Insulin Sensitivity, Leptin, Adiponectin, Resistin, and Testosterone in Adult Male and Female Rats After Maternal-Neonatal Separation and Environmental Stress.

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Running Head: Adult Insulin Resistance after Neonatal Stress

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ABSTRACT:

Care of premature infants often requires parental and caregiver separation particularly during hypoxic and hypothermic episodes. We have established a neonatal rat model of human prematurity involving maternal-neonatal separation and hypoxia with spontaneous hypothermia prevented with external heat. Adults previously exposed to these neonatal stressors show a sex difference in the insulin and glucose response to arginine stimulation suggesting a state of insulin resistance. The current study used this cohort of adult rats to evaluate insulin resistance (Homeostatic Model Assessment of Insulin Resistance [HOMA-IR]), plasma adipokines (reflecting insulin resistance states), and testosterone. The major findings were that daily maternal-neonatal separation led to an increase in body weight and HOMA-IR in adult male and female rats and increased plasma leptin in adult male rats only; neither prior neonatal hypoxia (without or with body temperature control) nor neonatal hypothermia altered subsequent adult HOMA-IR or plasma adiponectin. Adult male-female differences in plasma leptin were lost with prior exposure to neonatal hypoxia or hypothermia; male-female differences in resistin were lost in the adults with prior neonatal hypoxia allowing spontaneous hypothermia Exposure of neonates to daily hypoxia while preventing spontaneous hypothermia led to a decrease in plasma testosterone in adult male rats. We conclude that neonatal stressors result in subsequent adult sex-dependent increases in insulin resistance and adipokines, and that our rat model of prematurity with hypoxia with the prevention of hypothermia alters adult testosterone dynamics.
INTRODUCTION:

Neonatal stress has dramatic acute effects in the premature infant as well as long-lasting, metabolic effects that are evident in adulthood (4, 30-34, 56, 66, 79). Premature birth often requires separation from direct, tactile parental or caregiver care (i.e. incubator therapy) that can be mimicked in rodents by maternal-neonatal separation (1, 2, 7, 14, 38, 41, 45, 46, 57, 64, 85). Furthermore, premature infants often have periods of hypoxia due to apneas or immature lung function that may worsen during “kangaroo care” used to minimize the negative effects of neonatal separation (1-3, 7, 35, 45, 64, 71). In addition, premature infants can experience significant hypothermia even when normoxic (27, 40, 41, 55, 76). Finally, hypoxia in the neonate results in profound, endogenous hypothermia (19, 59). The consensus seems to be that isothermia should be maintained during periods of acute hypoxia in the neonate even though therapeutic hypothermia is commonly used to prevent the sequelae of hypoxia at birth, particularly in the central nervous system (73, 74).

Exploiting the altricial nature of the newborn rat, we have developed a rodent model of prematurity and its treatment. This is accomplished by exposing neonatal rat pups periodically separated from their dams to hypoxia to mimic the apnea of the prematurity, while allowing or preventing the spontaneous hypothermia during hypoxic exposure (9, 10, 23). We have previously shown a significant additional neonatal stress response due to the prevention of hypoxia-induced spontaneous hypothermia in the neonate (9, 10, 23). Furthermore, we have shown long-term alterations in adult physiology subsequent to exposure to maternal-neonatal separation without and with concomitant hypoxia (13, 20, 21).
Of relevance to the current study is our previous finding of sex differences in acute adult rat insulin and glucose responses to arginine after neonatal separation and the environmental stresses described above (20). In particular, the adult rats exposed to prior neonatal stress demonstrated hyperinsulinemia without hypoglycemia and greater weight gain reminiscent of the metabolic syndrome and early type 2 diabetes mellitus in humans (48, 62). We now follow up these prior studies by correlating the calculated Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), a validated index of insulin resistance in adult rats (11, 53, 54, 61, 68) with measurement of the adipokines leptin, adiponectin, and resistin which are known to reflect the existence of, and be involved in the adaptation to insulin resistance (5, 16, 39, 42, 50, 51, 69, 72, 77, 87). Furthermore, in order to explore the significantly larger insulin response to arginine in males but not females, we measured adult plasma testosterone levels by LC-MS/MS. We hypothesize that maternal-neonatal separation without (normoxic) or with application of neonatal environment stressors will result in related increases in adult insulin resistance and associated increases in plasma leptin and resistin, and decreases in adiponectin.

METHODS:

This is the second in a series of studies in this cohort of rats exposed to neonatal stress (20). Federal guidelines (http://grants1.nih.gov/grants/olaw/references/phspol.htm) for use and care of laboratory animals were followed and the protocols were approved by the Institutional Animal Care and Use Committee of Aurora Health Care. Timed pregnant Sprague–Dawley rats (N = 10) were obtained at 18 days of gestation and housed in a standardized environment (lights on 0600–1800 h). Rats were provided ad libitum
standard diet and water. Dams were delivered normally and cared for their pups until experimentation. Each litter was assigned to a unique treatment group to avoid cross-fostering (47). A total of 110 pups were studied as described below.

**Neonatal stressors.** Male and female rat pups were randomly assigned to different (90 min) morning neonatal treatments from postnatal day (PD) 2 to PD6 as described below:

1) **Normoxia-Unseparated** – the control group for normoxic separation: These pups (N = 23) were left undisturbed except for weekly cage changes.

2) **Normoxia-Separated** – the normoxic control for the environmental neonatal stressors listed below: These pups (N = 22) were separated from their dams and placed into an environmental chamber with bedding and a variable setting heating pad on the lowest setting required to prevent a decrease in basal body temperature during separation from the dams (23). Pups were allowed to huddle normally.

3) **Hypoxia allowing spontaneous, endogenous hypothermia:** These pups (N = 25) were separated from the dam and placed into a chamber with bedding and a variable setting heating pad on low heat. Hypoxia was induced by decreasing the chamber O\(_2\) concentration to 8% as described in detail previously (9, 10, 23). This results in a plateau nadir transcutaneous O\(_2\) saturation of approximately 80% (10). Body temperature was allowed to spontaneously decrease during hypoxia and was measured in a sentinel pup in each chamber as described previously (23). Sentinel pups were not used for the adult experiments. Body temperature had spontaneously decreased to 23.9±0.5° C (n = 10) after 90 min of hypoxia. After 90 min, the chamber was opened to room air and the pups were warmed to a normal body temperature range of 32–34 °C using a variable setting heating pad set on low before returning the pups to the nest.
4) Induced Hypothermia: These pups (N = 15) were separated from the dam and placed in a normoxic chamber with bedding on top of a cold plate (Model #AHP-1200CPV; TECALAB, Chicago, IL) set between 24 and 27 °C and adjusted depending on measured body temperature. Body temperature was measured in a sentinel pup using RET-30-Iso rectal probes and a BAT-12 digital thermometer connected to a SBT-5 switchbox (Physitemp Instruments, Clifton, NJ). Body temperature was decreased to 25 °C over 30 min and held at 25°C by adjusting the temperature of the cold plate. After hypothermia was completed, body temperature was allowed to increase as described for hypoxia above.

5) Hypoxia while maintaining isothermia: These pups (N = 25) were treated the same as the hypoxia pups except that body temperature was maintained at 32 °C with a heat plate (Model #AHP-1200CPV; TECALAB) as described previously (23).

At PD22, pups were weaned and housed by sex and treatment group. Weaned animals were given a standard diet and water ad libitum and handled only during weekly bedding changes.

Adult Measurements: These measurements were obtained after an overnight fast. Rats between PD105 and PD133 had been accustomed to daily handling for 5-10 min for several days before experimentation in order to obtain basal stress hormone levels on the day of experimentation (65). For plasma insulin and glucose measurements, blood was obtained by tail nick as described previously (84). These data have been reported previously and are provided here to support the HOMA-IR calculations (shown below). Rats were exposed to inhaled isoflurane for 5-10 seconds and then killed by rapid
decapitation to obtain trunk blood in EDTA-treated tubes to generate plasma for the
measurement of hormones described below:

Plasma immunoassays: Plasma leptin and adiponectin were measured by ELISA
(Crystal Chem #90040 and #80570, respectively, Downer’s Grove, IL)(8). Plasma
resistin was measured by ELISA (BioVendor #RD391016200R, Karasek, Czech
Republic). The limit of detection is 0.05 ng/ml and intraassay and interassay coefficients
of variation are 4.9-5.2% and 4.9-9.3%, respectively.

Plasma testosterone: Plasma testosterone was measured by LC-MS/MS. Plasma
samples, quality controls or standards (100 µl) were each combined with 100 ng/dl
deuterium labeled d3-testosterone internal standard (100 µl) and extracted with 0.4 ml of
acetonitrile; the organic phase was evaporated to dryness under nitrogen in a 50 ºC water
bath and reconstituted in 100 µl of water/methanol (50:50, v:v). Testosterone
measurements were performed using a 1290 Infinity HPLC (Agilent Technologies, Palo
Alto, CA, USA) and a Triple Quad LC-MS (Agilent Technologies, Palo Alto, CA, USA)
with an ESI ion source in positive mode. The two dimensional LC separation technique
was performed with a Zorbax SB-C8 (2.1 x 15 mm, 3.5 µm) loading column (Agilent
Technologies, Palo Alto, CA, USA) and a Poroshell 120, EC-C18 (2.1 x 50 mm, 2.7 µm)
analytical column (Agilent Technologies, Palo Alto, CA, USA) both maintained at 50º C
combined with a 0.3 µm inline filter. The injection volume and flow rate range while
injections were on the columns was 5 µl and from 0.3-0.6 ml/min, respectively.
Introduction of sample onto the loading column was performed by a gradient elution of
mobile phase consisting of 5 mM ammonium formate in water (solvent A) and 5 mM
ammonium formate in methanol (solvent B) at 0.5 ml/min A:B 50:50–41:59 (0-1.2). The
analytical pump transferred the sample to the analytical column and then introduced it to
the mass spectrometer with a gradient elution of A:B 45:55-2:98 (1.1-4.4 min). The flow
rate of the analytical pump increased from 0.3 ml/min to 0.6 ml/min (4-4.1 min).
Additional, gradients used for eluting waste components and equilibration of columns to
initial conditions. MassHunter software (Agilent Technologies, Palo Alto, CA, USA)
was used to control the instruments and for data analysis. The mass spectrometer scan
type utilized was multiple reaction monitoring with total testosterone quantified and
qualified by the ion transition \( m/z \) 289.2/97.1 and \( m/z \) 289.2/109.1, respectively. \( \text{d}^3 \)-
testosterone internal standard was analyzed by the ion transition \( m/z \) 292.2/97.1. The
following source conditions were used: gas temperature, 300 °C; gas flow, 5 l/min;
nebulizer pressure, 60 psi; sheath gas temperature, 400 °C; sheath gas flow, 11 l/min;
capillary voltage, 3,500 V; nozzle voltage, 0 V; and an electron multiplier voltage of 400
V. Fragmentor voltage was 123 V for all compounds. Collision energy was 20 V for
internal standard and testosterone quantifier ions and 24 V for testosterone qualifier ions.
The functional sensitivity is 0.7 ng/dl, and intraassay and interassay coefficients of
variation are 1.5-3.9% and 2.6-7.8%.

**Calculations and Statistical Analyses:** HOMA-IR, a validated index of insulin
resistance in rats, was calculated as the product of fasting plasma glucose and fasting
plasma insulin divided by a constant (11, 61). Data were analyzed by two factor analysis
of variance (ANOVA) followed by the Holm-Sidak multiple comparisons method
(Sigmaplot 12.5). Since pups had to be separated from their dams to expose them to the
environment stressors, two different null hypotheses were evaluated. For the comparison
of separated (normoxia) vs unseparated (control for normoxic separated), the two
ANOVA factors were sex and separation. For the comparison of environmental stressors (that required separation) to their control (normoxic separated), the two factors were sex and environmental stressor. Data are presented as median (25%-75% percentile) or mean ± standard error of the mean with P<0.05 considered significant.

RESULTS:

Table 1 shows the insulin, glucose and body weight results in adults previously exposed to neonatal stressors. Notice that the adult males in the untreated (unseparated) group and in several prior neonatal stress groups had higher fasting insulin concentrations and all had higher body weight than adult females. The male and female adult rats previously exposed to neonatal hypothermia had increased fasting plasma glucose relative to the normoxic (normothermic) unseparated control. The adult male and female rats previously exposed to maternal-neonatal separation had higher body weights as compared to the unseparated control. Adult males with prior exposure to neonatal hypoxia with spontaneous hypothermia allowed or prevented (isothermia) had lower adult body weights compared to their normoxic separation controls.

Adult results after maternal-neonatal separation vs. no separation:

Figure 1 shows the HOMA-IR calculations, a validated index of insulin resistance. Notice first that male adult rats, without or with previous maternal-neonatal separation, were more insulin resistant than the adult females. Male and female adult rats with prior maternal-neonatal separation had increased insulin resistance compared to the adult rats not separated from the dams as neonates.
Female adult rats had lower plasma leptin (Figure 2A), higher plasma adiponectin (Figure 2B), and lower plasma resistin (Figure 2C) than adult males. The only significant effect of maternal-neonatal separation was the increase in plasma leptin in adult male rats compared to adult males not separated as neonates (p=0.27).

The concentrations of plasma testosterone in females were extremely low (Figure 3). Although there was a tendency for prior maternal-neonatal separation to result in higher plasma testosterone in adult males, this was not statistically significant.

**Adult results after neonatal environmental stressors – hypoxia with spontaneous hypothermia, hypothermia (normoxic), and hypoxia with body temperature control (isothermic hypoxia) compared to the appropriate control (normoxic separation).**

The control group for all of the environmental stressors described below is the normoxic separation group because the application of these environmental stressors required maternal-neonatal separation.

Figure 4 shows the index of insulin resistance (HOMA-IR) in adult male and female rats exposed to neonatal environmental stressors. As in Figure 1, adult males were more insulin resistant than females. There were no effects of prior neonatal hypoxia without (Hx), or with the maintenance of body temperature (HxIt), or of normoxic hypothermia (Ht) on subsequent HOMA-IR compared to the neonatal separation normoxic control in adult male or female rats.

Figure 5 shows the plasma leptin (A), adiponectin (B), and resistin (C) concentrations in adult male and female rats previously exposed to neonatal normoxia (Normox control), hypoxia (Hypox), Hypothermia per se (Hypothrm), or hypoxia with
body temperature maintenance (Hypox-Isotherm). The difference between adult male
and female plasma leptin concentration in the Normox (control) was lost in adults
previously exposed to any of the neonatal environmental stressors. In comparison, there
was no effect of any of the neonatal environmental stressors on subsequent adult plasma
adiponectin concentrations. The only relevant effect on plasma resistin concentrations
was a loss of male-female differences in adults who were previously exposed to neonatal
hypoxia. This was due to lower plasma resistin in adult males exposed to neonatal
hypoxia.

The only significant effect of neonatal environmental stress on adult plasma
testosterone concentration was a decrease in adult males exposed to hypoxia with body
temperature maintenance (Hypox-Isotherm in Figure 6).

In order to provide a medium to visually appreciate the adipokine data, Figure 7
plots the adipokine data from Table 1 against body weight in male and females rats.
First, it is interesting that the adipokine data in the neonatal separation per se group
(normoxia) was shifted to the right of the unseparated (control for separation) group
suggesting that either the increase in adult body weight was due to tissues that are not
major sources of adipokine secretion or the cells producing adipokines are less secretory.
It is interesting that the adult males, but not females, had an increase in leptin associated
with the increase in body weight after neonatal (normoxic) separation. Otherwise, prior
neonatal hypoxia per se consistently shifted the relationships to the left of the normoxic
(separation) control suggested a potential increase in adipokine production normalized to
body weight. It is interesting that neonatal isothermic hypoxia seem to shift the
relationship to the left in males but not females suggesting, again, a sexual dimorphism in
DISCUSSION:

The purpose of this study was to evaluate the long-term, metabolic effects of daily maternal-neonatal separation during the first week of life in the newborn rat without or with concomitant induction of neonatal hypoxia (allowing spontaneous hypothermia or maintaining isothermia), or of normoxic hypothermia. The major findings that will be the focus of this discussion are as follows: Daily maternal-neonatal separation on PD2-PD6 led to an increase in body weight and insulin resistance in adult male and female rats and increased plasma leptin in adult male rats only. Neither neonatal hypoxia (without or with body temperature control) nor hypothermia altered subsequent adult calculated insulin resistance or plasma adiponectin. However, the adult male-female differences in plasma leptin were lost with prior exposure to hypoxia or hypothermia and the male-female difference in resistin were lost in the adults previously exposed to hypoxia with spontaneous hypothermia. Of special interest was that exposure of neonates to daily hypoxia while preventing spontaneous hypothermia led to a decrease in plasma testosterone in adult male rats.

HOMA-IR: HOMA-IR is an accepted and validated index of insulin resistance in the adult rat (11, 61). It is essentially the product of fasting plasma insulin and glucose divided by a constant. If fasting plasma insulin is increased and fasting glucose is not decreased, or if fasting glucose is increased and fasting insulin is not decreased, then the product of insulin and glucose and, therefore, calculated insulin resistance is increased.
Our findings clearly demonstrate that prior maternal-neonatal separation led to an increase in insulin resistance in adult male and female rats. That body weight was also increased is consistent with the notion that these adult rats had a phenotype similar to the metabolic syndrome (12). In fact, maternal-neonatal separation without or with concomitant mild neonatal stress have been shown to induce a juvenile and adult phenotype very similar to the metabolic syndrome (24, 37, 44, 49, 60).

Interestingly, we have previously inferred an increase in insulin resistance by an augmented insulin and glucose response to acute arginine stimulation in adult male rats that had been exposed to daily neonatal separation from their lactating dams (20). Although we did not previously show dramatic changes in islet cell type, it is possible that there were subtle changes in islet cell distribution or function because of neonatal stressors since this is a critical period of beta and alpha cell development (22, 28, 58, 89).

Since adult females did not show an augmented insulin response to arginine in our prior report (20), the HOMA-IR findings in females in the current study may represent a more subtle form of insulin resistance.

Adult male and female rats previously exposed to stress during maternal separation (PD 5-9) show an increase in the ratio of visceral to subcutaneous fat (25). The males had pronounced hyperinsulinemia consistent with the increase in HOMA-IR in our study. Of great interest to us is that an intervention (mechanical-tactile stimulation) can prevent these long-term metabolic effects suggesting that it may be possible to implement a change in clinical care in premature infants that could ameliorate some of the long-term effects (64). It has been shown that rats exposed to maternal deprivation respond quite differently to a high fat diet than controls and, more importantly, that the
response is sexually dimorphic (54, 63). In particular, hyperleptinemia occurs earlier in rats previously exposed to maternal deprivation rat and that the subsequent reduced insulin sensitivity occurs only in males (54), again in agreement with our data.

There was no effect of prior neonatal hypoxia or hypothermia on HOMA-IR. This is not surprising since we previously demonstrated that the increases in fasting glucose in adult male rats exposed to prior neonatal hypothermia and hypoxia with isothermia (Table 1) were accompanied by a smaller insulin response to arginine (20) suggested that these neonatal environmental stressors primarily affect the islet cell rather than the peripheral sensitivity to insulin. Since the rat is an altricial animal, we have proposed the hypoxia in the neonatal rat separated from its dam for short periods of time is a model of the apnea of human prematurity which may lead to subsequent insulin resistance in the adult (30, 32-34, 62, 79).

**Leptin, Adiponectin, and Resistin:** These hormones produced by adipose tissue are well known to be involved in the regulation of insulin sensitivity, to reflect insulin resistance, and to exhibit sex differences (75).

Leptin is increased with weight gain and insulin resistance and it, in turn, acts to ameliorate insulin resistance and the phenotype of type 2 diabetes mellitus (5, 52, 53). In adult rats, leptin levels are typically higher in males compared to females (54) as we demonstrated. Furthermore, this sex difference effect on leptin is exacerbated by a high fat diet (54). We found daily maternal-neonatal separation during the first week of life resulted in increased leptin in adult male, but not female rat further illustrated in Table 7 showing a shift upwards and to the right in males, but not females, in the relationship between leptin and body weight (54). A single 24 hr episode of maternal-neonatal
separation at a later age (PD9) has been shown to decrease leptin in adults of both sexes (54). This suggests that the model, duration, and timing of maternal-neonatal separation is critical and may be associated with a subsequent attenuation or increase in weight gain. In fact, there are striking differences in acute stress responses and hypothalamic-pituitary-adrenal axis control in the PD7-10 pup compared to earlier and later in development suggesting that PD9 may be a unique moment in development in the neonatal rat (6, 10, 36, 82, 83, 86). Our approach is designed to model human prematurity, hence why we performed daily maternal-neonatal separation in the youngest rat pups feasible.

The effect of neonatal hypoxia or hypothermia on adult plasma leptin was not dramatic – if anything, it minimized the sex differences found in adult rats exposed to maternal-neonatal separation alone. Interestingly, whereas neonatal hypoxia with spontaneous hypothermia shifted the adipokine to body weight relationships to the left in male and female rats, hypoxia with isothermia shifted the adipokine to body weight relationship to the left in male, but not female rats. Since there may be cardiovascular and metabolic sex differences in adult humans who were born prematurely (62, 78), our approach as a model of human prematurity should prove useful to study pathophysiological mechanisms that cannot be evaluated in humans.

Adiponectin is expressed in mature adipocytes and is an insulin sensitizer; decreased adiponectin contributes to insulin resistance and the metabolic syndrome (68, 77, 88, 90). Plasma adiponectin was higher in females than males as shown previously (68) and was unaffected by maternal-neonatal separation or neonatal environmental stress. This suggests that adiponectin is probably not involved in a major way in the increased insulin resistance we demonstrated with maternal-neonatal separation.
Resistin is produced by adipocytes and seems to be important in the regulation of glucose metabolism and the promotion of insulin resistance in rodents, although its function in humans is not yet clear (42, 50, 51). In our study, plasma resistin was higher in males than females consistent with previous studies (68). Furthermore, in our studies, plasma resistin did not seem to correlate with changes in HOMA-IR, the validated index of insulin resistance, suggesting that resistin may not be a critical factor in our animal model.

**Testosterone**

We found that daily maternal-neonatal separation during the first week of life resulted in a trend towards an increase in total plasma testosterone in adult males in a manner similar to previously reports with testosterone measured by immunoassay (17, 18, 80). It is important to point out that we measured testosterone by LC-MS/MS which is a much more accurate, specific and precise method (70). Regardless, it seems unlikely that plasma testosterone was responsible for the sex differences in the change adult insulin resistance (i.e. HOMA-IR) after maternal-neonatal separation. Again, this seems to relate more to total body weight in male and female adult rats.

Previous studies help to suggest a mechanism of the decreased testosterone we described that is worth pursuing in follow-up studies. It may be that post-natal hypoxia affects the development of the testes with alterations in the microscopy anatomy of the testes during maturation (43). Pre- and post-natal early life stressors such as hypoxia, mother-infant separation, ether stress, and hypothermia have been shown to reduce sexual behavior in the male rat (29, 81). Finally, an epidemiological study in humans demonstrated that men who were born prematurely have reduced serum testosterone
levels as adults that was associated with a smaller testicular volume (26). These data suggest that post-natal stressors can lead to long term changes in testicular function – a possibility we are currently investigating.

Sleep-disordered breathing is more common in adult males than females (15). Furthermore, total plasma testosterone appears to have a brisker response to hypoxia in adult males exposed to maternal-neonatal separation (17). Finally, there is a sex difference in adiposity in adults who were born prematurely who typically experience prior maternal-neonatal and/or neonatal hypoxia with maintenance of isothermia (78).

Our finding of lower total plasma testosterone in adult male rats exposed to maternal-neonatal separation and hypoxia with the prevention of spontaneous hypothermia is compelling in relation to typical management of the premature infant (4, 27, 41, 64, 85), and we are currently pursuing these results in subsequent studies.

**Perspectives and Significance:** We propose that daily maternal-neonatal separation without and with hypoxia and/or hypothermia - a model of the medical management of the human premature infant - will continue to be useful in the evaluation of the metabolic pathophysiology of the long-term effects of neonatal stress (30, 32-34). We found that maternal-neonatal separation alone resulted in an increase in insulin resistance and body weight in adult male and female rats confirming this model as a representation of the development of the adult metabolic syndrome and/or type 2 diabetes mellitus (48, 62, 78). Since tactile stimulation of the separated newborn can attenuate some of these effects (24, 25, 60), we feel that continued mechanistic studies in our rat model will prove useful to develop new approached to the management of the premature human infant to mitigate the long term effects. Only plasma leptin in the adult showed a sex difference in
response to maternal-neonatal separation. Adiponectin and resistin did not correlate with the large effect of maternal-neonatal separation on adult insulin resistance. The other compelling finding was a decrease in adult plasma testosterone in males with prior exposure to neonatal hypoxia while preventing spontaneous, endogenous hypothermia (i.e. with maintenance of isothermia). This finding correlates with a sex difference in adult humans who were born prematurely (78) which may reveal that the mechanism of this effect may be mediated through the hypothalamic-pituitary-gonadal axis. It is particularly interesting that this decrease in testosterone may represent the development of primary hypogonadism that has significant implications in adult males (26, 29, 43, 81).

Taken together with our previous findings and those of others also using animals models (9, 23, 67), we once again question the maintenance of isothermia during periods of hypoxia in the premature infant.

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Author Contributions: Conception and experimental design: HR, ALG; Performing experiments: BH, MJ, CL, ALG. Assays: All authors. Statistical analyses: HR, ALG. First Draft: HR; Revisions: All authors. Approval of final version: All authors.

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## Table 1: Basal plasma insulin, glucose, body weight, and adipokines in adult rats previously exposed to different neonatal stressors.

<table>
<thead>
<tr>
<th>Neonatal Treatment</th>
<th>Insulin (ng/ml)</th>
<th>Glucose (mg/dl)</th>
<th>Body Weight (g)</th>
<th>Leptin (ng/ml)</th>
<th>Adiponectin [(ng/ml)*10^3]</th>
<th>Resistin (ng/ml)</th>
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<tbody>
<tr>
<td><strong>Normoxia Unseparated</strong></td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>0.5 ± 0.1</td>
<td>74.1 ± 2.1</td>
<td>331 ± 6</td>
<td>1.8 ± 0.1</td>
<td>10.5 ± 1.2</td>
<td>14.5 ± 0.5</td>
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<tr>
<td>Female</td>
<td>0.3 ± 0.1^a</td>
<td>72.1 ± 2.1</td>
<td>207 ± 3^a</td>
<td>1.5 ± 0.1^a</td>
<td>17.5 ± 1.3^a</td>
<td>11.4 ± 0.5^a</td>
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<tr>
<td><strong>Normoxia Separated</strong></td>
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</tr>
<tr>
<td>Male</td>
<td>0.6 ± 0.1</td>
<td>83.6 ± 3.5</td>
<td>381 ± 6^b</td>
<td>2.3 ± 0.2^b</td>
<td>9.8 ± 0.5</td>
<td>14.9 ± 1.0</td>
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<tr>
<td>Female</td>
<td>0.4 ± 0.1</td>
<td>80.4 ± 3.7</td>
<td>229 ± 4^ab</td>
<td>1.5 ± 0.1^a</td>
<td>16.3 ± 1.4^a</td>
<td>10.5 ± 0.8^a</td>
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<td><strong>Hypoxia with Spontaneous Hypothermia</strong></td>
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<tr>
<td>Male</td>
<td>0.6 ± 0.1</td>
<td>85.8 ± 2.4</td>
<td>350 ± 4^bc</td>
<td>2.0 ± 0.1</td>
<td>9.1 ± 0.4</td>
<td>11.5 ± 1.0^c</td>
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<tr>
<td>Female</td>
<td>0.4 ± 0.1</td>
<td>75.3 ± 3.9</td>
<td>350 ± 4^bc</td>
<td>1.6 ± 0.3</td>
<td>15.6 ± 0.8^a</td>
<td>10.6 ± 0.6</td>
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<td><strong>Hypothermia (Normoxic)</strong></td>
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<tr>
<td>Male</td>
<td>0.7 ± 0.1</td>
<td>98.3 ± 3.0^b</td>
<td>351 ± 7^bc</td>
<td>2.1 ± 0.1</td>
<td>9.6 ± 0.4</td>
<td>15.1 ± 1.1</td>
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<tr>
<td>Female</td>
<td>0.4 ± 0.0^a</td>
<td>94.0 ± 3.6^b</td>
<td>351 ± 7^bc</td>
<td>1.9 ± 0.1</td>
<td>13.3 ± 1.1^a</td>
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<td><strong>Hypoxia with Isothermia Maintained</strong></td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>0.8 ± 0.2</td>
<td>99.5 ± 4.3^b</td>
<td>363 ± 4^bc</td>
<td>1.8 ± 0.1</td>
<td>9.5 ± 0.1</td>
<td>13.0 ± 0.5</td>
</tr>
<tr>
<td>Female</td>
<td>0.4 ± 0.0^a</td>
<td>97.2 ±10.3^b</td>
<td>228 ± 4^a</td>
<td>1.4 ± 0.1</td>
<td>15.5 ± 0.7^a</td>
<td>8.6 ± 0.9^a</td>
</tr>
</tbody>
</table>

Insulin, glucose, and body weight data were published previously (20) and are presented here for reference. Data are mean ± sem.

Normoxia Unseparated is the control group for comparison to Normoxia Separated. Normoxia Separated is the control group for the environmental stressors that were all separated from their dams for stress exposure. For males: Normoxia Unseparated (N=12), Normoxia Separated (N=13), Hypoxia (N=14), Hypothermia (N=8), Hypoxia Isothermia (N=14). For females: Normoxia Unseparated (N=11), Normoxia Separated (N=9), Hypoxia (N=11), Hypothermia (N=7), Hypoxia Isothermia (N=11). Data are...
presented as mean ± SE. *Female different from male within each row; †different from Normoxia Unseparated within column. ‡Different from Normoxia Separated within column
Figure Legends:

Figure 1: HOMA-IR (Homeostatic Model Assessment of Insulin Resistance) in adult male and female rats exposed to prior daily (PD2-PD6) maternal-neonatal separation (Sep) compared to unseparated (Unsep) controls [median (25%-75% percentile)]. For males: Unseparated (N=12), Separated (N=13). For females: Unseparated (N=11), Separated (N=9). *different from Unsep, †different from Male within treatment (P<0.05).

Figure 2: Fasting plasma leptin, adiponectin, and resistin in adult male and female rats exposed to prior daily (PD2-PD6) maternal-neonatal separation compared to unseparated controls (mean ± standard error). For males: Unseparated (N=12), Separated (N=13). For females: Unseparated (N=11), Separated (N=9). *different from unseparated control within sex, †different from Male within treatment, ‡different from Male within treatment by t-test (P<0.05).

Figure 3: Fasting total plasma testosterone in adult male and female rats exposed to prior daily (PD2-PD6) maternal-neonatal separation compared to unseparated controls (mean ± standard error). For males: Unseparated (N=12), Separated (N=13). For females: Unseparated (N=11), Separated (N=9). †different from Male within treatment (P<0.05).

Figure 4: HOMA-IR (Homeostatic Model Assessment of Insulin Resistance) in adult male and female rats exposed to prior daily (PD2-PD8) neonatal separation with Normoxia (N; control), Hypoxia with spontaneous hypothermia (Hx), Hypothermic
normoxia (Ht), and hypoxia with maintenance of body temperature (HxIt) [median (25%-75% percentile)]. For males: Normoxia (N=13), Hypoxia (N=14), Hypothermia (N=8), Hypoxia Isothermia (N=14). For females: Normoxia (N=9), Hypoxia (N=11), Hypothermia (N=7), Hypoxia Isothermia (N=11). a different from Male within treatment (P<0.05).

Figure 5: Fasting plasma leptin, adiponectin, and resistin in adult male and female rats exposed to prior daily (PD2-PD8) neonatal separation with Normoxia (Normox; control), Hypoxia with spontaneous hypothermia (Hypox), Hypothermic normoxia (Hypothrm), and hypoxia with maintenance of body temperature (Hypoxic-Isotherm) (mean ± standard error). For males: Normoxia (N=13), Hypoxia (N=14), Hypothermia (N=8), Hypoxia Isothermia (N=14). For females: Normoxia (N=9), Hypoxia (N=11), Hypothermia (N=7), Hypoxia Isothermia (N=11). a different from Normoxia within sex. b different from hypoxia with spontaneous hypothermia within sex. c different from Male within treatment. (P<0.05).

Figure 6: Total plasma testosterone in adult male and female rats exposed to prior daily (PD2-PD8) neonatal separation with Normoxia (Normox; control), Hypoxia with spontaneous hypothermia (Hypox), Hypothermic normoxia (Hypothrm), and hypoxia with maintenance of body temperature (Hypoxic-Isotherm) (mean ± standard error). For males: Normoxia (N=13), Hypoxia (N=14), Hypothermia (N=8), Hypoxia Isothermia (N=14). For females: Normoxia (N=9), Hypoxia (N=11), Hypothermia (N=7), Hypoxia
Isothermia (N=11). *different from Male within treatment. (P<0.05). †different from hypoxia with spontaneous hypothermia (Hypox-Isothrm) within male by t-test (P<0.05).

Figure 7: Leptin, adiponectin, and resistant data (mean ± standard error) plotted against respective body weights (mean ± standard error) in male and female rats. Except for the normoxia separated group, all other treatments required separation of the pups from their dams and placement in an environmental chamber. For males: Normoxia Unseparated (N=12); Separated: Normoxia (N=13), Hypoxia (N=14), Hypothermia (N=8), Hypoxia Isothermia (N=14). For females: Normoxia Unseparated (N=11); Separated: Normoxia (N=9), Hypoxia (N=11), Hypothermia (N=7), Hypoxia Isothermia (N=11).
Adiponectin (ng/mL)

Unseparated Separated

Resistin (ng/mL)

Leptin (ng/mL)

A

B

Males

Females

C

Factors

Significance
Unseparated Separated

Testosterone (ng/dL)

Males

Females

0 20 40 60 80 100 120 140

Unseparated Separated
Testosterone (ng/dL)

- Normox
- Hypox
- Hypothrm
- Hypox-Isothrm

- Males
- Females

- c

- d