Regulation of Corticoid and Serotonin Receptor Brain System following Early Life Exposure of Glucocorticoids: Long Term Implications for the Neurobiology of Mood

*Delia M. Vázquez\textsuperscript{1,2}, Charles R. Neal, Jr.\textsuperscript{4}, Paresh D. Patel\textsuperscript{2,3}, Juan F. López\textsuperscript{2,3}

\textsuperscript{1}Department of Pediatrics and Communicable Diseases, University of Michigan, Ann Arbor, MI 48109, USA
\textsuperscript{2}Psychiatry Department, University of Michigan, Ann Arbor, MI 48109, USA
\textsuperscript{3}Molecular and Behavioral Neuroscience Institute, University of Michigan, Ann Arbor, MI 48109, USA
\textsuperscript{4}Department of Pediatrics and Communicable Diseases, John A. Burns School of Medicine, University of Hawaii, Honolulu, HI 96813 USA

*corresponding author
Delia M. Vazquez, MD
Professor, Pediatric and Psychiatry Department
3737 Medical Science II
1241 East Catherine Street
University of Michigan
Ann Arbor, MI 48109-SPC5671
dmvazq@umich.edu
734-764-5175 (Office)
734-763-7475 (Fax)

Running Title:

Neonatal Steroid Effects: HPA Stress, Behavior and Brain Regulation in Adulthood
Abstract
Potent glucocorticoids (GC) administered early in life has improved premature infant survival dramatically. However, these agents may increase the risk for physical, neurological and behavior alterations. Anxiety, depression and attention difficulties are commonly described in adolescent and young adult survivors of prematurity. In the present study we administered vehicle, dexamethasone, or hydrocortisone to rat pups on postnatal days 5 and 6, mimicking a short term clinical protocol commonly used in human infants. Two systems that are implicated in the regulation of stress and behavior were assessed: the limbic-hypothalamic-pituitary-adrenal axis [LHPA, glucocorticoid and mineralocorticoid receptors within] and the Serotonin (5-HT) system. We found that as adults, pups treated with GC showed agent specific altered growth, anxious behavior, changes in corticoid response to novelty and gene expression changes within LHPA and 5-HT–related circuitry. The data suggest that prolonged GC-receptor occupation during the early neonatal period can contribute to the development of individual differences in stress response and anxiety behavior later in life.

Keywords
Dexamethasone/*administration & dosage/*adverse effects
Hydrocortisone/ *administration & dosage/*adverse effects
Body Weight/drug effects
Animals, Newborn/growth & development
Anxiety Behavior/Attention
Stress response
Adult
Rat
Introduction

Premature birth defined as birth prior to 37th week of gestation, accounts for approximately 11% of all births in the USA (Centers for Disease Control and Prevention, 2007). In 2007 this was equivalent to over 500,000 newborns (Centers for Disease Control and Prevention, 2007). Over the last two decades the survival of the most premature infants in this group has increased to approximately 40% at 24 weeks of gestation and 95% at 28 weeks (Hack and Fanaroff, 1999; Lorenz, 2001). Improvements in survival have created a population of extremely low birth weight premature infant survivors with unique physical and behavioral challenges (Hack and Fanaroff, 1999; Hack and Wilson-Costello et al., 2000). A significant aspect of prematurity is the fact that brain maturation after birth is very complex, corresponding to that which is occurring in utero during the late second and entire third trimester of human pregnancy. This “ex-utero” brain development remains the least understood in terms of its role in long-term neurodevelopmental outcome. Complicating this process, the brains of critically ill premature infants must develop while simultaneously experiencing multiple stressors (e.g. cold, light, noise, pain), as well as exposure to neuroactive agents, such as glucocorticoids (GC), and opiates.

The glucocorticoids dexamethasone (DEX) and hydrocortisone (HC) are likely the most frequently used neuroactive agents in the neonatal intensive care setting for multiple reasons, the most prominent of which are lung disease (Cummings and D'Eugenio et al., 1989; O'Shea and Kothadia et al., 1999) and refractory hypotension (Martens and Rijken et al., 2003). For the past two decades, DEX has been the glucocorticoid agent most often used in this clinical setting. Unfortunately, DEX administration has been associated with neurodevelopmental impairments among preterm infants (The American Academy of Pediatrics 2002). Neurodevelopmental difficulties described in the premature infant survivors who are not neurologically impaired vary
depending on the age of the child. At pre-school age (2–3 y), several reports indicate delayed psychomotor development and anxious/depressed and/or withdrawn behavior (Sajaniemi and Hakamies-Blomqvist et al., 2001; Stoelhorst and Martens et al., 2003; Stoelhorst and Rijken et al., 2003). At school age and adolescence, increased incidences of social competence problems, attention disorders and hyperactivity have been described (Pharoah and Stevenson et al., 1994; Stjernqvist and Svenningsen, 1995; Schothorst and van Engeland, 1996; Botting and Powls et al., 1997; Saigal, 2000; Hille and den Ouden et al., 2001). A systematic review of the DEX experience in neonates (Doyle and Davis, 2000; Barrington, 2001) led to a consensus statement from The American Academy of Pediatrics which recommended limited use of DEX in premature infants (American Academy of Pediatrics, 2002). During the past decade, hydrocortisone (HC), a less potent glucocorticoid agent that has the same chemical structure as the endogenous glucocorticoid (cortisol) in humans, has become a preferred agent in neonatal medicine for the treatment of hypotension and chronic lung disease (van der Heide-Jalving and Kamphuis et al., 2003). Premature infant survivors treated with HC during their neonatal period are at early school age now so less is known regarding the neurodevelopmental effects of hydrocortisone use in this population, compared to those children who are now adolescent and young adults that received DEX early in life. Immediate effects on neurodevelopment appear to be benign (Benders and Groenendaal et al., 2009), but dosing regimens with HC are markedly variable and there are mixed reports of the long term consequences (Lodygensky and Rademaker et al., 2005; Thompson and Wood et al., 2008).

The brain is clearly a target for GC action (De Kloet and Rosenfeld et al., 1988; Vazquez, 1998) and specific brain systems expressing receptor molecules that are under organization at the time of GC exposure may be at the root of the long term consequences observed in survivors of
premature birth. Pre-clinical animal models have been developed to study the long term effects of early life GC exposure on neurodevelopment, eliminating confounding perinatal and postnatal factors found in human studies (Kurosawa and Kageyama et al., 1980; Slotkin and Barnes et al., 1982; Kauffman and Seidler et al., 1994; Felszeghy and Gaspar et al., 1996; Ferguson and Holson, 1999; Felszeghy and Bagdy et al., 2000; Heine and Rowitch et al., 2009). However, few animal studies have administered GC agents in the same manner as performed in neonatal intensive units across the nation. Most recently, our laboratory reported neonatal DEX effects on neonatal neurodevelopment, adolescent rat behavior, and adult limbic neurochemistry using a pre-clinical animal model that mimics the prolonged DEX exposure that until 2002 was common in neonatal intensive care settings (Flagel and Neal et al., 2002; Neal and VanderBeek et al., 2004; Neal and Weidemann et al., 2004; Bhatt-Mehta and Huang et al., 2010). Long-term effects of neonatal treatments in any of these developmental animal models are interpreted under the assumption that, although timing differs significantly between species, the general sequence of brain growth is similar (Dobbing, 1981; Flagel and Neal et al., 2002). While much caution is necessary when extrapolating from animal models to the human condition, one can still take advantage of similarities in sequence and timing of brain development between species. In humans, excluding the cerebellum and hippocampus, neuronal proliferation is essentially completed before 24 wks of gestation (Dobbing, 1974). Beyond 24 wks of gestation, glia continues to proliferate and oligodendroglia maintain ongoing myelination, with a peak in brain growth occurring near term. In contrast to humans, *rodents experience their brain growth spurt after birth*. It is estimated that on postnatal day 10 the rodent brain is roughly equivalent to that of the full term human brain of 38 to 40 weeks post-conception (Dobbing, 1974, 1981; Hagberg and Bona et al., 1997). Extrapolating from this model, the brain of a rodent pup at birth (postnatal day
1 or PD 1) corresponds to that of a human fetal brain at or near 19–21 weeks of gestation (Dobbing, 1981; Whitelaw and Thoresen, 2000). The PD 3 corresponds with that of a 24-26 week human, and PD 5-6 approximates a 28-32 week human. Given these parallels, the neonatal rodent is an ideal model in which to investigate effects of a tapering course of neonatal GC exposure on the developing brain.

In the present study, our objective was to compare the effects of the two leading GC agents under clinical use in premature neonates over the last two decades (DEX and HC) on the developing central nervous system. We chose to focus on a short course of GC treatment given on PD 5 and PD 6 for several reasons. First, the majority of infants receiving GC for chronic lung disease will be those born at 23-25 weeks of gestation (Hack and Fanaroff, 1999; Lorenz, 2001). These infants will receive steroids for lung disease usually around 3-4 weeks of life. In addition, HC is often given to infants who are born at 24-28 weeks of gestation for refractory hypotension. Consequently, a relatively short course of HC treatment is used extensively in neonatal intensive units for non-pulmonary reasons. Second, the timing of GC treatment is critical, because normal development of the human limbic-hypothalamic-pituitary axis (LHPA) provides a low GC milieu during a period when elevated GC levels may negatively impact neuronal circuits (Watterberg and Gerdes et al., 2001). Third, we wished to focus on specific structures of the developing limbic system and brainstem, locations where there is a high density of corticoid and serotonin (5-HT) receptors and 5-HT producing cells, that have roles in the development of stress and emotional regulation (McEwen, 1987; Benesova and Pavlik, 1989; Weinstock and Matlina et al., 1992; Gunnar and Vázquez, 2006). Thus, in the present study our objectives were three fold: 1) to provide GC during a postnatal age in the rat that corresponds to the neurodevelopmental time point at which human premature infants commonly receive GC therapy, 2) to provide tapering
doses for both agents at a particular time after birth (from PD 5 to PD 6) with the specific goal to more closely mimic and compare GC protocols provided in the neonatal intensive care setting, where 3-7 day courses are administered (Halliday and Ehrenkranz et al., 2003), and 3) to ascertain the long term effect of each of these agents on: a) growth, b) hormonal response to stress, c) anxiogenic behavior and d) molecules relevant to GC action and the serotonin (5-HT) system. We hypothesized that somatic effects and emotional instability will be present in animals treated early in life with both glucocorticoid agents, with DEX having the greatest effect. In addition, we hypothesized that the behavioral phenotype will be associated with developmental adaptations of both the limbic-corticoid and serotonin receptor systems, to early life exposure of GC.
Material and Methods

Animals: Litter management and animal handling of neonatal rats in this study was similar to that reported from our laboratories previously (Flagel and Vazquez et al., 2002). Adult Sprague-Dawley rats (Charles Rivers, Wilmington, MA) were housed and treated according to Guide for the Care and Use of Laboratory Animals. All animals were kept under constant temperature (25 ± 2°C) and photoperiodicity (14:10h light-dark cycle) and provided with food and water ad libitum. Animals were mated using one to one mating system (1F:1M). Assuming a 21-day gestation, pregnant females were housed separately, starting on day 18. They were then checked twice daily until pups were born. The day of the birth of the pups was designated as postnatal day one (PD1).

On PD2, each litter was sexed and culled to 12 pups (6M:6F) that were randomly selected in male: female pairs from 3 different dams giving birth on the same day. This ensured both genetic diversity, equality in nutrition and maternal care within litters. On PD3, 4 pups representing both sexes were assigned to one of three treatment groups within each litter (4 pups x 3 treatment groups= 12 pups /litter): 1) Vehicle Controls (VEH), 2) Dexamethasone (DEX) and 3) Hydrocortisone (HC). Therefore, a total of 144 animals obtained from 12 different litters were utilized in the study. All animals were studied in the early ages of PD 5, 6, 7, 14, 20, and 33. At day 33, female animals were sacrificed and brains collected, as part of another experiment. Seventy-two males were studied at pre adolescence and adulthood on days PD 33, 60, 70 and 140 (n=24 for each treatment).

Drug Treatment: On the day of treatments, cages with the dam and pups were brought into a room adjacent to the animal colony and placed in a warm pad (temperature 30-35°C). Mothers were removed, placed in a holding cage and moved to a separate room sheltered from the noise
that may have been present in the treatment room. Pups were sexed, weigh and treated. Animals in the DEX group received an intramuscular (IM) injection of DEX in a tapering dose of 0.5 mg/kg on PD 5 and 0.1 mg/kg on PD 6. Animals in the HC group also received an IM injection of HC in a tapering dose of 5.0 mg/kg on PD 5 and 1.0 mg/kg PD 6. Animals in the vehicle (VEH) group received equivalent volume of IM sterile normal saline as the DEX and HC animals. The dam was then reunited with the pups. The procedure time was 5 minutes and these were performed between the hours of 1100 and 1300. The study protocol and days of treatment are depicted in Table 1.

Weaning: Weaning was performed at PD 21. Females were grouped housed as 6 animals per cage, then sacrificed on PD33. Males were grouped housed 6 animals per cage from PD 21 to PD 45, then separated to single housing due to their weight at PD 45 until the end of the experiments. This was done following the "Guidelines for the Care and Use of Laboratory Animals", which bases the number of animals in a cage on the size of the animals and expected growth without food restriction.

Somatic Growth: Lengths and weights were measured for each pup in each treatment group before treatments on PD 5 and 6. All animals were also measured and weighed on PD 7, 14, 20, 33. Male animals were weighed when 33, 60 and 140 days old. Length was not obtained at 60 days in the males due to a lab error. The weight and length of the female animals up to PD 33 is reported here for comparison purposes with the males (Figure 1). At each age, length was measured from the nose to the base of the tail (head-rump length).

Behavioral Testing: Light-Dark Preference Box- The light-dark preference procedure was used to evaluate the locomotion and investigatory behavior of the animals when 70 days old. Preference for darkness and decreased activity are gross measurements of anxiety (File, 1990;
Bourin and Hascoet, 2003; Ramos, 2008). Three days prior to testing (PD 67), animals were acclimated to the handling needed before the start of the procedure. Handling consisted in transporting the cage to the test room and removal from the cage at the expected time of testing. This was done for three consecutive days. The testing apparatus was a covered 30 x 60 x 30-cm Plexiglas shuttle-box with a computerized monitor. It has two equal sized compartments (light and dark) with a 12-cm wide opening and stainless steel grid floor suspended above the corncob bedding. The light compartment was constructed of white Plexiglas and brightly illuminated. The dark compartment was constructed of black Plexiglas and minimally illuminated. Fluorescent lights above the box provided the illumination. Half of the boxes had the light compartment on the right side, half on the left side; this controlled for lateralization across the groups. To start the session, each animal was placed in the dark compartment and the timer set to start. Each animal's locomotor activity, the time spent in each compartment, the number of transitions and the latency to leave the dark were scored. Locomotor activity as well as time spent in each compartment was monitored by photocells located on the wall of each box, with the number of photocell beams interrupted per unit time recorded with microprocessor. The number of transitions was recorded manually. Total testing time was 5 minutes (Flagel and Vazquez et al., 2002).

**Adrenocortical Response to Novelty Stress:** Immediately after light-dark preference testing was completed on PD 70, animals underwent a tail nick procedure to collect blood. Blood was collected from the tail vein at 15, 30 and 60 min after placing the rat in the dark compartment of the light-dark box. The last blood sample was collected 120 min after the start of the test at which time the animals were decapitated. The time of testing was equally randomized across the groups. A pre-stress blood sample was obtained the day prior to the light-dark box as the animal
was acclimated to handling. The time of the pre-stress sample corresponded to the same time of the start of the procedure on the following day. Blood samples were collected in pre chilled tubes containing EDTA, placed on ice and subsequently spun at 2000 rpm for 7 min. The plasma was separated and stored at -20°C until assayed for corticosterone hormone concentrations.

**Hormonal Assay:** Corticosterone (CORT) levels were measured using a commercially available corticosterone I¹²⁵ radioimmunoassay kit (Cat. #07-120102, MP Biomedicals LLC, Diagnostic Division, Orangeburg, New York). Un-extracted plasma samples were diluted to 1:200 in Phosphosaline gelatin buffer (pH 7.0). The intra- and inter assay CVs for corticosterone were 4.4% and 6.5%, respectively.

**Brain Sectioning:** Six animals per group were randomly selected for brain sectioning and anatomical analyses that followed. For this purpose, the blocks between Bregma +5.9mm to -2mm (“forebrain”) and between Bregma -2mm to Bregma -7mm (“raphe”, with DR cell groups typically between Bregma -4.1mm and -5.2mm) were frozen in dry ice-isopentane at -20°C followed by storage at -80°C. We generated two brain blocks per animal. The raphe block was sectioned at 12 µm and collected in sets of 5 deep (sections 1-5 on slides 1-5, respectively; sections 6-10 on slides on 1-5 again, etc, repeated until full, then on subsequent groups of 5 slides). This generates 5 “sets” of slides, each of which surveys the region at ~60 µm intervals. The forebrain block (containing dorsal hippocampus and amygdala in the same coronal plane) were acquired 8 sections deep. This sampling scheme generated approximately 250 sections per animal for the rostral raphe group, generating 9 slides per animal per set (x 5 sets), with each slide containing six sections. Three set of slides surveying raphe were analyzed separately by in situ hybridization (ISH) for 1) neuronal tryptophan hydroxylase (nTPH), 2) serotonin transporter (5-HTt), 3) 5-HT1a receptor mRNAs. Sections through the third ventricle captured the
periventricular nucleus of the hypothalamus (PVN) which was processed for corticotrophin releasing hormone (CRH) mRNA. Sections through the hippocampus were processed for 1) 5-HT1a mRNA, 2) mineralocorticoid receptor (MR) mRNA, and 3) glucocorticoid receptor (GR) mRNA. Table 2 depicts the riboprobes and anatomical areas used for these analyses.

Hybridization: Sections were removed from storage at −80°C and placed directly into 4% buffered paraformaldehyde at room temperature. After 60 min, slides were rinsed in isotonic phosphate-buffered saline and treated with proteinase K (1 μg/mL in 100 mmol/L TRIS/HCl, pH 8.0) for 10 min at 37°C. Subsequently, sections underwent successive washes in water (1 min), 0.1 mol/L triethanolamine (pH 8.0, plus 0.25% acetic anhydride) for 10 min and 2X SSC (0.3 mmol/L NaCl, 0.03 mmol/L sodium citrate, pH 7.2) for 5 min. Sections were then dehydrated through graded alcohols and air dried.

Postfixed sections were hybridized with 1.0 × 10⁶ dpm [³⁵S]UTP-labeled riboprobes in hybridization buffer containing 50% formamide, 10% dextran sulphate, 3X SSC, 50 mmol/L sodium phosphate buffer (pH 7.4), 1X Denhardt’s solution, 0.1 mg/mL yeast transfer RNA (tRNA), and 10 mmol/L dithiothreitol in a total volume of 25 μL. The probe was applied to sections on a glass coverslip and hybridized overnight at 55°C. Next day the sections were washed in 2X SSC for 5 min and then treated with RNase A (200 μg/mL in 10 mmol/L TRIS/HCl, pH 8.0, containing 0.5 mol/L NaCl) for 60 min at 37°C. Subsequently, sections were washed in 2X SSC for 5 min, 1X SSC for 5 min, and 0.5X SSC for 60 min at hybridization temperature, and 0.5X SSC at room temperature for 5 min, and then dehydrated in graded alcohols and air dried. For signal detection, sections were placed on Kodak XAR-5 X-ray film and exposed for 2 days at room temperature.

Microdensitometric Analysis: Autoradiograms generated from the ISH were analyzed using an automated image analysis system (Dage camera, Scion Image Beta 4.03; Scion Corporation).
Anatomical regions of interest were interactively selected and mean optical density measurements for each region were determined from at least six coronal sections in a single animal. This single data point was utilized in the statistical analyses. Hippocampus subfields were determined with reference to Nissl-stained sections and the anatomical atlas of Paxinos and Watson (Paxinos and Watson, 1986). Nonspecific labeling of [35S]-riboprobes was determined from an area of section exhibiting apparent lack of hybridization signal.

**Statistical Analyses:** Statistical differences were determined by analysis of variance (ANOVA). Significance was indicated by a p value p<0.05. Once significance was observed by ANOVA, the Fisher's least significant difference (Fisher’s PLSD) method was utilized for further pair wise comparisons.

### Results

**Somatic Growth:** ANOVA analyses revealed significant sex, treatment and age effect (sex, treatment and age, each p< 0.0001). Figure 1, Panels A and B shows weight and length progression on females from PD 5 to PD 33; Panels C and D depicts these on the males.

Compared to VEH animals the DEX-treated rats had a decrease in somatic growth at all early ages. The adult DEX-treated males were not different from VEH treated males for both weight and length at PD60 and 140. In contrast, HC-treated animals were not different from VEH at early ages until adulthood when both weight and length were significantly greater in the males.

**Anxiety Behaviors (Figure 2):** A treatment effect was evident on the parameters assessed with the L-D box test on PD 70 animals only (time to light p <0.05, time in light p<0.002, activity in light p<0.0005, time in dark p<0.002). Post hoc analyses revealed that as young adults (PD70), DEX-
and HC-treated animals show altered behaviors in the L-D box that were similar. Animals are placed in the dark compartment upon initiation of this test and HC-treated animals moved from the dark to the light compartment significantly faster when compared to VEH-treated animals (see Figure 2, time to light). This behavior was not observed in the DEX-treated animals. Compared to VEH controls, the DEX-treated animals showed significantly decreased time and exploratory behavior in the light compartment. DEX-treated animals spent an increased amount of time in the dark compartment when compared to VEH. The HC treated animal also had significantly decreased time and exploratory behavior in the light compartment and they spent an increased amount of time in the dark compartment when compared to VEH controls. The decreased time and activity in the light compartment of the HC-treated animals was somewhat less marked when compared to the DEX-treated animals.

**Stress Response (Figure 3):** A significant effect for treatment and time, with a treatment by time interaction was observed in the ANOVA analysis (treatment p < 0.0001; time p<0.0001; treatment:time, p<0.001). DEX-treated adult rats had a blunted adrenocortical response (CORT) to novelty stress compared to all groups. HC-treated animals were also different from VEH-treated animals. HC-treated animals had a lower peak response and faster CORT inhibition when compared to VEH.

CRH gene expression in the PVN was significantly decreased in adult animals treated with DEX on PD5 and 6 (p=0.0001, data not shown).

**Corticoid Receptors [glucocorticoid receptor (GR) and mineralocorticoid receptor (MR)] and Serotonin (5-HT) Molecules (Figure 4 and 5)**

**Hippocampus:** ANOVA revealed a treatment (p<0.01) and region (p<0.001) effect on GR gene expression. In the hippocampus, a significant region effect was observed for GR mRNA, thus the
ANOVA analysis was split by region. We found that adult DEX-treated rats had significantly decreased GR mRNA expression in the CA2 area of Ammon’s horn, and in dentate gyrus (DG) when compared to HC and VEH animals (CA2 p<0.0001; CA3, p=0.13; DG, p=0.01). Post hoc analysis demonstrated that DEX treated animals had down-regulation when compared to VEH control. No effect of DEX was found on the MR gene expression (ANOVA p=0.345) and no GR or MR mRNA changes were detected in the HC-treated animals compared to VEH controls.

The analysis of variance (ANOVA) of the 5-HT1a receptor mRNA levels in the hippocampus revealed a treatment (p<0.0001) and region effect (p=0.001). When split by region the effect was in CA1 and CA3 (CA1 p=0.006; CA2 p=0.62; CA3 p=0.05; DG p=0.63). Post hoc analysis using Fisher protected least significance difference (Fisher PLSD) showed significant down-regulation in the DEX and HC treated animals when compared to vehicle (VEH) [see Figure 4].

Raphe: In the raphe nucleus, ANOVA revealed no treatment (p=0.84) or region effects for 5-HT1a receptor gene expression (dorsal raphe dorsal, p=0.8 and dorsal raphe ventral, p=0.93; see Figure 5, Panels A and B, third row). Treatment and regional effects were observed in this structure for nTPH and 5-HTt gene expression measurements (ANOVA nTPH treatment= p<0.0001; region= p<0.05; ANOVA 5-HTt treatment= p<0.0001; region= p<0.005). In the DEX and HC -treated animals nTPH mRNA was significantly decreased in the ventral part of the Dorsal Raphe nucleus (DRN). HC -treated animals showed a trend towards down-regulation in the dorsal part of the DRN (p=0.06). Both DEX and HC treated animals also showed a modest but significant down regulation of the 5HTt mRNA levels in the dorsal area of the DRN when compared to VEH controls. Notice that compared to VEH-treated animals the 5-HT1a auto-receptor gene expression was found to be unchanged in the DRN of the DEX and HC treated animals.
Discussion

In the present study, we have investigated long-term effects of a DEX and HC treatment regimen given during the first week of life in the infant rat. We administered tapering doses of these agents on postnatal days 5 and 6 in an attempt to mimic short treatment regimens of DEX and HC that are commonly used in the neonatal intensive care setting in premature infants. The timing of drug administration is critical in our rat model, since it corresponds to late third trimester of human pregnancy (28 to 32 weeks of gestation), a period of growth and development that renders the brain highly vulnerable to insult in the human neonate (Dobbing, 1981). We found that our treatments had significant effects on somatic growth, affecting both length and weight in the developing organism. Beyond these somatic observations, we found that a short course of either DEX or HC administration in the postnatal period affects stress response, and results in anxiogenic behavior in the adult animal. Changes in the gene expression of corticoid receptors and serotonin related molecules were also observed in hippocampus and raphe of those animals experiencing anxiety-like behavior. These findings suggest that early short-term exposure to either DEX or HC may have long-lasting consequences even when a tapering dose regimen is implemented.

Dexamethasone or HC administered during early in life has a lasting impact on somatic growth. The effects were specific for each of the agents used, both in terms of time at which the growth was affected and the persistence of the change. Consistent with previous reports, we found that DEX treatment induced significant decreases in length and weight gain in the rat pup that persisted for up to two weeks after treatment (Flagel and Neal et al., 2002; Neal and Weidemann et al., 2004; Kanagawa and Tomimatsu et al., 2006; Kreider and Tate et al., 2006; Slotkin and Kreider et al., 2006). Catch up growth was consistently achieved by adulthood. In contrast, HC-
treated animals were not affected by this regimen during infancy, but were significantly larger, for both weight and length, than VEH and DEX treated animals at adulthood. The decreased somatic growth observed in the DEX-treated pups may be a result of inadequate nutritional intake during the postnatal period, due to inability of the pup to attach to the mother’s nipples, or poor suckling. Though this is a possibility, it has been shown that the dam spends more time providing nutrition, stimulation, and warmth to a litter that is perceived to have poor health (Lynch, 1976; Wiener and Fitzpatrick et al., 1977; Wiener and Levine, 1978; Brunelli and Shair et al., 1994; Stern, 1997). Direct effects of DEX on protein catabolism that result in reduced growth and lean body mass have also been described (Weiler and Wang et al., 1997; Leret and Peinado et al., 2004). Thus, it is likely that increased protein catabolism beyond the capacity for an anabolic state needed for growth is responsible for the growth deficits observed during the first 3 weeks of life in the DEX-treated animals. The adverse effects on weight gain in early neonatal stages likely contribute to long-term risks of GC use, as has been postulated in the ‘Barker Hypothesis’, relating early growth failure in human infants to subsequent elevated risk of cardiovascular and metabolic disorders (Lucas, 1991; Baker, 2000; Breier and Vickers et al., 2001). In both animal and human literature there is evidence to suggest that early life DEX treatment similarly increases the incidence of cardiovascular and metabolic disorders (Barrington, 2001; Seckl, 2008), despite the eventual recovery of body weight, as seen in our study.

Animals treated with postnatal HC have a unique pattern of growth when compared to DEX-treated animals: these do not have altered growth early in life, but show significant weight gain and linear growth in adulthood. The increased somatic growth is difficult to explain. It is possible that the potency of HC at the given doses was significantly less than that of DEX, as aspect that is currently under study in our laboratory. If the potency of HC dose used is considerably less
when compared to the DEX dose, it is conceivable that the known negative effects of glucocorticoids on bone growth plates was not fully achieved (Zhang and Wang et al., 2007). However, this does not explain the enhanced growth and weight gain later in life. A possible explanation is an effect of HC, at the given doses and developmental period, on insulin action. Glucocorticoid levels achieved by the early treatment could induce an insulin resistant state that promote adiposity later in life (Grino, 2005). Elevated insulin can act as a growth factor because of its structural similarity to insulin-like-growth factors directed by growth hormone (Johnson and Kamilaris et al., 1996; Dupont and LeRoith, 2001). The important distinction here is that the detrimental effects of the more potent DEX treatment given early in life on somatic growth is not observed in animals treated with the less potent glucocorticoid during the same developmental period. While this may indicate that HC is a ‘better’ glucocorticoid agent in early life, there are other concerns. The catch-up growth observed in our HC treated animals that favored visceral fat deposition could increase the risk for insulin resistance and subsequent diabetes later in life by promoting obesity in adulthood (Eriksson and Forsen et al., 2003; Langley-Evans, 2006). A thorough review of the postnatal HC literature in humans or animals treated with HC early in life revealed that this phenomenon has received little attention (Peltoniemi and Lano et al., 2009). But, it is possible that early life HC treatment also increases the risk for later metabolic disorders. Further research on this aspect of early life HC exposure is warranted.

Similar to the rodent, in the premature human infant the ability to mount a cortisol response to stress is limited (but present) (Davis and Townsend et al., 2004). In the human the long term effect of early glucocorticoid exposure has received limited attention. The research in the human has focused on the regulation of the LHPA in preemies that received antenatal glucocorticoid treatment. These studies indicate that baseline cortisol levels are profoundly suppressed for 2 to
7 days after birth, subsequently return to normal levels (Kauppila and Tuimala et al., 1978; Ballard and Gluckman et al., 1980; Dorr and Heller et al., 1989; Wittekind and Arnold et al., 1993). Antenatal glucocorticoid administration results in an impaired response to stress that last beyond the first week after treatment (Davis and Townsend et al., 2004). The duration of the suppression is not known. The human literature warns that future research is needed to explore whether the effects of prenatal corticosteroids persist and alter the developmental trajectory of the LHPA as well as related cognitive and emotion systems (Davis and Townsend et al., 2004). In the present study, the adult adrenocortical stress response was altered by early life GC treatments. Basal corticosterone levels were not different between groups. In response to novelty stress, the male animals exposed to HC or to DEX-early in life had a blunted adrenocortical response, with an adequate termination of the stress response. These patterns of stress response are consistent with other animal and human reports that indicate lifetime altered responses with early life DEX treatments (Felszeghy and Bagdy et al., 2000; Flagel and Vazquez et al., 2002; Karemaker and Kavelaars et al., 2008; Ng and Lam et al., 2008). Surprisingly, no other animal studies have investigated long-term response to early HC treatment.

Modulation of glucocorticoid receptors in hippocampus are linked to baseline corticosterone levels and the quality of the adrenocortical response [reviewed in (De Kloet and Vreugdenhil et al., 1998)]. Our findings of unchanged MR mRNA expression in the hippocampus are consistent with the basal corticosterone levels observed in both treatment groups. However, decreased GR mRNA expression but no change in MR mRNA expression in adult rat hippocampus would predict a delayed return to baseline in serum corticosterone after stress (reviewed in (De Kloet and Vreugdenhil et al., 1998)). This combination of corticoid receptor imbalance was found in our DEX-treated (but not the HC-treated) animals; however an adequate inhibition of corticosterone
secretion was present. One possible explanation is that a much stronger stressor is needed to challenge the DEX treated animals in order for this to result in a loss of regulatory feedback inhibition of corticosterone secretion. Neal and co-workers showed that peak corticosterone response to crowding stress in adult animals exposed to tapering doses of DEX from postnatal day 3 to 6 is no different from VEH control animals, but delayed return to baseline is noted in the DEX-exposed cohort (Neal and Weidemann et al., 2004). Taken together, these data indicate either that the type of stressor is important for the adrenocortical response observed or that the timing of GC exposure early in life is an important factor modulating long-term effects of neonatal exposure.

Increased anxiety-like behaviors were observed in both DEX and HC–treated animals when tested in the light-dark test. Of importance is the fact that all males were housed alone starting at adolescence. This factor may constitute an environmental modification; therefore there are a number of ways to interpret our study findings. One is that the behavioral manifestations (and stress response) seen in the GC treated animals emerges during the transition from adolescence to adulthood. It is also possible that our animals may have been affected by their single-housing environment at adolescence after PD 45. This raises the possibility that GC exposure early in life creates a vulnerable state in the animal, leading to alterations in limbic-hypothalamic-pituitary-adrenal function only after experiencing what could be construed as a prolonged social isolation. There is significant literature from the 1960’s that support the possibility of “isolation stress syndrome” in rodents. Animals exhibiting this syndrome are excessively reactive to handling, timid, or fearful (Ader and Beels et al., 1960 ; Stern and Winokur et al., 1960 ; Hatch and Wiberg et al., 1965). However, in 1990, Holson and co-workers were able to show that the isolation syndrome was dependent on environmental factors and not the isolation itself (Holson and Scallet...
et al., 1991). Animals isolated in hanging metal cages showed the behavioral and stress responses of profound fear, but this was not the case for littermates that were isolated and reared singly in plastic cages. The main difference was that animals in hanging metal cages were never touched by human caretakers, whereas rats reared in plastic cages were picked up and put in clean cages twice weekly as part of their usual animal care. In support of this observation, handling animals that were isolated in hanging-cages twice weekly to model the handling associated with the plastic cage changes completely protected against the “isolation stress syndrome”. Our animals were isolated in plastic cages but received care as described in Holson’s study. Despite this reassuring report, there is a recent publication that shows a lower level of exploratory behavior following a mild shock in 65 and 130-day-old rats isolated during the juvenile stage, but not in rats isolated after puberty (Arakawa, 2007). Although further studies comparing single and group housing are currently being performed in our laboratory to elucidate the importance of these housing conditions, we interpret the adult anxiogenic phenotype observed in our early life GC treated groups as one that is related to specific changes occurring early in life as a result of HC exposure. It is possible that this exposure renders vulnerability to the later exposure to an isolated environment, at puberty. Both DEX and HC cause long-term repercussions in behavior and in the serotonin (5-HT) system. Animals treated with our GC tapering paradigm exhibited, as adults (but not pre-adolescence(Bhatt-Mehta and Huang et al., 2010), anxiety-like behavior in the Light-Dark test. When compared to VEH control, both DEX and HC treated animals also showed a down-regulation of the expression of the rate limiting enzyme for 5-HT synthesis, nTPH and the molecule that transports the neurotransmitter serotonin from synaptic spaces into presynaptic neurons, 5-HTt. A down-regulation of the postsynaptic (but not the pre-synaptic) 5HT1a receptor was also observed when compared to VEH control. Since
nTPH is the rate-limiting enzyme in 5-HT biosynthesis, a decrease in nTPH expression, with a consequent decrease in 5-HT synthesis, may be responsible for the fear-related behavior we observed in adult animals exposed to neonatal GC treatments. The observed down-regulation of 5-HTt gene expression in these animals is also consistent with this hypothesis. Decreasing the molecules responsible for the reuptake of 5-HT may represent an adaptive neuronal mechanism to compensate for lower 5-HT levels. Interestingly, this “compensatory” down-regulation of the 5-HTt has been observed in the brains of depressed subjects as well as in suicide victims (Mann and Huang et al., 2000), and it is also thought to be a response to lower 5-HT levels. Compounding this problem is the simultaneous down-regulation of hippocampal 5HT1a, a receptor implicated in anxiety behavior, as well as in depression. We suggest that GC treatment within that specific developmental window may trigger unique adaptations of the 5-HT system that may have repercussions in behavior later in life. In adult animals these adaptations may be consistent with anxiety behavior (Blier and de Montigny, 1994; Zhuang and Gross et al., 1999).

Hydrocortisone treated animals did not exhibit a down-regulation of GR. Nevertheless, these animals showed behavioral and 5HT abnormalities similar to the DEX treated group. It may be that HC doses are low compared to DEX such that the HC doses may only lead to MR occupation. It is known that MR has a higher affinity for GC when compared to GR (Fischer and von Rosenstiel et al., 2002; Macleod and Johansson et al., 2003; Crochemore and Lu et al., 2005). In addition, the highest signal intensity for both hippocampal GR and MR is seen in the first weeks of life (Vázquez and López et al., 1993). Beyond this age, GR expression decreases to resemble the adult distribution (Rosenfeld and Sutanto et al., 1988; Vazquez, 1998), whereas MR continues to be very abundant in the hippocampus and cortical brain layers, and does not acquire the limited adult-like distribution until close to adolescence (Herman and Patel et al., 1989).
Of importance is the fact that MR occupation has been shown to be neuroprotective (Fischer and von Rosenstiel et al., 2002; Macleod and Johansson et al., 2003; Crochemore and Lu et al., 2005). If this is true in these animals, it would indicate that even if HC is relatively neuroprotective compared to DEX, it can still cause long term behavioral and neurochemical changes. Pharmacological studies are currently being designed in our laboratory to address both the issue of DEX/HC dose equivalence and the possibility of HC acting as a neuroprotective agent in the doses given at postnatal day 5 and 6.

In conclusion, the present study suggests that GC exposure during a specific time in the early postnatal period of the rat leads to critical changes in the hypothalamic-pituitary-adrenal axis and 5-HT circuitry in the neonatal animal that set up the stage for vulnerability later in life. The notion that the external environmental milieu influences permanent hardwiring in the stress responsive limbic-hypothalamic-pituitary-adrenal axis and serotonin brain system is not a novel concept (Lauder, 1990; Walker and Anand et al., 2001; Gaspar and Cases et al., 2002; Ansorge and Zhou et al., 2004). These systems are highly plastic during development and adverse levels of either stress or GC can have a profound impact on development (McEwen and Brinton et al., 1987; McEwen and Chao et al., 1987; Gould, 1994; McEwen, 1994) and plasticity in adults (Smythe and Murphy et al., 1997; McCullers and Herman, 1998; Spencer and Kim et al., 1998; McEwen, 2000). Our findings raise concerns about routine use of hydrocortisone in premature infants even when the potency of this agent is considerably reduced compared to dexamethasone, an agent associated with significant neurodevelopmental effects when used routinely for prolonged periods. Although to-date the immediate and short term effects of early hydrocortisone treatment in premature infants who are now in school age appears to be benign, subtle maladaptive behavioral strategies may not be recognizable until later in development.
Such effects may have important implications on learning, mood and ultimately quality of life in survivors of prematurity. Both DEX and HC should continue to be used with caution in this vulnerable population.
Animals received a single drug treatment (DEX or HC) and were matched within litter with animals that were treated with vehicle injections.

Table 1 Schematic of the procedures and age of the animals at which these were performed.

<table>
<thead>
<tr>
<th>Post-natal Day</th>
<th>PD2</th>
<th>PD5</th>
<th>PD6</th>
<th>PD7</th>
<th>PD14</th>
<th>PD21</th>
<th>PD33</th>
<th>PD70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug*</td>
<td>DEX 0.5 mg/kg</td>
<td>DEX 0.1 mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HC 5 mg/kg</td>
<td>HC 1 mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Procedure Or Test</td>
<td>Culled Sexed</td>
<td>Body Weight Length</td>
<td>Body Weight Length</td>
<td>Body Weight Length</td>
<td>Body Weight Length</td>
<td>Body Weight Length</td>
<td>Body Weight, Length, Brain Weight</td>
<td>-Light-Dark Preference</td>
</tr>
</tbody>
</table>

*Animals received a single drug treatment (DEX or HC) and were matched within litter with animals that were treated with vehicle injections.

Table 2 Riboprobes and anatomical areas surveyed in the study.

<table>
<thead>
<tr>
<th>System Studied</th>
<th>Probes</th>
<th>Anatomical Areas</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHPA Related</td>
<td>CRH</td>
<td>PVN, HC, Raphe</td>
</tr>
<tr>
<td></td>
<td>GR</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>MR</td>
<td>X</td>
</tr>
<tr>
<td>5-HT</td>
<td>5HT1a</td>
<td>X, X</td>
</tr>
<tr>
<td></td>
<td>5HT1</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>nTPH</td>
<td>X</td>
</tr>
</tbody>
</table>

LHPA= Limbic-Hypothalamic-Pituitary-Adrenal axis, PVN=ParaVentricular Nucleus, HC= Hippocampus, CRH=Corticotrophin Releasing Hormone, GR=Glucocorticoid Receptor, MR=Mineralocorticoid Receptor, 5-HT= Serotonin, 5-HT1a=Serotonin1a Receptor, 5-HTt= Serotonin Transporter, nTPH=neuronal Tryptophan Hydroxylase.
Figure 1  Effects of tapering doses of DEX or HC given at post-natal day 5 and 6 on body weight and length. Growth progression of females is observed in Panels A (weight) and C (length), while male growth is depicted in Panels B (weight) and D (length). Hydrocortisone treatment resulted in an increase growth in males at adulthood. Dexamethasone treated male animals had growth failure early, but catch-up growth by young adulthood. Data are expressed as means ± SD; n = 24 per group, per sex at every age. *p < 0.05 DEX vs HC and VEH; † p<0.05 vs VEH.
Figure 2  Adult behavior in Light:Dark preference box evaluated the locomotion and investigatory behavior of the animals in adulthood. Preference for darkness, delay to move to light compartment and decreased activity within the space chosen are gross measurements of anxiety. Data are expressed as means ± SD; n = 24 per group, per sex at every age. *P < 0.05, vs age-matched VEH by Fisher’s least significant difference.
Figure 3  Adrenocortical response to 5 minutes of novelty stress in adult animals treated with tapering doses of DEX or HC on post-natal day 5 and 6. VEH treated animals serve as the comparison group. *p<0.05 vs VEH, ** DEX and HC vs VEH; n=8/group by Fisher’s least significant difference.
Figure 4  Hippocampal GR (Panel A) and 5HT1a (Panel B) mRNA gene expression in adult animals treated with tapering doses of DEX or HC on postnatal day 5 and 6. The specific areas of regional significance are presented here. *p<0.05 vs VEH n=6-7/group by Fisher’s least significant difference.
Figure 5  Densitometric analyses of 5-HT related molecules in Dorsal Raphe (dorsal and ventral portions). The specific areas of regional significance are presented here. *p<0.05 vs VEH n=6-7/group by Fisher’s least significant difference. nTPH= neuronal tryptophan hydroxylase, 5-HTt= serotonin transporter, 5-HT1a= serotonin 1a receptor.
References


Halliday HL, Ehrenkranz RA, Doyle LW. 2003. Moderately early (7-14 days) postnatal corticosteroids for preventing chronic lung disease in preterm infants. Cochrane database of systematic reviews (Online) CD001144.


O'Shea TM, Kothadia JM, Klinepeter KL, Goldstein DJ, Jackson BG, Weaver RG, 3rd, Dillard RG. 1999. Randomized placebo-controlled trial of a 42-day tapering course of dexamethasone to reduce the duration of ventilator dependency in very low birth weight infants: outcome of study participants at 1-year adjusted age. Pediatrics 104:15-21.


