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Hypothermia Does Not Reverse Cellular Responses Caused by Lipopolysaccharide in Neonatal Hypoxic-Ischaemic Brain Injury

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Animal model · Apoptosis · Astrocyte · Microglia · Hypoxia-ischemia · Hypothermia · Infection · Lipopolysaccharide · Neuron · Neuroprotection

Abstract
Introduction: Bacterial lipopolysaccharide (LPS) injection prior to hypoxia-ischaemia significantly increases hypoxia-ischaemic brain injury in 7-day-old (P7) rats. In addition, therapeutic hypothermia (HT) is not neuroprotective in this setting. However, the mechanistic aspects of this therapeutic failure have yet to be elucidated. This study was designed to investigate the underlying cellular mechanisms in this double-hit model of infection-sensitised hypoxia-ischaemic brain injury. ***Material and Methods:*** P7 rat pups were injected with either vehicle or LPS, and after a 4-hour delay were exposed to left carotid ligation followed by global hypoxia inducing a unilateral stroke-like hypoxia-ischaemic injury. Pups were randomised to the following treatments: (1) vehicle-treated pups receiving normothermia treatment (NT) (Veh-NT; n = 40), (2) LPS-treated pups receiving NT treatment (LPS-NT; n = 40), (3) vehicle-treated pups receiving HT treatment (Veh-HT; n = 38) and (4) LPS-treated pups receiving HT treatment (LPS-HT; n = 35). On postnatal day 8 or 14, Western blot analysis or immunohistochemistry was performed to examine neuronal death, apoptosis, astrogliosis and microglial activation. ***Results:*** LPS sensitisation prior to hypoxia-ischaemia significantly exacerbated apoptotic neuronal loss. NeuN, a neuronal biomarker, was significantly reduced in the LPS-NT and LPS-HT groups ($p = 0.008$). Caspase-3 activation was significantly increased in the LPS-sensitised groups ($p < 0.001$). Additionally, a significant increase in astrogliosis (glial fibrillary acidic expression, $p < 0.001$) was seen, as well as a trend towards increased microglial activation (Iba 1 expression, $p = 0.051$) in LPS-sensitised animals. Treatment with HT did not counteract these changes. ***Conclusion:*** LPS-sensitised hypoxia-ischaemic brain injury in newborn rats is mediated through neuronal death, apoptosis, astrogliosis and microglial activation. In this double-hit model, treatment with HT does not ameliorate these changes.

Introduction

Perinatal hypoxic-ischaemic (HI) brain injury is one of the major causes of long-term neurological disability or death in term newborns. Perinatal infection is also known to increase the vulnerability of the newborn brain to hypoxia-ischaemia [1–5]. Therapeutic hypothermia (HT) is the standard treatment for term infants after peri-

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natal HI injury [6], as it has been shown to significantly reduce mortality and neurodevelopmental disability in survivors [7]. However, around 50% of cooled asphyxiated newborns still suffer poor outcomes [7], some of which may have been exposed to perinatal infection [8].

In a previous study [5], we showed that exposure of neonatal rats to bacterial lipopolysaccharide (LPS) 4 h prior to a mild hypoxia-ischaemia insult significantly increases brain injury and appears to abolish the neuroprotective effects of HT. The mechanisms behind this finding are not yet fully elucidated. HI insults induce an almost immediate inflammatory response in the neonatal brain mediated through activation of microglia and astrocytes, and subsequent production of cytokines [9, 10]. This neuroinflammatory response has a dual role in the brain [9, 11, 12]. Inflammatory cells participate in beneficial tissue remodelling after a hypoxic brain injury [13]. However, induction of the inflammatory cascade also leads to blood-brain barrier disruption, stimulation and infiltration of neutrophils and monocytes, gliosis and increased apoptosis in the ischaemic brain. All of these processes contribute to the aetiology of HI brain injury [9, 14]. Furthermore, HI brain injury will also initiate a complex bidirectional crosstalk between the brain and the systemic immune system, which can exacerbate brain injury [15].

This study was designed to investigate cellular responses and cell death in a double-hit model of LPS-sensitised HI brain injury in neonatal rats under both hypothermic and normothermic conditions. This can provide insight into why HT is not neuroprotective in this setting, as well as direct future research towards potential therapies for neonatal hypoxia-ischaemia in the context of perinatal infection.

Material and Methods

Procedures

All experiments were approved by the University of Oslo's Animal Ethics Research Committee. Experiments were performed on 7-day-old (P7) Wistar rats (Charles River, Sulzfeld, Germany) of both sexes, randomised prior to treatment across litter, sex and weight. All pups were kept in an animal facility with a 12:12-hour dark/light cycle at 19–21°C environmental temperature with food and water ad libitum, and were weighed and checked for health daily.

LPS-Sensitised HI Brain Injury

A total of 153 pups were randomised to the following four groups: (1) vehicle-treated pups receiving normothermia treatment (NT) (Veh-NT; n = 40), (2) LPS-treated pups receiving NT treatment (LPS-NT; n = 40), (3) vehicle-treated pups receiving HT treatment (Veh-HT; n = 38) and (4) LPS-treated pups receiving HT treatment (LPS-HT; n = 35). Of these, 66 animals were used for Western blot analysis and 87 for immunohistochemistry. The animals that served as temperature probes and those that died during experimental procedures are not included in the above numbers, and were excluded from the final analysis.

All experiments were performed as previously described [5]. Briefly, at the start of each experiment, animals were injected according to randomisation with a single intraperitoneal dose of either vehicle (0.9% NaCl) or LPS solution (*Escherichia coli* O55:B5, Sigma; 0.1 mg/kg), given in a volume of 10 μl/g of body weight. At this dose, LPS does not induce area loss unless combined with hypoxia-ischaemia [5]. After a 4-hour delay with their dams, pups were exposed to a mild HI insult (ligation of left carotid artery under isoflurane anaesthesia followed by exposure to 8% O_2 for 50 min). Immediately thereafter, pups received either of the 2 allocated treatments: 5 h of NT (T_{rectal} 37.0°C) or HT (T_{rectal} 32.0°C).

During treatment, the core and rectal temperature of two 'sentinel' pups, which were injected with the vehicle, was continuously recorded in each chamber. The temperature of the chamber was maintained within ±0.2°C of the target value using a continuous rectal probe temperature recording (IT-21; Physitemp Instruments, Clifton, N.J., USA), which servo-controlled a water-filled mat (CritiCool, MTRE, Yavne, Israel) on the floor of the chamber.

After the 5-hour treatment period, pups were returned to their dams. Pups were sacrificed on postnatal day (P) 8 or P14, according to the protocol.

Western Blotting

For Western blotting analysis, pups were sacrificed at either P8 or P14. Samples from P8 brains were analysed for cleaved caspase 3 (cCas3, a biomarker for apoptotic cell death) or ionised calcium-binding adapter molecule 1 (Iba1, a biomarker for microglia). Samples taken at P14 were analysed for neuron-specific nuclear protein (NeuN, a neuronal biomarker) or glial fibrillary acidic protein (GFAP, a biomarker of mature astrocytes). These specific time points were chosen because cCas3 [16] and Iba1 [17] peak around 24 h after HI brain injury (P8), and 1 week (P14) is the normal survival time in our model. Brains were collected by decapitation. Left and right hemispheres were snap frozen separately in liquid nitrogen and stored at –80°C until further processing. Frozen brain tissue was crushed and homogenised by sonication in ice-cold radioimmunoprecipitation assay buffer (RIPA; Millipore, Mass., USA) to which Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford, Ill., USA) was added. Samples were centrifuged at 4°C for 10 min (2,000 *g*), and the supernatant collected. Protein concentrations were determined with a micro-BCA protein assay kit (Thermo Scientific). Cell lysates (10 or 25 μg/lane) in Laemmli buffer were loaded on a 4–20% gradient polyacrylamide gel (Bio-Rad, Hercules, Calif., USA) and transferred to nitrocellulose membranes (Bio-Rad). Membranes were then incubated in blocking buffer, 5% non-fat dry milk in TBST (20 mM Tris–HCl, pH 7.6, 137 mM NaCl and 0.1% Tween-20), at room temperature for 1 h with agitation, followed by incubation with rabbit antibody against NeuN (1:2,000; Millipore, Billerica, Mass., USA), cleaved caspase 3 (1:2,000; Cell Signalling Technology, Beverly, Mass., USA) and Iba1 (1:1,000; WAKO, Tokyo, Japan), or mouse antibody against GFAP (1:1,000; Millipore). β-Actin (1:5,000; Abcam, Boston, Mass., USA) was used as an internal protein control. Following rinses with TBST, the membranes were incubated with peroxidase-conjugated goat anti-rabbit or anti-mouse secondary anti-

bodies (1:3,000 and 1:10,000; Southern Biotech, Birmingham, Ala., USA) in blocking solution for 1 h at room temperature. Bands were visualised using Pierce ECL2 Western Blotting Substrate (Thermo Scientific, Rockford, Ill., USA) according to the manufacturer's instructions. ImageQuant (ImageQuant TL, version 7.0; GE Healthcare, N.J., USA) was used to measure the optical densities of the protein signals on scans of X-ray films. The relative optical density was calculated using the optical density of protein signals divided by the optical density of a loading control (β-actin or Ponceau staining; the latter was used if the molecular weight of the examined protein was too similar to the molecular weight of β-actin). All values for a particular Western blot were normalised to the protein expression in the right hemisphere (unligated, exposed only to hypoxia) of the Veh-NT group, as this hemisphere was considered to act as an internal control [18].

Immunohistochemistry

For immunohistochemistry analysis, pups were sacrificed either at P8 (Iba1, NeuN) or P14 (NeuN). Transcardiac perfusion with 10% neutral-buffered formalin was performed under isoflurane/N_2O-anaesthesia. Brains were harvested and kept in 10% neutral-buffered formalin for 7 days until further processing. Coronal 3-mm blocks were cut through the brain using a standard matrix (ASI Instruments Inc., Warren, Mich., USA) and embedded in paraffin. Two 5-μm sections were cut from blocks, giving representative areas of the cortex, hippocampus, basal ganglia and thalamus. Paraffin-embedded tissue was deparaffinised in xylene and rehydrated in decreasing concentrations of ethanol. Antigen retrieval was then performed by citrate buffer solution pH 6.0, using a PT link instrument (Dako, Glostrup, Denmark). After blocking in 10% goat serum, primary rabbit antibody against Iba1 (1:1,000; WAKO), NeuN (1:500; Millipore) or caspase 3 (1:2,000; Cell Signalling Technology), or mouse antibody against NeuN (1:500; Millipore), was applied overnight at room temperature. In control brain sections, the primary antibodies were omitted. After rinsing with PBS, the slices were incubated for 1 h at room temperature with secondary Alexa Fluor 568 and/or 488 (Invitrogen, 1:500) antibodies. Finally, the slides were rinsed and coverslipped with ProLong Gold with DAPI (Invitrogen). Sections were scanned with a virtual microscopy scanner (Axio Scan.Z1; Carl Zeiss, Jena, Germany) using the fluorescence mode with plan apochromatic 20× lens. Virtual slides were exported as high-resolution tiff images for further analysis.

To evaluate the effect of different treatments on neuronal loss, neurons in the CA1 region of the hippocampus were counted, as this region is known to be particularly vulnerable to hypoxia at P7 [19]. Three non-overlapping fields of the CA1 region in both the right and left hippocampi (6 animals per treatment group) were assessed. Cells that were positive for both NeuN (neuronal marker) and DAPI (cell nucleus) were counted as neurons. Counting was performed by two individual observers blinded to the treatment group, and an average of the two was taken for further analysis. The total number of neurons across the three fields of each hippocampus was summed, and a ratio of L/R hippocampal neuron count was calculated.

Data Analysis

One-way ANOVA was used to examine differences across all treatment groups. To investigate the nature of any interaction between ipsi-/contralateral hemispheres, vehicle/LPS injection and NT/HT treatment on the expression of various Western blot biomarkers or hippocampal neuron count, we used a multivariate linear regression analysis (the 'Enter' method). To question whether there is an interaction between the combined effects of LPS and HT, a variable indicating this combination was used in the multivariate linear regression. If this variable showed significance in the linear regression model, this would indicate that the combined treatment acted synergistically to influence the severity of injury. A probability value of ≤0.05 was considered statistically significant. Graphical data are presented as means with 95% CI. SPSS software version 22 (SPSS Inc., Chicago, Ill., USA) was used for statistical analyses.

Results

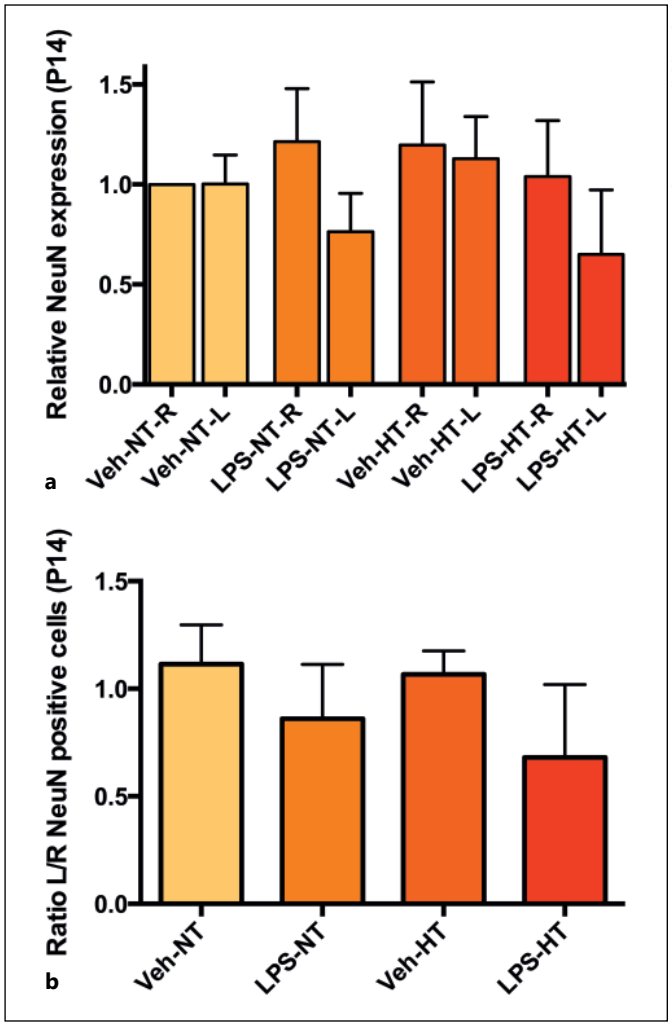
LPS Sensitisation Exacerbates Neuronal Loss

Figure 1a shows the relative expression of NeuN in the left (ipsilateral) and the right (contralateral) hemispheres, determined by Western blot analysis, across the 4 groups of animals 1 day after hypoxia-ischaemia (P8). One-way ANOVA analysis showed there were significant differences between the treatment groups ($p = 0.006$). In addition, linear regression analysis showed that the ipsilateral sides expressed significantly less NeuN compared to the contralateral sides ($B = -0.226$, $p = 0.003$). HT treatment by itself did not affect NeuN expression ($p = 0.9$), while LPS injection prior to hypoxia-ischaemia significantly decreased NeuN expression ($B = -0.146$, $p = 0.049$). Moreover, there was a significant synergistic interaction between LPS exposure and HT, with the combination being more detrimental than LPS alone ($B = -0.305$, $p = 0.036$).

Figure 1b shows the relative number of NeuN-positive cells in the CA1 region of left and right hippocampi, expressed as the mean of the L/R ratios for each group. One-way ANOVA showed significant differences between the treatment groups ($p = 0.008$). Linear regression analysis showed that HT treatment did not affect the L/R ratio of NeuN-positive cells ($p = 0.2$), while LPS exposure significantly decreased the ratio of NeuN-positive cells ($B = -0.315$, $p = 0.003$). No significant interacting effect between hypoxia-ischaemia and LPS on the number of NeuN-positive cells in the hippocampi was found.

LPS Sensitisation Increases Apoptosis

Western blot analysis showed significant differences in expression of cCas3 between the treatment groups ($p < 0.001$; fig. 2a). Ipsilateral hemispheres showed significantly more cCas3 expression compared to the contralateral sides ($B = 2.814$, $p < 0.001$). HT treatment did not significantly decrease cCas3 expression in this mod-



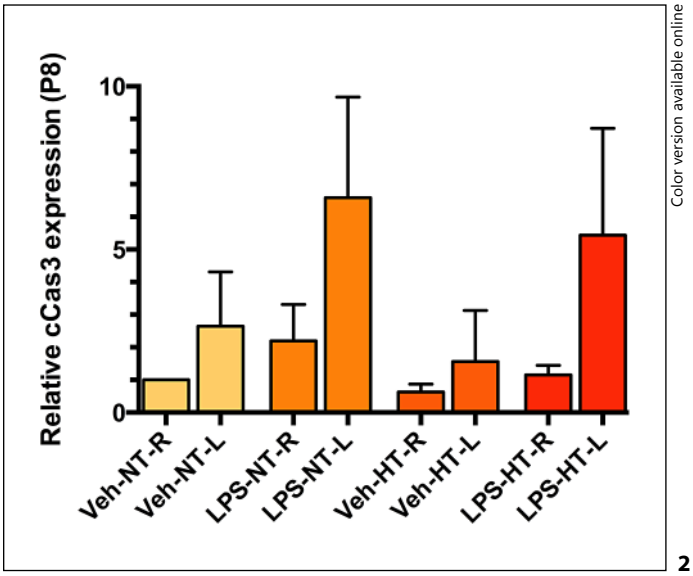


Fig. 1. a Western blot analysis of relative NeuN expression on P14, which is 7 days after hypoxia-ischaemia in the corresponding treatment groups. Ipsilateral hemispheres (L; left carotid ligation) and LPS sensitisation were both related to lower NeuN expression. **b** Ratios (L/R hemisphere) of NeuN-positive cells in the CA1 region of hippocampi 7 days after treatment. Fewer NeuN-positive cells were seen in the left hippocampal CA1 regions from LPS-sensitised animals. Bars show means with 95% CI. L = Left hemisphere; R = right hemisphere.

Fig. 2. Western blot analysis of cleaved caspase 3 expression 24 h after hypoxia-ischaemia. Ipsilateral hemispheres showed increased cCas3 expression, compared to contralateral hemispheres. LPS sensitisation significantly increased cCas3 expression. Bars show means with 95% CI. L = Left hemisphere; R = right hemisphere. Bar, 20 μm.

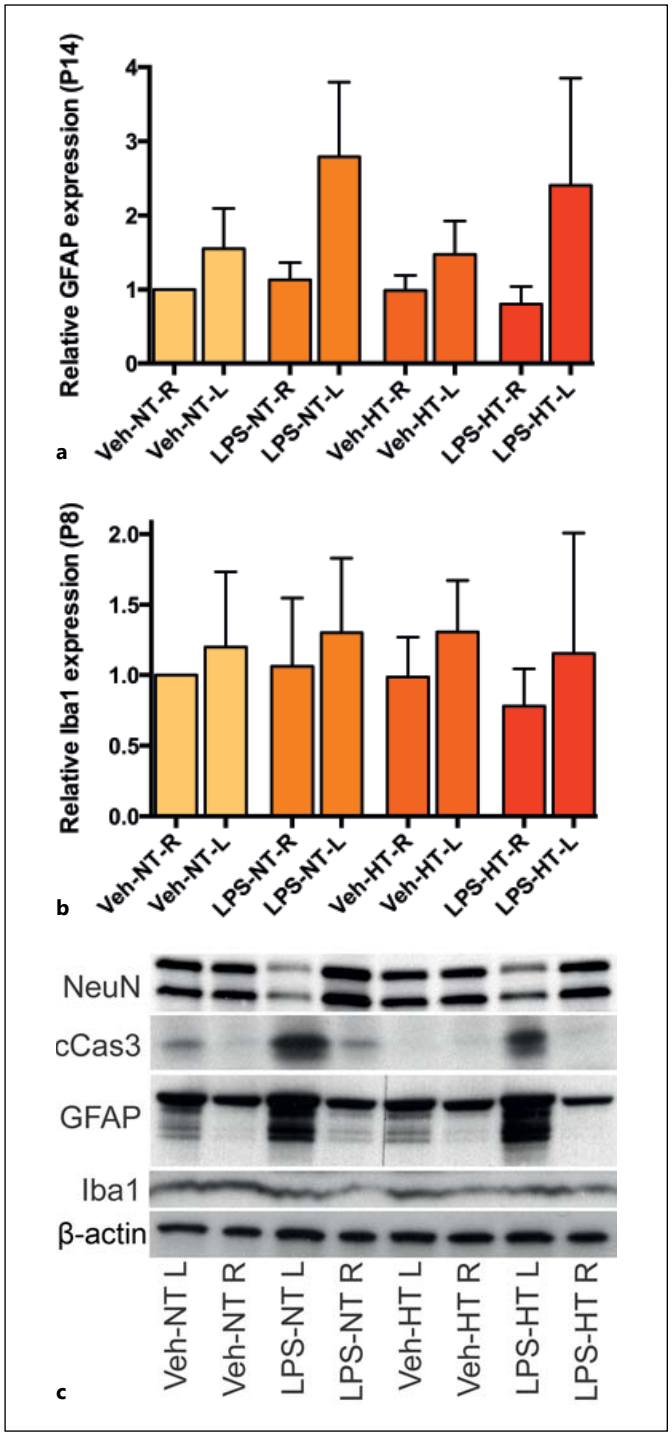
el (B = –0.911, p = 0.105). LPS injection prior to hypoxia-ischaemia significantly increased cCas3 expression (B = 2.386, p < 0.001). No significant interaction between LPS and HT was found in relation to cCas3 expression.

LPS Sensitisation Enhances Astrogliosis

There were significant differences between the treatment groups in Western blot expression of GFAP (p < 0.001; fig. 3a). Ipsilateral hemispheres showed significantly more GFAP expression compared to the contralateral sides (B = 1.049, p < 0.001). HT treatment had no significant effect on astrogliosis (p = 0.39). LPS injection prior to hypoxia-ischaemia significantly increased GFAP expression (B = 0.569, p < 0.006). There was no significant interaction between pre-hypoxia-ischaemia exposure to LPS and HT.

Microglial Activation after 1 Day of LPS Exposure and Hypoxia-Ischaemia

Western blot analysis with linear regression showed a trend towards higher expression of Iba1 in ipsilateral hemispheres, compared to contralateral hemispheres, although this was just above the significance level (p = 0.051; fig. 3b). There was no quantitative effect of LPS injection or HT on Iba1 expression. However, Iba1-positive cells were morphologically different in groups pretreated with LPS (fig. 4). Iba1-positive cells could be seen in both cerebral hemispheres in all groups, but they were most abundant in the ipsilateral hemispheres of animals in the two LPS-injected groups (fig. 4, LPS-NT and LPS-HT), and largely localised around the area of injury. Additionally, Iba1-positive cells in the ipsilateral sides in these groups showed a more activated morphology (fig. 4, LPS-NT and LPS-HT, inserts L).



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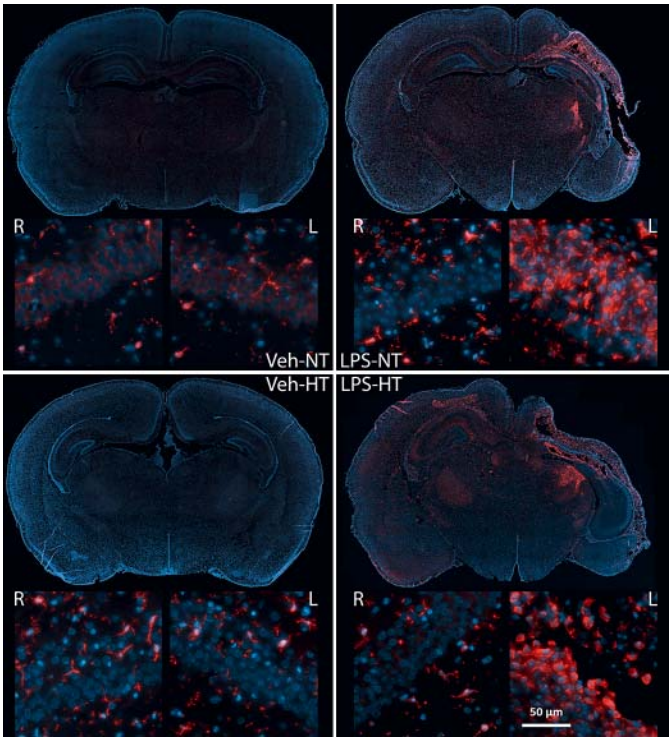

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Fig. 3. a Western blot analysis of GFAP on P14, 7 days after hypoxia-ischaemia. Ipsilateral hemispheres showed increased GFAP expression, with a significant effect of LPS sensitisation also seen. **b** Relative Iba1 expression 24 h after hypoxia-ischaemia. Ipsilateral hemispheres showed more Iba1 expression. **c** Composite image of various Western blot gels. Bars show means with 95% CI. L = Left hemisphere; R = right hemisphere.

Fig. 4. Representative immunohistochemistry slides showing Iba1 expression 24 h after hypoxia-ischaemia across the different treatment groups. At rest, microglia exhibit ramified cell morphology with numerous thin processes extending from their soma. Upon activation, these processes retract resulting in a more rounded amoeboid-like appearance. Ipsilateral hemispheres of the LPS pretreated animals (LPS-NT and LPS-HT inserts L) showed increased IBA1 expression and an activated morphology of microglial cells. Activated microglia were also found on the contralateral side of LPS-treated animals. Iba1-positive microglia with a more resting morphology were found also in the vehicle-injected animals (Veh-NT and Veh-HT). L = Left hemisphere; R = right hemisphere.

Discussion

The results of the present study suggest that sensitisation of the neonatal brain with LPS modifies multiple cellular responses to HI brain injury in P7 rats. In a previous study evaluating hemispheric brain area loss after hypoxia-ischaemia, treatment with HT was not found to be neuroprotective after LPS sensitised neonatal HI brain injury [5]. The findings of the current study further confirm this finding at a cellular level: HT neuro-

protection was lost, and HT may even be detrimental in this setting.

In the absence of inflammation, HT has a neuroprotective effect after hypoxia-ischaemia on several brain regions, such as the cerebral cortex, hippocampus, basal ganglia and thalamus, and improves long-term outcome [20]. However, the presence of inflammation changes the landscape of cell survival and the potential for neuroprotective intervention dramatically. Exposure to LPS, a pro-inflammatory constituent of the bacterial wall of Gram-negative bacteria, induces neurotoxicity in a region-specific manner, which is partly attributable to differences in the number of microglia present within the region and the levels of inflammatory mediators they produce [21]. Hippocampi seem to be particularly vulnerable. Neonatal LPS exposure in rats reduces both hippocampal volume and the absolute number of NeuN-positive cells, as well as increases axonal injury in the CA1 region, leading to long-term memory and behavioural problems [22]. The timing of LPS exposure is also a critical factor to consider. While exposure to LPS 4–14 h before hypoxia-ischaemia significantly worsens hypoxia-ischaemia-induced brain injury [4, 5, 23], exposure to LPS 24 h prior to hypoxia-ischaemia seems to precondition the brain, which causes a reduction in brain injury [23, 24] and reduces hypoxia-ischaemia-induced neuroinflammation [25], particularly if given in low doses such as 0.05 mg/kg [25].

Our findings are in accord with these observations, as LPS sensitisation resulted in decreased NeuN expression, both on a hemispheric level (Western blot analysis) as well as in hippocampi specifically (cell counting). On a cellular level, HT was not neuroprotective in this infection-sensitised model. For instance, a detrimental interaction between HT and LPS on levels of NeuN expression on Western blot was found. However, this observation was not uniformly seen throughout our experiments.

From the results of our study it is not possible to deduct the mechanisms behind the failure of HT to reduce brain injury in inflammation-sensitised neonatal rat brains, but it appears to at least in part occur through enhanced apoptosis. Increased levels of apoptosis on the ipsilateral ligated hemispheres (exposed to hypoxia-ischaemia) were seen in all groups, which is in accord with existing knowledge on cell death after hypoxia-ischaemia [26]. The neuroprotective effect of HT has been partly attributed to reduced apoptosis [27, 28], a trend which we also saw in the vehicle-treated animals in this study, although it was not statistically significant. However, the HI insult administered to the vehicle-injected rat pups was relatively mild (50 min of 8% O_2), and HT has been proven to have its best neuroprotective efficacy after moderate HI brain injury (usually 90–100 min of 8% O_2), where there is a higher potential for HT to rescue tissue [29]. In the neonatal rat brain, LPS exposure induces apoptotic pathways, the final common step of which is usually caspase 3 activation [30]. Post-HI treatment with immediate HT did not counteract the LPS-induced increase in apoptosis, which might partly explain the ineffectiveness of HT in this model. However, the mechanism by which LPS sensitisation neutralises the anti-apoptotic effect of HT remains to be elucidated.

After HI brain injury, microglial cells become activated, proliferate and release inflammatory mediators, leading to neuronal injury [31–33]. Nimmervoll et al. [34] have shown that LPS-induced microglial secretion of TNFα increases activity-dependent neuronal apoptosis in the neonatal cerebral cortex. Microglia may play a critical role in our model as well. We have found a trend towards increased Iba1 expression on Western blot analysis in the ipsilateral hemispheres of all groups. Immunohistochemistry showed that Iba1-positive cells were more abundant in the ipsilateral hemispheres of LPS-sensitised animals, with particular localisation to areas where the greatest histological tissue damage was seen. These cells also showed an activated morphology, suggesting an active role in the process of injury. Inflammation has a dual role in the neonatal brain, as certain pathways also contribute to tissue repair and regeneration [35].

Astrocytes are the most numerous cell type in the central nervous system and provide structural, trophic and metabolic support to neurons. Their function is impaired during brain ischaemia, which can significantly influence neuronal survival [36]. In a study by Barbierato et al. [37]. LPS-induced upregulation of proinflammatory genes and mediators in astrocyte/microglia co-cultures far exceeded that observed from cultures containing the same numbers of microglia only, which suggests that astrocytes play an active role in the inflammatory response. We have seen an increase in the mature astrocyte marker GFAP in the present study. The effect of increased astrogliosis in the interplay between HI brain injury, inflammation and HT needs further evaluation.

This study has some limitations. It was aimed at the neuropathological response on the cellular level, but was not designed to investigate these effects on a subcellular level, i.e. investigating inflammatory mediators released by microglial cells or looking at specific pathways involved in the injury process, such as cascades triggered by

the activation of the TLR4 receptor or activation of cell death/survival genes. This approach could provide additional insight into the observed cellular responses, as well as the absence of any neuroprotective effect of HT after LPS sensitisation. The exact mechanism of the failure of HT neuroprotection in infection-sensitised HI brain injury could not be uncovered by these findings. One could speculate that the reason lies in the perturbed fine balance between the beneficial and injurious aspects of neuroinflammation by a sufficiently strong inflammatory stimulus at a particularly vulnerable point in time. LPS exposure results in an inflammatory response that overrides the innate inflammatory response to hypoxia-ischaemia and its modulation by HT, resulting in the latter being ineffective. The complex interplay of the brain immune system with the peripheral immune system could also play an important role.

In conclusion, an inflammatory challenge before HI brain insult significantly influences cellular responses and increases brain injury. Although HT has important neuroprotective effects after HI insult in the absence of inflammation, it does not reverse cellular responses caused by LPS in a rat model of infection-sensitised neonatal HI brain injury.

Acknowledgements

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Disclosure Statement

The authors report no conflicts of interest.

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