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Deleterious Effect of Crossfostering in Rat Pups on Hypoxic-Ischaemic Injury Tolerance and Hypothermic Neuroprotection

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Keywords
Animal model · Hypothermia · Hypoxia-ischaemia · Neuroprotection · Neonatal

Abstract
We study the effect of hypothermia (HT) following hypoxic-ischaemic (HI) brain injury in postnatal day 7 (P7) rats. In 2015, new European Union animal transport regulations prompted a change in practice at the breeding facility, which henceforth crossfostered P3 litters to P8 older lactating dams prior to transportation. It is generally assumed that crossfostering does not significantly affect the experimental results. The aim of this study was to examine whether crossfostering affects our model consistency by modifying injury susceptibility and hypothermic neuroprotection. We analysed 219 pups from 11 experiments conducted between 2013 and 2015: 73 non-crossfostered and 146 crossfostered pups. At P7, all pups underwent unilateral common carotid artery ligation followed by 50 min of hypoxia (8% O_2, 36°C). Immediately after this mild insult, the pups were randomized to post-insult normothermia or HT treatment. Pups were culled at P14. Injury was assessed by area loss of the ipsilateral hemisphere and histopathology scoring of the hippocampus, cortex, thalamus, and basal ganglia. Crossfostered pups had double the injury compared to non-crossfostered pups irrespective of the treatment group. Hypothermic neuroprotection was statistically significant, but with a smaller and less consistent effect in crossfostered pups (relative neuroprotection 16% vs. 31% in non-crossfostered). These results demonstrate hypothermic neuroprotection following a mild HI insult. A representative subset of 41 animals was also assessed for evidence of microglial reactivity; however, no detectable difference in microglial reactivity was observed between any of the groups. In conclusion, crossfostering alters outcomes in our established model through reduced insult tolerance and variable neuroprotection. Crossfostering as a common breeding practice is a largely unexplored variable in animal research that may result in invalid research conclusions if inadequately adjusted for by larger group sizes. As a result, crossfostering is likely to be inconsistent with the principles of replacement, reduction, and refinement.

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Introduction

Animal studies ought to yield unambiguous and reproducible findings that have translational potential [1]. However, in many cases, animal models fall short of pre-

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dicting human outcome because of cross-species, genetic, physiological, and/or developmental differences [2–4]. Furthermore, findings can be affected by animal welfare standards, as stressed animals express atypical species-specific behaviours and abnormal physiology [5]. Therefore, experimental animal models require periodic re-evaluation to ensure that they meet the stringent ethical principles of refinement, replacement, and reduction (3 Rs) [6].

Perinatal hypoxic-ischaemic (HI) injury is a major cause of death and disability in term-born infants [7]. Hypothermia (HT) reduces death and disability and is clinically the standard of care for asphyxiated infants with moderate or severe encephalopathy [7–9]. However, the benefit of HT for infants with mild injury remains uncertain. The translational success of HT is owed to numerous animal experiments in different species [10–12], many of which were conducted in our lab over the past decades using the Vannucci model in rats [13] and global hypoxia model in newborn pigs [12]. Though it is the standard rodent model in the field, the Vannucci model is susceptible to large variability in the magnitude of neuroprotection, due to both known and unknown experimental variables [14]. Because mild injuries render a small effect size, demonstration of neuroprotection from HT requires a model with low variability. In the process of studying neuroprotection in a mild version of the Vannucci model, we noticed changes in variability over time, which apparently coincided with changes in practice of transportation of the rats.

Our laboratory in Oslo, Norway, imported neonatal rat pups with their lactating dam from Germany, as no breeding facility in Scandinavia had the capacity to accommodate our need for timed litters at postnatal day 7 (P7). In May of 2015, the transport of less than 7 days postpartum dams became prohibited [15], as the stress to the dam was deemed to violate welfare standards. To receive the pups in time for our experiments, P3 pups were removed from their biological dam and placed in the care of an older lactating dam who had given birth at least 1 week prior and was therefore eligible for transportation. The young dam and the older pups were culled. The older dam nursed the crossfostered litter until the termination of the experiment at P14. Under this practice, one could argue that there was a serious compromise of the 3 Rs, likely additional stress on the dam and pups, as well as an associated increased financial burden.

Reallocating pups from one lactating dam to another is known as crossfostering. Rats are especially suitable for this practice as the dam usually accepts the pups [16]. Crossfostering is common in breeding facilities to keep litters at a consistent size and reduce variability in weight gain due to litter size, though researchers are not explicitly informed of this practice. Crossfostering is most successful when done earlier during lactation [16] and to a dam close in lactational stage [17].

As we encountered altered breeding practices imposed by new regulations, we became interested in whether crossfostering affected injury susceptibility in the Vannucci model and whether post-insult HT remained neuroprotective. In this study, we revisited previously conducted experiments of induced perinatal HI brain injury in P7 rats. We analysed experiments conducted prior to 2015, where the litters were (to our knowledge) nursed by the biological dam (non-crossfostered, NCf) and experiments conducted after 2015, where the litters were crossfostered by an older dam (crossfostered, Cf). We hypothesized that crossfostering would increase susceptibility to HI injury, reduce neuroprotection from HT, and increase model variability.

Materials and Methods

Animals

We conducted 11 experiments on P7 Wistar rats (Charles River laboratories, Sulzfeld, Germany), collecting data on 259 rats from litter size of 10 pups (2013–2015). We examined the effect of HT following mild HI injury using a modified Vannucci model as described below. The University of Oslo's Animal Ethics Research Committee approved the experiments. Rats were kept in an animal facility with 12:12 h day:night cycle, at room temperature of 21°C with food and water ad libitum.

In each experiment, rats were randomly allocated to carry a skin or rectal temperature probe during the experiment. These were excluded from the analysis ($n = 28$) [18]. Five rats died during common carotid artery ligation, no rats died during hypoxia, 5 rats died in the cage in the subsequent days following the insult, and 2 rats were prematurely culled due to poor weight gain. In total, 12 pups (50% crossfostered) were excluded from the analysis due to premature death and 219 rats were analysed (NCf $n = 73$, Cf $n = 146$). The distribution of sex and treatment allocation was equal in each litter and experiment.

Study Design

All experiments were performed using a modified Vannucci model to achieve mild unilateral HI injury [19]. On P7, the pups underwent ligation of the left common carotid artery under anaesthesia (3% isoflurane in a 2:1 gas mixture of NO_2/O_2), followed by a brief recovery period until awake and alert, before being returned to the dam for feeding for at least 30 min. Pups were then placed in a specially designed hypoxic chamber that provided even heat distribution and continuously monitored levels of O_2 and CO_2. During the experiment, core temperature was continuously monitored in designated pups carrying a rectal temperature probe in each chamber [20]. The rectal temperature was maintained within

Table 1. Definition and criteria for histopathological scoring

Brain area	Grading	Percentage area affected	Morphological changes
Cortex, thalamus and basal ganglia	0	0	No visually visible injury
	1	<10	Small, patchy, complete, or incomplete infarcts
	2	20–30	Partly confluent, complete, or incomplete infarcts
	3	40–60	Large confluent complete infarcts
	4	>75	In cortex, total disintegration of the tissue, in thalamus and basal ganglia large complete infarcts
Hippocampus	0	0	No visually visible injury
	1	<20	Necrotic neurons only in the most lateral areas: CA_1–CA_2
	2	50	Patchy areas of necrotic neurons in CA_1–CA_4
	3	75	More extensive areas of necrotic neurons in CA_1–CA_4
	4	100	Complete infarction of hippocampus, including the dentate gyrus

Histopathological score. Regional pathology scoring was based on the morphology of necrosis in the injured hemisphere. The score used is a 9-point score, with increments of 0.5 (0.0–4.0) [11].

±0.2°C using a servo-controlled water-filled mat (Criticool, MTRE, Yavne, Israel). All pups were exposed to 8% O_2 for 50 min at 36.0°C. This duration of hypoxia produces a mild insult with approximately ≤10% hemispheric tissue loss [19], in contrast to the 40% tissue loss achieved after a moderate HI insult of 90 min [21]. To assess the neuroprotective effect of HT, the pups were randomized by litter, weight, and sex to 5-h treatment of either normothermia (NT), target 37°C ± 0.2°C rectal temperature, or HT, target 32°C ± 0.2°C rectal temperature. Immediately after hypoxia, the pups were transferred to the allocated NT or pre-cooled HT treatment chamber. Following 5 h of treatment, the cooled group was rewarmed at 1°C every 15 min until reached core temperature of 33–34°C. All pups were returned to the dam after the experiment, for a survival period of 7 days. The pups were weighed during the experimental period and monitored for inadequate weight gain defined as no weight gain during 2 consecutive days.

Tissue Sampling

Transcardiac perfusion with 10% phosphate-buffered formaldehyde (0.1 M) was performed at P14 under isoflurane anaesthesia. The brain was extracted and kept in formaldehyde for 4 days until further processing. Six coronal 3-mm blocks, numbered 1 (frontal) to 6 (caudal), were cut through the brain (ASI Instruments Inc., Warren, MI, USA) and embedded in paraffin. Slices (6 μm) were cut to include the following anatomical regions: basal ganglia (block 3), thalamus (block 4), and hippocampus (block 4). The injury is best represented in these blocks, as ligation of the left common carotid artery ceases blood flow predominantly to the vascular supply area of the left middle cerebral artery. Regions supplied by the left anterior or posterior branches are generally less injured.

Sections were stained with haematoxylin and eosin and assessed for injury, except 1 block which was damaged during preparation. For immunohistochemistry, a representative selection of all injury severities was chosen based on the global pathology score. Four sex-matched rats from each of the 4 groups (NT-NCf, HT-NCf, NT-Cf, and HT-Cf) were chosen from the 0th-centile, 50th-centile, and 75th-centile. Seven blocks went missing or damaged. In total, $n = 41$ were selected for immunohistochemistry.

Immunohistochemistry

Sections were deparaffinized at 60°C for 1 h followed by incubation in 100% xylene (15 min) and absolute ethanol (10 min), then rehydrated in graded alcohol concentrations at 5-min intervals in 96% ethanol, 80% ethanol, 70% ethanol, and washed in distilled water for 5 min. The sections were washed in a solution of 0.1% Tween 20-phosphate buffer saline (0.1% PBS-T). Antigen retrieval was performed by 25 min boiling in 10 mM citrate buffer (pH = 6.0). Endogenous peroxidases and phosphatases were blocked in dual enzyme block for 10 min (Dako, S2003, Agilent), followed by a 1-h incubation in 10% normal horse serum in 0.2% PBS-T. Primary antibodies against NeuN (mouse anti-rat; 1:1500, Abcam ab-104224) and Iba-1 (rabbit anti-rat; 1:750, Wako 013-27691) were applied for 48 h in 10% normal horse serum +5% bovine serum albumin in 0.2% PBS-T. Following incubation, primary antibodies were washed off in PBS-T (0.1%). Secondary antibodies were applied (horse-anti-rabbit HRP [brown] + horse-anti-rabbit AP [magenta] using the ImmPRESS Duet Double Staining Polymer kit MP-7724, Vector Laboratories). Visualization of epitopes was achieved with DAB and AP chromogens applied sequentially. Sections were counterstained in preheated methyl green at 60°C for 5 min and excess histochemical washed off in 10 min of distilled water. Sections were dehydrated in graded alcohols at 70% ethanol, 80% ethanol, 96% ethanol and cleared in absolute ethanol and 100% xylene before they were mounted with permanent mounting medium (DPX).

Histological Assessments

Two methods of assessment were used: area loss analysis in ImageJ (v.1.46r, National Institutes of Health, Bethesda, MD, USA) and classical histopathology. For area loss analysis, haematoxylin and eosin-stained slides from blocks 3 and 4 were scanned (Epson Perfection V750 Pro) to 600 dots per inch (dpi) images. The relative area loss of the ligated injured hemisphere was calculated using the unligated hemisphere as control by 2 blinded investigators and crosschecked. Pixel intensity threshold was applied to the image, and the hemispheric relative area loss was calculated by the formula (1-[left area/right area]) × 100. The average area loss

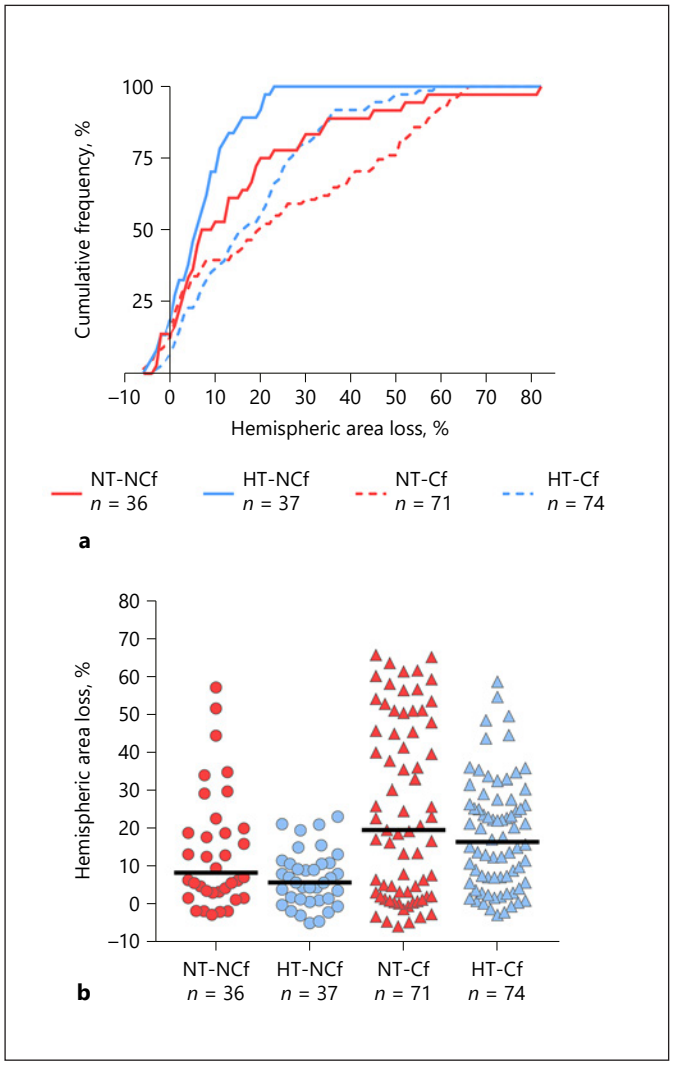

Fig. 1. Area loss. **a** Cumulative frequency distribution of hemispheric area loss (%). **b** Scatter plot of hemispheric area loss (%). Negative values are included to indicate the degree of stochastic variability between the hemispheres, either due to biological asymmetry, skewed cutting or tissue breakage during preparation.

of block 3 and 4 was then calculated. Some results are presented with negative values, meaning the ligated hemisphere is larger than the unligated, instead of set to 0 as previously done in published work [22], to show the stochastic variation in these measurements.

Classical histopathological assessment was conducted in the same sections as analysed for area loss. For this purpose, each section was scanned using a high-resolution microscopy scanner (Carl Zeiss Axioscan Z1, pixel resolution: 0.220 µm × 0.220 µm, objective: plan/apochromat 20×/0.8 M27). The investigator, blinded to the treatment, assessed the injury using a 9-point scoring system (Table 1) previously developed and validated [11, 23]. The cortex (mean of blocks 3 and 4), basal ganglia, thalamus, and hippocampus were scored individually. The global pathology score was calculated as the mean of all regions.

Similarly, sections stained with bright field were scanned in Zeiss Axioscan Z1 (pixel resolution: 0.220 µm × 0.220 µm, objective: plan/apochromat 20×/0.8 M27). Based on our preliminary results from the classical histopathological assessment, we chose the hippocampus as the main region of interest for assessment of microglial reactivity. Two independent blinded investigators scored the degree of microglial reactivity in the hippocampus based on the morphology of microglial cells, ramification complexity, and somal size [24]. Microglial surface area was calculated in ImageJ by selecting three areas (300 × 300 µm^2 per frame) of the different cornu ammonis regions of the hippocampus, and applying colour deconvolution and threshold to the image. Measurements were conducted on representative cells where the nucleus was visible. In total, n = 488 cells were analysed.

Statistical Analysis

Statistical analyses were performed in SPSS Statistics (v.26.0, Chicago, IL, USA) and GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA). Because the data were not normally distributed, we applied non-parametric statistics. For analysis of weight gain and microglia, we applied the Wilcoxon Mann-Whitney two-sample test to compare the observed medians between groups. For analysis of area loss and pathology score, we applied the Kolmogorov-Smirnov two-sample test to compare the cumulative frequency distributions between groups. The Kolmogorov-Smirnov D-statistic presented in the results is a measure of effect size and quantifies the maximum distance between the cumulative distributions. The same analyses were split by sex to examine any sex-dependent differences. We calculated the relative neuroprotection (%) per experiment and for the dataset as a whole, by calculating the per cent decrease in hemispheric area loss with formula $(NT_{median} - HT_{median}) \times 100/NT_{median}$ for the crossfostered and non-crossfostered group. Similarly, we calculated the absolute neuroprotection as the difference in median between the treatment groups: $NT_{median} - HT_{median}$.

We tested homogeneity of variance between the treatment groups for $\alpha = 0.05$. To assess equality of variance, we applied Levene's test. Correlations were examined using Spearman's correlation test. All figures were created in GraphPad. All values are presented as medians with interquartile range (IQR).

Results

Crossfostering Affects Weight Gain

Crossfostered pups had significantly lower initial weight (P6), end-weight (P14), as well as overall weight gain during the survival week compared to the non-crossfostered pups: NCf-P6 10.3 g (11.0–9.9) versus Cf-P6 9.5 g (10.6–8.2) ($p = 0.01$), NCf-P14 23.6 g (26.2–21.7) versus Cf-P14 20.7 g (23.1–18.0) ($p < 0.001$), and weekly weight gain NCf 13.3 g (16.1–11.6) versus Cf 11.2 g (12.7–9.6) ($p < 0.001$). We found no significant difference in weight gain between the treatment groups (NT vs. HT) in either crossfostered or non-crossfostered pups ($p > 0.50$).

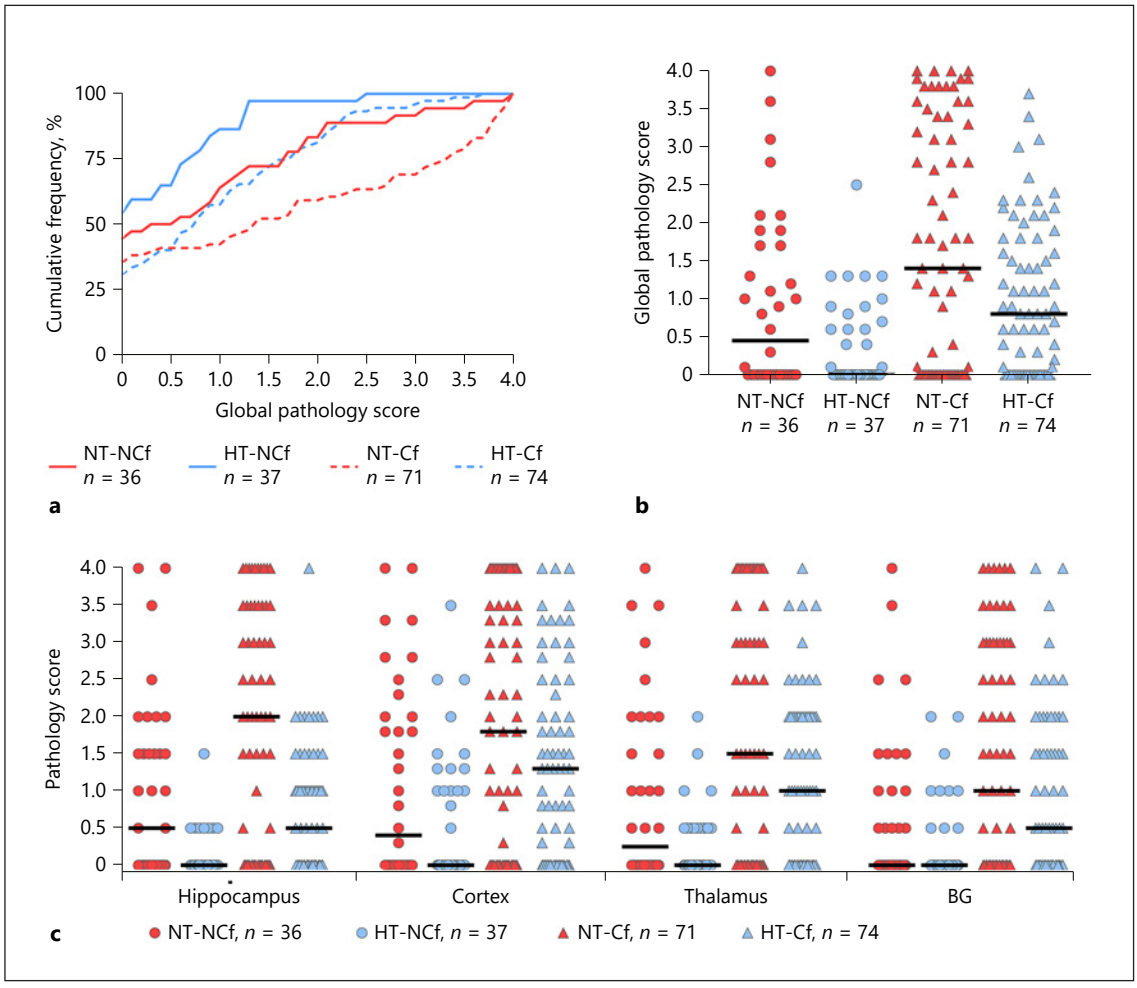

Fig. 2. Pathology score, with represented medians where relevant. **a** Cumulative frequency distribution of global pathology score. This distribution has a large cumulative percentage at zero global pathology score because many rats had no detectable injury. **b** Scatter plot of global pathology score. **c** Scatter plot of regional pathology score of the four brain regions assessed. The hippocampus had the greatest injury and greatest neuroprotection among the regions.

Crossfostering Increases Susceptibility to Injury

Crossfostered pups had significantly higher post-insult hemispheric area loss compared to non-crossfostered pups in both treatment groups (Fig. 1). In the crossfostered animals, the area loss was doubled in the NT-group, NT-NCf: 8.5% (3.2–22.1) versus NT-Cf: 19.8% (2.2–48.1) (D = 0.269 p = 0.06), and tripled in the HT-group HT-NCf: 5.8% (1.3–10.9) versus HT-Cf: 16.6% (5.8–26.9) (D = 0.419 $p < 0.01$). Hemispheric area loss assessment strongly correlated with global pathology score (Spearman's correlation coefficient: 0.887 $p < 0.001$). Crossfostered pups had greater injury in all regions and significantly higher global pathology scores than non-crossfostered pups, irrespective of treatment group (Fig. 2): NT-NCf: 0.45 (0.0–1.7) versus NT-Cf: 1.4 (0.0–3.4) (D = 0.283 $p < 0.05$), HT-NCf: 0.0 (0.0–0.75) versus HT-Cf: 0.8 (0.0–1.8, 3.7) (D = 0.319 p = 0.01).

HT Is Neuroprotective following Mild Insults

For the effect of HT, significant neuroprotection was detected by area loss in the crossfostered pups (D = 0.272, $p < 0.01$), while neuroprotection was not statistically significant in the non-crossfostered pups (D = 0.283, p = 0.108). Similarly, when assessed for global pathology score, neuroprotection was only significant in the crossfostered pups (D = 0.313, $p < 0.01$), and notsignificant in the non-crossfostered pups (D = 0.255, p = 0.188). There were no difference between the sexes when split by treat-

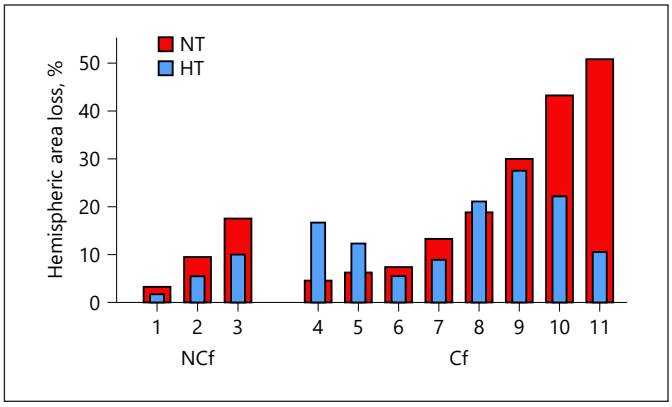

Fig. 3. Experimental outcome variability. Data are split by individual experiments and arranged by increasing median area loss (%) in NT-group to visualize the variability in outcome. The relative neuroprotection was variable in the crossfostered experiments, ranging from −249% to 79%, while the relative neuroprotection ranged from 40% to 42% in the non-crossfostered experiments. In three out of seven crossfostered experiments the HT group had greater injury than the NT group.

ment and crossfostering ($p > 0.50$). Regional histopathological assessment (Fig. 2) demonstrated significant neuroprotection in the non-crossfostered pups in the hippocampus ($D = 0.443$ $p < 0.01$) and close to significance in thalamus ($D = 0.309$ $p = 0.06$); however, no observed neuroprotection in the cortex ($D = 0.253$, $p = 0.19$) or the basal ganglia ($D = 0.135$, $p = 0.597$). Crossfostered pups had significant neuroprotection in all regions: hippocampus ($D = 0.443$, $p < 0.01$), cortex ($D = 0.227$, $p < 0.05$), thalamus ($D = 0.285$, $p < 0.01$), and basal ganglia ($D = 0.232$, $p < 0.05$).

Based on the Kolmogorov-Smirnov D-statistic, we hypothesized HT would be significantly neuroprotective in the non-crossfostered pups given equal sample size of the crossfostered group. To confirm this, we ran a simulation by doubling the number of observations in the non-crossfostered group by duplicating each case. Repeating the analysis, significant neuroprotection was detected both by hemispheric area loss assessment ($p < 0.01$) and global pathology score ($p = 0.02$).

Increased Variance and Inconsistent Neuroprotection in Crossfostered Pups

The crossfostered group had significantly greater statistical variance than the non-crossfostered group ($p < 0.001$), irrespective of the greater variance in the NT group as compared to HT group ($p < 0.001$): NT-NCf: 363.9 versus NT-Cf: 538.0, HT-NCf: 53.6 versus HT-Cf: 213.8. Across all experiments, the relative neuroprotection in hemispheric area loss was 31% in the non-crossfostered group and 16% in the crossfostered group. The absolute neuroprotection was 2.6% in the non-crossfostered group and 3.2% in the crossfostered group. The large variability in neuroprotection in the crossfostered group, ranging from −247% to 79% relative neuroprotection, is more evident when splitting the data by individual experiments (Fig. 3).

Crossfostering Does Not Affect Microglial Phenotype

Microglial reactivity grading correlated with cell surface area measurements (Spearman´s correlation coefficient: 0.625, $p < 0.001$). Microglia with increased somal size and ramification complexity were given a higher grade score and had increased surface areas (Fig. 4). We detected no differences between the crossfostered and non-crossfostered group for both cell surface area and microglial grade. In the non-crossfostered pups, microglial reactivity grade trended towards neuroprotection ($p = 0.16$); however, this was countered by no detectable effect of HT in the surface area measurements ($p = 0.54$). In crossfostered pups, no effect of HT was detected ($p > 0.5$).

Discussion

We have investigated the effect of crossfostering in neonatal brain-injury experiments conducted on P7 rats using a mild Vannucci model of HI injury. We have demonstrated hypothermic neuroprotection following a mild HI insult. The neuroprotection in the non-crossfostered pups was consistent across experiments and with previously published work. Despite of this, neuroprotection did not reach statistical significance at the current sample size when assessed for global injury. However, regional neuroprotection was significant in the hippocampus and thalamus.

Crossfostered pups had poorer weight gain, twice the brain injury, and more variable effect from HT as compared to non-crossfostered pups. Previous work by others has showed that randomized crossfostering of rat pups within 1 h of birth to older lactating dams results in poorer weight gain and increased mortality. With this mild insult, we did not observe any difference in mortality between the groups (NCf: 8% vs. Cf: 4%). No difference between the sexes was detected in either crossfostered or non-crossfostered pups [14]. Microglia, as early mediators in the response to hypoxia [25], may affect the susceptibility to HI injury [26]; however, we were unable to detect differences in microglial reactivity among the four groups.

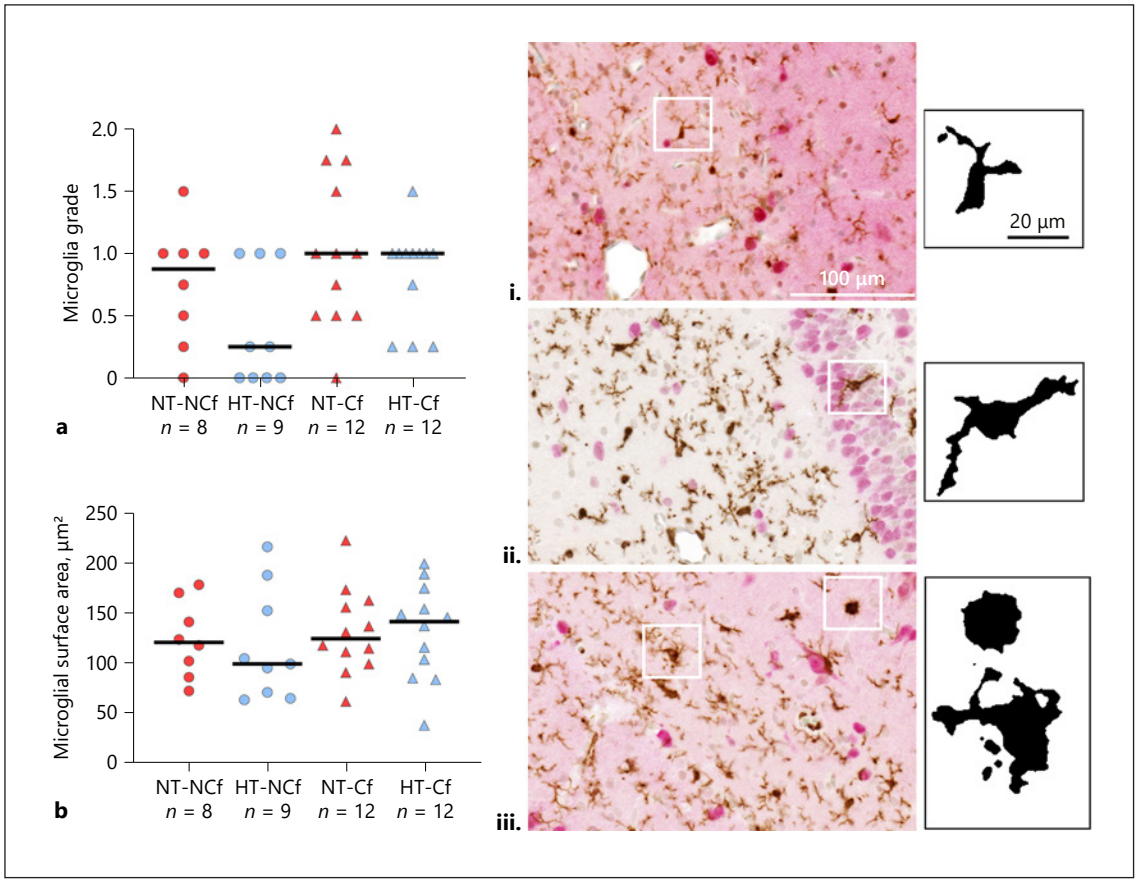

Fig. 4. Microglial reactivity. **a** Scatter plot of microglia reactivity grade in the hippocampus. **b** Scatter plot of microglial surface area measurements in the hippocampus. Representative images of the different surface area measurement showing the morphology of the microglia (mean surface area): (**i**) 73 µm^2, (**ii**) 138 µm^2, (**iii**) 174 µm^2.

It is unknown why crossfostering reduced tolerance to HI injury. For the dam, the stress of losing her litter may affect milk production and her willingness to care for and groom her litter. In addition, the milk of an older lactating dam may have an inadequate nutrient composition, as the macro- and micronutrients vary depending on the stage of lactation. In particular, the concentration of iron, copper, manganese, and zinc drops quickly during the first 10 days postpartum [27], which would coincide with the post-insult survival period. Rat pups receive maternal immunoglobulins through milk [28], and the relative composition of immunoglobins and other growth factors [29] may play a role in pathogenesis of HI injury. For the pups, the stress of maternal separation may affect feeding habits, tissue repair, and brain connectivity following HI injury [30]. Other types of stress have been shown to affect injury susceptibility in the same model [18].

Crossfostered pups had double the brain injury as non-crossfostered pups, despite being exposed to a mild HI model which normally results in ≤10% area loss. The increased susceptibility to injury if crossfostered makes it difficult to establish mild insults in rats and examine hypothermic neuroprotection, which is of great clinical interest to this field [31]. Clinically, HT is only offered following moderate or severe HI encephalopathy, with unknown benefits to infants with mild encephalopathy. Precise and reliable experimental animal models are required to accurately induce mild HI injury and detect neuroprotection, which is complicated by crossfostering.

A limitation of our study is being unable to match sample size between the crossfostered and non-crossfostered group as the change in practice was imposed suddenly, and we were unable to receive more non-crossfostered litters. This complicated the interpretation of the effectiveness of HT, as well as neuroprotection in the crossfostered group was perhaps easier to detect because the initial injury was twice the size. Furthermore, the small representative sampling for microglia assessment only allows

for limited interpretation of our findings. Another limitation is that some of the pups in the non-crossfostered group likely were crossfostered to same-age dams, as sex-balanced litters of 10 are otherwise impossible to obtain consistently. However, this effect is likely minor compared to the crossfostering of an entire litter to an older dam. Lastly, other unknown concurrent events may have change in 2015, mediating the effect of crossfostering. Factors such as research staff members, housing conditions and experimental protocol remained unchanged [19, 22], and to our knowledge, no other major changes occurred within this period.

Conclusions

In this study, we demonstrate neuroprotection by HT in a mild HI model. We show that crossfostering complicates the outcome and interpretation of neuroprotection studies in the Vannucci model, which may reduce the validity of the results. Furthermore, it must be highlighted that inconsistent results and greater variability in the data necessitate larger sample sizes to detect statistical significance, which could contribute to the wasteful use of animals in research and add an unnecessary financial burden. We argue that the practice of crossfostering is inconsistent the principle of the 3 Rs, and therefore that the grounds for prohibition of transporting recently postpartum dams should be revised.

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Statement of Ethics

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 Act of 1986, FOTS ID number: 4344.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

All authors fulfil the four ICMJE criteria with (1) Substantial contribution to the conception, design and analysis of the work (M.T., J.K.G., L.W., and T.R.W.), and acquisition and interpretation of data (M.T., J.K.G., L.W., T.R.W., and D.A.M.); (2) drafting the work or revising it critically for important intellectual content (M.T., J.K.G., L.W., T.R.W., and D.A.M.); (3) final approval of the version to be submitted (M.T., J.K.G., L.W., T.R.W., and D.A.M.); and (4) agreement to be accountable for all aspects of the work (M.T., J.K.G., L.W., T.R.W., and D.A.M.).

Data Availability Statement

The data generated and analysed during this study and included in this article are part of an ongoing PhD project (J.K.G.). Further enquiries can be directed to the corresponding author.

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