Falck, M., Osredkar, D., Maes, E., Flatebø, T., Wood, T., Walløe, L., Sabir, H., & Thoresen, M. (2018). Hypothermia is Neuroprotective after Severe Hypoxic-Ischaemic Brain Injury in neonatal rats pre-exposed to PAM3CSK4. *Developmental Neuroscience*, [201710007]. <https://doi.org/10.1159/000487798>

Peer reviewed version

Link to published version (if available):
[10.1159/000487798](https://doi.org/10.1159/000487798)

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Hypothermia is Neuroprotective after Severe Hypoxic-Ischaemic Brain Injury in neonatal rats pre-exposed to PAM₃CSK₄

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Key words: PAM₃CSK₄, perinatal infection, maternal pyrexia, perinatal asphyxia, encephalopathy, temperature, LPS

Abstract

Background: Pre-clinical research on the neuroprotective effect of hypothermia after perinatal asphyxia has shown variable results, depending on co-morbidities and insult severity. Exposure to inflammation increases vulnerability of the neonatal brain to hypoxic-ischaemic (HI) injury, and could be one explanation for those neonates whose injury is unexpectedly severe. Gram-negative type inflammatory pre-sensitisation with lipopolysaccharide (LPS) prior to a mild HI insult negates hypothermic neuroprotection. However, the neuroprotective effect of HT is fully maintained after gram-positive type pre-sensitisation with PAM³CSK⁴ (PAM) in the same HI model. *Whether HT is neuroprotective in severe brain injury with gram-positive inflammatory pre-sensitisation has not been investigated.*

Methods: 59 seven-day-old rat pups were subjected to a unilateral HI insult, with left carotid artery ligation followed by 90 min hypoxia (8% O₂ at T_{rectal} 36°C). An additional 196 pups received intraperitoneal 0.9% saline (control) or PAM_{1mg/kg}, 8 h before undergoing the same HI insult. After randomisation to 5 h normothermia (NT_{37°C}) or HT_{32°C}, pups survived one week before they were sacrificed by perfusion fixation. Brains were harvested for hemispheric (HEM) and hippocampal (HIP) area loss analyses at P14, as well as immunostaining for neuron count in the HIP CA1 region.

Results: Normothermic PAM animals (PAM-NT) had a comparable median area loss (HEM: 60% (95% CI 33-66); HIP: 61% (95% CI 29-67)) to vehicle animals (Veh-NT) (HEM: 58% (95% CI 11-64); HIP: 60% (95% CI 19-68)), which is defined as severe brain injury. Furthermore, mortality was low and similar in the two groups (Veh-NT 4.5% vs PAM-NT 6.6%). HT significantly reduced HEM and HIP injury in the Veh group (HEM: p=0.048; HIP: p=0.042) as well as in the PAM group (HEM: p=0.03; HIP: p=0.027).

Conclusion: In these experiments with severe brain injury, TLR-2 exposure prior to HI does not have an additive injurious effect, and there is a small but significant neuroprotective effect of HT. Hypothermia appears to be neuroprotective over a continuum of injury severity in this

- 1 model, and the effect size tapers off with increasing area loss. Our results indicate that gram-
- 2 positive inflammatory exposure prior to HI injury does not negate a neuroprotective effect of
- 3 HT in severe brain injury.

1 Introduction

2
3 Perinatal hypoxic-ischaemic (HI) brain injury remains a major cause of long-term neurological
4 disability and death in term newborns [1]. For term neonates with moderate and severe HI
5 encephalopathy (HIE), hypothermia (HT) treatment is standard of care, and it is currently the
6 only approved treatment option [2]. With a number needed to treat of 8, 45-50% of
7 encephalopathic term babies will still die or suffer from long-term disability despite HT therapy
8 [3]. However, in follow-up studies from large randomized clinical trials, infants with the most
9 severe HIE had the most severe neurological dysfunction, and have been hypothesised to
10 benefit less from HT [3]. Pre-clinical studies of hypothermic neuroprotection after severe HI
11 brain injury have shown conflicting results [4–6].

12 Perinatal infection is a wellrecognised risk factor for cerebral palsy (CP) and long-term disability
13 [7–9]. Systemic inflammation lowers the threshold at which an HI insult leads to permanent
14 neuronal injury [10–12]. In a study well before HT was introduced, Grether and Nelson
15 identified maternal pyrexia or chorioamnionitis during birth in 37% of patients who developed
16 the most severe form of cerebral palsy, compared to only in 3% in the general population [8].
17 In animal models of inflammation the toll-like receptor (TLR)-2 agonist PAM₃CSK₄ (PAM)
18 induces inflammatory activation which mimics gram-positive infection [13] – the most
19 frequently-isolated group of pathogens in term neonates with early onset sepsis (EOS) [14].
20 However, as it is cheap, readily available and easy to work with, the most commonly-used
21 inflammatory trigger in experimental research is *E. coli* lipopolysaccharide (LPS). LPS only
22 represents gram-negative type infections, and PAM and LPS act differently as inflammatory
23 activators. A systemic injection of either PAM or LPS prior to a mild unilateral HI insult in the
24 neonatal rat both sensitises the immature brain and increases injury severity [15–18]. However,
25 as opposed to after LPS sensitised HI brain injury, HT is still highly neuroprotective in PAM-
26 sensitised injury (previous data combined in Fig. 1) [17].

Whether HT is neuroprotective in the setting of a more severe inflammation-sensitised HI brain injury is unknown. We therefore aimed to investigate HT neuroprotective effect in a model of more severe brain injury, both with and without inflammatory pre-sensitisation, in the postnatal day 7 (P7) neonatal rat.

Figure 1. Hemispheric area loss (%) after pre-sensitisation and a mild HI insult.

Horizontal lines represent the median. Bars show median with 95% confidence interval. The graph compares left hemispheric area loss (%) after pre-sensitisation with lipopolysaccharide (LPS) or PAM₃CSK₄ (PAM), carotid artery ligation and 50 min hypoxia (8%O₂ at 36°C). Pups were randomised to 5 hours normothermia treatment (NT_{37°C}) or hypothermia treatment (HT_{32°C}). LPS NT: n=18; LPS HT: n=24; PAM NT: n=36; PAM HT: n=32. Mortality per group (%) is inserted above. HT provided significant neuroprotection after 1 week's survival in PAM-sensitised pups (p<0.0002), but not in LPS-sensitised pups. Modified and combined from Osredkar et al and Falck et al [16,17].

Material and Methods

Animals and injections

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 Laboratories, Sulzfeld, Germany) of both genders. Litters were culled to 10 pups. All pups were kept in an animal facility with a 12:12-hr dark/light cycle at 21°C environmental temperature with food and water *ad libitum*. Animals were randomised across litter, sex and weight before the experiments commenced.

To trigger inflammation we used the synthetically-manufactured TLR-2/1 agonist PAM₃CSK₄ (*PAM₃CSK₄ Vaccigrade, Sigma-Aldrich*) (PAM) in a dose of 1 mg/kg, dissolved in sterile LPS-free water, further diluted in 0.9% NaCl. The dose of PAM was based on previous publications on neonatal rodents [13,19,20], in combination with our own dose-response experiments where we compared post-insult hemispheric area loss according to our standard protocol [17]. We titrated the dose in combination with 50 minutes (min) of 8% hypoxia in order to produce what we have previously defined as a moderate degree of brain injury (around 40% area loss of the affected hemisphere), as we have done previously in the LPS-sensitised model [16]. Fig. 1 compares previously published data on LPS- and PAM-sensitised HI brain injury. Control groups received a single dose of vehicle (sterile physiological 0.9% NaCl) (Veh). All injections were given intraperitoneally in a volume of 10µl/g body weight.

In this model, PAM is injected 8 hours (h) prior to carotid artery ligation (compared to 4 h in the LPS model). This time-point was based on the post-injection temporal development of intracerebral inflammatory cytokines from a previous study [21]. IL-6 and TNF-α peaked 2 h after an LPS injection, whereas after an injection of PAM, IL-6 and TNF-α were significantly upregulated after 6 h, indicating a more delayed intracerebral inflammatory activation. A subsequent pilot experiment with different pre-sensitisation times made us chose an 8 h incubation period between injection and commencement of surgical procedures (Fig. 2).

Figure 2. Different pre-insult sensitisation time.

Inflammatory pre-sensitisation with PAM₃CSK₄ (PAM) was induced 4, 8 or 24 hours (h) prior to the hypoxic-ischaemic insult (n=10-11/group). Error bars show 95% CI of hemispheric area loss (%) after carotid artery ligation and 50 min hypoxia (8%O₂ at 36°C). Veh 4 h: Vehicle injected 4 h prior to the insult. PAM 4 h, PAM 8 h and PAM 24 h: PAM injected 4, 8 or 24 h respectively, prior to the insult.

Surgical Procedures and HI

Comparing results from different experimental laboratories are challenging due to variations in the methods used. In our laboratory we have administered the insult with three different severity levels, depending on the aim of the study. When inflammatory pre-sensitisation is added to the model, the injury is more severe, and the “mild insult” will result in a moderate injury (table 1).

Hypoxic insult:	50 min 8% O ₂ @ 36°C	90 min 8% O ₂ @ 36°C	150 min 8% O ₂ @ 37°C
Injury degree without pre-sensitisation / Veh groups	Mild injury: < 20% area loss	Moderate injury: ~ 40% area loss	Severe injury: ~ 60% area loss
Injury degree with inflammatory pre-sensitisation (LPS or PAM)	Moderate injury: ~ 40% area loss	Severe injury: ~ 60% area loss	

Table 1. Relationship between insult severity and degree of injury.

The table shows the median degree of injury we have seen traditionally in our lab after a mild, moderate or severe hypoxic-ischaemic insult. First row shows the standard model, the second row shows how the degree of injury changes with inflammatory pre-sensitisation.

However, the injury induced is not always in line with the insult severity, due to a variable vulnerability in the rat pups. Various factors might explain the well-known variability in the Vannucci model, and some have attributed it to a variability in communicational blood flow [22]. We have seen an increased vulnerability in the pups after the 2005 changes in EU-regulation for animal transport were enforced from 2015. The amendments have led to rat pups being cross-fostered to a dam which is not biologically theirs [23].

Therefore, to re-characterise the model as run in our laboratory, we first performed a series of two-group experiments without inflammatory pre-sensitisation. We used the experimental protocol planned for the current study, namely unilateral (left) carotid artery ligation followed by 90 min 8% O₂ at T_{rectal} 36°C (Fig. 3).

All surgical procedures were performed as previously described [17], but followed by a 90 min instead of a 50 min hypoxic insult. Briefly, at the start of each experiment, animals were injected with PAM or Veh according to randomisation. After an 8 h delay with their dams, pups underwent ligation of the left carotid artery under isoflurane anaesthesia followed by exposure

to 8% O₂ for 90 min at T_{rectal} 36.0°C, which without PAM exposure results in moderate brain injury in this model (table 1) [4]. Immediately thereafter, pups received either of the two allocated treatments: 5h of normothermia (NT); T_{rectal} 37.0°C or hypothermia (HT); T_{rectal} 32.0°C. During treatment, the core and surface temperature of two 'sentinel' pups from the Veh groups, were 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.

Figure 3. Model variability; Hemispheric area loss (%) after 50 or 90 minutes of hypoxia.

The floating bars show median area loss with 95% confidence interval from a series of experiments with different hypoxia times (the first two: 50 min, the next three: 90 min), producing a mild and moderate injury, respectively, with a degree of injury along a spectrum of severity. The red bars display normothermia (NT) treated groups, and the blue bars display hypothermia (HT) treated groups in the corresponding experiments. The HT-mediated reduction in median injury degree (%) per experiment is indicated by the numbers in squares.

1

2 ***Histopathology and Area loss analyses***

3 Pups were sacrificed at P14 by trans-cardiac perfusion-fixation with 10% neutral-buffered
4 formalin under isoflurane-N₂O-anaesthesia. Brains were harvested and kept in 10%
5 neutral-buffered formalin until further processing. Coronal blocks (3 mm) were cut using a
6 standard rat matrix (ASI instruments Inc., Warren, MI, USA), and embedded in paraffin.
7 Slices (5 µm) were cut from the two neighbouring blocks best representing cortex,
8 hippocampus, basal ganglia and thalamus. Sections were stained with hematoxylin and
9 eosin (H&E) and scanned (Epson Perfection V750 Pro). Virtual slides were exported with
10 600 dpi resolution. Optical density and hemispheric area was analysed using ImageJ
11 computer software (ImageJ, version 1.46r, National Institutes of Health, Bethesda, MD,
12 USA). The ligated side was compared to the non-ligated side, and area loss of the ligated
13 side calculated by the formula $(1 - (\text{area left}/\text{area right})) \times 100$. Percent hemispheric area
14 loss has previously been shown to correlate well with a formal 9-graded step neuropathology
15 score in this model [4].

16 Evaluation of hippocampal area loss was performed in the same way, and calculated as:
17 $(1 - (\text{area of left hippocampus}/\text{area of right hippocampus})) \times 100$. A subset of the H&E stained
18 sections were examined for hemispheric and hippocampal areas by two blinded assessors to
19 check for inter-rater reliability.

20

21 ***Immunohistochemistry***

22 Immunohistochemical staining was performed as described in previous publications using
23 the same material and antibodies [17]. Briefly, slides were prepared from paraffin-
24 embedded sections. Primary mouse antibody against NeuN (1:500; Millipore), was applied
25 overnight at 4°C. In control brain sections, the primary antibodies were omitted. Secondary
26 Alexa Fluor 568 (Invitrogen, 1:500) antibodies stayed on for 1 h at room temperature.

1 Finally, the slides were coverslipped with ProLong Gold with DAPI (Invitrogen). Sections
2 were scanned (Axio Scan.Z1; Carl Zeiss, Jena, Germany) using the fluorescence mode
3 with plan apochromatic 20X lens, and exported as high-resolution tiff images for further
4 analysis.

5 The hippocampus is known to be particularly vulnerable to hypoxia [24–26]. Therefore, to
6 evaluate the effect of different treatments on hippocampal neuronal loss, NeuN and DAPI-
7 positive cells in the CA1 region of the hippocampus were counted. Aiming for a
8 representative subset from each treatment group, the 10 animals closest to the median
9 hemispheric area loss, were selected for formal hippocampal neuron counting, as in previous
10 publications [5]. Three non-overlapping fields, each sized 200 μm x 200 μm , of the CA1
11 region in the left hippocampi were assessed. Counting was performed by two individual
12 observers blinded to the treatment groups, and an average of the two was taken. The total
13 number of neurons across the three fields of each hippocampus was summed and
14 compared across groups.

16 ***Statistical Data Analysis***

17 Statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software Inc.,
18 La Jolla, Ca, USA) and SPSS software version 22 (SPSS Inc., Chicago, IL, USA). As the data
19 are not normally distributed, non-parametric statistics were applied. The Kruskal-Wallis test
20 was used for comparisons across multiple treatment groups, and the Wilcoxon-Mann-Whitney
21 test was used for two-group comparisons to get two-tailed p-values. Linear regression analysis
22 was used to confirm correlation between hemispheric area loss (dependent variable) and
23 hippocampal area loss (independent variable). Graphical data are presented as median with
24 95% confidence intervals (CI) calculated by the exact method (Clopper & Pearson) [27]. A p-
25 value of <0.05 (two-sided) was considered statistically significant.

Results

Characterisation of the model

We and others have documented a high degree of variability in the Vannucci model [22]. Comparing results across laboratories is challenging, and the smallest changes in protocol or environment can have impact on injury severity. To examine the variability in our laboratory, we performed a series of experiments with identical hypoxia protocols of 90 min 8% O₂, and compared to previously-performed milder insults of only 50 min at 8%, conducted by the same researchers (Fig. 3). All experiments were followed by 5 h of NT or HT and 1-week survival before analysis of relative hemispheric area loss.

50 min of hypoxia induced a median injury degree of 4.1% (CI 0-19) in the first, and 10.4% (CI 2.1-37) in the second run, showing a trend towards statistically different level of severity ($p=0.06$). HT treated groups had a median area loss of 4.1% (CI 0-7.8) and 10.9% (CI 7.2-15.4) respectively, thus there was no significant neuroprotection detected with area loss analysis after these mild insults.

In two experiments using 90 min of hypoxia the median injury degree was 37.5% (CI 3-61.9) and 45.4% (CI 33.9-52.1) after NT survival. The corresponding HT treated groups had a reduced median area loss of 17.9% (CI 2.3-45.5) and 35.7% (CI 21.2-47.9) respectively. HT showed less neuroprotection with increasing injury severity (Fig. 3).

PAM-sensitised HI injury – Left Hemispheric area loss

HT provided significant neuroprotection in Veh-injected rat pups, and reduced brain injury from 57.6% (CI 53-61.8%) in the Veh-NT group to 50.1% (CI 18-54.7%) in the Veh-HT group ($p=0.049$). HT was equally neuroprotective in PAM-injected pups, reducing median brain injury from 60% (CI 43.9-63.5%) in the PAM-NT group, to 47% (CI 33.8-54.8%) in the PAM-HT group ($p=0.03$). There was no difference in hemispheric tissue loss between the PAM-sensitised NT treated pups compared to Veh-injected NT treated pups with Mann-Whitney to group

comparison (Fig. 4A). Areas of the un-ligated hemispheres were compared across the 4 groups using the Kruskal-Wallis test. There was no significant difference in tissue loss from the right hemispheres in this model ($p=0.9$). Mortality in the PAM-NT group was 6.6%, and 4.5% in the Veh-NT animals.

PAM-sensitised HI injury – Left Hippocampal area loss

Hippocampal area loss was also significantly reduced in the Veh-HT group with median 43% (CI 26-55%) tissue loss, compared to 59.5% (CI 42.9-64.3%) in the Veh-NT group ($p=0.042$) (Fig. 4A). The same was true for the PAM-injected pups, where median hippocampal area loss was reduced from 61.1% (CI 53-63.5%) in the PAM-NT group, to 38.5% (CI 27-56.3%) in the PAM-HT group ($p=0.027$).

There was no statistical difference between NT-treated pups pre-treated with Veh compared to those who received PAM, with respect to both hemispheric area loss (57.6% vs 59.9%), and hippocampal area loss (59.5% vs 61.1%). Linear regression analysis showed a significant correlation between hemispheric and hippocampal area loss ($R^2=0.7-0.9$, $p<0.0001$ for all four groups, $B=0.823$).

Figure 4. Area loss (%).

A: Aligned dot plot with horizontal lines representing the median. P7 rat pups were injected i.p. with vehicle (Veh) or PAM₃CSK₄ (PAM). All pups were subjected to left carotid artery ligation before 90 min of 8% hypoxia, and 5 hours of normothermia (NT_{37°C}) or hypothermia (HT_{32°C}) treatment, with 7 days' survival. HT provided significant reduction in hemispheric (Hem) and hippocampal (Hip) area loss in both Veh- and PAM-injected groups. *p<0.05.

B: Representative brain slices stained with Haematoxyline&Eosin, demonstrating the severity of left hemispheric tissue loss.

PAM-sensitised HI injury - Neuronal Rescue in the CA1 Hippocampal Region

The total number of neurons in three 200x200µm fields of the CA1 region of the left hippocampus were counted in a subset of animals from all 4 treatment groups (n=10-11 per group) (Fig. 5). The number of remaining hippocampal neurons was similar in PAM-NT animals (4, CI 0-110.5) to the number in Veh-NT animals (1.25, CI 0-53.5). Significant neuronal rescue was seen in the Veh-HT group (48.5, CI 3-132) compared to Veh-NT (p=0.03). In the PAM-HT group (74, CI 11-120.5) numbers did not reach statistical significance due to low numbers, but there was a trend towards neuronal rescue, compared to PAM-NT animals (p=0.06). The neuron count per three fields correlated inversely with hemispheric area loss ($R^2=0.24$, p=0.0016).

Figure 5. Neuron count in the hippocampal CA1 region

A: Three non-overlapping regions-of-interest (200 μm^2) were analysed from the left hippocampal CA1-region per brain. Each symbol represents the total number of hippocampal neurons counted in one animal. Horizontal lines show the median. The neuron count was not different in PAM₃CSK₄ (PAM) -injected animals compared to vehicle (Veh) -animals treated with normothermia (NT_{37°C}). Hypothermia (HT_{32°C}) provided neuronal rescue both in the Veh-group and after PAM sensitisation. *p<0.05. **B:** Representative images from the left hippocampal CA1 region are shown from each experimental group (**B**). Slides were stained with NeuN (green) and DAPI (blue). Neurons with green cytoplasm (arrows) were rarely seen in the Veh-NT and PAM-NT groups.

1 **Discussion**

2 This study demonstrates hypothermic neuroprotection in severe HI brain injury, as well as in
3 severe HI brain injury with prior PAM exposure.

4 The relatively low hypothermic neuroprotective effect (13% reduction in hemispheric area
5 loss, and a 28% reduction of hippocampal tissue) in the non-sensitised group is what we
6 would expect after such severe injury. We have traditionally seen a 25-50% degree of
7 neuroprotection in moderate brain injury [5,28]. In the mildest injuries, the method of
8 hemispheric area loss is unable to identify small changes. HT appears to be neuroprotective
9 over a continuum of injury severity in this model, and the effect tapers off with increasing
10 area loss (Fig. 3).

11 With a median area loss of near 60% in the current study, the injury is defined as severe.
12 The relation between hypoxia time and area loss severity is not linear, and the variability of
13 injury induced by the insult is large [22]. When a certain injury severity is reached, a further
14 duration of the hypoxic insult will not increase injury correspondingly. Therefore, upon
15 adjusting the model to examine severe injury, the intrahypoxic temperature has been
16 elevated by one degree Celsius. Previously-published data on the lack of HT neuroprotection
17 in severe brain injury in the Vannucci model (after 60 and 66% area loss respectively) [4,5],
18 comes from an experimental design where the hypoxic insult was administered at an
19 elevated temperature of 37°C rather than the standard 36°C. In addition a longer hypoxia
20 time was used (150 min vs 90 min). The importance of the intra-hypoxic temperature was
21 addressed by Busto *et al* in 1987, demonstrating that even minimal temperature changes will
22 have impact on injury severity [29]. Northington *et al.* describe a continuum phenotype of cell
23 death that varies on a cell-by-cell basis in the neonatal forebrain after an HI insult, and show
24 how apoptotic cascades are triggered simultaneously with mitochondrial structural and
25 functional failure. They suggest that the phenotype of cell death is dependent on the energy
26 available to drive the apoptotic pathways to completion [30]. When a higher intra-ischaemic

temperature alters the metabolic rate and increases energy demands during the injurious processes, this leads to further compromise in cellular energy reserves, which would then shift more cells towards the necrotic side of the cell death spectrum.

By changing the temperature by 1 degree Celsius in our model, although the medians remain the same, we see a clear reduction in variability of injury within the group, as well as increased mortality, among those who received the insult at a higher temperature (Fig. 6).

There are no surviving pups with injury in the mild range, and also fewer of those pups with very severe area loss, probably due to death.

Figure 6. Different distribution of injury with elevated intrahypoxic temperature.

Horizontal lines represent the median hemispheric area loss, bars show median with 95% confidence interval. P7 rat pups were subjected to left carotid artery ligation before 90 or 150 minutes of 8% hypoxia, administered at 36°C or 37°C. The left (black) bars display current data from the Vehicle-Normothermia group (hypoxia administered at 36°C). The right bars show unpublished data from our laboratory (M. Thoresen Bristol laboratory), where hypoxia was administered at 37°C. Both insults induced a median area loss of around 60%, but with different distribution and mortality (4.5 vs 28%).

1 The proportion of permeable mitochondria is thought to be pivotal with regards to necrotic
2 cellular death relative to the controlled necroptotic-apoptotic type [31–33], and Hagberg
3 suggests that mitochondrial permeabilisation would lead to injury beyond the point of no
4 return [34]. Hua *et al.* demonstrated reduced number of rat cortical neurons with
5 mitochondrial injury in culture exposed to hypoxia at 33°C compared to hypoxia at 37°C [35].
6 Taken together this suggests that an increased core temperature during the HI insult leads to
7 more severe mitochondrial injury and thereby more necrotic type cellular death, beyond the
8 point of hypothermic rescue.

9 Clinically, elevated intrahypoxic temperature could reflect fever. The strong association
10 between maternal pyrexia and severe CP was studied before the era when HT became a
11 treatment option [8]. The most common cause of fever in otherwise healthy adults is viral
12 infections [36]. In pre-clinical research, in addition to bacterial type pre-sensitisation through
13 TLR-2 (PAM) or TLR 4 (LPS), pre-sensitisation with Poly I:C (acts through TLR-3 and mimics
14 a viral infection) prior to an HI insult, also sensitises the immature brain to HI [16,37]. It is
15 therefore reasonable to hypothesise that any systemic inflammatory activation with an
16 elevated core temperature during an HI insult, could lead to a more severe and definite type
17 of neuronal injury, with less potency for hypothermic rescue. The lack of hypothermic
18 neuroprotection in a setting of elevated intrahypoxic temperature was already shown by
19 Yager *et al* in 1995 [24]. Furthermore, post-HI hyperthermia increased morbidity and
20 mortality in neonatal rat pups compared to normothermia treatment [38], supporting the role
21 of temperature on the extent of HI damage. The hypothesis poses the question of whether
22 strictly controlling maternal core temperature at normothermia (37°C) prior to and during
23 labour would improve neonatal outcome, and/or reduce the incidence of HIE. This remains to
24 be investigated.

25
26 In this study, mortality is low, similarly to that seen in the previous PAM-sensitised
27 experiments with only 50 min of hypoxia [17]. When inflammatory activation was induced by

1 LPS imitating a gram-negative type sepsis prior to the HI insult, mortality was very high (>
2 40%) after only 50 min of hypoxia [16]. Eklind *et al* reported in 2001 how LPS-treated pups
3 showed increasing mortality with increasing length of hypoxia, starting already at 20 min [10].
4 Administering as much as 90 min of hypoxia to P7 rat pups pre-treated with LPS was not
5 possible due to the high mortality at 50 min. This distinct difference between the two models,
6 as well as the difference in susceptibility to hypothermic neuroprotection (Fig. 1) [16], is in
7 line with other discrepancies between LPS- and PAM-triggered inflammation and how they
8 affect the immature brain even without the HI insult [21]. LPS induced significant brain
9 apoptosis, poor weight gain, and immediate loss of core temperature (median 31.2°C). PAM
10 did neither, and the pups remained at normothermia. The effect of the studied intervention on
11 thermoregulation is critical in pre-clinical models of neonatal HI and neuroprotection [39]. The
12 profound mortality among LPS-sensitised pups might partly be explained by the temperature
13 drop that LPS induces prior to the HI insult [21]. This means that during the insult, we impose
14 a much greater temperature elevation in LPS-sensitised animals than we do in PAM-
15 sensitised animals, and intrahypoxic temperature is relatively higher for the LPS pups. This
16 partly makes them similar to the pups in the previous experiments of severe HI injury, where
17 the intrahypoxic temperature was elevated. The mortality was high, and HT was not
18 neuroprotective.

19
20 PAM exposed pups do not have a higher level of injury than the control group in the current
21 study. However, we have robustly shown that PAM does sensitise the immature brain to a
22 mild HI insult, and increases area loss from 10% in the vehicle group to 36% in the PAM
23 sensitised group [17]. Furthermore, PAM is a highly stable synthetically manufactured
24 agonist to TLR2. PAM sensitisation reduced the threshold for cellular injury after a mild insult,
25 but our current results show that with longer hypoxia time and a more severe insult, the
26 vulnerability induced by PAM is no longer visible. This phenomenon has previously been
27 shown after LPS pre-treatment, however they still displayed increased mortality [10]. In the

1 model of with PAM exposure, in clear contrast to after LPS sensitisation, the injurious effect
2 of the HI insult appears to be more significant to the outcome than the inflammatory pre-
3 sensitisation, and the damage induced by the inflammatory response and HI are no longer
4 additive.

5 We have not investigated white matter injury, a limitation to a study on inflammatory-evoked
6 injury to the immature brain. Although also a part of term HI injury pattern, white matter injury
7 is more of a focus in injury to the preterm [9], while this being a model of late preterm to term
8 equivalent brain maturity. Additionally, the injury severity in our model makes investigations
9 on white matter challenging, as there is little remaining tissue on the injured side (Fig.4B).

10 HT is neuroprotective in the current study, with a 22% and 37% reduction of hemispheric and
11 hippocampal area loss respectively in the PAM-sensitised group. A plausible hypothesis
12 might be that the inflammatory activation in the absence of marked temperature changes
13 lowers the threshold to cellular injury from an HI insult, without leading to neuronal necrosis,
14 and thereby maintaining susceptibility to hypothermic rescue. The increased rate of infection
15 (6-13%) among HIE neonates, compared to 0.5-1% in the general population supports the
16 theory of elevated susceptibility to HI [40,41]. HT reduces mortality and severe neurological
17 morbidity in asphyxiated neonates, even though the investigated cohorts include infants with
18 severe encephalopathy of HI origin both with and without infectious pre-sensitisation [1,42].

19 With these data we conclude that there is no one level of HI-induced area loss where HT is
20 not a beneficial therapy in this model. Rather there is a continuum along which HT reduces
21 permanent neuronal injury. PAM exposure prior to a moderate HI insult did not sensitise to
22 increased brain injury severity. Based on our results from PAM-sensitised injury with a milder
23 HI insult, in combination with these current data, we suggest that inflammatory exposure
24 through the gram-positive route is not likely to negate hypothermic neuroprotection at any
25 level of severity in the term neonate.

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1 **Statement of Financial Support**

2 This study was supported by the Norwegian Research Council ([NFR 214356/F20](#)). We also
3 thank the Anders Jahre Fund, the German Research Council (H.S.), the University of Oslo
4 (T.W.) for additional funding, a private donation via SPARKS UK (M.T.) as well as financial
5 support from the Norwegian Cerebral Palsy Association.

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7 **Disclosure Statement**

8 The authors declare no competing financial interests.