Protective Effects and Magnetic Resonance Imaging Temperature Mapping of Systemic and Focal Hypothermia in Cerebral Ischemia

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Background and Purpose—Hypothermia is potentially the most effective protective therapy for brain ischemia; however, its use is limited because of serious side effects. Although focal hypothermia (FH) has a significantly lower stress profile than systemic hypothermia (SH), its efficacy in ischemia has been poorly studied. We aimed to compare the therapeutic effects of each treatment on various short- and long-term clinically relevant end points.

Methods—Sprague–Dawley rats were subjected to transient (45 minutes) occlusion of the middle cerebral artery. One hour after arterial reperfusion, animals were randomly assigned to groups for treatment with SH or FH (target temperature: 32°C) for 4 or 24 hours. Lesion volume, edema, functional recovery, and histological markers of cellular injury were evaluated for 1 month after ischemic injury. Effects of SH and FH on cerebral temperature were also analyzed for the first time by magnetic resonance thermometry, an approach that combines spectroscopy with gradient-echo–based phase mapping.

Results—Both therapeutic approaches reduced ischemic lesion volume (P<0.001), although a longer FH treatment (24 hours) was required to achieve similar protective effects to those induced by 4 hours of SH. In addition, magnetic resonance thermometry demonstrated that systemic hypothermia reduced whole-brain temperature, whereas FH primarily reduced the temperature of the ischemic region.

Conclusions—Focal brain hypothermia requires longer cooling periods to achieve the same protective efficacy as SH. However, FH mainly affects the ischemic region, and therefore represents a promising and nonstressful alternative to SH. (Stroke. 2016;47:2386-2396. DOI: 10.1161/STROKEAHA.116.014067.)

Key Words: focal hypothermia ▪ ischemia ▪ magnetic resonance imaging ▪ middle cerebral artery ▪ systemic hypothermia ▪ temperature

Hyperthermia is highly correlated with poor outcomes in patients with stroke,1,2 whereas therapeutic hypothermia is considered a promising therapeutic strategy in patients with ischemic stroke.3,4 However, systemic (whole body) hypothermia (SH) has not yet been implemented as a routine treatment in patients with ischemic stroke, largely because of side effects including shivering, hypotension, arrhythmia, or infection that complicate the clinical management of these patients.5 Therefore, new strategies to improve the management of therapeutic hypothermia and reduce its adverse effects are urgently needed. In this regard, brain focal hypothermia (FH) has been proposed as a promising alternative to SH6 because selective hypothermia induction in the focal ischemic area may provide similar protection while achieving faster cooling and avoiding systemic side effects. In fact, recent studies using novel devices to induce FH have shown promising results for stroke therapy.7,8 However, no study to date has evaluated the comparative efficacies of SH and FH in patients with ischemic stroke.

Previous experimental studies in this field have mainly focused on the optimization of individual parameters of SH treatment, such as the therapeutic time window or treatment duration.9-14 The results of these studies, as well as those of a meta-analysis study,15 indicate that a target temperature of 32 to 34°C should be induced as soon as possible after stroke onset. Thus far, only one previous work has suggested that FH...
requires at least 48 hours to elicit protective effects.2 However, many of these studies bear some limitations, most notably the use of invasive probes, which sample only a restricted local volume of tissue, to confirm the effects of hypothermia in the cerebral ischemic region. In addition, opening the skull for probe insertion may itself alter temperature dynamics. Thus, an accurate and noninvasive procedure is needed to measure the temperature in the ischemic area during therapeutic hypothermia.

The development of magnetic resonance thermometry (MRT) has shown that brain temperature can be measured noninvasively by means of magnetic resonance spectroscopy, in combination with magnetic resonance imaging (MRI) provides spatial temperature distribution data across the brain, including ischemic and normal tissue, with sufficient reliability for group comparisons.15,16 Utilizing the principle that the water frequency shift relative to N-acetyl aspartate, creatine, or choline is temperature dependent,15,16 MRT has been validated as a noninvasive assessment of brain temperature in patients with stroke. MRT provides fast temperature measurement with 3-dimensional imaging coverage, and an accuracy of 0.1/0.2°C, allowing for analysis of either changes in or absolute brain temperature.17,18

Therefore, the aim of the present work was to analyze the protective effects of optimized SH and FH protocols in a multimodal comparative fashion, using these clinically relevant MR-based techniques in an animal model of ischemia. We evaluated the long-term progress of animals undergoing each procedure using MRI, in combination with functional tests and histological analysis of molecular processes involved in the pathogenesis of ischemic injury, including neuronal loss, early inflammation, and lesion scar formation. We also determined the effects of each hypothermic treatment on brain temperature using both invasive (temperature probes) and noninvasive strategies (MRT).

Materials and Methods

Animals

All experimental protocols were approved by the local Animal Care Committee according to the guidelines established by the European Union (86/609/CEE, 2003/65/CE, and 2010/63/EU). Male Sprague-Dawley rats weighing between 280 and 330 g were used. Animals were housed individually at an environmental temperature of 23°C, with 40% relative humidity and a 12 hours light–dark cycle, and they were given free access to food and water.

Cerebral Ischemia

Transient focal ischemia (45 minutes) was induced by intraluminal occlusion of the middle cerebral artery, following the method described previously.19 Cerebral blood flow was monitored during surgery, and a baseline MRI evaluation was performed during the occlusion period, before hypothermic treatment. Details about surgical materials and protocols, as well as MRI, are described in the online-only Data Supplement.

Experimental Design

After arterial reperfusion, animals were randomly assigned to the following experimental groups: systemic control group, 4 hours of treatment duration (SC-4h; n=12); SH group, 4 hours of treatment duration (SH-4h; n=12); focal control group, 24 hours of treatment duration (FC-24h; n=12); and FH group, 24 hours of treatment duration (FH-24h; n=12). Using the same randomized protocol, a second study included the following experimental groups: focal control group, 4 hours of treatment duration (FC-4h; n=6) and FH group, 4 of hours treatment duration (FH-4h; n=6). In addition, 3 FC-24h animals and 3 FH-24h animals were included.

SH and FH protocols were selected based on previous studies9–11 and are described below and in the online-only Data Supplement. Treatments began 1 hour after reperfusion; the target temperature in both methods was 32°C. In all groups, MRI was used to determine the ischemic lesion volume and evaluate edema formation during the occlusion period, before hypothermia treatment (baseline lesion volume), and at 24 hours, 7 days, and 30 days after treatment. Functional outcomes were assessed in the SC-4h, SH-4h, FC-24h, and FH-24h groups using the cylinder test before surgery and 48 hours, 6 days, and 29 days after surgery. Three animals per group were euthanized for histological analyses at 24 hours, 7 days, and 30 days after surgery.

We also analyzed SH- and FH-induced changes in body and brain temperature using implantable probes and MRT in separate groups of animals. Body rewarming rates after SH were measured using implantable temperature sensors inserted into the peritoneal cavity (n=3 animals). To measure brain temperature during FH or SH, temperature probes were implanted into both hemispheres (n=3 per group). Detailed procedures for the above implantations are described in the online-only Data Supplement.

MRT was used to measure brain temperature in normothermic, SH, and FH groups of animals (n=3 per group) after middle cerebral artery occlusion. Ischemic lesion was confirmed by MRI in all animals 1 day after ischemic injury.

Exclusion Criteria

The following exclusion criteria were used: (1) <70% reduction in relative cerebral blood flow; (2) arterial malformations, as determined by magnetic resonance angiography; (3) baseline lesion volume of <25% or >45% with respect to the ipsilateral hemisphere, as measured using apparent diffusion coefficient maps; (4) absence of reperfusion or prolonged reperfusion (>10 minutes until achievement of at least 50% of the baseline cerebral blood flow) after filament removal; and (5) failure to complete treatment. All excluded or deceased animals were replaced until the total number of animals indicated for each group was attained.

SH Procedure

SH was induced in anesthetized animals 1 hour after reperfusion, for a 4-hour period, using a rectal thermostat-controlled electric pad (NeosBiotec, Pamplona, Spain). After treatment, animals were allowed to rewarm spontaneously. Details about the SH protocol and monitoring are described in the online-only Data Supplement.

FH Procedure

FH was induced 1 hour after arterial reperfusion, for 4 hours or 24 hours of periods, using a previously described cooling device,20 with some modifications (Figure I in the online-only Data Supplement). Cooling devices were removed immediately after the treatment period. Details about the FH cooling device, protocol, and monitoring are described in supplementary material.

Brain MRT

All MR measurements were performed using a 9.4 T MR system (BrukerBioSpin, Billerica, MA). Our MRT approach consisted of single-voxel magnetic resonance spectroscopy combined with chemical shift imaging21 and gradient-echo–based phase mapping,22 all of which rely on temperature-dependent shifts in the water resonance frequency. Details about experimental conditions, sequences, and data processing are described in the online-only Data Supplement and Figures II and III in the online-only Data Supplement.
MRI Assessment and Data Analysis
MRI assessments and analyses were performed according to a previously described protocol. In brief, a baseline MRI evaluation was performed during the occlusion period, including magnetic resonance angiography to confirm middle cerebral artery occlusion, and diffusion tensor imaging with a spin-echo echo-planar imaging sequence to obtain diffusion-weighted images and calculate apparent diffusion coefficient maps for measurement of baseline lesion volume. To analyze lesion volume evolution, T2-weighted images were acquired 24 hours, 7 days, and 30 days after ischemic injury. All image processing was performed with ImageJ (Rasband WS, National Institutes of Health, Bethesda, MD). Additional details about MRI sequences and data analysis are described in the online-only Data Supplement.

Functional Assessment
Functional outcome was evaluated using the cylinder test (online-only Data Supplement).

Brain Histological Analysis
Twenty-four hours after ischemic injury, neurons were immunofluorescently labeled with anti-NeuN antibodies and further stained via terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) to identify dying neurons. Seven days after injury, NeuN labeling was combined with labeling for Iba1, a microglia- and macrophage-expressed inflammatory marker. Finally, 30 days after ischemic injury, NeuN labeling was combined with labeling for the astrocyte marker glial fibrillary acidic protein (GFAP). In addition, lesion scar composition was assessed using glial and fibrotic markers (GFAP and vimentin, respectively). Detailed protocols are described in the online-only Data Supplement.

Statistical Analysis
All data are presented as the mean and SEM (mean±SEM). One-way or 2-way ANOVA followed by the post hoc Bonferroni test was used to identify significant differences in multiple comparisons. The Student t test was used to identify significant differences between the 2 groups. Statistical significance was set at P<0.05. Statistical analyses were conducted using SPSS Statistics for Macintosh, version 18.0 (IBM, Armonk, NY).

Results
A total of 121 animals were used in this study (Figure IV in the online-only Data Supplement): 100 to compare SH and FH and 21 to assess body and brain temperature during SH and FH procedures. A total of 34 animals were excluded from the comparative study. Twenty-seven animals were excluded after ischemic injury because of nonoptimal baseline lesion volume (17 animals; Figure VA through VC in the online-only Data Supplement), arterial malformations (2 animals; Figure VD in the online-only Data Supplement), or surgical complications (8 animals). Seven animals were excluded during the hypothermia procedures, 4 animals undergoing FH removed the cooling device, and 3 animals undergoing SH died from respiratory complications. No differences in blood glucose levels or weight were observed between the different experimental groups (data not shown).

Invasive Analysis of Body and Brain Temperature During SH
SH was rapidly induced with a decrease in rectal temperature from 37 to 32°C in <15 minutes. The average rectal temperature during SH was 32.3±0.3°C, whereas the average rectal temperature in the systemic control group was 36.9±0.5°C. After the SH procedure, animals were returned to their home cages to spontaneously rewarm. The rewarming rate, measured using an implanted temperature sensor was ≈0.03°C/min (Figure VI in the online-only Data Supplement). Approximately 150 minutes was required for recovery to normothermia. SH caused a reduction in whole-brain temperature that was similar to the observed decrease in body temperature (Figure VIIA in the online-only Data Supplement).

Invasive Analysis of Brain Temperature During FH
An implanted focal cooling device (Figure VIIB in the online-only Data Supplement) did not induce a significant alteration in cerebral temperature in the ipsilateral hemisphere compared with the contralateral hemisphere before cold-water perfusion began. However, when cold water was perfused through the device, a rapid decrease in temperature in the ipsilateral hemisphere was observed (35 to 32°C in 15 minutes). After this initial reduction, the temperature remained stable (31.9±0.4°C). Ipsilateral hemisphere cooling only minimally affected the temperature in the contralateral hemisphere and did not affect body temperature. When cold-water perfusion was stopped, the ipsilateral region spontaneously rewarmed to the baseline temperature in <7 minutes (approximated rewarming rate: 0.5°C/min).

MRT Analysis of Brain Temperature During SH and FH
The effects of normothermic conditions, SH, and FH on brain temperature were determined noninvasively by means of MRT. Similar to the invasive analysis results above, MRT demonstrated that the whole-brain temperature (measured in region of interests [ROIs] 1–3) was similar to the body temperature under normothermic conditions (Tbody=37.2°C; Figure 1A). However, SH reduced both the whole-brain (32–33°C) and body (Tbody=32.6°C) temperatures. By contrast, FH reduced the brain temperature from 33 to 32°C in the ischemic region where the cooling device was located (ROI3), but only minimally affected the healthy tissue (ROIs 1–2) or body temperature (Tbody=35.6°C).

Brain temperature changes because of normothermia, FH, and SH were also accurately determined using gradient-echo–based phase mapping, with a precision of±0.4°C (Figure 1B). In the normothermic group (ΔTbody=37.0–37.6°C), minimal temperature changes (ΔT) were observed (ΔTROI1 and ΔTROI2=0.3°C). In contrast, SH (ΔTbody=36.8 to 32°C) resulted in ΔTROI1=3.9°C and ΔTROI2=3.7°C. Finally, in the FH group (ΔTbody=36–35.7°C), a minimal temperature change was observed in the nonischemic region (ΔTROI1=1.1°C), in contrast to the larger change in the ischemic region (ΔTROI2=3.7°C).

Protective Effects of SH and FH Against Ischemic Damage
According to our inclusion criteria, the baseline lesion volume in each animal, determined using apparent diffusion coefficient maps, was between 25% and 45% of the ipsilateral hemisphere: 40.5±1.2% in the SC-4h group, 34.0±1.2% in the SH-4h group, 35.8±1.6% in the FC-24h group, and 34.7±1.8% in the FH-24h group (Figure 2A and 2B; Figure VIII in the online-only Data Supplement). Statistical analysis
of the infarct volumes in both SH- and FH-treated groups compared with their respective control groups showed a significant reduction at the different time points: at 24 hours, \( P<0.001 \); at 7 days, \( P<0.001 \), and at 30 days, \( P<0.01 \); this suggests that hypothermia-mediated protection persisted ≤30 days after ischemic injury. Expressing infarct volume as a percentage of basal lesion volume produced the same results (Figure IX in the online-only Data Supplement).

To determine whether 24 hours of FH were needed to achieve protective effects similar to those induced by 4 hours of SH, an independent group of ischemic animals were treated with 4 hours of FH. Infarct volume analysis showed that a 4 hours of FH treatment did not reduce infarct volume relative to the FC-4h group (\( P>0.05 \); Figure 2A and 2B; Figure X in the online-only Data Supplement).

**Effects of SH and FH on Edema Formation**

Edema formation was maximal 24 hours after ischemic injury (Figure 2C), and both SH (4 hours) and FH (24 hours) significantly reduced edema with respect to their control groups (\( P<0.05 \)). However, 4 hours of FH failed to reduce edema formation.

**Analysis of Functional Deficits**

Ischemic injury was associated with neurological deficits, which were evaluated using the cylinder test 48 hours, 6 days, and 29 days after ischemic injury (Figure XI in the online-only Data Supplement). Although not significantly different, cylinder test scores were asymmetrical, such that the SH-4h and FH-24h groups had better scores (ie, increased recovery) mainly at 29 days after injury relative to their respective control groups.

**Brain Histological Analysis After SH and FH**

A histological analysis performed 24 hours after ischemic injury revealed areas of TUNEL-positive (dead or dying) cells in the striatum and cortex in both control groups, whereas TUNEL-positive cells were mainly found in the striatum in both hypothermia-treated groups (Figure 3). The majority of TUNEL-positive cells colocalized with NeuN immunoreactivity, indicating neuronal death in the ischemic region. This distribution was consistent with the hyperintense areas observed in T2 maps and T2-weighted MR images recorded 24 hours after injury.

Seven days after ischemic injury, NeuN immunoreactivity had disappeared from the injured area (Figure 4), where dead cells had previously been observed (Figure 3). The distribution of Iba1 immunoreactivity corresponded to these NeuN-negative areas.

At 30 days after injury, the lesion cores in the control groups were negative for GFAP and NeuN immunoreactivity; however, the lesion cores in both hypothermic groups included GFAP-positive cells (Figure 5).

Lesion scars were also studied 30 days after injury (Figure 6). Markers of the 2 main components of the lesion scar were studied: GFAP as a marker of the glial component, and vimentin, an intermediate filament protein expressed in fibroblast-like cells, as a marker of the fibrotic component. Control animals showed a clear distinction between the vimentin-positive lesion core and the uninjured areas, where only GFAP immunoreactivity was observed. The peri-infarct region was characterized by the colocalization of these markers, indicating reactive astrogliosis. In contrast, the lesion cores in hypothermia-treated animals did not show this stratification. The area identified as the lesion core was positive for both markers, similar to the peri-infarct areas of untreated animals. Thus, the primarily fibrotic phenotype observed in the lesion cores of control animals was not observed in hypothermia-treated animals.

**Discussion**

SH is considered the most promising neuroprotective therapy for cerebral ischemia. However, clinical success in patients
with acute ischemic stroke has not yet been attained, most likely because of the considerable side effects associated with systemic cooling. Thermoregulation is a highly efficient physiological mechanism that remains intact even during severe pathophysiological processes, such as ischemic stroke. In consequence, therapeutic hypothermia is useful in patients who are anesthetized or in a deep coma, but it is complicated in most patients with ischemic stroke, mainly by the side effects of vasoconstriction and shivering, which may interfere with the beneficial effects of the treatment. In fact, induction of therapeutic hypothermia requires the use of additional pharmacological tools to inhibit thermoregulatory mechanisms. These clinical limitations could be observed in our study, since animals subjected to SH had to be anesthetized to induce SH treatment. Previous studies have also probed the effect of SH in awake animals using fans and fine water misters for cooling, and an overhead infrared lamp for warming, with beneficial results in ischemic animal models. However, like humans, adult rats have an efficient thermoregulation mechanism. In our hands, inducing SH in awake animals is technically demanding, and the environmental conditions are extremely stressful for the animals. Therefore, we decided to test SH under anesthetized conditions that allowed us to accurately control body temperature, and that reduced the hypothermic stress on the animals. In fact, this setup is more clinically relevant, and thus more easily translated, than awake-animal protocols.

Novel techniques mainly based on surface and endovascular cooling procedures have been developed and tested in

Figure 2. Magnetic resonance imaging (MRI) assessments of ischemic injury evolution. Apparent diffusion coefficient (ADC) maps were recorded before treatment to ensure that all animals included in the study were subjected to similar levels ischemic damage. Lesion volume evolution was assessed using T2-weighted images recorded 24 hours, 7 days, and 30 days after ischemia induction. A, MRI assessment of animals subjected to 4 hours of systemic hypothermia (SH) or 24 and 4 hours of focal hypothermia (FH). B, Quantitative analysis of lesion volumes of animals subjected to 4 hours of SH or 24 and 4 hours of FH. C, Edema evolution in animals subjected to 4 hours of SH or 24 and 4 hours of FH. Data are shown as mean±SEM. *P<0.05, **P<0.01, ***P<0.001; using 2-way ANOVA followed by the post hoc Bonferroni test (n=12 for baseline and 24-h assessments, n=9 for 7 d assessment, and n=6 for 30 d assessment in the systemic control (SC)-4h, SH-4h, focal control (FC)-24h, and FH-24h groups; n=6 in the FC-4h and FH-4h groups).
Figure 3. Histological marker expression 24 hours after ischemia. Immunofluorescent labeling of neurons (NeuN, red), dead cells (TUNEL, green), and nuclei (Hoechst, blue) 24 hours after ischemia in the systemic control (SC)-4 hours, systemic hypothermia (SH)-4 hours, focal control (FC)-24 hours, and focal hypothermia (FH)-24 hours groups. A, Whole-brain reconstructions from multiple (Continued)
patients with stroke to optimize SH protocols for clinical use. Several trials are ongoing, although no clear evidence has been obtained thus far. However, current research efforts aim to develop safer and more effective SH therapies, the alternative use of FH to rapidly induce hypothermia in the ischemic region had been poorly studied. In this study, we have compared SH and FH protocols featuring commonly used target temperature, therapeutic window, and duration parameters. We demonstrate that, when initiated 1 hour after arterial reperfusion, FH in awake animals induces similar protective effects to those of SH against ischemic damage. Efficacy is dependent on treatment duration; FH needs to be maintained for at least 24 hours to achieve protective effects similar to those of 4 hours of SH. Edema, which frequently occurs during the acute phase of stroke, hampers the effects of hypothermia and is closely related to patient outcome; however, it too was reduced by both methods.

Other beneficial effects of therapeutic hypothermia include reduction of neuronal death and attenuation of the inflammatory response. Thus, we analyzed neuronal loss and microglia and macrophage activation during the follow-up period in control and hypothermia-treated animals. Histological assessment of the brain samples confirmed similar protective efficacies for SH and FH. In addition, analysis of the residual ischemic lesion in the control groups at 30 days after the intervention showed that the strong fibrotic phenotype observed in the control animals, indicated by high vimentin immunoreactivity, was attenuated in both hypothermia-treated groups. Lesion scar development is a critical factor that influences the regenerative potential in the central nervous system. Scar formation seems to play dual roles in isolating the injured area and limiting inflammation; however, this isolation may also impede the regenerative process. In this regard, the hypothermia-induced decrease in fibrotic lesion scar formation may contribute to regeneration of the damaged tissue, as has been described by other similar studies.

In a separate comparison of hypothermia-induced temperature changes measured using MRT and invasive temperature probes, we showed that SH reduced both body and brain temperatures to 32°C, whereas FH induced a similar temperature reduction mainly in the ischemic region. As mentioned, FH required 6-fold longer treatment than SH to achieve a similar effect. Comparing local and SH is difficult because it is hard to perfectly match treatment protocols (same temperature profile in the brain, same cooling and rewarming rate, etc.). However, this disparity suggests that the protective effects of SH may include noncerebral physiological mechanisms, whereas the...
Figure 5. Histological marker expression 30 days after ischemia. Distribution of neurons (NeuN, red), astrocytes (GFAP, green), and nuclei (Hoechst, blue) 30 days after ischemia in the systemic control (SC)-4 hours, systemic hypothermia (SH)-4 hours, focal control (FC)-24 hours, and focal hypothermia (FH)-24 hours groups. A, Whole-brain reconstructions from multiple photomicrographs showing the general distribution of each marker, merged images, and correspondence with T2-weighted magnetic resonance images and maps. B, High magnification (×400) photomicrographs showing representative immunoreactivity patterns in the cortex and striatum. C, Neuron density in different brain regions. Scale bars, 50 μm. Data are shown as mean±SEM. GFAP indicates glial fibrillary acidic protein. **P<0.01; using 2-way ANOVA followed by the post hoc Bonferroni test (n=3, per group).
Figure 6. Histological examination of lesion scars 30 days after ischemia. Distribution of astrocytes (GFAP, red), fibroblast-like cells (vimentin, green), and nuclei (Hoechst, blue) 30 days after ischemia in the systemic control (SC)-4 hours, systemic hypothermia (SH)-4 hours, focal control (FC)-24 hours, and focal hypothermia (FH)-24 hours groups. A, Whole-brain reconstructions from (Continued)
effects of FH are mediated only through local effects in the ischemic region. Consistent with this, previous evidence has indicated that SH affects both the brain and peripheral organs.25 This difference may explain why FH requires a longer treatment period to achieve a protective effect. It is also possible that the protective effect of 4 hours of SH could be partly mediated by the use of anesthesia during the hypothermic treatment period because anesthesia is well known to protect against cerebral ischemia.26 However, a previous study in awake animals subjected to cerebral ischemia showed that 48 hours of focal cooling was needed to mitigate the ischemic injury, whereas 12 hours of SH was enough.12 These data indicate that anesthesia does not mediate the effect of SH. However, rewarming was faster in the local cooling group, an effect that is harmful for the ischemic tissues, and potentially could explain why longer cooling was needed. In fact, 1 recent study suggests that edema may be worsened by rewarming from focal cooling.27 In our study, systemic rewarming was slow, and focal rewarming was found to be difficult to control, as a minimal increase in the bath temperature (adjusted to 4°C) induced an immediate increase in brain temperature. Thus, one of the limitations of this study was the inability to accurately control this parameter. However, as 4 hours of FH did not increase the size of the ischemic region, we feel faster focal rewarming cannot explain the differences observed between 4 hours of SH and 24 hours of FH in our ischemic model.

Overall, hypothermia seems to be a complex therapy affecting multiple molecular components of the ischemic cascade, and which may be active throughout the body or in the lesioned area alone. In recent years, the pathways mediating hypothermia-mediated neuroprotection have attracted increasing interest.28 Understanding these mechanisms may facilitate the design of pharmacological alternatives with similar protective efficacy and without side effects.29 A limitation of this study is that FH application to the surface of the head is not a translational approach because the thickness of the skull would necessitate the use of skin-damaging low temperatures and long treatment periods to achieve the target temperature within the brain. In fact, the beneficial effects of selective brain cooling using a cooling helmet are more evident in infants because of their smaller head size and reduced skull thickness. However, the recent introduction of new approaches to cerebral FH induction, including the use of a cooling neck collar and intracarotid infusion of cold saline, has shown great promise.6,8,14 It should be noted that, in line with our results, hypothermia seems to be an effective strategy mainly in models of transient ischemia, suggesting that the combination of hypothermia and reperfusion therapies may be the most promising strategy in humans.30 Although some data suggest that FH could be beneficial in permanent ischemia,12 further studies are necessary to validate its use as a monotherapy.

Finally, magnetic resonance spectroscopy has been tested as a noninvasive and reliable tool for the measurement of brain temperature in both animal and human studies.31 This strategy provides temperature distribution data across the brain, including the ischemic and normal tissue, allowing us to determine the effects of FH on the ischemic regions. This study represents the first use of MR as a thermometric tool to determine and compare the effects of SH and FH on cerebral temperature. Using brain temperature mapping, we have demonstrated that SH affects the temperature throughout brain (healthy and ischemic regions), whereas FH reduces the temperature mainly in the ischemic region. These findings were verified by gradient-echo–based phase mapping, a novel but validated MR technique.

Conclusions

Focal brain hypothermia is an effective therapeutic alternative to SH that circumvents the side effects associated with systemic cooling, enabling better patient management, and allowing the design of personalized treatments according to the characteristics of the ischemic lesion.

Disclosures

None.

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SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Rat model of cerebral ischemia

All surgical procedures were performed under sevoflurane anaesthesia (6% induction and 4% maintenance in a mixture of 70% NO₂ and 30% O₂). Rectal temperature was maintained at 37 ± 0.5°C in all animals during surgery using a thermostat-controlled electric pad (Neos Biotec, Pamplona, Spain). The animals’ heads were placed on a porexpan plate in order to avoid direct contact between the pad and head.

Transient focal ischemia (45 min) was induced by intraluminal occlusion of the MCA, following the method described previously.¹ Occlusion was performed using commercially available sutures with silicone-rubber-coated heads (350 µm in diameter and 1.5 mm long; Doccol, Sharon, MA, USA). Cerebral blood flow was monitored with a Periflux 5000 laser Doppler perfusion monitor (Perimed AB, Järfälla, Sweden) by placing the Doppler probe (model 411; Perimed AB) under the temporal muscle at the parietal bone surface, near the sagittal crest. Once artery occlusion had been achieved, as indicated by Doppler signal reduction, each animal was carefully moved from the surgical bench to the MR system for baseline ischemic lesion assessment using ADC maps. MRA was also performed to ensure that the artery remained occluded throughout the MR procedure and to detect arterial malformations. Animals were then returned to the surgical bench and the Doppler probe was repositioned. Reperfusion was performed 45 min after occlusion onset. Tail blood samples were obtained before surgery, during arterial reperfusion, and 4 h, 24 h, 7 d, and 30 d after ischemia onset for assessment of glucose levels. Animal weights were determined before surgery and 24 h, 72 h, 7 d, and 30 d after ischemia onset.
Experimental groups

Following reperfusion, animals were randomly assigned to four experimental groups, using computer-generated random numbers, as follows:

1) Systemic control group (SC-4h; n = 12). One hour after reperfusion, anaesthetised animals were maintained under normothermic conditions (target rectal temperature: 37ºC) for 4 h and subsequently allowed to awaken.

2) Systemic hypothermia group (SH-4h; n = 12). One hour after reperfusion, anaesthetised animals were subjected to SH (target rectal temperature: 32ºC) for 4 h and subsequently allowed to awaken.

3) Focal control group, 24 h treatment duration (FC-24h; n = 12). Immediately after reperfusion, anaesthetised animals were placed in a stereotaxic frame (TSE Systems, Inc., Bad Homburg, Germany) for implantation of a cooling device over the skull in the ischemic brain region. One hour after reperfusion, animals were allowed to awaken from anaesthesia and move freely. The cooling device was connected to a thermal bath without water perfusion. These conditions were maintained for 24 h, after which the cooling devices were removed and MRI assessment was performed.

4) Focal hypothermia group, 24 h treatment duration (FH-24h; n = 12). These animals received the same treatment as the FC-24 group; however, the cooling device was connected to a thermal bath maintained at 4ºC during the 24 h treatment period.

To analyse the effect of 4 hours of focal hypothermia, in a separate study and following reperfusion, animals were randomly assigned to 2 experimental groups, using computer-generated random numbers, as follows:

1) Focal control group, 4 h treatment duration (FC-4h; n = 6). These animals received the same treatment as the FC-24h group; however, the cooling devices were removed after 4 h.
2) Focal hypothermia group, 4 h treatment duration (n = 6) (FH-4h; n = 6). These animals received the same treatment as the FH-24h group; however, the cooling devices were removed after 4 h.

In this separate study three animals with FC-24h and three with FH-24h were (randomized) also included to validate the effect of FH-4h vs FH-24h.

In all groups, ischemic lesion size was determined after 24 h, 7 d, and 30 d using MRI. In the SC-4h, SH-4h, FC-24h, and FH-24h groups, functional outcomes were examined using the cylinder test before surgery and 48 h 6 d, and 29 d after surgery. Histological analyses were performed 24 h, 7 d, and 30 d after surgery.

**SH procedure**

SH was induced using a rectal-thermostat-controlled electric pad (Neos Biotec). Initial rapid cooling was induced by alcohol application to the body surface. Rectal temperature was recorded throughout the treatment period and maintained using a secondary rectal probe (Perimed). Lower levels of anaesthesia levels during treatment were required in the SH-4h group (2% sevoflurane) than in the SC-4h group owing to the reduced breathing rate observed in these animals. After 4 h of hypothermia, animals were returned to their home cages to spontaneously rewarm.

The rewarming rate measured in a separate group of ischemic animals (n = 3) using implantable temperature sensors (Maxim Integrated, San Jose, CA, USA) inserted into the peritoneal cavity 1 week before ischemic surgery. One day after ischemia and the following 4 h SH treatment, the sensors were removed to obtain the temperature measurements, and ischemic lesion was confirmed by MRI.

To measure reductions in brain temperature induced by SH, temperatures were measured in both hemispheres in an independent group of three animals using invasive temperature probes. Two burr holes were made in the skull over each hemisphere.
(coordinates: +0.6 mm anterior and ±3 mm lateral to Bregma). Two thermocouples connected to a BAT-10 thermometer (Physitemp, Clifton, NJ, USA) were slowly inserted into the striatum to a depth of 5 mm. Analogue signal was converted to digital signal by an A/D converter (LabJack, Lakewood, CO, USA) and recorded with LJ LogUD software (LabJack).

**FH procedure**

FH was induced using a cooling device that has been described previously, with minor modifications. Briefly, a 23 G needle was warmed and bent to create a metallic spiral tube 5 mm in diameter and 1 mm thick (Supplementary Figure I). The tube was connected to a thermal bath using tubing of different diameters to gradually reduce the pressure from the thermal bath to the cooling coil. To facilitate movement in awake animals, a double connector was placed in the middle of the tubing.

Immediately after arterial reperfusion (45 min), the anaesthetised animals were placed in a stereotaxic frame for implantation of the cooling device. The cooling spiral was inserted between the skull and the right temporalis muscle over the ischemic region and secured with two surgical screws (Fine Science Tools, Inc., Heidelberg, Germany) and dental cement (Kerr, Orange, CA, USA). One hour after reperfusion, animals were allowed to awaken from anaesthesia while cold water began to flow through the cooling device. The temperature of the water bath was 4°C; the temperature of the water in the device was 13.5°C.

To measure reductions in brain temperature induced by the cooling spiral, temperatures were measured in both hemispheres in an independent group of three animals using invasive temperature probes located in both hemispheres, following the protocol described above for SH. The cooling device was inserted between the skull and the right temporalis muscle, as described above. After implantation, temperatures were recorded
in both hemispheres at three time points: 1) before cold-water perfusion, in order to
determine the thermal effects of the implanted device due to the passive heat-dissipating
capacity of the metal coil; 2) during cold-water perfusion; and 3) after cold-water
perfusion was stopped, allowing measurement of the rewarming rate.

**Brain MRT**

All MR studies were conducted using a 9.4 T MR system (Bruker BioSpin, Billerica,
MA, USA) with 440 mT/m gradients and a combination of a linear birdcage resonator
(7 cm in diameter) for signal transmission and a 2 × 2 surface coil array for signal
detection. A quadrature volume coil (7 cm in diameter) was also used in some cases.
Our MRT approach combined single-voxel MRS with chemical shift imaging (CSI) and
gradient-echo-based phase mapping, all of which rely on temperature-dependent shifts
in water resonance frequency. Phase mapping data were acquired using a multi-gradient
echo (MGE) method implemented with a flip angle (FA) = 52º, repetition time (TR) =
1000 ms and 12 echoes with echo spacing = 2.85 ms. One slice (2 mm thick) with a
field of view (FOV) of 32 × 32 mm² was covered by 128 × 128 points, resulting in an
in-plane resolution of 0.25 mm/pixel. The total acquisition time was 2 min. A point-
resolved spectroscopy (PRESS) sequence was used to acquire 1H-MRS spectra of the
ROIs (TR = 2500 ms, echo time [TE] = 60 ms, 2048 points, spectral width [SW] = 4
KHz, number of averages [NA] = 64–128, and acquisition time = 2:50–5:30 min).
Voxels with sizes of 3 × 3 × 3 or 3 × 8 × 3 mm³ were positioned in ipsilateral, central,
and contralateral areas of the putamen and parietal cortex. Chemical shift images were
acquired using a spin-echo CSI sequence with the following parameters: TR = 1000 ms,
TE = 60 ms, 2048 points, SW = 4 KHz, and NA = 2. One slice (2 mm thick) with a
FOV = 32 × 32 mm² was covered by 24 × 24 points (spatial resolution = 1.33
mm/pixel). The acquisition time was 14 min.
Data were processed using custom-written MATLAB scripts (Math Works, Inc., Natick, MA, USA) and MestReNova software (Mestrelab Research, Santiago de Compostela, Spain). Phase maps were calculated from gradient-echo data using all echoes and unwrapped using a temporal unwrapping algorithm.

Brain temperature change ($\Delta T$) maps were produced by subtracting phase images at two temperatures and converting phase difference to frequency difference maps and then to $\Delta T$ maps using known relationships between water resonance frequency and temperature. Using CSI and MRS data, absolute brain temperature was calculated based on the frequency difference between water and N-acetyl aspartate or creatine and previously described quantitative relationships. Voxel sizes varied between experiments.

MR spectra obtained from methanol phantoms were used as references to correct for frequency drifts arising from hardware instabilities. These spectra are sensitive to field disturbances and therefore can be used as indicators of magnetic field drift. A methanol phantom containing a temperature probe was placed in the scanner. The phantom consisted of a 1.5 mL microcentrifuge tube containing methanol (99.9%, Sigma-Aldrich, St. Louis, MO, USA) placed inside a 50 mL conical tube filled with phosphate-buffered saline (PBS; Sigma-Aldrich). The field drift was calculated in parts per million (ppm) from the temporal evolution of the methanol spectra measured with PRESS sequences in a localised voxel of $3 \times 3 \times 3$ mm$^3$ at constant room temperature. Field drift was calculated by subtracting the peak positions of the methanol spectra: $T_1 = 20.2 \pm 0.2^\circ C$ for PRESS 1 and $T_2 = 19.9 \pm 0.2^\circ C$ for PRESS 2, performed 120 min apart. The probe temperature measurements showed no heating during scans, with an average temperature $T_{\text{probe}} = 20.1 \pm 0.1 ^\circ C$. MR images and frequency spectra obtained from the methanol phantom are shown in Supplementary Figure X. The field drift was 0.012 ppm.
over 120 min, which could be erroneously interpreted as a temperature change of approximately 0.5°C/h in phase maps without drift correction. Because the brain temperature change maps in our experiments were obtained in less than 30 min, this field drift was taken into account in the analysis. However, the absolute temperature measurements were clearly unaffected by this drift. In order to validate this in vitro approach, body and cerebral temperatures were determined simultaneously in vivo (n=3) under normothermic and hypothermic conditions (see Supplementary Figure XI).

After the MRT protocol was established, brain temperatures were determined in groups of ischemic animals exposed to normothermic, SH, and FH conditions (n = 3 per group). MRT measurements were performed on the anaesthetised animals after arterial reperfusion; cerebral temperature maps were recorded under normothermic conditions ($T_{\text{body}} = 37^\circ\text{C}$) in the normothermic and SH groups, followed by SH conditions ($T_{\text{body}} = 32^\circ\text{C}$) in the SH group. In FH-treated animals, a plastic MR-compatible spiral tube was used to induce the head cooling. After reperfusion, the tube was implanted over the ischemic region and connected to a thermal bath, following a similar protocol as indicated for the FH group. MRT data were acquired under normothermic conditions ($T_{\text{body}} = 37^\circ\text{C}$) and then under FH conditions while maintaining $T_{\text{body}} = 37^\circ\text{C}$.

**MRI assessment and data analysis**

An initial MRI study was performed during the occlusion period to measure baseline lesion volumes. Time-of-flight MRA was first performed to confirm that the MCA remained occluded after moving the animal from the surgery bench. MRA images were acquired using a three-dimensional fast low-angle shot (3D-FLASH) sequence with TE = 2.5 ms, TR = 21 ms, FA = 20°, and NA = 1. One slice was recorded (12 mm thick) with FOV = 19.2 × 19.2 mm² and a resolution of 0.120 mm/pixel (160 × 160 × 100-point matrix). To measure lesion volumes, ADC maps were obtained min after
occlusion but before removing the occluding filament. The area of ischemic damage was determined by counting pixels with ADC values below a threshold in the ipsilateral brain hemisphere. The values of ADC in the healthy rat brain normally do not fall below $0.55 \times 10^{-3} \, \text{mm}^2/\text{s}$; therefore, this threshold provides a convenient means of segmenting abnormal tissue.\textsuperscript{6} Diffusion-weighted images (DWI) used to calculate ADC maps were acquired using diffusion-tensor imaging with a spin-echo echo-planar imaging sequence (DTI-EPI) with the following parameters: FOV = $24 \times 16 \, \text{mm}^2$, matrix = $96 \times 64$ points (in-plane resolution = $0.25 \, \text{mm/pixel}$), 14 consecutive slices (1 mm thick), TE = 27 ms, TR = 4 s, NA = 4, and diffusion b values of 300, 600, 900, 1200, 1600 and 2000 s/mm$^2$ applied in the z direction.

To analyse lesion volume evolution, RARE T2-weighted images were acquired 24 h, 7 d, and 30 d after ischemia induction using a multi-slice multi-spin-echo (MSME) sequence with the following acquisition parameters: TE = 9 ms, TR = 3 s, 16 echos, NA = 2, 14 consecutive slices (1 mm thick), FOV = $19.2 \times 19.2 \, \text{mm}^2$ and an in-plane resolution of $0.1 \, \text{mm/pixel}$ (192 × 192-point matrix). These data were also used to calculate T2 relaxation maps. All images were processed using ImageJ (Rasband WS, National Institutes of Health, Bethesda, MD, USA) on an independent computer workstation. Infarct volumes were determined from quantitative ADC maps and T2-weighted images averaged between the 4th to 8th echoes by a researcher blinded to the treatment groups. Oedema formation was measured by quantifying the midline deviation (MD), calculated as the ratio between ipsilateral and contralateral hemispheric volumes; lesion volumes were then calculated in $\text{mm}^3/\text{MD}$. Lesion volume as a percentage of the ipsilateral hemispheric volume was calculated as $(\text{lesion volume [mm}^3/\text{MD]}/\text{ipsilateral hemispheric volume [mm}^3]) \times 100$.

\textbf{Functional assessment}
Functional outcome was evaluated using the cylinder test.\(^7\) Exploratory behaviour in each animal was recorded for 10 min in a 20 × 30 cm cylinder in a darkened room with an infrared video camera (Sony, Tokyo, Japan). All functional assessments were conducted during the animals’ active periods (the first 6 h of the dark period). Scores were obtained from a total number of 10 full rears to control for differences in rearing between animals. Once scores had been acquired, forelimb asymmetry was calculated using the formula: \(100 \times (\text{ipsilateral forelimb use} + \frac{1}{2} \text{bilateral forelimb use}) / \text{total forelimb use observations}\).

**Perfusion and tissue processing**

Three animals in each of the six groups described above were sacrificed 24 h, 7 d, and 30 d after ischemic injury. Animals were deeply anaesthetised with sevoflurane (6% in a mixture of 70% NO\(_2\) and 30% O\(_2\)) and transcardially perfused with 100 mL of 0.1 M PBS (pH 7.4) followed by 150 mL of 4% formaldehyde. Brains were carefully removed from the skull and sectioned at 2 mm thick using a matrix. Slices were postfixed by immersion in 4% formaldehyde overnight, washed in PBS, and cryoprotected in a solution of 30% sucrose in PBS with 0.05% sodium azide. Slices were embedded in OCT compound (Sakura Finetek, Torrance, CA, USA), flash-frozen with liquid nitrogen, and cut into 8 µm-thick slices using a cryostat (Sakura Finetek).

**TUNEL and immunofluorescence**

Brain slices were dried at 38ºC for 3 h and the OCT was then removed by washing the slices twice in PBS. Antigen retrieval was performed by immersion of slices in a thermal bath of citrate buffer (pH 6.0) for 20 min at 99ºC.

To detect dead cells, a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay was performed. After antigen retrieval, slices were incubated for 30 min in a blocking solution containing 3% bovine serum albumin in 0.1 M Tris-buffered
saline (TBS; pH 7.5). After blocking, slices were washed in PBS and incubated for 1 h at 38°C with Fluorescein In Situ Cell Death Detection Kit mix (Roche, Basel, Switzerland). Slices were then washed and immunofluorescently labelled with anti-NeuN primary antibody, as described below.

Immunofluorescence was performed using antibodies against NeuN (ab104225; Abcam, Cambridge, MA, USA), GFAP (G3893; Sigma-Aldrich), vimentin (bs-0756R; Bioss, Woburn, MA, USA), and Iba1 (MABN92; EMD Millipore, Darmstadt, Germany). After antigen retrieval, slices were incubated overnight at room temperature with primary antibodies diluted in PBS with 0.2% Triton X-100 and 15% normal serum derived from the same species as the secondary antibody. Primary antibody concentrations were 1:1000 for NeuN and GFAP and 1:500 for vimentin and Iba1. Slices were then washed with PBS and incubated for 1 h at room temperature in the darkness with secondary antibodies: biotinylated horse anti-rabbit (BA-1100; Vector Laboratories, Burlingame, CA, USA), biotinylated horse anti-mouse (BA-2001; Vector Laboratories), DyLight 488 horse anti-mouse (IGGDY488H-OIMG-CUSTOM; Immunostep, Salamanca, Spain), or DyLight 488 goat anti-rabbit (DI-1488; Vector Laboratories). Secondary antibodies were diluted at 1:200 in PBS with 0.2% Triton X-100. Slices were then washed and incubated for 30 min in the darkness with DyLight 594 streptavidin (SA-5594, Vector Laboratories) at a concentration of 1:500 in PBS with 0.2% Triton X-100. Finally, slices were washed and incubated with Hoechst stain (Thermo Fisher Scientific, Waltham, MA, USA) at a concentration of 1:6000 in PBS for 10 min in the darkness. Slices were mounted with Aqua-Poly/Mount (Polysciences, Warrington, PA, USA) and photographed using a Leica DMI6000 B microscope with Leica Application Suite Advance Fluorescence software, version 1.0.0 (LAS AF; Leica Microsystems, Wetzlar, Germany).
TUNEL and NeuN quantification

A quantitative analysis of TUNEL and NeuN immunoreactivity was performed using three animals from each group. Photomicrographs were obtained from the striatum and the lateral and dorsal cortex in both hemispheres. Replicate photomicrographs were obtained from two consecutive slices containing the central part of the injured region (between 0.7 mm anterior and 0.3 mm posterior to Bregma). Positive nuclei were counted manually.
SUPPLEMENTARY FIGURES

**Supplementary Figure I.** Cooling device made of a metallic spiral tube used to induce FH.

**Supplementary Figure II.** Protocol diagram summarizing the number of animals included, with exclusion per group, for final analysis. Exclusion criteria were defined as described in Materials and Methods.
**Supplementary Figure III.** Representative ADC maps of animals included or excluded in the study. **A**– ADC map of an animal excluded owing to a baseline lesion volume smaller than 25% of the ipsilateral hemisphere. **B**– ADC image of an included animal with a baseline lesion volume within the accepted range (25–45% of the ipsilateral hemisphere). **C**– Representative ADC image of an animal excluded owing to a baseline lesion volume greater than 45% of the ipsilateral hemisphere. **D**– Reconstructed MRA image showing an arterial malformation found in two animals. Rows show the duplicated MCA in the ipsilateral hemisphere.

**Supplementary Figure IV.** Evolution of body temperature during the SH protocol, as measured using implantable temperature sensors (n = 3). Rewarming rate: 0.03°C/min.
Supplementary Figure V. A– Rectal and brain temperatures in normothermia and systemic hypothermia conditions (SH protocol) (n=3). B– Rectal and brain temperatures in normothermia and focal hypothermia conditions (FH protocol) (n=3). After the device was implanted, temperature was measured before (normothermia) and during cold-water perfusion (hypothermia). Data are shown as mean ± SD.*P<0.05 compared to the contralateral region using Student t-test.
**Supplementary Figure VI.** MRI assessment of lesion volume evolution. Antero-posterior brain sections corresponding to lesions at baseline (ADC images) and 24 h, 7 d, and 30 d after injury (T2-weighted images [T2-WI]).
Supplementary Figure VII. Relative infarct volumes respect to the basal lesion (considered as 100%) at 24 h, 7 d, and 30 d after ischemia induction of animals subjected to 4 h of SH 24 h of FH. Data are shown as mean ± SEM of infarct volumes respect to the basal lesion considered as 100%. *P<0.05, **P<0.01, ***P<0.001; using two-way ANOVA followed by the post-hoc Bonferroni test (n=12 for baseline and 24h assessments, n=9 for 7 d assessment, and n=6 for 30 d assessment in the SC-4h).

Supplementary Figure VIII. MRI assessments of ischemic injury evolution of animals subjected to 4 and 24 h of FH. Ischemic injury was determined before treatment (basal volume) and at 24 h, 7 d, and 30 d after ischemia induction. Data are shown as mean ± SEM.*P<0.05, **P<0.01, ***P<0.001; using two-way ANOVA followed by the post-hoc Bonferroni test (n=6 for 4 h of FH group and n=3 for 24 h of FH group).
Supplementary Figure IX. Assessment of sensorimotor function using the cylinder test. Functional tests were performed before ischemic injury (baseline) and 48h, 6 d, and 29 d after injury in A – systemic control (SC-4h) and systemic hypothermic (SH-24) groups; and B – focal control (FC-24h) and focal hypothermic (FH-24h) groups. Forelimb asymmetry (score asymmetry) was calculated using the formula: (contralateral forelimb use / total forelimb use observations) X 100 . Data are shown as mean±SEM and analysed using two-way ANOVA followed by the post-hoc Bonferroni test (n=9 for 48 h and 6 d assessments and n=6 for 30 d assessment).
**Supplementary Figure X.** Measurement of magnetic field drift. A RARE-T2 image of a methanol phantom, with a square ROI indicated (left); and two methanol frequency spectra (right) corresponding to PRESS 1 and 2 sequences, obtained 120 min apart, that indicate the presence of magnetic field drift.

![Methanol phantom and frequency spectra](image)

**Supplementary Figure XI.** Analysis of body and brain temperatures used to validate the MRT protocol. Body and brain temperatures were determined simultaneously in healthy animals (n = 3) under normothermic and hypothermic conductions. Body temperature was measured using a rectal probe; brain temperature was measured by 1H-MRS. Data are shown as mean ± SEM.

![Temperature vs. time graph](image)
SUPPLEMENTARY REFERENCES


