Neonatal immune challenge does not affect body weight regulation in rats

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Neonatal immune challenge does not affect body weight regulation in rats. Am J Physiol Regul Integr Comp Physiol 293: R581–R589, 2007. First published May 16, 2007; doi:10.1152/ajpregu.00262.2007.—The perinatal environment plays a crucial role in programming many aspects of adult physiology. Myriad stressors during pregnancy, from maternal immune challenge to nutritional deficiency, can alter long-term body weight set points of the offspring. In light of the increasing concern over body weight issues, such as obesity and anorexia, in modern societies and accumulating evidence that developmental stressors have long-lasting effects on other aspects of physiology (e.g., fever, pain), we explored the role of immune system activation during neonatal development and its impact on body weight regulation in adulthood. Here we present a thorough evaluation of the effects of immune system activation (LPS, 100 μg/kg ip) at postnatal days 3, 7, or 14 on long-term body weight, adiposity, and body weight regulation after a further LPS injection (50 μg/kg ip) or fasting and basal and LPS-induced circulating levels of the appetite-regulating proinflammatory cytokine leptin. We show that neonatal exposure to LPS at various times during the neonatal period has no long-term effects on growth, body weight, or adiposity. We also observed no effects on body weight regulation in response to a short fasting period or a further exposure to LPS. Despite reductions in circulating leptin levels in response to LPS during the neonatal period, no long-term effects on leptin were seen. These results convincingly demonstrate that adult body weight and weight regulation are, unlike many other aspects of adult physiology, resistant to programming by a febrile-dose neonatal immune challenge.

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selected time points bracketing P7 because of these earlier findings and including P14 because of our previously observed programming effects on other aspects of physiology.

Long-term changes have been seen in proinflammatory cytokine (TNF-α and IL-6) responses to LPS after an immune challenge at P14 (17). In the present investigation, we hypothesized that any alterations in body weight or feeding as a result of a neonatal immune challenge would be reflected in changes in another proinflammatory cytokine, leptin. Leptin has a well-established function in satiety signaling, and its absence, whether by a mutation in the gene encoding for leptin itself (ob/ob) or in the gene encoding for the receptor (db/db), leads to an obese phenotype (30, 60). It has also become evident that leptin is a proinflammatory cytokine released in response to LPS and that it plays an important role in modulating both the febrile and the anorectic responses to LPS (25, 49, 58). We therefore also examined changes in neonatal and adult leptin levels after LPS.

Thus, in the present investigation, we hypothesized that a neonatal immune challenge at a critical point in development may have long-term programming effects on body weight. We therefore conducted a careful analysis of body weight changes and indicators of body weight regulation in Sprague-Dawley male and female rats given an immune challenge (LPS) or pyrogen-free saline (as controls) at different time points during the neonatal period.

MATERIALS AND METHODS

Animals. Pregnant Sprague-Dawley rats (Charles River) mated between 8 and 9 wk were maintained at 22°C on a 12:12-h light-dark cycle (7:00 AM–7:00 PM) with pelleted 575 Purina rat chow and water available ad libitum. Litters were reduced to a maximum of 12 pups 2 days after birth, to standardize competition for food and water available ad libitum. Litters were chosen as a representative skeletal muscle as it has previously been shown to be altered by postnatal endotoxin given at

![Fig. 1. Body mass (A and B) and nasal-anal lengths (C and D) for the weeks following treatment in male (A and C) and female (B and D) rats treated at postnatal day (P) 3 (squares), P7 (circles), or P14 (triangles) with saline (Sal; white symbols) or LPS (black symbols). No significant differences were found between the groups. Data shown are means ± SE; n = 5–8/group.](http://ajpregu.physiology.org/)
P3 and P5 (39). The retroperitoneal fat was chosen as it is a reliably and easily dissected representative fat pad.

**Neonatal LPS: effects on adult feeding and LPS-induced anorexia.**

A separate group of litters was treated, at P14 only, with an intraperitoneal injection of LPS (100 μg/kg in 1 ml/kg pyrogen-free saline) or to an equivalent volume of pyrogen-free saline. For this experiment, we tested only P14 males as our other findings were indicating no effects of neonatal LPS, regardless of neonatal age. We were therefore of the opinion that including the entire cohort of groups would be an unnecessarily wasteful use of animals unless some changes were found. Female pups were similarly treated and kept for use in other (separate) experiments.

At 10 wk the rats were rehoused singly. At this time, rats were fed a standard powdered rat chow to allow for accurate measurement of daily food intake. The rats were allowed to acclimatize to the single-housing and powdered food for a period of 7 days prior to the experiment. Thereafter all rats were given 50 μg/kg LPS ip in 1 ml/kg pyrogen-free saline or pyrogen-free saline alone at the beginning of the dark cycle. This dose was chosen as the magnitude of fever it elicits is equivalent to that produced in the neonates by 100 μg/kg (9).

Body weight and food intake were assessed (by weighing the rats and remaining food) at 8 and 12 h postinjection. This experiment was conducted on 13 rats randomly selected from four litters.

**Neonatal LPS: acute effects on circulating leptin.** At P3, P7, or P14, the pups were removed from their home cage for ~10 min and subjected to an intraperitoneal injection of LPS (100 μg/kg in 1 ml/kg pyrogen-free saline) or to an equivalent volume of pyrogen-free saline. Heads were marked with permanent marker for identification. Injections were administered between 0800 and 1000. At 2.5 h after LPS or saline administration, pups were quickly decapitated and the blood collected in heparinized tubes. Efforts were made to complete sample collection from one litter very quickly to minimize the stress effects of sequential removal of pups from the litter. Blood was immediately centrifuged and the plasma aliquots snap frozen in liquid nitrogen and kept at −80°C until use. In the case of the P3 animals, where the plasma yield from a single pup was very small, two to three samples from the same treatment were combined for assay. Four to six litters were used for each treatment day to avoid litter-related confounding effects. These experiments were conducted on 124 animals from 14 litters yielding 84 plasma samples.

**Neonatal LPS: effects on adult leptin and LPS-induced leptin.**

Long-term effects of LPS on leptin were assessed using a fourth group of litters. These were injected on P3, P7, and P14 as described above and left undisturbed, except for weaning and the usual cleaning and feeding procedures, until early adulthood. At ~12 wk of age, each rat was briefly anesthetized with halothane (induced at 4% and maintained at 2%) and a silicone cannula (ID: 0.025 cm, OD: 0.047 cm) was implanted into the jugular vein. Each cannula was filled with heparinized saline containing gentamycin. The rats were housed separately after the surgery and allowed 7 days to recover before experimentation.

**Fig. 2.** Retroperitoneal fat-to-right tibialis anterior muscle (as %total body weight) ratios in 19-wk-old male (A, C, and E) and female (B, D, and F) rats treated at P3 (A and B), P7 (C and D), or P14 (E and F) with saline (white bars) or LPS (black bars). Data shown are means ± SE; n = 4–8/group.
On the day of experimentation, the cannula was attached via polyethylene tubing to a syringe outside the housing chamber, allowing blood sampling without handling. The rats were then left undisturbed for 90 min prior to LPS injection. Cannulas were set up between 0800 and 1000. Blood samples (0.5 ml, immediately replaced with an equal volume of heparinized saline) were collected onto ice from each animal 5 min before and 2.5 h after an intraperitoneal injection of LPS (50 μg/kg in 1 ml/kg pyrogen-free saline). Samples were quickly centrifuged, and the plasma then snap frozen in liquid nitrogen and stored at −80°C until use. Samples were collected from 70 animals from 14 litters.

Measurement of circulating leptin. Plasma leptin concentrations were measured using a leptin ELISA kit (LincoResearch, St. Charles, MO). The interassay variability for this ELISA was 3.0–3.9% coefficient of variation (CV), intra-assay variability 1.9–2.5% CV, and the lower limit of detection was 0.04 pg/ml. All samples were assayed in duplicate.

Data analysis. Organ weight results (expressed as a %total body weight) and LPS-induced body weight and anorexia, as well as adult and neonatal plasma leptin results, were analyzed using Student’s unpaired t-tests comparing saline and LPS-treated rats at each age for males and females separately. Preliminary analysis of the neonatal leptin data showed significant (P < 0.05) gender and treatment effects within the P14 group. However, post hoc comparisons revealed that these effects were between males and females of different neonatal treatments. This mixed interaction was not associated with the primary hypotheses of the present experiments, and thus we decided to analyze all of our data in separate gender groups specific for age. Scores for body weights and lengths were compared using a one-way repeated-measures ANOVA, with injection (LPS or saline) as the between factor, and time from treatment (week) as the repeated measures for each of the P3, P7, and P14 treatment days for males and females. Scores for the fasting/recovery experiment were compared using a one-way repeated-measures ANOVA, with injection as the between factor and time as the repeated measures for each of the treatment days for males and females. In each case, statistical significance was assumed when P < 0.05. Data are presented as means ± SE.

RESULTS

Neonatal LPS: effects on body weight, fasting-induced body weight changes, and LPS-induced anorexia. To determine whether neonatal LPS caused long-term programming effects on body weight, we monitored body weight and growth, as well as appetitive and body weight responses in the adult to fasting and inflammation. Body weights measured to 13 wk were not affected by neonatal treatment at any of the three ages in either males or females (Fig. 1, A and B; n = 5–8 per group; for this and subsequent data, rats are representatives of 4–6 litters per neonatal treatment age). Nasal-anal lengths mea-

Table 1. Brain and adrenal weights

<table>
<thead>
<tr>
<th></th>
<th>P3 Males</th>
<th>P3 Females</th>
<th>P7 Males</th>
<th>P7 Females</th>
<th>P14 Males</th>
<th>P14 Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain, saline</td>
<td>0.41±0.02</td>
<td>0.66±0.02</td>
<td>0.41±0.02</td>
<td>0.57±0.01</td>
<td>0.42±0.03</td>
<td>0.62±0.01</td>
</tr>
<tr>
<td>Brain, LPS</td>
<td>0.41±0.03</td>
<td>0.62±0.02</td>
<td>0.40±0.01</td>
<td>0.61±0.02</td>
<td>0.43±0.04</td>
<td>0.60±0.02</td>
</tr>
<tr>
<td>Adrenals, saline</td>
<td>0.010±0.001</td>
<td>0.020±0.002</td>
<td>0.010±0.0004</td>
<td>0.023±0.002</td>
<td>0.012±0.001</td>
<td>0.019±0.001</td>
</tr>
<tr>
<td>Adrenals, LPS</td>
<td>0.012±0.001</td>
<td>0.021±0.001</td>
<td>0.011±0.0004</td>
<td>0.021±0.001</td>
<td>0.011±0.001</td>
<td>0.019±0.001</td>
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Values are means ± SE of %total body weight in 19-wk-old male and female rats treated at postnatal days 3, 7, or 14 with saline or LPS.
sured to 5 wk were also very similar between groups (Fig. 1, C and D). Although statistical comparisons were not made (see Data analysis), male and female rats were very similar in body weight while together as a litter, and the males, as expected, started to outgrow their female counterparts ~2 wk after weaning.

The possibility of similar body weights masking differences in fat-to-muscle ratios was explored by examining the postmortem weights of the retroperitoneal fat pads and the tibialis anterior. No differences were found in the fat-to-muscle ratios between any of the treatment groups for males or females either in absolute values (data not shown) or when expressed as a %body weight (Fig. 2; n = 4–8 per group). No differences were found in either fat or muscle weights expressed independently (data not shown). Furthermore, no differences were seen in brain or adrenal gland weight between any of the groups (Table 1).

We further asked if a challenge in the form of food deprivation or immune stimulation would differentially affect rats given LPS or saline as neonates. The same rats as described above were deprived of food for 16 h overnight. All the rats, regardless of gender or treatment group lost between 5.4 and 6.6% of their body weights during the fasting period and had gained these weights back again by 24 h later (Fig. 3, A and B; n = 5–8 per group).

In addition, no differences were seen in LPS-induced body weight changes or LPS-induced anorexia between adult males treated with LPS at P14 and the P14 saline-treated controls (Fig. 3, C and D; n = 5–8 per group). With similar starting weights, all rats given LPS, regardless of neonatal treatment, gained ~5 g in the first 8 h following LPS-injection and did not further significantly gain weight (or lose it) in the 4 h after that. Saline-treated adult rats ate ~20 g of rat chow in the first 8 h of the dark cycle and ~25 g by 12 h (i.e., a further 5 g) regardless of neonatal treatment. LPS-injection resulted in the expected anorexia, reducing chow consumption to ~15 g by 8 h and 16 g (i.e., a further 1 g) by 12 h, but no differences were seen in chow consumption between the two P14-treated groups.

Neonatal LPS: acute effects on circulating leptin. Given no differences in body weight, growth, or in fasting or sickness-induced body weight changes, we wanted to also discount the possibility of more subtle changes to satiety signaling during the neonatal period. Because of the role of leptin in both modulation of immune responses and in regulating food intake, we therefore measured basal and LPS-induced plasma leptin immediately after a neonatal injection of LPS. In the neonatal males, basal (saline-treated) circulating leptin levels were ~2,000 pg/ml at all three age groups, and values were similar regardless of age. In the females, basal levels tended to be higher, ~3,000 pg/ml at P3 and P7, and lower, ~1,800 pg/ml, at P14 (Fig. 4; n = 5–12 per group). LPS-treatment (n = 5–16 per group) did not further elevate these levels in either males or females. However, a significant decrease in plasma leptin was seen with LPS in females at both P3 (t = 2.412, P = 0.037) and P14 (t = 2.374, P = 0.040) with a trend in the same direction at P7 (t = 1.675, P = 0.128; Fig. 4). In the males, no significant differences with LPS treatment were seen. However, there was a substantial trend toward a difference at P3 (t = 2.016, P = 0.069).

**Neonatal LPS: effects on adult LPS-induced leptin.** We also examined potential programming effects of a neonatal neuro-immune challenge by measuring basal and acute LPS-induced leptin levels in adulthood. Our results reveal no effects of a neonatal immune challenge at any of the ages tested on adult leptin levels, either basal or LPS-stimulated, in either males or females (Fig. 5; n = 4–7 per group).

**DISCUSSION**

In the present series of experiments, we have convincingly demonstrated that a neuroimmune challenge during the early postnatal period, at least at the time points and dose tested here, does not significantly affect growth and body weight of the male or female rat. In addition to basal growth and body weight, we tested changes in weight in response to a brief fasting period, as well as body weight and food intake to a febrile dose of LPS. We found identical responses regardless of neonatal treatment.

Our findings, in light of the existing literature, indicate that, while a prenatal immune challenge may affect subsequent body weight and growth, rats do show a resistance to programming effects of a similar challenge experienced postnatally. Other groups have shown that maternal exposure to multiple injections of endotoxin or some cytokines can lead to increases in body weight, adiposity, and circulating leptin levels in the
offspring (15, 40, 63). In contrast, multiple endotoxin administration during the very early postnatal period (at P3 and P5) has been reported to result in a very slight reduction (62) or no change (39, 61) in total body weight. We have now extended this neonatal period to time points that are very sensitive to other programming aspects and still find no effect on weight. It is possible that animals raised under different environmental conditions may display vulnerabilities in terms of their weight regulation that are not evident in our cohort of animals raised under standard husbandry conditions. For example, in the Walker study (62), the rats were housed singly, and neonatal LPS can, under some circumstances, affect adult weight in housing situations of four or more rats (57).

While it is yet preliminary to conclude that neonatally LPS-treated rats would not respond differently to other challenges to their appetite and weight regulation, we did not see differences with the challenges used here. As mentioned in the introduction, we have previously seen altered weight loss as a result of experimental colitis after P14 LPS. However, this stimulus is one which is relatively chronic, causing weight loss for at least 4 days after treatment. It also directly affects the gastrointestinal tract, probably involving mechanisms of weight loss independent of, or in addition to, suppression of appetite and feeding (56).

There is now considerable evidence that neonatal leptin, although most likely not acting as a satiety factor as it is known to in the adult, may be very important in maturation of the hypothalamic regulatory circuits involved in appetite regulation (1, 11–13, 37, 44, 54). Thus we were very interested in determining whether neonatal administration of LPS altered neonatal leptin levels. Previous reports indicate transient high neonatal leptin levels in mice (1, 16) and in Zucker fa/fa rats (47). However, we did not see that here, and, at the three times we collected plasma, leptin levels in the neonates were similar to what we measured in ad libitum fed adult rats. It has previously been demonstrated, at least in adult rodents, that a neuroimmune challenge in the form of a high dose of LPS can cause a two- to threefold increase in circulating leptin levels (6, 23, 33, 34, 50). It is interesting to note that, in our experiments, instead of causing an increase in circulating leptin as we expected, given these previous findings in adults, LPS during the neonatal period reduced circulating leptin levels. This was particularly true of the females, while the males did not show a significant response to LPS. While rats are still suckling, the
majority of their leptin comes directly from the mother through the milk (42), and it could be suggested that the leptin reduction we see in these rats is due to a sickness-induced decrease in food intake, possibly due to reduced success in competition for food with healthy littermates. However, there is no evidence that LPS-exposed neonates suckle less or receive less maternal attention than their saline-injected littermates at the times we collected plasma (3, 57) or that they gain less weight (Fig. 1. and Ref. 57). Also, 12-h maternal and food deprivation in P8 mouse pups does not alter plasma leptin (1). This reduction in plasma leptin could therefore reflect a reduction in the contribution coming from the pups themselves via an as yet unknown mechanism or an alteration in the absorption of leptin from the gastric tract (2). Whatever the mechanism of this significant reduction in circulating leptin, especially in the females, there appears to be no resulting alterations in related adult physiology, suggesting that the change in leptin we see here is either too short-lived or too small to result in long-term alterations.

Rats given LPS as adults show well-documented increases in circulating levels of the proinflammatory cytokines TNF-α and IL-6 (8, 17, 19). We have previously demonstrated significant reductions in the levels of the LPS-stimulated proinflammatory cytokines in LPS-treated adults given LPS at P14 (17). Thus we were very interested in determining whether leptin levels, which can also be increased after LPS (6, 23, 33, 34, 50), are similarly reduced. However, we did not see any increase in leptin with the dose of LPS used (50 μg/kg) in the adult rats. This dose was deliberately selected as we have previously used the same to demonstrate alterations in other systems, such as neuroimmune regulation and pain perception with a neonatal immune challenge (9, 10, 17, 18, 57). It is possible that either our dose of LPS to induce leptin release into the plasma is too low or that our time point for plasma sampling (2.5 h) is not optimal. For example, in the earlier studies in which LPS injection was shown to cause elevations in leptin, doses were either much higher, in a range known to produce sepsis; 5 mg/kg ip (6) or 150–600 μg/kg iv (33, 34) to rats or 1 mg/kg ip to mice (50). Thus, the present study does not rule out a potential long-lasting effect of higher doses of LPS on body weight. Also, in some cases, plasma samples were taken much later at 4–5 h (6, 50).

In the few studies where circulating leptin has been altered by high doses of LPS and plasma measured earlier, effects have been shown to occur by ~2 h (34), thus at approximately the same time as our sampling. Also in agreement with our findings, another recent study reported no elevation in plasma leptin levels in rats after 250 μg/kg LPS (22). It is difficult to understand therefore why, in several recent studies (25, 26, 49), febrile and anorectic effects induced by 100–250 μg/kg LPS were abrogated or reduced at similar time points by leptin antiserum. Further investigation will be required to determine how leptin antiserum can alter LPS-induced variables in the absence of a change in circulating levels. Exploration of these issues is beyond the scope of this communication but may provide fruitful avenues for future investigation.

In the present study, we investigated rats of young (P70)-to-mid (P133) adulthood and it could be argued that neonatal LPS may have programming effects that are revealed in later adulthood or old age. This suggestion is unlikely to be the case, however. Rats at P70 can be regarded as adults in the sense that they are sexually mature (32, 41) and capable of reproducing and successfully raising pups. Furthermore, body weight during childhood, adolescence, and early adulthood is an excellent predictor of body weight and propensity to obesity in later life (38, 59) even in humans (14, 21, 24). We are therefore confident that the lack of a programming effect of neonatal immune challenge we see in young adults is indicative that there would be a similar lack of effect on phenotype in later age.

The questions examined in the present investigation have significant relevance to human health and the impact of perinatal events on long-term phenotype is important. Therefore, it is essential to note that it is difficult to equate rat developmental stages with those of humans. It has been estimated that the rat brain at P7-P13 is approximately equivalent developmentally to the human brain at birth (5, 48), and therefore the time points selected in this study may more closely reflect that of at or around birth in humans.

Together these data convincingly indicate that the programming effects of a febrile dose of LPS do not affect all components of the host defense response in the adult. Whereas the febrile, neuroendocrine and sensory (hyperalgesia and allodynia) changes that occur subsequent to LPS administration are all altered by neonatal LPS (9, 10, 17, 18, 51, 52, 55, 56), the anorectic response, examined here, is not. If this is indeed dependent upon circulating leptin, as is proposed from other studies (25, 49), it seems that a febrile dose of LPS, given to the neonate when this system is not yet active in regulating food intake and metabolism, does not cause a programming effect. Thus the critical window that we have demonstrated for neonatal programming of febrile responses (approximately P14-P21; Ref. 57) might be different from that for the LPS-induced anorexia. Our results do convincingly indicate that a brief neonatal inflammation at the time points examined here does not impact upon a number of indicators of growth and body weight regulation in adulthood, and this has positive implications for humans concerned about possible outcomes of an early-life immune challenge on later susceptibility to disorders in satiety signaling.

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