Evidence for a Hypo-Responsive Limbic-Hypothalamic-Pituitary-Adrenal Axis Following Early-Life Repetitive Hypoglycemia in Adult Male Rats

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ABSTRACT:
The developing limbic-hypothalamic-pituitary-adrenal (LHPA) axis is highly vulnerable to programming by early-life environmental factors, including exposure to synthetic glucocorticoids and nutrient deficiencies. Early-life repetitive hypoglycemia (RHG) is a common complication of insulin therapy for type-1 diabetes that may have long-term consequences in adulthood. Recent observations in a rat model of early RHG suggest persistent changes in LHPA axis function, including changes in relevant hormones and affective behaviors, that support a hyper-responsive LHPA axis. Thus, we hypothesized that early RHG would alter the expression of key genes regulating LHPA axis function in adulthood. The present study employed a rat model of insulin-induced RHG spanning postnatal days (P)24-28, a neurodevelopmental equivalent of early childhood in humans, to assess the long-term effects on mRNA levels for proteins relevant to the LHPA function and the corticosterone responses to ACTH stimulation of dispersed adrenocortical cells in vitro and restraint stress in vivo at adulthood. This early RHG model resulted in a hypo-responsive LHPA axis characterized by impaired corticosterone response, increased hippocampal glucocorticoid and mineralocorticoid receptor (GR and MR), decreased hypothalamic corticotropin releasing hormone (CRH), increased adrenal steroidogenic-acute-regulatory protein and GR, and decreased adrenal MR, melanocortin-type-2 receptor (MC2R) and low-density lipoprotein receptor (LDLR) expression. Our findings highlight a complex environmental-gene interaction between RHG and LHPA axis during development that influences regulation of this axis in adulthood. The findings are consistent with the developmental origins of disease and underscore the influences of early-life events on the programming of a major regulatory system.
KEY WORDS:

Hypoglycemia, Limbic-Hypothalamic-Pituitary-Adrenal (LHPA) Axis, Developmental programming, Corticosteroid receptors, low-density lipoprotein receptor (LDLR).
INTRODUCTION:

During development, many hormonal systems are susceptible to programming by the environment. Through such programming, environmental factors may permanently alter the morphology and physiology of organ systems. For example, protein and energy insufficiency during human fetal-neonatal development lead to impaired cardiovascular function and metabolic disorders in adulthood (1, 18, 33).

The mammalian system for glucose regulation is vulnerable to programming by glucose deprivation during postnatal development. Glucose delivery from the blood to the mammalian brain requires its transport across the endothelial cells of the microvessels of the blood-brain barrier, which is predominantly mediated by the facilitative glucose transporter GLUT1, and across the neuronal plasma membranes, which is mediated by GLUT3 (36, 42). Chronic hypoglycemia in adult rats has been shown to upregulate expression of GLUT1 and GLUT3 (8, 9, 40) and causes a redistribution that increases their concentrations at the luminal or cell surface, thereby enhancing glucose transport into the brain and neuron (35). Since there is evidence that GLUT1 expression is upregulated in the developing brain after recurrent hypoglycemia (37); repeated bouts of insulin-induced hypoglycemia during early postnatal development are known to facilitate the subsequent ability to cope with a severe hypoglycemic challenge in adult male rats (38). Thus there could be a beneficial adaptive effect derived from early-life repetitive hypoglycemia (RHG) due to alteration of regional expression of glucose transporters in the brain. Glucose deprivation during development, however, has also been shown to have deleterious, acute and long-term effects in both rats and humans, including regional-specific neuronal injury and alterations in cerebral function, cognition, and affective behavior (11, 25, 29, 32). Thus, a
continuously sufficient supply of glucose is requisite for normal development (41). Unfortunately, RHG often occurs asymptptomatically in children undergoing insulin treatment for type-1 diabetes and in children with several metabolic disorders (15, 29).

Another system that is highly susceptible to programming during development is the limbic-hypothalamic-pituitary-adrenal (LHPA) axis (23, 30). Early-life adversity that can program the LHPA axis can also lead to long-term, often maladaptive alterations in sensitivity and reactivity to stress (19, 24). Epidemiological and experimental studies show that LHPA functioning and associated behavior are programmable early in development by numerous environmental factors, including prenatal maternal stress, exposure to synthetic glucocorticoids, nutrient deficiency, neonatal handling, and infection (23). For example, adverse maternal-infant interactions and perinatal exposure to infections result in impaired stress responses and altered emotional behavior (19, 20, 22). Prolonged, early maternal separation results in adult rats with hyper-reactive LHPA activity, increased hypothalamic corticotrophin releasing hormone (CRH), and heightened anxiety (19, 31).

Hypoglycemia is a potent physiological stressor, inducing large increases in plasma adrenocorticotrophin hormone (ACTH) and corticosterone levels in both young and adult rats (4, 26). Thus repeated hypoglycemic insults during early postnatal development may exert a programming effect on the adult LHPA axis. Indeed, Moore and colleagues (25) found that insulin-induced RHG during early development (postnatal day (P) 10-19) in male rats leads to increased behavioral and hormonal responsiveness to stress (i.e., exaggerated acoustic startle
reflex, increased fear-potentiated startle, and increased behavioral and corticosterone (CORT) responses to restraint stress) that persist into early adulthood.

To gain insights into the long-term programming effects of early RHG on the regulation of the LHPA axis at the molecular level, the present study employed a developmental model of insulin-induced RHG (spanning P24-28) in rats and then assessed the long-term effects (at P90) on transcript levels of key proteins relevant to LHPA axis function as well as the CORT responses to ACTH stimulation of harvested adult adrenal tissue and restraint stress of adult male rats. Rats of this age are used to model the effects of insulin-induced RHG in young children with type-1 diabetes (45, 46). It is worth noting that repeat bouts of hypoglycemia have been associated with long-term neurological deficits in human infants (10, 21). Based upon the LHPA axis effects of other stressors (19) and the behavioral and hormonal effects Moore and colleagues (2009) observed following early RHG, we hypothesized that early RHG would program the LHPA axis to have a higher than normal basal level and a hyper-reactive stress response. However, the early RHG model used in the present study led to a hypo-reactive LHPA axis in adulthood evidenced by attenuated CORT response to ACTH-challenged of dispersed adult adrenocortical cells or following restraint stress of adult male rats. These effects were accompanied by the upregulation of hippocampal corticosteroid receptors (GR and MR) and adrenal steroidogenic-acute-regulatory protein (StAR) and GR as well as downregulation of hypothalamic corticotropin releasing hormone (CRH) and adrenal MR, melanocortin-type-2 receptor (MC2R) and low-density lipoprotein receptor (LDLR) in adult rats. Additionally, early RHG led to regional dysregulation of glucose transporters with reduced adult hypothalamic and pituitary GLUT3, and increased pituitary GLUT1.
MATERIALS and METHODS:

Animals

Gestational day 2 pregnant Sprague-Dawley rats were purchased (Charles River Laboratories, Raleigh, NC) and allowed to deliver spontaneously. Dams and pups were housed in a temperature- and humidity-controlled animal care facility with 12-hr/12-hr light/dark cycle and were allowed food and water *ad libitum*. Cages were changed weekly by Research Animal Resources’ personnel. Litter size was culled to 8 on P3 with six males and two females. Four litters were used for gene expression analysis and in vitro ACTH stimulation. Another four litters were used for restraint stress study. Pups were weaned on P21 and littermates were housed together with 4 rats/cage until P65, at which time they were split to 2 rats/cage. Rats were tracked by ear punches. Only male offspring were used in experiments. The University of Minnesota Institutional Animal Care and Use Committee approved all experimental protocols.

Induction of hypoglycemia

Hypoglycemia was produced once daily between 8 AM and 10 AM from P24 to P28 (totaling 5 episodes) as described previously (11) with modifications. Rats were not fasted prior to induction of hypoglycemia. Half the litter (3 males/litter) was randomly selected for injection with human regular insulin (Novo Nordisk Inc., Clayton, NC) at a dose of 10 IU/kg i.p. (RHG group). The target blood glucose concentration was <40 mg/dl, a value conventionally used to defined hypoglycemia during development. The other half of the litter was injected with equivalent volume of 0.9% saline (control group). Rats in both groups were fasted following insulin injection (water available) and maintained at an ambient temperature of 34.0 ± 1.0°C.
Blood glucose concentration was measured every 30 min using a glucometer (Accu-Chek® Compact; Roche Diagnostics, Indianapolis, IN). Insulin injected rats were closely monitored for signs of seizures (rhythmic tail flicks, jerking of limbs or arching), and 10% dextrose was administered in a dose of 200 mg/kg s.c. if the blood glucose was <20 mg/dL to prevent seizures and death. Hypoglycemia was terminated 120 min after insulin injection by administering 200 mg/kg of 10% dextrose i.p. All rats (control and hypoglycemic) were injected with 10% dextrose and introduced to food immediately following injection. All rats were confirmed for euglycemia (blood glucose >50 mg/dL) and normal activity 2 hr after returned to their home cages with *ad libitum* food.

*Tissue collection*

Rats were killed on P90 using sodium pentobarbital (100 mg/kg i.p.). Following the removal of brains from the crania, pituitary glands were collected. Hypothalami were dissected using the optic tract and mammillary recess as the anterior and posterior limits, respectively, and the brains were microdissected for hippocampi. Collected tissues were immediately flash-frozen in liquid nitrogen and stored at -80°C for RNA isolation. Adrenal glands were removed and one gland was flash-frozen while the other was immersed in cold PBS for cell dispersion.

*Quantitative RT-PCR (qPCR)*

The qPCR protocols were carried out as described previously (39) with n=8/group (2 rats from each of the four litters). For frozen tissues (hippocampus, hypothalamus, pituitary and adrenal gland), total RNA was isolated using an RNA-isolation kit (Zymo Research, CA) and concentrations were measured by absorbance at 260 nm (A<sub>260</sub>) using a NanoDrop ND-1000
(NanoDrop Technologies, Inc., Wilmington, DE). The cDNA was generated from 500 ng of total RNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) per manufacturer recommendation. The resulting cDNA was diluted 10-fold to give a 200 µL final volume. For dispersed adrenocortical cells, total RNA was isolated using RNaqueous™ RNA isolation kit (Ambion Inc., Austin, TX); 80 ng total RNA was used for cDNA synthesis in a 40 µL total volume, which was used for qPCR analysis without further dilution. All qPCR experiments were performed with the use of Taqman® Gene Expression Assay probes, which are presented in Table 4. Ribosomal protein S18 was used as an internal control. Thermocycling was carried out according to the manufacturer’s protocol using a MX3000P instrument (Stratagene, La Jolla, CA).

Adrenal cell dispersion

Experiments were carried out using previously described methods (39) with modifications. In brief, following removal of fat and capsule from the adrenal glands, adrenal cortices were detached and placed into a few drops of dispersion media (Dulbecco’s Modified Eagle Media [DMEM], 0.32% collagenase [type I, Gibco BRL], 4% BSA [Sigma], 0.1% DNAse [Sigma]). Tissues were then minced with surgical scissors, transferred to dispersion media and incubated for 90 min in a 37°C and 10% CO₂ cell culture incubator with trituration at 15 min intervals. Dispersed cells were filtered through a 100 µm wire mesh into wash media (DMEM, 0.4% BSA, 0.28% HEPES) and centrifuged at 200 g for 5 min. Following removal of the supernatant, cells were rinsed in wash media and resuspended in incubation media (Wash media + 7.65mM CaCl₂). Cells were seeded at 8,000 cells/well in a 96-well plate and incubated at 37°C and 10% CO₂ for 2 hr. For each group (n=8), left adrenals were collected from each rat and divided into two
subgroups to prepare two separate batches of dispersed cells. Cells were then stimulated with ACTH at 5, 10, 25, 50, 100, 250, and 500 pg/ml (1-39, Tocris Bioscience, Ellisville, MO), angiotensin II at 5, 10, 25, 50, 100, 250, and 500 ng/ml (Ang II; Sigma), or potassium (K⁺) at 5.3, 7.3, 9.3, 13.3, and 17.3 mM, and incubated overnight. Following overnight incubation, media were collected for corticosterone measurements and cells were lysed in RNA lysis buffer for RNA isolation (RNaqueous™, Ambion Inc., Austin, TX). Cell stimulations were carried out in triplicates for each dose sequentially using the same batch of dispersed cells. Cell stimulations were repeated to confirm findings using another batch of dispersed cells.

**Restraint Stress**

P90 control and early RHG rats (n=8/group) were restrained in pairs (one control and one RHG) for 30 min, a duration described in (25), using plastic tubes with a narrow end that exposed rat’s head. Pre-stressed sera were collected by tail bleed immediately following the placement of rats into restraint devices. Post-stressed sera were collected from trunk blood following decapitation at conclusion of the restraint period.

**CORT measurements (RIA)**

The corticosterone concentration was determined using a commercially available radioimmunoassay kit (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA). The intraassay and interassay CVs for CORT were 9.7 and 11.6%, respectively.

**Statistical methods**
Mean difference between groups for plasma CORT and mRNA levels were analyzed using Unpaired t-test. Levels of plasma CORT were analyzed by 2-way ANOVA to test for the effects of group and stressor. Levels of CORT in culture media and mRNA measured from dispersed adrenocortical cells were analyzed by 2-way ANOVA to test for effects of group and ACTH stimulation. Statistical analyses were performed and graphs were generated with GraphPad Prism (GraphPad Software Inc., San Diego, CA). Significance was set at alpha <0.05.

RESULTS:

**Blood glucose and growth measures**

The target blood glucose concentration for hypoglycemia (<40 mg/dl) was achieved within 30 min of insulin administration and maintained until 10% dextrose administration (120 min post-insulin injection; resulting in at least 90 min of hypoglycemia) on each of the 5 days (P24-28) in the RHG group. Compared with the saline-injected control group, the blood glucose concentration was lower in the RHG group during the period of observation (Table 1). The blood glucose concentrations were similar in the control and RHG groups 2 hr after animals were returned to their home cages (Table 1). Early RHG did not affect the rate of growth, and body weights were comparable at adulthood in the two groups (Table 1). Three rats in the RHG group had clinical evidence of seizures during hypoglycemia and were excluded from further analysis.

**Increased adult hippocampal GR and MR levels following early RHG**

We reasoned that if early RHG led to a hyper-reactive LHPA axis in adult animals, it would also affect the expression of adult hippocampal corticosteroid receptors, which mediate glucocorticoid feedback inhibition (13, 34). We measured transcript levels of *GR* and *MR* to test
the effects of early RHG on the expression of hippocampal corticosteroid receptors in adulthood.

Compared with saline-injected controls, early RHG rats showed increased mRNA levels of GR and MR in the adult hippocampus (Table 2 and Figure 1A).

**Altered adult hypothalamic and pituitary expression of glucose transporters and stress regulators following early RHG**

To determine the effects of early RHG on expression of mediators for glucose metabolism and stress responses in the adult hypothalamus and pituitary, we compared transcripts levels of relevant genes in adult RHG and control rats. Following early RHG, the level of neuronal glucose transporter GLUT3 mRNA was lower in the adult hypothalamus and pituitary compared with saline-injected control rats (Table 2 and Figure 1B-C). The level of GLUT1 mRNA was higher in the adult pituitary following early RHG compared with control rats (Table 2 and Figure 1B), but was comparable between the groups in the adult hypothalamus (Table 2). Both CRH and GR mRNA levels were lower in the adult hypothalamus of the early RHG group (Figure 1B). Hypothalamic Avp, MR and GLUT4 mRNA levels were not different between groups. Adult transcript levels of GR, MR, GLUT4, and proopiomelanocortin (POMC) in the pituitary were not different between groups (Table 2).

**Lower LDLR, MR, and MC2R accompanied by higher GR and StAR levels in adult adrenal glands following early RHG**

To examine whether early RHG also has specific effects on steroidogenesis in adult adrenal glands, we assessed mRNA levels of cholesterol transporters, steroidogenic enzymes, ACTH receptor (MC2R), and corticosteroid receptors in adult rats. The adult adrenal glands following
Early RHG showed lower levels of LDLR, MC2R, and MR, accompanied by higher mRNA levels of StAR and GR compared with those of saline-injected control rats (Table 2 and Figure 2). The mRNA levels in the adult adrenal glands for genes encoding the P450 steroidogenic enzymes (i.e., $3\beta$-HSD, CYP11A1, CYP11B1, CYP11B2, CYP17A1, and CYP21A1) were not different between groups (Table 2).

Attenuated CORT response to ACTH in dispersed adult adrenocortical cells following early RHG

To assess whether early RHG alters the capacity of adult adrenocortical cells to respond to stress, we stimulated dispersed adrenocortical cells (from fasciculata and reticularis zones) with ACTH and measured the level of secreted CORT. The CORT response of adult adrenals to ACTH stimulation following early RHG was blunted compared with that of control rats (Figure 3A). As negative controls, both Ang II and K$^+$ did not elicit a CORT response from these dispersed adrenocortical cells in either group following overnight incubation (Data not shown). To identify possible factors contributing to the attenuated steroidogenic response to ACTH, we quantified the mRNA levels of LDLR, MC2R, and StAR from ACTH-stimulated dispersed adrenocortical cells. The transcript levels of LDLR and StAR showed a blunted response to ACTH in early RHG compared with that of control rats (Figure 3B, C), whereas the MC2R transcript level was similar between the two groups (Data not shown).

Early RHG led to lower CORT response in restraint stress of adult male rats

To confirm the effect of early RHG on stress-induced CORT responsiveness in vivo, we subjected P90 rats to a 30 min restraint, which activated the stress responses and induced CORT
secretion (Figure 4A). While pre-stress plasma CORT levels were not different, albeit trending lower in RHG group (P =0.08), post-stress levels were lower in RHG compared to control group (Figure 4B). Analysis of variance identified significant effects for both stressor and group (Table 3).

DISCUSSION:
The present study utilized a model of recurrent insulin-induced hypoglycemia in young rats demonstrated that early RHG leads to altered hypothalamic and pituitary expression of glucose transporters, increased hippocampal GR and MR, increased adrenal StAR and GR, and decreased adrenal LDLR, MC2R and MR expression in adulthood. Contrary to our hypothesis that early RHG would program a hyper-responsive LHPA axis, the present study shows that early RHG led to attenuated CORT responses to the ACTH stimulation of dispersed adult adrenocortical cells and restraint stress of adult rats, suggesting that our early RHG model programmed a hypo-responsive LHPA axis. These findings suggest a complex environment-gene interaction between early RHG and LHPA axis development, similar to the LHPA programming found in adult rats handled during early postnatal periods (7).

The upregulation of adult pituitary GLUT1 following early RHG in the present study is likely serving to facilitate glucose transport. A similar effect has been demonstrated at the blood brain barrier level following chronic hypoglycemia in adult rats (37). Thus there could be a beneficial adaptive augmentation of glucose transport derived from early RHG through alteration of regional expression of glucose transporters that may provide neuroprotection during future episodes of hypoglycemia. The lack of GLUT1 upregulation in the hypothalamus is not
surprising, given the low glucose requirements of this brain region (11, 27). Unlike the extensive
data on GLUT1, the effect of RHG on brain GLUT3 remains controversial with data showing
both increased (9, 40) or no change (35) in expression following RHG in adult rats. The
observed downregulation of adult hypothalamic and pituitary GLUT3 in the present study
suggests a persistent reduction in neuronal glucose transport. This downregulation may result
from attenuated adrenal function following early RHG (5). Another possibility is that dextrose
infusions received at the end of the hypoglycemic period could alter GLUT regulation.
However, this is a remote possibility, since acute or chronic hyperglycemia does not alter GLUT
protein expression in adult rats (35). While our early RHG rat model is the first to demonstrate
long-term regional changes in GLUT regulation, additional studies are needed to unequivocally
establish such effects.

The hypo-responsive LHPA axis of adult rats following early RHG could be a beneficial
adaptation that could spare the animal from the negative effects associated with LHPA
hyperactivity (23). Our finding differs from those of Moore and colleagues (25), which suggest
a hyper-responsive LHPA axis following early RHG. These different effects likely stem from
variations in the timing and bouts of RHG. The model of early RHG in the present study was
neither as early in development (P24-28 vs. P10-19) nor as frequent (5 vs. 20 episodes) as the
study conducted by Moore and colleagues (25). These variables would produce different effects
of circulating glucocorticoids on the developing LHPA axis. In particular, hippocampal MR and
GR are most sensitive to circulating CORT during P18-P28 (43), which overlaps extensively
with our early RHG model. Moreover, studies of neonatal rats separated from their mothers
indicate that prolonged periods of maternal separation results in adult rats with hyper-reactive
LHPA and heightened anxiety, whereas brief periods of maternal separation results in adult rats that are generally stress-resistant, with hypo-reactive LHPA (19, 31). Thus, the differences between the timing and severity of hypoglycemia in the present study and that by Moore and colleagues (25) may explain the disparate programming effects of RHG on the LHPA axis. These differences emphasize the need to use developmentally appropriate and sensitive models when studying the developmental origins of disease, particularly when relating the model effects to human developmental time periods (28). For example, juxtaposing the current study with the Moore et al (2009) findings would suggest that repetitive hypoglycemia occurring in a 2-5 year old child (i.e. P24-28 in the rat) due to insulin treatment may have greater long-term ramifications than similar repetitive hypoglycemia in a neonate (i.e. P9-10 in the rat). We postulate that the recurrent RHG-induced elevations in plasma CORT during the developmental period P24 to P28 (44) and the lesser degree of hypoglycemic severity in the present study are responsible for programming a hypo-responsive LHPA axis. Future studies should seek to characterize the parameters of such a critical period and the role of RHG severity and duration in programming the LHPA axis.

The observed lower hypothalamic CRH and higher hippocampal GR and adrenal StAR support the conclusion of a hypo-responsive LHPA axis following this model of early RHG. The effect is consistent with the increased hippocampal GR seen in rats with hyporesponsive LHPA axes (7, 19). Each of these alterations could be a compensatory adaptation to impaired CORT production. Hippocampal GR is normally upregulated by feedback when corticosteroid levels are low (3). Thus, the attenuated CORT production in adult adrenals following early RHG could have caused an upregulation of GR to facilitate more effective glucocorticoid feedback.
Likewise, the upregulation of adult adrenal Star, a necessary carrier for cholesterol transport (2), suggests an increased availability for transporting cholesterol to be used in steroidogenesis. Finally, reduced in vitro CORT response to ACTH would normally be associated with a lower Star expression (12). Decreased Star expression following ACTH stimulation was indeed observed in the adult adrenocortical cells of early RHG compared to that of control rats (Figure 3C).

Higher hippocampal Mr and lower adrenal Mc2r and Ldlr in adulthood following early RHG may underlie the impaired CORT production. Increased hippocampal Mr would enhance its action in modulating inhibitory tone over the LHPA axis, thereby maintaining a low level of LHPA activity at basal conditions as well as during stress responses (7). These are consistent with the lower pre- and post-stress plasma CORT levels in early RHG compared to control rats (Figure 4B). Lower adrenal Mc2r may reflect an adaptive response to perturbations in systemic glucose homeostasis in the developing adrenal cortex, because MC2R function is important for adrenal development and regulation of gluconeogenesis (6). Finally, a 49% lower adult adrenal Ldlr level following early RHG is key in attenuated adrenal CORT production, which would reduce the endosomic uptake of cholesterol precursors, necessary substrates for adrenal steroidogenesis (14, 16, 17). Also, adrenal LDLR expression is regulated by ACTH (17). The muted LDLR response to ACTH stimulation in dispersed adult adrenocortical cells derived from early RHG rats suggests a programming effect on LDLR regulation. Other contributing factors may include the alteration of neuropeptides and neuromodulators (e.g., substance P, vasoactive intestinal polypeptide, neuropeptide Y, and cholecystokinin) that mediate CORT production (3). Future studies could focus on how early RHG effects the regulation of these factors.
The finding of lower adult hypothalamic $GR$ level following early RHG is unexpected because downregulation of $GR$ is normally associated with resistance to glucocorticoid feedback inhibition and lower hypothalamic GABAergic-mediated inhibition (7). This finding suggests that enhanced negative feedback at the hypothalamic level may involve an overriding action of MR-mediated inhibitory input from extra-hypothalamic networks in these animals (7). Another potentially relevant finding is the changes in adult adrenal $GR$ and $MR$ levels of early RHG rats. Whether these changes are important factors in producing a hypo-responsive LHPA axis will require more studies to establish if early RHG alters the cytoarchitecture of the limbic-hypothalamic inputs and the role of glucocorticoid receptors in adult adrenal glands.

Perspectives and Significance

Contrary to our initial hypothesis, we found evidence of a hypo-responsive LHPA axis with altered expression of stress response regulators and glucose transporters in adult rats of an early RHG model. Our findings underscore the need to consider developmentally appropriate and sensitive models when studying the developmental origins of disease, especially when relating the model effects to human developmental time periods. The present study demonstrates the persistent effects of early RHG into adulthood and pinpoints molecular substrates for future studies to delineate the underlying mechanisms of early RHG-mediated developmental programming of the LHPA axis. The insights gained from this study could lead to novel strategies in the development of therapeutic interventions aimed at reversing the long-term impact of an adverse perinatal environment on the postnatal development of the major physiologic regulatory systems.
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DISCLOSURES:

The authors have no financial interest or potential conflict of interest to disclose.
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16. **Kovanen PT, Faust JR, Brown MS, and Goldstein JL.** Low density lipoprotein receptors in bovine adrenal cortex. I. Receptor-mediated uptake of low density lipoprotein and utilization


FIGURE LEGENDS:

Figure 1: Altered transcript levels in P90 adult hippocampus, hypothalamus, and pituitary following early RHG. A) GR and MR mRNA levels in the adult hippocampus. B) GLUT3, GR and CRH mRNA levels in the adult (P90) hypothalamus. C) GLUT1 and GLUL3 mRNA levels in the adult pituitary. Pituitary POMC and GR mRNA levels were not different between groups. Data are normalized to the saline-injected control group. Values are mean ± SEM, n=5-8/group. Asterisk denotes significant p values (*\(P<0.05\), **\(P<0.01\)).

Figure 2: Altered adult adrenal mRNA levels of LDLR, MC2R, StAR, GR, and MR of early RHG male rats. Data are normalized to the saline-injected control group. Values represent mean ± SEM, n=4-6/group. Asterisks denote significant differences (*\(P<0.05\)).

Figure 3: CORT response and mRNA levels of LDLR, StAR and MC2R in adult dispersed adrenocortical cells following ACTH stimulation. A) Attenuated ACTH-induced CORT production of dispersed adult adrenocortical cells derived from early RHG group (\(P<0.01\), 2-way ANOVA, treatment and group effects). B) Decreased LDLR mRNA levels in cells derived from early RHG compared with control group (\(P<0.001\), 2-way ANOVA, treatment and group effects). C) Reduced StAR mRNA levels in cells derived from early RHG compared with control group (\(P<0.01\), 2-way ANOVA, treatment and group effects). MC2R mRNA levels were not different between groups.

Figure 4. Plasma CORT levels of P90 male rats before and after 30 min restraint stress. A) Changes in CORT levels in individual rats following restraint stress. B) CORT levels within
groups before (Pre) and after (Post) restraint stress. Values are mean ± SEM, n=7/group;

*P<0.05, **P<0.01, Unpaired t-test.
Table 1: Serum glucose and growth measurement.

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<th>Variables</th>
<th>Control</th>
<th>P24-28 Repetitive Hypoglycemia</th>
<th>Unpaired t-test P-values</th>
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<td>Blood glucose during hypoglycemia (mg/dl)</td>
<td>132 ± 12 (n=12)</td>
<td>31 ± 15 (n=12)</td>
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<td>Blood glucose 2 hr after hypoglycemic cessation (mg/dl)</td>
<td>139 ± 18 (n=8)</td>
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<td>Adult body weight (g)</td>
<td>345 ± 23 (n=8)</td>
<td>359 ± 32 (n=8)</td>
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Values are mean ± SD (n); ns, not significant.
Lehmann et al., FIGURE 1
Lehmann et al., FIGURE 2
Lehmann et al., FIGURE 3
Table 2: Quantitative measurements of mRNA in the LHPA axis of adult male rats. Data are normalized to control.

<table>
<thead>
<tr>
<th>Transcript by Region</th>
<th>Ratio of Control</th>
<th>Unpaired t-test P values</th>
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<td>GR</td>
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<td>MR</td>
<td>1.00 ± 0.08 (6)</td>
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<td>Avp</td>
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<td>GLUT1</td>
<td>1.00 ± 0.13 (5)</td>
<td>1.19 ± 0.07 (5)</td>
</tr>
<tr>
<td>GLUT3</td>
<td>1.00 ± 0.18 (5)</td>
<td>0.78 ± 0.10 (6)</td>
</tr>
<tr>
<td>GLUT4</td>
<td>1.00 ± 0.14 (5)</td>
<td>1.02 ± 0.12 (5)</td>
</tr>
<tr>
<td>POMC</td>
<td>1.00 ± 0.31 (5)</td>
<td>0.82 ± 0.15 (5)</td>
</tr>
<tr>
<td><strong>Adrenals</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GR</td>
<td>1.00 ± 0.13 (4)</td>
<td>1.49 ± 0.26 (4)</td>
</tr>
<tr>
<td>MR</td>
<td>1.00 ± 0.20 (4)</td>
<td>0.54 ± 0.12 (4)</td>
</tr>
<tr>
<td>StAR</td>
<td>1.00 ± 0.15 (4)</td>
<td>1.53 ± 0.26 (5)</td>
</tr>
<tr>
<td>MC2R</td>
<td>1.00 ± 0.09 (4)</td>
<td>0.84 ± 0.07 (4)</td>
</tr>
<tr>
<td>LDLR</td>
<td>1.00 ± 0.24 (5)</td>
<td>0.61 ± 0.23 (6)</td>
</tr>
<tr>
<td>3β-HSD</td>
<td>1.00 ± 0.25 (4)</td>
<td>1.30 ± 0.21 (4)</td>
</tr>
<tr>
<td>CYP11A1</td>
<td>1.00 ± 0.26 (4)</td>
<td>0.97 ± 0.20 (6)</td>
</tr>
<tr>
<td>CYP11B1</td>
<td>1.00 ± 0.51 (6)</td>
<td>0.95 ± 0.57 (6)</td>
</tr>
<tr>
<td>CYP11B2</td>
<td>1.00 ± 0.40 (5)</td>
<td>1.02 ± 0.66 (4)</td>
</tr>
<tr>
<td>CYP17A1</td>
<td>1.00 ± 0.76 (5)</td>
<td>0.98 ± 0.84 (4)</td>
</tr>
<tr>
<td>CYP21A1</td>
<td>1.00 ± 0.16 (4)</td>
<td>1.14 ± 0.11 (5)</td>
</tr>
</tbody>
</table>

Values are mean ± SD (n); ns, not significant.
Lehmann et al., Figure 4
Table 3: Plasma CORT of restraint stress of adult P90 male rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>CORT (ng/ml)</th>
<th>2-Way ANOVA P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-stress</td>
<td>Post-stress</td>
</tr>
<tr>
<td>Control</td>
<td>140 ± 47.1</td>
<td>729 ± 78.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(n=7)</td>
<td>(n=7)</td>
<td></td>
</tr>
<tr>
<td>P24-28 Repetitive Hypoglycemia</td>
<td>103 ± 18.4</td>
<td>609 ± 77.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(n=7)</td>
<td>(n=7)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SD (n); a>b with P<0.01, Bonferroni post-hoc t-test.
Table 4: Identification of transcripts assessed.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene bank accession #</th>
<th>ABI Assay ID</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR</td>
<td>NM_012576</td>
<td>Rn00561369_m1</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>MR</td>
<td>NM_013131</td>
<td>Rn00565562_m1</td>
<td>Mineralocorticoid receptor</td>
</tr>
<tr>
<td>GLUT1</td>
<td>NM_138827</td>
<td>Rn01417099_m1</td>
<td>Blood-brain barrier glucose transporter</td>
</tr>
<tr>
<td>GLUT3</td>
<td>NM_017102</td>
<td>Rn00567331_m1</td>
<td>Neuronal glucose transporter</td>
</tr>
<tr>
<td>GLUT4</td>
<td>NM_012751</td>
<td>Rn00562597_m1</td>
<td>Insulin-regulated glucose transporter</td>
</tr>
<tr>
<td>Avp</td>
<td>NM_016992</td>
<td>Rn00566449_m1</td>
<td>Arginine vasopressin</td>
</tr>
<tr>
<td>CRH</td>
<td>NM_031019</td>
<td>Rn01462137_m1</td>
<td>Corticotropin releasing hormone</td>
</tr>
<tr>
<td>POMC</td>
<td>NM_139326</td>
<td>Rn00595020_m1</td>
<td>Pro-opiomenocortin</td>
</tr>
<tr>
<td>StAR</td>
<td>NM_031558</td>
<td>Rn00580695_m1</td>
<td>Adrenal mitochondrial cholesterol transporter</td>
</tr>
<tr>
<td>LDLR</td>
<td>NM_175762</td>
<td>Rn00598442_m1</td>
<td>Low density lipoprotein receptor</td>
</tr>
<tr>
<td>MC2R</td>
<td>NM_001100491</td>
<td>Rn01491505_m1</td>
<td>Adrenal ACTH receptor</td>
</tr>
<tr>
<td>3β-HSD</td>
<td>NM_001042619</td>
<td>Rn01789220_m1</td>
<td>Steroidogenic enzymea</td>
</tr>
<tr>
<td>CYP11A1</td>
<td>NM_017286</td>
<td>Rn00568733_ml</td>
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<td>CYP11B1</td>
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<td>Rn02607234_gl</td>
<td>Steroidogenic enzymec</td>
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<td>CYP11B2</td>
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<td>Rn02396730_g1</td>
<td>Steroidogenic enzymed</td>
</tr>
<tr>
<td>CYP17A1</td>
<td>NM_012753</td>
<td>Rn00664858_ml</td>
<td>Steroidogenic enzymee</td>
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<td>CYP21A1</td>
<td>NM_057101</td>
<td>Rn00588996_g1</td>
<td>Steroidogenic enzymef</td>
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<tr>
<td>S18 ribosomal</td>
<td>NM_213557</td>
<td>Rn01428915_g1</td>
<td>Internal control</td>
</tr>
</tbody>
</table>

a Converts pregnenolone to progesterone  
b Converts cholesterol to pregnenolone  
c Converts 11-deoxy-corticosterone to corticosterone  
d Converts corticosterone to aldosterone  
e Converts pregnenolone prenenolone to 17a-hydroxypregnenolone and then to DHEA, converts progesterone to 17a-hydroxy-progesterone and then to androstenedione  
f Converts progesterone to 11-deoxy-corticosterone, converts 17a-hydroxy-progesterone to 11-deoxy cortisol