Impact of Hyperthermia on Inflammation-Related Perinatal Brain Injury

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Abstract
In a rat model of perinatal inflammation and hypoxia, we investigated the impact of hyperthermia on the deleterious events which are commonly associated with chorioamnionitis. Late-pregnancy gestational day 20 rats received a single injection of either lipopolysaccharide (LPS) *Escherichia coli* endotoxin or saline. The offspring were born 24–36 h later at full term. The pups underwent hypoxia on the first postnatal day (PND1) immediately after which they were maintained at a planned target temperature for 2 h, before being returned to the dams. The pups were sacrificed on PNDS and the brain tissue was examined. Results showed that LPS alone or in combination with hypoxia was well tolerated. The additional stress of moderate hyperthermia (39°C for 2 h) on PND1 resulted in (a) a significant increase in brain reactive nitrogen species (RNS), (b) a significant increase in caspase-3 activity, (c) a significant increase in c-jun, bax and bcl-2 gene expression and (d) a significant increase in apoptotic cells in the CA1 region of the hippocampus. Hyperthermia was also associated with reduced growth over the ensuing 4 days in a small number of pups. In this model of perinatal inflammation, we demonstrated that brief hyperthermia when superimposed on a perinatal inflammation stimulus and hypoxia led to brain injury while either inflammation alone, or combined inflammatory stimulus and hypoxia did not cause significant damage.

Introduction
Identifiable perinatal brain insults such as ischemia and hemorrhage account for less than a quarter of cerebral palsy cases. However, amongst a group of other poorly defined prenatal and perinatal factors [1], epidemiologic and experimental studies have implicated an infection of the fetal membranes, namely chorioamnionitis, in the pathogenesis of a range of neurodevelopmental disabilities [2, 3]. In patients with chorioamnionitis, both hypoxia and fever are common. Animal studies indicate that exposure to bacterial endotoxin prior to birth sensitizes the immature brain to a subsequent hypoxic-ischemic event [4, 5]. Intrapartum fever is associated with increased risk of neonatal seizures [6], neonatal encephalopathy [7], and cerebral palsy in offspring [8]. The details of the degree, timing and duration of temperature eleva-
tion relative to any associated brain damage are not well described, including in animal models. We wished to explore brain effects of hyperthermia in a perinatal inflammation setting. We hypothesized that hyperthermia has deleterious brain effects when combined with other insults such as inflammation and hypoxia. In order to address this hypothesis, we modified a commonly used rat model of perinatal inflammation involving lipopolysaccharide (LPS) injection in late pregnancy. We then applied a clinically relevant brief hypoxic stress and hyperthermic stress on postnatal day 1 (PND1). Having previously reported that moderate (39°C) brief hyperthermia amplified the pro-inflammatory cytokine and reactive nitrogen species (RNS) response in rat pup brain after a single prenatal dose of LPS [9], our goal in the current study was to determine if hyperthermia exposure, in a setting of inflammation and hypoxic stress, would result in death of brain cells.

**Materials and Methods**

**Animals**
Timed pregnant Sprague-Dawley rats (Charles River, St. Constant, Que., Canada), based on vaginal plug, were shipped 10 days prior to the expected date of parturition and housed in pairs. The animals were maintained in standard care conditions (ambient temperature 22°C, 12-hour light/12-hour dark cycle, lights on at 06.00 h, with free access to food and water). The procedures were approved by Queen’s University Animal Care Committee in accordance with guidelines of the Canadian Council on Animal Care.

**Procedure**
At gestational day 20, the dams received a 125 μg/kg i.p. dose of LPS *Escherichia coli*, O55:B5 from Sigma-Aldrich. Control animals were injected with an equivalent volume of saline (range 200–250 μl) and all were returned to standard care conditions. The dams were injected with LPS between 08.00 and 09.00 h.

The dams delivered at term 24–36 h later. Pups were carefully labeled and litters were cross-fostered, being evenly distributed to balance maternal nurturing effect and litter size effect. Pups were labeled with code numbers with a marker and were checked daily to ensure the numbers were still legible. If they were beginning to wear off, or being licked off, the mark was clarified. In order to avoid neuroprotective effects resulting from the physiologic low temperature observed in nesting newborn rats, we had 2 control groups – both untreated and LPS-treated dams. Four experimental groups of pups were established – a control group of pups where the dam had been injected with saline and the pups remained with her throughout; a maternal LPS group that received a postnatal hypoxic stress, after which the pups were returned to the dam; a maternal LPS group that underwent postnatal hypoxia, after which the pups’ core temperature was maintained at 39°C for 2 h before returning them to the dam. Three days later, at PND5, pups from each group were sacrificed by rapid decapitation and brains were promptly removed and stored appropriately for later analysis.

**Hypoxia Exposure.** Six to twelve hours after birth, all pups scheduled for hypoxia exposure were placed in a Plexiglas chamber containing an oxygen analyzer (MiniOX, Ohio Medical). The chamber was flushed with 95% N₂/balance CO₂ to provide oxygen concentrations of 0.5–1% for 10 min during which pups were observed to have stage 2 hypoxic response with reduced respiratory rate and short apneas for up to 10 s [10]. Their temperature was maintained at physiologic 33–34°C with a nearby heat lamp. The pups were exposed to hypoxia and hyperthermia between 08.00 and 11.00 h.

**Hyperthermia.** Following hypoxia, the pups’ body temperature was stabilized for 2 h at 3 different levels while monitoring with rectal thermistor probe (Thermalert TH-5, Physitemp). Internal control pups were returned to their dams and rectal temperature in the ensuing 2 h ranged from 33.0 to 33.5°C, which was the expected temperature range for newborn rats [9] and the same as for the control pups with saline-injected dams. For the hyperthermia groups, the desired temperature was achieved by placing the pups in a plastic container in a water bath at an appropriate temperature and humidity to quickly achieve target temperatures, i.e. 37°C (mild hyperthermia) or 39°C (moderate hyperthermia). Temperature was measured with a rectal thermometer with probe being inserted 0.5–1.0 cm. The control pups that were with a dam throughout had a single measurement. The hypoxic (37 and 39°C) pups had temperature measurements until the target was reached and stabilized (3–4 measurements over 10 min) and then again at 1 h and at the end of the 2-hour hyperthermia. Pups were then returned to the dam.

**RNS Measurement.** Elevated RNS plays an important role in mediating tissue stress and damage. A modification of the Griess assay was used to quantify nitrate and nitrite levels in brain tissue. Frozen PND5 brain tissue samples were homogenized in HEPES buffer, centrifuged for 10 min and the supernatant was collected. Nitrate was catalytically converted to nitrite by the addition of vanadium trichloride according to the method of Miranda et al. [11]. Samples were mixed with Griess reagents (naphthylethylenediamine dihydrochloride and sulfanilamide in phosphoric acid) and incubated at 37°C for 1 h. Absorbances of samples were measured using a microplate reader at wavelength 540 nm.

**Caspase-3 Activity Measurement.** Hypoxic neuronal death in developing brain is predominantly apoptotic. Caspase-3 activity is specific for apoptosis. Frozen brain tissue samples were homogenized and cells lysed in lysis buffer (Biovision) on ice for 10 min, followed by centrifugation at 13,000 g for 10 min. Caspase-3 activity was determined by colorimetric assay (Biovision) using the peptide DEVD-pNA as a substrate. Samples were incubated at 37°C for 4 h and absorbances were measured at wavelength 405 nm.

**Gene Expression in the Hippocampus.** The immediate early gene, c-jun, together with the pro-apoptotic gene, bax, and the anti-apoptotic gene, bcl-2, were interrogated by quantitative RT-PCR. Hippocampi were rapidly removed from pups sacrificed on...
PND5 and promptly stored in liquid nitrogen. Total RNA from the frozen hippocampal tissue was isolated using Trizol reagent (Invitrogen-Life Technologies) according to the manufacturer’s instructions. DNA in the samples was eliminated by using DNase I digestion. RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and oligo(dT) [11]. Real-time RT-PCR was performed using a LightCycler 480 system (Roche Applied Science, Laval, Que., Canada). Primers were used as shown in table 1. SYBR Green I Master mix was supplied by Roche. Thermal cycling conditions were as follows: 95 °C for 5 min followed by 40 cycles of amplification at 95 °C for 15 s, 60 °C for 15 s and 72 °C for 15 s. Gene expression results were normalized relative to multiple control genes as described by Vandesompele et al. [12]. The internal control genes Gapdh and HprtI were selected for their high expression and low sample-to-sample variability.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Staining. We assessed PND5 cortical and hippocampal tissue for histologic evidence of apoptosis. Four-percent paraformaldehyde-fixed brain slices were submitted to in situ terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using an anti-BrdU-based kit (TACS XL-Blue label, Trevigen Inc.) according to the supplier’s instructions. Sections were counterstained with nuclear fast red. Both TUNEL-positive and -negative cells were manually counted within 8–10 sequential adjacent fields of cortex and 6–7 adjacent fields in CA1 hippocampus at a magnification of ×250. TUNEL positivity was calculated from the number of TUNEL-positive cells as a percentage of total cell count in each field and expressed as mean percent ± standard deviation (SD).

Statistical and Histological Analysis
Data were reported as mean ± SD. For body weights, caspase-3, gene expression and for histopathological studies, the data were analyzed by Student’s t test and analysis of variance. Regarding nitrite/nitrate values, the data distributed from normality and variances were not equal. Nonparametric statistics were therefore used and data were analyzed by the Kruskal-Wallis test. Differences were considered statistically significant at p values less than 0.05.

**Results**

**Response of Dams to LPS**
All pregnant animals which received LPS exhibited reduced movement and piloerection within 2 h. Dams with sustained inactivity or mild drop in core temperature (not less than 36.0°C) received a saline bolus in the hours immediately following injection to stabilize their circulation. Dams which became hypothermic or appeared very ill were either euthanized or excluded from further study. The reported data are from one set of experiments with the exception of the following mortality and morbidity data which were calculated from all three sets of replicate experiments. The overall mortality of dams was 15%. Surviving dams recovered to baseline within 12 h and were again active, eating and grooming. Both control and LPS-injected dams went on to deliver 24–36 h later at gestational day 21–22 (term). Litter size ranged from 11 to 13 and did not differ significantly between the LPS-treated and control groups. Stillbirth occurred in 4% of pups from the LPS-treated dams. No stillbirths were observed in control dam litters. While no control LPS-plus-hypoxia-treated pups died, the mortality rate of LPS-, hypoxia- and hyperthermia-treated pups was 4%.

**Effects of Exposures on Growth of Rat Pups**
Pups were weighed on PND1 and PND5. Pup weight and average weight gain of pups from each fostering dam were not significantly different (table 2). Maternal LPS alone did not affect birth weight or growth relative to saline or no intervention controls. Although there were no statistically significant differences in average pup growth from birth through PND5, there were outliers with re-

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Table 1. Primer sequences used for RT-PCR

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Accession No.</th>
<th>Orientation</th>
<th>Sequence (5′-3′)</th>
<th>Product size, bp</th>
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<tbody>
<tr>
<td>gapdh</td>
<td>NM017008</td>
<td>Fw</td>
<td>CTCTGCTCCTCCCTGTGTTCTA</td>
<td>99</td>
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<tr>
<td></td>
<td></td>
<td>Rev</td>
<td>GCCAAATCGTTCACACCAC</td>
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</tr>
<tr>
<td>hprtI</td>
<td>NM012583</td>
<td>Fw</td>
<td>CTTCTCTCTTCAGACGGTTTT</td>
<td>80</td>
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<td></td>
<td></td>
<td>Rev</td>
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</tr>
<tr>
<td>c-jun</td>
<td>NM021835</td>
<td>Fw</td>
<td>CGACCAGAACGAGTTGCTTT</td>
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<td></td>
<td></td>
<td>Rev</td>
<td>CGGAGCACTACAGAAAGCAT</td>
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<tr>
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<td>GCAGAGGATGATTGCTGACG</td>
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<td></td>
<td></td>
<td>Rev</td>
<td>TCCAGTGTCGAGCCCAT</td>
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<tr>
<td>bcl-2</td>
<td>NM016993</td>
<td>Fw</td>
<td>GGAACCGAGGCTGGGTGTG</td>
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<td></td>
<td></td>
<td>Rev</td>
<td>TATTGGTTGCGGCAGGTTCT</td>
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</table>

Fw = Forward primer; Rev = reverse primer; gapdh = glyceraldehyde-3-phosphate dehydrogenase; hprtI = hypoxanthine phosphoribosyltransferase 1.
tarded growth which came from the mild (37°C) and moderate hyperthermia (39°C) groups (table 2).

**RNS Production**

In our previously reported study, elevated levels of nitrite in neonatal rat brains were observed 3 and 24 h after hyperthermia treatment following maternal LPS administration but not with hyperthermia alone [9]. To investigate the longer-term effects of hyperthermia on brain RNS, in this study the nitrate and nitrite concentrations in the cortex were measured 4 days following hyperthermia treatment. As shown in figure 1, brain cortex of the moderate hyperthermia group of pups at PND5 showed an increase in nitrite/nitrate levels relative to normothermia pups, which was, however, not statistically different from controls (saline, no other intervention). Mild hyperthermia did not significantly alter nitrate/nitrite levels. LPS administration alone revealed lower RNS, although this was not statistically significant.

**Caspase-3 Activity**

The activity of the pro-apoptotic enzyme, caspase-3, was also measured in pup brain 4 days after the exposures. In the moderate hyperthermia (39°C) group, caspase-3 activity was significantly increased compared to the control group and the normothermia/LPS/hypoxia-treated group. Caspase-3 activity was lower in the normothermia group and higher in the mild hyperthermia-treated group when compared to control, but neither was statistically significant (fig. 2).

### Table 2. The effect of maternal LPS and postnatal hypoxia and hyperthermia on rat pup growth

<table>
<thead>
<tr>
<th>Experimental procedure</th>
<th>PND1 (g)</th>
<th>PND5 (g)</th>
<th>Weight gain (PND5 – PND1)</th>
<th>Number of pups that gained &lt;1 g</th>
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<tbody>
<tr>
<td>Control (n = 15)</td>
<td>6.49±0.39</td>
<td>9.7±0.45</td>
<td>3.2±0.42</td>
<td>0</td>
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<tr>
<td>LPS + hypoxia with dam (n = 14)</td>
<td>6.74±0.56</td>
<td>9.89±0.9</td>
<td>3.16±0.92</td>
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<tr>
<td>LPS + hypoxia 37°C (n = 11)</td>
<td>6.56±0.47</td>
<td>9.39±1.11</td>
<td>2.71±0.91</td>
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<tr>
<td>LPS + hypoxia 39°C (n = 19)</td>
<td>6.53±0.55</td>
<td>9.12±1.59</td>
<td>2.66±1.51</td>
<td>3</td>
</tr>
</tbody>
</table>

Data from 5 litters in which 2 dams were injected with saline and 3 dams were injected with LPS. Columns labeled PND1 and PND5 indicate weight in grams (mean ± SD). The last column indicates the number of pups that gained less than 1 g in 4 days.

![Fig. 1. Boxplot representation of nitrite and nitrate production in neonatal rat brain 4 days after they received hypoxia treatment followed by either normothermia (LHN), n = 6, mild hyperthermia (LH37), n = 5, or moderate hyperthermia (LH39), n = 6. Values expressed as means ± SD. ** p < 0.01 compared with normothermia. Control n = 4.](image1)

![Fig. 2. Caspase-3 activity in neonatal rat brain 4 days after they received hypoxia treatment followed by either normothermia (LHN), n = 6, mild hyperthermia (LH37), n = 5, or moderate hyperthermia (LH39), n = 6. Control represents pups from saline-injected dams, n = 4. Values expressed as means ± SD * p < 0.05; ** p < 0.01.](image2)
Gene Expression in the Hippocampus

Quantitative real-time PCR showed that hyperthermia resulted in significantly increased hippocampal mRNA levels of c-jun and bax and also led to an increase in the anti-apoptotic gene, bcl-2 (fig. 3). Levels of c-jun mRNA in LPS/hypoxia/hyperthermia treatment groups were greater than those in the control or LPS/hypoxia/normothermia treatment groups. Bax mRNA was elevated in both moderate hyperthermia and mild hyperthermia treatment groups compared to the control. However, only in the moderate hyperthermia combination group was bax mRNA significantly higher than in the normothermia group. Bcl-2 mRNA levels were significantly elevated in the moderate hyperthermia/LPS/hypoxia treatment group when compared to either control or normothermia groups. However, bcl-2 mRNA levels were not changed in response to mild hyperthermia.

Cell Death

To quantify cell death, TUNEL was performed on PND5 cortical and hippocampal regions to assess the percentage of cells undergoing apoptosis. In the cortical regions, combined LPS and hypoxia showed a trend towards an increase in TUNEL-positive cells. A statistically significant increase was seen in the 37°C group (fig. 4). In the hippocampus, apoptosis in the LPS/hypoxia group immediately returned to the dam was not statistically significant. Cell death was similar in the 37°C and control pup hippocampus, but moderate hyperthermia pups (LPS/hypoxia/39°C) had a significantly higher percentage of apoptotic cells in the hippocampus both when compared with untreated controls (p < 0.05) and internal control, LPS/hypoxia control groups (p < 0.01).

All three sets of completed experiments showed the same pattern of findings. Those few pups in the hyperthermia groups that had poor growth had similar changes in RNS, caspase and TUNEL patterns to those within the group which had normal growth (data not shown).

Discussion

In this rat model of combined late-pregnancy LPS exposure and early postnatal hypoxia, we found that an additional stress in the form of a 2-hour period of moderate hyperthermia caused significant neonatal brain disruption with increases in RNS and caspase-3 activity. Furthermore, while combined maternal LPS and postnatal hypoxia did not cause cell death, the three perturbations together increased apoptotic cell death in the CA1 region of the hippocampus significantly. Hyperthermia was thus the key compounding factor in mediating brain damage in this triad of perinatal stressors.
Elevated temperature is known to impair brain development. It is teratogenic during the first half of fetal life [13] manifesting as major structural brain defects [14]. Animal studies indicate that hyperthermia impairs fetal and newborn tolerance to hypoxia-ischemia. Small increases of 1–2°C in brain temperature during hypoxia-ischemia in 7-day rat pups worsened subsequent behavioral deficits [15]. The normal temperature of newborn rat pups is known to be 33–34°C. Establishing what is abnormally elevated temperature is somewhat complicated. We were concerned that if we only chose 37°C as hyperthermia, this may equate to simply removing a potentially neuroprotective effect of the naturally low core temperature in pups. We thus included a 39°C group. In 10-day rat pups, a core body temperature of 37.5°C compared with 36.0°C for 4 h immediately after hypoxia-ischemia increased the extent of neuronal injury [5]. Marked hyperthermia present during an insult has also been studied. A temperature of 42°C during 15 min of severe hypoxia-ischemia was associated with caspase-3 activation and cerebral apoptosis [16]. Furthermore, seizure-associated spontaneous hyperthermia occurring after ischemia in neonatal rat pups led to worsening of brain damage. This did not occur when hyperthermia was prevented [17]. Interestingly, in the latter study, the worsening of cell death was found in the hippocampus, similar to what we observed.

We noted several interesting features in the pattern of RNS and caspase-3 changes following insult. Elevated nitrate levels found in the hyperthermia group 4 days after stress were somewhat surprising as the RNS surge might be expected to have dissipated by then. It suggests that an ongoing pro-inflammatory process may have maintained or resurgent inflammation may have been triggered by the brief period of hyperthermia resulting in ongoing stress manifest as elevated free radical production.

Similar to RNS patterns, caspase-3 was elevated at a time point remote from the hyperthermia stress. Although caspase-3 might be expected to peak at 24–48 h after insult, and then decline, our finding of elevated caspase-3 at PND5 supports the view of either persistent or delayed activation of pro-apoptotic cell death pathways. Post-insult neuronal death in developing brain is predominantly apoptotic and increased caspase-3 activity is specific for apoptosis. We noted incremental increases in caspase-3 activity as more stressors were applied. The significantly increased caspase-3 activity with hyperthermia indicated that these newborn animals can tolerate this level of insult well but hyperthermia was the critical component in the cumulative increase in stressors in vulnerable brain areas. Of note, the pattern of caspase-3 activity with increased stressor exposure mirrored the pattern of increased TUNEL positivity in the hippocampus.

Both pro- and anti-apoptotic genes were elevated in the hippocampus, which at first glance appears contradictory. However, a compensatory increase in immediate early genes and in anti-apoptotic genes in more resistant cells within the region to counter the apoptosis which has taken place in the hippocampus, seems plausible. This interpretation is supported by the observation that bcl-2 expression was not significantly elevated in experimental groups where apoptosis was not increased.

We noted a small decrease in RNS 4 days after LPS when compared to saline-treated controls, which might suggest that rat offspring adapt quickly to a pro-inflammatory stimulus to the mother. It may have been due to rapid induction of anti-oxidant enzymes. Further studies, however, are needed to explore the reason for this observation.

What is the mechanism of hyperthermia-associated brain damage? Intra-ischemic hyperthermia results in enhanced release of neurotransmitters, exaggerated oxygen radical production, reduced blood-brain barrier competence, enhanced inflammatory responses, impaired recovery of energy metabolism [18] and protein synthesis, and worsening of cytoskeletal proteolysis. Caspase activation is likely a final common pathway to cell death [19]. The exacerbating effects of hyperthermia may simply be the converse of the neuroprotective effect of cooling as observed in hypoxia-ischemia [20–22], but this has not been confirmed.

Do our data have implications for human infants? Approximately 7% of women have fever during labor [8]. Fever is a known risk factor for neonatal encephalopathy [7, 23], neonatal seizures [6], cognitive impairment [24] and cerebral palsy [25]. Maternal temperature greater than 38°C in labor is associated with increased risk of unexplained cerebral palsy (odds ratio = 9.3; 95% CI = 3.7–23) [25]. It is not known if regulated, cytokine-mediated, fever as might occur with infection and a non-regulated environmental hyperthermia from any cause has different outcomes. We speculate that the outcome is similar since both regulated and nonregulated fever may be felt by the fetus as pure environmental hyperthermia, with the fetal core temperature being approximately 0.5°C higher than the mother’s core temperature [26].

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In a randomized clinical trial on cooling for hypoxic-ischemic encephalopathy, Laptook et al. [27] noted temperature elevations in the nonintervention group of infants. After control for many key variables, temperature elevations were associated with significant increases in poor outcomes. For every 1°C increase in the mean of the highest quartile for temperature, death or moderate to severe disability was increased 4-fold [27]. One might conclude that spontaneous or environmental hyperthermia following insult may be deleterious and should be avoided.

The model provides the ability to study the impact of late-pregnancy inflammation and to adjust the intensity while controlling and monitoring a key variable in the offspring, namely, core temperature. Previous models looking at the effect of hyperthermia have used relatively prolonged hyperthermia exposures or severe cerebral ischemia [16]. Our experiments attempted to closely mimic some common perinatal events in humans. Although we designed our experiments as a potential model for cerebral palsy, it appears to manifest in more subtle yet significant brain damage originating during the perinatal period. The three stressors were individually mild and one would not be expected to independently cause brain injury. However, a combination of all three perturbations is deleterious. A longer-term follow-up study of neurobehavior and neuropathology would be informative but was beyond the scope of this study.

A proportion of pups were assigned to each of the different groups from each litter to ensure the different dams contributed pups to the different postnatal treatments. We were concerned that dams that had received LPS may not have fully recovered, which might impair their milk supply or nurturing capabilities. In hindsight, this was probably unnecessary as the dams appeared to be healthy after they gave birth.

This study explored the contribution of hyperthermia to brain damage in offspring, particularly when it occurs around the time of a combined perinatal inflammation and hypoxia. Future long-term studies using this model to assess neuropathology and behavior will provide further insight as to the degree of damage and functional impairment. Given that newborn rat brain ontogeny broadly parallels those of third-trimester human fetus, our data may be relevant to inflammation-related, fever-associated events in late pregnancy.

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References


