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Alterations in stress responses of the hypothalamic-pituitary-adrenal axis in small for gestational age infants

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Summary Mounting epidemiologic evidence and animal models suggest that stressful conditions during the intrauterine period may increase susceptibility to several adult conditions, including metabolic syndrome, cardiovascular disease, and psychiatric disorders. Increased cortisol levels due to alterations in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis are believed to be one mediating mechanism. Infants born after significant exposure to stressful conditions are often small for gestational age (SGA) based on standardized growth norms. Lifelong programming of the HPA axis has been proposed as a mechanism to explain the association between SGA infants and adult disease. However, few studies have measured HPA axis function proximal to birth as done in this study of SGA infants during the first week of life. Participants included 37 infants in two groups based on birth size (gestational age range: 34—41 weeks). SGA infants were <10th percentile for age (n = 21) and appropriate for gestational age (AGA) infants (n = 16) were from 20 to 90th percentile for age. Cortisol response to a heel lance for blood collection was measured for all infants. Hierarchical Linear Modeling was used to test the effect of AGA/SGA group status on cortisol trajectories in response to the stressor. Group was a significant predictor of quadratic slopes (t = 2.84, χ^2 = 8.19, p = .004) after controlling for the effect of group on intercepts and linear slopes. Predicted growth curves for ln-cortisol were plotted for each group based on regression coefficients. The predicted curves capture the significant group difference in trajectories, as well as the blunted response for the SGA group and the robust peak in cortisol production in response to the stressor for the AGA group. This evidence suggests SGA neonates have blunted HPA axis responses to stressors in comparison to AGA infants. These findings are consistent with animal models showing that adverse intrauterine conditions can result in blunted cortisol responses to acute stressors and may provide a mechanism for adult susceptibility to disease for individuals that are SGA at birth.

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1. Introduction

Mounting epidemiologic evidence and animal models have demonstrated that stressful conditions during intrauterine life may have a substantial impact on susceptibility to several adult conditions including metabolic syndrome, cardiovascular disease, and psychiatric disorders such as depression (Barker et al., 1989a,b; Kajantie et al., 2002; Matthews, 2002; Newsome et al., 2003; Ward et al., 2004). These associations have been found to be independent of adult size or lifestyle. One mediating mechanism is believed to be intrauterine exposure to stress leading to alterations in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis resulting in increased cortisol levels in both mothers and infants. Over time, increased cortisol levels have been shown to negatively affect the functioning of biologic systems (Barker et al., 1989a,b; McEwen, 2008). Infants who are born after significant exposure to adverse conditions are often small for gestational age (SGA) based on standardized growth norms. Lifelong programming of the HPA axis has been proposed as a mechanism to explain the association between low birth weight infants and later development of the metabolic syndrome and hypertension as adults (Barker et al., 1989a,b; Phillips et al., 1998; Reynolds and Phillips, 1998). Although most of the previous epidemiologic research uses low birth weight as a measure of reduction in intrauterine growth, it is important to distinguish between those infants that are smaller than expected size for their gestational age norms. SGA infants are defined unequivocally as those $<$10th percentile for gestational age which more accurately identifies a population of fetuses experiencing adverse intrauterine conditions. By including all low birth weight infants, one fails to distinguish premature infants from those who are potentially full term, but smaller than expected for age. With the inclusion of only SGA infants, one may expect to more clearly identify infants who had reduction in intrauterine growth as compared to others of the same gestational age.

Programming implies that an insult at a critical period of development results in permanent adaption of the organism's physiology or structure, in this case, altering the regulation of the HPA axis. While these theoretical proposals are biologically plausible, studies of adults who were low birth weight as infants provide only partial evidence for the lifelong programming hypothesis. These studies have not measured HPA axis function proximal to birth in SGA infants. The present study measured HPA axis function in SGA infants during the first week of life to address this gap in the literature.

Overactivity of the HPA axis has been associated with increased central adiposity, a known risk factor for cardiovascular disease (Larsson et al., 1992). Abnormally increased concentrations of glucocorticoids from pharmacologic administration of steroids or from conditions such as Cushing's syndrome are associated with increased abdominal fat deposition, hypertension, and glucose intolerance. The fetus is usually protected from normal levels of active glucocorticoids during pregnancy by the placental enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) which converts glucocorticoids to an inactive form (Clark et al., 1996). In a rat model, reduction in enzyme activity is associated with reductions in birth weight of offspring, supporting the hypothesis that increased exposure to active maternal steroid hormones due to either very high maternal levels that cannot be handled by the enzyme barrier or less efficient production of 11β-HSD may be one mechanism leading to small size at birth and the potential for long lasting effects on the HPA axis (Lindsay et al., 1994).

Despite the well-supported argument that chronic stress increases vulnerability to disease by increasing the activity of the HPA axis, there is a growing body of evidence demonstrating contradictory findings with stress-induced declines in cortisol. The possibility that chronic stress may both increase and decrease HPA activity exists, but likely at different time points over the course of the stressful period. Early in the course of a stressful situation, the HPA axis will be activated, elevating cortisol output. After a period of time if stress persists, the body will mount a counter-regulatory response, resulting in blunted cortisol output. Thus, after chronic stress, rather than an acute event, a period of hypoactivity is expected (Miller et al., 2007). Once the chronic stress is alleviated, measures may reveal that the axis has been reset at a higher level of activity (Gunnar and Vazquez, 2001).

This study evaluated stress reactivity in SGA neonates after birth to more fully understand the development of patterns of fetal neuroendocrine regulation that have been proposed as a predisposing factor in the pathogenesis of disease for former low birth weight infants. There are few studies to date that have examined HPA reactivity in SGA human newborns. Previous work by Schaffer et al. demonstrated a blunted cortisol response to a heel lance stressor in small for gestational age infants (Schaffer et al., 2009). Current evidence suggests that SGA infants have higher cortisol levels as adults, however, there is no data on when the actual shift from hyporesponsive to hyperresponsive cortisol reactivity occurs. In previous work by Grunau et al. (2004, 2007), salivary cortisol levels in preterm infants at 3 months corrected gestational age were lower than term infants, however, by 8 and 18 months corrected gestational age, there was a shift to significantly higher levels of salivary cortisol in the preterm groups. These results suggest a long term resetting of endocrine stress systems in these preterm infants possibly due to physiologic immaturity at birth with the cumulative stress of hospitalization, as well as the normal developmental changes in the fetal adrenal gland. This study hypothesized that late preterm and term SGA infants would also demonstrate resetting of endocrine stress systems similar to preterm infants.

2. Methods

2.1. Participants

The Institutional Review Board for the protection of human subjects approved all aspects of this study protocol. Informed consent was obtained from one or both parents prior to enrollment in this study.

The study sample comprised 37 participants who were born at a university-based children's hospital in the Midwest. Subjects were recruited serially into two groups based on size at birth. Group one (n = 21) included small for gestational age (SGA) infants with birth weight $<$10th percentile for gestational age based on the Fenton Growth Curve for Preterm and Term Infants. Group two (n = 16) included appropriate for

gestational age (AGA) infants with birth weight between the 20th and 90th percentiles. Gestational age was based on menstrual dating in conjunction with a <20 week ultrasound when available (n = 32 infants). All infants were between 34 and 41 weeks gestational age in order to select an appropriate medically well population with no previous antenatal betamethasone exposure.

Because cortisol levels increase over the last trimester of pregnancy, attempts were made to recruit equal numbers of SGA neonates and AGA neonates of the same gestational age. Infants in the SGA group had a mean gestational age of 36.3 weeks with a range from 34 to 41 weeks. Infants in the AGA group had a mean gestational age of 36.0 weeks with a range from 34 to 41 weeks. 56% of AGA infants were born prematurely at <37 weeks gestation and 57% of the SGA infants were born prematurely. Male and female participants were balanced with 50% of the AGA group male and 43% of SGA group male (chi-square p = 0.27, ns).

All SGA and AGA infants between 34 and 41 weeks gestational age without the following exclusion criteria were eligible for participation. Infants were excluded if prenatal or postnatal corticosteroid treatment was given. Also excluded were infants with any major chromosomal or genetic abnormality (e.g., trisomy 21), major neonatal illness (e.g., sepsis), severe hypoglycemia, intraventricular hemorrhage, mechanical ventilation, maternal adrenal illness, maternal diabetes, or maternal substance abuse (nicotine, alcohol). Clinical information for both groups is described in Table 1. There was no difference between the mothers in the two groups in age at delivery, race, whether prenatal care was received during the first trimester, or maternal education. Infants did not differ in Apgar scores, mode of delivery, or twin versus singleton delivery.

Table 1 Clinical characteristics of study sample.

	SGA (n = 21)	AGA (n = 16)	p-Value[a]
Maternal age	25.7, *SD* 3.4	26.1, *SD* 4.7	.76
Race			.57
Caucasian	13	11	
African American	4	3	
Hispanic	1	—	
Native American	3	2	
1st Trimester prenatal care	18	14	.88
Maternal education			.39
High school or equivalent	5	6	
University degree	13	9	
Post graduate degree	3	1	
Mean Apgar 1 min, 5 min	7, 8	6, 8	.20
Mode of delivery			.27
Vaginal	12	11	
Cesarean section	9	5	
Twins	6	4	.8

[a] Maternal age, prenatal care, Apgar score, and frequency of twins were compared using two-sample t-tests and race, maternal education, and mode of delivery using chi square test.

2.2. Procedures

The glucocorticoid hormone cortisol is the endpoint of HPA axis activation. Salivary cortisol was measured because it is a non-invasive, reliable method to measure the biologically active, non-protein bound plasma fraction of cortisol (Kirshbaum and Hellhammer, 1989). Salivary cortisol levels have been highly correlated with serum cortisol levels in premature and full term newborns (Gunnar, 1992; Calixto et al., 2002). The primary outcome measured in this study was change in salivary cortisol level in response to a physiologic stressor, a heel lance for blood collection.

Baseline salivary cortisol was collected between 2 and 5 days of age to allow the initial cortisol response to birthing to resolve. Samples were collected between 7 am and 9 am to standardize the results although previous research has shown that newborns have not yet coordinated circadian rhythms with time of day. Saliva was collected using a hydrocellulose microsponge device Sorbette by Salimetrics LLC. This device permits reliable collection of saliva volumes between 50 and 200 μL within a 1—2 min collection time (DeWeerth et al., 2007). Because the presence of a quiescent state is a critical control in infancy, collections were timed about 2 h after the last feeding and 1 h without infant handling according to procedures previously validated to examine HPA responsiveness by Davis et al. (2004), Gunnar (1989) and Gunnar et al. (1992, 1996). Infants were generally asleep at least 20—30 min prior to the baseline saliva collection.

Infants were weighed and measured and salivary cortisol was collected immediately prior to a heel lance for blood collection done for medically indicated tests such as the newborn metabolic screen or screening bilirubin. Repeat salivary cortisol was collected 20 min after the heel lance to determine the response to the stressor, and then at 45 min after the stressor to determine the recovery from the stressor.

The saliva samples were frozen and stored at −20 °C until analysis in an endocrine assay laboratory. After thawing, saliva samples were centrifuged at 2000 × g for 10 min, which resulted in a clear supernatant of low viscosity. 100 μL of saliva was used for duplicate analysis. Cortisol levels were determined employing a competitive solid phase time-resolved fluorescence immunoassay with fluorometric end point detection (DELFIA). 96-well-Maxisorb microtiter plates were coated with polyclonal swine anti-rabbit immunoglobulin. After an incubation period of 48 h at 4 °C plates were washed three times with wash buffer (pH 7.4). In the next step the plates were coated with a rabbit anti-cortisol antibody and incubated for 48 h at 4 °C. Synthetic saliva mixed with cortisol in a range from 0 to 100 nmol/L served as standards. Standards, controls (saliva pools) and samples were given in duplicate wells. 50 μL of biotin-conjugated cortisol was added and after 30 min of incubation the non-binding cortisol/biotin-conjugated cortisol was removed by washing (3×). 200 μL europium—streptavidin (Perkin Elmerc, Liefe Science Turku, Finland) was added to each well and after 30 min and 6 times of washing 200 μL enhancement solution was added (Pharmacia, Freiburg, Germany). Within 15 min on a shaker the enhancement solution induced the fluorescence which can be detected with a DELFIA-Fluorometer (Wallac, Turku, Finland). With a computer-controlled

program a standard curve was generated and the cortisol concentration of the samples was calculated. The intra-assay coefficient of variation was between 4.0% and 6.7%, and the corresponding inter-assay coefficients of variation were between 7.1% and 9.0% (Dressendorfer et al., 1992).

2.3. Statistical analyses

Cortisol variables were not normally distributed, so a natural logarithm transformation was applied to all cortisol variables in statistical analyses. We report analyses on 32 infants with cortisol data (only 1 missing sample at Time 1, 2 at Time 2, 1 at Time 3 from different infants; we did not use imputation methods given that less than 5% of data were missing). Two infants had entirely missing data due to difficulty in obtaining enough saliva and therefore could not be included. Additionally, 3 SGA participants had higher cortisol levels at baseline than other subjects (>2 *SD*s above the mean, $M = 8.3$ nmol/L, $SD = 9.34$ nmol/L), but the overall pattern of AGA/SGA group differences in cortisol trajectories was the same with and without them, thus we included them in all analyses (for predicted growth curves with these 3 infants, see Figs. 1 and 2; without them, see Fig. 3). We used Pearson correlations to examine the relationship between the cortisol response and body weight, and a *t*-test to examine whether the SGA and AGA groups were different at baseline. We then used Hierarchical Linear Modeling (HLM) implemented in the R software to model the cortisol trajectories of the two groups and examine static predictors of these trajectories (group, sex). The Level 1 model included time and time^2 as fixed effects to model the shape of the cortisol curve across time: $\text{LnCort}_{ij} = \beta_{1i} + \beta_{2i} \times \text{time}_{ij} + \beta_{3i} \times \text{time}_{ij}^2 + e_{ij}$. The Level 2 model included sex and group as predictors of intercepts and slopes

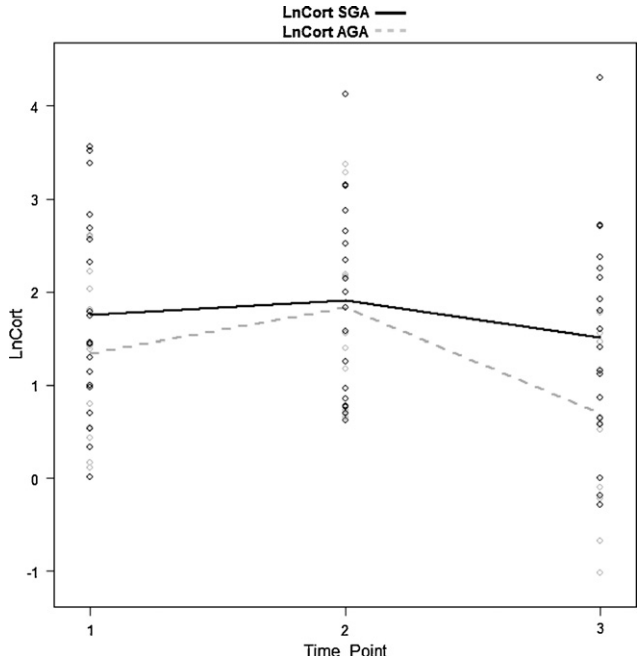

Figure 1 Group mean ln-cortisol values by AGA/SGA group status at each time point. Time 1 was the baseline measurement, Time 2 was 20 min from baseline, and Time 3 was 40 min from baseline.

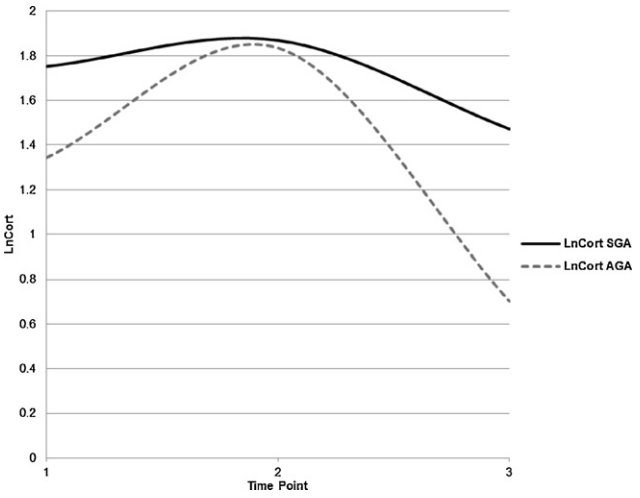

Figure 2 Predicted quadratic growth curves for ln-cortisol by AGA/SGA group status based on HLM regression coefficients.

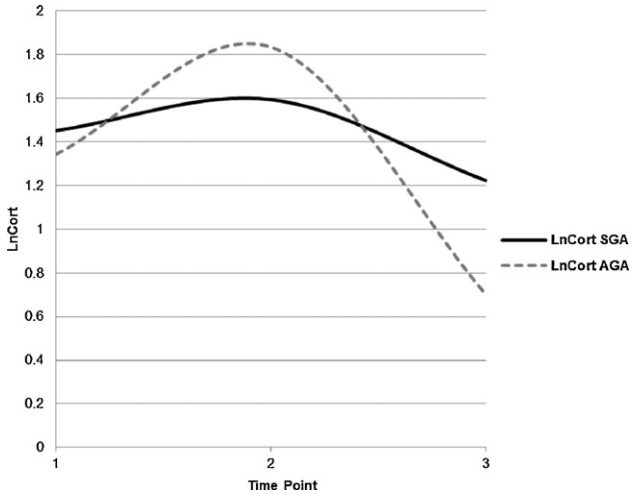

Figure 3 Predicted quadratic growth curves for ln-cortisol by AGA/SGA group status based on HLM regression coefficients, excluding 3 high baseline SGA subjects.

from Level 1 ($\beta_{1i} = \beta_1 + \beta_4 \times \text{sex}_i + \beta_7 \times \text{group}_i + b_{1i}$, $\beta_{2i} = \beta_2 + \beta_5 \times \text{sex}_i + \beta_8 \times \text{group}_i + b_{2i}$, $\beta_{3i} = \beta_3 + \beta_6 \times \text{sex}_i + \beta_9 \times \text{group}_i + b_{3i}$). We used a step-up analysis using nested χ^2 tests to examine the significance of each parameter and select the best-fitting model for the data at each level (steps consistent with HLM proposed by Raudenbush (2001), and Raudenbush and Bryk (2002). We first tested the Level 1 effects, then the random effects, and ultimately tested the Level 2 static predictors.

3. Results

Participants in the two groups were matched in gestational age (no group differences, $t(30) = 0.06$, $p = .96$), but the SGA group was significantly smaller in body weight ($t(30) = 3.97$, $p < .001$). Body weight was positively correlated with ln-transformed cortisol reactivity in the entire sample ($r = .38$, $p = .048$), but not within each of the groups. As will be shown

later, this may be due to the fact that the AGA group was characterized by both higher body weight and higher cortisol reactivity. However, it could also be the case that we had a lack of adequate statistical power to detect the correlation between body weight and cortisol reactivity in each of the two smaller groups, and the effect could only be detected in the larger sample. There were no baseline differences in cortisol levels between the two groups ($t(29) = 1.07$, *ns*).

To further explore group differences in the cortisol trajectories across the experiment (using normalized ln-transformed cortisol values), we used HLM. We first tested the fixed effects of time and time^2 for Level 1 of the model. There was a significant improvement in the intercept-only model by adding the linear time term ($p = .003$) and the quadratic time term ($p < .001$). The parameter estimates for the quadratic model are presented in Table 2. We then tested whether a random effect was needed for the linear slope, but since adding a random effect for the linear slope did not significantly improve the model ($\chi^2 = 4.68$, $p = .10$), we retained the model without it.

In the next step we tested Level 2 parameters by adding static predictors to the quadratic curve model to explain the differences in intercepts and slopes. Sex was not a significant predictor of intercepts ($\chi^2 = .05$, *ns*) and adding sex as a predictor of linear and then quadratic slopes did not improve model fit ($\chi^2 = .34$, .09, *ns*). Thus, we excluded sex from all future models.

We then tested the effect of AGA/SGA group status. Mean ln-cortisol growth curves for each group are presented in Fig. 1. Group was not a significant predictor of intercepts and adding it to the model did not improve model fit ($\chi^2 = 1.25$, *ns*). Group was also not a significant predictor of linear slopes ($\chi^2 = 2.38$, *ns*), but was a significant predictor of quadratic slopes ($t = 2.84$, $\chi^2 = 8.19$, $p = .004$) after controlling for the effect of group on intercepts and linear slopes. We plotted predicted growth curves for ln-cortisol for each of the two groups based on the regression coefficients (see Fig. 2). The predicted curves capture the significant group difference in trajectories, as well as the blunted response for the SGA group and the robust peak in cortisol production in response to the stressor for the AGA group. We report estimates for parameters in the final model in Table 3.

Table 2 Estimates for Level 1 parameters.

	Estimate	Std. error	*t*-Value
Intercept	0.37	0.40	0.92
Time	1.70	0.41	4.11
Time^2	−0.48	0.10	−4.68

Table 3 Estimates for parameters in final HLM model.

	Estimate	Std. error	*t*-Value
Intercept	−0.77	0.61	−1.27
Group	1.89	0.78	2.43
Time	2.93	0.61	4.79
Time^2	−0.81	0.15	−5.37
Group × time	−2.04	0.79	−2.59
Group × time^2	0.55	0.19	2.84

It must be noted that gestational age was not a significant predictor of the cortisol trajectory, since adding GA as a predictor of intercepts, linear, and quadratic slopes in our basic model of cortisol over time did not yield a significant improvement in model fit ($\chi^2 = 1.25$, $p = .23$). Furthermore, we found the same significant effect of AGA/SGA group status on quadratic slopes ($\chi^2 = 8.18$, $p = .004$) when including GA in the model to control for its effects on the intercept, the linear, and the quadratic slope. We also added an interaction term (GA × group) as a predictor of intercepts, linear and quadratic slopes, in addition to the effects of GA and group separately and there was no significant improvement in model fit ($\chi^2 = 1.46$, $p = .69$). Thus we excluded GA from our final model and growth curve estimates in Figs. 1–3 since the results hold with or without controlling for this potential confound.

4. Discussion

We provided evidence that SGA neonates have blunted HPA axis responses to stressors in comparison to AGA controls. These findings are consistent with previous animal studies showing that adverse intrauterine conditions can result in low cortisol responses to acute stressors, likely through alterations in the sensitivity and set points of the fetal HPA axis, which may impair feedback regulation (Murphy, 1982). Animal studies suggest that this process is likely mediated by alterations in the pituitary glucocorticoid receptor and adrenal ACTH receptor. However, we can only speculate on the mechanisms involved in this effect in human SGA newborns.

The concept of lifelong programming of the HPA axis has been proposed as a plausible mechanism to explain the association between SGA infants and later development of adult cardiovascular and metabolic disease with the implication that an insult during the critical window of fetal development resulted in permanent adaptation of the HPA axis. Adults born low birth weight have consistently demonstrated elevated cortisol responses; however, we provide evidence that SGA infants in the newborn period have blunted cortisol responses. Although these results may seem contradictory, likely they indicate a transient down-regulation of the HPA axis in response to chronic interuterine stress. Given the adult data on individuals who were SGA at birth, it seems that at some point this blunted response resolves and shifts to a higher set point for the axis. When and how this happens is uncertain, but as noted, Grunau et al. (2004, 2007) noted a shift between 3 and 8 months from hypo- to more hyperactivity of the axis in preterm infants. When and if this shift occurs for SGA infants and whether the influence of environmental factors such as parental sensitivity can moderate the HPA effects postnatally will be important to examine in the future.

There were several limitations in our study. The sample size is relatively small. The differences at varying gestational ages may be difficult to elucidate with this small sample size. Although we attempted to control for resting state and timing of feeding, there were a few infants unable to maintain a quiescent baseline which may have altered their cortisol results. Furthermore, it must be recognized that SGA status was used as a proxy for suboptimal gestational health, but it is neither a necessary nor sufficient condition

for identifying intrauterine stress exposure. It is possible for infants to experience prenatal adversity without being small for gestational age, as well as for infants to be small due to less detrimental circumstances (e.g., maternal stature). Future research should also examine infants at the other end of the continuum, who are large for gestational age, as this is also considered an important risk factor. Another factor that may limit our generalizability is the high level of education in our sample (70% of mothers have at least a University degree). Future studies should expand the range of SES investigated, especially given the widely acknowledged social stratification of health.

There were also several major strengths of this study including the ability to rule out alternative causes such as antenatal corticosteroid administration, maternal diabetes, and maternal smoking, all of which have been demonstrated to alter HPA activity in the newborn. Furthermore, we used a standard stressor paradigm (the heel lance), which has been shown in multiple studies to effectively activate the newborn HPA axis although with some degree of individual variation. There were three infants (all in the SGA group) who exhibited a very high level of baseline cortisol with a lack of a peak response to the stressor. We report the results both with and without these infants as they may represent interesting individual differences in HPA axis function, while supporting the overall pattern of results. While it is possible that these high baseline infants reacted to the novel testing environment and were not able to reach a quiescent baseline before the stimulus, it is also possible that these infants represent a different type of dysregulation with either very high basal cortisol output or hyper-reactivity to novelty accompanied by poor recovery over time.

In the future, it will be important to study these infants longitudinally to determine when and how the adaption from hypoactive to hyperactive HPA activity develops. We have provided evidence that the stress response in SGA infants may appear blunted as early as the first week of life. This study sheds light on the development of the HPA axis early in human ontogeny, providing critical information about the role of exposure to stressors and allowing the empirical test of programming hypotheses. This study also represents a successful translational effort from animal models to human development. Future studies should strive to identify factors that promote plasticity in the HPA axis and to continue to study mechanisms that may mediate the links between chronic intrauterine stress and risk for disease in adulthood.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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