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Effects of Prenatal Dexamethasone on Hipopcampal Serotonin 1A Receptors in Adult Male Rats

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EFFECTS OF PRENATAL DEXAMETHASONE ON HIPPOCAMPAL 5HT1A RECEPTORS IN ADULT MALE RATS

By

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I, Rahul Vijay, do grant permission for my thesis to be copied.

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ABBREVIATIONS

ACTH	Adrenocorticotropic Hormone
AVP	Arginine Vasopressin
ADX	Adrenalectomy
B_{max}	Binding Maximum
CBG	Corticosterone Binding Globulin
CNS	Central Nervous System
CORT	Corticosterone
CRF	Corticotrophin Releasing Factor
DEX	Dexamethasone
DG	Dentate Gyrus
EC_{50}	Effective Concentration 50
EPM	Elevated Plus Maze
GC	Glucocorticoid
GD	Gestation Day
GDP	Guanosine Diphosphate
GPCR	G Protein Coupled Receptor
GR	Glucocorticoid Receptor
GTP	Guanosine Triphosphate

[^{3} H] MPPF	2'-methoxyphenyl-(*N*-2'-pyridinyl)-*p*-fluoro-benzamidoethyipiperazine
HPA	Hypothalamo-Pituitary-Adrenal
LHPA	Limbic Hypothalamo Pituitary Adrenal
MR	Mineralocorticoid Receptor
mRNA	Messenger Ribonucleic Acid
NIH	National Institute of Health
PVN	Paraventricular Nucleus
RIA	Radio Immunoassay
RT	Room Temperature
sc	subcutaneous
sGC	Synthetic Glucocorticoid
SSRI	Selective Serotonin Reuptake Inhibitor
WAY 100635	N-[2-[4-(2- Methoxyphenyl)-1-piperazinyl] ethyl]-N-2-pyridinylcyclohexanecarboxamide maleate salt
5HT	Serotonin
5HT1A	Serotonin 1A
5HT1B	Serotonin 1B
5HT2A	Serotonin 2A

8-OH-DPAT	8-hydroxy-*N*,*N*-dipropyl-2-aminotetralin
11-β-HSD	11-β- Hydroxysteroid Dehydrogenase

ABSTRACT

The main activation route for the stress response is the hypothalamo-pituitary-adrenal axis (HPA) and the sympatho-adrenomedullary system. The HPA axis is a neuroendocrine feedback loop mediated by an array of tissue specific hormones, receptors and neurotransmitters that regulate glucocorticoid (GC) release. GCs are steroidal hormones produced by the adrenal glands and are key players in a negative feedback loop controlling HPA activity. They influence the HPA axis through glucocorticoid receptors in the hypothalamus and pituitary and through both glucocorticoid (GR) and mineralcorticoid receptors (MR) that are co-localized in the hippocampus. Repeated or chronic stress exerts a negative influence on these HPA axis regulatory sites and contributes to potentially pathological conditions, especially during early development. For example, chronic stress promotes increased maternal adrenal gland secretion of glucocortiocoid, leading to abnormally high concentrations of GC in the fetal environment. The timing and maturation of the HPA axis relative to birth is highly species specific and is closely linked to landmarks in fetal development. In rats this development of the HPA axis takes place *in utero* and continues even shortly after birth. It is likely that the maternal endocrine environment will affect fetal development during this critical time point and may alter the overall set point for the expression of genes and their protein products that mediate fetal HPA axis function. Dexamethasone (DEX) is a synthetic glucocorticoid (sGC) and is a consensus treatment in preterm

pregnancies used to expedite fetal lung development. However it has been shown that DEX causes long term physiological and behavioral disorders in prenatally-exposed laboratory animals. Previous studies have also shown that it alters the MR: GR receptor ratio in the hippocampus. Taking into consideration corticosteroid regulation of serotonin receptors, especially 5HT1A receptors and their putative interaction with glucocorticoid receptors in the hippocampus, we hypothesized that prenatal DEX exposure would lead to changes in the expression and function of 5HT1A receptors in the hippocampus. We administered DEX to rat dams during the last trimester of gestation and investigated the changes in these receptors in the adult rat offspring. Radioligand receptor binding assays were used to study hippocampal 5HT1A receptor binding affinity and number. Our results demonstrate that hippocampal 5HT1A receptors are increased in the DEX animals compared with controls by 36%, with no change in binding affinity. The efficiency of ligand-induced receptor signal transduction via G-protein activation was also studied using [^{35}S]GTPγS incorporation assay. Using this technique, we showed that there was no significant difference in the maximum ligand mediated stimulation (E_{max}) of 5HT1A receptors between control and dex exposed animals. However, the intracellular signalling efficiency of hippocampal 5HT1A receptors was diminished, since a significant increase in EC_{50} values was obtained with the dex exposed group showing a value 51% higher EC_{50} than controls. Taken together these data illustrate a considerable change in the 5HT1A component of the serotonergic system following prenatal DEX exposure.

INTRODUCTION

STRESS AND HPA AXIS

Stress, originally defined by Hans Selye as a "non specific response of the body to a demand" may also be defined as any environmental change: either internal or external that disturbs the maintenance of homeostasis (Leonard, 2005). The stress response contributes to the maintenance of homeostasis (Sapolsky, 2003) which includes a series of physiological reactions such as endocrine activation and cardiovascular changes that normally do not cause any psychopathological problems. It is only when a prolonged and sustained stimulation exceeds the body capacity to maintain homeostasis that stress can have psychopathological sequelae (Chrousos & Gold, 1992).

When an organism is exposed to a stressor, several mechanisms are activated to restore homeostasis, most importantly, in the paraventricular nucleus of the hypothalamus. When this brain region is stimulated by stress, it releases corticotrophin releasing hormone (CRH) and its cosecretagogue arginine vasopressin (AVP) which induce the release of adrenocorticotropic hormone (ACTH) into the circulation (Chrousos & Gold, 1992). ACTH then stimulates adrenocortical cells in the rat adrenal glands to secrete corticosterone (CORT) into the blood (Palkovits , 1987). Thus corticosteroids are the main effectors of the HPA axis and their action mediated by corticosteroid receptors which include both MR and GR (Herman *et al.*,1989). Using autoradiographic and immunohistochemical techniques it has been shown that the hippocampus contains high

concentrations of both these receptors in comparison to other brain regions which contain only GR (Zhong & Ciaranello, 1995). The co-localization of these receptors in the hippocampus allows this region to play a key role in the regulation of negative feedback on HPA axis activity. This feedback system mediated by a delicate balance between MR and GR activation in the hippocampus keeps HPA activity in check and thus acts to mitigate psychopathological diseases.

LIMBIC MODULATION OF HPA AXIS

Evidence clearly shows that the limbic system is a central modulator of the HPA axis (Jacobsen *et al* 1991 and Reul *et al* 1990) and that it is highly responsive to fluctuations in circulating corticosteroids (Matthews, 2002 ; McEwen, B.S, 1991 and Weinstock, 1997). As previously stated, corticosteroid receptors are highly expressed in the limbic system particularly in the hippocampus (McEwen *et al.,* 1968 and Sapolsky *et al.,* 1983) where GR and MR are colocalized (Van Eekelen *et al.,*1988 and Van Haarst *et al.,* 1997). Corticosterone exerts its feedback regulation of the HPA axis through GR mediated inhibitory action at the level of the pituitary gland and the paraventricular nucleus (PVN) in the hypothalamus. At a higher integrative level, corticosterone exerts its effects on both hippocampal MR and GR depending on CORT concentration. Under basal conditions, hippocampal neurons exert an inhibition of HPA axis activity via a MR mediated mechanism. It follows that hippocampal GRs are activated by removing the tonic influence of ligand activated MRs which results in a stimulatory influence on HPA axis activity (De Kloet, 1991 and De Kloet *et al*., 1986).

Serotonergic pathways originating in the midbrain raphe nuclei provide a widespread innervation of corticolimbic structures such as the hippocampus, amygdala, septum and frontal cortex (Abrams *et al.,* 2005; Engin & Treit, 2007) and the activity in these regions is integrated with that of the HPA axis in the control of glucocorticoid secretion and the stress response. Interestingly, animal studies have shown that corticosteroids can also alter several elements of serotonergic neurotransmission. For example, removal of circulating corticosteroids by adrenlectomy results in anatomically specified decreases in the indices of serotonin metabolism while stressful procedures, which raise corticosteroid levels, produce corresponding increases in the turnover of serotonin (5HT) (Curzon *et.al* 1972 and Van Loon, G.R *et.al* 1981); however, corticosteroids may also act to directly modulate serotonergic transmission via serotonin receptors (Watanabe *et al.,* 1993). Further investigations confirmed the sensitivity of 5HT1A receptors to circulating corticosteroid levels (De Kloet *et al*.1986) and indicate that specific hippocampal subfields are exquisitely sensitive to adrenal steroids. In addition, electrophysiological studies have shown a suppression of 5 HT induced hyperpolarization within hippocampal Ammon's horn 1 (CA1) pyramidal cells after brief application of steroids (Joel *et al*. 1991). These data indicate that there is a functional coupling between glucocorticoid and serotonergic function within the hippocampus. It is possible that the ascending serotonergic neurons originating in the midbrain raphe nuclei and projecting to postsynaptic 5HT receptors in the hippocampus may interact functionally at this site with glucocorticoid actions mediated by the balance between activated MR and GR. Thus, the hippocampus represents a key anatomical

structure involved in the central control of limbic hypothalamo pituitary adrenal (LHPA) axis function and limbic circuitry.

In the brain 5HT is synthesized from the essential amino acid L-tryptophan exclusively in serotonergic neurons located within the midbrain raphe nuclei and secretion of 5HT exerts a wide influence over many brain functions. Seven different families of 5HT receptors with a total of 14 subtypes have been identified and six among the major seven are G-protein coupled heterotrimeric receptors (GPCRs) with seven transmembrane α helices (Hoyer *et al.,* 2002). Most serotonergic receptors are located postsynaptically, but 5HT1A and 5HT1B receptors are also located in the presynaptic neuron and function as autoreceptors (Boess *et al.,* 1994). 5HT receptors are coupled to various effector systems mainly via G- proteins composed of the G_i /G_o type which act as inhibitors of adenylate cyclase (Raymond, *et al.,* 1986). Stress activates the serotonergic neurons projecting to the hippocampus and amygdala through cortical association areas and through ascending catecholaminergic neurons from the brain stem (Feldman *et al.* 1998 and Koob & Heinrichs, 1999). Interestingly, stress activation of the serotonergic system may stimulate both anxiogenic and anxiolytic pathways depending on the type of serotonin receptors stimulated. Moreover, perturbations in serotonergic activity have been closely linked to the pathogenesis of anxiety and other psychaitric disorders (Totterdell, 2006; Firk & Markus, 2007; Engin & Treit, 2007). It has also been postulated that the serotonergic innervation of the amygdala and hippocampus mediates the anxiogenic effects of the transmitter by activating the 5HT2A receptors whereas activation of the 5HT1A receptors, which are highly expressed in the CA1 and CA3

regions of the hippocampus as well as in the dentate gyrus (DG) (Chalmers and Watson, 1991; Pucadyil *et al.,* 2005; Tokugawa *et al.,* 2007), suppresses these anxiogenic effects (Graeff *et al.,* 1993). In support of this hypothesis 5HT1A knock out mice show increased anxiety and fear, while the chronic administration of the 5HT1A partial agonist, 8-OH-DPAT, exerts anxiolytic effects both in rodents and in patients with generalised anxiety disorder (Ramboz *et al.,* 1998). However studies conducted specifically on the autoreceptors of the presynaptic neuron and the postsynaptic population of 5HT1A receptors conclude that stimulation of presynaptic 5HT1A receptors induces anxiolytic effects through a reduction of 5HT release (Picazo *et al.,* 1995; File *et al.,* 1996; King *et al.*, 1997; Millan *et.al., 1999;* Romaniuk *et.al.,* 2001) whereas the administration of the 5HT or the 5HT1A agonist 8-OH-DPAT into the amygdala (Hodges *et al.,*1987; Gonzalez *et al.,* 1996) and the dorsal hippocampus (Romaniuk *et al.,* 2001) produces anxiogenic effects.

The stress response and its subsequent glucocorticoid output exert major effects on the expression of 5HT1A and 5HT2A receptors. Moreover, the HPA axis mediates the stress response, and its output is under tonic inhibition by adrenal steroids in the hippocampus and elsewhere in the brain where mineralocorticoid receptors are expressed (Lopez *et al.,* 1998). It has been demonstrated that the density of 5HT1A receptors decreases in response to chronic stress or the administration of glucocorticoid and increase after adrenalectomy. In contrast, 5HT2A receptor expression is increased by chronic stress or chronic glucocorticoid administration and decreased in response to adrenalectomy (Watanabe *et al.,* 1993). In general, glucocorticoid hormone increases

tryptophan hydroxylase activity, restores the decreased 5HT that occurs after adrenalectomy to more normal levels (Azmitia & McEwen, 1974), and regulates the synthesis and release of corticotropin-releasing factor (CRF) (Paull & Gibbs, 1983; Plotsky & Sawchenko, 1987; Akana *et al.,* 1992). It has also been shown that glucocorticoid hormones, especially CORT, can selectively down regulate 5HT1A receptor mRNA expression in hippocampal areas, but not in the raphe nuclei (Neumaier *et al.,* 2000), suggesting the the 5HT1A autoreceptors are not involved in this glucocorticoid action. Moreover, evidence suggests that stress modifies the functionality of 5HT1A receptors. For instance, a variety of stimuli can induce desensitization of these receptors located in the dorsal raphe nuclei (Laaris *et al.,* 1999; Lanfumey *et al.,*1999), an effect mimicked by the injection of high doses of 5HT1A agonists (Kennett *et al.,* 1987; Beer *et al.,* 1990; Seth *et al., 1997*). Overall, these findings suggest that 5HT1A receptors are influenced by glucocorticoid action and may mediate both glucocorticoid and serotonergic modulation of HPA axis activity.

HPA AXIS DEVELOPMENT AND PRENATAL PROGRAMMING

Cumulative evidence supports the claim that the HPA axis is highly susceptible to programming during development (Matthews, 2002; Meaney, 2001; Weinstock, 2001; Welberg and Seckl, 2001; Schneider et *al.,* 2002; Sloboda *et al.,* 2002). In the adult there is clear association between HPA function, glucocorticoids, and behavior, particularly in behavioral responses to stress (De Kloet *et al.,* 1998). As a result, a number of research groups have investigated the influences of the perinatal environment on neonatal, juvenile

and adult behaviors (Matthews, 2002; Meaney 2001; Weinstock, 2001; Welberg & Seckl, 2001; Schneider *et al.,* 2002).

The timing and maturation of the HPA axis relative to birth is highly species specific and is linked to landmarks of brain development (Dobbings & Sands, 1979). Growth factors, transcription factors and nutrients are known to affect brain development and steroids in particular have powerful brain programming properties (Matsumoto & Arai, 1997). The HPA axis is shown to have the greatest plasticity during the third trimester of gestation, a time when set points for basal expression of genes regulating the HPA axis are programmed according to genotype. If the fetal endocrine environment is abnormal, for example if the GC levels is in excess, genetic programming may be reset toward HPA axis hyperactivity.

Maternal stress during gestation may lead to a variety of behavioral, neuroendocrine, and neuroanatomical alterations in the offspring. In the absence of direct neural connections between the developing fetus and the dam, maternal hormones have been hypothesized to mediate the effects of prenatal stress, particularly through alterations in the maternal HPA axis. The role of glucocorticoids as mediators of prenatal stress effects has been investigated by administering corticosterone (or synthetic analogues e.g DEX) to pregnant dams and results from these studies suggest that fetal glucocorticoid exposure mediates prenatal stress and programming of the offspring (Maccari *et al.,* 2003; Welberg & Seckl, 2001). Results from a few well designed human studies are concordant with decreased adaptation to novelty, altered attention and

increased emotionality (Huizink *et al.,* 2004). Although the range of behavioral abnormalities is much more limited in animals than in humans, animal models allow for control of environmental factors and hypothesis testing based on manipulation of both the prenatal and postnatal enviroment (Weinstock, 2001). Animal models are therefore of great value in order to identify behavioral domains or physiological systems that are particularly vulnerable to prenatal stress, to investigate whether individual sensitivity plays a role, and to determine which physiological mechanisms mediate the effects of prenatal stress (Huizink *et al.,* 2004). The adult offspring of rat dams subjected to stressors during gestation display increased anxiety-related behaviors, e.g., suppressed exploration of the open areas of elevated plus maze (Rimondini *et al.,* 2003; Zimmerberg & Blaskey, 1998) and increased defensive withdrawal (Ward *et al.,* 2000). An important and more noticeable feature of prenatally stressed offspring is a hyperactive HPA axis with an elevated basal corticosterone level (Weinstock, M., 1997). However contrary to the aforementioned finding by Weinstock, there are reports that prenatal exposure to betamethasone decreases anxiety in developing rats which is attributed to the increase in the expression of neuropeptide Y in the hippocampus that is anxiolytic in nature (Velisek, 2006)

Glucocorticoids have become primary candidates for programming the fetal HPA axis in response to prenatal stress. They are essential for normal brain development and exert a wide spectrum of organizational effects. Under normal circumstances access of maternal endogenous glucocorticoid to the fetus is low for two reasons: (1) expression of 11β hydroxysteroid dehydrogenase in the placenta reduces glucocorticoid-action by

(Burton & Wadell, 1999) converting cortisol and corticosterone to inactive products - cortisone and 11- dehydrocorticosterone respectively and (2) corticosteroids are substantially complexed with proteins such as corticosteroid binding globulin (CBG) that render it incapable of penetrating the adult blood brain barrier or the feto-placental barrier (De Kloet *et al.,* 1998 and Owen D. 2002).

PRENATAL GLUCOCORTICOID THERAPY

Extensive study by Liggins and Howie in the early 1970's led to the widespread use of synthetic glucocorticoid (sGC) as a life saving treatment to prevent respiratory distress syndrome, by enhancing fetal lung development in those pregnancy cases in which preterm delivery is imminent (Liggins & Howie, 1972). Preterm delivery occurs in approximately 7- 10% of all births in North America and is responsible for about 75% of neonatal deaths (Effects of corticosteroid for fetal maturation and perinatal outcomes., NIH Consensus, 1995). Neonatal morbidity was also found to be very high in surviving preterm infants and complications such as respiratory distress syndrome, intraventricular hemorrhage and necrotizing enterocolitis are also common. An NIH consensus conference published a statement in 1994 indicating that one-time corticosteroid administration is safe and without side effects for prevention of respiratory distress syndrome in prematurely born neonates. This conclusion was based on a more positive outcome for the immediate perinatal health from numerous studies using this treatment (MacArthur *et al.,*1981; Schmand *et al.,*1990; Smolders-de Hass *et al.,*1990). The trend in clinical practice then progressed to a protocol involving repeat injections of synthetic

corticosteroid to pregnant women at the risk for premature delivery if delivery did not occur in 7-10 days after the initial treatment. This protocol creates an intrauterine environment where exposure to synthetic corticosteroids is long lasting. Interestingly, in most of these prenatal glucocorticod therapy protocols the synthetic glucocorticoid employed is dexamethasone.

By the late 1990's emerging evidence, particularly from animal studies, have suggested that there may be long term consequences on early brain development following multiple exposures to sGC (Smith *et al.,* 2000). These consequences of repeated DEX administration were unexpected since it was thought that maternal corticosteroids are unavailable to the fetal brain due to CBG and 11β hydroxysteroid dehydrogenase (11 β HSD) activities. However it was found that CBG cannot bind DEX nor is the latter a good substrate for 11 β HSD (Owen D, 2002). Thus it was finally concluded that prenatal exposure of sGC increases the level of glucocorticoid in the fetal blood. Excess GC significantly alters the fetal endocrine environment and interferes with the programming of the CNS especially in areas such as the hippocampus which ultimately regulate the HPA axis and incoming serotonergic projections involved in modulating its activity.

SPECIFIC AIMS OF THE STUDY.

This study was designed to test the hypothesis that prenatal dexamethasone treatment alters 5HT1A receptor function and may be associated with a hyperactive HPA axis. Due to the increase in occurrence of preterm fetal delivery dexamethasone has been increasingly used during late gestation (G.C, Liggins; R.N, Howie, 1972). Although the use of prenatal dexamethasone therapy has been shown to augment the fetal lung development, newer research conducted during the late 1990's and early 2000 showed that prenatal glucocorticoid therapy has long term consequences for fetal brain development (G.N, Smith *et al.* 2000 ; Matthews S.G, 2000) and that it alters the hippocampal drive on HPA axis (Shoener *et al.* 2006; Weinstock, M., 1997). As stated previously, studies also suggest that fetal glucocorticoid exposure is part of the mechanism by which prenatal stress programs the offspring (Maccari, *et al.* 2003 & Welberg L.A., Seckl J.R, 2001). In fact, the hyperactivity of HPA axis following prenatal dexamethasone exposure is believed to be due to changes in negative feedback mediated through the corticosteroid receptors especially in the MR/GR ratio. In addition, although ADX studies and neuronal lesions have shown that circulating corticosteroids exert a negative regulation on the serotonergic system (Curzon *et.al* 1972 and Van Loon, G.R *et.al* 1981), activity of the serotonergic system is also mediated through the different types of serotonin receptors in the hippocampus and higher cortical centers. 5HT1A and 5HT2A receptors by virtue of their capability to acts as anxiolytic and anxiogenic mediators have been a focal point for research (Graeff *et al.* 1993; Ramboz *et al.*1998). Studies show that the expression of the 5HT1A receptor is under tonic inhibition by

adrenal steroids in the hippocampus and elsewhere in the brain where mineralocorticoid receptors are expressed (Lopez *et al.* 1998) and that glucocorticoid action on serotonergic function may be involved in limbic system dysfunction. For example, a deficiency in brain serotonergic activity has been proposed to increase vulnerablitiy to major depression (Asberg *et al.* 1986). It is possible that a diminished availability of 5-HT precursors such as L-tryptophan, impairments in 5-HT synthesis, release or metabolism, and changes in 5-HT receptor function could underly this affect disorder.

In summary this study will attempt to answer the following questions: 1) Does prenatal dexamethasone treatment alter the binding affinity of 5HT1A receptors in the hippocampus 2) Is there a change in 5T1A receptor density in this region of the brain following the treatment 3) Does prenatal dexamethasone treatment alter intracellular signalling from these receptors upon stimulation?

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS AND DEX TREATMENT

Pregnant female Sprague-Dawley dams were obtained on the 9th day of gestation from Hilltop Laboratories. Since the fetal HPA axis develops only after gestation day (GD) 12 -13, the stress due to transportation of the dams is less likely to affect the former. All animals were maintained on standard rat chow providing 3.85kcal/g (dry weight) and at conditions of controlled lighting (0600-1800 hours) and temperature (23°C). These rats were given a week of acclimatization before treatment. They were randomly divided into two groups namely the control and dex. The treatments began on GD 14 with the dex group receiving daily injections (sc) of dexamethasone @ 150µg/kg/day: Sigma St.Louis, MO) and the control group receiving vehicle (0.9% saline + 0.4% ethanol) until GD 19. Data from earlier studies prove that DEX treatment @200µg/kg/day or greater resulted in significant growth retardation and lethality in offspring. Offspring weighed at birth were distributed in such a way that each dam nursed 10 pups, which ensured that the pups were neither overfed nor underfed. The offspring were sexed and weaned on postnatal day 21 and females were terminated. The males were then distributed at the rate of 2/cage according to litter preserving the treatment groups. The offspring were also fed the same standard rat chow (3.85kcal/g). At 90 days of age the control and the dex animals were terminated. All animals were asphyxiated with CO_2 in a pre-charged chamber and terminated by guillotine. Terminations were

staggered between the two groups to prevent variability in resting periods. Immediately followed by each termination, the hypothalamus and the hippocampus were excised and stored in eppendorf tubes at -80ºC. Additional trunk blood samples were collected immediately upon termination and were centrifuged at 1300 rpm for 5 min at 4ºC. Serum aliquots were stored at -80ºC for CORT assay using radioimmunoassay.

TISSUE GRINDING.

The hippocampus stored at -80°C was ground into fine suspension using a polytron tissue homogenizer in 50mM Tris buffer (at 4°C, pH 7.4). The tissue homogenate from this first round of grinding was then centrifuged using Beckman Coulter centrifuge at 18000rpm (40000 g) at 4°C for 20 minutes. The resulting tissue pellet was then subjected to another round of grinding and centrifugation followed by a final grinding using 20mM Tris buffer (at RT, pH7.4). During this round of grinding the volume of the buffer was closely monitored to make the final tissue concentration to 2mg/ml (concentration used during the radioligand receptor binding assay). A portion of the final homogenate was used for the radioligand receptor binding assay and the other was saved at -80ºC for [^{35}S] GTPγS incorporation assay.

RADIOLIGAND RECEPTOR BINDING ASSAY

The radioligand receptor binding assays are typically used to measure the binding affinity of the receptor for its ligand as well as to determine the density of receptors in the

tissue used. This assay makes use of a radiolabelled ligand (agonist or an antagonist), specific for the receptor in question. Incorporation of any nonspecific binding was avoided by using a selective receptor antagonist along with the radiolabelled ligand.

In this study the 5HT1A receptor antagonist [H^3] MPPF (2'-methoxyphenyl-(*N*-2'-pyridinyl)-*p*-fluoro-benzamidoethyipiperazine) (Perkin Elmer, USA) (specific activity of 78.3 Ci/mmol) was used as the radioligand. Serial two fold dilutions of the radioligand were made using 20mM Tris buffer (at RT, pH7.4) from 10.56 nM up to 8 dilutions. Assay tubes were also labeled from 1 to 8 in triplicates. 250µL of the ligand from each dilution was then pipetted into each corresponding triplicate assay tubes. This was followed by the distribution of the tissue homogenate into all assay tubes at 500µL per tube. To make up the volume to 1 ml per tube another 250 µL of 20mM Tris buffer (at RT, pH7.4) was added to each tube. The tubes were then incubated at 25°C in a water bath for one hour. The same set up was followed for estimating the nonspecific binding using WAY-100635 (*N-[2-[4-(2-Methoxyphenyl)-1-piperazinyl] ethyl]-N-2-pyridinylcyclohexanecarboxamide maleate salt*) (Sigma, St.Loius, MO) at 4µM concentration (in 20mM Tris) using another set of 8x3 assay tubes that received same respective dilutions of the radioligand, except that instead of adding the final 250µL of the 20mM Tris buffer for volume make up, 250µL of 4µM WAY-100635 was used which was also incubated at 25°C in a water bath for one hour.

Also to another set of 8x2 scintillations vials, 500µL of the respective radioligand dilutions were added to get the total count of radioactivity added to each assay tube.

Cell Harvesting and Scintillation Counting

The reaction between cell membranes and the radiolabeled ligand was terminated by rapid filtration through Whatman GF/B glass fiber filter soaked in polyethylene glycol using a 24 tube Brandel cell harvester. The filter with the harvested radiolabelled cell membrane was then allowed to dry for an hour. The dried filter was cut out into circles corresponding to the number of the respective assay tubes which were then distributed into same numbered scintillation vials and 3ml of scintillation flour was added into each vial. Scintillation vials were left undisturbed for about 24 hours for proper integration of the flour with the filter after which they were taken into a liquid scintillation counter for reading the residual radioactivity in cpms. The liquid scintillation counter was programmed to read only ^{3}H channel for a preset time of 1 minute per sample. Data was then analyzed using the non linear curve fitting program LIGAND (Munson & Rodbard, 1980).

[^{35}S]GTPγS INCORPORATION ASSAY

The homogenate saved after tissue grinding was used to study the intracellular signalling from the 5HT1A receptors upon activation by a ligand (agonist). This study made use of the fact that the 5HT1A receptors are G-protein coupled receptors.

Three fold serial dilutions of the 5HT1A receptor agonist 8-OH-DPAT(*8-hydroxy-2-(di-n-propylamino) tetralin*) (Sigma, St.Loius, MO) were prepared in 50mM

Tris (@RT, pH 7.4) starting at 10 μM up to 6 dilutions. The 4X GTP buffer was made by mixing 400mM NaCl (Sigma, St.Loius. MO), 0.8mM EGTA (Sigma, St.Loius. MO) and 12mM $MgSO_4$ (Sigma, St.Louis. MO) in 50mM Tris buffer (pH 7.4, R.T). 120mM GDP buffer was made by dissolving GDP (Sigma, St.Loius. MO) in 4X GTP buffer. After calculating the radioactivity using the radioactivity decay calculator a 400pM stock of the [^{35} S] GTPγS (specific activity of 1250Ci/mmol) was made in 120mM GDP buffer. Unlabeled GTPγS (40μM; Sigma, St.Loius. MO) was also prepared in 50mM Tris buffer (Sigma, St.Loius. MO) (pH 7.4, R.T).

The assay set up consist of triplicates of 6 assay tubes, another of basal and yet another of nonspecific. 250μL of the drug (8-OH-DPAT) from each dilution were pipetted into the 6 triplicates of corresponding assay tubes. To the basal tubes 250μL of 50mM Tris buffer (pH 7.4, R.T) was added. To the nonspecific 250μL of the 40μM unlabeled GTPγS was added. All the tubes received 250μL of [^{35} S] GTPγS and followed by the addition of 500μL of the tissue homogenate at 1mg/mL.

Cell Harvesting and Scintillation Counting

All the tubes were incubated exactly for 45 minutes at 30°C and the reaction was terminated as before by rapid filtration onto a Whatman GF/B glass fiber filter as before using the cell harvester. Filter with the harvested radiolabelled cell membrane was then allowed to dry for an hour. The dried filter was cut out into circles corresponding to the number of the respective assay tubes which were then distributed into same numbered scintillation vials and 3ml of scintillation flour was added into each vial. Scintillation

vials were left undisturbed for about 24 hours for proper integration of the flour with the filter after which they were taken into a liquid scintillation counter for reading the residual radioactivity in cpms. The liquid scintillation counter was programmed to read only ^{35}S channels for a preset time of 1 minute per sample. The results obtained were used to calculate stimulation maximum (E_{max}) and EC_{50} values of the ligand for the receptor, from linear regression plots with the reciprocal of ligand (8-OH-DPAT) concentrations on the X axis and reciprocal of percentage stimulation on the Y axis.

This study was conducted in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals, and the protocols have been approved by the Animal Care and Use Committee of Bucknell University

RESULTS

Body Weight and Energy Intake

Dams that had been treated with 150µg/kg DEX daily from GD14-19 had offspring with significantly reduced birth weights (Fig.1, Table.1; 6.79 ± 0.18 g, n= 5 for dex group; versus 8.00 ± 0.21 g, n=4 for control; P=0.004), which is in agreement with previous studies (Keller-Wood & Dallman, 1984; Kinnunen *et al.,* 2003; Shoener *et al.,* 2006). In addition prenatal DEX exposed groups also continued to show reduced body weight at weaning (Fig 1., Table.1; 27.76 ± 0.34 g, n=5 for dex group; versus 34.32 ± 1.5 g, n=4 for control; P <0.0005*),* puberty (Fig. 2., Table.1; 337.7 ± 3.4 g, n=5 for dex group; versus 374.4 ± 5.8 g, n=4 for control; P<0.0005) and on the day of sacrifice (Fig 2., Table.1; 521.2 ± 5.8 g, n=5 for dex group; versus 569.0 ± 10 g, n=4 for control; P <0.0005). These results suggest a permanent metabolic disturbance in the treated animals which was supported by the significantly reduced daily energy intake in these animals (Fig. 3. Table.1; 184.9 ± 0.61 kcal/day, n=5 for dex group; versus 192.3 ± 0.76 kcal/day, n=4 for control; P<0.0005)

Binding Affinity and Receptor Density of Hippocampal 5-HT1A Receptors

In order to study the binding affinity and receptor density of hippocampal 5HT1A receptors, radio-ligand receptor binding assays were done as described in the materials

and methods section. We found that prenatal dex exposure significantly increased the number of 5HT1A receptors (B_{max}) in the rat hippocampus by 35% (Fig.4., Table.2; 6.1379 ± 0.355 g, n=5 for dex group; versus 4.5868 ± 0.298 g, n=4 for control, *P*=0.002). However these changes in receptor density occurred with no significant change in 5HT1A receptor affinity (K_d) (Fig 5). The K_d value for the dex group was 2.53 ± 0.143 nM versus 2.298 ± 0.156 nM for control group (*P*=0.292). A Scatchard plot of the specific binding is given in Fig.6. This figure illustrates that the regression lines are parallel, indicating that there is no change in receptor affinity, whereas increased intercept on the horizontal axis indicates a large increase in $B_{max\ of}$ 5HT1A receptors in treatment group (dex animals).

G protein activation of 5HT1A Receptors.

The differences in the intracellular signalling of 5HT1A receptors were measured in terms of [^{35} S] GTPγS incorporation and the E_{max} and $EC_{50\ values}$ were calculated. The results showed that there is no significant difference in ligand mediated maximum stimulation (E_{max}) of hippocampal 5HT1A receptors between control and dex group (Fig.7., Table.2; 35.91 ± 2.0 % for dex group; versus 30.83 ± 1.8 % for control, *P*=0.069). However the EC_{50} values of 5HT1A receptors in dex group was higher than in the control by around 51% (Fig.8., Table.2; 0.2258 ± 0.02 μM for dex group; versus 0.1095 ± 0.0155 μM for control, *P*=0.002) indicating that the former required a higher concentration of the ligand (8-OH-DPAT) for saturating 50% of its hippocampal 5-HT1A

receptors compared to the control. A double reciprocal plot is given in Fig 9. This figure illustrates that there is no change in E_{max} values between control and dex animals, whereas a higher intercept for the dex compared to control on the X-axis indicates a larger EC_{50} value for dex exposed animals.

DISCUSSION

In the present study we demonstrate, for the first time, that prenatal exposure to dexamethasone alters the hippocampal 5HT1A receptor number and the efficiency of ligand mediated G-protein activation in adult male rats. Earlier studies from our lab and others show that rats exposed to excess levels of maternal and synthetic glucocorticoid *in utero* experience changes in hippocampal MR and GR expression that leads to destabilization of HPA axis regulation (Takahashi *et al.,* 1991; Henry *et al.,* 1994; Shoener *et al.,* 2006). Additional studies have also shown that HPA axis activity is modulated by serotonergic function (Klaassen *et al.,* 2002) .

The relationship between corticosteroids and the serotonergic system was first reported following autoradiographic studies which identified increased 5HT1A receptor binding in the rat hippocampal formation one week after bilateral adrenalectomy (Biegon *et al.,* 1985). This report emphasized the responsiveness of the 5HT1A receptor to CORT. In addition, several investigators have reported increases in 5HT1A receptor binding and gene expression in adrenalectomised (ADX) rats demonstrating that these receptors are under tonic inhibition from corticosterone. Cumulatively, these studies emphasize the relationship between the serotonergic system and corticosteroids, however, it is important to note that these findings were in response to only postnatal alterations in the level of coritcosteroids. To our knowledge only a few studies have investigated the effects of prenatal glucocorticoid exposure on the serotonergic sysytem. Since

glucocorticoids and serotonergic function interact to control hippocampal negative feedback regulation of HPA axis activity, we investigated the effect of prenatal DEX (a synthetic glucocorticoid) on 5HT1A function by measuring receptor number, binding affinity and ligand-mediated receptor G- protein activation. The key findings of our study are that: (1) prenatal DEX administration increases the total 5HT1A receptor number in the hippocampus (2) the functionality of these receptors is reduced in experimental animals as evidenced by the increased EC_{50} values compared to controls and (3) DEX rats consistently showed an attenuated growth rate and energy intake throughout the study. This latter finding is consistent with other reports, one of which followed the same paradigm of *in utero* DEX-exposure (Shoener *et al.,* 2006) and the other that adopted a gestational stress model (Baker, et al., 2009). However, our data showing increased 5HT1A binding concurrent with a reduction in ligand-mediated G-protein activation is novel and warrants further investigation.

The 5HT1A receptors are expressed in two distinct populations in the brain: as somatodendritic autoreceptors on serotonin containing neurons of the raphe nuclei and as a heteroreceptor on the postsynaptic membrane of non-serotonin containing neurons in the cerebral cortex and limbic systems. In the raphe nuclei, locally released 5HT acts on autoreceptors to inhibit neuronal firing (Wang & Aghajanian, 1977; Celada *et al.,* 2001) and reduces 5HT release in all projection targets. 5HT1A heteroreceptors, which are more concentrated in the cortex and hippocampus, are also inhibitory. For example, the bindng of [^{3}H]-8-OH-DPAT, a 5HT1A receptor agonist, was reduced in both frontal cortex and hippocampus following chronic treatment with an antidepressant, a 5HT

reuptake inhibitor, imipramine (Mizuta & Segawa, 1989), presumably due to the down regulation of postsynaptic 5HT1A receptor population owing to the increased concentrations of 5HT available for binding at the synaptic cleft. This suggests that 5HT1A heteroreceptors are responsive to varying levels of 5HT release and together, these data underscore the integrated actions of both 5HT autoreceptors and heteroreceptors in serotonergic signalling. Recent studies (Slotkin *et al.,* 2006) reported an increase in 5HT1A receptor binding in the cortex in response to prenatal doses of 0.05mg Dex/kg to 0.8mg/kg from GD 17-19. Our data are in line with these results since we found a significant increase in hippocampal 5HT1A receptor binding, although our protocol involved a 0.15 mg DEX/kg from GD 14-19. Together these data are mutually supportive since the functions of the 5HT1A receptor in these brain regions are overlapping.

When considering the interactions between glucocorticoid exposure and serotonergic function, it is important to recognize that CORT action is mediated by two types of intracellular receptors, the mineralcorticoid receptor and glucocorticoid receptor. While CORT binding to MR is crucial to the tonic activation and regulation of circadian driven processes, CORT binding to GR is most influential during the circadian peak. Many studies have shown that these receptors act together to regulate the HPA axis rather than one of the receptors alone (De Kloet *et al.,* 1986; Gesing, *et al.,* 2001). Interestingly, the MR and GR colocalization has been detected only in the hippocampus and frontal cortex making them the most studied brain regions in relation to the HPA axis and its negative feedback mechanisms. In addition to its MR/GR activities, CORT selectively

down regulates 5HT1A receptor mRNA expression in hippocampal areas, but not in dorsal raphe nulei (Neumaier *et al.,* 2000), presumably due to a higher CORT receptor density in post compared to presynaptic sites (Reul & De Kloet, 1985). However this finding by Neumaier et al cannot be used to predict the changes in hippocampal 5HT1A receptors following prenatal exposure to glucocoticoids, especially dexamethasone. This is because the timing of brain growth spurt is critical and during this period, brain development is very susceptible to external manipulation. Therefore, manipulation of the fetal environment during late gestation will impact on later stages of brain and HPA development. So findings from our study emphasize the impact of prenatal programming of the brain in response to synthetic glucocorticoid exposure. Our study shows that following prenatal DEX exposure there is an increase in the number of hippocampal 5HT1A receptors coupled to a decrease in their function. Thus we state that, in addition to the earlier findings about the decrease in GR and MR receptors following prenatal exposure to dexamethasone, there is an overall decrease in function of hippocampal 5HT1A receptors.

However, the increase in 5HT1A receptors in response to CORT found in other studies must be clarified in the context of this receptor's function. For example, it has been previously shown that 5HT1A receptor knockout mice showed increased anxiety-like behavior, in three paradigms: open field, EPM, and novelty suppressed feeding (Ramboz *et al.,* 1998; Gross *et al.,* 2000), however, these mice could be "rescued" if their 5HT1A receptor expression was restored during adult stage. Thus, when 5HT1A function is lost in these knock-out mice, a propensity for anxiety-related behaviors

ensues. It could be hypothesized that this serotonin receptor, in part, mediates emotionality which is associated with changes in HPA axis activity. A previous study from our lab (Shoener *et al.,* 2006) showed that prenatal exposure to DEX alters hippocampal drive which resulted in HPA axis hyperactivity in DEX-exposed animals. After considering the results from both of our studies it could be assumed that the hyperactivity of HPA axis observed in our prenatally DEX exposed rats could be, in part, due to a decrease in the 5HT1A receptor functions. Although the DEX exposed animals in our study exhibit an increase in 5HT1A receptor density, the concomitant increase in the EC_{50} value of these receptors suggests that the dose of agonist required to activate the receptors and induce GTP incorporation was significantly higher. These findings support the hypothesis that 5HT1A receptor function has been impaired by exposure to prenatal DEX.

Although serotonin release is a key component of serotonergic function, a study by Slotkin et al (2006) found that alterations in presynaptic serotonin level and turnover are not the sole driving force behind the effects of DEX on 5HT1A receptors since upregulation was seen regardless of whether serotonin levels were increased, decreased or remain unchanged. In fact, increases in 5HT1A receptors in the face of a rise in presynaptic serotonin release appears particularly puzzling, since ordinarily one would expect to see compensatory downregulation of the postsynaptic receptors. This disconnection however provides important clues as to potential mechanisms underlying the effects of DEX on serotonergic function. For example, a similar pattern of postsynaptic receptor upregulation concomitant with higher presynaptic activity has also

been reported with neuroteratogens unrelated to DEX and typically reflects loss of postreceptor-signalling capabilities (Shahak *et al.,* 2003). In such a situation the failure of synaptic signals to activate postsynaptic 5HT1A receptor signalling might lead to both an increase in presynaptic 5HT release due to reduced effectiveness of 5HT1A autoreceptors as well as an upregulation of 5HT1A heteroreceptors in an unsuccessful attempt to compensate for the underlying deficiency. In light of these observations, the increase in hippocampal 5HT1A receptor number in DEX animals could be an unsuccesful attempt to compensate for less functional receptors and loss of signal transduction as indicated by the [^{35}S]GTPγS incorporation assay. The role of 5HT1A autoreceptors and heteroreceptors on anxiety and depression are quite controversial. However, a considerable body of evidence suggest that these receptors are anxiolytic. For example, heteroreceptors appear to play an important role in effects of drugs such as SSRI, which are used as antidepressants. In particular, experiments consisting of the selective regional rescue of 5HT1A receptors in 5HT1A knockout mice clearly showed that postsynaptic 5HT1A heteroreceptors in hippocampus are required for the anti-immobility effect of SSRI treatment in animals subjected to the tail suspension test (Overstreet *et al.,* 2003): a finding which supports the anxiolytic role of this subset population of 5HT1A receptors. Activation of both receptor populations results in membrane hyperpolarization and decreased neuronal excitability. For instance activation of 5HT1A autoreceptor by 5HT will result in an inhibitory negative feedback on the 5HT producing neurons in the raphe nuclei by decreasing the neuronal excitability . This reduction in excitability would then result in decreased 5HT output into the synaptic cleft and a resulting decrease in 5HT

binding and activation of postsynaptic 5HT1A receptors which would in turn reduce their hyperpolarizing effects. Our findings are in partial accordance with this account and in addition, recent findings show that ADX (no circulating CORT) diminishes the affinity of 5HT1A receptors in the dorsal raphe nuclei (autoreceptors) and CORT increases the affinity for 5HT (Bellido et al., 2004). Thus the increase in CORT in the cited study (Bellido et al., 2004) results in decreased firing of the presynaptic serotonergic neurons in the dorsal raphe nulceus and a consequent decrease in 5HT available for binding to the postsynaptic receptors including 5HT1A heteroreceptors. This reduction in 5HT1A receptor activity results in putative anxiogenesis. Our data agrees with these findings since a significant decrease in the functionality of 5HT1A heteroreceptors was measured following prenatal exposure to DEX.

It is likely that the prenatal exposure to DEX in our study, in part, results in a perturbation of 5HT1A receptor function and provides the ground for anxiogenic outcomes. This interpretation is further supported by current studies in our lab conducted by Joseph Donohue (unpublished). He has shown that although there is no obvious differences between control and Dex-exposed adult offspring under basal conditions, a brief exposure to stress results in a significant increase in anxiety-like behavior in the elevated plus maze ($P < 0.03$) and a significant increase in circulating CORT ($P = 0.003$).

A vast body of evidence suggests that actions of 5HT mediated through the 5HT1A receptors in the dentate gyrus may stimulate the production of neurons in the same region. In contrast to neurons in other brain regions the granule cell layer of the

dentate gyrus enjoys an extended period of development from gestation and continuing into adulthood, with a continual migration of granule cell precursors into the hippocampus from the lateral ventricles (Altman & Bayer, 1990; Rickmann *et al.,* 1987). Moreover, the role of hippocampal formation in learning and memory has been recognized for decades (Squire & Zola, 1996) and many abberations in the hippocampus have been associated with alterations in these attributes. It is possible that defects in learning and memory exhibited by mice and rats following prenatal glucocorticoid therapy (Emgard *et al.,* 2007; Noorlander *et al.,* 2007) could be further illuminated by our findings which support the hypothesis that prenatal dexamethasone induced hippocampal 5HT1A dysfunction may result in suppression of hippocampal neurogenesis and subsequent deficits in memory and learning.

Future Directions:

There is growing evidence that 5HT1A receptors together with 5HT2A receptors are responsible for the regulation HPA axis. Extensive studies have shown that 5HT1A receptor agonists have effects similar to 5HT2A receptor antagonists in a variety of systems (Darmani *et al.,* 1990; Meltzer & Maes, 1995). Additional evidence about the difference in function of these two classes of 5HT receptors comes from the observation that administration of CORT or ACTH alters the numbers of both 5HT1A and 5HT2A receptors quite substantially in opposite directions. In the light of all these observations from previous studies, our lab is currently preparing to explore the effects on 5HT2A receptors in the hippocampus as well as cortical tissue following prenatal DEX exposure.

The hippocampus is divided into two functionally different regions, the dorsal and ventral hippocampus. Whereas the ventral part of the hippocampus is primarily implicated in emotional processing, the dorsal part is mainly linked to learning and memory (Bannerman, et al., 2004). Since our study did not measure the effect on 5HT1A receptors separately on the ventral and dorsal hippocampus, it would be interesting to investigate the effect of prenatal DEX exposure on these receptors in these individual regions.

As mentioned earlier Bellido *et al* (2004) has reported an increase in 5HT1A autoreceptors in response to corticosterone. Since neuronal firing mediated by serotonin will depend upon the action of the entire 5HT1A receptor subfamily, comprising both autoreceptors and heteroreceptors, the hyperactivity of HPA axis observed in a previous study by Shoener *et al* (2006) cannot be explained with the changes in the heteroreceptor population alone. A more comprehensive picture could be drawn if the effect on 5HT1A autoreceptors could be studied following the same stress paradigm.

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Table 1. ***Effects on weight at birth, weaning, puberty and on the day of sacrifice following prenatal exposure to DEX***

Group	Birth weight(g)	Weaning weight (g)	Weight at puberty (g)	Sacrifice weight (g)	Energy intake, (kcal/day)
Control	8.003±0.21	34.32±1.5	374.4±5.8	569.0±10	192.3±0.76
Dex-exposed	6.791±0.18*	27.76±0.34*	337.7±3.4*	521.2±5.8*	184.9±0.61*
	P =0.004	*P* < 0.0005	*P* < 0.0005	*P* < 0.0005	*P* < 0.0005

Values are means ± S.E. *P*=0.004, *P<0.0005* indicate significant difference between dexamethasone (DEX)- exposed animals compared with controls using 1-way ANOVA for weights.

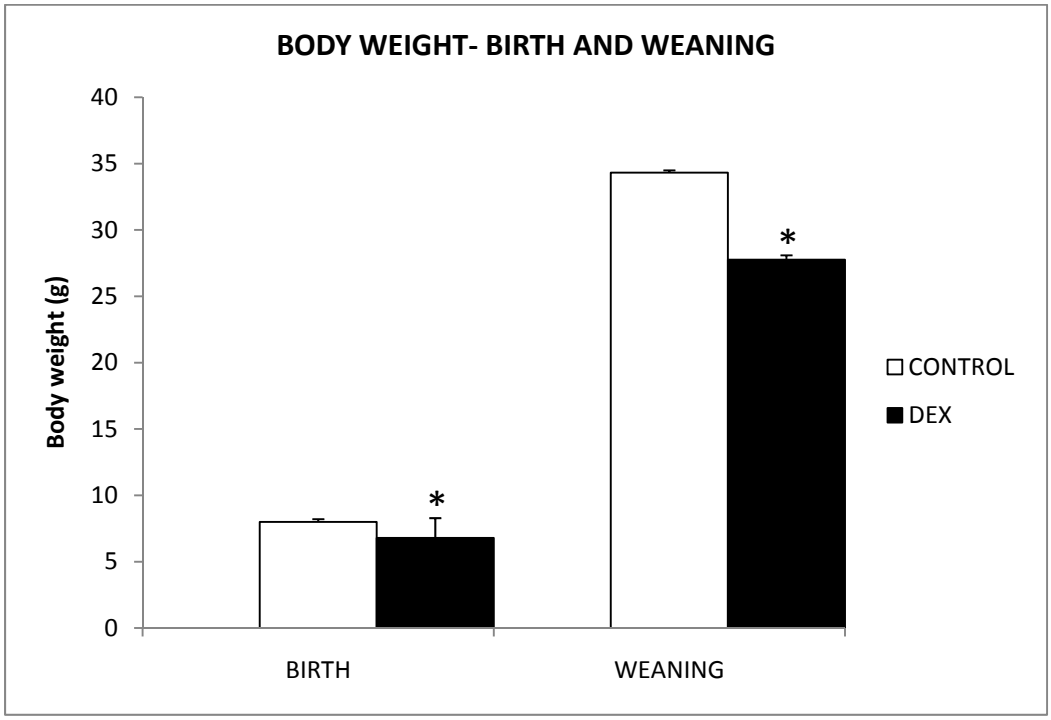

Figure.1. Difference in the average weight at birth and weaning of dex exposed and control animals. The birth weight of dex animals was significantly lower than control by 15%; P= 0.004. The weaning weight of dex animals was significantly lower than control by 19%; P<0.0005.

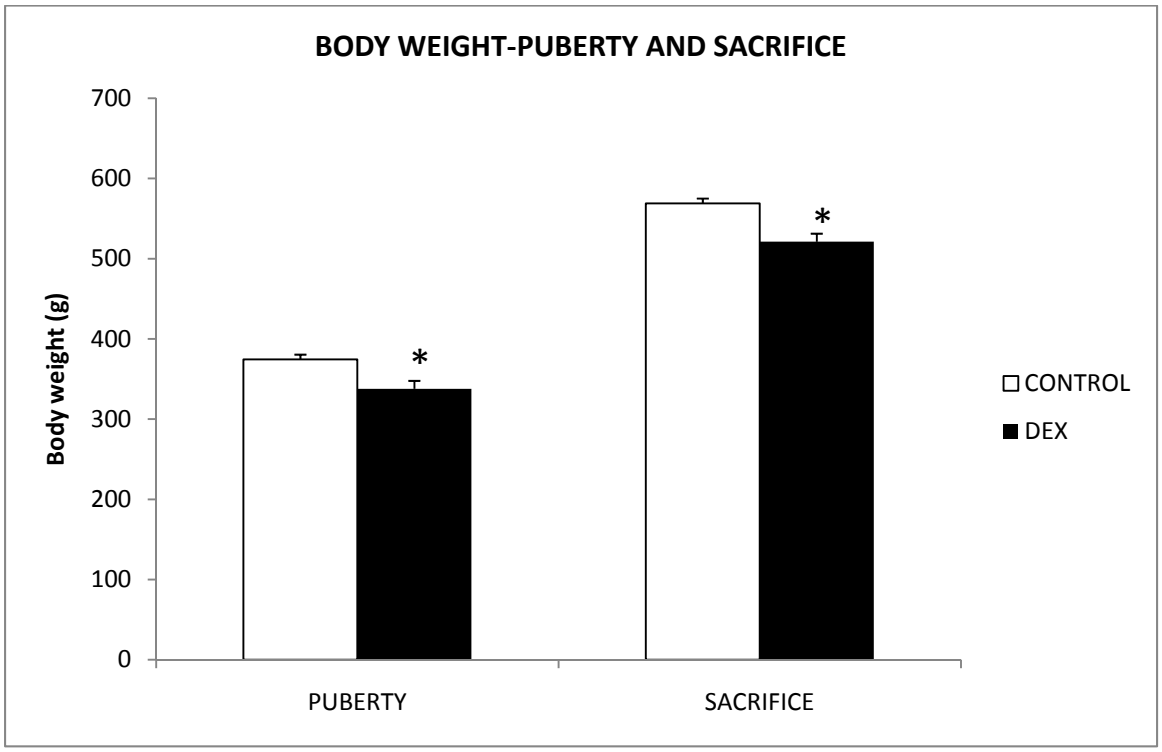

Figure.2. Difference in the average weight at puberty and sacrifice of dex exposed and control animals. The pubertal weight of dex animals was significantly lower than control by 10% (*P<0.0005)*. The terminal weight of dex animals was significantly lower than control by 8.5% (*P*<0.0005).

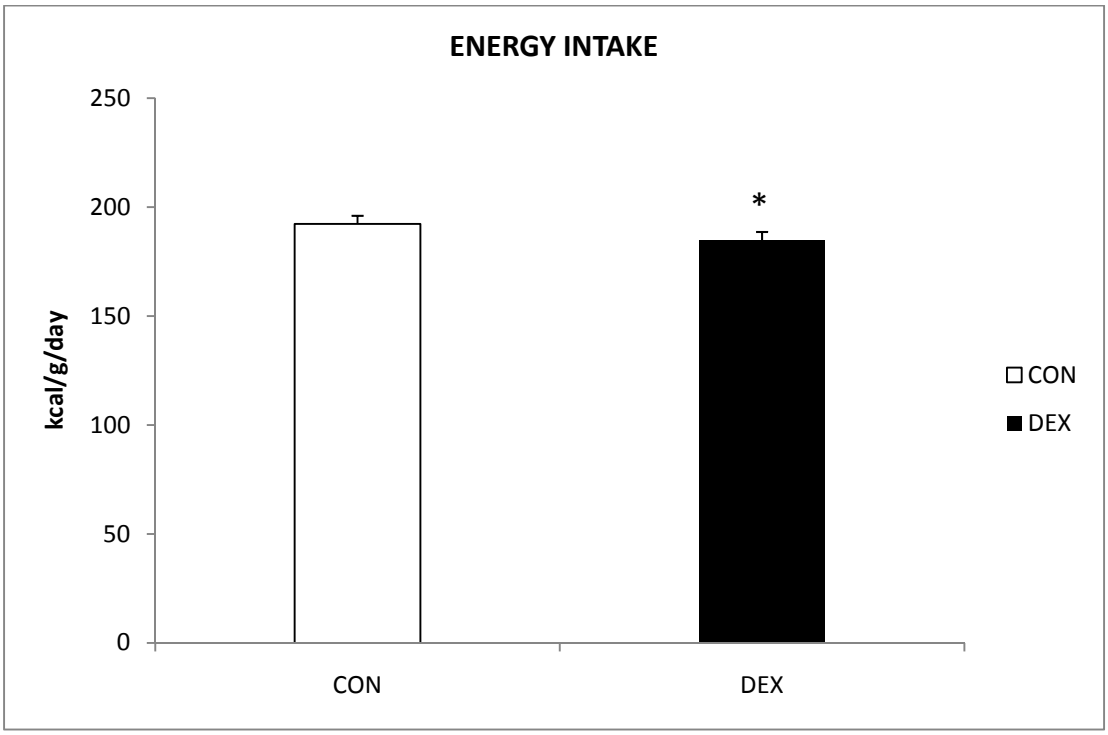

Figure.3. Difference in the average daily energy intake of dex exposed and control animals. The energy intake of dex animals was significantly lower than control by 3.8% (*P<0.0005)*

Table 2. ***Values for Dissociation constant, Binding Maximum, Stimulation Maximum, and Effective concentration$_{50}$ values (EC_{50}) for hippocampal 5HT-1A receptor in Control and Dex-exposed animals***

Group	K_d (nM)	B_{max}(fmol/mg tissue)	Percent Stimulation Max (E_{max})	EC_{50} (µM)
Control	2.298±0.156	4.587±0.298	30.83±1.8	0.119±0.0155
Dex-exposed	2.531±0.143	6.138±0.355*	35.91±2.0	0.226±0.02*
	P = 0.292	*P = 0.002*	*P = 0.069*	*P =0.002*

Using the radioligand receptor assay, no significant differences were found in the binding affinity, K_d, (*P*=0.292), whereas a significant increase in binding maximum, $B_{max,}$ was observed in the Dex-exposed animals (*P*= 0.002). In addition, no significant difference in maximum stimulation ($E_{max)}$ of 5HT1A receptor and GTPγS incorporation was detected (*P*=0.069). However, the effective concentration of 5HT1A receptor agonist required for maximal stimulation was almost two-fold higher in the Dex-exposed group (*P*= 0.002) Values are mean ± S.E.M.

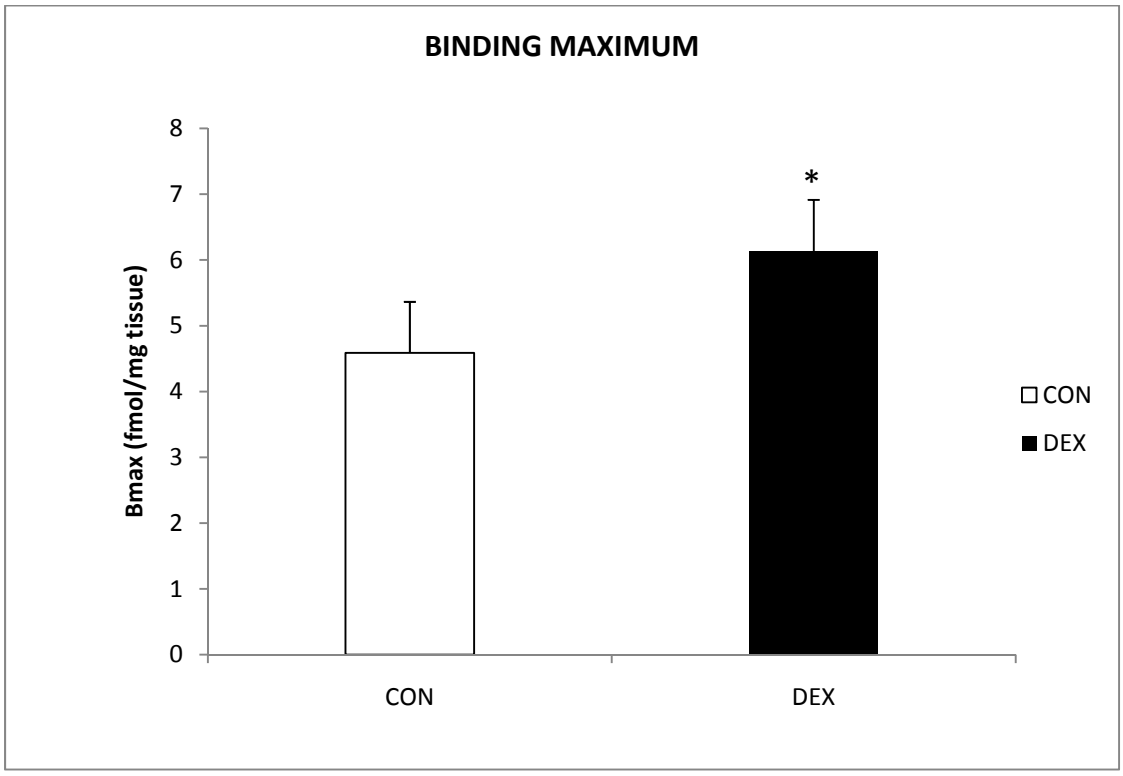

Figure.4. Difference in the B_{max} values of control and dex exposed animals. Increased B_{max} in dex exposed animals indicate an increase in 5HT1A receptor density. Dex animals showed a receptor density 35% higher than the control animals; *P*=0.002.

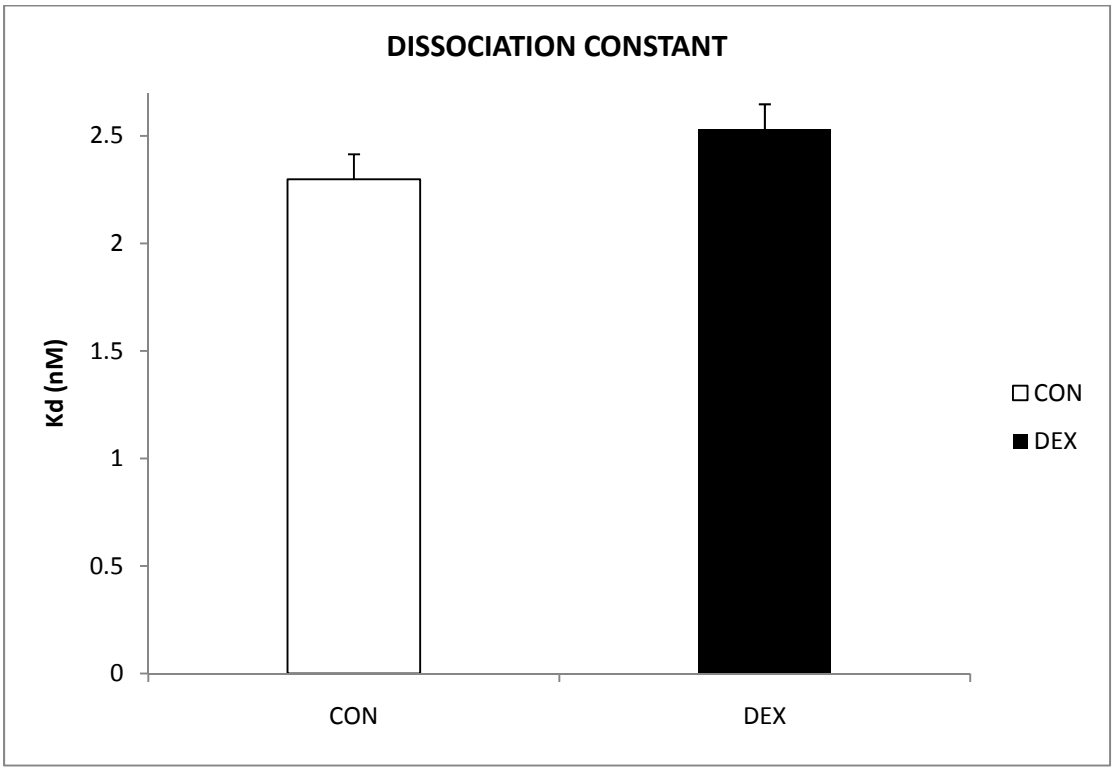

Figure.5. This graph illustrates the values for the dissociation constant (K_d) which reflect the binding affinity of the 5HT1A receptor for its ligand ([H^3] MPPF). No significant difference in the affinity was found following prenatal dexamethasone exposure; *P*=0.292.

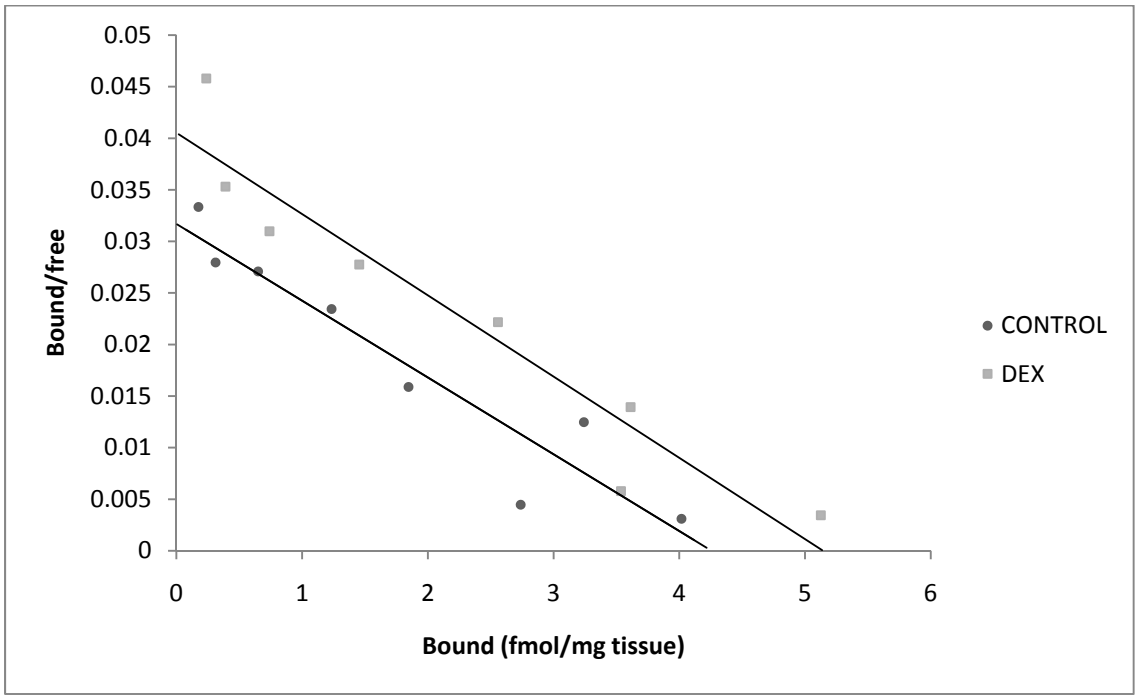

Figure 6. Scatchard analysis of [^{3} H] MPPF binding to rat hippocampal 5HT-1A receptors in Control and Dex animals. Each regression line is based on one pair of representative animals.

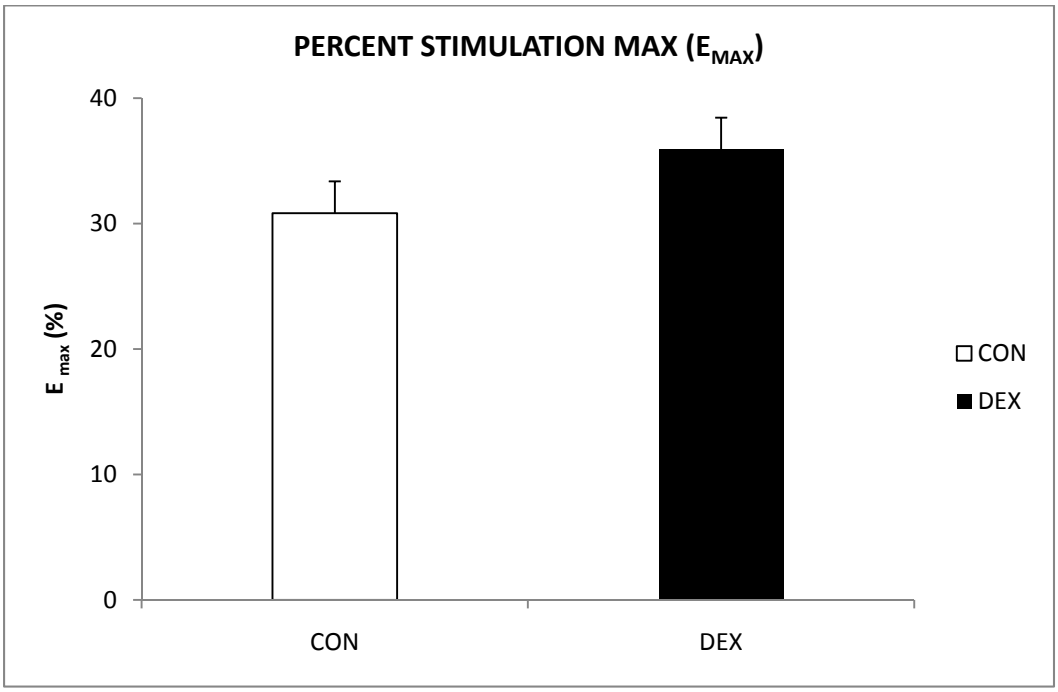

Figure.7. This graph shows the values of Stimulation max (Emax) which is a measure of the maximum stimulation of [^{35} S] GTPγS incorporation assay following ligand (8-OH-DPAT) mediated activation of 5HT1A receptor. No significant difference in the maximum stimulation was found owing to prenatal dexamethasone exposure; *P*=0.069.

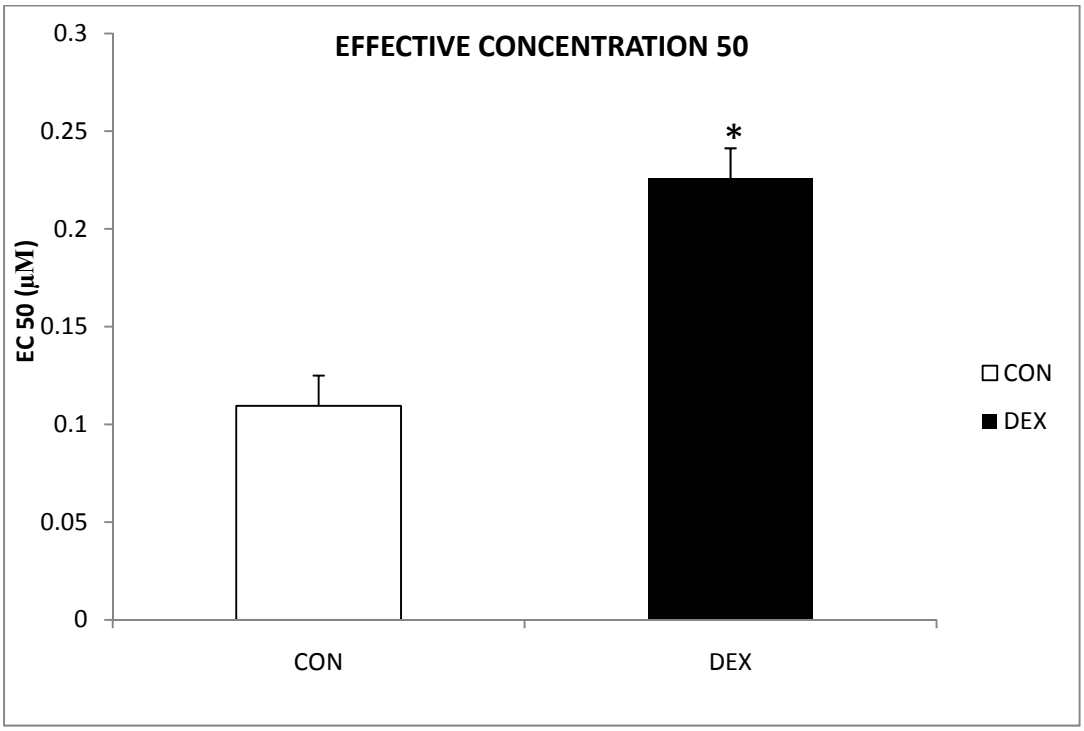

Figure.8. Effects of prenatal exposure of dexamethasone on the EC_{50} value of 5HT1A receptor. The EC_{50} values were determined by measuring the extent of incorporation of GTPγS following ligand (8-OH-DPAT)-mediated stimulation of the 5HT1A receptor). The dex animals showed a 51% increased EC_{50} value compared to controls; P=0.002.

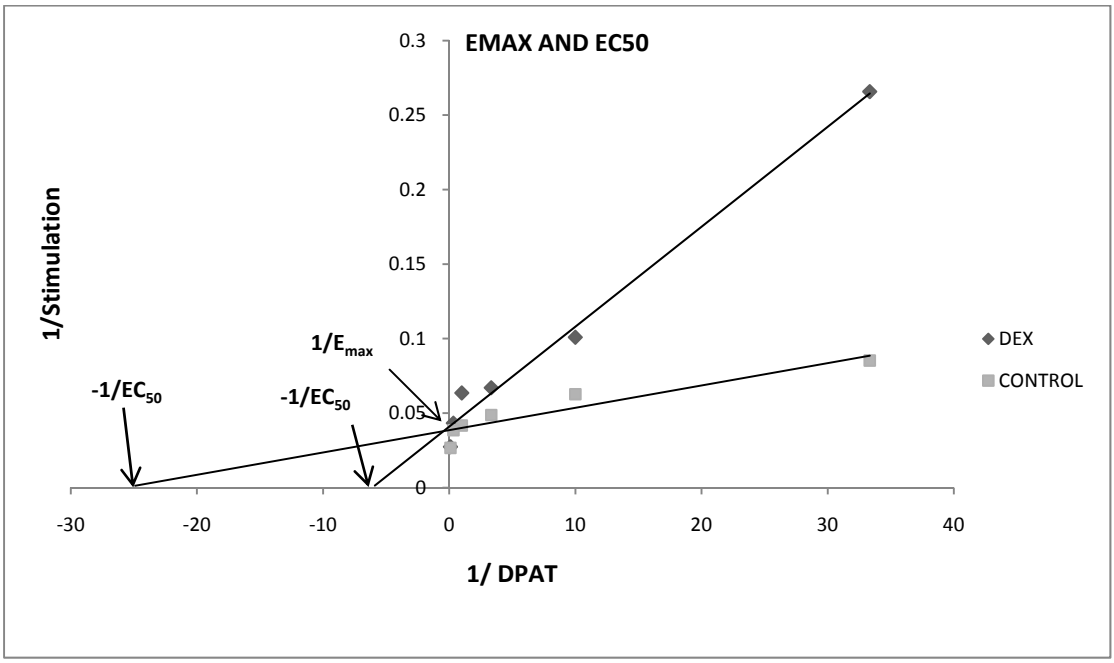

Figure.9. Double reciprocal plots illustrate stimulation of the 5HT1A receptor at increasing agonist concentrations, 8-OH-DPAT, between dex exposed and control group. Each regression line is from one representative animal.