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Hypothermic Neuronal Rescue from Infection-sensitised Hypoxic-ischaemic
Brain Injury is Pathogen Dependent

Running title: Pathogen-Dependent Hypothermic Neuroprotection

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Perinatal infection increases the vulnerability of the neonatal brain to hypoxic-ischaemic (HI) injury. Hypothermia Treatment (HT) does not provide neuroprotection after pre-insult inflammatory sensitisation by lipopolysaccharide (LPS), a gram-negative bacterial wall constituent. However, early-onset sepsis in term babies is caused by gram-positive species in more than 90% of cases, and neuro-inflammatory responses triggered through the gram-negative route (toll-like receptor 4; TLR-4), are different from those induced through the gram-positive route via TLR-2. Whether gram-positive septicaemia sensitises the neonatal brain to hypoxia and inhibits the neuroprotective effect of HT is unknown.

Seven-day-old (P7) Wistar rats (n=178) were subjected to intraperitoneal injections of PAM3CSK4 (1 mg/kg, a synthetic TLR-2 agonist) or vehicle (0.9% NaCl). After an 8-hour delay, the left carotid artery was ligated followed by 50min of hypoxia (8% O2) at Trectal36°C. Pups received 5h treatment of normothermia (NT, 37°C) or HT (32°C) immediately after the insult. Brains were harvested after seven days' survival for hemispheric and hippocampal area loss analyses and immunolabelling of microglia (Iba1) and hippocampal neurons (NeuN). Normothermic PAM3CSK4-animals showed significantly more brain injury than vehicle animals (p=0.014). Compared to NT, HT significantly reduced injury in the PAM3CSK4-injected animals, with reduced area loss (p<0.001), reduced microglial activation (p=0.006), and increased neuronal rescue in the CA1 region (p<0.001). Experimental induction of a sepsis-like condition through the gram-positive pathway sensitises the brain to HI. HT was highly neuroprotective after the PAM3CSK4-triggered injury, suggesting HT may be neuroprotective in the presence of a gram-positive infection. These results are in strong contrast to LPS-studies where HT is not neuroprotective.
**Introduction**

Perinatal hypoxic-ischaemic (HI) brain injury remains one of the major causes of long-term neurological disability or death in term newborns [1]. Perinatal infection is a risk factor for cerebral palsy (CP) and long-term disability [2–4], and systemic inflammation also lowers the threshold at which an HI insult leads to permanent neuronal injury [5–7]. Several small and large animal studies have demonstrated the infection-induced vulnerability of the brain to hypoxia, and investigated the mechanisms behind [8–11]. Interestingly, a generalised systemic inflammatory activation seems to be sufficient to cause this sensitisation, even in the absence of the pathogen itself. Chau *et al* showed that meningitis is not a prerequisite to increase susceptibility of the brain to HI, and most clinical studies linking severity of brain injury to perinatal infection have instead examined pro-inflammatory cytokines or clinical signs of perinatal infection, such as maternal pyrexia or clinical chorioamnionitis [3,12].

Furthermore, the success rate of pathogen isolation from the blood of neonates with clinical infection is poor at only 6% [13]. Pre-clinical animal models of simulated infection in the setting of HI injury often use inflammatory triggers like lipopolysaccharide (LPS), a constituent of gram-negative bacterial membrane, in place of the complete bacteria [8,9,14,15]. Systemic activation of immune cells will not only induce an inflammatory cascade in peripheral blood, but also induces inflammatory activation in brain tissue. The elevated cytokines and activated microglia elicit what is referred to as the infection-sensitised immature brain [7,15,17].

For term neonates with moderate and severe hypoxic-ischaemic encephalopathy (HIE) as a result of HI brain injury, hypothermia treatment (HT) is standard of care, and our only current treatment option [18]. With a number needed to treat of 8, 45-50% of encephalopathic term babies will still die or have long-term disability despite active HT therapy [19]. Based on a diverse range of clinical and pre-clinical studies, doubt exists as to whether HT is neuroprotective in infants with HIE where perinatal infection is a co-morbidity [6,20,21]. We recently showed experimentally that HT is not neuroprotective after pre-insult inflammatory
sensitisation with LPS in a post-natal day 7 (P7) rat model of unilateral HI brain injury [8].

LPS triggers the immune system primarily by binding to toll-like receptor (TLR) 4, but is likely to only represent infections caused by gram-negative bacteria which contain LPS in their cell wall [22]. However in term neonates in developed countries where HT is standard of care, culture positive sepsis has been shown to be caused by gram-positive bacterial species in >90 % of cases [13]. Peptidoglycans and lipoteichoic acid on the wall of gram-positive bacteria trigger human immune responses by binding to TLR-2, and thereby induce a different pathway to inflammatory activation [23–25]. We previously investigated the neuro-inflammatory responses in neonatal rat pups receiving systemic LPS, compared to those receiving the synthetic TLR-2 agonist PAM3CSK4 (PAM). Profound differences in temporal core temperature development, as well as in brain cytokine expression and inflammatory and apoptotic signal molecules were found, in response to the two different types of systemic inflammation [26].

Whether gram-positive septicaemia sensitises the neonatal brain to HI the way gram-negative septicaemia does, and whether it abolishes the neuroprotective effect of HT (as seen in LPS sensitization), is not known.

We therefore investigated the sensitising effect of systemic TLR-2 activation on the neonatal rat brain, as a model of gram-positive systemic inflammation in the setting of HI brain injury, in the P7 rat. Additionally we investigated whether HT is neuroprotective in this double-hit setting.
Materials and methods

Animals and injections

All experiments were approved by the University of Oslo's Animal Ethics Research Committee and performed by individuals holding an approved license according to the Animal (Scientific Procedures) Act of 1986. Experiments were performed on 7-day-old (P7) Wistar rats (Charles River Laboratories, Sulzfeld, Germany) of both sexes. All pups were kept in an animal facility with a 12h:12h-hour dark:light cycle at 19-21°C environmental temperature with food and water ad libitum.

To trigger inflammation through the TLR-2 pathway we used a synthetically-manufactured TLR-2 agonist (N-palmitoyl-S(2,3-bis(palmitoyloxy)-(2R,S-propyl))-cysteinyl-seryl-(lysyl)3-lysine, \textit{PAM}_3\textit{CSK}_4 or \textit{PAM}, Vaccigrade, Sigma-Aldrich) at a dose of 1 mg/kg body weight. PAM was initially dissolved in sterile LPS-free water, and then diluted in sterile physiologic saline (0.9% NaCl). The dose of PAM was based on previous publications on this agonist used in neonatal rodents [27–29], in combination with our own dose-response experiments (data not shown). Control groups received a single dose of sterile saline vehicle. All injections were given intraperitoneally (i.p.) in a volume of 10 µl/g body weight.

Animals were randomised across litter, sex and weights before the experiments commenced, to one of the following treatment groups; vehicle injection (Veh) and normothermia treatment (NT) (Veh-NT), PAM injection and NT (PAM-NT), Veh injection and hypothermia treatment (HT) (Veh-HT), and PAM injection and hypothermia treatment (PAM-HT).

Surgical Procedures

All experiments were performed as previously described for the LPS-sensitised modification of the Vannucci model of unilateral HI [8]. Briefly, at the start of each experiment, animals were injected with PAM or Veh according to randomisation. After an 8-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% O₂ 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 surface temperature of two 'sentinel' pups from the Veh groups, was continuously recorded in each chamber. Rectal temperature was maintained within ±0.2°C of the target value using a continuous 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 h treatment period, pups were returned to their dams. Pups were sacrificed on postnatal day (P) 14 for further analyses.

**Histopathology and Area loss analyses**

At P14, animals were sacrificed by trans-cardiac perfusion-fixation with 10% neutral-buffered formalin under isofluraneN₂O-anaesthesia. Brains were harvested and kept in 10% neutral-buffered formalin until further processing. Three mm coronal blocks were cut using a standard rat matrix (ASI instruments Inc., Warren, MI, USA), and embedded in paraffin. Five µm slices were cut from the two neighboring blocks best representing cortex, hippocampus, basal ganglia and thalamus. These were stained with hematoxylin and eosin (H&E) and scanned (Epson Perfection V750 Pro). Virtual slides were exported with 600 dpi resolution. Optical density and hemispheric area was analysed using ImageJ computer software (ImageJ, version 1.46r, National Institutes of Health, Bethesda, MD, USA). The ligated side was compared to the non-ligated side, and area loss of the ligated side calculated by the formula (1 – (area left/area right))*100. Percent hemispheric area loss at this level has previously been shown to be highly correlated with a formal neuropathology score and global degree of injury in this model [30].
Evaluation of hippocampal area loss was performed in the same way, and calculated as:

\[ (1 - \frac{\text{area of left hippocampus}}{\text{area of right hippocampus}}) \times 100 \]

A subset of the H&E stained sections were examined for hemispheric and hippocampal areas by two blinded assessors to check for inter-rater reliability.

**Immunohistochemistry**

For immunohistochemistry analysis, slides were prepared from paraffin-embedded sections as for H&E staining. Antigen retrieval was then performed in citrate buffer solution at pH 6.0, using a PT link instrument (Dako, Glostrup, Denmark). After blocking with 10% goat serum, primary rabbit antibody against Iba1 (1:1,000; WAKO), or mouse antibody against NeuN (1:500; Millipore), was applied overnight at 4°C. 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 20X lens. Virtual slides were exported as high-resolution tiff images for further analysis.

To evaluate the effect of different treatments on neuronal loss, NeuN and DAPI-positive cells in the CA1 region of the hippocampus were counted, as this region is known to be particularly vulnerable to hypoxia at P7\[31,32\]. Aiming for a representative subset from each treatment group, the 10 animals closest to the median hemispheric area loss, were selected for formal hippocampal neuron counting. Three non-overlapping fields of the CA1 region in the left hippocampi were assessed. Counting was performed by two individual observers blinded to the treatment group, and an average of the two was taken. The total number of neurons across the three fields of each hippocampus was summed and compared across groups.
To investigate inflammatory activation, staining for ionised calcium-binding adapter molecule 1 (Iba1, a microglial specific biomarker) was performed. Iba1 positive cells were separated from background and analysed by ImageJ. The summed colour intensity detected was calculated as a L/R hemispheric ratio and normalised to cross-sectional area before comparison across groups. The results from two blinded assessors were compared by calculating their correlation coefficient to validate the method.

Data Analysis

Statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software Inc., La Jolla, Ca, USA) and SPSS software version 22 (SPSS Inc., Chicago, IL, USA). As data were not normally distributed, the Kruskal-Wallis test was used for four-group treatment comparison, and Mann-Whitney U-test was used for two-group comparisons to get exact two-tailed p-values. Graphical data are presented as median with 95% confidence intervals (CI). A p-value of <0.05 was considered statistically significant.
Results

Mortality and exclusions

Of the 218 pups initially included no mortality was seen from injections alone. Four pups died during experimental surgical procedures and hypoxia. Pups carrying temperature probes in each experiment were excluded from further analysis (n=16), because the stress of carrying the probe could influence the outcome [33]. A total of 198 pups were therefore included in analysis. 20 pups served as juvenile controls, without receiving an injection.

Hemispheric Area loss after Systemic injections without HI

To make sure a systemic inflammation triggered by injection of PAM does not create brain tissue loss on its own, P7 rat pups received injections according to the above mentioned protocol with Veh (n=22) or PAM (n=23). Juvenile control (JC) animals of equivalent age (n=20) were used as controls. Total cross sectional area was compared across groups, as well as right hemispheric area and left hemispheric area separately. There was no statistical difference between treatment groups in any of the analyses (Kruskal-Wallis test; p=0.4, 0.5 and 0.3 respectively) (data not shown).

Hypothermic neuroprotection in PAM sensitised HI injury

In the NT treated groups, animals receiving a single i.p. injection of PAM prior to the HI insult were more vulnerable to 50 minutes of hypoxia and had significantly increased hemispheric area loss (35.8%, CI 20.4-48.6) compared to Veh-injected pups (10.4%, CI 2.1-37) (p=0.01). Treating PAM-injected pups with HT reduced median area loss (6.6%, CI 4.4-18.8), and thereby showed a significant neuroprotective effect compared to PAM animals treated with NT treatment (p=0.0002) (Figure 1).

Also hippocampal area loss was significantly higher in PAM-NT pups (55.5%, CI 26.3-
69.2) compared to Veh-NT (13.4%, CI 3.1-40.2) (p=0.03). Significant hypothermic neuroprotection in the hippocampal region was seen in PAM-HT animals (8.2%, CI 3.6-18.2) (p=0.003) (Figure 2). In the cortical area loss analyses we found the same differences, with PAM-NT pups having significantly more cortical tissue loss (50.4%, CI 25.1-67.6) compared to the Veh-NT group (18.5%, CI 2.4-54.2) (p=0.03). Significant neuroprotection in the cortical region was seen in PAM-HT animals (8.2%, CI 3.6-18.2) (p=0.003). Thalamic area loss was not significantly increased in the PAM-sensitised pups (28.8%, CI 13.2-46.3) compared to Veh-NT animals (14.2%, CI 7-32.1). There was, however, significant neuroprotective effect on thalamic tissue in the PAM-HT group (9.3%, CI 3.2-18) (p=0.01).

There was no statistical difference between Veh-injected pups treated with NT and Veh-animals receiving HT treatment, neither with respect to hemispheric (10.4%, CI 2.1-37 vs 10.9%, CI 7.2-15.4) area loss, nor to hippocampal (13.4%, CI 3.1-40.2 vs 7.6% CI 0.5-29.4), cortical (18.5%, CI 2.4-54.2 vs 15.9%, CI 9.8-20.5) or thalamic (14.2%, CI 7-32.1 vs 9.3%, CI 4.5-12.6) area loss.

Linear regression analysis showed a highly significant correlation between area loss in all three regions and hemispheric area loss (Hippocampus: $R^2=0.77$, p<0.0001; Cortex: $R^2=0.89$, p<0.0001; Thalmus: $R^2=0.78$, p<0.0001), with hippocampal and cortical loss tending to be greater (B=1.32 and B=1.27 respectively) than hemispheric area loss, while thalamic loss is slightly lower (B=0.92) (Figure 3). This is in accordance with regional analysis of vulnerability in the Vannucci rat model when exposed to HI only [31].

**Hypothermia Provides Neuronal Rescue in the CA1 Hippocampal Region.**

The total number of neurons in the CA1 region of the left hippocampus were counted in a subset of animals from all 4 treatment groups (n=10-11 per group) (Figure 4). The
number of neurons after a short HI insult was significantly lower in PAM-NT animals (44, CI 0-103) compared to Veh animals (122, CI 73-135) (p=0.01). Significant neuronal rescue was seen in the PAM-HT group (107, CI 94-141) (p=0.0008). There was no difference between the two Veh-injected groups (NT: 121.8, CI 73-135 vs HT: 114, CI 99.5-128).

PAM induced microglial activation

Iba1 upregulation was more pronounced, and greater relative to the amount of remaining tissue, in PAM-NT animals (p=0.035). Microglial activation was reversed in the PAM-HT group, with significantly reduced Iba1 immunolabelling (p=0.006) (Figure 5). Microglial activation, with bigger somas and retracted dendritic processes, was morphologically visible around the left hemispheric lesions of all animals (Figure 6).

Discussion

This study investigated the sensitising effect of systemic TLR-2 activation on the immature rat brain, combined with a mild unilateral HI insult. The motivation was to improve our pre-clinical model of infection-sensitised HI brain injury to more closely resemble the clinical situation in term asphyxiated neonates, where gram-positive infections predominate. Here we provide evidence that PAM, a TLR-2 agonist, does sensitise the immature brain when injected systemically. Importantly, PAM injected animals are equally vulnerable to HI as those sensitised by LPS [8]. However, HT still provides >80% neuroprotection of hemispheric area loss in animals administered PAM. This is in stark contrast to studies on LPS-induced sensitisation, where HT was ineffective.

Analyses of hippocampal area loss and neuron count in the CA1 region gave similar neuroprotection of HT after sensitisation with PAM. In the PAM groups, treatment with HT
resulted in an 85% reduction of total hippocampal tissue loss, and 2.4-fold higher number of surviving neurons in the CA1 region of the hippocampus. Tissue loss in the cortex followed the same pattern across the groups, with again significant neuroprotection in PAM-HT animals. Interestingly, thalamus is a less vulnerable area to the sensitizing effect of PAM, without worse outcome in the PAM-NT group compared to Veh-NT. Still hypothermia reduces injury significantly also in this region. Additionally, microglial activation relative to cross-sectional area was increased in PAM-sensitised brains. This was reversed by HT, with a 55% reduction of Iba1 expression.

Injection of PAM or of LPS, alone results in neuro-inflammatory alterations that differ highly depending on the inflammatory stimulus [34]. Our own experiments have shown that LPS-injected rat pups become hypothermic soon after injection, with a spontaneous core temperature drop of 3.5°C, from 35°C down to 31.5°C [26]. This decrease in core temperature was not seen in animals injected with PAM, which were not different from Veh animals. Intra-hypoxic temperature is known to have large impact on the susceptibility of the neonatal rat brain to HI injury [35]. At higher core temperatures during hypoxia, neonatal rats are more susceptible to brain injury, and vice versa, at lower body temperature it is more challenging to create a lesion [36]. The drop in core temperature we have seen after LPS injection, which is still present when the experiment commences, might partly be the reason for their increased vulnerability compared to Veh animals [5]. In studies on LPS-sensitisation the rats receive the insult at the same intra-hypoxic temperature as control groups (36°C) [5,8], meaning their temperature during hypoxia is rapidly increased by 4.5°C when placed in the hypoxia chamber. The temperature of Veh pups or juvenile control animals on the other hand, is only increased by up to 1°C during the hypoxia period. This does, however, not explain why PAM injected pups, which maintain the same core temperature as Veh animals post-injection, present a vulnerability to hypoxia similar to that of LPS-injected pups. This suggests that other mechanisms are as important as temperature when it comes to the brain’s resistance to an HI insult.
HI brain injury without systemic inflammation induces a lowering of core temperature [35,37]. During hypoxia there is also reduced metabolism and heat production [38]. The detailed mechanism behind this phenomenon, however, is not fully understood, and an innate neuroprotective defense mechanism has been suggested [35]. Experimental HI brain injury without infectious pre-sensitisation, is where HT has repeatedly been shown to be neuroprotective [39]. We therefore speculate whether the reduced core temperature found after injection of LPS might be more of a pathologic response, with disturbance of the thermoregulatory center in hypothalamus. Linthorst et al. have demonstrated several disturbances in the thalamic preoptic area after i.p. LPS administration, which substantiates this theory [40]. These changes have to not been investigated after PAM sensitisation. A study comparing these responses after PAM or LPS would help elucidate these mechanisms and their influence in infection-sensitised brain injury.

Resting microglia are activated in response to HI injury [41–43]. Studies on LPS-sensitised HI injury have demonstrated microglia to be both more numerous and in a more activated state around the site of the lesion [17,44,45]. The sensitising effect of LPS on the immature brain has been attributed to the number of activated microglia. Here we demonstrate a similar microglial response after PAM, with comparable increased neuronal vulnerability. This could suggest that microglial activation and proliferation is involved in the pathogenic inflammatory activation and brain sensitisation due to the combination of PAM and HI. It is however noteworthy that both area loss and microglial activation after PAM-sensitised HI is largely reversed by HT, while this is not the case after LPS. Microglial activation seems to be non-specific to the pathogen, and part of a more distal common pathway of neuro-inflammatory responses. In both models, the increased microglial response in sensitised brains is associated with a higher median degree of neuronal injury, due to the “double hit” insult, compared to controls. A constituent tonic inhibition of microglial activity occurs through ligand-receptor pairs from neurons, requiring direct cell-cell contact [43]. Even in the absence of damage-related signals, loss of neuronal integrity can induce a rapid microglial response.
The upregulation of Iba1 could therefore represent a response to a more comprehensive injury, which occurred prior to the microglial activation. As these microglia are stained after 7 days' survival, and are not phenotyped, their activation state is likely to be towards what was previously defined as the M2 phenotype on the classification spectrum, and a sign of inflammatory repair mechanisms [47]. The dramatic difference in sensitivity to HT indicates that other, earlier, inflammatory events are more important in the mechanistic explanation of inflammatory pre-sensitisation to neuronal Hi injury.

A limitation to this study is the lack of significant HT neuroprotection in the Veh-injected animals. We do however see a somewhat lower median hippocampal area loss in the Veh-HT group compared to the Veh-NT group, although not statistically significant. This goes well with how the hippocampus has been shown to be the most sensitive area to HI, but also the most sensitive to HT neuroprotection [48]. The lack of difference in hemispheric area loss may be due to the low degree of injury in this cohort as a result of the short hypoxia period (50 minutes compared to 90 minutes in our standard HI injury model without presensitisation) [30,49]. In our experience HT has not been neuroprotective after mild brain injury, defined in the Vannucci model as a median area loss below 25% [50,51]. A moderate degree of injury (30-60% tissue loss) is required to see neuroprotective effect of HT in this model [30,52]. Whether mild injury should be eligible for cooling in neonates is still debated, as the neuroprotective effect of HT has not been clarified for these patients [53]. Rat pups are highly variable in how much hypoxia they can withstand before cellular death is seen, and the same is likely to be the case for human neonates. The well-described variability of injury in the Vannucci model demands a substantial sample size, and the careful use of non-parametric statistical approaches. However, this model has been an important part of translating therapeutic hypothermia from bench to bedside [54], and harboring such variability might be part of what makes the model translatable. When we modify the model to include systemic infectious inflammatory activation, chances are high that processes and pathways are induced that still remain to be uncovered. The immune system is far from fully-mapped, and
furthermore, studies on translation of immune responses across species are scarce [55].

Though the focus of research on pre-sensitisation has primarily been based around bacterial infections, general signs of infection are mostly non-specific, and could be associated with other infectious agents. Some of the studies associating severity of brain injury to systemic inflammation used maternal fever as a sign of infection [2,3], but, the most common cause of fever is viral infections including influenza, rhinovirus, enterovirus and coronavirus [56]. The pre-sensitising effect of viral-induced materno-fetal inflammation has not been well investigated clinically. A study by Stridh et al on neonatal mice demonstrated significantly increased infarct volume after pre-sensitisation with an agonist to TLR-3, the pathogen recognition receptor of viral RNA [57]. Fever is induced by raised circulating levels of certain cytokines, specifically IL-6 [58], which occurs during both bacterial and viral illnesses [59]. A well-known consequence of intrapartum maternal infection is the fetal inflammatory response syndrome (FIRS), also characterised by elevated IL-6 levels in the fetus [60]. Whether this is dependent on the pathogen is not known, and could indeed include maternal viral infections as well as bacterial chorioamnionitis. With respect to how viral infections may interact with HT treatment in asphyxiated neonates, it is interesting to note that infections with common cold viruses increase in the winter season, and cold viruses have been shown to replicate better at cold environmental temperatures like in the nasal cavity (33-35°C) than at normal core temperature (37°C). The mechanism behind this is not yet elucidated, but is thought to be due to diminished antiviral immune responses at these lowered temperatures [61]. Cooling neonates with a viral infection might therefore bring them to a temperature that promotes the growth of certain viruses. The downstream effects of that are unknown.

Eklind et al. developed a modification of the Vannucci model with systemic inflammation to pre-sensitise the brain to HI [5]. They used lipopolysaccharide (LPS) as a systemic inflammatory trigger, and thereby modelled a gram-negative bacterial infection. This finding was particularly important with respect to the prematurely born population [62,63].
and furthermore to populations of less developed parts of the world, where the incidence of gram-negative infections is higher, and HT was [64]. Our group found the same HI-sensitising effect of LPS on the brain, however we showed that HT neuroprotection was negated in LPS-sensitised rat pups using that model [8,17]. Studies on HT in low-income settings have not been able to find neuroprotective effect [20], and HT is to date standard of care only in western high-income countries. On this basis, and knowing our target patient group to mostly have infections caused by gram-positive bacteria, we further-developed a model of gram positive infection, using a synthetic TLR-2 antagonist, as described. Activation of TLR-2 triggers inflammatory activation through the same pathway that initiates sepsis from gram positive species [25,65]. Surprisingly, and in opposition to the results seen in the LPS model, we demonstrate a neuroprotective effect of HT. This might not uncover the whole story, but it does underline the importance of tailoring our pre-clinical models as thoroughly as we can to the clinical scenario we aim to mimic.

With these data we can only conclude that HT treatment can be highly neuroprotective in inflammatory pre-sensitised HI injury, but the neuroprotective effect might depend on the pathogen. With current knowledge, our results in combination with clinical infection demographics suggests that we should continue to treat encephalopathic neonates who fulfill the cooling criteria, regardless of infectious status.
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Conflict of interest statement

The authors declare no competing financial interests.

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Figures legends

Figure 1. Hemispheric area loss (%).
Bars show median with 95% confidence interval. Postnatal day 7 (P7) rat pups were injected intraperitoneally with vehicle (Veh) or PAM3CSK4 (PAM). After an 8-hour-delay all pups had their left carotid artery ligated before 50 minutes of 8% hypoxia. Pups were randomized to 5 hours of normothermia treatment (NT) (37°C) or hypothermia treatment (HT) (32°C), with 7 days’ survival. PAM injected animals treated with NT (PAM-NT) had significantly more injury compared to the Veh-NT group. HT provided significant neuroprotection in PAM-injected group (PAM-HT). *p=0.01, ***p=0.0002.

Figure 2. Hippocampal area loss (%).
Bars show median with 95% confidence interval. PAM3CSK4-injected (PAM) pups receiving normothermia treatment (NT) (PAM-NT) lost significantly more hippocampal tissue than vehicle-injected pups (Veh-NT) receiving the same treatment. Hypothermia treatment (HT) was significantly neuroprotective in PAM-injected pups (PAM-HT) *p=0.03, **p=0.003.

Figure 3. Correlation between regional and hemispheric area loss.
Symbols represent unique animals, with lines denoting the correlation between hemispheric area loss and area loss in hippocampus (circles) ($R^2=0.77$), thalamus (squares) ($R^2=0.78$) or cortex (triangles) ($R^2=0.89$).

Figure 4. Hippocampal neuroncount.
Symbols represent the number of hippocampal neurons in the hippocampal CA1 region of each animal. Lines show the median. The neuroncount was significantly lower in PAM3CSK4-injected (PAM) animals compared to vehicle-injected (Veh) animals in the normothermia (NT)
groups. Hypothermia (HT) provides significant neuronal rescue after PAM sensitization.

*p=0.01, **p=0.008 (A).

Representative images from the hippocampal CA1 region are shown from each experimental group (B).

**Figure 5. Iba1 density (microglial activation) relative to cross-sectional brain area.**

PAM₃CSK₄-injected (PAM) animals treated with normothermia (NT) (PAM-NT) have a greater degree of microglial activation relative to remaining tissue, compared to vehicle-injected (Veh) pups receiving the same treatment (Veh-NT). Hypothermia treatment (HT) counteracts this effect. *p=0.035, **p=0.006.

**Figure 6. Representative images of microglia stained for Iba 1.**

*Above:* A vehicle-injected (Veh) normothermia treated (NT) (Veh-NT) animal showing ramified resting microglia with a small soma and long slender branched processes. *Below:* A PAM₃CSK₄-injected (PAM-NT) animal demonstrating proliferation and upregulation of activated microglia, with big round soma and retracted processes.