsi que la sensibilité à la douleur étaient mesures. Pour ce dernier test, nous avons utilisée une plaque chauffée. Il n'y avait pas de changements sous l'effet du traitement dans tous les paramètres sauf dans la sensibilité à la douleur thermale où le groupe qui a reçu le véhicule avait un seuil nociceptif plus bas que les groupes ayant reçu le fluoxétine et les groupes n'ayant aucun traitement. Ces résultats étaient obtenus en combinant les groupes de mâles et femelles agés de 9 semaines. La réduction du seuil de nociception après l'administration prénatale d'un véhicule est en accord avec d'autres études confirmant la diminution du seuil nociceptif sous l'effet du stress prénatal. Nous avons trouvé que l'exposition à la fluoxétine des cobayes enceintes a un effet anti-nociceptif chez les rejetons, ce qui est une nouveauté en accord avec d'autres études démontrant cet effet chez des animaux adultes.

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A. Rationale and Hypothesis

The aim of this study is to test the hypothesis that the administration of antidepressants during pregnancy has long-term effects on central nervous system functioning in the resulting offspring. Our concern comes from recent changes in the pattern of antidepressant therapy. The introduction of new medication with better outcome and fewer side effects has yielded a pattern of increased prescriptions of antidepressants. Furthermore, this pattern suggests that women of childbearing age and their offspring are being increasingly exposed to antidepressant medications.

In recent decades, there have been increasing rates of diagnosis of affective disorders that have been reported in epidemiological studies and particularly disconcerting is that these trends seem to affect particularly women of childbearing age (Klerman et al., 1989, Stoll et al., 1993). Furthermore, there is increase in the prescription of antidepressants for the treatment of affective disorders once the physician has made the diagnosis. (Olfson et al., 1993).

Next, in 1988, the introduction of specific serotonin reuptake inhibitors (SSRIs) has provided medical practitioners with a newer class of antidepressant medications which is though to be free of serious side effects, hence resulting in increased patient compliance and increased prescription. Finally, in addition to depression, SSRIs are being increasingly used for a wide variety of disorders, including amongst them obsessive-compulsive disorders and social phobia (Van Vliet et al., 1994, Fineberg et al., 1992). Furthermore the larger proportions of subjects diagnosed with these disorders are female and the length of therapy is relatively long (Goldstein and Rampey, 1994). Hence, there is strong evidence that indicates that women of childbearing age are being increasingly

prescribed antidepressant medication. Evidence also suggests that about half of pregnancies in North America are unplanned (Sophocles et al., 1986). This information together with studies showing increasing prescription of antidepressants, especially in women indicates that there may be high rates of in utero exposure to SSRIs. Moreover, SSRIs like fluoxetine have long half-lives, requiring a period of weeks to months before the drug is eliminated after long-term treatment (Altamura et al., 1994). Hence even if therapy is discontinued when pregnancy is discovered, fetal exposure may not be avoided.

In addition to unintentional exposure, antidepressants are knowingly prescribed during pregnancy. Most antidepressants are placed under pregnancy category B (U.S. Food and Drug Administration). This means "reproduction studies in animals indicate either no or some evidence of teratogenicity". Unfortunately according to this categorization, the only requirement in ruling out drug teratogenicity is the demonstration of no gross morphological changes in the tissues of these animals. We feel that this is inadequate due to the fact that certain psychiatric disorders, such as schizophrenia, are known to be caused by more subtle changes in brain structure and function.

Whether prenatal exposure to SSRIs produces long-term effects on specific neurotransmitter systems has not yet been adequately tested in animals. Our approach has been to develop an appropriate small animal model, using the guinea pig, for antidepressant drug administration during pregnancy. The first studies we completed with this model were pharmacokinetic studies, to determine whether blood levels of antidepressants achieved are similar to therapeutic blood levels in humans. Following this, we performed experiments to determine whether in utero antidepressant exposure

produces long-term alterations in some central nervous system (CNS) outcome measures related to known actions of these drugs. The main outcomes in our study were measures of the densities of 5-HT_{1A} (serotonin 1A) and 5-HT_{2A} receptors in the brains of the offspring and assessment of three 5-HT mediated behaviors: pain sensitivity, acoustic startle response and prepulse inhibition of acoustic startle.

B. Background

B-1) Use of antidepressants in women of childbearing age

Many epidemiological studies suggested that depression is increasing at a significant rate. A review of epidemiological studies from the U.S., Sweden, Germany, Canada and New Zealand indicates an increasing rate of depression for all ages across the 20^{th} century (Klerman et al., 1989). Data from six North American psychiatric teaching hospitals showed diagnoses of major affective disorders increased from 10% in 1972 to 44% in 1990 (Stoll et al., 1993). Furthermore, the tendency is for depression to be associated with decreased age of onset, with 2-3x higher risk in females compared to males (Klerman et al., 1989). Moreover, lifetime prevalence rates are the highest for females aged 20-39 (i.e. women of childbearing age).

Antidepressant medications, especially SSRIs, are being increasingly prescribed for depressed persons. An analysis of data from the U.S. National Center for Health Statistics provided estimates of volume of office based care in the US through sampling of representative psychiatrists (Olfson et al., 1993). This study estimated that the prescription of antidepressants with each visit to the psychiatrist grew from 2.5 million in 1980 to 4.7 million in 1989. Furthermore, the majority of visits that included an

antidepressant prescription were for white females under age 51, hence women of childbearing age. Hence, evidence indicates that women of childbearing age are increasingly being prescribed SSRIs and there might be an increase in the amount of in utero exposure to these agents.

Although unplanned use of antidepressants during pregnancy is of concern, many women are knowingly being prescribed antidepressants during pregnancy. A study investigating the use of medication in a general population shows that up to 5.4% of woman may be using psychoactive drugs, including antidepressants, during pregnancy (Arpino et al., 1995). This pattern in drug intake can be explained by the increase risk of major depression in women of childbearing age. An epidemiological study has shown that the lifetime risk for depression in community samples varies from 10% to 25% for women, with peak prevalence at age 25 through 44 years (Goldstein and Marvel, 1993).

Available guidelines published by American Psychiatric Association (1993) concerning the use of antidepressants during pregnancy recommends: "The relative risks and benefits of prescribing antidepressants must be particularly weighed in treatment of a pregnant woman. In patients whose safety and well-being require antidepressant medications, a tricyclic or any of the newer antidepressant compounds may be justifiably used, after the first trimester if possible" (Karasu, 1993). Furthermore, in a recent consensus survey of expert opinion consisting of psychiatrists, the expert panel agreed (general agreement at 76%) that pregnant women having an episode of severe depression should be treated with SSRIs in combination with psychotherapy (Altshuler et al., 2001). However, in milder forms of depression, psychotherapy was preferred. These recommendations indicate that, although the benefits of using antidepressants have been

investigated, there remains uncertainty on the possible risks involved in their use. Hence, our study will investigate the possible effects of SSRIs on the offspring of animals treated with the drug throughout pregnancy.

For our current proposal, we will confine our studies to effects of fluoxetine. This antidepressant was chosen because it is the most commonly prescribed SSRI and its long half-life may expose the fetus unwillingly in unplanned pregnancies. Furthermore, fluoxetine and its metabolite, norfluoxetine, being soluble molecules of molecular weight <600, readily cross the placenta, hence exposing the fetus to the drug (Mirkin, 1974). In a recent study by Hendrick et al. (2003) maternal and umbilical cord samples were obtained from 38 women taking SSRIs, including fluoxetine, during pregnancy and they reported that antidepressant and metabolite concentration was found in 86.8% of umbilical cord samples. In addition, a study looking at infants exposed to fluoxetine throughout gestation show abnormalities at birth, including increased premature delivery, lower birth weight, poor neonatal adaptation and increased minor physical anomalies (Chambers et al., 1996).

B-2) Fluoxetine during pregnancy in humans

Fluoxetine was initially approved for treatment of depression in the United States in 1987. Since then, it has been marketed in more than 90 countries. At the present (2003), Eli-Lilly, the manufacturer of the antidepressant estimates that the population of patients treated with fluoxetine is approximately 40 million worldwide. Form 1989 until 2000, fluoxetine was the most prescribed antidepressant drug in the United States (Galewitz, 2000). By 1989, fluoxetine was prescribed in 29.6% of all psychiatric visits

that included an antidepressant prescription. In addition to depression, fluoxetine has efficacy in the treatment of a variety of other mental disorders including panic disorder (Jobson et al., 1995), obsessive-compulsive disorder (Fineberg et al., 1992), social phobia (van Vliet et al., 1994), and bulimia nervosa (Goldbloom et al., 1993). Hence there is a strong indication that fluoxetine is increasingly prescribed to the population of which women of childbearing age constitute a large proportion (Klerman et al., 1989, Stoll et al., 1993).

In a retrospective study of fluoxetine use during pregnancy, Chambers et al. (1996) reported that a sample of 73 newborn infants exposed to the drug throughout the 3 trimesters of pregnancy had increased risk for premature delivery, lower birth weight, poor neonatal adaptation including respiratory difficulty, cyanosis on feeding and jitteriness. The incidence of 3 or more minor physical anomalies was also greater in infants exposed to fluoxetine throughout pregnancy vs. control.

However, in another study, the Mother Risk group (Nulman and Koren, 1996) reported no anomalies in IQ, language or behavioral development in a group of children between 16 and 86 months of age born to 55 women who took fluoxetine during pregnancy. A shortcoming of this study was that children exposed to fluoxetine during the first trimester only were combined with those exposed throughout pregnancy and only 18 out of the 55 children were exposed to fluoxetine throughout pregnancy. A recent meta-analysis reviewing epidemiological studies looking at fluoxetine use during the first trimester of pregnancy has concluded that there were no adverse effects on human infants (Addis and Koren, 2000). However, the major limitation in this study was the small sample sizes of each study included in the meta-analysis.

In contrast, a recent study examined obstetric and neonatal records of infants exposed to fluoxetine in utero anytime during gestation and found that although there were no differences in birth weight and acute neonatal outcomes, there was a higher frequency of special care nursery admissions in infants exposed to fluoxetine in late pregnancy; although special care nursery admission could not be attributed to a specific factor (Cohen et al., 2000). Even though there seems to be controversy on the neonatal effects of fluoxetine exposure during pregnancy, it still remains unclear whether children exposed to fluoxetine **throughout pregnancy** will display long-term neurodevelopmental abnormalities. Furthermore, prenatal exposure to SSRIs might affect regulation of specific transmitter systems with no gross CNS alteration. Given that large numbers of developing humans are exposed to SSRIs, and there is evidence that indicates adverse effects of fluoxetine exposure throughout gestation on humans at birth, an analysis of long term effects of prenatal fluoxetine on brain development and function is required.

B-3) SSRI function in the CNS

Clinically effective SSRIs share the ability to enhance 5-HT transmission and 5-HT is thought to be an important regulator of early brain development. Acutely, SSRIs have been shown to inhibit 5-HT re-uptake; enhanced extracellular concentrations of 5-HT due to 5-HT reuptake inhibition by the SSRIs also decrease firing rate of 5-HT neurons via activation of somatodendritic 5-HT1A autoreceptors. Furthermore, chronic administration of SSRIs, has been shown 1) to enhance 5-HT transmission, measured electrophysiologically in rat hippocampus (Chaput et al., 1988), 2) to enhance extracellular 5-HT measured by microdialysis in rat frontal cortex (Bel et al., 1993) and

3) to enhance evoked release of 5-HT from slices of rat hypothalamus (Moret et al., 1990), dorsal raphe and suprachiasmatic nucleus (O'Connor et al., 1994) and guinea pig hippocampus, frontal cortex and hypothalamus (Blier and Bouchard 1994). The mechanism by which chronic SSRIs enhance 5-HT transmission appears to involve activativation of post-synaptic 5-HT_{1A} receptors (Haddjeri et al., 1998), desensitization of somatodendritic and terminal 5-HT autoreceptors (Berqvist et al., 1999; Blier and de Montigny, 1999) and desensitization of the 5-HT transporter (Pineyro et al., 1994).

While acute SSRI administration depresses neuronal firing rate, desensitization of somatodendritic 5-HT_{1A} autoreceptors with chronic SSRI treatment restores neuronal firing rat to pre-drug levels (Blier et al., 1994, Welner et al., 1989). Chronic SSRIs also produce greater release of 5-HT per action potential via desensitization of release-regulating 5-HT_{1D} autoreceptors in guinea pig hippocampus and hypothalamus, but not in frontal cortex (Blier et al, 1994) and through desensitization of 5-HT_{1B} autoreceptors in the rat hippocampus (Chaput et al., 1991, Pineyro et al., 1988) and hypothalamus (Moret et al., 1990) and of 5-HT_{1D} autoreceptors in rat raphe (Pohland et al., 1989).

SSRIs used during pregnancy might affect 5-HT transmission in offspring since lipid soluble molecules of molecular weight < 600, such as antidepressants, cross the placental barrier (Mirkin, 1974, Pohland et al., 1989). Furthermore, SSRIs during gestation could have extensive effects on the fetus since 5-HT is though to be an important regulator of early brain development. It is thought that the 5-HT system is one of the first neurotransmitter systems to develop during ontogeny (Tillet, 1988, Whitaker-Azmitia et al., 1989). 5-HT neurons are detectable as early as day 13 in rats and synthesize 5-HT before synapses are formed (Tillet, 1988). Both 5-HT receptors and 5-

HT re-uptake sites are present in rat brain prenatally (Hellendall et al., 1993, Hillion et al., 1993, Azmitia et al., 1990). Also, 5-HT has been shown to act as a differentiation signal of CNS target cells and to autoregulate growth of 5-HT neurons in culture (McGuirk and Silverstone, 1990). These observations suggest that 5-HT acts to regulate early brain development and hence, can be affected by prenatal exposure to SSRIs. Furthermore, SSRIs also have peripheral effects such as, placental vasoconstriction and alteration of maternal food intake, which may indirectly affect the development of the fetus (Wong et al., 1988, Bross and Hoffer, 1995, Lightowler et al., 1996).

B-4) Animal Studies on fluoxetine during pregnancy

There exist five studies on the effect of gestational fluoxetine on the resulting offspring and all of them are on a rat model. Del Rio et al (1994) administered fluoxetine in drinking water to pregnant rats from gestation day 6 to birth (day 22) at daily dosing of 2.5 mg/kg. Decreases in 5-HT mediated phosphoinositide hydrolysis, a measure of post-synaptic 5-HT receptor efficacy, in cortical slices and in [^{3}H] imipramine binding were observed in offspring at day 25. In a second experiment from a different group, Vorhees at al (1994) administered fluoxetine by gastric lavage to pregnant rats on day 7-20 of gestation at 12 mg/kg/day. Fluoxetine caused maternal weight loss, reduced litter sizes and increased neonatal mortality. However, they found no effect of fluoxetine on several neurobehavioral tests in surviving offspring and concluded that "the data suggest that fluoxetine is not developmentally neurotoxic in the rat".

In a series of experiments, Cabrera and Battaglia (1994) administered fluoxetine (10 mg/kg/d) subcutaneously for only 8 days, from gestation day 13-20 to pregnant rats.

In the first of these studies, they found reduced density of hypothalamic 5-$HT_{2A/2C}$ receptors, and a decreased ACTH response to a 5-$HT_{2A/2C}$ receptor agonist, in offspring at day 70, but not at day 25. Furthermore, using the same methods, Cabrera-Vera and Battaglia (1998) showed that prenatal exposure to fluoxetine significantly altered the density of 5-HT transporters in thethe hypothalamus, hippocampus and amygdala of prepubescent rats (25 days) but not in the adult rats (70 days). Again using the same methods, Cabrera-Vera et al. (1997) demonstrated that gestational exposure to fluoxetine significantly reduced 5-HT content in the frontal cortex of prepubescent but not adult male offspring. However, no differences in 5-HT content were seen in the midbrain at both ages. These series of experiments accentuates the importance of examining long-term effects of prenatal drug exposure to fluoxetine and the importance of observing effects in different age groups.

As mentioned in some previous studies, prenatal fluoxetine exposure is associated with lasting changes in 5-HT-mediated processes in rat brain. However these few studies have many notable limitations. First, the above rat studies administered fluoxetine only from day 6-7 of gestation until birth. Thus these studies have not tested effects of SSRIs administered to rats either during very early pregnancy or during the brain growth spurt, a sensitive period for CNS effects, which occurs around day 7-10 postnatally in the rat (Montero et al., 1990). In humans, the exposure to fluoxetine is likely to occur early in pregnancy such as in the case of unplanned pregnancy and, exposure during the last trimester (when the brain growth spurt occurs in humans) is likely during prescribed medication. Therefore, studies must be designed to administer fluoxetine at times during gestation when human fetuses are more likely to be exposed.

Secondly, the rat model is not as appropriate for the study of the effects of prenatal drug exposure as the guinea pig. The first reason is the structure of the placenta. The human and the guinea pig placenta are of the hemomonochorial type, since they contain a single trophoblast layer. In contrast, the rat and the mouse possess a hemotrichorial placenta, which contains three layers of trophoblast cells. Thus, it has been mentioned that the guinea pig is the animal of choice for studies of placental physiology (Dawes, 1968). A second advantage with the guinea pig is the fact that it is neurologically mature at birth (Fink et al., 1996), while the brain of the rat only at postnatal day 10-20 reaches maturity equivalent to human at birth. Finally, the guinea pig is increasingly used for studies in 5-HT transmission and on antidepressant action (Blier and Bouchard, 1994, Hoyer and Middlemiss, 1989, Lauder et al., 1982), since 5-HT release-regulating terminal autoreceptors are of the 5-HT_{1D} type in both human and guinea pig brain but almost exclusively 5-HT_{1B} in rat brain (Buhlen et al., 1995, Lauder et al., 1982). Thus, due to the similar barrier to drug transport through the placenta and comparable receptor characteristics as compared to humans, we aimed to develop the more appropriate guinea pig model for studies on gestational effects of fluoxetine.

B-5) 5-HT receptors and fluoxetine

The 5-HT system is involved with higher cognitive functioning and is the target of many antidepressant therapies, including fluoxetine. Chronic use of these drugs results in receptor function and density changes. The investigation of these changes is important in understanding the behavioral effects seen in humans and animals treated with these drugs. Furthermore, gestational exposure to these drugs might inadvertently modulate the

densities of the receptors in the fetuses. Therefore, it is important to investigate these receptors in offspring exposed to gestational fluoxetine (specific to our study).

5-HT_{1A} receptor

The 5-HT1A receptor belongs to the group of G-protein coupled receptors that consists of 7 distinct hydrophobic amino acid sequences which form 7 trans-membrane α-helices. The two regions with the highest density of these receptors in the CNS are in the hippocampus and the dorsal raphe nuclei. Furthermore, the localization of this receptor within the cell is different in the given regions, being somatodendritic in the raphe nuclei and postsynaptic in the hippocampus (Olivier et al., 1999).

The effects of treatment of adult animals with fluoxetine on the 5-HT_{1A} receptor have been extensively investigated. Welner et al. (1989) reported that a 21-day treatment of fluoxetine (10 mg/kg/day) significantly reduced 5-HT_{1A} receptor binding levels in the dorsal raphe nucleus of treated rats, but did not affect binding in the hippocampus. Furthermore, in a study by Lepoul et al. (1995) fluoxetine or paroxetine, injected daily (5mg/kg, i.p.) for various time periods up to 21 days, produced a functional desensitization of somatodendritic 5-HT_{1A} autoreceptors. This effect also increased along the course of the treatment from approximately 40% on the 3rd day to 60-80% on the 21st day. However, the same group also reported no alteration in densities of 5-HT receptor binding sites (including 5-HT_{1A}) following 3 week treatment with fluoxetine.

Klimek et al. (1994) tested the effects of chronic treatment (11days) with fluoxetine and citalopram and reported a differential regulation of 5-HT_{1A} receptor in the rat brain. Their effects were compared with those of other antidepressants: imipramine,

mianserin and levoprotiline. The density of 5-HT_{1A} receptors in the rat hippocampus was enhanced after chronic citalopram, imipramine and levoprotiline, but not altered after fluoxetine administration. In contrast with chronic treatment, acute administration of fluoxetine resulted in a decreased density of 5-HT_{1A} receptors in the hippocampus. Interestingly though, when chronic fluoxetine treatment was compared with acute fluoxetine treatment, a 24% increase in the number of [^{3}H] 8-OH-DPAT (a selective 5-HT_{1A} ligand), binding sites in the hippocampus was observed. In contrast to these findings, a recent study by Subhash et al. (2000) reported that chronic administration of fluoxetine (10mg/kg) significantly decreased the densities of 5-HT_{1A} receptor sites in the cortex and the hippocampus of the rat brain.

Overall, the literature indicates that chronic fluoxetine administration to adult animals enhances 5-HT transmission by increasing functional sensitivity of 5-HT_{1A} autoreceptors. However inconsistent results have been obtained with respect to 5-HT_{1A} receptor density, with studies showing increases, decreases and no change in post-synaptic 5-HT_{1A} receptor densities in the hippocampus and reductions or no change in somatodendritic 5-HT_{1A} receptor densities in the dorsal raphe, following chronic fluoxetine treatment in adult rats. No studies on effects of maternal fluoxetine treatment during pregnancy on 5-HT_{1A} receptor density in offspring have been published. However the above data from studies with adult animals raise the possibility that prenatal fluoxetine may alter 5-HT_{1A} receptors in offspring.

5-HT_{2A} receptor

Serotonin $5HT_{2A}$ receptors have important implications in physiological responses in both the central and peripheral nervous systems. These processes include smooth muscle contraction, platelet aggregation and the modulation of mood and perception in the CNS. The present review will focus on CNS implications of the 5-HT_{2A} receptor.

The 5-HT_{2A} receptor belongs to the G-protein-coupled receptor superfamily. The human 5-HT_{2A} receptor is 87% homologous with the rat counterpart. The CNS distribution of 5-HT_{2A} receptor has been mapped extensively by receptor autoradiography. High levels of 5-HT_{2A} binding sites are found in many forebrain regions, but particularly cortical areas including the neocortex, entorhinal and pyriform cortex, claustrum, caudate nucleus, nucleus accumbens, olfactory tubercle and hippocampus (Lopez-Gimenez et al., 1997).

Previous studies investigating fluoxetine effects on the 5-HT_{2A} receptors in the hypothalamus suggest that fluoxetine gradually increases the coupling of 5-HT_{2A} receptors to G-proteins when [^{125}I] DOI is used as the 5-HT_{2A} receptor ligand, but does not up-regulate 5-HT_{2A} receptors using [^{3}H] ketanserin (Li et al. 1997). In contrast, Klimek et al. (1994) demonstrated that the density of 5-HT_2 receptors using [^{3}H] ketanserin binding in the rat cerebral cortex was increased after 11 day fluoxetine administration. However, it is important to note that [^{3}H] ketanserin binds 5-HT_{2A} receptors both coupled and uncoupled to G-protein.. Furthermore, binding in the two experiments was at different sites (hypothalamus vs cortex).

In an experiment analyzing the effects of gestational fluoxetine administration, Cabrera and Battaglia (1994) administered fluoxetine (10 mg/kg/d) subcutaneously from

gestation day 13-20 to pregnant rats. They found reduced density of hypothalamic 5-$HT_{2A/2C}$ receptors, and a decreased ACTH response to a 5-$HT_{2A/2C}$ receptor agonist, in offspring at day 70, but not at day 25. This result Indicates that gestational fluoxetine can have long term effects on 5-$HT_{2A/2C}$ receptors, a least in the hypothalamus. Although the analysis of the effects of fluoxetine on the 5-HT_{2A} receptor is not as exhaustive as on the 5-HT_{1A} receptor, there exists inconsistencies in the literature as to whether the densities of the 5-HT_{2A} receptor change following fluoxetine administration to the adult animal. However, Cabrera and Battaglia (1994) clearly demonstrated the possibility of changes in receptor density that might occur after gestational exposure to fluoxetine.

B-6) 5-HT, fluoxetine, prenatal stress and behavior

The 5-HT system has been shown to affect many behavioral functions such as temperature regulation, feeding behavior, sexual behavior, response to painful stimuli, sensorimotor gating, escape behavior, and stress. Therefore, since fluoxetine is a selective serotonin reuptake inhibitor affecting 5-HT transmission, this drug can have potential influences on these behaviors.

Feeding behavior

Animal models have demonstrated that increased transmission of 5-HT with SSRIs, such as fluoxetine and sertraline, may have a role in the inhibitory control of feeding (Simansky et al., 1996) Behavioral studies have shown that these drugs reduce the rate of eating and size of meals in a manner suggesting that increased serotonergic transmission may terminate feeding by specifically enhancing satiation (McGuirk et al

1992). Analysis of the 5-HT receptor subtypes involved, using selective agonists and antagonists, has showed that 5-HT_{1B}, 5-HT_{2C}, and 5-HT_{2A} receptors have an influence in regulating food intake. Furthermore these receptor subtypes produce different behavioral outcomes. 5-HT_{1B} and 5-HT_{2C} receptors appear to be involved in regulating meal size, whereas 5-HT_{2A} receptors disrupt the continuity of feeding (Simansky and Vaidya 1990). Furthermore, the administration of fluoxetine has been shown to reduce hunger and food intake in humans (McGuirk et al., 1990). In addition, the use of fluoxetine during pregnancy has been shown to depress maternal weight gain, in humans (Chambers et al., 1996) and animals (Da Silva et al., 1999). Hence, these finding indicate that the administration of fluoxetine during pregnancy may change the eating behavior of dams exposed to the drug and furthermore influence the development of the resulting offspring.

Pain Sensitivity

In addition to treating depression, antidepressant drugs are increasingly used as co-analgesics in clinical management of migraine and neuropathic pain. The rationale is that these drugs increase the pain threshold in patients with chronic pain (Ansari, 2001). Although the exact mechanism of action is presently unknown it has been shown that these therapeutic benefits can be directly attributed to the effects of the drugs on the 5-HT system. Singh et al. (2001) explored the possible mechanism of fluoxetine's antinociceptive properties in animals. Acetic acid-induced writhing, hot plate and tail-flick tests were performed to assess fluoxetine-induced antinociception. Fluoxetine (5-20 mg/kg) administered intraperitoneally produced a significant and dose-dependent antinociceptive effect against acetic acid-induced writhing in mice. Fluoxetine (20

mg/kg) also exhibited antinociceptive effects in the tail flick and hot plate tests. Most importantly, fluoxetine (10 mg/kg) did not exhibit any antinociceptive effect in serotonin-depleted animals. These data suggest that fluoxetine-induced antinociception involves serotoninergic pathways since the effects of fluoxetine weren't seen in 5-HT depleted animals. The possible implications of the antinociceptive effect of fluoxetine for our experiment are two-fold. Firstly, it is possible that this effect can reduce the pain experienced by the dams during gestation hence diminishing the stress experienced in the pregnant animals. Secondly, fluoxetine might alter 5-HT functioning in the resulting offspring, hence changing their pain sensitivity, which as mentioned previously is partly regulated by the serotonin system.

Sensorimotor gating and acoustic startle response

Sensorimotor gating is a measure of the ability of a subject to inhibit a response to a stimulus after being given an environmental cue that the stimulus will be initiated. One of the tests used to measure the capacity of sensorimotor gating in animals is prepulse inhibition (PPI) of acoustic startle. In this test an animal is presented with an initial loud (high intensity) acoustic stimulus for which an acoustic startle response (ASR) is measured with mechanical transducers. On subsequent trials, the animal is given prepulse tones at differing low intensities to warn the animal of the pending loud startle noise. The ability of the animal to inhibit the startle to the high intensity stimulus is measured at different prepulse intensities.

PPI and ASR are influenced by glutaminergic, dopaminergic and serotonergic modulation (Koch, 1999, Swerdlow et al., 2001). More of interest to our study is the

latter system, which is altered with long-term fluoxetine administration and may thus affect ASR or PPI outcome measures. The effects of fluoxetine on ASR have been investigated in an experiment by Dow-Edwards (1996). Male and female rats received 25 mg/kg/day fluoxetine HCl or vehicle SC during postnatal days 11-20. At 75 days of age, animals were tested for startle responses on 2 consecutive days. Fluoxetine exposed males showed a significant increase in startle response compared to the rats receiving only vehicle. These results suggest that administration of fluoxetine during the postnatal period has lasting effects on ASR by modulating the 5-HT system. Although fluoxetine affects ASR, it doesn't seem to modulate PPI, at least when administered acutely to adult animals. Martinez & Geyer (1996) administered fluoxetine (10 mg/kg) to adult rats prior to testing and found no effect on PPI. Unfortunately, the long-term effects of fluoxetine administration on PPI outcome measures have not yet been investigated.

Overall, the literature suggests that modulation of the 5-HT system by fluoxetine administration in adult animals has effects on 5-HT receptors and behaviors regulated by 5-HT. Hence, it is possible that similar changes may be seen in offspring in response to gestational fluoxetine.

C. Experimental Design

The experiments in this thesis, all carried out in guinea pigs, were designed:

1. To determine the amount of fluoxetine that is needed to inject in guinea pigs, in order to mimic the therapeutic plasma concentrations obtained in humans.
    a. to determine the half-life of fluoxetine and its metabolite, norfluoxetine in the guinea pig.

b. to evaluate the fluctuation of drug levels in a given day after plateau drug levels had been reached during a regimen of repeated daily fluoxetine injections.

c. to determine the concentration of fluoxetine that was needed to be administered via osmotic pumps to obtain steady state levels of fluoxetine and norfluoxetine of 350 mg/nl in the plasma (therapeutic levels in humans)

2. To determine whether the administration of fluoxetine via daily injections throughout pregnancy would affect 5-HT brain receptor densitiess in the resulting guinea pig offspring

   a. to investigate the short-term effects (14-day old pups) of fluoxetine administration during pregnancy via injections in the resulting offspring, specifically looking at 5-HT_{1A} receptor densities in hippocampal subregions.

   b. to investigate the long-term effects (9-weeks old pups) of fluoxetine administration during pregnancy via injections in the resulting offspring, specifically looking at 5-HT_{2A} receptor densities in the frontal cortex and striatal subregions.

3. To study the effects of gestational fluoxetine administration via osmotic pumps on pregnancy parameters and on behavioral performance of the resulting offspring.

a. to test the effects of gestational fluoxetine via osmotic pumps on maternal weight gain through pregnancy, pup weight gain (at 2 weeks and 9 weeks), number of stillborns and live litter size between treatment groups.

b. to measure the thermal pain tolerance of the guinea pig offspring (at both 2 weeks and 9 weeks) using a hot-plate test.

c. to measure the acoustic startle response and prepulse inhibition of acoustic startle in guinea pigs (at 2 weeks and 9 weeks) exposed in utero to fluoxetine, vehicle or no treatment via osmotic pumps.

Section II: Methodology

A. Introduction

B. Preliminary pharmacokinetics experiments

1) Half-life of fluoxetine after a single acute injection in the female guinea pig
2) Daily fluctuations in plasma fluoxetine during a regimen of repeated daily fluoxetine injections
3) Plasma fluoxetine levels during continuous administration of fluoxetine by osmotic mini-pump

C. Effects of gestational fluoxetine administered by subcutaneous injection on the CNS 5-HT_{1A} receptors and 5-HT_{2A} receptors in the offspring

1) Experimental groups
2) Mating protocol
3) Gestational period
4) Autoradiography and image analysis of 5-HT_{1A} receptors
5) Autoradiography and image analysis of 5-HT_{2A} receptors

D. Effects of gestational fluoxetine administered by subcutaneous osmotic pump on the offspring's performance in two behavioral tests

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4) Gestational period and parturition

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1) Pharmacokinetics of fluoxetine in guinea pigs

2) Steady state levels of fluoxetine and norfluoxetine after repeated injections of fluoxetine HCL in female guinea pigs

3) Determination of plasma levels of fluoxetine and norfluoxetine after the administration of fluoxetine HCl via osmotic mini pumps

4) 5-HT_{1A} and 5-HT_{2A} receptor densities

5) Maternal and offspring weight gain

6) Length of pregnancy, # of live births and # of still births

7) Acoustic startle response

8) Pre-pulse inhibition

9) Hot-plate test

A. Overall Design

The aim of the current project was to examine whether fluoxetine administration to guinea pigs during pregnancy has long-term effect on 5-HT receptors and 5-HT-modulated behaviors in the resulting offspring. The study was divided into two separate experiments, each with different and independent outcome measures. The first experiment consisted of treating pregnant guinea pigs with a daily dose of fluoxetine injected subcutaneously. The second experiment consisted of administering the fluoxetine to pregnant guinea pigs via Alzet osmotic mini-pumps also placed subcutaneously, thus delivering the drug at a steady rate and avoiding the larger daily fluctuations in plasma drug levels obtained with daily injection. In each of these experiments, a vehicle and control group were included for investigation.

In the injection study we chose to investigate the density of the 5-HT_{1A} and 5-HT_{2A} receptors in the offspring of the treated dam. In previous studies in our lab, 9 week old offspring from dams administered fluoxetine by injection throughout pregnancy, under conditions identical to those described here (once daily injections of 7 mg/kg fluoxetine throughout pregnancy), showed a near significant ($p<.07$) increase in 5-HT_{1A} receptor binding in the CA1 region of the hippocampus compared to the vehicle group (S. Malik, R. Vartazarmian, Y. Zhang and P. Boksa, unpublished observations). In the present study, hippocampal 5-HT_{1A} receptors were measured in 2 week old pups to determine if these receptors might be significantly (and transiently) altered in offspring at an age younger than 9 weeks, after maternal fluoxetine treatment. We measured 5-HT_{2A} receptors in adult (9 week old) offspring, in order to test if gestational fluoxetine had long-term effects on this receptor sub-type in offspring.

In the pump study, three separate behavioral tests were performed on the offspring at 2 weeks and at 9 weeks of age, i.e. measurement of acoustic startle responses, prepulse inhibition of

acoustic startle and thermal pain threshold. Before engaging in these experiments, preliminary studies were performed to determine the pharmacokinetics of fluoxetine. These studies and the previously mentioned experiments are described below.

B. Preliminary Pharmacokinetics Experiments

The primary aim of these experiments was to determine the amount of fluoxetine that was needed to inject in guinea pigs in order to mimic the therapeutic plasma concentrations obtained in humans. Therapeutic doses of fluoxetine used in major depression range from 20mg/day to 80 mg/day. For our study we attempted to achieve blood levels similar to those obtained in humans after the oral administration of 40mg of fluoxetine daily. Similar doses were shown to produce prenatal effects in human foetuses (Chambers et al., 1996). The levels of steady state plasma fluoxetine and norfluoxetine measured in humans receiving 40 mg of fluoxetine a day for 21-30 days were 91-302 ng/ml fluoxetine and 72-258 ng/ml norfluoxetine (Baker et al., 1992). With this data, we decided that plasma blood concentrations of about 200 ng/ml for fluoxetine and 150 ng/ml for norfluoxetine, would approximate therapeutic blood levels in humans, for our experiments with guinea pigs. However, we anticipated that the half-life of fluoxetine would be considerably shorter in guinea pigs compared to humans (as it did turn out to be, see below). Therefore we chose to use the combination of both fluoxetine and its metabolite, to obtain a desired plasma level of about 350 ng/ml fluoxetine + norfluoxetine. Evidence has shown that norfluoxetine effectively inhibits the uptake of 5-HT from the synapse; thus it is an active metabolite (Wong et al., 1993).

B-1) Half-life of Fluoxetine after a Single Acute Injection in the Female Guinea Pig

The purpose of this experiment was to determine the half-life of fluoxetine and its metabolite, norfluoxetine in the guinea pig. Although the plasma half-lives of this drug and its metabolite are well studied in rats (Caccia et al., 1990) and in humans (Altamura et al., 1994), these measurements are unknown in the guinea pig. Furthermore, the half-life measurement of fluoxetine will allow us to approximate the time needed to reach steady state levels of the drug in the bloodstream when administering the drug chronically. The time that steady state levels are obtained is in approximately 4 half-lives.

For this study, we measured blood plasma levels of fluoxetine and norfluoxetine after a single injection of fluoxetine in the non-pregnant female guinea pig. Although, a half-life study in pregnant guinea pigs would have been a more accurate indicator for the drug levels obtained during the main experiment, we were unable to perform this study due to financial constraints. The blood was collected from individual animals at 5 min, 15 min, 30 min, and 1,2,3,5,10,24,36,48 hours after subcutaneous administration of 10mg/kg of fluoxetine. There were six individual animals in each time group except at 24 hours, when only 5 animals were sampled. Separate animals were used for each time point due to the fact that the blood was collected by cardiac puncture. (This route of blood sampling was necessary in order to obtain a sufficient volume of blood from the guinea pig for analysis of plasma fluoxetine). At 1 PM, animals were injected with fluoxetine and then anesthetized with isofluorane before the collection of blood by cardiac puncture (approx. 2ml/animal). Blood was collected in ice-cold, saturated EDTA-containing tubes and subsequently centrifuged at 1500 r.p.m. for 15 min, at 4°C. The supernatant, consisting of plasma, was stored in Eppendorf tubes at –80°C. The plasma concentrations of fluoxetine and its main metabolite, norfluoxetine, were measured by Dr. Glen

Baker (University of Alberta) using gas chromatography with electron capture (Torok-Both et al., 1992).

B-2) Daily Fluctuations in Plasma Fluoxetine during a Regimen of Repeated Daily Fluoxetine Injections

The aim of this study was to evaluate the fluctuation of drug levels in a given day after plateau drug levels had been reached during a regimen of repeated daily fluoxetine injections. Non-pregnant female guinea pigs were injected once every 24 hours for a total of 9 days (9 injections) with fluoxetine at a concentration of 5mg/kg/day. Animals were anesthetized with isofluorane and blood was drawn by cardiac puncture at 5minutes prior to the scheduled daily injection and at 15min, 30min, 5hrs and 10hrs after the injection. Three animals were used in each time group and separate animals were used for each time point. With this study we concluded that 7 mg/kg/day should be injected daily to attain fluoxetine + norfluoxetine blood levels comparable to human levels.

B-3) Plasma Fluoxetine Levels during Continuous Administration of Fluoxetine by Osmotic Mini-pump

The purpose of this experiment was to determine the concentration of fluoxetine that was needed to be administered to guinea pigs via osmotic pumps to obtain steady state levels of fluoxetine + norfluoxetine of 350 ng/ml in the plasma. See Methods section D3 below for detailed description of the method used for surgical implantation of the osmotic pump. For this experiment, non-pregnant guinea pigs were implanted with osmotic pumps containing fluoxetine; on the day of blood sampling, animals were anesthetized with isofluorane and blood

samples were obtained by cardiac puncture for measurement of plasma fluoxetine and norfluoxetine

The administration of 3.5mg/day in five non-pregnant female guinea pigs for 12 days (using 14-day osmotic pumps) gave a mean level of fluoxetine + norfluoxetine in the blood plasma that was lower than the target of 350 ng/ml. We subsequently doubled the concentration of fluoxetine administered by the pump to attempt to reach the required plasma levels. As predicted, the infusion of fluoxetine at 7 mg/kg/day for 21 days (using 28-day osmotic pumps) in four non-pregnant guinea pigs yielded combined mean fluoxetine + norfluoxetine levels relatively close to our desired target. Since the combined levels of fluoxetine + norfluoxetine of the last experiment were close to the desired levels, in subsequent experiments with pregnant females, we chronically administered fluoxetine using osmotic pumps at a rate of 7mg/kg/day.

C. Effects of Gestational Fluoxetine Administered by Subcutaneous Injection on CNS 5-HT_{1A} receptors and 5-HT_{2A} Receptors in the Offspring

The aim of this experiment was to determine whether the administration of fluoxetine (by injection) throughout pregnancy would affect 5-HT receptor densities in the brains of resulting guinea pig offspring. This consisted of administering daily fluoxetine to pregnant guinea pigs, raising the resulting offspring, and analyzing the 5-HT receptors in the brain of these offspring using receptor autoradiography.

C-1)Experimental groups

Group 1: Fluoxetine-injected

This group of pregnant guinea pigs received once daily injections of subcutaneous fluoxetine HCl (7mg/kg; dissolved in distilled water) from day one of gestation until parturition.

Group 2: Vehicle-injected controls

This group of pregnant guinea pigs received once daily subcutaneous injections of water from the first day of gestation until parturition.

Group 3: Non-injected controls

This group of guinea pigs weren't injected or handled throughout gestation.

C-2) Mating protocol and housing conditions

The animals were mated using a technique that uses the vaginal membrane opening found in guinea pigs as a marker of possible pregnancy. One of the advantages of using guinea pigs for breeding is the fact that they lose or break their vaginal membrane when they're in estrous, allowing for precise timing of pregnancy. The mating procedure was as follows:

1. The females were classified as being in estrous or not by noting the presence or absence of the vaginal membrane, respectively.
2. The females in estrous were housed in groups in the absence of males.
3. Groups of three guinea pigs were formed combining two females, who were not in estrous, with one male.

4. The animals in the harem were kept in place until they completed the estrous stage. Once the membrane was completely closed, indicating that the animal was pregnant if mating was successful, chronic administration of fluoxetine (pumps or injections) was begun. (Considering that the estrous period lasts for three days and that mating usually occurs during the first 12 hours of the given period, all pregnant animals start being treated with the drug at least by day 2 of pregnancy and some (probably most) start receiving treatment on day 0 or day 1 of pregnancy.)
5. The animals were continued to be checked daily for the possible reopening of the vaginal membrane, which indicates the mating was unsuccessful. If so, the administration of the drug was discontinued and the animal was sacrificed. The success rate of this mating method was between 75-80%.

The guinea pigs in this study were group housed during the first 53 days of their pregnancy. At gestation day 54 (2 weeks prior to the expected day of delivery, gestation day 68), each pregnant guinea pig was individually housed to prevent mixing between mothers and litters, and to prevent cannibalism of the newborn pups. When the guinea pigs gave birth, daily fluoxetine injections were stopped and the pups were weaned at 2 weeks of age. Only male offspring were retained for this study, and these were group-housed until behavioral testing or sacrifice for measurement of 5-HT receptors at 9 weeks of age. Due to technical and economic constraints, the pups weren't cross-fostered. However it is likely that effects on the offspring of altered maternal care due to fluoxetine would be minor since guinea pigs are born relatively neurologically and behaviorally mature. In fact, guinea pigs pups can be weaned within 3-4 days after birth.

C-3) Autoradiography and Image Analysis of 5-HT_{1A} receptors

The purpose of this study was to investigate the short-term effects (2 week old pups) of fluoxetine administration during pregnancy on hippocampal 5-HT_{1A} receptors in the resulting offspring. Offspring from dams administered fluoxetine throughout pregnancy (once daily injections of 7 mg/kg fluoxetine) were sacrificed at 2 weeks of age. The intact brain was collected and placed in –50°C isopentane for 5 seconds. The brain was then removed from the isopentane, wrapped in aluminum foil, and stored at –80°C until analysis of 5-HT receptors was performed.

The following protocol was used for the investigation of the 5-HT_{1A} receptor using [^{3}H] 8-hydroxy-2- [di-N-propylamino] tetralin ([^{3}H] 8-OH-DPAT).

Brains were serially cut into 20 micrometer thick coronal sections using a cryostat. The sections were mounted on slides coated with 0.2% gelatin/0.033% chromium potassium sulfate, and kept at –80°C until use. On each slide there were 3 sections, corresponding to consecutive coronal planes at 500-600 micrometer intervals. The tissue sections were preincubated for 30 min at 25°C in 0.17 mM Tris-HCl buffer at 7.4.pH. Incubation was performed with the same buffer containing 2 nM [^{3}H] 8-OH-DPAT (NEN/DuPont, Boston, MA; specific activity 234 Ci/mmol) for 60 min at room temperature. Non-specific binding was determined in adjacent sections by incubating with buffer containing 2 nM [^{3}H] 8-OH-DPAT plus 10 micromolar (final concentration) of unlabeled 5-HT (Sigma). The slides were then washed in ice-cold buffer (2 times for 5 min), rinsed in cold distilled water and dried under a stream of air.

The sections and [^{3}H] Microscales standards (Amersham, Arlington Heights, IL) were exposed to tritium-sensitive Kodak Biomax MR films. Densitometric measurements of the films

was carried out with an MCID image analysis system (Imaging Research, St-Catherines, Ontario). Standard curves generated from [^{3}H] Microscales was used to convert optical densities into fentomoles of ligand bound per milligram of protein (fmol/mg protein). The different anatomical structures were defined according to the atlases of Lehman (1974) and that of Paxinos and Watson (1986). Structures analyzed were subregions of the hippocampus (CA1, CA2, CA3, Dentate Gyrus).

C-4) Autoradiography and Image Analysis of 5-HT$_{2A}$ receptors

The purpose of this study was to investigate the long-term effects (9 week old offsprings) of fluoxetine administration during pregnancy (once daily injections of 7 mg/kg fluoxetine) on 5-HT$_{2A}$ receptor densities in the resulting offspring. Although analyzing a short-term effect (2 week offspring) would have added an interesting chronological comparison, we were unable to execute these experiments due to a low sample size. [^{125}I] (2,5-dimethoxy-4-iodophenyl)2-aminopropane ([^{125}I] DOI) is considered to be a relatively selective agonist of the 5-HT$_{2A}$ receptor and hence was used to analyze the density of these receptors. Brains were serially cut into 20 micrometers thick coronal sections using a cryostat; sections were mounted on slides coated with 0.2% gelatin/0.033% chromium potassium sulfate, and kept at –80°C until use. On each slide there were 3 sections, corresponding to consecutive coronal planes at 500-600 micrometer intervals. The tissue sections were preincubated for 30min at 25°C in 50 mM Tris-HCl buffer at 7.4. pH containing 4mM CaCl, 0.1% ascorbic acid, and 0.1% bovine serum. They were then incubated in the same buffer containing 200 pM [^{125}I] DOI (NEN/DuPont, Boston, MA; specific activity 2200 Ci/mmol) and 30 nM of unlabeled 5-HT for competitive binding, for 90 min at room temperature. Non-specific binding was determined by adding 4 mM unlabeled 5-

HT (Sigma) to the incubation solution. The slides were then washed in ice-cold buffer (4 times for 2 min), rinsed in cold distilled water and dried under a stream of air.

The sections and [^{125}I] Microscales standards (Amersham, Arlington Heights, IL) were exposed on Kodak Biomax MS films. Densitometric measurements of the films was carried out with an MCID image analysis system (Imaging Research, St-Catherines, Ontario). Standard curves generated from [^{125}I] Microscales were used to convert optical densities into fentomoles per milligram of protein (fmol/mg protein). The different anatomical structures were defined according to the atlases of Lehman (1974) and that of Paxinos and Watson (1986). Two structures containing relatively high densities of 5-HT_{2A} receptors were analyzed, the frontal neocortex and the dorsal striatum. Although the hypothalamus and the nucleus accumbens also contain appreciable amounts of 5-HT_{2A} receptors, it was not possible to analyze these structures due to the poor quality of brain slices in these regions.

D. Effects of Gestational Fluoxetine Administered by Subcutaneous Osmotic Pump on the Offspring's Performance in Two Behavioral Tests.

The aim of this experiment was to study the effects of gestational fluoxetine administration via osmotic pumps on the behavioral performance of the resulting offspring. The study design consisted of delivering fluoxetine to pregnant guinea pigs using Alzet osmotic mini-pumps, then raising the resulting offspring and performing two behavioral tests on them. Furthermore, once the behavioral tests were completed the brains of the offspring were removed and placed in storage for further investigation (not included in this thesis due to time constraints).

D-1) Experimental groups

Group 1: Fluoxetine - via osmotic pump

This group of pregnant guinea pigs received fluoxetine via Alzet osmotic mini pump through the gestation period. The pumps administered fluoxetine HCl (7mg/kg/day) dissolved in DMSO (see section D-3 below for concentrations of DMSO used) and distilled water from the first day of gestation until parturition.

Group 2: Vehicle - via osmotic pump

This group of pregnant guinea pigs received vehicle via Alzet osmotic mini pump through the gestation period. The pumps administered DMSO (concentrations identical to those used for the fluoxetine group) in distilled water from the first day of gestation until parturition.

Group 3: No osmotic pump - controls

This group of pregnant guinea pigs weren't operated on or handled throughout gestation.

D-2) Mating protocol

The mating protocol was similar to the one used in the injection study (described in Methods section C above).

D-3) Surgical implantation of osmotic pumps and housing of pregnant dams

The chronic administration of the antidepressant fluoxetine was done using Alzet osmotic mini-pumps. To administer drug throughout gestation, an osmotic mini-pump capable of delivering drug for 28 days drug was implanted on day 1 of pregnancy; this pump was replaced by a second pump on gestation day 22, followed by a third pump on day 44. For the first pump,

fluoxetine was dissolved in 10% dimethyl sulfoxide (DMSO) and 90% water. Due to the increasing weight of the pregnant guinea pigs, we were required to increase the percentage of DMSO to 15% for the second pump. For the third pump the DMSO concentration was further increased to 20% DMSO in 80% water. The surgical procedure to place the pump subcutaneously was as follows:

1. The animal was anesthetized using 4% isofluorane and 1l/min oxygen.
2. Once anesthetized, the animal was shaved in the mid-dorsal area.
3. The fur was then cleared away and an alcohol swab was passed over the shaved area.
4. A 2 cm long incision was made using a scalpel, following the longitudinal axis of the animal.
5. A hemostat was used to create a subcutaneous space big enough for the pump to fit comfortably, minimizing the pressure on the skin.
6. The pump was then inserted into the space and the wound was closed using suture clips.
7. A topical antibiotic, cicatrin 5%, was then applied to the wound to prevent infection.

The animal was then placed for 30 minutes in a recovery cage where warmth was applied using a heat lamp. The guinea pig was then placed in its regular cage.

The pregnant dams were housed in groups of six per cage and were identified with non-toxic ink markers in the dorsal aspect of the outer ear. As in the previous experiment the dams were housed individually two weeks prior to their due date. Once the dams gave birth, the fluoxetine and vehicle treated dams were sacrificed and the control dams were used as surrogate mothers.

D-4) Cross-fostering

At the date of delivery all pups, including the control untreated pups, were cross-fostered with a surrogate untreated control dam; this cross-fostering procedure controls for maternal behavioral changes due to different treatment groups that can potentially affect our outcome measures in the pups. Each dam received 3 pups, preferably one from each treatment group. At 2 weeks of age, some male pups from each treatment were behaviorally tested and were subsequently sacrificed and brains were flash-frozen and stored. The pups belonging to the 9 week group were weaned and placed in group housing, with 3 animals of the same sex per cage. At 9 weeks, the remaining - offspring were behaviorally tested and were then sacrificed and brains stored at $-80^{\circ}C$.

D-5) Hot Plate Test

The purpose of this test was to measure the thermal pain tolerance of the guinea pigs by placing them on a hot plate and measuring the amount of time it took for them to lift one of the paws. The apparatus consisted of a thin metal plate surrounded by Plexiglas, hence preventing the animal from escaping and also preventing water from entering the apparatus, keeping the animals dry. The plate was submerged in temperature-controlled water. The temperature of the plate was set at $55^{\circ}C$ (Rochford and Chatigny, 1993). Prior to testing, the animals were habituated to the testing room for 20 minutes. Once habituated, the animals were carefully placed on the hot plate and the latency to lift either a hind or front paw was noted. If the animal didn't respond, it was removed from the plate at 90 seconds and given a score of 90 seconds, which was included in the analysis.

D-6) Acoustic Startle Response and Prepulse Inhibition

Startle reactivity was measured using two SR-LAB startle response chambers (San Diego Instruments, San Diego, CA). Each sound-attenuated and ventilated chamber contained a Plexiglas cylinder, whose diameter (12.6 cm) allowed the animal to move but not to turn around. The cylinder was mounted atop a piezoelectric transducer, which detected vibrations caused by movement of the animal. An SR-LAB calibration unit was used to produce consistent response sensitivity between chambers and across days of testing. A sound generation system produced continuous background white noise at 70 dB and the required acoustic stimuli. Sound intensity within the chambers was calibrated using a Radio Shack digital sound level meter (A scale). A microcomputer control unit digitized and stored startle responses and also controlled timing and presentation of acoustic stimuli. Startle amplitude was defined as the average of 100 readings taken at 1-msec intervals, beginning at stimulus onset.

Startle testing took place between 8:00 h and 17:00 h. The startle session began with a 5-min acclimatization period in the presence of 70 dB background noise, that continued throughout the session. After this habituation period, the animals were presented with two orienting pulse alone trials (120 dB, 30 msec); data from these two trials was discarded. Next, five blocks of trials were delivered. Each of these blocks consisted of the following eight trials: two pulse alone trials, five prepulse + pulse trials and one no-stimulus trial, in pseudo-random order. The pulse alone trial consisted of a 120 dB pulse for 30 msec. The five prepulse + pulse trials consisted of a 30-msec prepulse at 3, 6, 9, 12, or 15 dB above background followed by a 70-msec delay and then a startle pulse (120 dB, 30 msec). The intertrial interval was an average 15 sec (range 10-20 sec).

ASR was defined as the mean startle amplitude averaged from the 10 pulse alone trials. %PPI was defined as [1 - (mean startle amplitude on prepulse + pulse trial)/ASR)] × 100.

E. Data Analysis

E-1) Pharmacokinetics of fluoxetine in guinea pigs

Plasma level of fluoxetine and norfluoxetine, determined after a single injection of fluoxetine HCl at 10 mg/kg per female guinea pig, are expressed as ng of drug /ml of plasma in Figure 1. Best-fit trend lines were determined to calculate the half-lives of both fluoxetine and norfluoxetine.

E-2) Steady state levels of fluoxetine and norfluoxetine after repeated daily injections (5mg/kg/day) of fluoxetine HCl in female guinea pigs

The study consisted of injecting 5 mg/kg/day of fluoxetine for 9 days to non-pregnant guinea pigs. Figure 2 shows plasma levels of fluoxetine and norfluoxetine, in ng/ml, at different time points before and after the last injection. The minimum and maximum concentrations were taken from the graph to extract information for future experiments

E-3) Determination of plasma levels of fluoxetine and norfluoxetine after the administration of fluoxetine HCl via osmotic mini pumps

In the first experiment, the delivery of fluoxetine at 3.5 mg/kg/day via osmotic pumps was done for a period of 12 days (using 14 day pumps). The plasma concentrations of fluoxetine and norfluoxetine, in ng/ml, were measured and then added. In the subsequent experiment, the delivery of fluoxetine was increased to a concentration of 7 mg/kg/day. Furthermore, two

different pumps were used; 14-day pumps and 28-day pumps. As in the first experiment, the plasma concentrations of fluoxetine and norfluoxetine were measured and then added. These results are summarized on Table 1.

E-4) 5-HT_{1A} and 5-HT_{2A} receptor densities

Computerized image analysis (MCID System, Imaging Research, Saint Catherines, Ontario) was used to analyze the autoradiograms. Binding was analyzed in the right and left hemispheres of various brain regions identified according to Paxinos and Watson (1986). For each brain region, 9 sections measuring total binding and 6 sections measuring non-specific binding were analyzed for each animal; means of the values from the total binding sections were used for total binding for that animal and similarly, for non-specific binding. The ^{125}I and ^{3}H microscale standards for autoradiography are calibrated for the auto-absorptive features of intact brain gray matter, to produce a standard curve in which optical density is converted to nCi radioligand/ mg tissue. Thus, data are expressed as fmol/mg of tissue +/- standard error of mean. For each brain region, statistical comparisons were performed using 2-way ANOVA with treatment and hemisphere as independent variables. If there was no effect of hemisphere and no interaction between hemisphere x treatment, then the data over hemispheres were combined and one-way ANOVA with treatment as independent variable was performed. Post-hoc Newman-Keuls tests was performed, as appropriate. The accepted level of statistical significance was $p<0.05$. Results on the 5-HT_{1A} and 5-HT_{2A} receptors are shown on Figure 3 and Figure 4, respectively.

E-5) Maternal and offspring weight gain

The maternal weight gain was measured at day one (0 weeks), 3 weeks and 6 weeks into gestation. The offspring weight gain was measured at 2 weeks and 9 weeks after parturition. Mean weights in grams with standard errors of means are summarized in Figure 5. Statistical analysis of the maternal weight gain was performed using repeated measures 2-way ANOVA, with treatment as between subject factor and time as repeated measure. If there was an interaction of treatment x time, one-way ANOVA was performed at each time point, followed by post-hoc Newman-Keuls if required. In the statistical analysis of the offspring weights, one-way ANOVA was performed at the different time point due to the combination of all groups of animals (2 week males, 9 week females, 9 week males) in the 2 weeks result analysis. This was followed by post-hoc Newman-Keuls if required. Furthermore, the two 9 weeks group were combined and analyzed by one-way ANOVA. Statistical significance was considered at $p<0.05$. Results are shown on Table 3.

E-6) Length of pregnancy, # of live births and # of still births

The data for length of pregnancy, # of live births and # of stillbirths were analyzed independently. The means and standard error of the mean of each parameter for each treatment group are indicated in Table 2. The statistical analysis was conducted using one-way ANOVA with the independent variable being the treatment. Post-hoc Newman-Keuls tests were performed if required, and $p<0.05$ was considered significant.

E-7) Acoustic startle response

The acoustic startle response (ASR) was defined as the mean startle amplitude averaged from the 10 pulse alone trials. The mean startle responses and the standard errors of the means are indicated in Figure 6. A one-way ANOVA was performed for the effect of treatment on the mean acoustic startle response.

E-8) Pre-pulse inhibition

The % PPI was defined as [1 - (mean startle amplitude on prepulse + pulse trial)/ASR)] × 100. The mean % PPI and the standard errors of the means are indicated in Figure 7. Statistical comparison was performed using 2-way ANOVA, with treatment as between subject factor and pre-pulse intensity as repeated measure. If there was a significant interaction between treatment x pre-pulse intensity a one-way ANOVA was performed at each pre-pulse intensity, followed by post-hoc Newman-Keuls tests, as required. $P < 0.05$ was considered significant.

E-9) Hot-plate test

The mean response times to the thermal pain stimulus and standard errors of the means in the three treatment groups are indicated on Figure 8. Data for 2-month female and 2-month male groups were first analyzed separately. Since the trend in findings was similar for both sexes, data for the two sexes were combined and further analysis performed on this data set to increase the n and hence the statistical power. Statistical comparisons of the three treatment groups were performed using one-way ANOVA followed by post-hoc Newman-Keuls tests, if required. Statistical significance was set at $P < 0.05$.

Section III: Results

A. Preliminary Experiments

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2) Steady state levels of fluoxetine and norfluoxetine after repeated daily injections of fluoxetine HCl (5mg/ kg/day) in non-pregnant female guinea pigs
3) Plasma levels of fluoxetine and norfluoxetine after the administration of Fluoxetine HCl via Osmotic Mini Pumps in non-pregnant female guinea pigs

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2) Figure 2: Plasma levels of fluoxetine and norfluoxetine in adult female guinea pigs after once daily injections of fluoxetine (5 mg/kg), for a period of 9 days
3) Table 1: Plasma levels of fluoxetine and norfluoxetine following administration of fluoxetine to non-pregnant female guinea pigs via osmotic mini-pump
4) Figure 3: 5-HT_{1A} receptor binding in four subregions of the hippocampus from 14 day old male guinea pigs born from dams receiving no treatment (control) or receiving once daily injections of fluoxetine (7 mg/kg, n=7) or vehicle from gestation day 1 until parturition
5) Figure 4: 5-HT_{2A} receptor binding in the striatum and frontal cortex of 14 day old male guinea pigs born from dams receiving no treatment (control) or receiving once daily injections of fluoxetine (7 mg/kg, n=5) or vehicle from gestation day 1 until parturition
6) Table 2: Pregnancy length, live litter size at postnatal day 2 and number of stillborns for guinea pig dams receiving no treatment

(control) or receiving either fluoxetine (7 mg/kg/day) or vehicle via osmotic pumps from gestation day 1 until parturition

7) Table 3:Weights of male pups at 2 weeks and 9 weeks from guinea pig dams receiving no treatment (control) or receiving either fluoxetine (7 mg/kg/day) or vehicle via osmotic pumps from gestation day 1 until parturition
8) Figure 5: Weight of pregnant guinea pigs receiving fluoxetine (7 mg/kg/day) or vehicle via osmotic pumps from day 1 of gestation to parturition
9) Figure 6: Acoustic startle responses in 2 week male, 9 week male, an 9 week female guinea pigs born from dams receiving no treatment (control) or receiving either fluoxetine (7 mg/kg) or vehicle via osmotic pump from gestation day 1 until parturition
10) Figure 7: Prepulse inhibition in 2 week male, 9 week male, and 9 week female guinea pigs born from dams receiving no treatment (control) or receiving either fluoxetine (7 mg/kg) or vehicle via osmotic pump from gestation day 1 until parturition
11) Figure 8: Thermal pain threshold in 2 week male, 9 week male, and 9 week female guinea pigs born from dams receiving no treatment or receiving either fluoxetine (7 mg/kg) or vehicle via osmotic pump from gestation day 1 until parturition

A. Preliminary Experiments

A-1) Half-lives of fluoxetine and norfluoxetine in non-pregnant female guinea pigs

Plasma levels of fluoxetine and norfluoxetine measured after a single injection of fluoxetine HCl at 10 mg/kg per female guinea pig are shown in Figure 1. Blood samples were taken 5 min, 15 min, 30 min, 1hr, 2 hrs, 3 hrs, 5 hrs, 10 hrs, 24 hrs, 36 hrs and 48 hrs after the fluoxetine injection. The sample size at each time point was n=6, except for the 36 hrs time point where n=5. The best-fit trend line for the change in fluoxetine over time demonstrates a logarithmic relationship over time points 30 minutes to 48 hours. The equation of this line is $y = -307.98 \text{ Ln } (x) + 2313.9$, where y represents fluoxetine concentration in ng/ml and x represents the time in minutes. Addition of 30 minutes to the half-life calculated (since the trend line was fitted only starting at 30 min) gives a $t_{1/2}$ of approximately 3.6 hours for fluoxetine and a $t_{1/2}$ of 29.8 hours for norfluoxetine.

A-2) Steady state levels of fluoxetine and norfluoxetine after repeated daily injections of fluoxetine HCl (5mg/ kg/day) in non-pregnant female guinea pigs.

The study consisted of administering 5 mg/kg/day of fluoxetine for 9 days to non-pregnant guinea pigs. Figure 2 shows plasma levels of fluoxetine and norfluoxetine at different time points on day 9. The time points selected were 5 minutes before the last injection, and 15 min, 30 min, 5 hrs and 10 hrs after the last injection on day 9. The sample size is n=6, except for the fluoxetine measure 5 minutes prior to the last injection and the norfluoxetine measure 30 minutes after the last injection (n=5). The fluoxetine concentration reached a maximum of 429.4 ng/ml at 30 minutes after injection and minimum of 19.8 ng/ml 5 min prior to the last injection. The norfluoxetine levels were

the highest 5 hours after the last injection at 235 ng/ml and were the lowest 5 minutes prior to the last injection at 114.5 ng/ml. Since the target blood level of combined fluoxetine and norfluoxetine for our study was 350 ng/ml, the combined steady state levels of these two compounds were assessed to help establish the dosing necessary to achieve this target. With daily injections of 5 mg/kg of fluoxetine, the combined fluoxetine + norfluoxetine levels went from a high of 626.2 ng/ml at 30 min after injection, to 208.2 ng/ml at 10 hrs after injection, to a low of 139.1 ng/ml at 5 min prior to last injection. Since these latter two levels were considerably below the 350 ng/ml target levels, the daily dose of fluoxetine concentration to be administered was raised from 5 mg/kg/day to 7 mg/kg/day for the subsequent experiments.

A-3) Plasma Levels of Fluoxetine and Norfluoxetine after the Administration of Fluoxetine HCl via Osmotic Mini Pumps in non-pregnant female guinea pigs.

After delivery of fluoxetine at 3.5 mg/kg/day via osmotic pumps (using 14 day pumps) for a period of 12 days, the combined mean plasma concentration of fluoxetine + norfluoxetine was 186.6 ng/ml in the non-pregnant female guinea pig (Table 1; n=5). This plasma level was below the target of 350 ng/ml, so the concentration administered in the subsequent experiment was raised to 7 mg/kg/day. Furthermore, a 28-day pump was added to the experiment to compare the efficacy of 14-day and 28-day pumps. Blood samples taken at 22 days in the guinea pigs (n=4) implanted with the 28 day pumps yielded a combined mean plasma concentration of fluoxetine and norfluoxetine of 347.7 ng/ml which was close to the target concentration (350 ng/ml) found in humans taking fluoxetine therapeutically. Furthermore, the use of the 28-day pump, compared to the 14-

day pump, reduced the number of operations needed to administer fluoxetine throughout pregnancy. It is unclear why the blood levels obtained were different between the two types of pumps but this may be related to the performance characteristics of the pumps. In both groups steady state levels should have theoretically been reach in an interval of 4 times the half-lives from the beginning of drug administration. In future experiments testing effects of prenatal fluoxetine administered by osmotic pump on CNS function in offspring, we chose to administer 7.0 mg fluoxetine/kg/day using 28-day mini-pumps.

B. Injection Study: Receptor Autoradiography in Offspring

B-1) 5-HT_{1A} Receptor Density in the Hippocampus

The aim of this study was to test if once daily injections of fluoxetine administered throughout pregnancy in the guinea pig affect levels of hippocampal $5HT_{1A}$ receptors in male offspring at 2 weeks of age. Guinea pig offspring were born from dams that received either no treatment, once daily injection of fluoxetine (7mg/kg) or vehicle from day one of gestation until delivery. Male pups from each treatment groups were sacrificed at 14 days of age and their brains taken for analysis. Autoradiography was performed on sections including the hippocampus, using [^{3}H] 8-OH-DPAT for analysis of the 5-HT_{1A} receptor. Specific [^{3}H] 8-OH-DPAT levels (fmol/mg of tissue) in hippocampal sub-regions is reported in Figure 3. Two-way ANOVA for each subregion show no significant effects of treatment (CA1: $F_{2,32}$=12.91, p=0.1078; CA2: $F_{2,32}$=13.07, p=0.0981; CA3: $F_{2,32}$=3.82, p=0.5314; dentate gyrus (DG): $F_{2,32}$=15.35, p=0.0658), no significant effects of hemisphere, (CA1: $F_{1,32}$=0.03, p=0.9220; CA2: $F_{1,32}$=2.37, p=0.3484; CA3: $F_{1,32}$= 1.07, p=0.5527; DG: $F_{1,32}$= 0.60, p=0.6331) and no significant

group x hemisphere interactions (CA1: $F_{2,32}$=0.61, p=0.8934; CA2: $F_{2,32}$= 0.19, p=0.9643; CA3: $F_{2,32}$=0.02, p=0.9959; DG: $F_{2,32}$=1.55, p=0.7425). Combining data for the right and left hemispheres in each subregion, and subsequently conduction one-way ANOVA, also indicates no significant treatment effects in all subregions (CA1: $F_{2,16}$=1.410, p=0.2759; CA2: $F_{2,16}$=1.754, p=0.2048; CA3: $F_{2,16}$=0.4207, p=0.6636; DG: $F_{2,16}$=1.781, p=0.2002). Therefore, prenatal fluoxetine exposure had no effect on 5-HT_{1A} receptor binding in hippocampal regions of young male guinea pig offspring.

B-2) 5-HT_{2A} Receptor Density in the Striatum and Frontal Neocortex

The aim of this study was to test if once daily injections of fluoxetine administered throughout pregnancy in the guinea pig affect levels of hippocampal $5HT_{2A}$ receptors in male offspring at 9 weeks of age. Guinea pig offspring were born from dams that received either no treatment, once daily injections of fluoxetine (7mg/kg) or vehicle from day one of gestation until delivery. Male pups from each treatment groups were sacrificed at 63 days of age, and their brains taken for analysis. Autoradiography was performed on sections including the caudal striatum and the frontal cortex on the 9 week brains, using [^{125}I] DOI for analysis of the 5-HT_{2A} receptor. Receptor levels (fmol/mg of tissue) in striatum and frontal cortex region is reported in Figure 4. Two-way ANOVA in the striatum and frontal cortex regions in 9 week offspring shows no significant effect of treatment (Dorsal Striatum(St): $F_{2,32}$=4.86, p=0.4219; Frontal Cortex (FC): $F_{2,32}$=5.97, p=0.3534), no significant effect of hemisphere (St: $F_{1,32}$=0.01, p=0.9521; FC: $F_{1,32}$=4.70, p=0.2028) and no significant treatment x hemisphere interaction (St: $F_{2,32}$=1.66, p=0.7411; FC: $F_{2,32}$=0.32, p=0.9442). Combining data for the right and left hemispheres

in each region, and subsequently conduction one-way ANOVA, also indicates no significant group effects in all regions (St: $F_{2,17}$=0.3214, p=0.7294; FC: $F_{2,17}$=0.7975, p=0.4666). Therefore, prenatal fluoxetine exposure had no effect on 5-HT_{2A} receptor binding in striatum and frontal cortex regions of the 9 week male guinea pig offspring.

C. Osmotic Pump Study: Pregnancy Parameters and Behavioral Studies

The aim of this study was to test if fluoxetine administered via osmotic mini-pump throughout pregnancy affects pregnancy parameters in the guinea pig dam and performance on three 5-HT-modulated behaviors in offspring as adults. The behavioral tests assessed were acoustic startle responses, pre-pulse inhibition of acoustic startle and pain threshold in the hot plate test.

C-1) Effects of Gestational Fluoxetine on Pregnancy Outcomes

a) Maternal weight gain during gestation

The maternal weight gain during gestation of guinea pigs receiving either no treatment, fluoxetine (7 mg/kg/day) with osmotic mini-pumps, or vehicle with osmotic pumps is shown in Figure 5. Weights in these animals were recorded at day 1, 3 weeks and 6 weeks in the gestation period. Two-way repeated measures ANOVA indicates a significant effect of time ($F_{2,63}$=59.98, p<0.00001), but no significant effect of treatment ($F_{1,63}$=0.16, p=0.6151), and no significant treatment x time interaction ($F_{2,63}$=0.34, p=0.7631). This indicates that there was no significant difference between treatment groups on maternal weight gain during pregnancy.

b) Weight of offspring at 2 weeks and 9 weeks post-parturition

The weights of male offspring born from guinea pig dams receiving either no treatment, fluoxetine (7 mg/kg/day) with osmotic mini-pumps, or vehicle with osmotic pumps is shown in Table 2. A one-way ANOVA for the 2 week-old animals shows a significant effect of treatment ($F_{2,83}$=3.353, p=0.0398). However, the post-hoc Newman-Keuls Multiple Comparison Test showed no significance between treatment differences (p>0.05). The one-way ANOVA for the 9 week-old animals showed no significant effect of treatment ($F_{2,62}$=0.9749, p=0.3829).

c) Length of pregnancy, # of live births and # of still births

Length of pregnancy of guinea pigs receiving either no treatment, fluoxetine (7 mg/kg/day) with osmotic mini-pumps, or vehicle with osmotic pumps throughout pregnancy is shown in Table 3. Analysis of the data on length of pregnancy using one-way ANOVA reveals no significant effect of treatment ($F_{2,45}$=1.097, p=0.3412). One-way ANOVA also show no significant treatment effect for the number of live births ($F_{2,54}$=0.2846, p=0.7535) and number of still births ($F_{2,54}$=0.6855, p=0.5082).

C-2) Behavioral Effects in the Offspring

a) Acoustic Startle Response

Acoustic startle responses in offspring of guinea pigs receiving either no treatment, fluoxetine (7 mg/kg/day) with osmotic mini-pumps, or vehicle with osmotic pumps throughout pregnancy is shown in figure 6. Male offspring were tested at 2 weeks and at 9 weeks of age, and female offspring were tested at 9 weeks of age. Analysis of the data

using one-way ANOVA shows no significant effect of treatment in the 2 week old males ($F_{2,18}$=0.6517, p=0.5330), in the 9 week males ($F_{2,30}$=1.007, p=0.3773) and in the 9 week females ($F_{2,23}$=0.3203, p=0.7290).

b) Pre-pulse Inhibition

Pre-pulse inhibition of acoustic startle in offspring of guinea pigs receiving either no treatment, fluoxetine (7 mg/kg/day) with osmotic mini-pumps, or vehicle with osmotic pumps throughout pregnancy is shown in Figure 7. Male offspring were tested at 2 weeks and at 9 weeks of age, and female offspring were tested at 9 weeks of age. Data were analyzed using 2-way ANOVA with treatment as between-subject factor and pre-pulse intensity as repeated measure. These analyses show significant effects of pre-pulse stimulus intensity for males (M) at both ages (2weeks:2W or 9 weeks:9W) and for females (F) (9WM: $F_{4,150}$=53.56, p<0.0001; 9WF: $F_{4,115}$=61.84, p<0.0001; 2WM: $F_{4,90}$=45.04, p<0.0001), no significant effect of treatment (9WM: $F_{2,150}$=0.92, p=0.4103; 9WF: $F_{2,115}$=2.18, p=0.1361; 2WM: $F_{2,90}$=0.03, p=0.9746), and no significant treatment x pre-pulse stimulus intensity interactions (9WM: $F_{8,150}$=1.49, p=0.1665; 9WF: $F_{8,115}$=0.07, p=0.9997; 2WM: $F_{8,90}$=0.93, p=0.4966).

c) Hot-Plate Test

Figure 8 shows pain threshold in the hot-plate test for guinea pigs born from dams receiving either no treatment, fluoxetine (7 mg/kg/day) with osmotic mini-pumps, or vehicle with osmotic pumps throughout pregnancy. The hot-plate test was performed by measuring the amount of time (seconds) the guinea pig took to lift a paw from the hot

plate in response to a thermal pain stimulus. Male offspring were tested at 2 weeks and at 9 weeks of age, and female offspring were tested at 9 weeks of age. One-way ANOVA of the results show no significant effects of treatment for males at both ages as well as for females (9WM: $F_{2,30}$=1.978, p=0.1559; 9WF: $F_{2,28}$=1.907, p=0.1673; 2WM: $F_{2,17}$=0.9674, p=0.6344). However, since the data for males and females showed similar (near significant) trends for decreased pain threshold in the vehicle treatment groups, data for 9 week old male and female animals were combined to increase statistical power, and re-analyzed. One-way ANOVA on data for the combined (males and females) 9 week-old groups shows a significant effect of treatment ($F_{2,61}$=4.112, p=0.0211). Post-hoc Newman-Keuls Multiple Comparison Tests show significant differences between vehicle vs. control ($p<0.05$) and between vehicle vs. fluoxetine ($p<0.05$) groups.

Figure 1

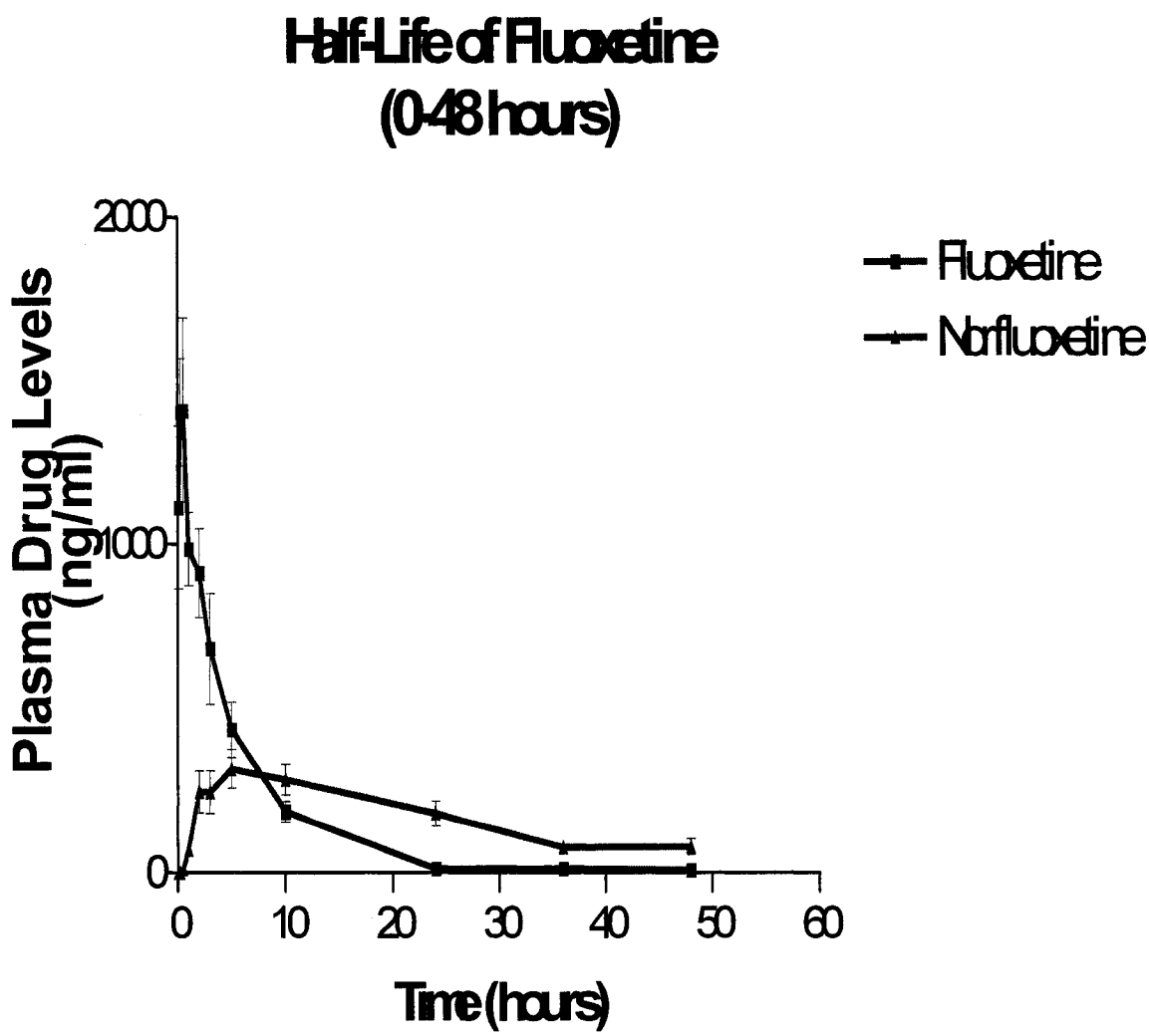


Figure 1. (A) Plasma levels of fluoxetine and norfluoxetine, at various time points, in non-pregnant adult female guinea pigs following a single subcutaneous injection of fluoxetine (10 mg/kg). A sample size (n) of 6 was employed for each time point unless otherwise specified. The plasma levels were measured at 11 different time points: 5 min, 15 min, 30 min, 1 hr, 2 hrs, 3 hrs, 5 hrs, 10 hrs, 24 hrs, 36 hrs (n_{flu}=5, n_{nor}=5) and 48 hrs. Peak fluoxetine levels were obtained at 30 min (1413.0 ng/ml +/- 281.0), while for norfluoxetine the maximum level was reached at 5 hours (314.9 ng/ml +/- 59.7). The data yield a half-life of approximately 3.6 hours for fluoxetine and 29.8 hours for norfluoxetine.

Figure 2

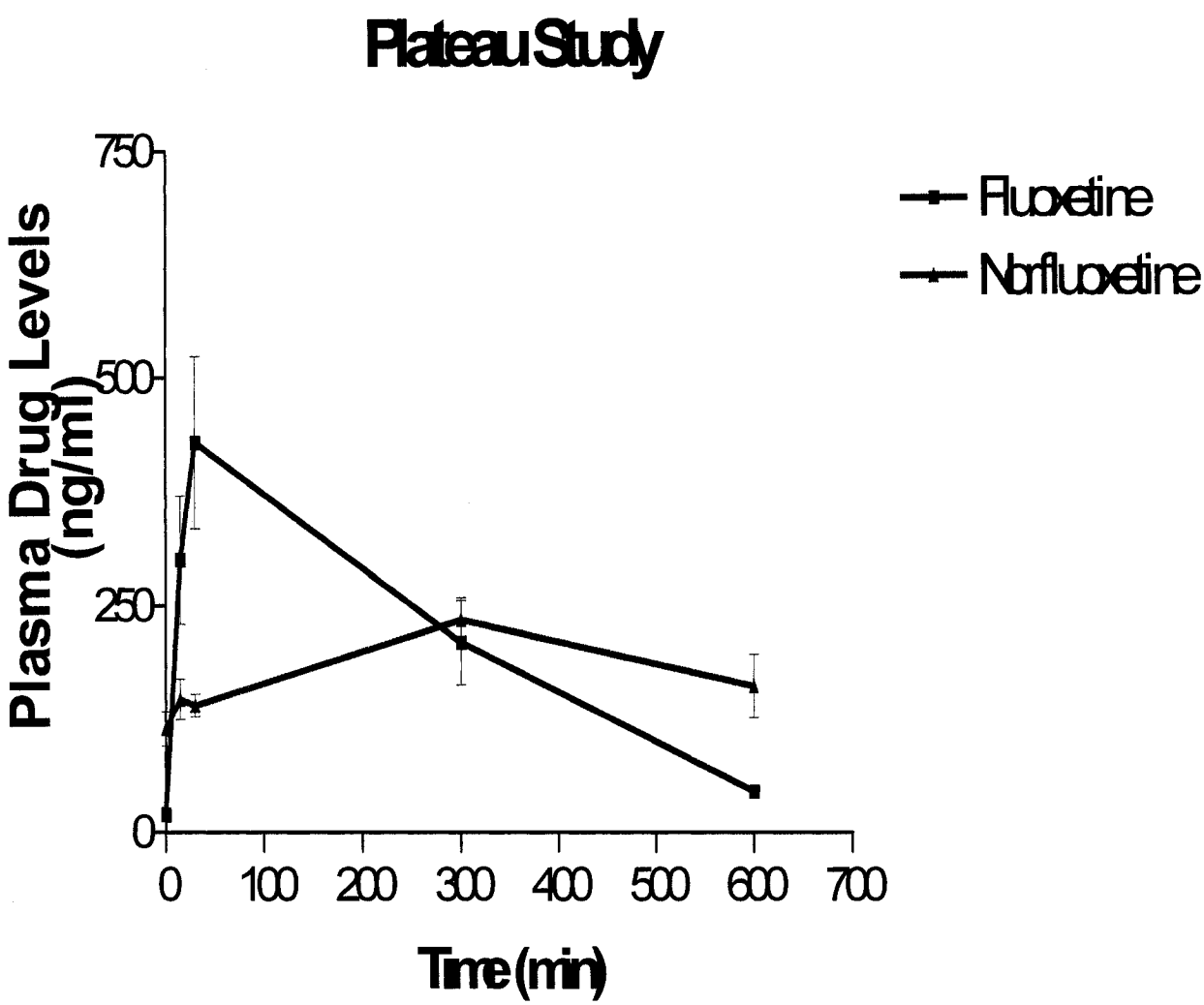


Figure 2. Plasma levels of fluoxetine and norfluoxetine in adult female guinea pigs after once daily injections of fluoxetine (5 mg/kg), for a period of 9 days. Blood samples were taken from non-pregnant female guinea-pigs at 5 minutes before the 9th injection, and 15 min, 30 min, 5 hrs and 10 hrs after this injection. The sample size for each time point was n=6, except at 5 min before injection (fluoxetine, n=5) and 30 min post injection (norfluoxetine, n=5). A maximum concentration of 429.4 ng/ml +/- 94.3 at 30 min, and 235.1 ng/ml +/- 24.3 at 5hrs, were attained for fluoxetine and norfluoxetine, respectively. The lowest levels of fluoxetine (19.8 ng/ml +/-8.1) and norfluoxetine (114.5 ng/ml +/- 18.9) were both obtained 5 minutes prior to injection.

Table 1

Table 1. Plasma levels of fluoxetine and norfluoxetine following administration of fluoxetine to non-pregnant female guinea pigs via osmotic mini-pump. Three different treatment protocols are analyzed.

Treatment	Sample Size	Mean Plasma Fluoxetine (ng/ml) +/- SEM	Mean Plasma Norfluoxetine (ng/ml) +/- SEM	Mean Plasma Fluoxetine & Norfluoxetine (ng/ml) +/- SEM
A	5	41.5 +/- 4.8	145.0 +/- 31.5	186.6 +/- 35.9
B	5	87.9 +/- 18.5	146.5 +/- 40.8	234.4 +/- 57.8
C	4	116.3 +/- 21.2	231.4 +/- 61.8	347.7 +/- 82.9

Treatment A: Animals were treated via 14 day osmotic mini-pumps delivering 3.6 mg/kg of fluoxetine per day. Plasma was sampled 12 days after pump implantation.

Treatment B: Animals were treated via 14 day osmotic mini-pumps delivering 7.0 mg/kg of fluoxetine per day. Plasma was sampled 12 days after pump implantation.

Treatment C: Animals were treated via 28 day osmotic mini-pumps delivering 7.0 mg/kg of fluoxetine per day. Plasma was sampled 22 days after pump implantation.

Figure 3

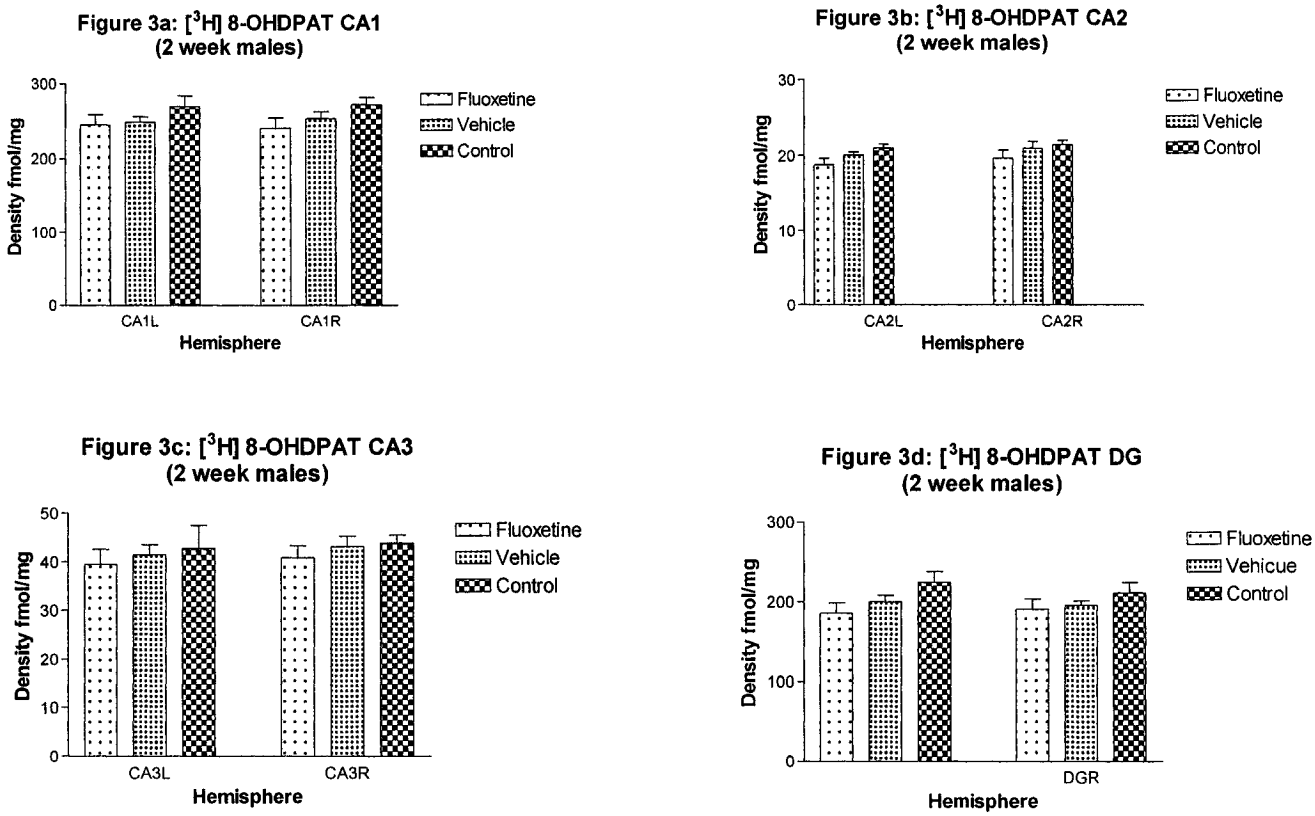
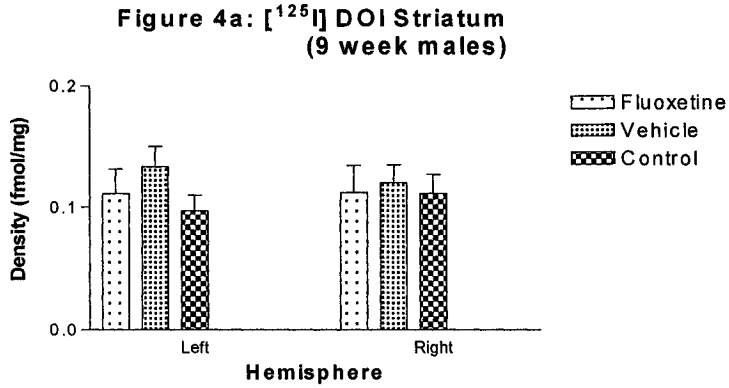


Figure 3. 5-HT_{1A} receptor binding in four subregions of the hippocampus from 14 day old male guinea pigs born from dams receiving no treatment (control, n=4) or receiving once daily injections of fluoxetine (7 mg/kg, n=7) or vehicle (n=8) from gestation day 1 until parturition. Autoradiography of these receptors was performed using [^{3}H] 8-OH-DPAT. Right (R) and Left (L) hemispheres were analyzed separately. Values for the 5-HT_{1A} receptor binding per region are means +/- SEM and are expressed as fmol specific binding / mg protein. The subregions of the hippocampus examined are shown in the above figures: a) CA1, b) CA2, c) CA3, and d) dentate gyrus (DG). There were no significant differences in 5-HT_{1A} receptor binding between the 3 treatment groups in any subregion.

Figure 4



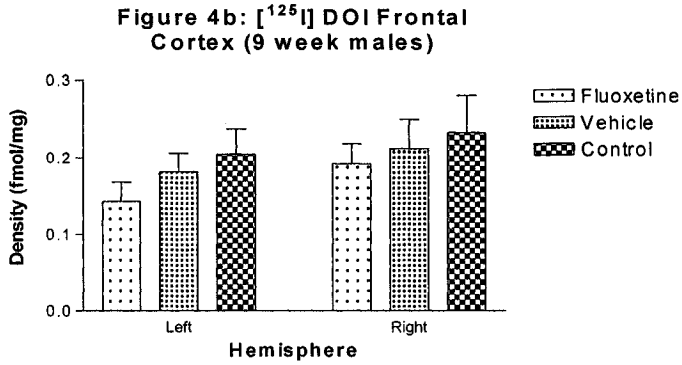


Figure 4. 5-HT_{2A} receptor binding in the a) striatum and b) frontal cortex of 14 day old male guinea pigs born from dams receiving no treatment (control, n=6) or receiving once daily injections of fluoxetine (7 mg/kg, n=5) or vehicle (n=9) from gestation day 1 until parturition. Autoradiography of these receptors was performed using [^{125}I] DOI. Right (R) and Left (L) hemispheres were analyzed separately. Values for the 5-HT_{2A} receptor density per region are means +/- SEM and are expressed as fmol specific binding / mg protein. There were no significant differences in 5-HT_{2A} receptor binding between the 3 treatment groups in both regions.

Table 2

Treatment	Sample size (n)	Mean Pregnancy Length (days) +/-SEM	Mean Number of Pups born Live/Litter +/- SEM	Mean Number of Stillborns/Litter +/-SEM
Fluoxetine	12	66.11 +/- 0.57	2.50 +/- 0.38	0.50 +/- 0.34
Vehicle	15	65.25 +/- 0.75	2.20 +/- 0.34	0.53 +/- 0.32
Control	30	63.31 +/-1.73	2.53 +/- 0.28	0.93 +/- 0.26

Table 2. Pregnancy length, live litter size at postnatal day 2 and number of stillborns for guinea pig dams receiving no treatment (control) or receiving either fluoxetine (7 mg/kg/day) or vehicle via osmotic pumps from gestation day 1 until parturition. There were no significant differences among the 3 treatment groups in pregnancy length, live litter size and number of stillborns.

Table 3

Treatment Group	Mean weight (grams) at 2 weeks +/-SEM	Mean weight (grams) at 9 weeks +/-SEM
Fluoxetine	211.35 +/- 4.26 (n=26)	567.38 +/- 12.75 (n=21)
Vehicle	225.92 +/- 4.31 (n=27)	595.75 +/- 18.56 (n=20)
Control	226.36 +/- 4.82 (n=33)	582.5 +/- 10.74 (n=24)

Table 3.Weights of male pups at 2 weeks and 9 weeks from guinea pig dams receiving no treatment (control) or receiving either fluoxetine (7 mg/kg/day) or vehicle via osmotic pumps from gestation day 1 until parturition. Values shown are mean weight in grams +/- SEM. The sample size per treatment group is specified in the table. No significant differences between treatment groups were found for the 2 week old and 9 week old animals.

Figure 5

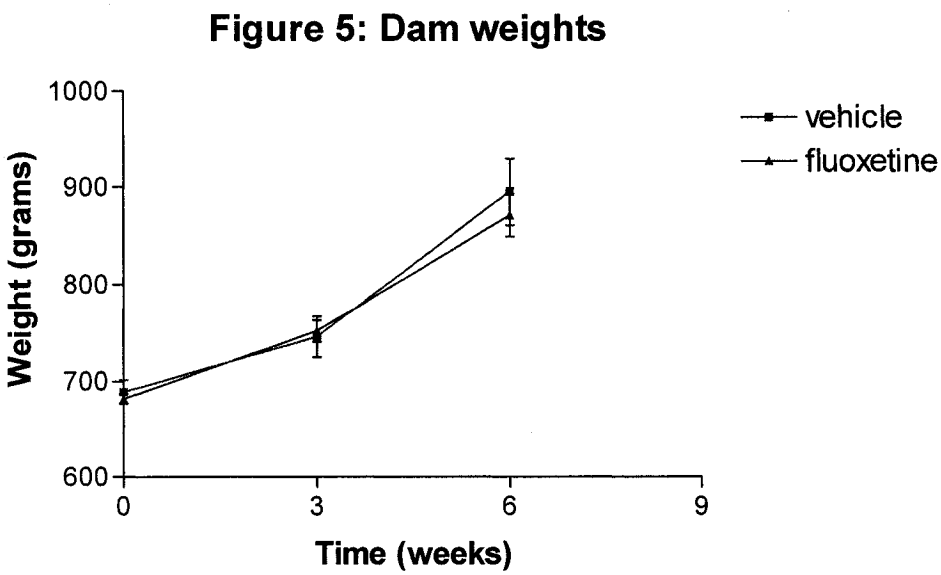
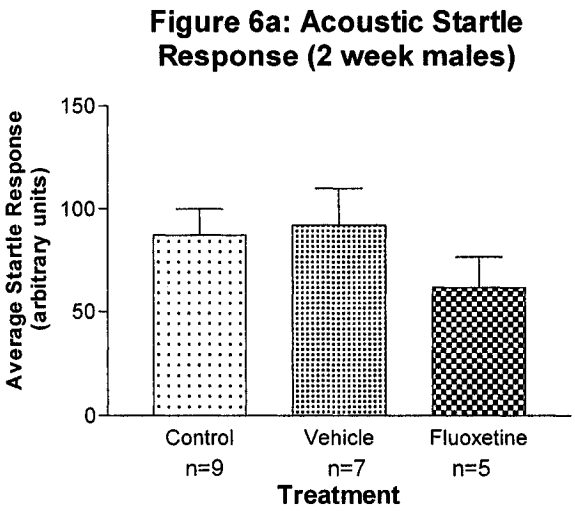
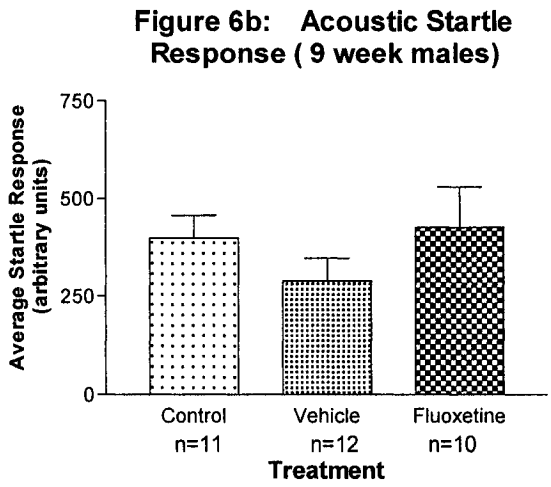


Figure 5. Weight of pregnant guinea pigs receiving fluoxetine (7 mg/kg/day) or vehicle via osmotic pumps from day 1 of gestation to parturition. Values shown are mean weight in g +/- SEM. The sample sizes for the fluoxetine and vehicle groups are n=11 and n=12, respectively. There were no significant differences in maternal weight between the treatment groups.

Figure 6





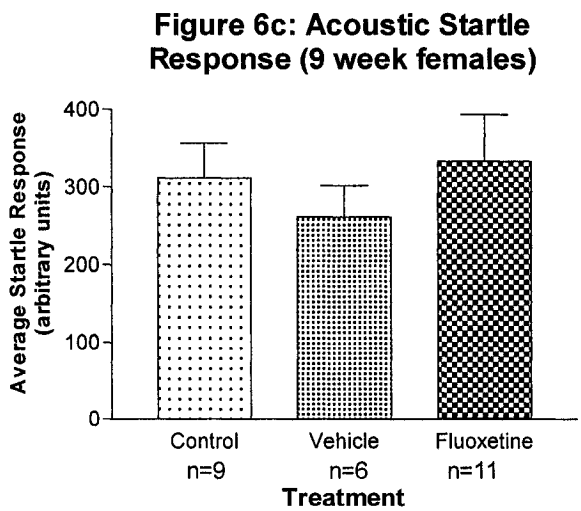


Figure 6. Acoustic startle responses in (a) 2 week male, (b) 9 week male, and (c) 9 week female guinea pigs born from dams receiving no treatment (control) or receiving either fluoxetine (7 mg/kg) or vehicle via osmotic pump from gestation day 1 until parturition. There were no significant differences between the 3 treatment groups, for males at 2 or 9 weeks of age or for females at 9 weeks.

Figure 7

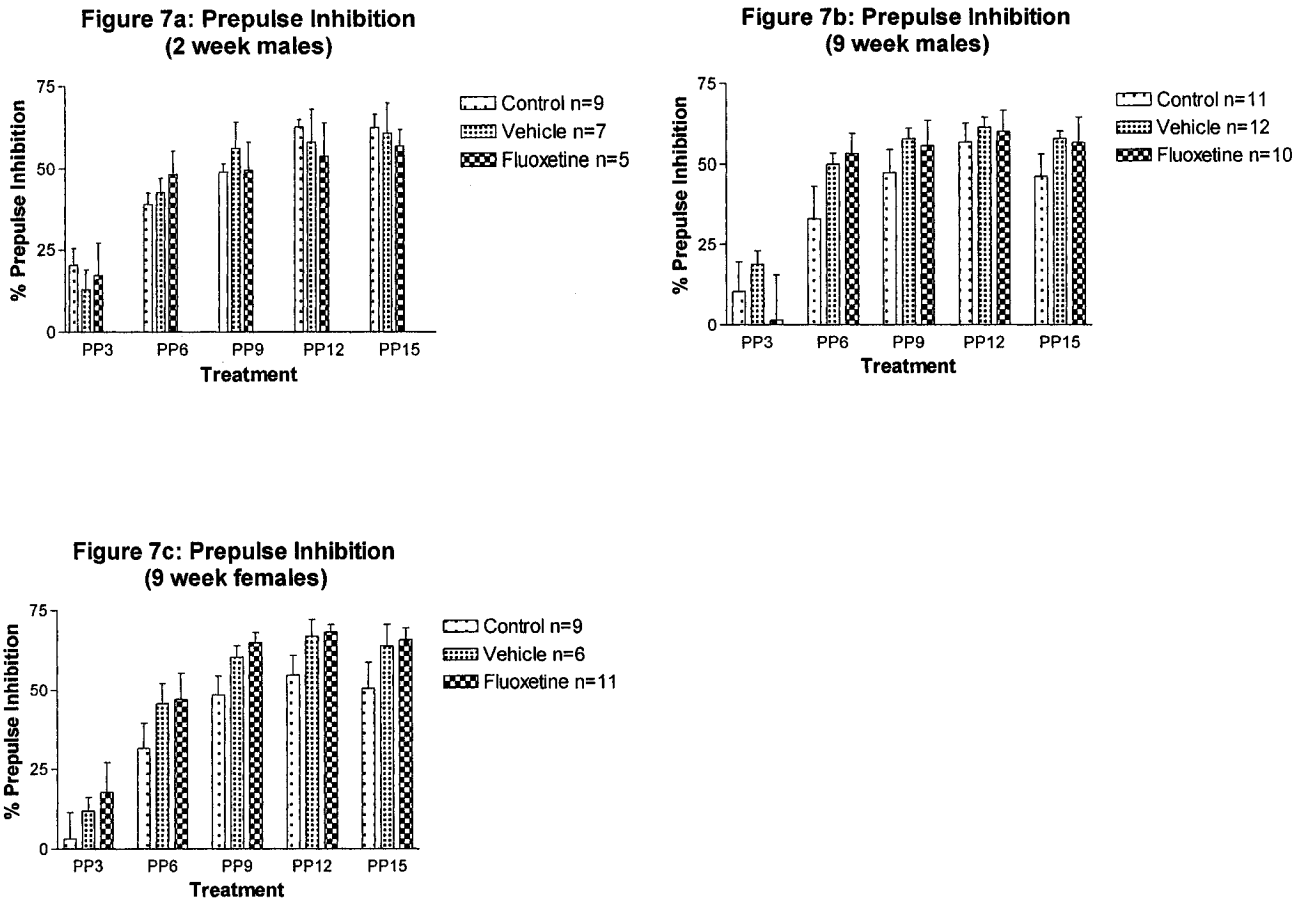


Figure 7. Prepulse inhibition in (a) 2 week male, (b) 9 week male, and (c) 9 week female guinea pigs born from dams receiving no treatment (control) or receiving either fluoxetine (7 mg/kg) or vehicle via osmotic pump from gestation day 1 until parturition. Mean % PPI + SEM is shown at each of five pre-pulse intensities: PP3, PP6, PP9, PP12 and PP15 = pre-pulse intensities of 3,6, 9, 12 and 15 dB above background, respectively. There were no significant differences between the 3 treatment groups for males at 2 or 9 weeks of age and 9 week female offspring.

Figure 8

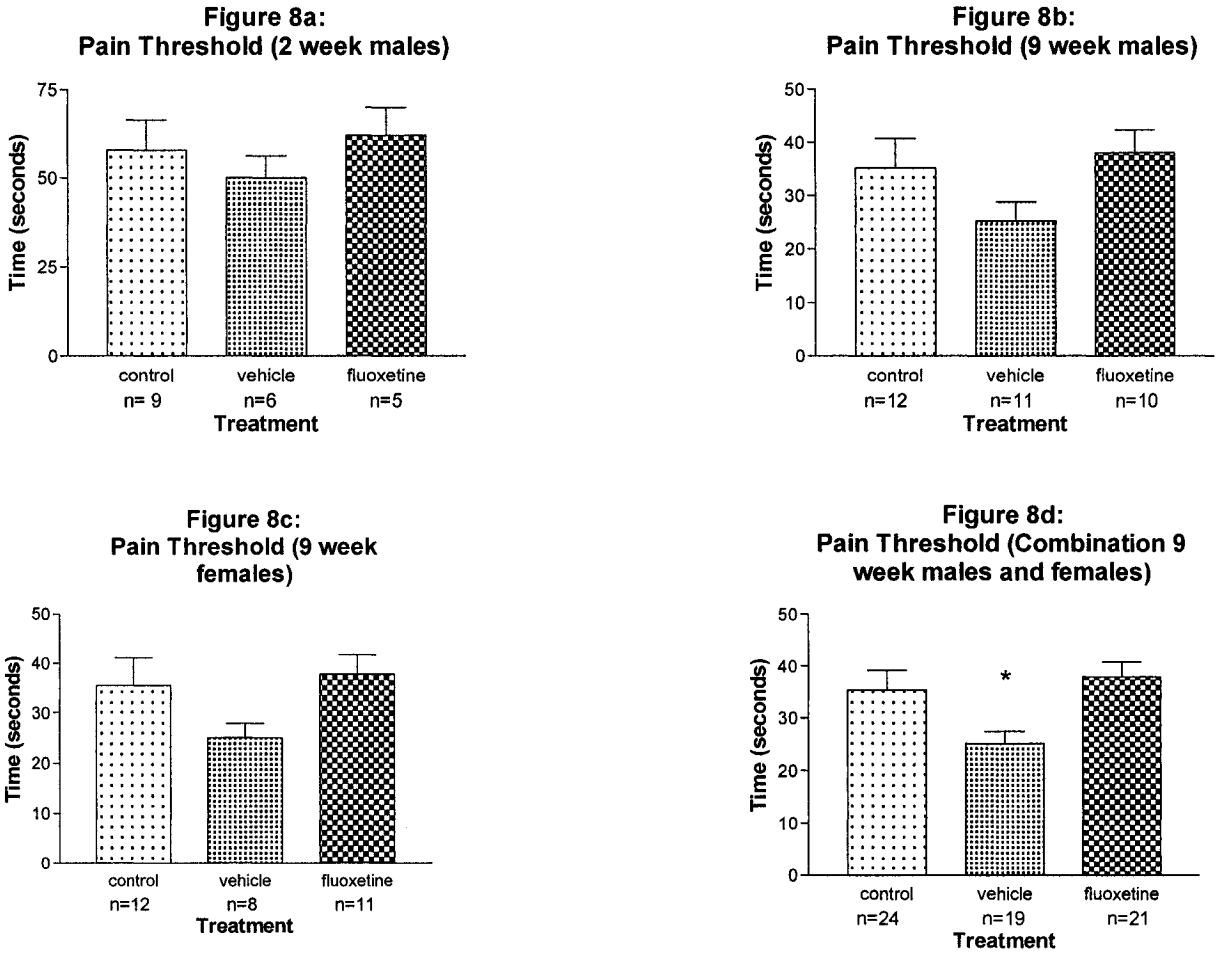


Figure 8. Thermal pain threshold in (a) 2 week male, (b) 9 week male, and (c) 9 week female guinea pigs born from dams receiving no treatment or receiving either fluoxetine (7 mg/kg) or vehicle via osmotic pump from gestation day 1 until parturition. Mean latency to lift a paw from a 55°C hot plate is shown + SEM. There were no significant differences between the 3 treatment groups for males at 2 or 9 weeks of age or for females at 9 weeks. However, since there was a similar trend for lower values in the vehicle-treated group for both males and females, data for 9 week old males and females were combined and reanalyzed. For the 9 week old males and females combined (d), the vehicle group had a significantly lower thermal pain threshold compared to both control and fluoxetine groups. * $p<0.05$ versus both control and fluoxetine.

Section IV: Discussion

A. Half life of fluoxetine and norfluoxetine and plasma levels after repeated daily injection or continuous administration by osmotic pump

1) Half-life
2) Plateau levels

B. Effects of gestational fluoxetine administered by daily injection, on 5-HT_{1A} receptors and 5-HT_{2A} receptors

1) The 5-HT_{1A} receptor
2) The 5-HT_{2A} receptor

C. Effects of gestational fluoxetine administered by osmotic pump, on dam and pup weight and pregnancy outcomes

D. Effects of gestational fluoxetine administered by osmotic pump, on behavioral measures in the offspring

1) Acoustic Startle Response
2) Prepulse Inhibition
3) Hot-Plate Test

A. Half Life of Fluoxetine and Norfluoxetine and Plasma Levels after Repeated Daily Injection or Continuous Administration by Osmotic Pump

A-1) Half-Life

The acute half-life study of fluoxetine at 10 mg/kg in the adult female guinea pig resulted in the calculation of fluoxetine and norfluoxetine half-lives. The half-life for fluoxetine was 3.6 hours and for norfluoxetine it was 29.8 hours. When compared to data for humans, the plasma half-lives of these compounds in guinea pigs is considerably shorter (human fluoxetine t ½: 1-3 days and norfluoxetine t ½: 7-15 days; Altamura et al., 1994). The difference in half-lives between the guinea and human has implications for comparisons of effects of fluoxetine exposure between the two species. First, the fluctuation of blood levels in guinea pigs given once daily doses of fluoxetine are likely greater than that in humans taking fluoxetine once a day. The difference in fluoxetine half-life is likely due to a higher metabolic rate in guinea pigs compared to humans, and not specific to differences in hepatic metabolism or different carrier proteins in plasma. Furthermore, after discontinuation of drug administration the time required to washout the drug and its metabolite from the system in the guinea pig is shorter than in humans. The former difference across species was addressed by developing an osmotic pump protocol that reduced the daily drug fluctuations in the guinea pig model.

A-2) Plateau Levels

The administration of fluoxetine by subcutaneous injection at 5 mg/kg daily, allowed us to establish the levels achieved after 9 days of administration. The plasma

fluoxetine levels ranged from 19.8 ng/ml, five minutes prior to the last injection to a maximum of 429.4 ng/ml at 30 minutes post-injection. Similarly, norfluoxetine levels ranged between a maximum of 235.1 ng/ml five hours post injection to a minimum of 114.5 ng/ml, five minutes prior to the last injection. Unfortunately, this fluctuation of blood levels doesn't mimic the changes seen in administration of fluoxetine in humans, due to a longer in half-life of fluoxetine and norfluoxetine seen in humans (Altamura et al., 1994). A possible solution would have been to inject the animals twice a day, however this wasn't feasible due to time requirement both in the length of pregnancy and the number of animals that where to be injected. Another solution to the problem of fluctuation was to administer fluoxetine via osmotic pump, which was done in the second part of our experiments. In these experiments, the osmotic pumps were delivering fluoxetine at a steady rate, which aided at reducing the fluctuations seen in the injection study.

B. Effects of gestational fluoxetine administered by daily injection, on 5-HT_{1A} receptors and 5-HT_{2A} receptors

B-1) The 5-HT_{1A} receptor

Overall, our results indicate that there are no significant differences in levels of hippocampal 5-HT_{1A} receptors between treatment groups in 2-week male guinea pigs exposed to prenatal fluoxetine. The male guinea pigs were either born from a dam receiving once daily injections of 7 mg/kg fluoxetine, daily injections of vehicle, or no treatment, throughout pregnancy. The regions specifically analyzed were the subregions of the hippocampus (CA1, CA2, CA3 and dentate gyrus) due to the high density of 5-

HT_{1A} receptors in these areas. These findings are consistent with previous animals studies that suggested that chronic antidepressant treatment with fluoxetine administered to adult rats does not change 5-HT_{1A} receptor densities in several brain regions (Bijak et al., 1996; Dremencov et al., 2000). Although these results demonstrate no significant changes in hippocampal 5-HT_{1A} densities in offspring (2 weeks old) from dams receiving fluoxetine by daily injection, a previous study done in our lab demonstrated an (almost significant) trend for an increased density of 5-HT_{1A} receptors in the CA1 subregion of the hippocampus in 9 week offspring treated with gestational fluoxetine compared to vehicle and control. The possibility remains that there may be a progressive change over time where a significant difference may be seen in animals older than 9 weeks postpartum. Moreover, we are presently investigating the density of 5-HT_{1A} receptors in the dorsal raphe (the major region containing serotonergic cell bodies with their 5-HT_{1A} autoreceptors and known for its high density of 5-HT_{1A} receptors; unable to include in this thesis due to time constraints), which will give us a broader sense of possible changes seen in other brain regions.

B-2) The 5-HT_{2A} receptor

The present study indicates that, for both the dorsal striatum and the frontal cortex, there are no significant differences in 5-HT_{2A} receptors between treatment groups in 9-week male guinea pigs exposed to prenatal fluoxetine. The male guinea pigs were either born from a dam receiving once daily injections of 7 mg/kg fluoxetine, daily injection of vehicle, or no treatment, throughout pregnancy. The regions specifically analyzed were the dorsal striatum and the frontal neocortex, due to the high density of 5-

HT_{2A} receptors in these areas. Although there aren't any previous studies investigating 5-HT_{2A} receptors densities in the striatum and frontal cortex of animals exposed to prenatal fluoxetine, Cabrera and Battaglia (1994) have demonstrated a reduction in hypothalamic 5-$HT_{2A/2C}$ receptors in male rat offspring that were exposed to prenatal fluoxetine. Although we had initial intentions to measure the density of the 5-HT_{2A} receptor in the hypothalamus, this was not possible due to technical constraints. However it would be important to include this analysis in subsequent studies. Hence, although no significant treatment effects on 5-HT_{2A} receptors were found in the cortical and subcortical areas we examined, there may be differences found in other regions where a high density of these receptors exist (e.g. hypothalamus, nucleus accumbens). Unfortunately, these regions weren't included in the analysis due to technical difficulties that compromised the quality of brain sections obtained.

C. Effects of Gestational Fluoxetine Administered by Osmotic Pump, on Dam and Pup Weight and Pregnancy Outcomes

Administration of fluoxetine by osmotic pump throughout gestation in guinea pigs had no significant effect on weight of the offspring at 2-week post-parturition and at 9-weeks post-parturition. However, fluoxetine treated animals at 2-weeks of age had a trend towards lower weights that just missed significance. This interpretation is based on the comparison of offspring born to dams being administered fluoxetine (7 mg/kg day) via osmotic pump throughout gestation, vehicle treated dams and control dams. These results with osmotic pump delivery of fluoxetine are consistent with the results observed

previously in our lab with offspring from dams receiving once daily injections of fluoxetine (7/mg/kg/day) throughout pregnancy. In these experiments, there were no significant effects of gestational fluoxetine on weight in the offspring at 2 weeks or at 9 weeks of age. In the present literature, there exists an inconsistency in birth weights after gestational exposure to fluoxetine in humans, one reporting lower birth weight in newborns (Chambers et al 1996), while others reporting no changes in weight (Cohen et al, 2000; Nulman et al 1997).

The investigation of weight changes in the dams administered fluoxetine by osmotic pump throughout gestation yielded no significant effects. These measures were taken at day 1, 3 weeks and 6 weeks in gestation. These results are consistent with a report by Nulman et al., 1997 that showed no change in maternal weight in humans taking fluoxetine during pregnancy. However, the results are in contrast to another human study (Chambers et al., 1996) and several animal studies using rats (Da Silva et al., 1999; Byrd et al., 1994, Hoyt et al., 1989) that have shown that fluoxetine therapy depressed maternal weight gain. However, these changes were observed in animals given more than 7.4 mg/kg of fluoxetine daily. Furthermore, the above-mentioned studies used other species including rats and rabbits.

The finding on the pregnancy parameters was that the administration of fluoxetine (7 mg/kg/day) by osmotic pump throughout gestation in guinea pigs had no significant effect on pregnancy parameters, such as gestation length, # stillborn births and litter size. These findings are consistent with previous results obtained in our lab on pregnancy parameters in guinea pigs injected with fluoxetine daily throughout pregnancy. However,

these results are in contrast to rat studies that have demonstrated negative pregnancy outcomes (Vorhees et al., 1994, Hoyt et al., 1989).

D. Effects of Gestational Fluoxetine Administered by Osmotic Pump, on Behavioral Measures in the Offspring

D-1) Acoustic Startle Response

The results obtained on the acoustic startle response showed that fluoxetine during gestation had no significant effect on the startle response in the resulting offspring at adulthood. This interpretation is based on the comparison of offspring born to dams being administered fluoxetine (7 mg/kg/day) via osmotic pump throughout gestation, vehicle treated dams and control dams. Furthermore, three different groups were investigated independently for these outcomes (9-week old males, 9-week old females and 2-week old males). Although there wasn't any previously published study looking at effects of prenatal exposure to fluoxetine on acoustic startle response, Dow-Edwards (1996) investigated the effects, on the acoustic startle response, of fluoxetine administered acutely to normal adult male rats. The results indicated that male rats receiving fluoxetine at 10 mg/kg showed a significant increase in startle response compared to the rats receiving vehicle. Although these results suggest acute effects of fluoxetine on acoustic response, conclusions cannot be drawn on the chronic effects of fluoxetine on acoustic startle response. Therefore, investigation of chronic fluoxetine in normal animals might have been an interesting addition to the present study and might have given insight to the outcomes obtained in the offspring exposed to prenatal

fluoxetine in our study by allowing us to compare known 5-HT functional changes seen in fluoxetine administration to ASR outcomes in these animals.

D-2) Prepulse Inhibition

The results obtained on prepulse inhibition of acoustic startle showed no significant effect of prepulse inhibition in 2 week males, 9 week males and 9 week females. This interpretation is based on the comparison of offspring being born to dams that were administered fluoxetine (7 mg/kg day) via osmotic pump throughout pregnancy, vehicle treated dams and control dams.

The trend for increased %PPI in the vehicle and fluoxetine treated groups compared to control may be explained by maternal stress imposed on the vehicle and fluoxetine treatment groups. These stressors might include the anxiety caused by the three operations for implantation of osmotic pumps during gestation, by the administration of isoflurane anesthesia during each operation and by the irritation afforded by the implanted pump and vehicle administration. Consistent with this, in an experiment examining effects of prenatal stress on PPI outcome measures, Lehmann et al. (2000) showed that adult rats exposed to prenatal stress had enhanced prepulse inhibition of acoustic startle.

Although PPI is modulated partly by the 5-HT system (reviewed in Geyer et al., 2001) gestational fluoxetine treatment had no effect on the PPI outcome measures in the resulting offspring. It is possible that the regions known to exert 5-HT modulation of PPI outcomes, such as, the ventral pallidum, caudate nucleus (Swerdlow et al., 2001), nucleus accumbens and striatum (Sipes and Geyer, 1997) had no alteration in their function or

morphology due to prenatal fluoxetine. Analyzing 5-HT markers, such as receptor density changes, could have verified this likelihood, however, time constraints limited us to this behavioral study. One also cannot exclude the possibility that compensatory modulation of PPI by other systems known to regulate this behavior (e.g. dopaminergic, glutamatergic, cholinergic) could counteract alterations in 5-HT modulation of PPI caused by prenatal fluoxetine.

D-3) Hot-Plate Test

The result obtained in the hot plate test show a significant effect of treatment on thermal pain tolerance, when data for 9 week old male and female offspring are combined. These results were based on the comparison of offspring born to dams that were administered fluoxetine (7 mg/kg day) via osmotic pump throughout pregnancy, vehicle treated dams and control dams. Significant differences were seen between control vs. vehicle and fluoxetine vs. vehicle groups, with the vehicle group showing reduced pain threshold relative to the other two treatment groups. Previous investigations (Butkevich et al., 2001) have demonstrated an increase in pain sensitivity to noxious stimuli in adult rats that were exposed to prenatal stress by having the pregnant dams placed in a tube for 30 min twice a day through gestation. These observations support the idea that the reduced pain threshold (increased sensitivity) seen in the vehicle treated group compared to controls in our study may be due to the prenatal stress of pump implantation, isoflurane anesthesia and/or vehicle administration. Furthermore, there is evidence that fluoxetine has anti-nociceptive properties when administered to adult animals (Singh et al., 2001, Jett et al., 1997, Dirksen et al., 1998, Belcheva et al., 1995).

This anti-nociceptive action of fluoxetine is consistent with our observation that prenatal fluoxetine reversed the decrease in pain threshold produced by prenatal vehicle treatment in guinea pigs. Although others have previously demonstrated anti-nociceptive effects of fluoxetine when administered to adult animals, ours is the first study to observe that prenatally administered fluoxetine has lasting anti-nociceptive effects in offspring as adults.

Regions in the CNS where pain is modulated by the 5-HT system have been shown to be at the level of the dorsal raphe and the periaqueductal gray (PAG) matter in the brainstem. Electrostimulation of the raphe nuclei and the PAG have been shown to be antinociceptive in rats (Warner et al., 1990, Fardin et al. 1984). This analgesic effect induced by electrostimulation of the PAG and raphe nuclei has been thought to result from activation of descending fibers of nucleus raphe magnus (NRM) traveling in the dorsolateral funiculus (DLF) of the spinal cord (Guimaraes and Prado 1999). Many of these fibers descend through the DLF and innervate the dorsal horn, which receives somatosensory input including pain, and are thought to be serotonergic (Gao and Mason, 2000). Since fluoxetine has been shown to desensitize 5-HT_{1A} receptors in the dorsal raphe after chronic administration to adult animals, prenatal fluoxetine exposure might also produce effects in the descending serotonergic fibers that modulate pain in guinea pig offspring. An interesting experiment to add to the pain sensitivity study would have been to measure the desensitization of these latter receptors and correlate the results with the behavioral study. Furthermore, the analysis of density changes of the 5-HT_{1A} receptor in the dorsal raphe, which have been shown to be affected by fluoxetine exposure in adult animals, would have supplemented further this behavioral study. In

addition, an important experiment to validate the effectiveness of the hot-plate test in measuring pain threshold in guinea pigs would have been to test the efficiency of different amounts of analgesics in attenuating the response to thermal pain stimuli. However, due to time constraints we were unable to perform these experiments.

E. Summary

The administration of fluoxetine (7mg/kg/day) via once daily injections through pregnancy in the guinea pig had no significant effect on hippocampal 5-HT_{1A} receptors in the resulting 2 week old offspring. Furthermore, no significant effect was found in the analysis of the striatal and cortical 5-HT_{2A} receptors of the 9 week old offspring using the same route of drug administration.

The administration of fluoxetine (7mg/kg/day) thought gestation via osmotic mini-pumps to pregnant guinea pigs yielded a significant difference in the pain sensitivity test in the 9 week animals, in which vehicle treated pups had lower pain threshold as compared to both control and fluoxetine treated offspring. No significant difference between treatment groups was seen in two other behavioral measures (PPI, ASR), offspring weights (2 week old and 9 week old) and pregnancy parameters (maternal weight gain, #stillbirth, # pups born, pregnancy length).

F. Conclusion

From the information gathered in our experiments, besides the pain threshold difference seen in 9 week old offspring receiving fluoxetine via osmotic pumps, it appears that fluoxetine doesn't cause any long-term or short-term effects in guinea pig offspring exposed to fluoxetine through gestation. This, however, doesn't imply that

fluoxetine is entirely safe to use during pregnancy in humans. Initially, our main concern regarding the use of this drug during pregnancy was that the changes produced in the 5-HT system of subjects administered prenatal fluoxetine would be amplified or would developmentally alter the brain of the budding fetus; this due to the importance of this system in normal brain development. We also hypothesized that evidence of such a change might appear in subtler neurochemical or behavioral outcomes as opposed to gross morphological changes in brain structure.

Hence, we set out to determine whether markers of 5-HT function, such as receptor density changes, would be altered with prenatal exposure to fluoxetine. Although no significant changes in receptor densities were observed in our study, we strongly believe that our model can be used in a more comprehensive analysis of receptor densities for a range of 5-HT receptor sub-types in additional brain regions. Furthermore, due to the known actions of fluoxetine on 5-HT uptake, it would be of interest to analyze this function in different brain regions from guinea pigs prenatally exposed to the drug. In addition, measurement of levels of monoamines, including 5-HT, would allow us to investigate neurotransmitter systems that may be affected by gestational fluoxetine.

Although our results don't point to an alarming indication to discontinue fluoxetine during pregnancy, in our behavioral analysis of prenatally treated fluoxetine pups, we discovered a potentially therapeutic effect of fluoxetine during pregnancy. The effects of stress induced with the procedure of drug or vehicle administration may have been altered by the administration of fluoxetine to the pregnant dam. Although very speculative, this might have implications in the therapeutic management of pregnant women that are severely depressed or anxious during pregnancy. It indicates that it may

be favorable to administer fluoxetine to these women to avoid harmful effects that stress during pregnancy may bear on their offspring. Furthermore, although behavioral testing of guinea pigs is often technically challenging, we believe that further behavioral experiments such as a resident-intruder aggression test or fighting behavior should be investigated due to their known modulation by the 5-HT system.

In conclusion, we believe that further investigation is warranted on the effects of antidepressants use during pregnancy in humans before claims can be made on its safety. Although in general it is recommended to avoid using these drugs during pregnancy, in some case where depression is severe and sometimes life threatening, a psychiatrist has little choice in prescribing these medications. Hence, this subject matter remains of great importance in the therapeutics of depression. Furthermore, we believe that our experiments using the guinea pig model has yielded a valid methodology that can be used in assessing the safety of other antidepressant that can potentially be used in the treatment of depression during pregnancy.

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APPENDIX

A. Research Compliance Certificates for Animal Subjects

B. Research Compliance Certificates for use of Radioactive Material

RADIOISOTOPE LICENCE | PERMIS DE RADIO-ISOTOPE | Licence Number / Numéro de permis

I) LICENSEE

The Atomic Energy Control Board issues this licence to:

Hôpital Douglas/
Douglas Hospital
6875 LaSalle Boulevard
Verdun, QC
H4H 1R3

hereinafter «the licensee».

This licence replaces licence 04-07438-98 (REV 2).

II) PERIOD

This licence is valid from: February 17 2000 to April 30 2002.

III) LICENSED ACTIVITY

This licence is issued for the POSSESSION, IMPORTATION and USE of the radioactive prescribed substance or the device containing the radioactive prescribed substance described in Section IV for:

laboratory studies: 10 or more laboratories where radioisotopes are used or handled (836)

IV) RADIOACTIVE PRESCRIBED SUBSTANCE

ITEM	*DESCRIPTION*	*POSSESSION LIMIT UNSEALED SOURCES*	*MAXIMUM ACTIVITY SEALED SOURCES*	*TYPE OF DEVICE*
1	Hydrogen 3	4 GBq	n/a	n/a
2	Carbon 14	1 GBq	n/a	n/a
3	Iodine 125	1 GBq	n/a	n/a
4	Cesium 137	n/a	1480 kBq	n/a
5	Radium 226	n/a	400 kBq	n/a
6	Calcium 45	40 MBq	n/a	n/a
7	Phosphorus 32	400 MBq	n/a	n/a
8	Sulfur 35	1 GBq	n/a	n/a
9	Phosphorus 33	1 GBq	n/a	n/a

Bq = becquerel kBq = kilobecquerel MBq = megabecquerel
GBq = gigabecquerel TBq = terabecquerel PBq = petabecquerel
g = gram kg = kilogram Mg = megagram
SQ = scheduled quantity

The amount of radioactivity for the radioactive prescribed substance or substances referred to in each item, shall not exceed the possession limit for unsealed sources, or the maximum activity per sealed source or device in accordance with the provisions of the above table.

«Sealed Source» means a radioactive prescribed substance in a capsule that is sealed or in a cover to which the substance is bonded, where the capsule or cover is strong enough to prevent contact with and dispersion of the radioactive prescribed substance under the conditions of use for which the capsule or cover is designed.

When a device is listed opposite a radioactive prescribed substance, the said substance is to be used only in that device.

V) LOCATION

Subject to the conditions of this licence, the radioactive prescribed substance(s) may be

used or stored at:
6875 LaSalle Boulevard
Verdun, QC

VI) CONDITIONS

AECB 53 Rev. 1979/CCEA 53 Rév. 1979

In addition to the Atomic Energy Control Regulations and the Transport Packaging of Radioactive Materials Regulations, the licensee shall comply with the following conditions:

1. Staff Training
The licensee shall ensure that only persons properly trained to work with radioactive prescribed substances and informed of the hazards involved are permitted to work with radioactive prescribed substances or operate devices containing radioactive prescribed substances.
(565-2)

2. Storage
When in storage, radioactive prescribed substances or devices containing radioactive prescribed substances shall be stored in an area, room, or enclosure that:
(a) is accessible only to personnel authorized by the licensee;
(b) has affixed to its exterior a clearly visible and legible radiation warning sign, and the name or job title and phone number of a 24 hour contact in case of emergency;
(c) does not have, at any occupied location outside the area, room or enclosure, a dose rate that exceeds 2.5 μSv/h.
(575-2)

3. Laboratory Design
Each laboratory constructed or renovated after January 1, 1986, in which more than one scheduled quantity of an unsealed radioactive prescribed substance is used;
(a) shall conform to the requirements of Sections 3 and 4 of AECB Regulatory Document R-52 (Rev. 1) "Design Guide for Basic and Intermediate Level Radioisotope Laboratories"; and
(b) shall, for Intermediate and High Level Radioisotope Laboratories, be approved in writing by the AECB prior to commencing use with radioactive prescribed substances.

(1108-1)

4. Laboratory Lists
A list of all designated radioisotope laboratories shall be maintained including the designation level of each laboratory as defined in Table 1 of the Regulatory Document R-52 (Rev. 1). All laboratories shall be decommissioned in accordance with criteria set out in this licence prior to removal from the list. When any location is decommissioned, the AECB shall be notified in writing by the licensee within seven days.
(569-1)

5. Laboratory Procedures
Handling procedures in each designated radioisotope laboratory shall be in accordance with the appropriate safety poster (Basic INFO-0142-1/Rev. 2 or Intermediate INFO-0142-2/Rev. 2 or a version approved in writing by the AECB). In all cases, this poster shall be prominently posted in the radioactive work area.
(570-1)

6. Licence Posting
This licence, or a copy thereof, shall be conspicuously posted at all specific locations listed in Section V and shall be available at all other locations where the radioactive prescribed substances listed in Section IV are used or stored.
(573-1)

7. Exposure Monitoring
The licensee shall ensure that persons are monitored for radiation exposure from the licensee's possession or use of radioactive prescribed substances in accordance with the AECB Regulatory Document R-91, "Monitoring and Dose Recording for the Individual". Specifically, if monitoring is required it shall be performed as follows:

(a) Measurement of external doses of radiation shall be by means of a personal dosimeter
(i) supplied by an agency approved by the AECB
(ii) suitable for the type of radiation exposure

(iii) worn in the manner and for the duration specified by the supplier such that the dose recorded is indicative of the external dose received by the wearer.

(b) Determination of the uptake of
(i) iodine-125 and iodine-131 shall be in accordance with the AECB Regulatory Guide R-58 "Bioassay Requirements for I-125 and I-131 in Medical, Teaching and Research Institutions" and
(ii) other radionuclides shall be by means of bioassay methods approved by the AECB.
(46-2)

8. Exposure Reporting
Where the licensee becomes aware of a dosimetry result for a person exceeding any limit in Schedule II of the Atomic Energy Control Regulations for that person, the licensee shall inform the AECB within 24 hours and the person promptly. The licensee shall investigate the causes and circumstances contributing to that dosimetry result and shall provide a written report on the investigation and corrective actions to the AECB within ten days.
(567-1)

9. Contamination Criteria
The licensee shall ensure that:
(a) on all normally accessible working surfaces in any area, room or enclosure where a radioactive prescribed substance is used or stored, non-fixed contamination does not exceed 5 Bq/cm2 of substances that emit only beta or gamma radiation or 0.5 Bq/cm2 of substances that emit alpha radiation averaged over an area not exceeding 100cm2,
(b) on all other surfaces, and prior to decommissioning any area, room or enclosure where a radioactive prescribed substance has been used or stored, non-fixed contamination does not exceed 0.5 Bq/cm2 of substances that emit only beta or gamma radiation or 0.05 Bq/cm2 of substances that emit alpha radiation averaged over an area not exceeding 100 cm2,
(c) the dose rate due to contamination does not exceed 0.5 μSv/h at 0.5 metre from any surface,
(d) records of all measurements shall be maintained for a least three years.
Any other maximum contamination criteria will require specific written approval of the AECB.
(571-2)

10. Disposal
Subject to any other condition of this licence respecting the disposal of specific radioactive prescribed substances, all radioactive prescribed substances shall be disposed of by:
(a) making prior arrangements and returning to the supplier, or
(b) making prior arrangements and sending to Atomic Energy of Canada Limited or
(c) making prior arrangements and sending to a facility possessing an appropriate AECB Prescribed Substance Licence or a Waste Facility Operating Licence, or
(d) release through the municipal garbage system provided the radioactive prescribed substance(s) is in the solid form, is uniformly distributed in the waste, and that the concentration is less than 1 scheduled quantity per kilogram of waste material or
(e) release through the municipal sewage system provided the radioactive prescribed substance(s) is water soluble and that the concentration in the sewer at the property line for the facility is less than 0.01 scheduled quantity per litre of effluent based upon a yearly average, or
(f) release to the atmosphere provided the radioactive prescribed substance(s) is in the form of a vapour or gas and that the concentration at the point of release is less than 0.001 scheduled quantity per cubic metre of air when averaged over a 1-week period.
Any other waste disposal method will require specific written approval of the AECB.
(576-1)