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Postnatal Stress Produces Hyperglycemia in Adult Rats Exposed to Hypoxia-Ischemia

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ABSTRACT: Fetal or early postnatal stressors may predispose infants to develop diabetes, metabolic syndrome, or stroke. We hypothesized that postnatal stress will predispose animals to develop metabolic syndrome and impair the physiologic response to hypoxic-ischemic brain injury. We characterized the short- and long-term physiologic responses to postnatal stress by examining corticosterone (CS), glucose metabolism, and brain injury in neonatal and adult rats exposed to hypoxia-ischemia (H-I). Rat pups were divided into three levels of postnatal stress from postnatal day (P) 3 to P7. All rats underwent unilateral brain injury on either P7 or P134. We measured brain injury, growth, blood pressure, urine/plasma CS, plasma leptin, insulin, and glucose before and after H-I. Postnatal stress increased neonatal CS production, exacerbated neonatal white matter injury, and was associated with adult hyperglycemia after H-I despite increased insulin production. There were no group differences in adult weight, blood pressure, or leptin. Postnatal stress exacerbated brain injury and produced adult hyperglycemia, triggered after hypoxia exposure, consistent with the hypotheses that neonates exposed to early stress are more vulnerable to hypoxia and may be predisposed to develop metabolic syndrome in adulthood. Prolonged maternal separation produced more hyperglycemia than did brief daily handling. **(*Pediatr Res* 66: 278–282, 2009)**

Prolonged hospitalization in a neonatal intensive care unit is stressful because preterm infants experience prolonged isolation, gavage feedings, and multiple painful procedures. These postnatal stressors may perturb early brain development and permanently impact brain function by affecting neuronal apoptosis, neurogenesis, synaptogenesis, or vascular development. For example, neonatal isolation permanently impairs hippocampus-dependent learning and memory (1), promotes depression (2), and disrupts fear conditioning in rats (3). In addition, because prenatal events can exacerbate neonatal brain injury (4), it is possible that postnatal stress may also exacerbate neonatal H-I. Surprisingly, it is still unknown whether postnatal stress has acute or lasting effects on physiologic responses to H-I.

Heart disease, cerebrovascular disease, and diabetes are among the top causes of death in the United States (CDC), and these disease processes can be influenced by early life experiences. The observation that diabetes is more prevalent in infants born to diabetic mothers (5–7) has prompted the development of models of gestational diabetes. A variety of experimental manipulations find that prenatal hyperglycemia leads to abnormal glucose homeostasis in the adult animal (8–11). Moreover, experimental IUGR produces defects in insulin secretion and hyperglycemia in the exposed adult rats (12,13). The fact that prenatal exposure to dexamethasone increases fetal hepatic glucocorticoid receptor expression and produces adult hyperglycemia in rats strongly suggests that fetal stress may program adult function (14). Although our laboratory has found adult cognitive deficits in rodents exposed to postnatal stress (15,16), we have yet to examine the effects of postnatal stress on adult glucose metabolism.

We hypothesized that postnatal stress would disrupt glucose metabolism and produce adult hyperglycemia. We also hypothesized that postnatal stress would exacerbate the brain injury due to neonatal H-I. In addition, we tested whether postnatal stress-induced effects are dose dependent by comparing brief handling and prolonged maternal separation. Lastly, to model some of the indices that define human metabolic syndrome, we measured corticosterone (CS), glucose, and insulin before and after H-I in neonatal and adult rats as well as adult weight, blood pressure, and plasma leptin. This study further characterizes the short- and long-term effects of postnatal stressors. An improved understanding of the consequences of postnatal stress may reveal important therapeutic opportunities.

METHODS

Animals. The University of Washington IACUC approved all animal procedures. Pregnant Sprague-Dawley rats were purchased from Harlan. Birth was recorded as postnatal day 1 (P1), and pups were assigned to treatment groups on P3. Neonatal rats were housed in the laboratory and adults were transferred to a vivarium facility.

Neonatal treatment groups. Postnatal stress procedures were modified from our published model (15). Three levels of postnatal stress were applied from P3 to P7: 1) controls were dam reared and were not handled except for weekly cage cleaning; 2) brief separation/pain involved temporary (0.5 h/d) maternal separation during which the animals were weighed and injected with 50 μL saline s.c. to simulate procedural pain; 3) prolonged separation/pain involved weighing, isolation into cups in a veterinary warmer for 8 h/d with three gavage feedings (0.1 to 0.3 mL/feeding) of rat milk substitute (17) and a saline injection.

Unilateral brain injury. The right common carotid artery (CCA) was ligated, and the animals were exposed to hypoxia. Neonatal rat lesions (n = 151) were performed on P7, and the pups were killed 72 h later. Adult lesions (n = 75) occurred on P134, and the rats were killed 2 weeks later. During surgery, rats were anesthetized (2.5% isoflurane), the CCA cauterized, and were exposed to 8% O_2 for either 120 m (neonates 35°C) or 60 m (adults room temperature) 1 h later.

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Abbreviations: CCA, common carotid artery; **CS,** corticosterone; **H-I,** hypoxia-ischemia; **P,** postnatal day

Physiologic measures. Dam-reared controls were handled minimally and only weighed on P10. Other groups were weighed daily. Mortalities occurred during postnatal stress, surgery, or after lesion.

Blood glucose and plasma insulin, leptin, CS (adults), and urine CS (neonates) concentrations were measured. Adults tail vein samples were collected before CCA surgery and at death. Plasma was prepared, and all samples were frozen at −80°C until assayed. In neonates, urine was obtained immediately before CCA surgery and shortly after recovery from hypoxia exposure on P7. Urine was obtained after tactile stimulation of the genital region or by needle aspiration from the bladder. Repeated urine sampling was nonlethal. A baseline for neonatal rat urine CS concentrations was determined using six nonstudy rats. ELISA kits were used to quantify CS (DSLabs Inc.), insulin (Bachem), and leptin (SPIbio). Blood glucose concentrations were measured at death using a One Touch Ultra meter (LifeScan Inc.). Blood pressure was measured at P120 using a noninvasive tail cuff (Kent Sci.).

Brain injury scoring. All rats were killed by overdose with Euthasol (2.2 mL/kg i.p.; Delmarva Labs, Inc). Brains were removed, weighed, and grossly examined for injury and scored on a 5-point scale: 0 = no injury; 1 = mild edema/atrophy with <25% lesion; 2 = moderate atrophy, 25–50%; 3 = severe atrophy with cystic cavitation, 50–75%; and 4 = severe atrophy, >75%. Coronal blocks containing hippocampus were cut, fixed, and processed to H&E stain 5 μM sections. Blinded images were captured for each hemisphere and scored by two individuals. The area of hippocampus was outlined, and the injured/uninjured hemisphere ratio was calculated using software (AnalySIS). In neonates, the cortex, thalamus, and internal capsule were also scored 0–4 for structural injury or gliosis as previously described (18–20).

Statistical analysis. ANOVA or nonparametric analyses and *post hoc* comparisons were made using SPSS software. Levene's test for homogeneity was evaluated. Parametric data were expressed as means and include SE (SEM). Nonparametric Mann-Whitney *U* tests with Bonferroni corrections were used. Two-tailed *post hoc* tests included *t* test (two groups) or Dunnett's (three groups). χ^2 test compared rates. Alpha was 0.05 (uncorrected).

RESULTS

Neonatal measures. We implemented a procedure to permit longitudinal measurement of CS production in neonatal rats. Figure 1 illustrates the daily CS concentrations measured from urine samples repeatedly collected from P2 to P17. Repeated sampling of neonatal urine worked well, and CS concentrations were detectable and exhibited an abrupt increase from P10 onward. Next, we sought to identify whether the handling and separation procedures had acute effects on neonatal rat CS production. Figure 2 shows the urine CS concentrations measured from a subset of rats 45, 120, and 300 m after exposure to brief or prolonged separation on P6. Urine from prolonged-separation rats contained higher concentrations of CS than rats given brief separation, indicating that the increased severity of neonatal maternal separation quickly produces a greater CS response. Figure 3 compares the mean urine CS concentrations in P7 rats, before and after H-I, and illustrates that postnatal stress elevated basal urine CS before CCA ligation and H-I triggered an acute CS response that was elevated by prolonged separation.

We examined whether postnatal stress modulated neonatal brain injury on P10. The control group showed mild injury (mean brain injury score ± SEM: 1.1 ± 0.2, n = 22), and there were no effects of stress on gross brain injury. Brain injury scores were 0.8 ± 0.4, n = 14 and 1.1 ± 0.5, n = 12 in the brief and prolonged separation groups, respectively.

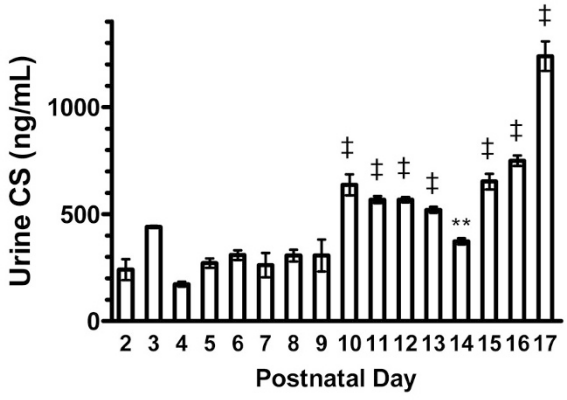

Figure 1. Mean (± SEM) CS concentrations in neonatal rat urine sampled daily from P2 to P17. These data define the expected range for urine CS values in neonatal rats. Repeated measures (RM)-ANOVA found effects of time (F_{15} = 24.5, $p < 0.001$ and Dunnett's *post hoc* comparisons to P2 are indicated as ‡$p < 0.001$ and **$p < 0.05$. n = 3–6/time point.

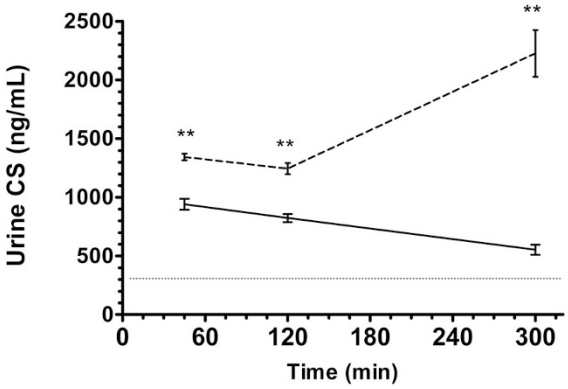

Figure 2. Mean (± SEM) urine CS concentrations measured on P6 from rats after exposure to brief handling (*solid line*) or during prolonged maternal separation (*dashed line*). These data identify that prolonged separation is more stressful than brief handling as indicated by an immediate increases in CS production. RM-ANOVA found effects of time ($F_{2,8}$ = 12.8, $p < 0.01$) and separation ($F_{1,4}$ = 77.1, $p < 0.001$) and a separation × time interaction ($F_{1,4}$ = 45.8, $p < 0.01$) with n = 3/group. *Post hoc* comparisons between the groups are indicated (**$p < 0.01$). The *horizontal dotted line* at 308 ng/mL is the baseline P6 CS level from Figure 1.

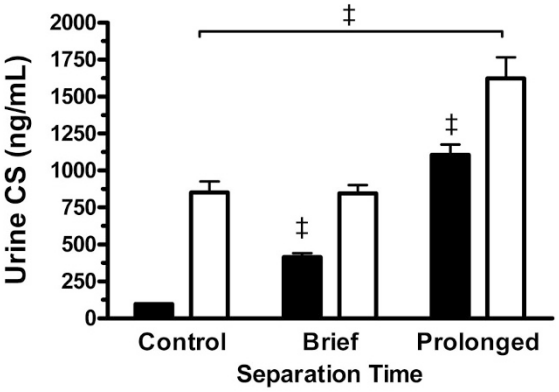

Figure 3. Mean (± SEM) urine CS concentrations from groups of P7 rats before (*filled bars*) and shortly after (*unfilled bars*) unilateral hypoxic-ischemic lesion. RM-ANOVA$_{2 \times 3}$ found significant effects of hypoxic-ischemic lesion ($F_{1,34}$ = 219.8, $p < 0.001$) and separation ($F_{2,33}$ = 47.9, $p < 0.001$) and a separation × lesion interaction ($F_{2,33}$ = 6.5, $p < 0.01$). Five days (P3–P7) of brief handling or prolonged separation increased all the before lesion values compared with control. Urine CS was increased after lesion for all groups ($p < 0.001$). As indicated by the bracket, the after lesion urine CS values for the prolonged separation group were significantly higher than control. There were no differences due to sex, so data from males and females were combined. Dunnett's *post hoc* comparisons to controls at the same before/after time are indicated as ‡$p < 0.001$. n = 12/group.

There were also no differences in hippocampal injured/uninjured area ratios (range 78–90% of the intact hemisphere) between the treatment groups (data not shown). Figure 4 displays the injury scores assigned in neonatal cortex (top panel), thalamus (center panel), and internal capsule (bottom panel). There were no group differences in cortical injury scores, and this is consistent with the gross injury and hippocampal volume data. However, postnatal stress increased the degree of injury in thalamus and internal capsule. These data demonstrate that gross injury scores may only provide information about cortical injury and may not reflect internal brain injury. These data also indicate that postnatal stress can specifically exacerbate certain types of hypoxic-ischemic injury. In particular, the white matter of the internal capsule seems to be very sensitive to neonatal handling and separation.

There were no differences between the groups for neonatal mortality, neonatal growth, or neonatal brain weights at death (data not shown).

Adult measures. Rats were lesioned at P135 and killed 2 weeks later to identify whether postnatal stress altered adult physiologic responses to H-I. Apart from expected sex differences, there were no group differences for adult body weights (means ± SEM, male: 374 ± 6 g, $n = 19$; female: 255 ± 3 g, $n = 24$) or blood pressures (means ± SEM male: 158/115 ± 3/2, $n = 19$; female: 137/97 ± 3/2, $n = 24$) measured 2 weeks before lesion at P120. Comparing measurements before and after H-I, plasma glucose and insulin, but not CS and leptin, were significantly elevated after lesion. Table 1 lists the plasma glucose for adult rats before and after H-I. Hypoxia-ischemia elevated glucose in all groups, and this effect was significantly greater in stressed rats compared with controls. There were no differences due to sex for plasma glucose. Table 2 shows that plasma insulin concentrations were also elevated postlesion; however, there were no differences between the treatment groups. Table 3 lists the plasma corticosterone and Table 4 lists the plasma leptin concentrations measured before/after lesion, and there were no group differences in either of these indices before/after lesion. Similar to the neonatal data, there were no differences in the degree of gross brain injury or in hippocampal injured/uninjured area ratios (range 69–79% of the intact hemisphere) between the groups (data not shown).

DISCUSSION

This experiment exposed rats to three levels of postnatal stress and then evaluated physiologic responses to H-I at both neonatal and adult ages. The goal was to understand whether postnatal stress produces effects that interact with H-I. The

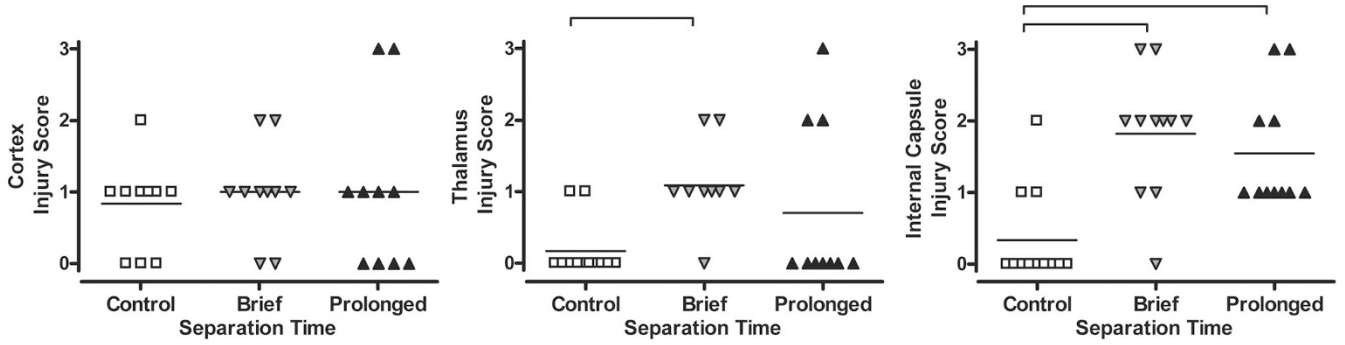

Figure 4. Regional injury scores of H&E-stained slides from cortex (*top panel*), thalamus (*center panel*), and internal capsule (*bottom panel*) identify that neonatal maternal separation exacerbates white matter injury in groups of neonatal rats killed at P10. Individual scores are shown and the mean is indicated by the horizontal line. Nonparametric analysis identified significant effects of separation on injury scores in thalamus ($p < 0.05$) and internal capsule ($p < 0.001$). Bonferroni-corrected *post hoc t* tests compared brief and prolonged separation to control and significant differences are indicated by the brackets. $n = 10–12$/group.

Table 1. *Mean ± SEM (N) adult plasma glucose (mg/dL) before and after hypoxic-ischemic lesion*

	Female		Male	
Separation time	Before lesion	After lesion*	Before lesion	After lesion*
Control	106.3 ± 9.4 (8)	139.9 ± 6.8 (7)	102.2 ± 4.6 (6)	143.3 ± 4.8 (6)
Brief	98.1 ± 5.7 (8)	167 ± 7.4 (6)†	108.7 ± 6.5 (6)	154.8 ± 11 (5)
Prolonged	108.1 ± 6.3 (8)	196.7 ± 8.3 (7)‡	112.3 ± 8 (7)	194.3 ± 8.2 (7)‡

ANOVA found effects of lesion ($F_{1,32} = 316.9$, $p < 0.001$), group ($F_{2,32} = 15.2$, $p < 0.001$), and a lesion by group interaction ($F_{2,32} = 14.6$, $p < 0.001$). All glucose values were increased after lesion (*$p < 0.001$). Significant group comparisons to control are indicated (†$p < 0.05$, ‡$p < 0.001$).

Table 2. *Mean ± SEM (N) adult plasma insulin (pg/mL) before and after hypoxic-ischemic lesion*

	Female		Male	
Separation time	Before lesion	After lesion*	Before lesion	After lesion*
Control	909.6 ± 150.8 (8)	2379.9 ± 400.6 (8)	1254.1 ± 302 (5)	2640.2 ± 426.2 (5)
Brief	1025.1 ± 204.2 (8)	2123.4 ± 342.8 (8)	897.9 ± 166.4 (6)	4040.9 ± 1631.9 (6)
Prolonged	1233.5 ± 214.2 (8)	2458.1 ± 297 (8)	1224.8 ± 278.7 (7)	2427.3 ± 441.3 (7)

ANOVA found insulin increased after lesion ($F_{1,36} = 28.1$, *$p < 0.001$) but there were no group differences.

Table 3. *Mean ± SEM (N) adult plasma corticosterone (ng/mL) before and after hypoxic-ischemic lesion*

	Female		Male*	
Separation time	Before lesion	After lesion	Before lesion	After lesion
Control	784.5 ± 126.4 (8)	592.7 ± 129.6 (8)	211.4 ± 44.2 (5)	348.8 ± 50.3 (5)
Brief	798 ± 151.9 (8)	1109.6 ± 222.9 (8)	341.6 ± 49.1 (6)	523.4 ± 297.8 (6)
Prolonged	976.6 ± 119.1 (8)	969.6 ± 179 (8)	201.4 ± 40.1 (7)	679.1 ± 269.3 (7)

ANOVA found sex differences ($F_{1,36} = 17.9$, $^*p < 0.001$) but there were no group differences.

Table 4. *Mean ± SEM (N) adult plasma leptin (pg/mL) before and after hypoxic-ischemic lesion*

	Female		Male	
Separation time	Before lesion	After lesion	Before lesion	After lesion
Control	2799.4 ± 432.8 (8)	1848.4 ± 522.8 (8)	3053.7 ± 316.2 (5)	3292.4 ± 429.2 (5)
Brief	2266.1 ± 251.7 (8)	2086.5 ± 475.7 (8)	2031 ± 540.9 (6)	1848.7 ± 371.6 (6)
Prolonged	2856.7 ± 375 (8)	4317.2 ± 2340.3 (8)	3073.2 ± 162.3 (7)	3444.5 ± 431 (7)

There were no differences due to lesion, group, or sex for leptin values.

major findings of this experiment are as follows: 1) while both brief and prolonged daily handling are stressful to neonatal animals, prolonged maternal separation is more stressful than brief separation as evidenced by increased neonatal CS production; 2) postnatal stress changes the vulnerability of neonatal animals to subsequent white matter and deep gray matter injury; and 3) postnatal stress alters adult glucose homeostasis in a dose-dependent manner.

Experiments examining effects of neonatal stress recognize that early life stress may produce life-long effects that increase risk for certain types of adult disease (13,21,22). Most notably, maternal separation stress enhances the hypothalamic-pituitary-adrenal stress response as indicated by circulating CS concentrations (23–27). Urine CS measurements have previously been reported as a reliable method by which to assess stress in adult rats (28), but no such data exists for neonatal animals. We now report that urine CS concentrations are also a valid, reliable, minimally invasive (and nonlethal) method for measurement of the stress response in tiny neonatal rodents. We have shown that CS production increases during early development, more prolonged separation induces greater CS production than does brief handling, and postnatal stress elevates CS production during the neonatal period. We note also that the abrupt increase in urine CS detected after P10 agrees with the observation that mouse plasma CS increases on P12 (29) but challenges the interpretation that the later postnatal increases in plasma CS are due to decreased clearance (30). Although postnatal stress elevated CS production acutely, this effect did not persist into adulthood, and these new adult data agree with our prior adult plasma CS measurements (15).

The overproduction of steroids during fetal or neonatal stress may reprogram organ tissues and produce abnormalities in adult organ function or metabolism (31). The metabolic abnormalities may be changes in basal function or changes in function during physiologic challenges such as H-I (32). If true, postnatal stress may alter susceptibility to later injury. To examine these possibilities, we measured weight and blood pressure in adults and also measured adult plasma glucose, insulin, CS, and leptin before/after exposure to H-I. There were no group differences in weight, blood pressure, leptin, or CS and thus no evidence of obesity, hypercortisolemia, or hypertension in the adult rat exposed to 1 week of brief or prolonged postnatal stress.

However, adult hypoxic-ischemic lesion was associated with elevated glucose in rats that had experienced prolonged postnatal stress, but not in controls. Given that insulin production increased after lesion in all groups, it is possible that the postlesion glucose elevation reveals an underlying insulin resistance. By this interpretation, we have identified that postnatal stress induces mild insulin resistance, which manifests after an adult hypoxic-ischemic insult. We qualify this interpretation by pointing out that the glucose was elevated beyond the maximum of the normal published range (33), but still below the 250 mg/dL cutoff for hyperglycemia in rats. These data complement reports demonstrating that experimental IUGR produces progressive adult insulin resistance and hyperglycemia in rats (12,13). Our postlesion glucose values at 21 wk of age closely match the glucose values seen at 15 wk of age in the IUGR model. Our findings also support observations that perinatal steroid exposure produces hyperglycemia in animals (14,34) and humans (35), and thereby strengthen the concern that perinatal glucocorticoids program adult disease states (36). Specifically, our findings are consistent with the hypothesis that neonates exposed to early stress may be predisposed to develop a prediabetic syndrome in adulthood.

We also examined whether neonatal brain injury was affected by prior postnatal stress. Although there were no effects of prolonged stress on neonatal mortality, growth, brain weight, or gross injury scores after H-I, there were effects on regional brain injury scores. In particular, neonatal stress increased the susceptibility to white matter injury in the internal capsule and to thalamic injury. Perhaps the cognitive deficits seen after neonatal pain and stress in animal models (15,16,37,38) and recently in preterm infants (39) are related to increased vulnerability of specific brain regions.

The observation that postnatal stress enhances white matter vulnerability is particularly salient for preterm infants. Between 25 and 48% of extremely low birth weight infants have substantial neurodevelopmental impairment (neurosensory abnormality and/or mental developmental index score <70)

(40–43). Some preterm infants exhibit reduced MRI gray and/or white matter volume at term corrected age when no known risk factors were present (44–46). Prolonged postnatal steroid therapy is a risk factor for cerebral palsy (47). We speculate that the increased steroid production associated with neonatal stress may exacerbate white matter vulnerability and contribute to periventricular leukomalacia and poor neurodevelopmental outcomes. There is still concern that neonatal pain and stress may contribute to abnormal neurodevelopment in preterm infants (48–50). In summary, the data presented support concerns about reducing neonatal stress to reduce risk of neonatal brain injury and adult diabetes.

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