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Darshan S. Shah
Bucknell University, dss022@bucknell.edu

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ALTERATION IN 5HT1A RECEPTOR ACTIVTY FROM A PRENATAL EXPOSURE TO DEXAMETHASONE IN A STRESSED AND NON-STRESSED ADULT MALE RAT

By:

Darshan S. Shah

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Approved:

_____
Dr. Kathleen Creed Page
Advisor

_____
Dr. Charles Clapp
Director Cell Biology/Biochemistry

_____
Dr. Jack Gallimore
Honors Committee Member

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Abbreviations

ACTH	Adrenocorticotropic Hormone
AVP	Arginine Vasopressin
B_{max}	Binding Maximum
CBG	Corticosterone Binding Globulin
$Control_{tot}$	Control Stressed+ Control Non-Stressed
CORT	Corticosterone
CRH	Corticotrophin Releasing Hormone
DEX	Dexamethasone
DEX_{tot}	DEX Stressed + DEX Non-Stressed
EC_{50}	Effective Concentration 50%
E_{max}	Excitation Maximum
GC	Glucocorticoid
GDP	Guanosine Diphosphate
GPCR	G-Protein Coupled Receptor
GR	Glucocorticoid Receptor
GTP	Guanosine Triphosphate
$GTP\gamma S^{35}$	Guanosine Triphosphate gamma $Sulfur^{35}$
K_d	Receptor Affinity
$[H^3]$MPPF	2'-methoxyphenyl-(*N*-2'-pyridinyl)-*p*-fluoro-benzamidoethyipiperazine
HPA	Hypothalamic-Pituitary-Adrenal
MR	Mineralocorticoid Receptor

$Non\text{-}Stressed_{tot}$	DEX Non-stressed + Control Non-stressed
PVN	Paraventricular Nucleus
RT	Room Temperature
sc	Subcutaneous
$Stressed_{tot}$	Control Stressed +DEX Stressed
WAY100635	N-[2-[4-(2- Methoxyphenyl)-1piperazyinyl] ethyl]-N-2-pyridinylcyclohexanecarboxamide maleate salt
5HT	Serotonin
5HT1A	Serotonin 1A Receptor
8-OH-DPAT	8-hydroxy-*N*,*N*-dipropyl-2-aminotetralin
11-β-HSD	11-β-Hydroxysteroid Dehydrogenase
11-β-HSD-1	11-β-Hydroxysteroid Dehydrogenase Type 1
11-β-HSD-2	11-β-Hydroxysteroid Dehydrogenase Type 2

Abstract

Synthetic glucocorticoids (GC) are used as a clinical therapeutic to stimulate lung development in fetuses that present the risk of preterm delivery. Previous studies have shown that a prenatal exposure to Dexamethasone (DEX) causes a disturbance in normal GC mediation of neuritic outgrowth, cell signaling, and serotonergic systems. Our hypothesis is that a prenatal exposure to DEX during the third trimester of pregnancy alters 5HT1A receptor function. Pregnant dams were injected daily with 150μg/ml/kg of DEX from gestation day 14 through 19. Control dams were treated with and equal volume of saline. Swim stress followed by elevated plus maze testing was conducted on male rats an hour and a half prior to being sacrificed to induce postnatal acute stress. The non-stressed group was also tested and allowed to return to baseline before sacrifice. Hippocampi were analyzed using a radioligand-receptor binding assay and $GTP\gamma S^{35}$ incorporation (3H-MPPF antagonist and 8-OH-DPAT agonist, respectively). A significant increase in K_d was found in non-stressed DEX-exposed animals compared to non-stressed controls ($p<0.05$). Moreover, K_d decreased significantly in the DEX-exposed stressed rats when compared with their non-stressed cohorts ($p<0.05$). When the K_d of all Dex-treated animals (DEXtot) animals was compared to the K_d of all control animals ($Control_{tot}$) animals, the K_d of the DEX_{tot} group was significantly increased. In contrast, the K_d measured in all animals exposed to stress ($stressed_{tot}$) was significantly decreased in comparison to all non-stressed animals ($non\text{-}stressed_{tot}$)**.** The B_{max} increased significantly in response to prenatal DEX when DEX_{tot} were compared to $Control_{tot}$ ($p<0.05$). However, the B_{max} significantly decreased in response to postnatal stress when combined stressed groups ($stressed_{tot}$) were compared combined non-stressed groups **(**$non\text{-}stressed_{tot}$) ($p<0.05$). In addition, a postnatal acute stressor caused a significant increase in the E_{max} for both the prenatal DEX treated and control animals as

compared to their unstressed littermates ($p<0.05$). Based on these results it is possible to say that a prenatal exposure to Dexamethasone significantly increases the receptor K_d in the adult male rats under resting conditions. This loss of affinity may initiate a compensatory action which would include an increase in receptor density. However, under periods of stress, both control and prenatally DEX treated animals showed an increase in receptor affinity. The increased E_{max} observed in stressed animals could be due to a shift in 5HT1A receptor population from an inactive to active state. Our results suggest that there are differential changes in 5HT1A receptor density and activity in the hippocampus of the male rat in response to a prenatal exposure to DEX as well as an acute stressor in adulthood.

Introduction

Throughout our lives we face stress in all different forms. These stressors can be physical, chemical, and even emotional. Regardless of the stressor, they elicit a similar response from the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis (HPA) (Munck, Guyre, & Holbrook, 1984). This "stress response" allows the body to adapt to challenge and maintain homeostatic balance which ensures that vital functions such as blood pH and hormone release will be regulated in response to the body's ever-changing needs.

Stressors are detected via a sensory system that transmits signals to the hypothalamus, the major integration center of the brain. Specifically the signals are sent to the parvocellular neurons of the paraventricular nucleus (PVN), which is the integration site for the stress response within the hypothalamus (Lopez, Young, Herman, Akil, & Watson, 1998). Activation of the PVN differentially modulates a number of physiological systems. For example, the immune system and the inflammatory response are suppressed whereas the production of arginine vasopressin (AVP) and corticotropin-releasing hormone (CRH) are increased in response to PVN activation (Munck, Guyre, & Holbrook, 1984) (Lopez, Young, Herman, Akil, & Watson, 1998). AVP decreases the amount of water lost during renal excretion, while CRH initiates the HPA axis cascade that ultimately leads to the release of cortisol in human and corticosterone in rats (Lopez, Young, Herman, Akil, & Watson, 1998). It is this latter component and its ramifications that will be a central focus for this research.

After the stress signal is received and processed by the hypothalamus, CRH is released. CRH then acts on the anterior pituitary and promotes the release of adrenocorticotropin hormone (ACTH). ACTH is a trophic hormone that enters the blood stream and acts on the adrenal gland which produces the final product of the HPA axis, cortisol (Miller & O'Callaghan, 2002). In rats

the final product of the HPA axis is corticosterone (CORT) which is very similar in structure to cortisol, the human stress hormone.

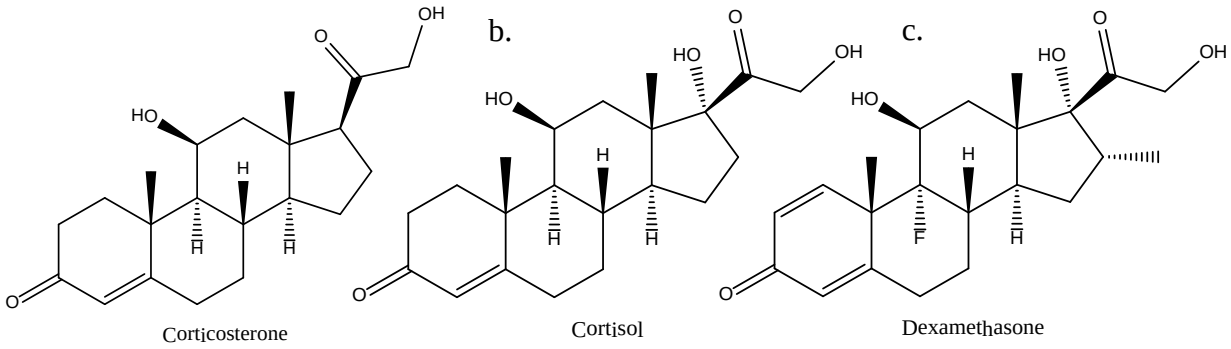

Figure 1. Structures showing similarities between a. corticosterone, b. cortisol, and c. Dexamethasone.

Naturally occurring glucocorticoids, such as CORT, are steroid hormones synthesized in the adrenal cortex and are involved in the regulation of mineral and salt secretion and in regulation of glucose metabolism via binding of mineralocorticoid (MR) and glucocorticoid (GR) receptors, respectively. CORT acts on a wide range of organs including several brain regions, particularly limbic regions such as the hypothalamus and hippocampus (Miller & O'Callaghan, 2002). The HPA-mediated release of CORT is controlled further through a negative feedback loop. Negative feedback loops occur when the product of the cycle acts to inhibit the components involved in producing it. In other words, the end product of HPA axis activation, CORT, acts directly on the anterior pituitary and hypothalamus to inhibit the production of CRH and ACTH, respectively. Moreover, CORT acts back on mineralocorticoid receptors and glucocorticoid receptors in the hippocampus in order to change the central drive on the HPA axis. The hippocampus is known to be the central “pulse generator” which establishes the neuroendocrine set point for this system during development. It should be noted that

neuroendocrine loops are continuously interacting and it has been shown that serotonergic activity and the release of serotonin, can have inhibitory effects on the HPA axis by inducing negative regulation of CORT (Gold & Chrousos, 1999).

Both MR and GR receptors are involved in the regulation of the HPA axis feedback. Activation of these receptors initiates the negative feedback mechanism of the HPA axis. MR receptors in the brain have ten times higher affinity for glucocorticoids than GR receptors. This suggests that under basal conditions the cell's CORT responses are dictated by MR, and only during times of increased HPA axis activity and increased CORT levels will there be appreciable levels of GR activation (de Kleot ER, 1998). Although it is well known that GR is involved in peripheral glucose metabolism and nutrient mobilization, differential activation of MR and GR are key determinants of transcriptional regulation for genes that regulate glucose metabolism, mineral retention, memory formation, memory processing in the brain (Lu, Wardell, Burnstein, Defranco, FULLER, & Gigure, 2006).

GR is expressed in most brain regions and is found almost equally in both neurons and glial cells (de Kloet, 1991). MR and GR are co-expressed in very specific brain regions, most importantly the hippocampus, which relates to its involvement in memory formation.

Prolonged increases in CORT levels due to conditions such as HPA axis hyperactivity have been shown to have negative impacts on memory formation and neuronal potentiation. However, when the HPA axis activity is up-regulated for only short periods of time, preferential binding to MR receptors has been observed over GR (McEwen & Saplosky, 1995). During short periods of high CORT, the enhanced MR activation and low levels of GR activation may have positive impacts on the potentiation of memory, but when high serum CORT levels are circulating for an extended period of time, inhibitory effects on these memory processes are

observed (McEwen & Saplosky, 1995). Therefore, due to the biphasic binding model of CORT to MR and GR the short term effects of HPA axis up regulation can have positive effects on the consolidation of memory, while long term effects can have deleterious effects on the same system (Reul & de Kloet, 1985) (Oitzl & de Kloet, 1992).

HPA axis set points vary during the lifetime of an individual. During development, specifically during the third trimester, the fetal neuroendocrine system is highly plastic, and any prolonged elevations or deprivations of hormones or other necessary components during this time period can have long lasting effects on the individual (Pariante & Lightman, 2008) (Takahashi, Turner, & Kalin, 1998). Increases in fetal CORT levels rise in response to stressors imposed on the mother during pregnancy. These stressors can come from mental, physical, emotional stressors, or even the administration of synthetic glucocorticoids, but all of these challenges have been shown to induce significant change in both maternal and fetal corticosterone levels (Douglas, 1975) (Barbazanges, Piazza, Le Moal, & Maccari, 1996) (Ward & Weisz, 1991).

Of interest to our study is the fact that DEX is a synthetic glucocorticoid administered in approximately 10% of human pregnancies in the United States (NIH Consensus, 2000). DEX is administered to mothers that are at risk for preterm deliveries. One of the main reasons for this is that glucocorticoids have been shown to promote surfactant production in the fetus. This is critical because without surfactant the surface tension around the alveoli would be too great and it would lead to alveolar collapse (Kattner, Metze, & Waisse, 1992). However, despite being a powerful tool in surfactant stimulation, prenatal DEX exposure has unwanted side effects. For example, DEX administration decreases maternal CORT levels, but increases total levels of circulating glucocorticoids, as DEX is a glucocorticoid. This increase in glucocorticoid

concentration in the prenatal environment has been associated with low birth weight offspring and other neurophysiological disturbances (Shoener, Baig, & Page, 2005) (de Kleot ER, 1998). These disturbances may be due to the inability of the body's defense mechanisms to recognize DEX. For example, adult neuronal cells have a membrane pump, mdr1a P-glycoprotein, in the blood brain barrier that opposes glucocorticoid transport into the brain and protects maternal neurons from elevated glucocorticoid levels. However, during the third trimester the fetal blood-brain barrier is not fully developed and the underdevelopment of the mdr1a P-glycoprotein defense mechanism during the time of DEX administration allows DEX to interact with the developing fetal brain (Owen, 2002). In addition, the effects of DEX are more effective compared to those of CORT because DEX has a longer half-life and a much higher affinity for GR receptors than does CORT. Thus, it is degraded more slowly and binds more effectively to GR receptors in comparison to endogenous CORT (Orth & Kovac, 1998) (Rupprecht, Reul, & Van Steensel, 1993). If DEX binds GR with a higher affinity and disturbs the MR/GR functions during times when MR should be controlling the CORT response, a dysfunctional neuroendocrine regulatory loop will be established in the developing fetus (Rupprecht, Reul, & Van Steensel, 1993). This is underscored by the fact that the DEX/MR receptor ligand complex is very unstable and this instability leads to weak activation of downstream gene transcription regulators mediated by MR (Ruel, Gressing, Droste, & al, 2000).

To protect the fetus from the effects of increased corticosterone, the maternal-placental barrier has proteins to prevent the flow of active CORT to the fetus. For example, the active from of CORT, corticosterone, can be converted to a biologically inactive form, 11-dehydrocorticosterone, by 11-Beta-hydroxysteroid dehydrogenase (11β-HSD) (Yang, 1995). Two forms of 11β-HSD exist at the fetal/placental barrier; however their transcriptional

regulation and function are different. The two forms of 11β-HSD are 11β-HSD-1 and 11β-HSD-2. During development, 11β-HSD-2 is localized to the fetal placental barrier while 11β-HSD-1 has more broad reaching effects. The 11β-HSD-2 isoform has a two to five-fold higher affinity for corticosterone but a very weak affinity for DEX. Similarly, 11β-HSD-1 has lowered affinity for CORT and no measurable affinity for DEX. In essence, DEX circulating in the maternal blood system is capable of passing through the fetal placental barrier almost unchecked (Yang, 1995).

Another regulatory component for the control of circulating corticosterone is corticosterone binding globulin (CBG). Essentially, CBG binds circulating corticosterone and sequesters it so that it becomes incapable of binding its MR and GR receptor targets. Although CBG is a very powerful regulator of corticosterone action, it has been shown that chronic stress and the consequent increase in corticosterone levels causes a decrease in circulating maternal CBG. As a result of this down regulation of CBG in the maternal environment, the fetus is more exposed to increases in corticosterone levels experienced by the mother (Takahashi, Turner, & Kalin, 1998). It should be noted that CBG is similar to 11β-HSD-2 in that it has a very high affinity for corticosterone and a very low affinity for DEX. Since DEX is not tightly regulated by 11β-HSD-1, 11β-HSD-2, or CBG especially during maternal stress, it easily passes from the maternal environment to the fetal environment through the fetal placental barrier (Weisner, Do, & Feldman, 1979).

As mentioned previously, the down regulation of CORT occurs due the similarities between DEX and CORT and the synthetic steroid's capacity to reduce CORT synthesis and release following DEX-induced negative feedback. When MR and GR in response to DEX or CORT receptors are activated, they act on a number of systems, including the regulation of

serotonin, 5HT. In fact, the balance between activation of glucocorticoid receptors and 5HT receptors ultimately controls the HPA axis negative feedback system (Gold & Chrousos, 1999). For example, when synthetic glucocorticoids are added, they inhibit endogenous CORT and drive 5HT regulation.

Serotonin is a tryptophan derived neurotransmitter and its actions are associated with numerous neurological regions (Jacobs & Azmitia, 1992). 5HT released from the presynaptic neurons, binds a number of cell surface receptors and generates a wide variety of second messengers (Barnes & Sharp, 1999). The activation of these receptors has been linked to many cognitive behaviors including learning (Meneses, 1999). Specifically, in regards to memory, the 5HT1A receptor has been associated with the regulation of working memory (Dourish, Ahlenius, & Hutson, 1987). The highest levels of 5HT1A receptors are found in the limbic regions of the brain such as the hippocampus, raphe nuclei, amygdala, hypothalamus, and cortex. Of these the hippocampus has been found to be very important in the processing of new memories (Kung, Zhuang, Frederick, & Kung, 1994). Since this receptor is expressed very early during development, it is a target for modulation by a number of prenatal effectors such as stress or DEX exposure (del Olmo, Lopez-Gimenez, Vilaro, Mengod, Palacios, & Pazos, 1998).

The 5HT1A receptors in the hippocampus are tightly regulated by glucocorticoids as shown by adrenalectomy. This loss of the adrenal gland essentially removes the glucocorticoid release in response to ACTH and is associated with a greater number of 5HT1A receptors as well as an increase in 5HT1A receptor mRNA transcripts. However, when a glucocorticoid such as CORT or DEX was introduced into the system, the 5HT1A receptor density and mRNA expression returned to that of control animals (Zhong & Ciaranello, 1995). This suggests that CORT negatively regulates the gene regulation of 5HT1A receptors in adult animals.

In this study the aim is to explore 5HT1A receptor function in the adult rat after changes to HPA axis function have occurred during development. Prenatally stressed male rats show HPA axis hyperactivity and an alteration in MR and GR density in adulthood (Kalinyak, Griffin, Hamilton, Bradshaw, Perlman, & Hoffman, 1989) (Meaney, Sapolsky, & McEwn, 1985).

The vast majority of the 5HT receptors belong to a large class of signaling receptors called G-protein coupled receptors (GPCRs). These GPCRs are seven membrane pass proteins that have their N-terminus on the extracellular domain and the C-terminus on the cytoplasmic side. This general structure is true for all 5HT receptors except 5HT3 (Pierce, Permont, & Lefkowitz, 2002).

The binding of serotonin to the extracellular domain of the GPCR causes the receptor to undergo a conformational change which then causes the intracellular G-protein to be activated. Both the intracellular region and extracellular region have three looping regions. The current model has the binding of the intracellular heterotrimeric G-protein occurring at the intracellular loops 2 and 3, with intracellular loop 2 causing the activation of the G-protein (Horn, Van der Wenden, Oliveiera, Ijzerman, & Vriend, 2001) (Bourne, 1997). The G-protein is a heterotrimeric protein with an α, β, and γ subunit. The α subunit contains the GTP/GDP binding domain from which the receptor and protein derive their name. The α subunit also contains a GTPase which hydrolyzes the GTP to GDP to inactivate the protein, while the receptor acts as the GDP to GTP exchange factor. Once the receptor has been activated and the GDP has been exchanged for GTP on the α subunit, the α subunit goes on to activate a set of downstream targets, while the βγ subunit dissociates from the heterotrimer and activates its own downstream targets (Clapman, 1996).

The overall goal of this study is to evaluate potential changes in the density (B_{max}), affinity (K_d) of the 5H1A receptor. Thus, a radio- labeled binding assay was performed in order to measure the density and affinity of receptors. In addition, the GTPγS incorporation assay was conducted in order to measure potency, which reflects the effective concentration of agonist required to stimulate the incorporation reaction to 50% maximum (EC_{50}), and efficacy (E_{max}) or effectiveness of the 5HT1A receptor transduction activity in the hippocampi of the prenatally DEX treated rats..

The binding assay operates on the assumption that when the reaction is terminated by filtration, the system has reached steady state equilibrium with the receptor (R), ligand (L) and receptor ligand complex (LR) (Scheme 1). The steady state can be represented as:

Scheme 1: Steady state equation for binding assay

$$L + R \Leftrightarrow LR$$

The measure of affinity is determined by the receptor K_d. The K_d is the ratio of the association constant (K_1) and the dissociation constant (K_2) (Scheme 2) (Zettner, 1973).

Scheme 2: Kinetics of K_d

$$L + R \overset{K1}{\rightarrow} LR$$

$$LR \underset{K2}{\rightarrow} L + R$$

$$\frac{K2}{K1} = K_d$$

The K_d is also equal to the concentration of ligand required to occupy 50% of the total receptors available for binding (B_{max})

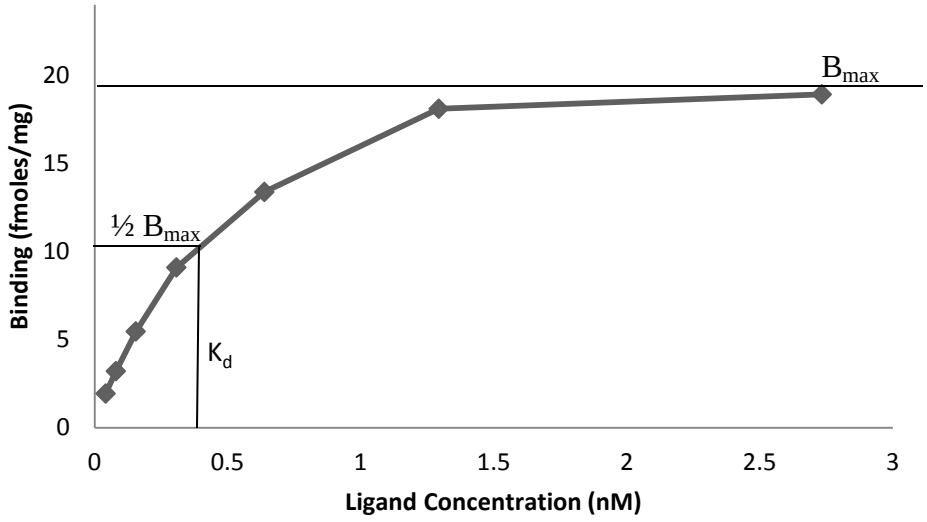

Figure 2. Representative binding curve showing B_{max} and K_d.

An important assumption that is made in the calculations for K_d and B_{max} is that when the steady state of a system is reached, the concentration of ligand is unchanged. In order for this assumption to be true, the concentration of ligand must be in large excess, relative to the receptor concentration, during the course of the reaction. When this is true, we are allowed to make the assumption that (Zettner, 1973):

$$[L]_{total} = [L]_{free} - [LR]$$

This allows us to say that the concentration of free ligand $[L]_{free}$ is equal to the concentration of ligand that was added originally, if and only if the concentration of receptor is negligible in relation to the receptor concentration. When conducting binding studies, the ligand that is used is very critical. Three major classes of ligands exist. The first class includes agonists. The function of agonists is to bind the receptors in the active conformation and elicit the intended response. The second class is antagonists. A pure antagonist does not only bind the receptors in the active conformation but also binds the receptors in the inactive conformation. By binding both conformations of the receptor, antagonists give a better representation of the total receptor count.

The third class of ligand is the inverse agonist. This class of receptors also binds both the active and inactive conformations of the receptor, but also causes the receptor activity to be driven in reverse. In this experiment, the antagonist 2'-methoxyphenyl-(*N*-2'-pyridinyl)-*p*-fluoro-benzamidoethyipiperazine (MPPF) was used as it is highly selective for the receptor of interest, the 5HT1A receptor (Foster, et al., 1995). A tritium tag was added to the MPPF so its presence could be detected through the use of liquid scintillation.

One common problem that arises in the use of binding assays is non-specific binding. Non-specific binding occurs when the ligand, in this case [H^3 MPPF], binds to another receptor in its family or to other constituents of the tissue. To block against non-specific binding, a non-labeled ligand with a higher affinity for the 5HT1A binding site and at a concentration that is large enough to block all specific binding sites within the sample is incubated with the labeled ligand. This then forces the labeled ligand to bind to non-specific sites. This can then be accounted for in the analysis so only specific binding sites are counted (Zettner, 1973). In this experiment, WAY100635 was used as the non-specific antagonist (Foster, et al., 1995).

To analyze the EC_{50} and E_{max} of the 5HT1A receptors in the hippocampus, a GTPγS^{35} assay was used. The GTPγS^{35} incorporation assay allows measurement of absolute levels of GTPγS^{35} incorporation into the alpha-subunit following activation of a single receptor, in this case, the 5HT1A. This assay unlike the binding assay relies on the irreversible incorporation of the non-hydrolysable GTP analog GTPγS (Scheme 3, 4) (Odagaki & Fuxe, 1995a).

Scheme 3: Reactions representing the interactions between G-proteins and GTP

$$(Gprotein - GDP_{bound}) + GTP_{free} \xrightarrow{Binding} (Gprotein - GTP_{bound}) + GDP_{free}$$

$$\xrightarrow{Hydrolyzation} (Gprotein - GDP_{bound}) + Pi_{free} \xrightarrow{Reset} (Gprotein - GDP_{bound}) + GTP_{free}$$

Scheme 4: Reaction representing the interaction between G-proteins and GTPγS

$$Gprotein + \text{GTP}\gamma\text{S}^{35}_{free} \xrightarrow{binding} Gprotein - \text{GTP}\gamma S^{35}_{bound}$$

In the reaction with $\text{GTP}\gamma\text{S}^{35}$, the molecule is non-hydrolysable due to the gamma phosphate having a sulfur atom in place of oxygen. The sulfur atom is both larger and less electronegative. This renders it a poor electron acceptor during the hydrolysis process (Harrison & Traynor, 2003).

Since the binding of the $\text{GTP}\gamma\text{S}^{35}$ is irreversible, it makes sense that the assay is based on absolute binding, not equilibrium. As a result, this assay is highly time sensitive. The incorporation of the $\text{GTP}\gamma\text{S}^{35}$ is linear therefore the total time the reaction proceeds is not critical, it is only critical that all experiments utilize the same amount of time (Alper & Nelson, 1998).

In order to stimulate the 5HT1A receptor to bind the $\text{GTP}\gamma\text{S}^{35}$ the 5HT1A selective receptor agonist ±7-(Dipropylamino)-5, 6, 7, 8-tetrahydronaphthalen-1-ol (8-OH-DPAT) was used (Alper & Nelson, 1998). Since 8-OH-DPAT is a receptor agonist, it will only bind only receptors that are in the active conformation.

Like the binding assay, the $\text{GTP}\gamma\text{S}^{35}$ binding assay is also prone to non-specific binding. To account for this, an excess of unlabeled GTPγS is added to the reaction and binds all of the G-protein binding sites. This forces the radioactively labeled $\text{GTP}\gamma\text{S}^{35}$ to bind only non-specific sites. This can then be subtracted out the same way it is in the binding assay (Alper & Nelson, 1998).

The E_{max} is the maximum response that can be elicited from a receptor population and it is a measure of the drug (agonist) efficacy, while the EC_{50} is the concentration that is halfway

between the maximum and minimum activation during the specified time course of the experiment (Figure 2) (Sjogren, Csoregh, & Sven ningsson, 2008).

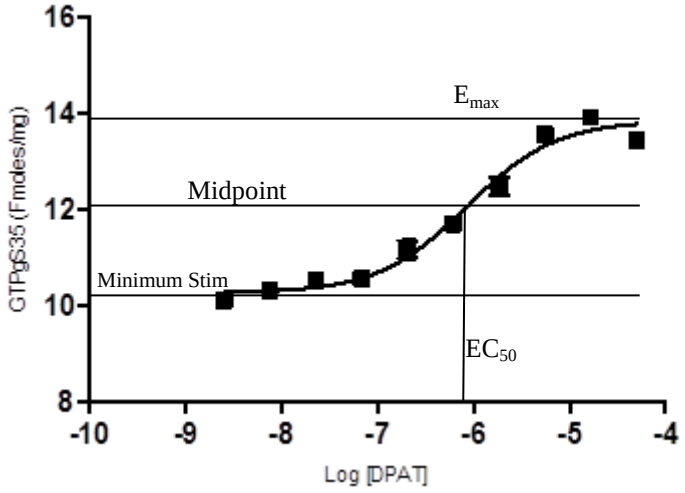

Figure 3. Representative GTPγS^{35} incorporation curve showing E_{max} and EC_{50}.

Specific Aims of Study

Using these techniques, the goal of this investigation was to analyze the effects of a prenatal exposure to Dexamethasone on the 5HT1A receptor number and activity. In addition, the effect of acute stress on receptor function was also examined. As Dexamethasone is used clinically to stimulate lung development, it is important that the long term effects of this drug are analyzed (Kattner, Metze, & Waisse, 1992) (NIH Consensus, 2000). Previous studies have shown that a prenatal exposure to DEX increases hippocampal drive and HPA axis activity which affects normal corticosterone rhythms. It is very likely that the disturbance is causing problems in neurophysiology as the HPA axis and receptor development are closely related (Shoener, Baig, & Page, 2005). Although the circulating glucocorticoids levels are closely controlled by regulatory proteins in the maternal fetal-placental barrier, DEX seems to be unaffected by these protective regulatory proteins due to low binding affinity (Weisner, Do, & Feldman, 1979). This results in a glucocorticoid excess in the form of DEX in the fetal environment, which alters the hippocampal MR/GR balance, and disturbs the set point for negative feedback regulation of CORT after birth. The excess DEX also attenuates maternal corticosterone which regulates the developing serotonergic system and influences serotonin and 5HT1A receptor relationships that are established in utero (Dallman, et al., 1992) (Ratka, Sutanto, Bloemers, & de Kloet, 1989) (Zhong & Ciaranello, 1995).

Acute stressors applied postnatally have been shown to cause improvement in memory consolidation (Oitzl & de Kloet, 1992). Unlike chronic stress which is associated with major gene transcription, acute stress in the postnatal environment is more likely to engage immediate receptor responses associated with 2nd messenger signaling.

Our aim was to determine whether prenatal Dexamethasone treatment elicits alterations in 5HT1A receptor density, extracellular binding affinity, potency of signal transduction, and intracellular incorporation of $GTP\gamma S^{35}$ into the alpha subunit of an activated G-protein. In addition we investigated these properties of the 5HT1A receptor following acute stress.

Materials and Methods

Materials

Tris(hydroxymethyl)aminomethane (Sigma-Aldrich, St Louis, MO), Guanosine triphosphateγS^{35} (Perkin Elmer, San Jose, CA), Guanosine diphosphate (Perkin Elmer, San Jose, CA) Guanosine diphosphate (Sigma Aldrich, St. Louis MO), Way100635 (Sigma Aldrich, St Louis MO), 2'-methoxyphenyl-(*N*-2'-pyridinyl)-*p*-fluoro-benzamidoethyipiperazine ($MPPF^{3H}$) (Perkin Elmer, San Jose, CA), EcoLume Scintillation Fluid (Beckman Coulter, Brea, CA), polyethylenimine (Sigma-Aldrich, St. Louis, MO), Dexamethasone (Dex) (Sigma-Aldrich, St Louis, MO), Guanosine triphosphate gamma sulfate (Sigma-Aldrich, St. Louis, MO), 7-(Dipropylamino)-5,6,7,8-tetrahydronaphthalen-1-ol (8-OH-DPAT) (Sigma-Aldrich, St Louis MO)

Animal Treatment

Time mated Sprague-Dawley Dams (n=17, DEX=9, Control=8) were obtained (Hilltop Laboratories) on gestation day 7. After transport, the rats were given one week of acclimation before DEX treatment was started. During the third week of gestation, dams were randomly assigned to receive daily sub cutaneous injections of DEX (150 $\mu g \cdot kg^{-1} \cdot day^{-1}$; n= 8) or vehicle (saline + 0.4% ethanol, n=9) during days 14-19 of gestation. Day 0 is defined as the morning of appearance of the vaginal plug and rat gestation lasts 21-22 days. All animals were maintained under 12 hour light (0600 to 1800) and constant temperature of 23°C. Water and food were provided *ad libitum*. Male offspring were weaned on postnatal day 21 and housed 2 per group according to litter and treatment. Non-stressed animals were euthanized following brief exposure to CO_2 in a pre-charged chamber and rapid decapitation by guillotine one week after behavior testing. Stressed animals were euthanized one and a half hours after a second exposure to the elevated plus maze to induce a postnatal stressor. This was done one week after initial behavior

testing was complete. Following decapitation trunk blood was collected and both hippocampi were extracted and frozen at -80°C and trunk blood was taken and stored at -80°C.

Tissue Preparation

Hippocampus samples were removed from -80°C freezer, weighed, and placed in 5mL of 50mM Tris pH 7.4 at 4°C. Samples were homogenized using a chilled polytron probe for 10 seconds in polypropylene oak ridge tubes. Residual sample from probe was recovered with a 3 second recovery in fresh 5mL of 50 mM Tris pH 7.4 at 4°C. Samples were mixed and spun for 20 minutes at 18,000 rpm in a Beckman J2000 centrifuge using the JA-20 rotor. Homogenization and centrifugation were then repeated with fresh 5mL of 50 mM Tris pH 7.4 at 4°C. Final homogenization was performed with room temperature (RT) 20 mM Tris pH 7.4.

Binding Assay

After final homogenization 20 mM Tris pH 7.4 at RT was added in sufficient volume to bring tissue concentration to 4 mg/ml. A 2 fold serial dilution for 2.64 nM $MPPF^{3H}$ was conducted such that the most dilute concentration of $MPPF^{3H}$ was 0.0413 nM. Each assay contained, 500 µL of tissue, 250 µL of 20 mM Tris pH 7.4 at RT, and 250 µL $MPPF^{3H}$. Total activity tubes received 250 µL of appropriate $MPPF^{3H}$ dilution. Non-specific binding was achieved using 2 µM Way 100635. For non-specific tubes, 250 µL of Way 100635 replaced the 250 µL of 20 mM Tris pH 7.4 at RT. Way 100635 was diluted to 2 µM using 20 mM Tris pH 7.4 at RT. Reaction was incubated for 1 hour in a 25 °C water bath. Reaction was stopped by rapid filtration using a Brandel Cell Harvester (Brandel, Gaithersburg, MD). A Whatman FP-100 glass filter membrane was used (Brandel, Gaithersburg, MD). Membrane was soaked in a polyethylenimine solution for 1 hour prior to harvesting. Filters were washed 3 times with 20 mM Tris pH 7.4 at 4°C. Filters were then dried at room temperature for one hour and retained

radioactivity was counted using a Perkin Elmer Tri-Carb (Perkin Elmer, San Jose, CA). Scintillation vials were filled with 3 mL of scintillation fluid. Data was analyzed Graphpad software using a specific + non-specific non-linear global fit. Group sizes were control non-stressed (n=9), control stressed (n=18), DEX non-stressed (n=9), and DEX stressed (n=18).

GTPγS Incorporation Assay

Tissue frozen at -80°C from binding assay with concentration 4 mg/mL was further diluted to 1 mg/mL using 50 mM Tris pH 7.4 at RT after being thawed on ice. For non-specific binding 500µL of tissue was combined with 250µL of 43µM GTPγS and 250µL of 100pM $GTP\gamma S^{35}$. Remainder of assay tubes contained 500µL of tissue, 250µL of 8-OH-DPAT (concentrations ranging from 1.25E-5M to 6.35E-10M), and 250µL 100pm $GTP\gamma S^{35}$. Basal activity was measured by substituting 50mM Tris pH 7.4 at RT for the drug. Assay was carried out in triplicates and was incubated for exactly 45 minutes in a 27°C water bath. Reaction was stopped by rapid filtration using a Brandel Cell Harvester (Brandel, Gaithersburg, MD). A Whartman FP-100 glass filter membrane was used (Brandel, Gaithersburg, MD). Filters were washed 3 times with 20 mM Tris pH 7.4 at 4°C. Filters were then dried at RT for one hour and retained radioactivity was counted using a Perkin Elmer Tri-Carb (Perkin Elmer, San Jose, CA). Scintillation vials were filled with 3 mL of scintillation fluid. Data was analyzed Graphpad software using a 3-parameter curve fit. Group sizes for incorporation data were control non-stressed (n=9), control stressed (n=9), DEX non-stressed (n=8) and DEX stressed (n=9)

Statistical Analysis

Analysis of variance (ANOVA) for non-repeated measures was used to analyze all variables (K_d, B_{max}, EC_{50}, and E_{max}). Post hoc comparisons were conducted using the Bonferroni analysis. Effects were considered statistically significant if $p<0.05$.

Results

Binding Assay

The 5HT1A receptor is an important receptor associated with learning and memory (Dourish, Ahlenius, & Hutson, 1987). The receptor induces the activation of a heterotrimeric G-protein that inhibits downstream signaling molecules (Pucadyil, Kalipatnapu, & Chattopadhyay, 2004). To study the receptor, a 5HT1A binding assay and GTPγS^{35} incorporation assay were performed to analyze the receptors affinity (K_d), total receptor count (B_{max}), potency (EC_{50}), and efficacy (E_{max}).

Changes in K_d in response to Prenatal DEX and Acute Stress

The K_d data from the binding experiment with the 5HT1A selective receptor antagonist [^{3}H] MPPF showed that the affinity at which the 5HT1A receptor bound its ligand decreased in response to prenatal DEX under resting conditions. This resulted in a significantly higher K_d value in the DEX non-stressed animals (DEX non-stressed: 0.71±0.02 nM, Control non-stressed: 0.58± 0.04 nM, $p<0.05$; figure 5).

In addition to the significant changes in K_d associated with prenatal DEX exposure, postnatal acute stress also elicited significant changes in the receptor K_d. In response to stress the K_d for the DEX treated animals significantly decreased in comparison to their non-stressed cohorts (DEX non-stressed: 0.71±0.02 nM, DEX stressed: 0.561±0.019 nM, $p<0.05$, figure 5). This change was not observed in control groups, as the K_d for the stressed group of control animals decreased, but not by a significant amount (figure 5).

Overall a prenatal exposure to DEX caused a significant change in K_d as DEX_{tot} (DEX stressed+ DEX non-stressed) had a significantly higher K_d than $Control_{tot}$ (Control stressed+ control non stressed) (DEX_{tot}: 0.61±0.02 nM, $Control_{tot}$: 0.53±0.02 nM, $p<0.05$, figure 6). In

contrast, the acute stressor caused a significant decrease in K_d for stressed$_{tot}$ (Control stressed+ DEX stressed) in comparison to the non-stressed$_{tot}$ animals (Control non-stressed+ DEX non-stressed) (stressed$_{tot}$: 0.532±0.012 nM; non-stressed$_{tot}$: 0.64± 0.03 nM, $p<0.05$, figure 6).

These results show that a prenatal treatment with DEX causes attenuation in receptor affinity under non-stressed conditions, but when the receptor is in a stressed environment the receptor K_d decreases for both groups. The order of receptor affinities from least to greatest is as follows: DEX Non-stressed< DEX Stressed = Control Non-Stressed < Control stressed. If we compare these results to the binding results listed in the following section, the trend indicates that the population with the most receptors has the highest K_d while the population with the fewest receptors has the lowest K_d.

Effects of Stress and Prenatal DEX on B_{max}

As shown in figure 7 a prenatal exposure to DEX or an acute exposure to stress did not cause a significant change in B_{max} when comparing the two groups. However, when the entire prenatal DEX population DEX$_{tot}$, is compared to the entire control population, Control$_{tot}$, there is a significant increase in the B_{max} of the prenatally DEX treated animals (DEX$_{tot}$: 25.2±0.6 fmoles/mg, Control$_{tot}$: 22.5±0.6 fmoles/mg, $p<0.05$, figure 8). Additionally, the stressed$_{tot}$ animals had a significantly lower B_{max} in comparison to the non-stressed$_{tot}$ group (stressed$_{tot}$: 23.2±0.6 fmoles/mg, non-stressed$_{tot}$: 25.2± 0.7 fmoles/mg, $p<0.05$, figure 8). These results tell us that a prenatal exposure to DEX is associated with a significant increase in the density of 5HT1A receptors, while an acute stressor induces a significant decrease in 5HT1A density (figure 7). The ranking of receptor densities from least dense to most dense seems to be Control stressed< Control Non-stressed< DEX stressed< DEX non-stressed.

Incorporation Assay

Given the results of the binding assay which showed that a prenatal exposure affected both the density and affinity of the 5HT1A receptor in the hippocampus, the next step was to examine potential changes in the downstream signaling. To measure these values a $GTP\gamma S^{35}$ incorporation assay was used. This assay allows measurement of the receptors potency (EC_{50}) and the efficacy (E_{max}) (figure 9).

EC_{50} changes in response to stress and prenatal DEX

The results of the $GTP\gamma S^{35}$ incorporation assay showed that there were no statistically significant changes in the E50 for any of the groups regardless of treatment (figure 10 and 11).

E_{max} changes in response to stress and prenatal DEX

Although there was no change in the EC_{50} for the 5HT1A receptor in any of the conditions, the E_{max} indicates that the changes in receptor K_d and B_{max} in response toacute stress influence G-protein signaling linked to the 5HT1A receptor. In response to stress both the control and the DEX treated animals exhibited a significant increase in their respective E_{max} (Control Non-stressed: 7.0±0.4 fmoles/mg, Control Stressed: 9.8±0.5 fmoles/mg, $p<0.05$, figure 12) (DEX Non-Stressed: 7.7± 1.1 fmoles/mg, DEX Stressed: 11.2±0.3 fmoles/mg, $p<0.05$, figure 12). Further analysis of the stressed data shows that the $stressed_{tot}$ group had a significantly higher E_{max} in comparison to the $Non\text{-}stressed_{tot}$ group ($Stressed_{tot}$: 10.5±0.3 fmoles/mg, $Non\text{-}stressed_{tot}$: 7.3±0.6 fmoles/mg, $p<0.05$, figure 13).

In contrast, a prenatal exposure to Dexamethasone was not associated with significant changes in E_{max} when comparing non-stressed or stressed animals. This data shows that stress has a greater impact on the 5HT1A receptor efficacy compared to the effects of a prenatal exposure to DEX.

Discussion

In our study we were able to show that a prenatal exposure to DEX as well as a postnatal exposure to acute stress in adulthood results in a significant alteration in the 5HT1A receptor K_d. More specifically it was found that prenatal DEX exposure was associated with a significant increase in the K_d of the 5HT1A receptor, which indicates a reduction in receptor affinity for its ligands. In contrast, the opposite trend in receptor affinity was observed in both DEX and control animals in response to stress. The effects of an acute stressor applied 1.5 hours prior to sacrifice reduced the 5HT1A receptor K_d in both DEX and control hippocampi which suggests that receptor affinity was increased (figure 5, 6).

It could be said that the effect of stress on K_d was not unexpected since it is known that learning is enhanced when the task being learned is coupled to a stressful event. Ultimately, stressors such as injected glucocorticoids or environmental stress are linked to increases in plasma CORT (Sandi, Loscertales, & Guaza, 1997), but the physiological link between high CORT levels and increases in 5HT1A receptor affinity remains unclear.

One plausible explanation that is gaining popularity is the idea that there are changes in lipid raft composition in association with membrane cholesterol in regions where 5HT1A receptors are located. It has been shown that increases or decreases in membrane cholesterol concentrations can have a direct impact on the affinity of the 5HT1A receptors. For example, increases in cholesterol are associated with an increase in receptor affinity, while sequestering cholesterol has the opposite effect (Pucadyil & Chattopadyay, 2004). It is also known that cholesterol in the membrane is not randomly distributed, but rather localized into raft regions that contain special lipids such as sphingolipids and phosphoglycerolipids. These are different than the typical glycolipids found on the other parts of the plasma membrane because these have

completely saturated acyl chains. This allows for greater hydrogen bonding and aggregation of these special lipids. In these domains cholesterol is found with sphingolipids and phosphoglycerolipids in a 1:1:1 ratio (Edidin, 2003). Although the link between acute stress and cholesterol distribution in the membrane has not been studied extensively, data has shown that increases in corticosterone are coupled to increases in plasma cholesterol (Barker, 1995). Whether plasma cholesterol is incorporated into the membrane is doubtful as it has also been shown that all cholesterol in the brain is produced locally (Jurevics & Morell, 1995). However the relationship between corticosterone production and potential elevations in brain cholesterol levels could provide insight into the increased receptor affinity associated with acute stress found in our study. The idea of cholesterol contributing to 5HT1A receptor function has been reported in the literature and is further supported by the fact that 5HT1A receptors are GCPRs that require cholesterol for proper function (Pucadyil & Chattopadyay, 2004) (Pierce, Permont, & Lefkowitz, 2002).

In contrast to the stress induced increase in 5HT1A affinity, prenatal DEX-exposure was associated with a significant attenuation in receptor affinity (figure 5, 6). However, the proposed cholesterol explanation could also be used to support this latter finding as follows: Prenatal exposure to DEX has been shown to reduce maternal circulating CORT, and yet, it increases plasma glucocorticoids since the administered DEX is a glucocorticoid. The high levels of circulating DEX induce negative feedback by interacting with GR receptors, the primary receptor involved in CORT feedback, which lowers the maternal and fetal corticosterone levels following DEX administration during the third trimester (Nyirenda, Welberg, & Sekel, 2001) (Rupprecht, Reul, & Van Steensel, 1993). It is highly possible that if membrane cholesterol is driven by circulating CORT, and these levels are reduced in DEX-treated animals, then the

lowering of maternal and fetal CORT levels could be coupled to cholesterol reduction. Particularly neuronal cholesterol since its level is set late in gestation (Turley, Bruns, & Dietschy, 1998). This putative disturbance in the set-point of neuronal cholesterol could be associated with the increased K_d and decreased affinity observed for 5HT1A receptors in the DEX-exposed non-stressed animals (Figure 5). As mentioned, neuronal cholesterol levels are determined late in gestation, during what is clinically known as the third trimester. Since reduced circulating prenatal and maternal cerebral corticosterone have been observed during this time, it is possible that this change is involved in the altered cholesterol set point in the developing DEX-treated fetus (Nyirenda, Welberg, & Sekel, 2001) (Takahashi L. K., 1998) (Turley, Bruns, & Dietschy, 1998).

In addition to the changes in receptor affinity we also observed significant changes in 5HT1A receptor density (B_{max}) when comparing the total populations of prenatally DEX treated animals (DEX_{tot}) to the total control population ($Control_{tot}$) and between the total stressed group ($stressed_{tot}$) and the total non-stressed group ($non\text{-}stressed_{tot}$) (figure 8). The significant up regulation in B_{max} detected in DEX-treated animals is supported from previous studies that measured the concentration of 5HT1A receptor post gestation in the prefrontal cortex. However, in that particular study there was no significant decrease in 5HT levels or turnover coupled to the increase in the 5HT1A receptor density (Slotkin, Kreider, Tate, & Seidler, 2006). This suggests that there is a change in the postsynaptic transmission. It is possible that the up-regulation of 5HT1A receptor is a compensatory response due to the lack of downstream signaling (Shahak & Slotkin, 2003) (Slotkin, Kreider, Tate, & Seidler, 2006). Our model fits this hypothesis since we found a decrease in receptor affinity in prenatally DEX-treated animals which is coupled to a significant increase in 5HT1A receptor density (figure 6, 8). Thus the decrease in ligand

effectiveness and reduction in downstream activation would trigger up regulation of the receptor number in order to compensate for the loss in efficacy.

A second possible explanation for the increase in 5HT1A receptor expression can be constructed from the changes in HPA axis programming which occurs during the time of DEX administration. DEX-exposure during the third trimester elicits a number of regulatory issues for the developing neuroendocrine system (Shoener, Baig, & Page, 2005). (1) DEX administration causes a significant decrease in the circulating levels of maternal and thus fetal CORT, (2) DEX preferentially binds to GR receptors that under basal CORT conditions would not be activated due to MR's higher affinity for DEX (Nyirenda, Welberg, & Sekel, 2001) (Rupprecht, Reul, & Van Steensel, 1993). This preferential binding of GR receptors by DEX alters the negative-feedback regulation of 5HT1A expression that is typically mediated by MR under basal conditions (Meijer & De Kloet, 1994) (Kuroda, Watanabe, Alkbeck, Hastings, & McEwen, 1994). Therefore, if the MR negative feedback regulation is less effective during the time of neuroendocrine set point determination, the fetal brain could be set to have a less effective regulation of 5HT1A receptors during adulthood. As regulatory influence is decreased, an elevation in 5HT1A receptor number may result. This is what was observed in our experiment (figure 8).

Not only did a prenatal exposure to DEX cause a significant increase in the density of the 5HT1A receptor, but an acute stressor administered 1.5 hours prior to sacrifice significantly decreased the 5HT1A receptor density. Previous studies have shown that CORT exerts a direct negative regulation of 5HT1A receptors by acting through its MR receptors. Activated MR then acts to inhibit 5HT1A mRNA and induces degradation or internalization of existing 5HT1A receptors (Meijer & De Kloet, 1994) (Kuroda, Watanabe, Alkbeck, Hastings, & McEwen, 1994).

Since CORT levels are significantly increased in response to stress it is likely that its effect on MR activation would serve to reduce receptor density and lower B_{max} as was observed in our experiment (Barker, 1995) (figure 8). In addition, studies have shown that hippocampal 5HT1A receptors can begin to change in response to adrenalectamy in as little as one hour (Zhong & Ciaranello, 1995). From this it is reasonable to assume that within 1.5 hours, enough time has passed for changes to have occurred which lead to a decrease in 5HT1A receptors in response to elevated CORT .

In our study, the increase in receptor affinity that is associated with acute stress seems to be coupled to an increase in E_{max} as the stressed groups of Control and DEX all showed a higher E_{max} figure 12, 13). Studies have shown that acute stress is associated with an increase in memory consolidation and that this process may result from an increased 5HT1A activation in response to stress since the 5HT1A receptor is associated with new memory formation (Douglas, 1975) (Ohno & Watanabe, 1996) (Oitzl & de Kloet, 1992). In our study, 5HT1A receptor potency, EC_{50}, did not change but a significant decrease in the total number of 5HT1A receptor was detected in response to stress. This would possibly lead one to think that the changes in receptor activation were due to the increased affinity during stress. However, when the E_{max} is measured, the effect of affinity is made negligible by increasing the concentrations to a saturation point for the receptor system. One possible explanation then for the change in receptor activation in response to stress could again be the increased membrane cholesterol that is being hypothesized. Since receptors are present in both the active and inactive conformation, it is possible that due to increased membrane cholesterol and therefore increased concentrations of lipid rafts, a greater number of receptors are present in the active conformation. By having a

population shift toward more receptors in the active conformation, the cell is then capable of activating more G-proteins in response to ligand binding.

The cholesterol hypothesis further supports the stress mediated increase in E_{max} because lipid raft formation is crucial for the proper activation of the GPCR class of receptors which includes the 5HT1A receptor (Pierce, Permont, & Lefkowitz, 2002). Therefore as a result of an increase in circulating corticosterone, the corresponding increase in membrane cholesterol allows for higher affinity of the 5HT1A receptor for its ligand, which is possibly associated with a population shift toward more receptors in the active conformation, which leads to increased levels of downstream signaling in response to a postnatal stressor.

Conclusions and Future Directions

From the results of this study we are able to conclude that a prenatal exposure to Dexamethasone causes a significant decrease in 5HT1A receptor affinity under resting conditions. The change in receptor affinity then seemed to elicit an increase in the receptor B_{max} as a means of compensation to restore "normal" amounts of downstream signaling as was indicated by the E_{max} which showed that prenatally DEX treated animals showed no significant change in downstream signaling in response to the alterations to K_d and B_{max}.

However, under stressed conditions the DEX and control groups indicated that elevated levels of CORT can be correlated to an improved $K_{d,}$ a lowered B_{max}, and also an increased E_{max}. If the changes in receptor affinity are linked to membrane cholesterol, which has been shown to increase in the plasma in response to circulating CORT, it is possible that the increase in E_{max} during an acute stress response could be due to the increases in CORT (Barker, 1995). The decreases in B_{max}, though they do not seem to alter the effect of stress on E_{max} could also be related to CORT increases during stress, as CORT has been shown to cause a decrease in 5HT1A receptor expression (Zhong & Ciaranello, 1995).

From the results of this experiment, the next area of investigation would logically be to test the effects of stress on membrane cholesterol and lipid raft composition as this may be the missing link between our results and what is currently in the literature.

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Figures

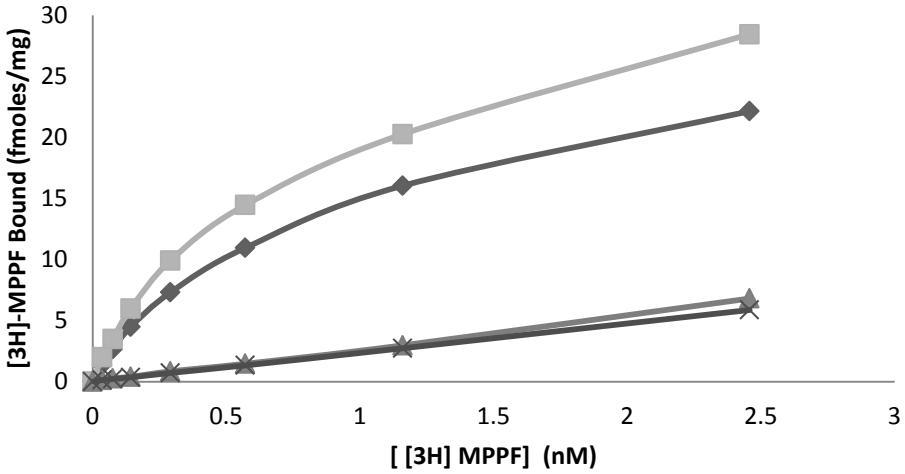

Figure 4. Representative Binding assay for control stressed (n=18), control non-stressed (n=9), DEX stressed (n=18), and DEX non-stressed (n=9) adult male rats. (■) indicates curve for DEX treated animals (non-stressed or stressed), (◆) represents control (non-stressed or stressed) animals, (▲) represents control non-specific, and (x) represents DEX non-specific binding. Specific binding was performed using dilutions of [^{3}H] MPPF, a 5HT1A receptor antagonist (ligand dilutions used: 0.0413, 0.0825, 0.165, 0.33, 0.66, 1.32, and 2.64). Non-specific binding was carried out using Way100635 at a concentration of 2 µM. Left hippocampus was used for all samples.

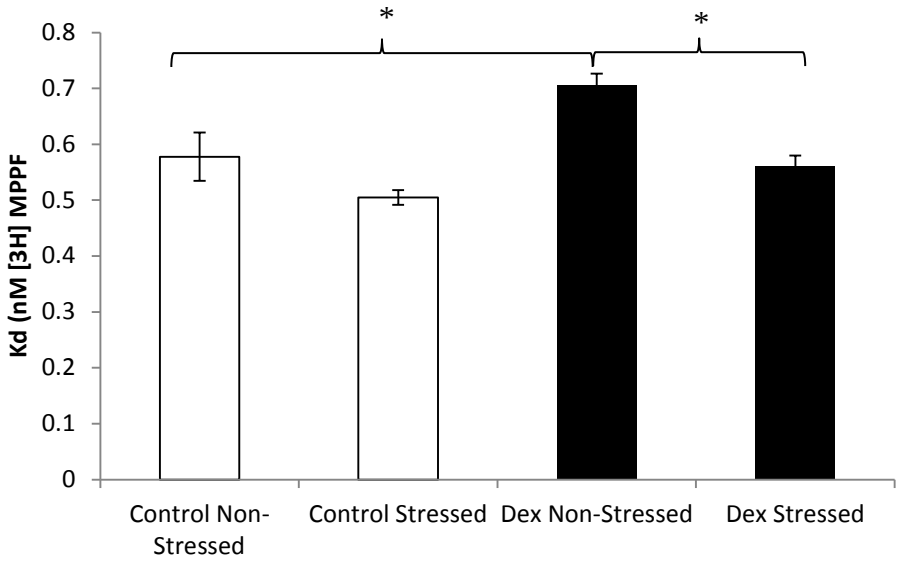

Figure 5. The K_d for 5HT1A receptors using [^{3}H] MPPF, a 5HT1A receptor antagonist, in the left hippocampus of adult control stressed (n=18), control non-stressed (n=9), DEX stressed (n=18), and DEX non-stressed (n=9) adult male rats. DEX non-stressed rats showed a significant increase in K_d (decrease in affinity) in comparison to Control non-stressed and DEX stressed groups (DEX non-stressed: 0.71±0.02 nM, Control non-stressed: 0.58± 0.04 nM, DEX stressed: 0.561±0.019 nM , $p<0.05$). Specific binding was performed using dilutions of [^{3}H] MPPF, a 5HT1A receptor antagonist (ligand dilutions used were: 0.0413, 0.0825, 0.165, 0.33, 0.66, 1.32, and 2.64 nM nM). Non-specific binding was carried out using Way 100635 at a concentration of 2μM. Left hippocampus was used.

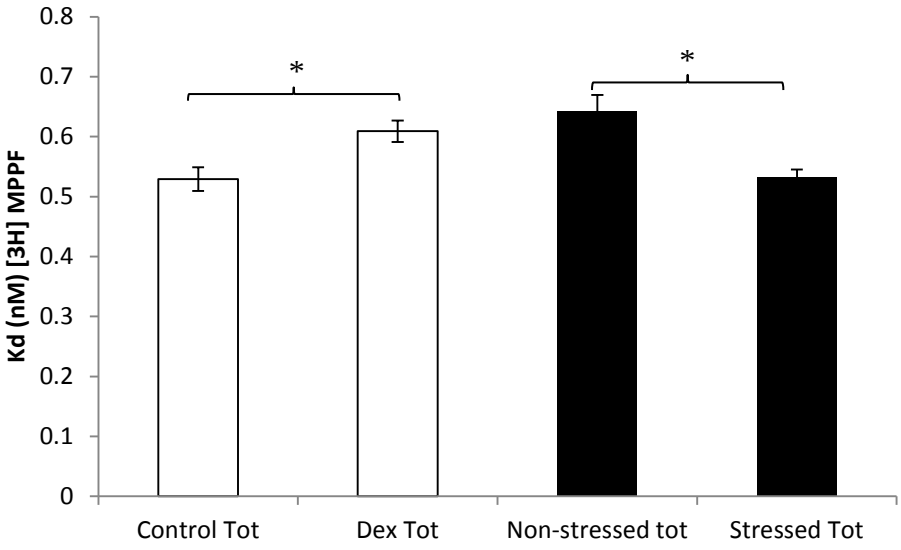

Figure 6. The K_d values for 5HT1A receptor binding using antagonist [H^3] MPPF in the left hippocampus of adult male rats. Results were grouped by treatment, $Control_{tot}$ (n=27), DEX_{tot} (n=27), non-$stressed_{tot}$ (n=18), and $stressed_{tot}$ (n=18). There was a significant increase in K_d in prenatally DEX treated animals in comparison to control animals (DEX_{tot}: 0.61±0.02 nM, $Control_{tot}$: 0.53±0.02 nM, $p<0.05$). In addition, $stressed_{tot}$ animals had a significantly lower K_d compared to the non-stressed $_{tot}$ animals (Non-$stressed_{tot}$: 0.64±0.03 nM, $stressed_{tot}$: 0.523±0.012, $p<0.05$). Binding was performed using dilutions of [^{3}H] MPPF and Way100635 (specific and nonspecific, respectively), as shown in Figure 5.

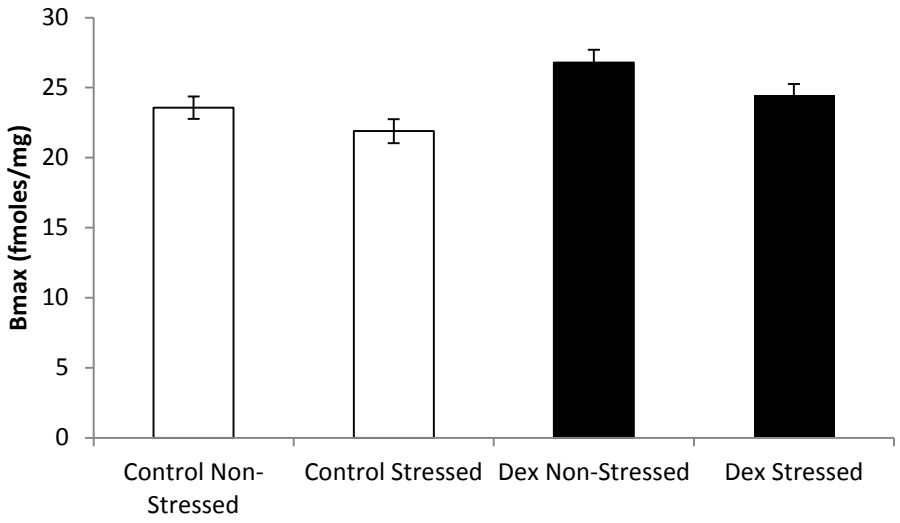

Figure 7. Maximum binding (B_{max}) of 5HT1A receptors in the left hippocampus in control non-stressed (n=9), control stressed (n=18), DEX non-stressed (n=9), and DEX stressed (n=18) adult male rats using a specific 5HT1A receptor antagonist [^{3}H] MPPF. No significant changes were detected in response to prenatal DEX or an acute stressor. Binding was performed using dilutions of [^{3}H] MPPF and Way100635 (specific and nonspecific, respectively), as shown in Figure 5.

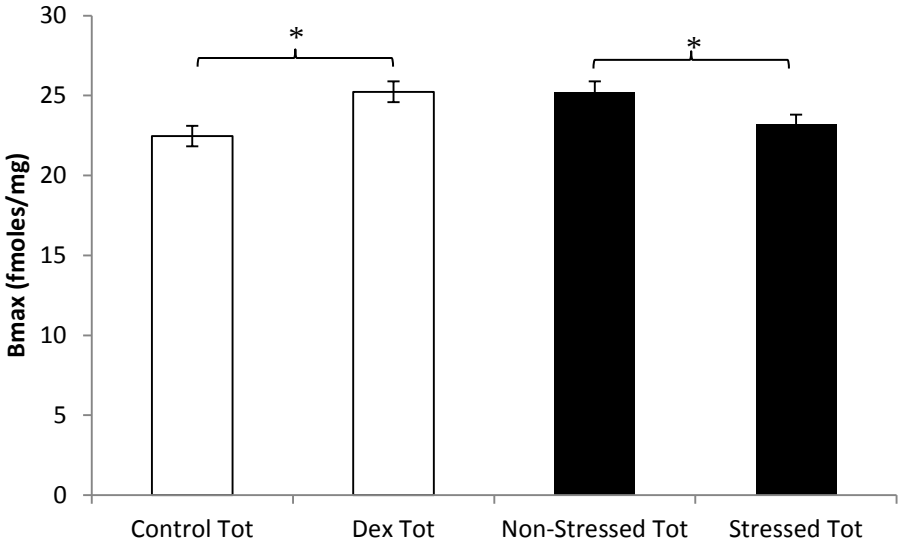

Figure 8. Binding Maximum (B_{max}) for left hippocampus from drug treated ($Control_{tot}$ (n=27), DEX_{tot} (n=27)) and stress challenged (non-$stressed_{tot}$ (n=18), and $stressed_{tot}$ (n=18)) animals. To measure 5HT1A binding, a selective receptor antagonist [H^3]MPPF was used. The DEX_{tot} group had a significantly higher B_{max} in comparison to the $Control_{tot}$ group (DEX_{tot}: 25.2±0.6 fmoles/mg, $Control_{tot}$: 22.5±0.6 fmoles/mg, $p<0.05$). A significant result was also found in the comparison between non-$stressed_{tot}$ and $stressed_{tot}$ groups ($stressed_{tot}$: 23.2±0.6 fmoles/mg, non-$stressed_{tot}$: 25.2± 0.7 fmoles/mg, $p<0.05$). Binding was performed using dilutions of [^{3}H] MPPF and Way100635 (specific and nonspecific, respectively), as shown in Figure 5.

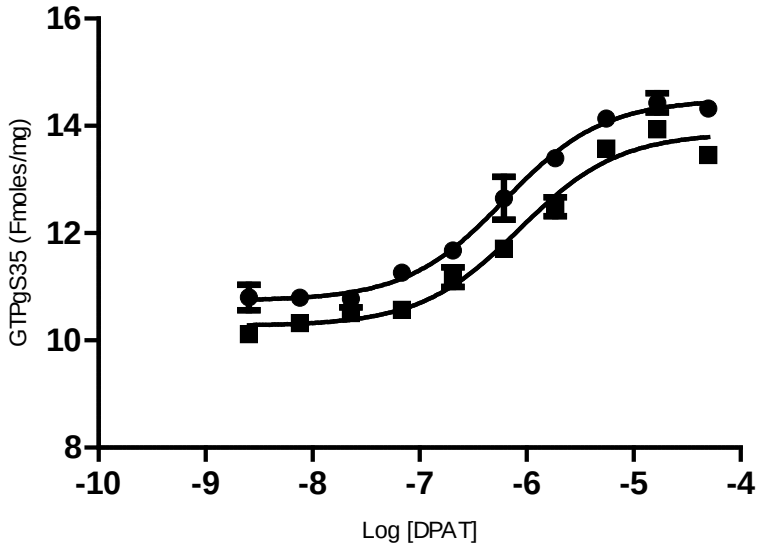

Figure 9. Representative GTPγS^{35} incorporation assay for DEX treated animals compared to control animals. (●) indicates curve for DEX (stressed) treated animals and (■) represents control (stressed) animals. 5HT1A receptors were activated using 8-OH-DPAT, a 5HT1A specific agonist. Agonist concentrations used were: 1.25E-5, 4.175E-6, 1.39E-6, 4.625E-7, 1.543E-7, 5.15E-8, 1.715E-8, 5.725E-9, 1.905E-9, and 6.35E-9 M. The Log [DPAT] concentration was plotted on the x-axis. Non-specific binding was measured using 43 μM unlabeled GTPγS. Left hippocampus was used. Activity of receptor was recorded using 100pM GTPγS^{35}.

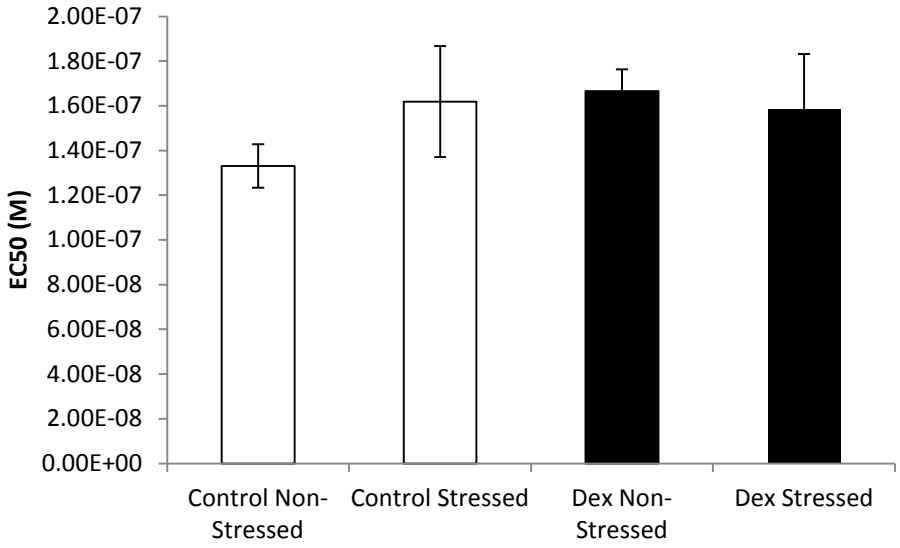

Figure 10. EC_{50} values for control non-stressed (n=9), control stressed (n=9), DEX non-stressed (n=8), and DEX stressed (n=9) adult male rats. Left hippocampus was tested using $GTP\gamma S^{35}$ as a marker to measure activation of the 5HT1A receptors by 8-OH-DPAT, a 5HT1A agonist. There were no significant changes in any of the treatment groups. Binding was performed as described in figure 9.

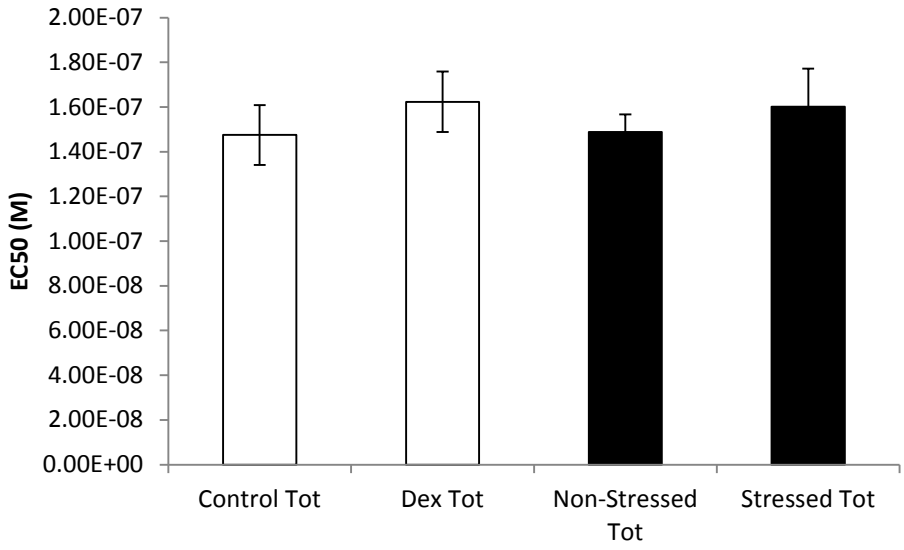

Figure 11. EC_{50} values for $control_{tot}$ (n=18), DEX_{tot} (n=17), $non\text{-}stressed_{tot}$ (n=17), and $stressed_{tot}$ (n=18) adult male rats. Left hippocampus was tested using $GTP\gamma S^{35}$ as a marker to measure activation of the 5HT1A receptors by 8-OH-DPAT, a 5HT1A agonist. There were no significant changes in any of the treatment groups. Binding was performed as described in figure 9.

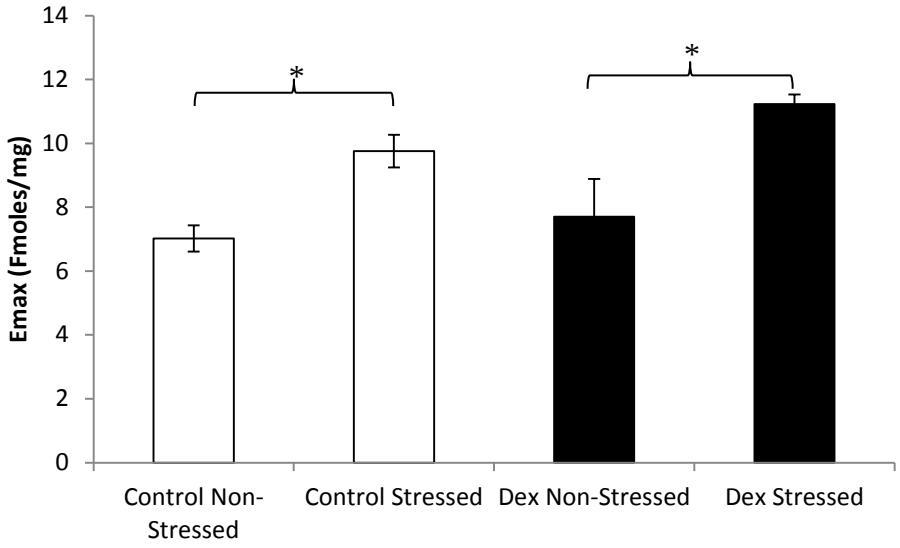

Figure 12. E_{max} values for control stressed (n=9), control non-stressed (n=9), DEX stressed (9), and DEX non-stressed (n=8) adult male rats. Left hippocampus was tested using GTPγS^{35} as a marker to measure activation of the 5HT1A receptors by 8-OH-DPAT, a 5HT1A agonist. A significant increase in the E_{max} for the Control stressed and DEX stressed animals in comparison to their respective non-stressed group (Control non-stressed: 7.0±0.4 fmoles/mg, Control stressed: 9.8±0.5 fmoles/mg, DEX non-stressed: 7.7± 1.2 fmoles/mg, DEX stressed: 11.2±0.3 fmoles/mg, $p<0.05$). Binding was performed as described in figure 9.

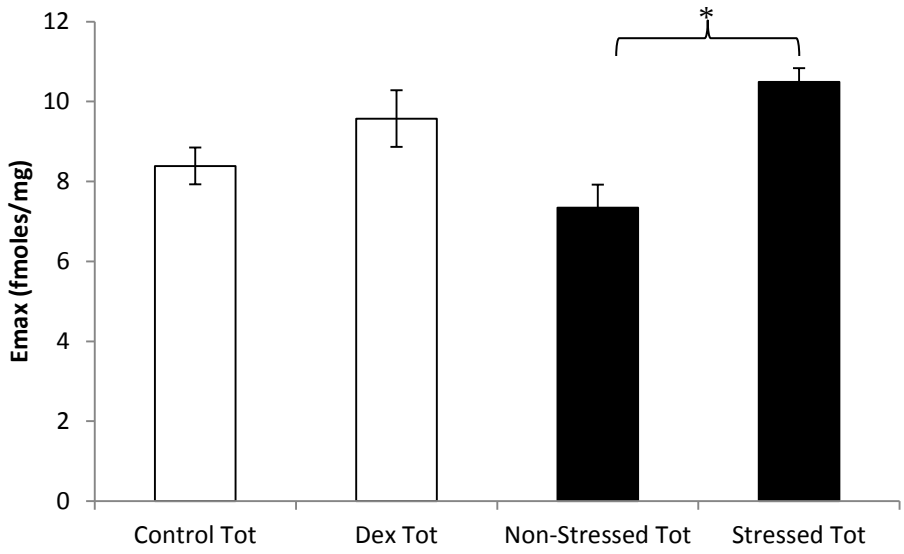

Figure 13. E_{max} values for $control_{tot}$ (n=18), DEX_{tot} (n=17), $non\text{-}stressed_{tot}$ (n=17), and $stressed_{tot}$ (n=18) adult male rats. Left hippocampus was tested using $GTP\gamma S^{35}$ as a marker to measure activation of the 5HT1A receptors by 8-OH-DPAT, a 5HT1A agonist. The E_{max} for the stressed group was significantly higher compared to the non-stressed group ($stressed_{tot}$: 10.5±0.3 fmoles/mg; $non\text{-}stressed_{tot}$: 7.3±0.6 fmoles/mg, $p<0.05$). No significant change was observed in prenatally DEX treated animals compared to the control group. Binding was performed as described in figure 9.

Table 1. Summary of binding and incorporation results for control non-stressed, control stressed, DEX non-stressed, and DEX stressed adult male rats (* indicates significance between control vs. DEX within same stress paradigm, $p<0.05$. ¥ indicates significance between non-stressed vs stressed within same drug treatment group, $p<0.05$.

	Control Non-Stressed	DEX Non-Stressed	Control Stressed	DEX Stressed
Kd (nM)	0.58 ± 0.04	0.71 ± 0.02*	0.5 ± 0.01¥	0.56 ± 0.02*¥
Bmax (fmol/mg)	23.6 ± 0.8	26.8 ± 0.9	21.9 ± 0.9	24.5 ± 0.8
EC50 (μM)	0.133 ± 0.010	0.167 ± 0.010	0.162 ± 0.02	0.158 ± 0.02
Emax (fmol/mg)	7.0 ± 0.4	7.7 ± 1.3	9.8 ± 0.5¥	11.2 ± 0.3

Table 2. Summary of binding and incorporation results for $Control_{tot}$, DEX_{tot}, non-$stressed_{tot}$, and $stressed_{tot}$ (* indicates significance between $Control_{tot}$ and DEX_{tot}, $p<0.05$, ¥ indicates significance between $stressed_{tot}$ and non-$stressed_{tot}$, $p<0.05$).

	$Control_{tot}$	**DEX_{tot}**	**Non-$Stressed_{tot}$**	**$Stressed_{tot}$**
Kd (nM)	0.53 ± 0.02	0.61 ± 0.02*	0.64 ± 0.03	0.53 ± 0.01¥
Bmax (fmoles/mg)	22.5 ± 0.6	25.2 ± 0.6*	25.2 ± 0.7	23.2 ± 0.6¥
EC50 (µM)	0.147 ± 0.013	0.162 ± 0.014	0.149 ± 0.008	0.160 ± 0.017
Emax (fmoles/mg)	8.4 ± 0.5	9.6 ± 0.7	7.4 ± 0.6	10.5 ± 0.3¥