The neurodevelopmental effects of synthetic glucocorticoid at different time point on stress and metabolism gene expression in the developing hypothalamus

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Abstract

The clinical use of synthetic glucocorticoids (sGC) to improve acute respiratory status in newborns with bronchopulmonary dysplasia, have been shown to have the undesired effects of increasing the risk of developing metabolic and neuropsychiatric disease in adulthood. Current data indicate that critical periods of sensitivity exist in fetal development during which exposures, such as sGC use, are more likely to result in long-term disease. In this study, we hypothesize that exposure to the sGC dexamethasone (DEX) at different time points during early development will result in unique expression profiles of hypothalamic genes in the adult rats. Sprague Dawley rat pups were treated with 0.2 mg/kg DEX beginning on postnatal day (PND) 4-6. Brain tissue from offspring was harvested at PND 7, 21, 90 and quantitative real-time PCR (RT-qPCR) was performed to measure the mRNA level of hypothalamic genes involved in metabolic and behavioral regulation. Results were also compared to a previous study in which pregnant Sprague Dawley dams were treated prenatally with DEX (gestational day 18-21). Of the genes we measured, thyrotropin releasing hormone (*Trh*) expression was decreased in the adult animals when DEX was administered either prenatally or postnatally. Subsequent examination of brain sections by immunohistochemistry (IHC) showed decreases in fiber and neuron counts that were only seen in the offspring treated with DEX prenatally. Further evidence suggesting a critical window of exposure include observations that mRNA coding for somatostatin and oxytocin, and plasma levels of the protein IGF-1 decreased only in the animals treated with DEX postnatally. Collectively, these data demonstrate that permanent effects of sGCs on hypothalamic gene expression are dependent upon the timing of the exposure

Introduction/Significance

Adverse fetal environments are hypothesized to increase the risk for metabolic and neurological disease in adulthood. Support for this hypothesis includes evidence that intrauterine growth restriction (IUGR) arising from maternal malnutrition predisposes the offspring to metabolic and neurological disorders in adulthood (1-3). IUGR can result from elevations in glucocorticoids during prenatal life from multiple causes such as prenatal stress, prenatal inflammation, malnutrition, etc. In addition, glucocorticoids are commonly used in the clinic to treat women at risk for preterm delivery or fetuses at risk for masculinization following CAH. The mechanism for programming of physiology by prenatal GCs is not known. But a possible explanation is the influx of GC may be causing down regulation of Glucocorticoid receptors (GR) in the developing brain hypothalamus leading to changes in critical events of neurodevelopment (8,32).

A common variable that influences the type and severity of disease in adulthood is the timing of the exposure to the fetus (3-4). Current hypotheses suggest that periods of rapid cell division represent the time at which a developing tissue is most vulnerable to an adverse fetal environment (29). For the developing brain, studies indicating that the third trimester in humans represents a period of great risk, since synaptogenesis, gliogenesis, myelination, and angiogenesis are occurring (5). However, exact periods of vulnerability during the third trimester which predispose the offspring to metabolic or neurological disorders have yet to be determined. Thus, the importance of recognizing these critical periods can be helpful in sculpting future guidelines for the use of synthetic GCs during pregnancy and also help predict which children are more prone to metabolic or psychiatric disease in the future by locating the time of insult to the fetus.

Background/Impact

Synthetic Glucocorticoid Use

Since the 1970s, steroids have been used to improve acute respiratory status in newborns with bronchopulmonary dysplasia (8). It has been estimated that more than 500,000 babies are born preterm in the US each year. However, this practice has decreased since these newborns are at

increased risk of cerebral palsy, short term neurodevelopment delay, impaired growth, decreased gray matter volumes, hyperglycemia, hypertension, cardiac hypertrophy, and GI hemorrhages due to the administration of sGC (8). At preschool age, children exposed to early sGC have also been shown to exhibit anxiety, depression, or withdrawn behavior (9). Furthermore, since many of these individuals are now reaching adulthood, it is unknown if they continue to be at increased risk of anxiety, depression, and metabolic disease in adulthood. Despite the potential risks arising from postnatal sGC administration, these compounds are still used to accelerate lung development in preterm infants and for the treatment of congenital adrenal hyperplasia. However, current recommendations set forth by the American Academy of Pediatrics (AAP) only suggest the use of systemic glucocorticoid therapy in infants over two to three weeks of age with severe bronchopulmonary dysplasia who require sustained substantial maximal ventilator and oxygen support (10).

Models of GC Exposure

One proposed mechanism of early exposure to excess sGC leading to neurologic disorders has been the upregulation and downregulation of Glucocorticoid receptors (GR). It has been shown that GR is elevated in the liver but down-regulated in the hippocampus of adult dex-treated offspring (33)

To study the developmental effects of sGC use in preterm infants, many different types of animal models have been used. Our laboratory has traditionally focused on the effects of DEX administration during GD 18-21 in rats (pre-DEX model), thus mimicking late second or very early third trimester in humans. However, it is estimated that the brain growth spurt occurring at birth in humans is centered around 1 week postnatal in rats, leading to the notion that the last trimester of human gestation corresponds to postnatal day (PND) 1–10 in rats (6,7). A more recent study on the neurotransmitter GABA (gamma-Aminobutyric Acid) refines this time period, suggesting that PND 2–7 in rat corresponds to the human third trimester, and human birth at 36 week gestation translates to PND 12–13 in rats (12, 19). Therefore, subcutaneous injection of DEX to pups on PND 4-6 is similar to a premature baby being injected with synthetic GC upon premature labor. We employed this post-DEX model in the present studies to test the

hypothesis that exposure during this time frame may result in consequences distinct from those observed using the pre-DEX model.

Critical Windows of Vulnerability

Fully understanding the effects of synthetic GC exposure on the development of preterm infants also includes identifying the periods of greatest vulnerability. These times are the critical periods of neuroplasticity when neuron formations are being established. In humans, neuronal proliferation and migration starts at gestational weeks (GW) 10 and ends around GW 24. Synaptogenesis, gliogenesis, and programmed neuronal cell death starts around GW 20, and myelination starting at 36 GW. It is thus plausible that an environmental exposure during these periods may lead to abnormal neurodevelopment.

Neuronal maturational processes occur at different times in almost every brain region and neural circuit, and the timing of developmental events varies with neural substrate. Therefore, critical periods of development might be specific for each brain region or neurotransmitter systems based on susceptibility to outside influences (7). An adverse environment or stimulus, such as GC, may produce alterations in chromatin, gene expression, and cell cycle, all contributing to the adult phenotype (5-7). An example of this critical window is perinatal asphyxia in preterm vs. full-term infants which results in brain damage to different areas. Specifically, asphyxia-induced brain lesions in preterm infants are usually localized in the periventricular regions. However, in full-term infants, the cortical areas, thalamus, basal ganglia and brainstem particularly vulnerable to asphyxia-induced injury (5). The existence of critical windows of susceptibility have also been documented in animal models such as the Guinea pig, in which prenatal stress at gestation days (GD) 50 -52 resulted in different effects compared to stress at GD 60-62. Specifically, Guinea pigs stressed later in gestation exhibited lower plasma estradiol levels, reduced ovary weight, and increased glucocorticoid receptor mRNA in the paraventricular nucleus (11).

The notion that critical windows of vulnerability exist is widely recognized, but the precise timing of these periods is not well characterized. However, determining the exact periods of vulnerability are important in helping to shape future guidelines of synthetic GC use

in premature infants. Furthermore, knowing these periods will assist future research looking into the long term neurodevelopment effects of adverse fetal environments, such as GC overexposure.

Target Genes Involved in Growth, Metabolism, and Stress

The choice of genes chosen for measurement was based on the major peptide gene expression involved in the Hypothalamic/Pituitary/Target Organ Pathway; specifically genes/proteins that were involved with the Hypothalamus. Some downstream products of the Hypothalamic/Pituitary/Target Organ Pathway were chosen as well. Most notably, Insulin-Like Growth Factor-1 (Igf-1), an insulin like molecule with well described roles in growth was also examined since a previous study (Carbone et al, 2012) showed that it is downregulated by prenatal DEX exposure.

Thyroid releasing hormone (*Trh*), Growth hormone releasing hormone (*Ghrh*), somatostatin (*Ghih*), and somatomedin C (*Igf-1*)) are genes that contribute to the regulation of growth and metabolism in the body. TRH produced by neurons that reside in the parvocellular division of the hypothalamus which are neurons of the PVN that project to the median eminence where their neurosecretory nerve terminals release their hormones to the anterior pituitary gland. TRH regulates the secretion of hormones into the systemic circulationis the key peptide hormone responsible for hypothalamic–pituitary–thyroid (HPT) axis regulation. TRH is therefore indirectly involved in the regulation of thyroid hormones triiodothyronine (T3) and thyroxine (T4) by inducing TSH stimulation from the pituitary. T3 and T4 regulate protein, fat, and carbohydrate metabolism, affecting how human cells use energetic compounds. TRH neurons also receive afferent connections from catecholamine neurons in the brainstem, and these play a significant role in the up regulation in *Trh* gene expression during cold exposure (15, 18)

The peptides GHRH and GHIH play opposing roles in the regulation of anterior pituitary growth hormone (GH) secretion. GHRH neurons are located in the arcuate nucleus of the hypothalamus (ArcN) and their secretory product stimulates GH release from the anterior pituitary. By contrast, GHIH is found in neurons located in the paraventricular nucleus of the

hypothalamus (PVN) and it acts upon the pituitary to inhibit GH release. Ultimately GH acts upon the liver to cause the synthesis and secretion of insulin-like growth factor-1 (IGF-1) which circulates throughout the body and targets specific cells for growth.

Corticotropin-releasing hormone (CRH) is another neuropeptide that is involved in controlling metabolism. It is secreted by parvocellular neurons in the PVN in response to stress. The primary role for CRH is to stimulate release of Adrenocorticotropic hormone (ACTH) from the pituitary gland. ACTH then acts on the adrenal glands to stimulate the release of cortisol, glucocorticoids, mineralocorticoids and DHEA into the general circulation. Other important components of the stress response include oxytocin (OT) and Vasopressin (AVP). Exogenous administration of OT can produce decreases in sympathetic activity and inhibit the secretion of cortisol. Vasopressin acts in a synergistic fashion on the corticotroph cells of the anterior pituitary to increase pro-opiomelanocortin (POMC) to cause an increase of ACTH release from the pituitary (14). Any permanent changes in expression of these key genes involved in the stress axis may help explain why premature babies exposed to sGC are at increased risk of neuroendocrine regulation, metabolism and stress responses later in adulthood.

Aims/Goals/Hypothesis

A comparison of DEX exposure at different developmental time points was performed to test the hypothesis that pathology arising from DEX exposure is dependent upon the window of exposure. Previous research conducted by our laboratory measured the effects of prenatal (GD 18-21) DEX exposure on gene expression in adulthood (23,24). However, given the possibility that such exposure differentially affects the offspring based on the window of exposure, we hypothesized that DEX exposure during a later period of development results in gene expression patterns distinct from our previous observations. To test this hypothesis, we exposed rats to DEX at PND 4-6 and compared the effects of this exposure on gene expression with our previously published results.

Research Methods and Materials

Animals

Timed pregnant female Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA, USA) and delivered to the Arizona State University laboratory animal research facility at 7 days gestation. They were not acclimated. This age was chosen to limit any effect of the stress from transport on development of the hypothalamus. Pregnant dams were monitored daily to record the litter's birth date which was designated as PND 0.

At parturition, each of the 25 litters were randomly culled into groups of 10, each consisting of 5 males and 5 females, and returned to the same dam. Male and female neonates were subcutaneously injected with a daily dose of DEX (0.2mg/kg in 100µl safflower oil) from PND 4 through 6, while the control groups received 100µl safflower oil. Our DEX dose was selected because it falls within range of a typical human exposure (0.1–0.5 mg/kg) while remaining consistent with doses used in Sprague–Dawley rats, which reportedly cause abnormal neurodevelopment of the offspring (0.2–0.8 mg/kg) (23,24). All animal protocols were approved by the Arizona State University Institutional Animal Care and Use Committee, under subcontract from the University of Arizona College of Medicine-Phoenix and were carried out in accordance with published National Institutes of Health guidelines.

Gene Expression - RT-PCR

Brains to be used for gene expression studies were rapidly removed from the skull after decapitation and snap-frozen in 2-methylbutane (-20°C) followed by transfer to dry ice. Rats were not given anesthesia prior to decapitation. All brains were stored at -80°C until cryosectioning and microdissection. Brains were similarly harvested from subsequent cohorts at PND 21 and PND 90. These brains were cryosectioned at 75µm (PND 7) and 150µm (PND 21 and PND90) using a Leica Model CM3050S Cryostat (Buffalo Grove, IL, USA). The paraventricular nucleus (PVN) and the arcuate nucleus (ArcN) were harvested individually using a 1mm diameter tissue punch.

Using the protocol of Chomczynki and Sacchi (25) total RNA was isolated from the rat brain tissue using phenol/chloroform/isoamyl alcohol extraction. Spectrophotometry was then

used to discern RNA concentration and purity. RNA was reverse transcribed to cDNA using an iScript cDNA synthesis kit (BioRad, Hercules, CA) according to manufacturer's instructions. Concentration of cDNA was measure using Oligreen ssDNA Quantitation Reagent and Kit (Molecular Probes, Inc. Eugene, OR, USA). Gene expression was measured by real-time quantitative PCR (RT-qPCR) with a Roche 480 LightCycler and SYBR green chemistry using custom intron-spanning primers designed for rat (Table 1) and qPCR was conducted using a LightCycler 480 thermal cycler with SYBR green detection reagent (Roche Diagnostics, Indianapolis, IA). Absolute quantification of the mRNA was calculated by using standard curves created with purified PCR product. Gene expression is reported as a ratio of target gene (fg) per total cDNA per reaction (pg), thus controlling for variations in the amount of cDNA per PCR reaction.

Immunohistochemistry

Prior to tissue harvest for Immunohistochemistry (IHC), eight rats(2 female control, 2 male control, 2 female treated, and 2 male treated) per time period were anesthetized using cryoanesthesia (PND7) or isoflurane (PND 21 and 90), and intracardially perfused with 4% neutral-buffered paraformaldehyde. Brains were removed and placed in 4% paraformaldehyde overnight at 4°C for postfixing and then were saturated with 30% sucrose in phosphate buffered saline (PBS for cryoprotection prior to freezing the tissue. Formaldehyde-fixed brains were cryosectioned at 35 μm sections using a Leica CM3050s cryostat (Leica, Buffalo Grove, IL, USA).

Immunohistochemical detection of ppTRH was performed using antibody targeting the preproTRH 178-199. This antibody was previously validated for IHC detection of ppTRH (16). Neurons and neuron fibers were visualized with a Zeiss Axioskop light microscope equipped with Neurolucida v.7 Software (MicroBrightField, Williston, VT, USA) and soma/fibers were counted bilaterally throughout the PVN in every fourth section. Data are reported as an estimate of ppTRH neurons/fibers per PVN.

Accession Number	Gene	Description	Primer	Sequence (5'-3')
NM_013046	*Trh*	Thyrotropin Releasing Hormone	Forward	ACCTTCCTGGCCTGGAGAATGTT
			Reverse	TGTCCTCCTCATCTGCCCATGAAT
NM_012659	*Ghih*	Growth Hormone Inhibiting Hormone (Somatostatin)	Forward	GCTCTGCATCGTCCTGGCTTT
			Reverse	ATCGTTCTCTGTCTGGTTGGG
NM_031577	*Ghrh*	Growth Hormone Releasing Hormone	Forward	GGGCCAATTATATGCCCGCAAACT
			Reverse	CACTCTGTCCAAATGGCGGTTGAA
NM_016992	*Avp*	Arginine Vasopressin	Forward	TGCTACTTCCAGAACTGCCCAAGA
			Reverse	AGGAAGCAGCCCAGCTCGT
NM_012996	*Oxt*	Oxytocin	Forward	TGGATCTCGGACTGAACACCAA
			Reverse	TTCTCCTCCTGGCAGCGCA
NM_031019	*Crh*	Corticotropin Releasing Hormone	Forward	AGGTACCTCGCAGAACAACAGT
			Reverse	ACAGAGCCACCAGCAGCAT
NM_022673	*Mecp2*	Methyl CpG Binding Protein 2	Forward	TCTGGTCGCTCTGCTGGAAAGTAT
			Reverse	TCATTAGGGTCCAAGGAGGTGTCT
NM_001003957	*Dnmt3a*	DNA Methyltransferase 3a	Forward	CATCCAAGTGGACCGCTACATC
			Reverse	GATGTAGCGGTCCACTTGGATG
NM_001003959	*Dnmt3b*	DNA Methyltransferase 3b	Forward	TAGTGACCAGTCCTCGGACACGAAG
			Reverse	TTAGACAGCCGTGAGCTTGATCTG

Table 1. Primer sequences for RT-qPCR.

Western blot analysis

16 rats (4 female control, 4 male control, 4 female treated, 4 male treated) were decapitated and 5 ml of trunk blood per rat were collected in the presence of aprotinin and EDTA to prevent proteolysis and clotting, respectively. Trunk blood was centrifuged to collect plasma, which was supplemented with a protease inhibitor cocktail (Sigma-Aldrich Chemical Co., St. Louis, MO) in preparation of western blot analysis for IGF1. Following standard SDS-PAGE of the plasma, protein was transferred to nitrocellulose and probed for IGF-I using monocolonal antibodies (Thermo Scientific, Freemont, CA), followed by visualization with a fluorescent secondary antibody (LiCOR Biosciences, Lincoln, NE). Plasma IGF-I immunoreactivity was quantified using an Odyssey imaging system (LiCOR Biosciences). Immunofluorescence intensity is reported in arbitrary units based on all six samples per treatment group.

Statistical Analysis

Statistical comparisons of gene and protein expression measurements were performed using GraphPad Prism v5.0b (GraphPad Software, Inc., La Jolla, CA). Two-way ANOVA was used to measure effects of sex and fetal DEX exposure on these parameters, and post hoc comparison of the data was performed using the method of Bonferroni. In all cases, differences were deemed significant if $p < 0.05$.

Results

Trh mRNA expression in the PVN

Two-way analysis of variance (2-way ANOVA) of *Trh* mRNA levels on PND7 (Figure 1A) indicated a main effect (a significant difference between Dex animals vs Vehicle treatment.) of DEX exposure [F(1, 26)=5.694; p=0.0246]. P*ost hoc* analysis of these data revealed a significant effect in the female offspring only, although no main effect of sex was observed by two –way ANOVA. Analysis of *Trh* mRNA levels at PND21 (Figure 1B) indicated a main effect of DEX exposure [F(1, 26)=7.297; p=0.0120]. P*ost hoc* analysis of these data revealed that an effect that was only significant in the male offspring, although no main effect of sex was observed by 2 Way ANOVA. Two-way ANOVA of TRH expression at PND90 (Figure 1C) indicated a main effect of DEX exposure [F(1, 28)=9.167; p=0.0052].

Ghih (Somatostatin) mRNA expression in the PVN

Two-way ANOVA of *GHIH* levels on PND 21 (Figure 2A) showed a significant interaction between sex and treatment [F(1,27) = 5.991; P = .0212]. P*ost hoc* analysis comparison revealed that decreased *Ghih* mRNA was more pronounced in males. A sex effect was also observed [F(1,27) = 9.831; P = .0041]. Two-way ANOVA of *Ghih* expression at PND 90 (Figure 2B) showed a main effect of DEX exposure [F(1,24)=21.76; P=.0001]. P*ost hoc* analysis of these data indicated a decrease in expression of *Ghih* in male and female offspring.

Oxytocin mRNA expression in the PVN

Two-way ANOVA of *Oxt* mRNA expression at PND21 (Figure 3A) indicated a main effect of DEX exposure [F(1, 24)=8.981; p=0.0063], and *post hoc* comparison of these data revealed that this effect was more pronounced in the male offspring, although no sex effect was observed. Two-way ANOVA of *Oxt* expression at PND90 (Figure 3B) did not indicate any effect.

IGF-1 Plasma

Relative levels of IGF-1 were determined by western blotting, followed by densitometric analysis. Two-way ANOVA of IGF-1 immunoreactivity revealed a sex effect (Figure 4)

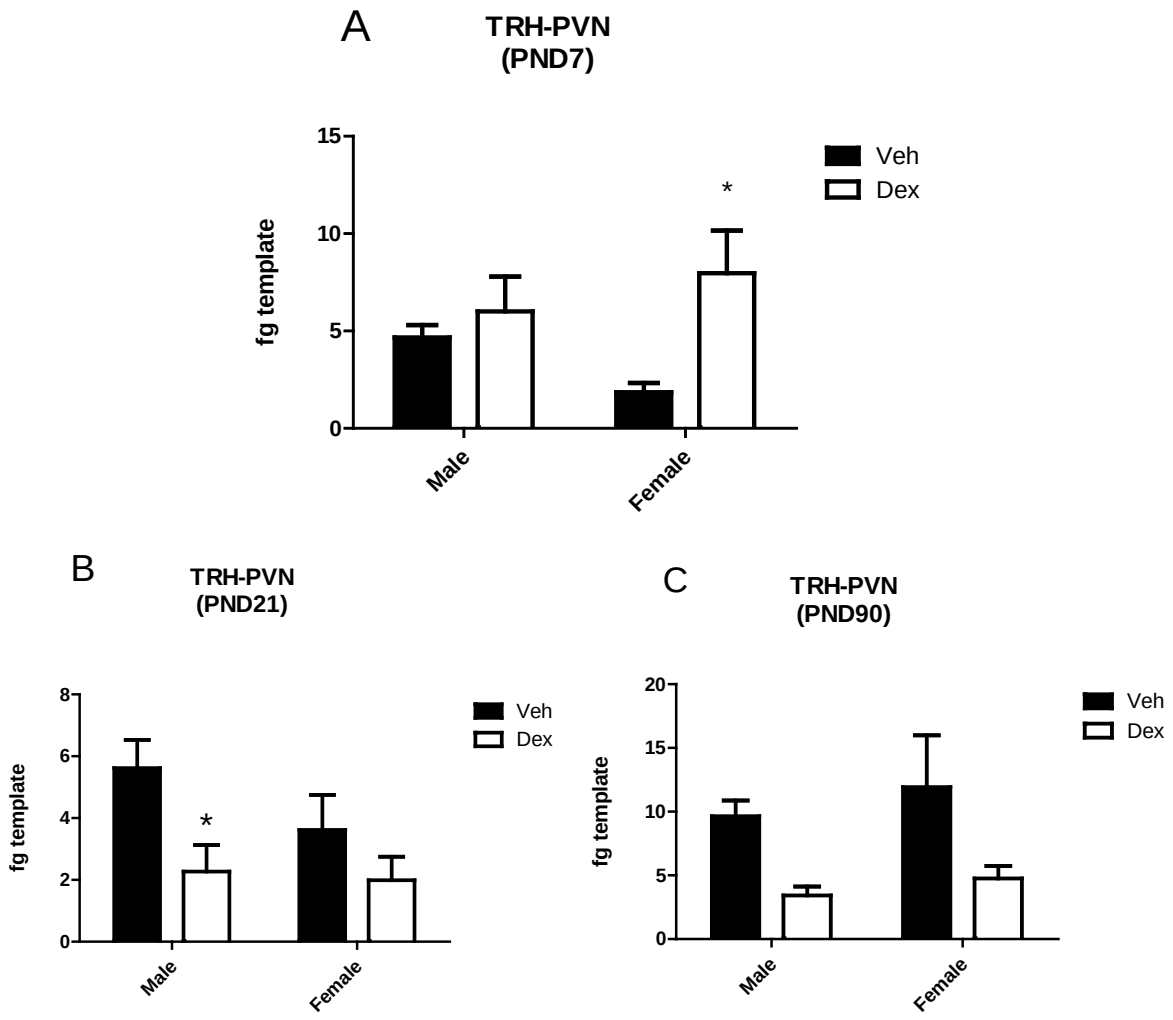

Figure 1 - *Trh* hypothalamic gene expression in response to Postnatal DEX exposure. ppTRH mRNA levels are reported as a ratio of target gene (fg) per pg total cDNA per reaction. Each bar represents the mean ± SEM of 8 animals. Statistical significance ($p<0.05$) between vehicle and DEX-exposed groups is indicated by asterisk. Panel A shows Trh mRNA levels in PND7 animals. Panel B shows TRH mRNA in PND 21 animals. Panel C shows TRH mRNA levels in PND 90 animals.

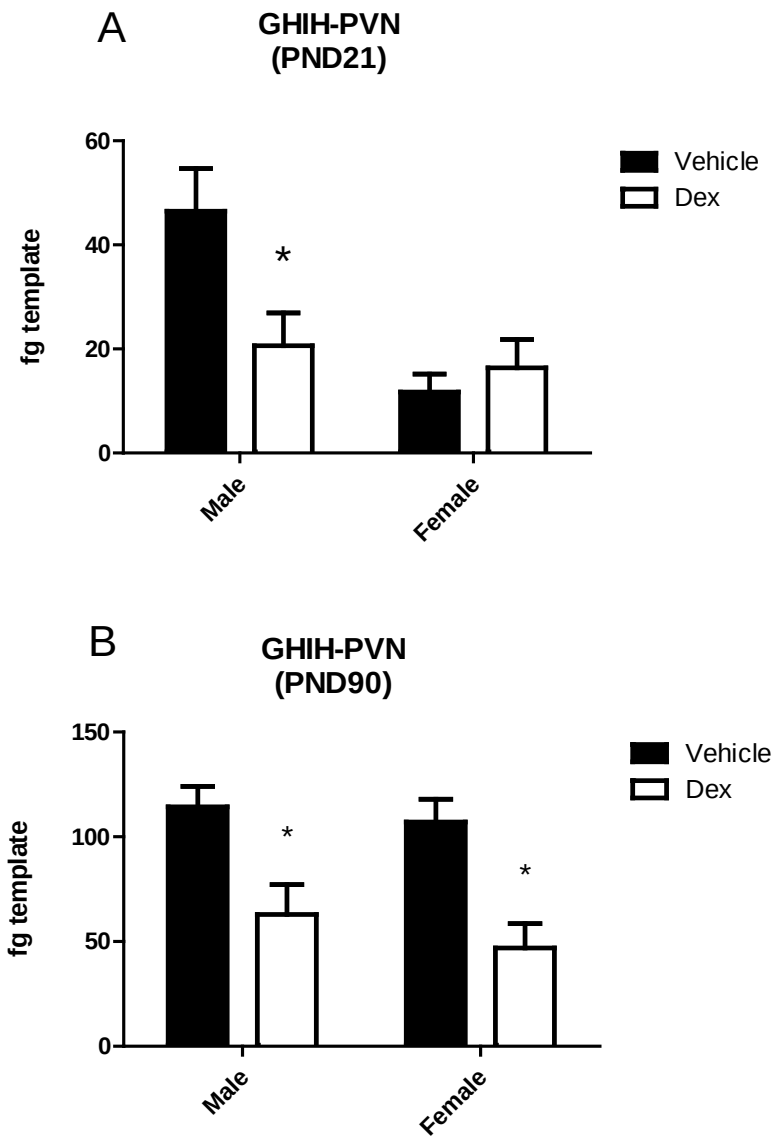

Figure 2 – *Growth Hormone Inhibitiing Hormone (Ghih)* gene expression in response to Postnatal DEX exposure. Gene expressionlevel is reported as a ratio of target gene (fg) to total cDNA per reaction (pg). Each bar represents the mean ± SEM of 8 animals. Statistically significant differences ($p<0.05$) between vehicle and DEX-exposed groups is indicated by asterisk. Panel A shows Ghih mRNA levels in PVN of PND21 animals. Panel B shows GHIH mRNA levels in PVN of PND 90

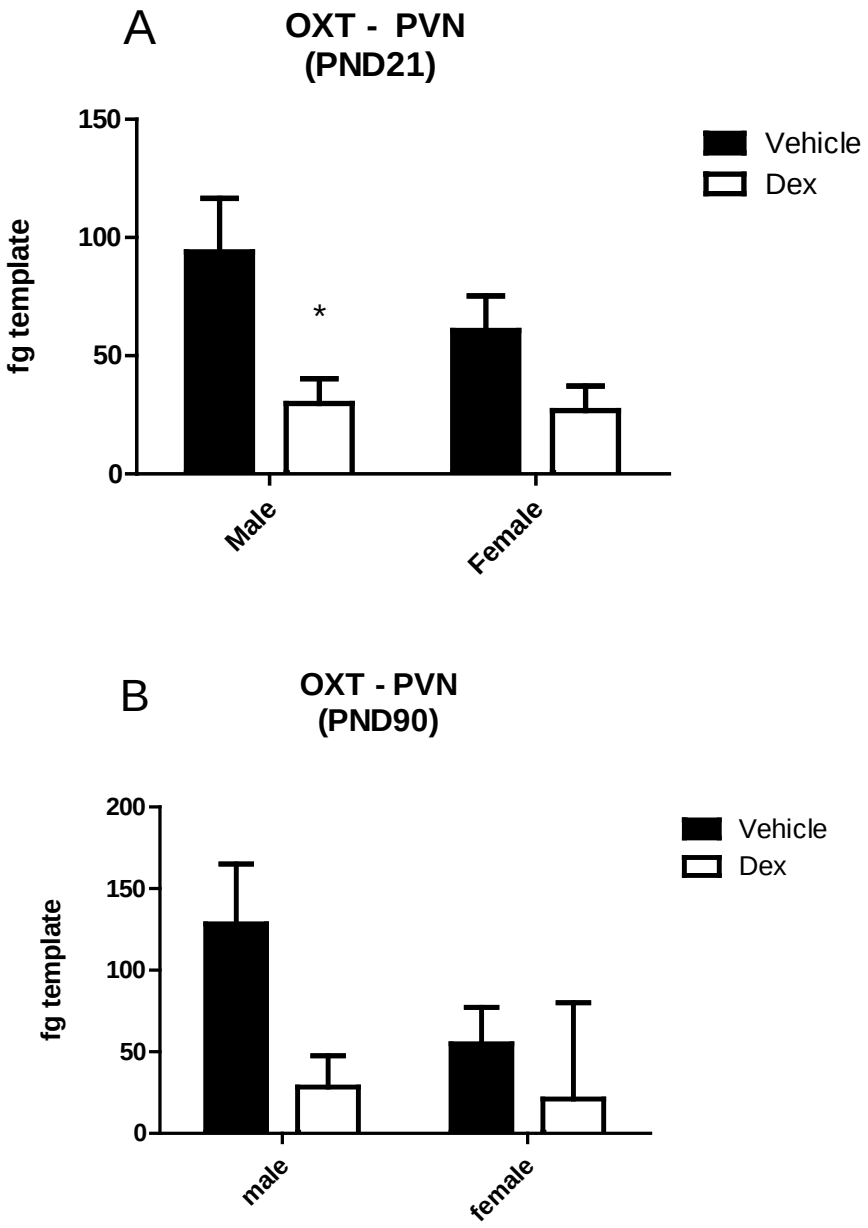

Figure 3 - *Oxytocin* mRNA expression in PND 21 (panel A) and PND 90 (panel B) animals following postnatal DEX exposure. Gene expression is reported as a ratio of target gene (fg) to pg total cDNA per reaction. Data are represented as the mean ± SEM of 8 animals, Statistical significance ($p<0.05$) between vehicle and DEX-exposed groups is indicated by an asterisk.

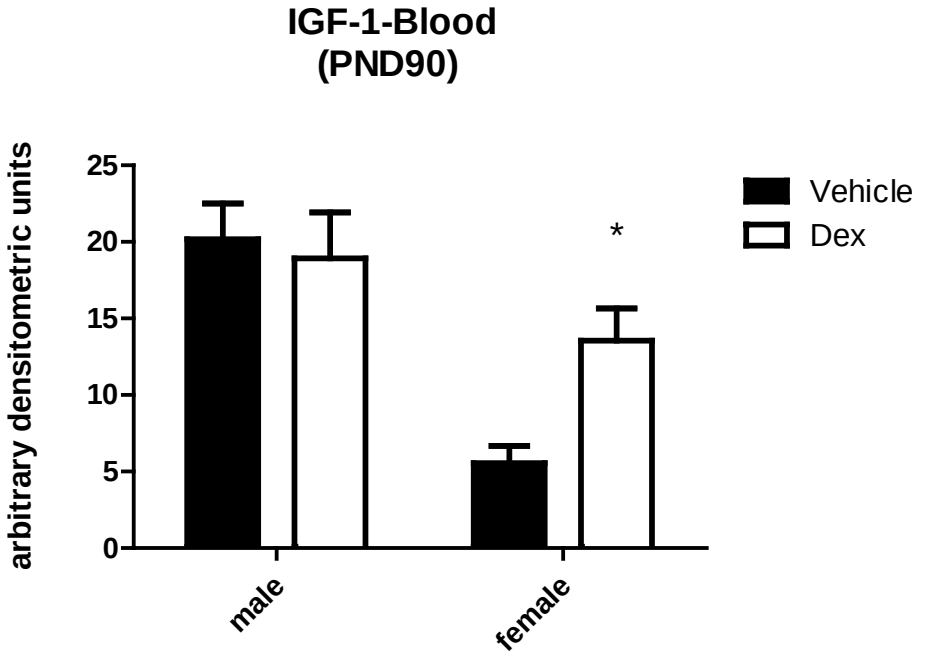

Figure 4 – IGF-1 levels in plasma of PND 90 animals following postnatal exposure to DEX. Relative levels were measured using Western blot analysis and protein expression is reported as arbitrary densitometric units. Each bar represents the mean ± SEM of 4 animals. Statistically significant differences ($p<0.05$) between vehicle and DEX-exposed groups are indicated by asterisk.

[F(1, 11)=18.77; p=0.0012], and *post hoc* analysis indicated a increase on IGF-1 in the females which were exposed to DEX.

Expression of ppTRH neurons and fibers in the PVN

PreproTRH immunoreactivity (ir) was measured bilaterally through the PVN in brains harvested from adult male and female rats that were exposed during fetal development to either DEX or vehicle (Figure 5 A-D). Two-way ANOVA did not reveal any treatment or sex interaction in either neuron or fiber count (Figure 5 E-F).

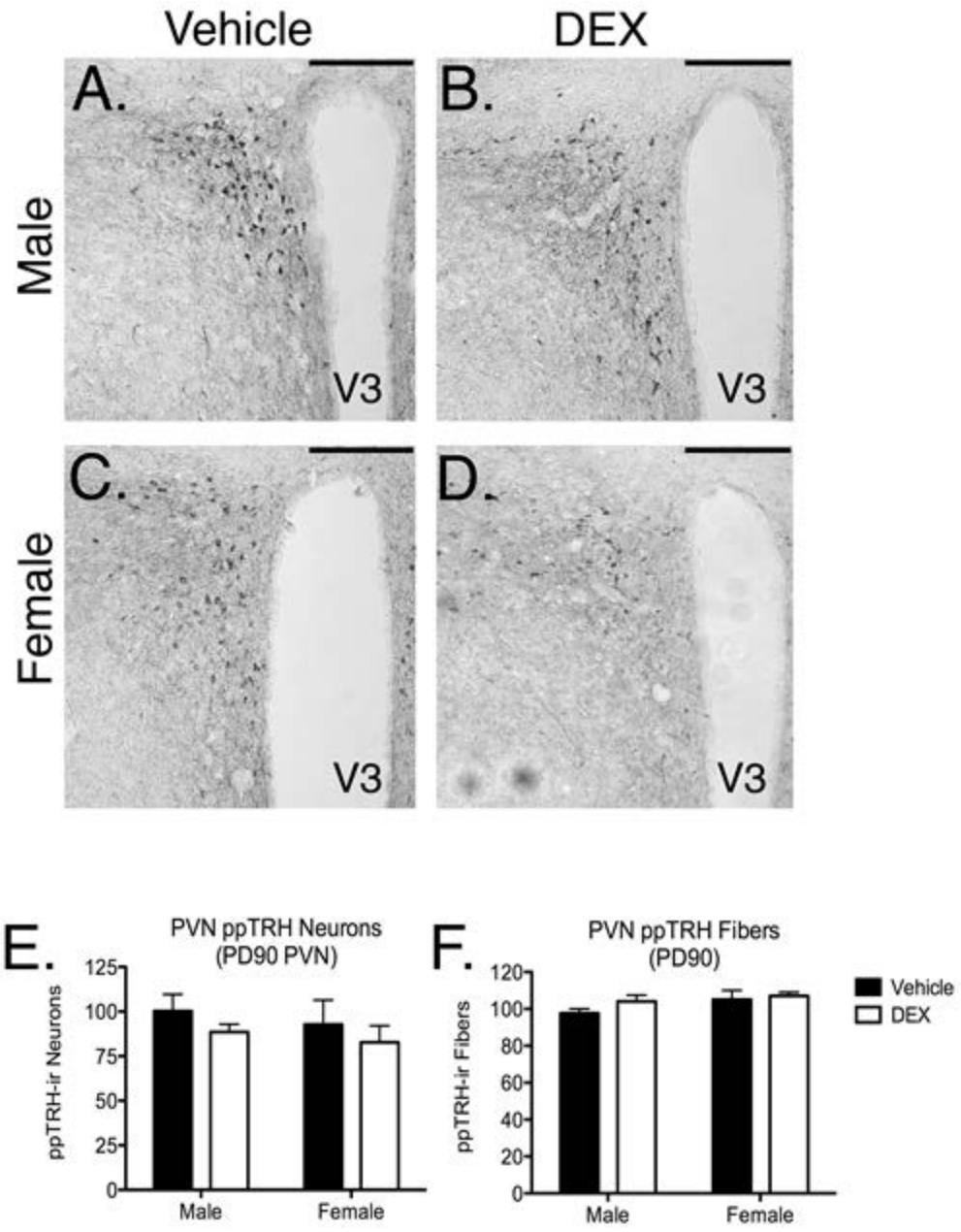

Figure 5. Photomicrographs showing ppTRH-ir neurons and fibers within the PVN of adult male and female rats that were exposed to postnatal DEX. Each bar represents the mean ± SEM of 6 animals per group. Males are shown in (Panels A–B); female (Panels C–D. Bilateral neuron counts (Panel E) or ppTRH-ir fiber counts (Panel F) were taken through the PVN in adult offspring (Panel E).

Discussion

In this experiment, we hypothesized that different critical windows of vulnerability exist in regards to metabolism and stress genes expression in the hypothalamus. We expected to see different results in rats that were exposed to dexamethasone in prenatal GC exposure at late gestation (GD 18-21) compared to postnatal injection (PND 4-6). Our results showed that thyrotropin-releasing hormone expression was decreased in the adult animals when DEX was administered either prenatally or postnatally. Examination of brain sections by immunohistochemistry (IHC) showed decreases in *Trh* fiber and neuron counts that were only seen in the offspring treated with DEX prenatally. In addition, somatostatin and oxytocin, and plasma levels of the protein IGF-1 decreased only in the animals treated with DEX postnatally.

Metabolism Genes

The genes *Trh, Ghih,* and the protein IGF-1, were measured due to their roles in regulating metabolism. In the post-DEX rats, a treatment effect was seen at all three time points (Table 2). Treatment effects were also seen in Pre-DEX treated rats as well. At PND7, Post-DEX treated female rats showed higher levels of *Trh* expression. This differed when compared to Pre-DEX rats which showed increased *Trh* expression in the vehicle rats at PND7.

This brings up the first difference of gene expression when comparing the two different timing paradigms. Why the post DEX had higher levels of *Trh* possibility is the acute nature of the dexamethasone, since the rats were injected with DEX within 24 hours prior to being tissue harvest. The influx of sGC could have acutely affected the TRH expression. This notion is supported by data indicating TRH neurons receive afferent connections from catecholamine neurons from the brainstem. These catecholamines play a significant role in the up regulation in the TRH gene expression during cold exposure (26). Therefore, GC could stimulat catecholamine neurons to upregulate *Trh* expression acutely and that is why higher levels were seen in the Post-DEX treated animals. This scenario is also supported by the discrepancy with *Trh* expression in the Pre-DEX model, since over 24 hours had passed prior to harvest, thus limiting any acute DEX effects.

	TRH		
	Sex	Treatment	Sex x Treatment
Pre-Dex			
PND7	NS	Up	NS
PND21	NS	Down	NS
PND 90	NS	Down	NS
Post-Dex			
PND0	**	**	**
PND 7	(F)	Down	NS
PND 60	NS	Down	NS

Legend

1) Down - Denotes lower levels in Dex when comparted to Vehicle
2) Up - Denotes higher level in Dex when compared to Vehicle
3) M - Denotes higher level in Male when compared to Female
4) F - Denote higher level in Female when comparted to Male
5) ** - Not done
6) NS- Not significant

Table 2. Comparison of *Trh* in Post DEX treated rats vs Pre DEX treated rats.

At PND 21 and 90 in Post-DEX-Rats and the Pre-DEX rats at PND 60, DEX treated rats showed lower levels of *Trh* expression when compared to vehicle rats (Table 2). This may suggest that the critical window of TRH expression is from at least GD18 to PND7, since similar results were seen in Pre and Post DEX treated animals.

Regarding the lower levels of *Trh* in Post and Pre DEX treated animals; multiple mechanisms could be at play. Postnatal exposure to excess GC may have altered corticosterone signaling in adults which results in abnormal Glucocorticoid Receptors (GR) expression in the PVN (26). Transcription factors responsible for the regulation of the *Trh* gene and its promoter region may have been changed by DEX through interaction with GR. Effects of developmental DEX exposure on corticosterone signaling are also supported by studies indicating that the amplitude of the corticosterone surge that effects TRH expression may be lowered in DEX treated rats (24). In addition, signaling factors from the peripheral circulation including leptin, triiodothyronine (T3), melanocyte-stimulating hormone (MSH), neuropeptide Y (NPY), and Agouti-related protein (AgRP) have all been documented to play a role in the regulation of proTRH production (26), although future studies are needed to determine if any of these signaling factors are changed in response to DEX exposure.

Another interesting phenomenon is the sexual dimorphism in response to pre- and postnatal DEX exposures. The Post-DEX rats exhibited effects primarily in the male at PND 21 and in female and male at PND 90. In comparison, the female predominant effects seen in the Pre-DEX rats at PND 60 suggest that timing of the DEX treatment affects *Trh* programming in a sex-specific fashion. Different surges of androgens during sensitive periods may be responsible for these differences. It has been shown that the timing of gonadal surges can change brain development and organize the brain in a sexually dimorphic manner during the perinatal sensitive period. Then in adulthood, gonadal hormones can activate this organized neurocircuitry to express appropriate sex-specific behavioral phenotypes (27). This fits with the observed data that in Pre-DEX treated rats, a female predominant treatment effect was seen, while in the Post DEX model, the Male rats were more affected.

A possible explanation for permanent *Trh* dysregulation by DEX exposure is the role of epigenetics and modulation of gene promoters. Existing studies have shown that there are changes in methylation patterns of certain gene promoter areas (14) that occur in a sex specific fashion. The permanent decrease of *Trh* expression in both the Pre and Post DEX rats suggests that epigenetic changes are present.

Epigenetics is the interaction between genes and phenotype, in which the genome, but not the DNA structures itself, changes. One mechanism of epigenetic regulation is DNA methylation, which occurs in GC-rich regions, or CpG islands, of a gene promoter area. Methylation of a gene promoter prevents transcription, thus inhibiting expression of the gene. The family of enzymes that are responsible for imprinting methylation are referred to as DNA methyltransferases (DNA MTase), and catalyze the transfer of a methyl group to cytosine nucleotides. The classes of DNA MTases include DNMT3a and DNMT3b, which methylate cytosine residues de novo, thus establishing methylation patterns. These patterns are maintained by DNMT1, which replicates methylation patterns by the methylation of DNA when one strand is already methylated. Methylation in turn recruits methyl-binding proteins, such as methyl CpG binding protein 2 (MECP2), which is thought to obstruct the binding of transcription factors and polymerase enzymes, thus preventing gene transcription (5).

To test whether pre- or postnatal DEX exposure may influence methylation, we measured expression of *Dnmt3a*, *Dnmt2b*, and *Mecp2* in PVN harvested from animals exposed to these scenarios which revealed no significant changes in the expression of these genes in Post-DEX animals (Table 3). However, minor changes were seen in the Pre-DEX animals. *Mecp2* levels were decreased at PND60, and *Dnmt3a* levels were decreased at PND0. These data suggest that if epigenetic changes are responsible, the window for potential epigenetic changes is prior to PND4, which is supported by a study conducted by Murtagoyd and Spengler (2011), in which stress spanning from PND1 to PND10 resulted in abnormal methylation patterns (14). Given the existence of CpG islands in the *Trh* promoter regions, the possibility exists that developmental DEX exposure alters the expression of this gene by altering methylation patterns, although further studies are needed to confirm this.

MeCP2

	Sex	Treatmen	Sex x Treatment
Post-Dex			
PND7	NS	NS	NS
PND21	NS	NS	NS
Pre-Dex			
PND 60	NS	Up	NS

DNMT3b

	Sex	Treatment	Sex x Treatment
Post-Dex			
PND7	NS	NS	NS
PND21	NS	NS	NS
Pre-Dex			
PND0	(M)	NS	NS
PND 7	NS	NS	NS
PND 60	NS	NS	NS

DNMT3a

	Sex	Treatment	Sex x Treatment
Post-Dex			
PND7	NS	NS	NS
PND21	NS	NS	NS
Pre-Dex			
PND0	NS	Down	NS
PND 7	NS	NS	NS
PND 60	NS	NS	NS

Table 3. Comparison of *Epigenetic gene expression* in Post DEX treated rats vs Pre DEX treated rats.

IHC-TRH

Analysis of ppTRH expression in neurons and fibers (Table 4) throughout the PVN indicated different effects of the pre- and postnatal DEX exposure. No effect of postnatal DEX exposure was seen on ppTRH expression. However, in the prenatal DEX exposure resulted in decreased numbers of ppTRH-expressing neurons and fibers in the PVN. Although it is unclear whether these neuronal populations are hypophysiotropic (TSH-stimulating) or non-hypophysiotropic, future studies identifying these neurons would be useful in determining the functional consequences of fetal DEX exposure. Multiple mechanisms potentially explain these results. During neurodevelopment, impaired migration of TRH neurons destined for the PVN may have occurred (24). However previous studies have shown TRH-expressing neurons reportedly achieve their adult location and have undergone differentiation by GD15 in rats (24). It is possible that TH feedback on the hypothalamus is altered in offspring following fetal DEX exposure, although additional studies are needed to explore this possibility.

In regards to the other metabolism genes *Ghih* and IGF-1, significant results were seen in the Post-DEX rats; i.e. lower levels of *Ghih* at PND 21 and 90 in DEX treated animals (Table 5 and 6), while none were seen in Pre-DEX animals. This may suggest that the critical window of *Ghih* expression is from at least from PND4 to 7. The same factors mentioned above regarding the *Trh* genes can be at play here, such as epigenetics or GC receptors regulation. Future studies looking IHC of GHIH neurons in the PVN may be interesting to see the effects of dexamethasone on migrating neurons.

Stress genes (AVP + CRH)

Vasopressin acts in a synergistic fashion with CRH on the corticotroph cells of the anterior pituitary to increase pro-opiomelanocortin (POMC) expression and cause an increase of ACTH release from the pituitary (14). ACTH then acts on the adrenal glands to stimulate the release of cortisol, While CRH stimulates the release of Adrenocorticotropic hormone (ACTH) from the pituitary gland. In other words an increase of AVP or CRH could be one explanation why preemies that were give sGC are more prone to neuropsychiatric disease such as depression

TRH-IHC-Neurons

	Sex	Treatment	Sex x Treatment
Pre-Dex			
PND 90	NS	NS	NS
Post-Dex			
PND 60	NS	Down	NS

TRH-IHC-Fibers

	Sex	Treatment	Sex x Treatment
Pre-Dex			
PND 90	NS	NS	NS
Post-Dex			
PND 60	NS	Down	NS

Table 4. Comparison of *TRH Neuron and Fiber count* in Post DEX treated rats vs Pre DEX treated rats.

Somatostatin and IGF-1

	GHIH		
	Sex	Treatment	Sex x Treatment
Post-Dex			
PND7	NS	NS	NS
PND21	(M)	NS	Down
PND 90	NS	Down	NS
Pre-Dex			
PND0	**	**	**
PND 7	**	**	**
PND 60	NS	NS	NS

Table 5. Comparison of *Ghih gene expression* in Post DEX treated rats vs Pre DEX treated rats.

	Plasma IGF-1		
Post-Dex	Sex	Treatmen	Sex x Treatment
PND 90	(M)	NS	NS
Pre-Dex			
PND 60	NS	NS	NS

Table 6. Comparison of *IGF-1* in Post DEX treated rats vs Pre DEX treated rats.

and anxiety. However, in both the Pre and Post DEX models, no changes in *Avp* or *Crh* gene expression were seen. This was interesting because studies which mice were exposed to early life stress (ELS), defined as by being separated by their mothers, which from PND 1 -10 showed increase levels of *Avp* and *Crh* (14). One possibility is that models of ELS are not mediated by glucocorticoids. Those other factors are being changed by ELS. Another possibility is if ELS is mediated by GC, than the timing of the insult started earlier (PND1) than our Post-DEX model and later than our Pre-DEX model. The Pre and Post DEX models might have missed this critical window.

Possible future studies looking to reproduce the change to *Avp* or *Crh* gene expression is warranted to narrow down the critical window of exposure, possibly by changing the Post-DEX treatment paradigm earlier to PND 1.

Oxytocin

Oxytocin (Oxt) was decreased in Post-DEX animals at PND 21 and 90 while no significant effects were seen in the Pre-DEX animals (Table 7). Because these results were not seen in Pre-DEX animals, our data indicate that the critical window for influencing *Oxt* expression must occur postnatally. As discussed above, *Oxt* plays a protective role on the stress axis by decreasing sympathetic activity and inhibiting the secretion of cortisol (28). Decreased *Oxt* levels in DEX treated animals may provide an explanation why DEX treated rats is more likely to exhibit anxiety like behavior in adulthood. Interesting future studies using IHC to measure OXT neuron populations should be performed to see if loss of expression of death of OXT neuron has been affected by the dexamethasone.

Weakness of current study

The original design of this experiment was initially to check AVP expression and epigenetics machinery genes. In the postnatal study, PND 7, 21, 90, was chosen because it mimicked another study. The methods after birth of the liter of the Postnatal study was done in the same way as the prenatal study with the exception of chosen time points. In the prenatal study, Time points of PND 0,7,60 were chosen instead. In future studies, it would be ideal if the same time

	OXT		
	Sex	Treatmen	Sex x Treatment
Chong			
PND7	NS	NS	NS
PND21	NS	Down	NS
PND 90	NS	NS	NS
Carbone			
PND0	(M)	NS	NS
PND 7	NS	NS	NS
PND 60	(F)	NS	NS

Table 7. Comparison of *Oxt* gene expression in Post DEX treated rats vs Pre DEX treated rats.

points were used in both studies. However, the aim of the study was to check the acute changes, and to see the changes into adulthood of the rats. Postnatal day 60 and 90 both represented persistent change into adulthood.

Future Directions

Our data suggest that critical windows of development may exist but this does not fully explain the exact mechanism of why exposure in specific time frames leads to lasting changes in function, one possible mechanism that should be further explored is the role of epigenetic marks in the persistence of these effects into adulthood. Studies measuring methylation of CpG islands in *Trh, Ghih and Oxt* promoters regions might help us understand the result. It would also be interesting to see if the changes in gene expression correlated with changes in neuron migration or proliferation. IHC of neuron population of GHIH or OXT in the PVN may show changes in neuron population. Lastly, The Pre and Post DEX models exposure periods differed by 10 day so it is hard to narrow down the exact critical window of each gene. Repeating this experiment with additional exposure points such as GD 21 to birth, and PND 1 to PND 3 can help narrow down the exact critical window of vulnerability for each of the genes measured in this paper.

Conclusion

It is clear that different critical windows exist for dexamethasone exposure effects of gene expression on genes in the hypothalamus. *Trh*, TRH neurons, *Oxt*, *Ghih*, IGF-1 levels all seem to be affected differently from changes of DEX timing. Our data demonstrate the existence of critical windows during certain stages of development, during which expression of specific genes is vulnerable to abnormal programming. Future studies showing the exact window and the mechanisms behind the window of the genes mentioned above would be interesting and help future research in finding ways to reduce metabolic and psychiatric disorders in these populations treated with sGC in the neonatal period.

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