

Early-Life Immune Challenge: Defining a Critical Window for Effects on Adult Responses to Immune Challenge

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Many aspects of mammalian physiology are functionally immature at birth and continue to develop throughout at least the first few weeks of life. Animals are therefore vulnerable during this time to environmental influences such as stress and challenges to the immune system that may permanently affect adult function. The adult immune system is uniquely sensitive to immune challenges encountered during the neonatal period, but it is unknown where the critical window for this programming lies. We subjected male Sprague–Dawley rats at postnatal day (P)7, P14, P21, and P28 to either a saline or lipopolysaccharide (LPS) injection and examined them in adulthood for differences in responses to a further LPS injection. Adult febrile and cyclooxygenase-2 responses to LPS were attenuated in rats given LPS at P14 and P21, but not in those treated at P7 or P28, while P7-LPS rats displayed lower adult body weights than those treated at other times. P28-LPS rats also tended to display enhanced anxiety in the elevated plus maze. In further experiments, we examined maternal–pup interactions, looking at the mothers' preference in two pup-retrieval tasks, and found no differences in maternal attention to LPS-treated pups. We therefore demonstrate a 'critical window' for the effects of a neonatal immune challenge on adult febrile responses to inflammation and suggest that there are other critical time points during development for the programming of adult physiology. We also show that the neonatal LPS effects on the adult immune system are not likely due to overt differences in maternal attention.

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INTRODUCTION

It has been established previously in this laboratory that exposure to an immune challenge in the form of the bacterial endotoxin lipopolysaccharide (LPS) at postnatal day (P)14 results in altered brain cyclooxygenase (COX)-2 and an attenuated response to LPS in adulthood. The febrile response to adult LPS is smaller, as is LPS-induced COX-2 in the hypothalamus (Boisse *et al*, 2004; Ellis *et al*, 2005). These attenuated responses to LPS are not seen when the prior LPS exposure is experienced as an adult (Boisse *et al*, 2004), demonstrating the importance of the early period of development in modifying the adult immune system.

While we have seen effects on the adult of an immune challenge administered at P14, similar changes in other areas of adult physiology have been seen when the neonatal inflammation is given earlier in the neonatal period. For

example, Shanks *et al* (2000) found that exposure to LPS at P3 and P5 reduces inflammatory responses to experimental arthritis and alters hypothalamic–pituitary–adrenal (HPA) axis function in the adult (Shanks *et al*, 1995, 2000). Hodgson *et al* (2001) found changes in resistance to adult tumor colonization, natural killer cell activity and weight gain, as well as corticosterone responses to stress after successive immune challenges administered at P1, 3, 5, and 7. There therefore remains a question as to what is the critical period in the animal's development for the establishment of these long-term effects. This information is crucial, as the brain undergoes significant developmental changes during the first few weeks of life that are potentially influenced by an immune challenge. For example, various neurotransmitters such as GABA and catecholamines (Herlenius and Lagercrantz, 2001), components of the respiratory control system (Carroll, 2003), pain processing (Nandi and Fitzgerald, 2005; Kostarczyk, 1999), and the stress response systems (Levine, 2001; Seckl and Meaney, 2004) are all functionally immature at birth and continue to develop throughout the first few weeks of life.

With this in mind, we therefore asked where the critical window lies in the animal's development for this programming of the adult responses to an immune challenge. Thus, at P7, P14, P21, and P28, male Sprague–Dawley rats were

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subjected to either an intraperitoneal (i.p.) injection of LPS or an equivalent volume of pyrogen-free saline. These time points were chosen to encompass the stress hypo-responsive period through to postweaning. The rats were then examined in adulthood for changes in febrile and hypothalamic COX-2 responses to a further injection of LPS.

Responses to LPS also include a spectrum of other physiological changes collectively known as 'sickness behavior'; these include alterations in food intake, activity, and indices of emotion. Early-life manipulations such as a neuroimmune challenge have been shown in some studies to affect activity and anxiety responses to mildly stressful situations in adulthood; however, there are some discrepancies between the findings (Walker *et al*, 2004; Breivik *et al*, 2002; Spencer *et al*, 2005). One major factor in this may be the timing of the early challenge. We have previously shown that LPS given at P14 does not affect basal exploration of the elevated plus maze, an assessment of activity and anxiety; however, two studies which involved an endotoxin exposure at earlier time points have demonstrated effects on plus maze responses in adults (Breivik *et al*, 2002; Walker *et al*, 2004). Thus, we also assessed activity and anxiety responses in the elevated plus maze. As an additional indicator of lasting effects of neonatal sickness and because the early environment is known to be crucial to the development of feeding circuits and satiety (Elmqvist and Flier, 2004), we also examined body weight in adulthood.

It is also now apparent that neonatal programming of a number of adult stress responses is influenced significantly by maternal interactions with the neonates (eg reviewed in Mirescu *et al* (2004); Sanchez *et al* (2001)). While the mechanisms for these changes are not yet fully described, differences in the quality of maternal care can have long-lasting influences on rat pups that manifest themselves as altered HPA axis responses to psychological stressors in the adult (Levine, 2001; Champagne and Meaney, 2001). As it is not yet known if maternal-pup interactions are affected by an immune challenge to the pup, we also asked, in a parallel series of experiments, if mothers interacted differently with LPS-treated neonates than with their saline-injected littermates.

MATERIALS AND METHODS

Animals

Pregnant Sprague-Dawley rats (Charles River) were maintained at 22°C on a 12 h light/dark cycle (0700–1900 hours) with pelleted rat chow and water available *ad libitum*. At 5 days after birth, that is P5, litters were culled to 12 pups except in the case where one dam gave birth to only 10 pups. All litters for adult experimentation were weaned at P21 and the male rats were kept and housed three to four animals per cage until they reached approximately 10 weeks of age. All procedures were conducted in accordance with the Canadian Council on Animal Care regulations and were approved by the local University of Calgary Animal Care Committee.

Adult Responses to an Early-Life Immune Challenge

Early-life immune challenge. At P7, P14, P21, and P28, the rats were all removed from their home cage for approxi-

mately 5 min. On these days, approximately two male rats were selected from each litter and were subjected to i.p. injections of LPS (*Escherichia coli*; serotype 026:B6, L-3755; Sigma, St Louis, MO; 100 µg/kg) in 1 ml/kg pyrogen-free saline (each rat had one treatment only). A control group of rats from the same group of litters was given an equivalent volume of pyrogen-free saline at P14 only. Additional saline groups were not included as we have previously shown that neonatally saline-treated animals are identical to untreated animals in their adult responses to a variety of tests including febrile and brain COX-2 responses to adult LPS (unpublished observations). An additional two female rats were injected at the same time as the males to ensure that on any one injection day approximately four rats from each litter were sick. Ears were clipped for identification. Pups were then returned to their home cages where nest temperatures approximate 30°C, a temperature at which the animals are capable of maintaining normal body temperature and developing a fever (Boisse *et al*, 2004). After the final injection day they were left undisturbed, except for the usual cleaning and feeding procedures, until experimentation. Care was taken to ensure animals from each group were representative of each litter. Experiments were conducted on 63 animals selected from nine litters over a period of 6 months.

Implantation of temperature monitors. When the animals reached approximately 10 weeks of age, they were briefly anaesthetized with halothane (induced at 4% and maintained at 2%) and sterilized silicone-coated precalibrated temperature data loggers (SubCue Data loggers; Calgary, Alberta, Canada) were implanted into the abdominal cavity. Briefly, a small incision was made in the skin and muscle using sterile techniques and a data logger inserted. The muscle layer was then sutured and the skin clipped. Each surgery took approximately 5 min. SubCues were recovered at the termination of the experiment.

Behavioral testing in the elevated plus maze. After approximately 7 days recovery from the SubCue implantation, activity and anxiety-related behaviors were assessed in a subgroup of the animals using the elevated plus maze. Behavioral testing was conducted during the light phase of the circadian cycle, between 0800 and 1100 hours. All tests were conducted under brightly lit conditions and all scores were made by an examiner who was blind to the type of early-life treatment the animals had received. The plus maze was made of wood, painted black, and was raised 50 cm above the floor. It consisted of two opposite open arms of 50 × 15 cm and two closed arms of the same dimensions with 15 cm high walls. Each rat was placed in the center of the plus maze and observed for the latency to move into an arm, as well as the number of entries into and percentage time spent in each of the open and closed arms. The animal was regarded as having moved into an arm when all four paws had crossed the threshold of the arm. Each trial lasted 5 min. The maze was thoroughly cleaned between each test.

Adult immune challenge. At 2 days after the elevated plus maze trial, the rats were subjected to i.p. injections of LPS (50 µg/kg). In a separate series of experiments, we determined that elevated plus maze testing had no

significant effect upon pyrogenic responses to LPS 2 days later (unpublished observations). Approximately half of the animals were left for 48 h to allow us to assess febrile responses to the LPS. At 2 h after the adult LPS injection, a time at which febrile temperatures and brain COX-2 levels should be reaching their peak (Mouihate and Pittman, 2003), the remainder of the rats were deeply anaesthetized with sodium pentobarbital (80–100 mg/kg *i.p.*) and perfused with 4°C phosphate buffered saline via the left cardiac ventricle. The right half of the hypothalamus, including the preoptic area, was quickly removed over ice, snap-frozen in liquid nitrogen and stored at –80°C until ready for use.

Semiquantitative western blot for COX-2. Tissue was homogenized, proteins extracted, and homogenates (30 µg protein per well) were separated by 10% SDS polyacrylamide gel electrophoresis as previously described (Mouihate and Pittman, 2003; Boisse *et al*, 2004). Proteins were then transferred to a nitrocellulose membrane and incubated for 2 h at room temperature in 5% fat-free milk in Tris-buffered saline, containing Tween 20 (TBS-T). The membrane was then incubated overnight at 4°C in a 1:3000 affinity purified rabbit anti-COX-2 antibody (Cayman Chemical, Ann Arbor, MI) in a 5% fat-free milk/TBS-T solution and washed for 30 min in TBS-T before being incubated for 1 h in goat anti-rabbit IgG horseradish peroxidase conjugate (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature. We have previously shown that this antibody is specific for COX-2 as labeling is eliminated with preabsorption (Mouihate *et al*, 2002). Bound antibodies were revealed using an enhanced chemiluminescence assay (Pierce, Rockford, IL). After detection of COX-2, membranes were stripped with β-mercapto-ethanol, incubated with rabbit anti-actin antibody (1:10 000; Sigma) and processed as described above. All samples were run twice. Densitometric analysis of both the COX-2 and actin bands was conducted and COX-2 to actin ratios calculated to enable a semi-quantitative analysis of COX-2 levels.

Mothers' Interactions with Immune-Challenged Pups

Housing and early-life immune challenge. In a separate set of experiments, mothers' interactions with immune-challenged pups were examined. Each dam was placed with her pups in a clear plastic cage of dimensions 41 × 39 × 20 cm, equipped with plentiful shredded paper for a nest. At P5, P10, or P14, half of the pups from each litter were subjected to *i.p.* injections of LPS (100 µg/kg) and the remainder received an equivalent volume of pyrogen-free saline. These time points were selected to bracket the P7 time point representing the period when the mother is particularly vigilant of her pups and when our preceding observations indicated altered adult neuroimmune responses. At time points later than P14, the pups are sufficiently mobile to return to the nest without assistance, thus this was the oldest age at which these experiments could be carried out. The ears were clipped and pups were marked with a colored line on the back for identification. As in the first series of experiments, care was taken to ensure that approximately equal numbers of pups per litter received LPS and saline.

All behavioral testing was conducted during the light phase of the circadian cycle, under brightly lit conditions,

between 1000 and 1600 hours. Pups were weighed in half-litter groups with their similarly treated siblings before treatment and following the 24 h post-treatment test to minimize isolation and stress on the pups.

Behavior in the home cage. At 1, 2, 3, 4, and 24 h postinjection, the dam was briefly isolated in an open cylinder in the home cage. Her pups were removed from the nest and immediately reintroduced into the cage in a random distribution around the periphery. The cylinder isolating the dam was removed, and over the next 15 min a record was kept (under video observation and direct recording) of the order in which pups were retrieved to the nest. These experiments were carried out on 12 litters over a period of 3 months.

Behavior in the Y-maze. In another series of experiments, we used a Y-maze, each arm of which was 46 cm long, 20 cm wide and 31 cm deep, to force the dam to actively seek out and retrieve pups. Each litter was given an initial trial where the dam was allowed 5 min to explore the maze alone, followed by one 15 min retrieval trial that was not scored. At 1, 2, 3, 4, and 24 h postinjection, the dam was placed with some of her nesting material in one arm of the Y-maze with a barrier preventing her entering the other arms until the pups were placed. Her litter was then placed in the Y-maze with the LPS-injected pups in one arm and the saline-injected pups in the other arm. Pups were placed in a small container that the dam could easily enter, but from which the pups could not escape. A video record of the experiment as well as direct recording allowed us to note the order in which she retrieved the pups to the first arm over 15 min. At each of the five trials, the location of the LPS- and saline-injected pups was switched between the left and right arms to avoid any potential left/right bias. These experiments were carried out on 13 litters over a period of 2 months.

Data Analysis

Scores for the body weights at P74, elevated plus maze, and COX-2 to actin ratios, as determined by the area under the intensity profile curve of each band, were compared using one-way analyses of variance (ANOVA) with early-life treatment as the between factor. Where a significant main effect was found, this was followed by a Dunnett's *post hoc* test comparing each LPS-treated group to the saline group. Fever data were calculated as a fever index (°C × hours) for the period between 1 and 6 h after adult injection. To calculate this, the mean baseline temperature for each animal for the hour before the injection was subtracted from the 15 min temperature records after the injection and these were then summed for the 5 h to enable us to determine the area under the curve. Thus, for each animal, a value was obtained that reflects both the height and duration of the LPS-induced febrile response that follows the early, nonspecific, injection-induced hyperthermia. These data were also compared using a one-way ANOVA as above.

In the tests for mothers' interactions with immune-challenged pups, change in body weights for each neonatal treatment day were compared between LPS and saline-

treated half-litters using a Student's unpaired *t*-test. Pup retrieval was compared by assigning a number to each pup, so that the first pup retrieved in each litter was given a score of 12, the second 11 and so on. The mean of the scores for each of the LPS- and saline-treated groups was compared at each treatment day using a one-way ANOVA with time as the repeated measure. As no statistical significance was found, no further analyses were conducted.

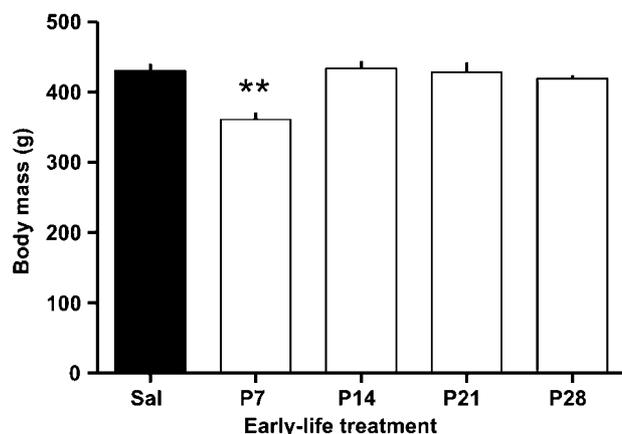


Figure 1 Body mass at postnatal day (P)74 of animals treated at P7 ($n=5$), P14 ($n=6$), P21 ($n=6$) or P28 ($n=4$) with lipopolysaccharide (LPS) or at P14 with saline (Sal; black bar; $n=6$). ** $P<0.01$ vs P14-Saline.

In each case statistical significance was assumed when $P<0.05$. Data are presented as mean \pm SE of the mean.

RESULTS

Adult Responses to an Early-Life Immune Challenge

Body mass. In animals treated with LPS at P7 ($n=5$), P14 ($n=6$), P21 ($n=6$), or P28 ($n=4$), body mass was measured at P74 and compared with the body mass from those treated with saline at P14 ($n=6$). A one-way ANOVA revealed a significant difference between the groups ($P<0.001$) and a Dunnett's *post hoc* test demonstrated that those animals treated with LPS at P7 weighed significantly less than those treated in early life with saline (361 ± 10 g compared with 430 ± 11 g; $P<0.01$; Figure 1). No other differences in body mass were found.

Elevated plus maze. Despite a tendency for animals treated with LPS at P28 to spend less time in the open arms, as well as show reduced numbers of open arm entries (Figure 2b and c), and an increased number of defecations (data not shown) in the elevated plus maze compared with rats treated at the other time points, no statistically significant differences were found between the five groups. Thus, no differences were seen between the groups treated with LPS at P7 ($n=8$), P14 ($n=6$), P21 ($n=6$), P28 ($n=7$), or with saline at P14 ($n=6$) in the latency to move into an arm

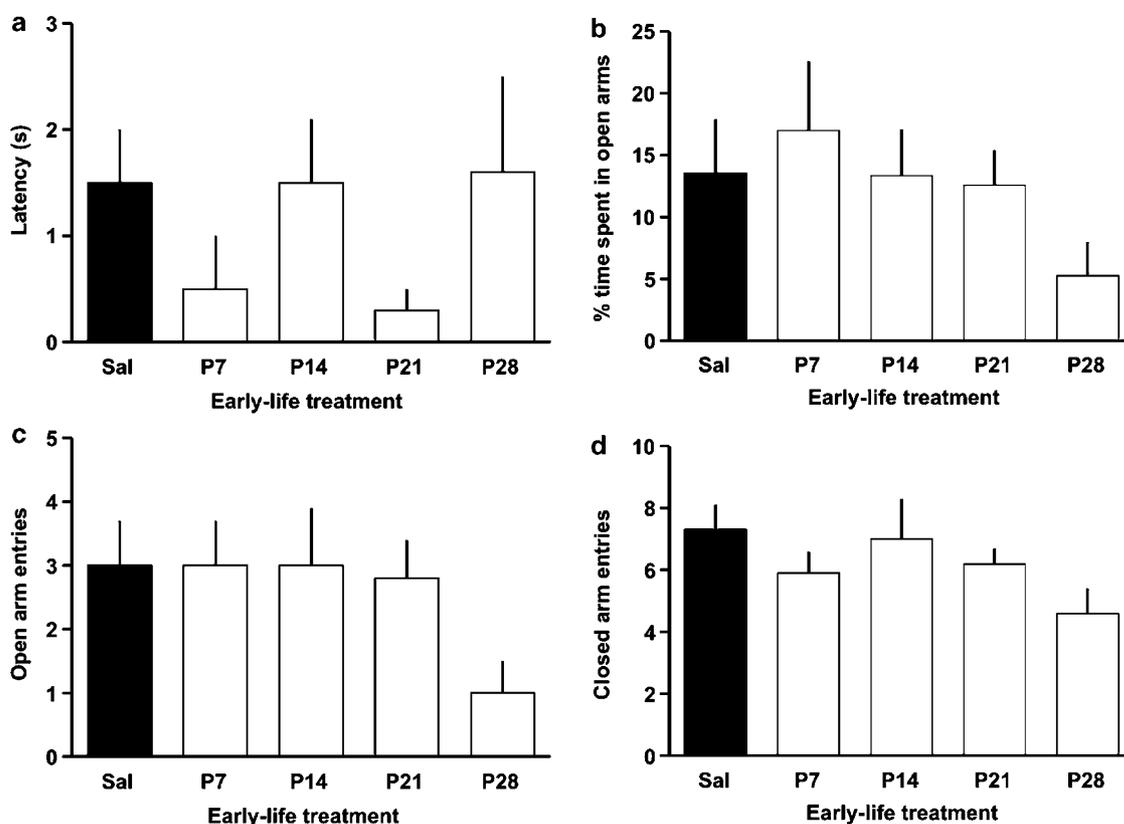


Figure 2 Elevated plus maze behavior in animals treated at postnatal day (P)7 ($n=8$), P14 ($n=6$), P21 ($n=6$) or P28 ($n=7$) with either lipopolysaccharide (LPS) or at P14 with saline (Sal; $n=6$). (a) Latency to move into an arm. (b) Percentage time spent in open arms. (c) Number of entries into open arms. (d) Number of entries into closed arms. Animals treated at P28 with LPS showed a tendency to enter and spend less time in the open arms than the rats treated at other time points, but no statistically significant differences were seen between the groups.

($P=0.296$), in the percentage time spent (open, $P=0.273$; closed, $P=0.301$), in the number of entries into either of the open ($P=0.118$), or closed arms ($P=0.124$; Figure 2).

Temperature. Prior to adult treatment with LPS, that is at baseline, temperatures and circadian rhythms of the rats were identical irrespective of early-life treatment. The adult LPS resulted in the expected injection-associated rapid transient increase in body temperature in all animals, which peaked at approximately 30 min after the injection. This initial response was followed by a prolonged hyperthermic response in all animals that peaked at approximately 3 h after injection (Figure 3a). This pattern of response did not differ among the five experimental groups.

Consistent with previous findings from this laboratory, the magnitude of this febrile response to adult LPS did, however, differ depending upon the early-life treatment to which the rats had been exposed ($P<0.01$). Rats treated at P14 with LPS ($n=7$) had an attenuated hyperthermic response to the adult LPS compared with those treated at P14 with saline ($n=7$; $P<0.05$; Figure 3). Rats treated at

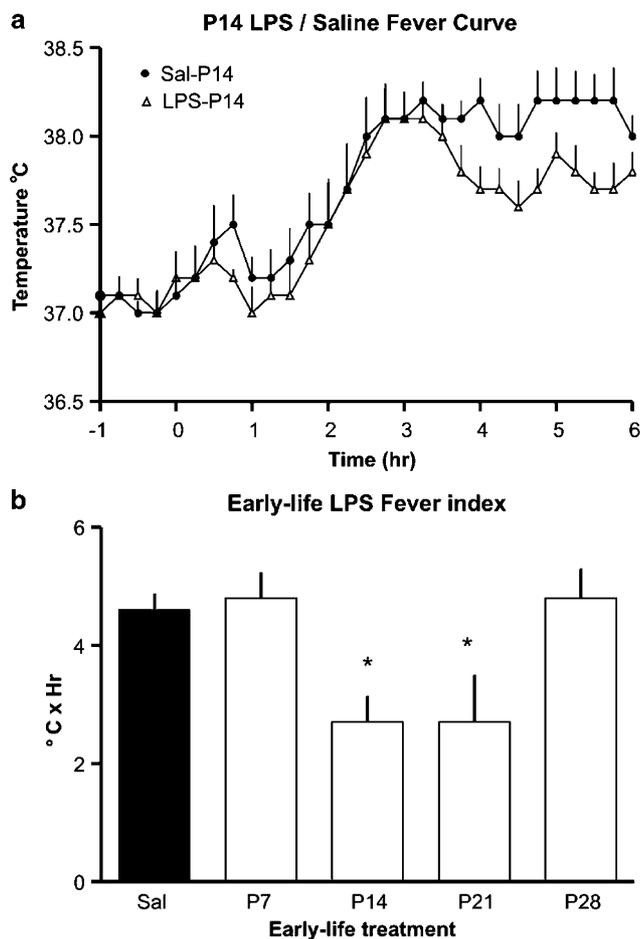


Figure 3 Body temperatures after adult lipopolysaccharide (LPS) of animals treated at postnatal day (P)7 ($n=8$), P14 ($n=7$), P21 ($n=4$) or P28 ($n=4$) with LPS or at P14 with saline (Sal; $n=7$). (a) Temperature curve of P14-LPS (open triangle) and P14-saline (filled circle) animals before and 6 h after adult LPS. Time 0 h = adult LPS injection. (b) Fever index for animals treated at P7, P14, P21 or P28 with LPS or at P14 with saline, 1–6 h after the adult LPS injection. * $P<0.05$ vs P14-Saline.

P21 ($n=4$) displayed a similar attenuation of the adult LPS-induced hyperthermia compared with saline-treated controls ($P<0.05$; Figure 3b). No differences were seen between early-life saline-treated rats and those treated at P7 ($n=8$) or P28 ($n=4$; Figure 3b).

Hypothalamic COX-2. Semiquantitative densitometric analysis ($n=6$ for all) of the Western blots for COX-2 revealed a nonstatistically significant attenuation of the LPS-induced COX-2 in the hypothalamus of adult animals previously exposed to LPS at P14 compared with P14 saline-treated animals ($P=0.202$; Figure 4). This attenuation is consistent with previous findings from this laboratory; however, in the present study, which makes comparisons between five groups, this difference in COX-2 levels did not reach statistical significance with the one-way ANOVA. Levels of COX-2 in animals treated at P21 with LPS were very similar to those of the P14 LPS-treated animals, while COX-2 levels of the P7 and P28 LPS-injected rats were more similar to those seen in the early-life saline-treated animals (Figure 4).

Mothers' Interactions with Immune-Challenged Pups

Body mass. All pups (weighed in half-litter groups with their similarly treated siblings) gained weight in the 24 h after neonatal treatment. Furthermore, irrespective of treatment day (P5, $P=0.071$; P10, $P=0.355$; and P14, $P=0.766$), this weight gain was similar between the saline and LPS groups (Table 1). A trend towards less weight gain in P5 LPS-treated pups was seen, however this difference

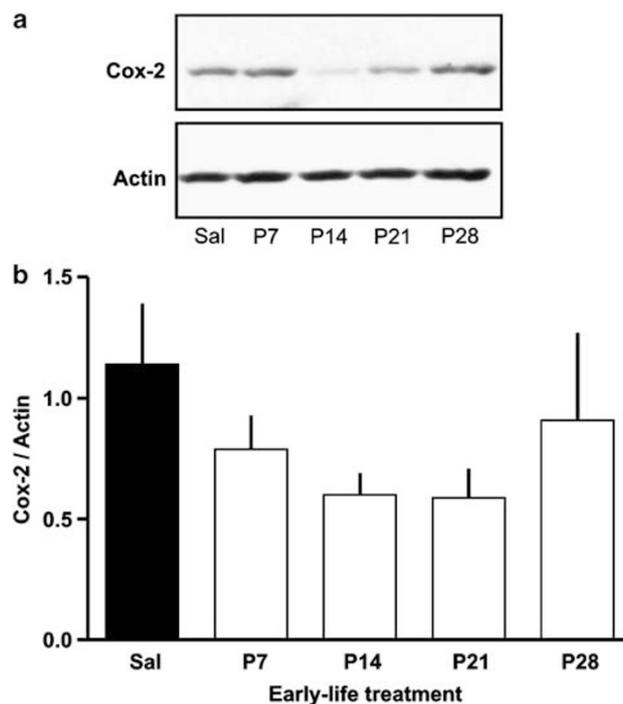


Figure 4 Adult hypothalamic cyclooxygenase (COX)-2 expression as identified by Western blots with semi-quantitative densitometric analysis. (a) Sample Western blot of hypothalamic COX-2. (b) Semi-quantitative densitometric analysis of Western blots ($n=6$ for all) after adult lipopolysaccharide (LPS) in animals treated at postnatal day (P)7, P14, P21 or P28 with LPS or at P14 with saline (Sal; black bar).

was not statistically significant. These data suggest that LPS-injected pups did not have less opportunity to suckle than the saline-treated pups, and digestion was not compromised by the LPS treatment.

Pup retrieval. When pups were given injections at P5 or P10, dams retrieved pups in the home cage with no difference observed whether the pups had been LPS-injected or saline-injected (Table 2). This finding was true at all time points after the injection, whether at the height of the immune response (calculated to be about 3–4 h after the injections (Heida *et al*, 2004)) or after a 24 h interval. At P14, pups were sufficiently mobile that they returned to the nest of their own accord, making it impossible to determine maternal attention.

When dams were required to actively retrieve pups from the arms of the Y-maze, results for pups injected at P5 and P10 were similar to those seen in the home cage (Table 2). That is, no differences were seen in order of pup retrieval by the mother. We also observed no differences in sequence of retrieval from the arms of the Y-maze between LPS- and saline-injected animals treated at P14.

DISCUSSION

In the findings of the present investigation, we demonstrate a critical window during development for the effects of an

immune challenge on adult febrile and COX-2 responses to LPS. However, other aspects of adult physiology may be affected by manipulations at different critical time points. We have shown that LPS when administered at P14 or P21, but not at either P7 or P28, significantly attenuated fever responses to adult LPS. Hypothalamic COX-2 also shows a tendency to be attenuated in these groups of animals. However, when LPS is administered at P7, adult body weights are significantly reduced, while when it is given at P28, but not at P7, 14, or 21, there is a tendency, albeit nonsignificant, towards more anxious behavior displayed in the elevated plus maze. Thus, there is no single 'critical window' in development for the transition from pup to adult. Different aspects of adult physiology are affected by challenges at different crucial stages of development.

LPS at P7 Alters Adult Body Weight

This investigation is, to our knowledge, the first to identify effects on adult body weights of a neonatal immune challenge, in that rats given LPS at P7 had significantly reduced body weights as adults while their littermates injected at other times did not. There are several possible mechanisms to account for this. There is considerable evidence that adult weight can be influenced by neonatal nutrition and food intake (Srinivasan *et al*, 2003). Thus, it is possible that the animals treated at P7 were relatively more sick than those treated at other ages and were therefore less able to compete for milk or even to suckle. In adult rats, LPS effects include decreased food intake in the hours after the LPS exposure (reviewed in Dantzer (2001)) and a similar effect may occur in neonates. However, when we examined weight changes 24 h after LPS at P5, P10 and P14, there was no difference between LPS and saline-injected animals at any time point examined, including those bracketing the P7 time point. It is also interesting that a localized painful inflammatory stimulus in litters of P3 pups has been associated with increased total nursing time for the subsequent 40 h (Anseloni *et al*, 2005). However, these experiments entailed subjecting the entire litter to the painful inflammatory stimulus; in our experiments only half the litter received a much more diffuse neuroimmune

Table 1 Change in Body Mass (g) in the 24 h after Neonatal Treatment with Lipopolysaccharide (LPS) or Saline

	nSal	nLPS
P5	9.4 ± 1.0	6.8 ± 0.9
P10	12.6 ± 1.1	11.3 ± 0.8
P14	9.3 ± 0.7	9.6 ± 0.7

There was no difference in the amount of weight gained in the 24 h after neonatal treatment between the neonatally LPS-treated (nLPS) and saline-treated (nSal) animals, irrespective of age at treatment. Pups were weighed in half-litter groups with their similarly treated siblings. Postnatal day (P)5 $n = 9$ litters, P10 and P14 $n = 8$ litters.

Table 2 Retrieval of Pups after Neonatal Treatment with Lipopolysaccharide (LPS) or Saline

	1 h Postinjection		2 h Postinjection		3 h Postinjection		4 h Postinjection		24 h Postinjection	
	nSal	nLPS	nSal	nLPS	nSal	nLPS	nSal	nLPS	nSal	nLPS
<i>Home cage retrieval</i>										
P5	7.6 ± 0.7	6.7 ± 0.7	6.4 ± 0.8	6.3 ± 0.8	7.3 ± 0.4	6.8 ± 0.5	7.6 ± 0.2	6.1 ± 0.6	7.6 ± 0.3	6.7 ± 0.5
P10	7.1 ± 0.5	6.7 ± 0.3	7.4 ± 0.3	6.3 ± 0.4	6.2 ± 0.2	7.3 ± 0.2	7.1 ± 0.2	6.6 ± 0.5	6.5 ± 0.7	8.0 ± 0.6
<i>Y-maze retrieval</i>										
P5	7.1 ± 0.9	7.3 ± 0.6	7.7 ± 0.7	7.5 ± 0.8	9.2 ± 0.8	8.7 ± 1.2	7.8 ± 1.0	8.1 ± 0.6	7.3 ± 0.7	7.0 ± 0.6
P10	7.1 ± 0.2	6.3 ± 0.4	6.5 ± 0.6	7.0 ± 0.5	7.0 ± 1.0	8.1 ± 1.0	6.1 ± 0.7	7.0 ± 0.7	6.6 ± 0.4	6.7 ± 0.2
P14	7.0 ± 0.4	6.6 ± 0.2	7.8 ± 0.6	6.8 ± 0.6	7.0 ± 0.6	7.0 ± 0.5	8.0 ± 1.0	7.3 ± 1.3	7.2 ± 0.6	6.5 ± 0.9

There were no differences in the sequence of pup retrieval in either the home cage (postnatal day (P)5 and P10 $n = 4$ litters) or the Y-maze (P5 $n = 5$ litters, P10, P14 $n = 4$ litters) between the LPS-treated (nLPS) and saline-treated (nSal) animals at any of the treatment days or time points tested.

stimulus. Based upon the weight gain data, our results do not indicate that the LPS-injected animals nursed more than their saline-injected counterparts. It is therefore unlikely that the attenuated gain in body weight seen in the adult P7 LPS-injected animals here is due to lack of maternal attention or nursing.

The differences in body weights seen with the P7-LPS animals may be due to downstream actions of LPS on factors affecting body weight or growth. For instance, LPS, as well as various cytokines are known to influence levels of leptin (Sarraff *et al*, 1997; Sachot *et al*, 2004), and this effect is reciprocal, with leptin regulating proinflammatory cytokine production (Loffreda *et al*, 1998). As leptin provides critical control of satiety and long-term body weight (Otero *et al*, 2005), increased leptin at P7, occurring in response to either LPS directly or to the subsequent cytokines produced, could lead to lasting alterations in satiety, feeding circuits, and adiposity. Leptin has recently been shown to alter the synaptic profile of hypothalamic neurons particularly those of the arcuate nucleus, which has a critical role in feeding (Pinto *et al*, 2004; Bouret and Simerly, 2004), and it has been suggested that there is a critical time during development for this programming. During the period from P7 to P10, a surge in leptin occurs (Ahima *et al*, 1998) that may lead to the establishment of a 'set point' for future levels of satiety and weight gain (Elmqvist and Flier, 2004). This leptin surge, coincident with an additional cytokine and LPS-induced leptin increase, could lead to permanent alterations in feeding pathways that would not be seen with similar LPS-induced effects on leptin at other time points in development.

In addition, there have been several reports of alterations in other hypothalamic regulatory peptides as a result of early neonatal intervention. For example, neuropeptide Y, also known to be important in appetite regulation (Kalra and Horvath, 1998; Schwartz *et al*, 2000), is increased in adults rats following a neonatal manipulation (Grove *et al*, 2001; Jimenez-Vasquez *et al*, 2001; Kozak *et al*, 2005).

LPS does not Significantly Alter Adult Behavior in the Elevated Plus Maze

Despite a strong tendency for those animals treated at P28 with LPS to display increased anxiety in the elevated plus maze compared with LPS injections at other time points, no statistically significant differences in behaviour were seen between the groups. We have previously seen minimal differences in adult behaviors in tests other than the elevated plus maze of rats treated at P14 with LPS. For instance, in the open field and in a forced swim paradigm, no behavioral differences were seen, although the P14 LPS-treated rats did show reduced exploration when exposed to novel objects (Spencer *et al*, 2005). Some alterations in performance in the elevated plus maze have been observed with a neonatal LPS challenge in investigations from other laboratories. However, at least one of these studies used the hypersensitive Fischer 344 rat (Walker *et al*, 2004), indicating a possible strain difference on behavioral consequences of neonatal LPS exposure. Also, in each of these studies, the LPS was delivered at earlier time points P3 and/or P5 (Walker *et al*, 2004; Breivik *et al*, 2002; Anseloni *et al*, 2005). It is possible that a significant effect on

behavior could be seen with an early-life LPS challenge at a different time point to those tested here, or that a different behavioral test would be more suitable for identifying differences.

LPS at P14 and P21 Alters Adult Febrile Responses to LPS

Consistent with previous findings from this laboratory (Boisse *et al*, 2004), rats in this investigation treated at P14 with LPS displayed attenuated fever responses to LPS in adulthood, as well as a tendency towards attenuated hypothalamic COX-2 expression compared with saline-treated rats. As we have previously discussed in great detail (Boisse *et al*, 2004; Ellis *et al*, 2005), these results are consistent with a mechanism whereby LPS in the neonate programs the HPA axis to interfere with corticosterone production and the elaboration of cytokines in later life. We have also demonstrated here that a similarly attenuated response is seen when the rats are administered LPS at P21, but not when treated at P7 or P28.

Our results are perhaps surprising given previous investigations into the effects of neonatal inflammation on the adult. For example, Shanks *et al* (1995) and Shanks *et al* (2000) found differences in adult HPA axis function and responses to a model of experimentally induced arthritis when the rats were given LPS at P3 and P5. Hodgson *et al* (2001) also found alterations in adult physiology with a series of immune challenges given in the first week of life. Another series of investigations has chronicled long-term alterations in pain sensitivity after short-lasting neonatal local inflammatory insult to P3 rats, an effect that was not seen when animals were similarly treated at P12 (Anseloni *et al*, 2005). These investigations all examined somewhat different end points from those looked at here and our present results indicate that this might be an important reason why these studies have found effects on the adult using such different preconditioning time points. In addition, the studies of Hodgson *et al* (2001), Shanks *et al* (1995) and Shanks *et al* (2000) used a multiple injection paradigm perhaps leading to effects on very different mechanisms.

The finding that the rats' adult febrile responses to LPS are affected by a neonatal immune challenge at P14 and P21 but not at P7 or P28 suggests that there is a discrete 'window' in which the innate immune response is subject to alteration, and that this window is likely very different for the development of different aspects of an animal's physiology. The lack of an altered response after an injection at P28, along with our previous observations that adults pretreated with LPS do not show this long-term attenuation in response to LPS (Boisse *et al*, 2004) suggest that this is indeed a developmental phenomenon. Whether this programming is specific to LPS or represents a more general change in coping remains to be determined.

During the third week of life, the rats are still with their mother and suckling, but P14 marks the end of the stress hyporesponsive period where they are incapable of mounting a normal stress response (reviewed in Levine (2001)). Perhaps the usual response associated with LPS is mitigated by either tactile stimulation from the mother, or the mother's milk and the effect would therefore not be seen

after weaning. In support of this, there is now considerable evidence that grooming by the mother, as well as feeding and passive maternal contact can act to downregulate HPA axis activity (reviewed in Levine (2001); Champagne and Meaney (2001)). It was possible that the attention of the mother subsequent to the challenge with LPS could aid to permanently attenuate responses to later challenges of the same type. We therefore attempted to quantify maternal interest in her litter at various developmental ages.

Mothers' Interactions with Immune-Challenged Pups

In these experiments, we subjected our animals to two different paradigms that could potentially reveal altered maternal-pup interactions. As each litter in our experimental paradigm contained both saline- and LPS-treated pups, we were precluded from observing direct maternal interactions with their pups, such as time spent nursing (Anseloni *et al*, 2005) or licking (Champagne and Meaney, 2001). The pup-retrieval tests allowed us to examine the possibility that maternal attention was affected by the pups displaying sickness or fever, perhaps due to changes in odor or vocalization. At none of the time points investigated was there any difference in the sequence the mothers retrieved her LPS- and saline-treated pups. This observation, along with the finding that LPS-treated pups appear to compete equally well with saline-treated pups for milk, as revealed by similar weight gains 24 h after treatment, suggests to us that there are not significant differences in maternal behavior that could account for the differential effect of LPS exposure at different ages. In addition, these observations likely indicate that differences in maternal behavior do not account for the differences in adult febrile responses we have observed here and previously in response to neonatal LPS (Boisse *et al*, 2004; Ellis *et al*, 2005). Further evidence for this is that maternal behavior has been shown to be critical for adult emotionality in rats. For instance, separation from the mother has been shown to alter responses in behavioral tests of anxiety including the elevated plus maze (Daniels *et al*, 2004; Kalinichev *et al*, 2002). As we saw no significant differences in the anxiety responses elicited by the elevated plus maze in the present study, it is unlikely that there were marked differences in maternal behavior directed towards LPS- or saline-injected pups.

Conclusions

In the present investigation, we have identified that a 'critical window' for the effects of an early-life challenge with LPS on the adult febrile response to LPS lies between P14 and P21, but that there are also other critical time points during development for the programming of other aspects of adult physiology, such as body weight and possibly behavioral responses. Differences in maternal attention or in access to milk do not appear to be responsible for the effects of neonatal LPS on adult neuroimmune responses. These findings indicate that the postnatal immune environment is crucial in the rats' development. These results have serious implications for mothering in humans, as well as for understanding and treatment of adult disorders based upon neonatal experi-

ences. They also illustrate that the timing is critical for the effects of physiological challenges, such as with a bacterial infection, on adult physiology.

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