MEDICAL SCHOOL UNIVERSITY OF PÉCS

Translational research of hypothermia: therapeutic use in humans and pharmacological induction in experimental models

DOCTORAL (PHD) THESIS

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List of abbreviations

cCT cerebral computed tomography

CI confidence interval

CNS central nervous system

COIN cooling index

DHCA deep hypothermic circulatory arrest

GCS Glasgow coma score

i.c.v. intracerebroventricular(ly)

i.p. intraperitoneal(ly)

i.v. intravenous(ly)

KO knockout

OR odds ratio

RCT randomized clinical trail

T_a ambient temperature

T_{ab} abdominal temperature

T_b body temperature

T_{sk} tail skin temperature

TBI: traumatic brain injury

TRP transient receptor potential

TRPA1 transient receptor potential ankyrin-1

TRPV1 transient receptor potential vanilloid-1

VO_2 oxygen consumption

Introduction

1. Hypothermia

Thermoregulation is a dynamic, homeostatic vital function of the autonomic nervous system in response to cold and heat stress, to maintain a stable, physiological temperature. Thermoregulatory physiology sustains health by keeping core body temperature (T_b) within the range of a degree or two of 37°C, which enables normal cellular functions. Heat production and dissipation are dependent on a coordinated set of autonomic responses. Failure of neural thermoregulatory mechanisms or exposure to extreme or sustained temperatures that overwhelm the body's thermoregulatory capacity can also result in potentially life-threatening deviations from normothermia. Hypothermia, defined as a core temperature of <35.0°C, may present with shivering, respiratory depression, cardiac dysrhythmias, impaired mental function, mydriasis, hypotension, and muscle dysfunction, which can progress to cardiac arrest or coma. Management includes warming measures, hydration, and cardiovascular support. Deaths from hypothermia are twice as frequent as deaths from hyperthermia.

Therapeutic use of hypothermia has returned to the frontline in the prevention and in mitigation of neurologic impairment in different pathological conditions over the past decade. The application of hypothermia is considered as a successful therapeutic measure not just in neuro- or cardiac surgeries, but also in various (*e.g.*, traumatic) states causing brain injury or damage.

In general, hypothermia is defined as a core body temperature of 35°C or less and it can be either induced (*i.e.*, therapeutic) or accidental. Guidelines distinguishing the depth or stages of hypothermia have differed between authors, and as a result there are various definitions within the published literature. The American Heart Association has arbitrarily adopted the definition of Polderman *et al*. (2009), which defines mild hypothermia as temperatures down to 34°C, while the hypothermia is moderate at 34-30°C and severe at <30°C (Polderman & Herold, 2009). In accidental cases, a single measurement of core temperature is often used to classify hypothermia as mild, moderate or severe. The treatment of patients with accidental hypothermia depends on the severity level, therefore, as our understanding of the pathophysiological mechanisms of hypothermia improves, it is important to have a general consensus on the stages so that treatment is appropriately targeted.

1.1 Accidental hypothermia

Accidental hypothermia is traditionally referred to as the unintentional lowering of deep T_b to below 35°C. The causes of accidental hypothermia are multifactorial and can be subdivided based on the circumstances of the cold exposure.

Acute hypothermia occurs when there is a sudden and severe exposure to a cold stress which is so great that the body's cold defense mechanisms are overwhelmed and the body cools before energy reserves are exhausted. In these circumstances the victim will rewarm once removed from the cold stress. This is most commonly seen following

immersion in cold water, or being hypothermic whilst under the influence of alcohol at low ambient temperatures (T_as) (Lloyd, 1979).

In subacute (exhaustion) hypothermia, the cold stress is less severe, and cooling only occurs when energy reserves are exhausted. Spontaneous rewarming is less certain in these circumstances and therefore every route of heat loss must be prevented. This type of hypothermia is most commonly found in mountain climbers exposed to the elements (moderate cold and wind/rain), hikers, in endurance sport or in people who have been immersed in temperate water.

In chronic hypothermia, a person has been exposed to a moderate cold stress for a prolonged period of time (days). This is typically found in the elderly, in whom core temperature decreases over time, when exposed to mild cold stress.

It should be mentioned that submersion hypothermia also exists, when the hypothermia occurs as a result of full body immersion in cold water. A number of reports have shown that in such cases survival of the patients is possible without oxygen for up to 60 minutes following submersion (Avellanas *et al*., 2012; Lloyd, 1996). In severe forms, accidental hypothermia is further complicated by risk of ventricular arrhythmias and cardiac arrest, which contribute to the high mortality rates among these patients.

1.2 Induced hypothermia

In contrast to accidental hypothermia, therapeutic hypothermia is a clinician-driven modality aimed at intentionally decreasing a patient's core T_b. The idea of using

hypothermia therapeutically is not a new concept and has been around for centuries, however many practiotioners abandoned the idea due to its potential adverse effects (Alzaga *et al*., 2006). The current clinical application of therapeutic hypothermia stems in part from experimental observations of Bigelow and associates in the 1950s. Their work demonstrated a potential beneficial effect of therapeutic hypothermia for the brain and heart during cardiac surgery in canine models (Bigelow *et al*., 1950). Based on later trials (Bernard *et al*., 2002; Holzer & Sterz, 2003), which were the largest to date, included several hundred patients, and found significant neurological protection in patients cooled at 32-34˚C, the American Heart Association and European Resuscitation Council recommended therapeutic hypothermia in such patients due to the favourable outcome.

The beneficial effect of hypothermia as a neuro-protectant is the result of a reduction in tissue oxygen consumption and metabolic demand. Therapeutic hypothermia is also used to varying degrees in other conditions such as aortic arch surgery. Cerebral protection has been the cornerstone of successful aortic arch surgery for almost 40 years and deep hypothermic circulatory arrest (DHCA), with temperatures as low as 15-20˚C, has been a main strategy to achieve sufficient cooling (Di Luozzo & Griepp, 2012).

Cooling the brain down to hypothermic temperatures is sufficient to reduce brain metabolic requirements to such an extent that blood flow can be completely interrupted. Most notably, DHCA offers surgeons a bloodless operating field and extended surgical time limit while meeting the body's metabolic demands. While other perfusion techniques have been developed, many doctors are in favor of hypothermia as the preferred technique due to the relative ease at which it can be carried out. It provides cerebral protection while

it also minimizes problems with perfusion techniques. Its use has been supported by follow up studies involving more than 400 patients that demonstrated positive clinical results, as well as low rates of side effects (*e.g.*, stroke and seizures) accompanied by a good cognitive function (Gega *et al.*, 2007). In recent years there has been growing interest into the beneficial effects of therapeutic hypothermia in different pathological conditions, such as ischaemic stroke and traumatic brain injury (TBI) (Basto & Lyden, 2018). In the former, the protective mechanisms of hypothermia affect the ischaemic cascade across several parallel pathways and it is therefore a condition where cooling may increase positive outcomes.

2. Therapeutic hypothermia in severe traumatic brain injury

TBI is recognized as a significant cause of mortality and morbidity predominantly in the young population (Yokobori & Yokota, 2016). Worldwide, TBI is estimated to affect 10 million people annually and nowadays it is one of the major causes of death and disability, posing a global health and financial burden for the society (Hyder *et al.*, 2007). Among the leading causes of TBI are motor vehicle accidents in both more and less developed countries, most frequently involving young males (Hyder *et al.*, 2007; Taylor *et al.*, 2015). Mild head injuries may recover fully without any specific treatment, whereas severe injuries are often rapidly fatal or leave survivors with disabilities (Finfer & Cohen, 2001). In accordance with the poor outcomes, severe TBI constitutes a major health and socio-economic problem worldwide (Maas *et al.*, 2008). Any potentially beneficial

interventions should be implemented in order to mitigate the burden of TBI on the patients and on healthcare, as well as to improve the outcome of the disease.

Therapeutic hypothermia has been well established as a treatment in this scenario for over 40 years (Fox *et al*., 2010; Sadaka & Veremakis, 2012). It is based on the principle that cooling prevents dangerously high intra-cranial pressure and reduces the damage to neural tissue from hypoxic and metabolic mechanisms. However, in recent years there has been growing evidence that therapeutic hypothermia is associated with unfavourable long-term outcomes when applied clinically (Crossley *et al*., 2014; Honeybul, 2016). This has been shown in both meta-analysis of previous studies (Shaefi *et al*., 2016) and in a large-scale clinical trial (Andrews *et al*., 2015b), which was prematurely terminated, because the clinical outcome did not improve in the hypothermia arm of the trial as compared to the normothermia arm.

The pathomechanisms underlying severe TBI are complex and often involve focal as well as diffuse changes (McGinn & Povlishock, 2016). Focal lesions include contusional damages of the brain parenchyma, most commonly in the frontal and temporal lobes, as well as disruptions in vasculature, resulting in intracerebral and extracerebral heamatomas (Maas *et al*., 2008; McGinn & Povlishock, 2016).

In a typical case like the one presented in Figure 1, focal lesions are diagnosed with routine imaging techniques, such as computed tomography (CT). This case study was collected at the St. Joseph's Hospital and Medical Center, Phoenix, Arizona, USA and presents a 35-year-old-man with severe TBI due to motor vehicle collision.

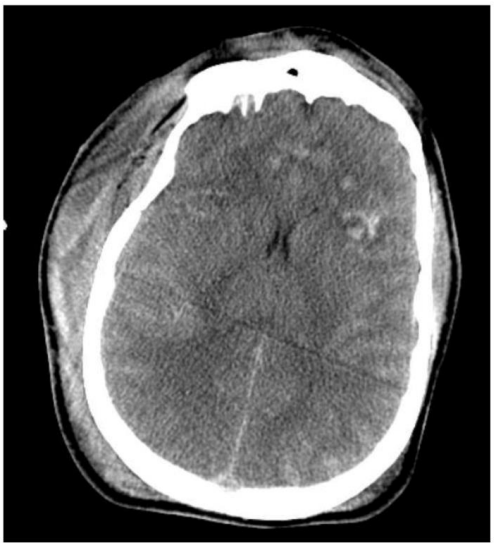

Figure 1 Axial CT image of a patient admitted with severe TBI, obtained at time of arrival to level 1 trauma center. TBI comprised diffuse bilateral subarachnoid hemorrhage, bilateral frontal contusions, small right subdural hematoma, and effacement of basal cisterns secondary to cerebral edema (Olah *et al*., 2018).

Many of the pathological mechanisms associated with TBI are temperature-sensitive (Stocchetti *et al*., 2017), suggesting that at a lower T_b the adverse processes can be decelerated and neuroprotective effects can be achieved. As a consequence, therapeutic hypothermia has been broadly investigated as a possible neuroprotective strategy to attenuate the harmful effects of severe TBI. In animal models of TBI, the beneficial effects of therapeutic hypothermia have been shown repeatedly (Bramlett & Dietrich, 2012; Feng *et al*., 2010; Sahuquillo & Vilalta, 2007; Zhao *et al*., 2017), however different clinical studies provided contradictory results. The first report of the use of hypothermia in TBI was in 1943 (Dietrich *et al*., 2009), while randomized controlled studies appeared only at the end of the 1990s (Fay, 1943). As attempts to ultimately answer the question of whether therapeutic hypothermia improves the outcome of TBI, several meta-analyses have also been performed over the last 25 years (Crompton *et al*., 2017; Harris *et al*., 2002;

Henderson *et al*., 2003; Li & Yang, 2014; McIntyre *et al*., 2003; Peterson *et al*., 2008; Zhu *et al*., 2016). Strikingly, the different analyses provided contradictory results. While, about 50% of them showed that therapeutic hypothermia might be efficient in the treatment of TBI and could decrease mortality (Crompton *et al*., 2017; Li & Yang, 2014; McIntyre *et al*., 2003; Peterson *et al*., 2008), the other 50% indicated that it did not reduce the rate of death (Harris *et al*., 2002; Henderson *et al*., 2003; Zhu *et al*., 2016). It could be assumed that the high between-study variability in the statistical and clinical designs of the trials (*e.g*., randomization), inclusion criteria of patients, and the applied cooling protocols varied substantially among the studies, which differences altogether could be responsible for the contradictory findings in the human studies. Indeed, high inter-study heterogeneity was reported in all of the performed meta-analyses so far (Crompton *et al*., 2017; Harris *et al*., 2002; Henderson *et al*., 2003; Li & Yang, 2014; McIntyre *et al*., 2003; Peterson *et al*., 2008; Zhu *et al*., 2016).

To examine and amalgamate the different findings and opinions, it was necessary to systematically review, categorize, and analyze the results, which is described in the first part of my dissertation. In addition to whether therapeutic hypothermia is beneficial or not, it was important to evaluate how it was implemented, since there are various physical devices and protocols for complete or partial body cooling in the clinical practice for this purpose.

3. Methods to induce hypothermia

In the clinical setting, the induction of hypothermia can be classified into: surface and internal cooling methods, whole-body, and localized cooling (Basto & Lyden, 2018). Whole-body cooling can be achieved by endovascular or surface methods, while localized cooling can be performed by endovascular infusions of cold saline or by focal cooling with an intracranially implanted device. Pharmacological strategies should be also mentioned among the hypothermia-inducing methods, but it must be noted that, as of today, the available thermopharmacological methods to induce a targeted and controlled drop in deep T_b are very limited, mostly due to the lack of a safe and usable substance for that reason in humans. Each method has advantages and problems that make them suitable for different situations (Basto & Lyden, 2018).

3.1 Surface cooling

Cooling of the surface of the skull was the earliest method of applying therapeutic hypothermia for stroke, but it was also used in the trials for cardiac arrest and neonatal hypoxic encephalopathy. Surface cooling is based on heat reduction from the head via skin convection. The induction and maintenance of hypothermia through surface cooling can be performed using fans, cold water and alcohol baths, ice packs, cooling blankets, and cooling pads. Drawbacks include the lack of an internal feedback loop (difficult accurate temperature management), a high incidence of overcooling, the need for high vigilance and experience to

maintain the goal temperature, and the difficulty in controlling the rate of rewarming (De Deyne, 2010).

3.2 Endovascular cooling

Endovascular cooling is the most widely studied method used in prior trials of therapeutic hypothermia for ischemic stroke. In these cases, an intravascular catheter with a heat exchange element is positioned into the inferior vena cava and connected to an external control unit. Then, some chilled solution is circulated into the heat exchange element in a closed circuit, allowing effective heat transfer from blood without infusion of the fluid into the patient. An advantage of this method is that it also allows simultaneous surface warming in order to avoid the shivering (*i.e.*, cold-defense response) of the patients. The cooling unit monitors temperature continuously and it can control the rate of cooling and rewarming according to the actual needs. COOL AID II and the ICTuS established the feasibility of this endovascular technique (De Georgia *et al.*, 2004; Lyden *et al.*, 2005).

3.3 Pharmacologic strategies

As an alternative approach to physical cooling, pharmacological agents may be also used to decrease deep T_b. To date, there are eight classes of hypothermia-inducing drugs, but none of them is routinely used in human patients: cannabinoids, opioid receptor activators, transient receptor potential vanilloid-1 (TRPV1) ligands,

neurotensins, thyroxine derivatives, dopamine receptor activators, hypothermia-inducing gases, and adenosine nucleotides. Antishivering effects were also described in case of opioids, TRPV1 ligands, neurotensin, and thyroxine families, while other thermoregulatory actions were reported for cannabinoid and opioid families (Feketa & Marrelli, 2015; Liu *et al*., 2016; Zhang *et al*., 2013). These drugs specifically target critical pathophysiologic steps such as mitochondrial dysfunction, excitotoxicity, endoplasmic reticulum stress, and neuroinflammation, all of which can be also manifested in case of TBI. The thermopharmacological agents may avoid the negative effects of physical cooling; however, flow redistribution, blood-brain barrier function, and hypoperfusion limit the penetration of most substances into the ischemic area. Moreover, the action mechanisms of these agents are not only restricted to induction of hypothermia, therefore side-effects such as sedation, immobility, depressed metabolism, and cardiovascular function may occur during their use. Nevertheless, the use of the thermopharmacological agents in combination with physical cooling can be an effective intervention to potentiate the molecular effects of therapeutic hypothermia. Before purely pharmacologically induced hypothermia could be routinely used in human patients with TBI, further studies are needed to discover the exact mechanisms of hypothermia induced by different agents in preclinical (animal) experiments, as well as in controlled clinical trials.

Despite the different available methods, the specific logistic details for optimal hypothermic therapy in humans have yet to be defined (including optimal time of onset, duration, and target temperature).

The main reason for the lack of thermopharmacologically induced hypothermia is that the target molecules which could produce direct and controlled hypothermia are largely unknown. In this aspect, it is of crucial interest, that Blackstone *et al*. (2005) reported that the inhalation of hydrogen sulfide (H_2S) induces concentration-dependent hypometabolism and hypothermia in mice without causing behavioral or functional damages to the animals. It was concluded that the thermoregulatory effect was triggered by the H_2S-induced inhibition of the terminal enzyme complex in the electron transport chain, thus the authors named the condition „suspended animation-like state" (Blackstone *et al*., 2005). Similar results were also obtained with sodium hydrosulfide (NaHS), a fast-releasing H_2S donor, in anesthetized rats (Aslami *et al*., 2010). Interestingly, the thermal effect of H_2S was debated later, because other authors could not reproduce the H_2S-induced hypothermia (Hemelrijk *et al*., 2018). In the end, the molecular site of action and the thermoeffector mechanisms underlying H_2S-induced hypothermia have remained unclarified.

Thermosensors, some of which belong to the group of temperature-activated TRP channels, play a key role in mediating alterations of deep T_b. Among the thermo-TRP channels, for the present work, the TRP ankyrin-1 (A1) ion channel has to be highlighted, which can be activated by many substances, also including H_2S, in addition to cold stimuli. The TRPA1 channel can be of crucial, and yet undiscovered, importance for the thermal

action of H_2S, since TRPA1 channel-mediated effects of sulfide donors and polysulfides were identified in different experimental models. An extensive list of studies that describe TRPA1-mediated effects of H_2S was recently collected by Pozsgai *et al* (2019), but thermoregulatory effects were not mentioned by the authors.

The diverse existence of H_2S-induced TRPA1 activation in different homeostatic processes may suggest that it could also be involved in thermoregulation. The investigation of the action mechanisms of H_2S-induced hypothermia constitued the basis of the the second part of my dissertation.

Aims

The ultimate goal of the present work was to evaluate the clinical importance of therapeutic hypothermia in human patients with TBI, and then to identify a potential pharmacological target for the induction of hypothermia in animal experiments. The main topics discussed in this thesis are as follows:

1. Therapeutic hypothermia was investigated repeatedly as a tool to improve the outcome of severe TBI, but previous clinical trials and meta-analyses found contradictory results. We aimed to determine the effectiveness of therapeutic whole-body hypothermia on the mortality of adult patients with severe TBI by using a novel approach of meta-analysis.
2. The cooling protocols of TBI patients widely differed among the studies, thus we aimed at developing a novel measure for the overall extent of the cooling. We calculated the integrated measure of therapeutic hypothermia from cooling parameters and introduced it as the cooling index (COIN), then we studied its relation to mortality in TBI.
3. H_2S has been shown in previous studies to cause hypothermia and hypometabolism in mice, however, the molecular target through which H_2S triggers its effects on deep T_b has remained unknown. We investigated the thermoeffector mechanisms of the hypothermic response to fast- (Na_2S) and slow-releasing (GYY4137) H_2S donors in C57BL/6 mice, and then tested whether their effects depend on the TRPA1 channel in *Trpa1* knockout (*Trpa1*$^{-/-}$) and wild-type (*Trpa1*$^{+/+}$) mice.
4. We also studied *Trpa1* expression in thermoregulation-related brain nuclei to explore the possible site of action for the hypothermic effect of H_2S.

Materials and Methods

1. Analysis of human data

1.1 Data extraction

In our meta-analysis (Olah *et al*., 2018), we searched the PubMed, EMBASE, and Cochrane Library databases from inception to February 2017. The identified human studies were evaluated regarding statistical, clinical, and methodological designs to ensure inter-study homogeneity. We extracted data on TBI severity, T_b, mortality, and cooling parameters. Our meta-analysis was conducted in accordance with the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis Protocols (Moher *et al*., 2015). The analysis was based on the Participants, Intervention, Comparison, Outcome (PICO) model: in severe TBI population, we aimed to assess the effect of therapeutic whole-body hypothermia compared to no cooling on the mortality ratio. The meta-analysis was registered with PROSPERO International Prospective Register of Systematic Reviews (CRD42017056535).

Only those studies were included in the meta-analyses, in which the effect of therapeutic hypothermia was compared with no cooling interventions on the mortality ratio of adult patients with severe TBI. Nineteen randomized clinical trails (RCTs) (Andrews *et al*., 2015a; Clifton *et al*., 1993; Clifton *et al*., 2001; Clifton *et al*., 2011; Harris *et al*., 2009; Hashiguchi *et al*., 2003; Jiang *et al*., 2000; Lee *et al*., 2010; Liu *et al*., 2006; Marion *et al*., 1997; Polderman *et al*., 2002; Qiu *et al*., 2007; Shiozaki *et al*., 2001; Shiozaki *et al*., 1993;

Smrcka *et al*., 2005; Suehiro *et al*., 2014; Tang *et al*., 2017; Zhao *et al*., 2011; Zhi *et al*., 2003) and eight non-RCT articles (Balvers *et al*., 2016; Bukur, Hadjibashi, *et al*., 2012; Hifumi *et al*., 2016; Liu *et al*., 2015; Maekawa *et al*., 2015; Rincon *et al*., 2014; Suehiro *et al*., 2015; Tohme *et al*., 2014) were eligible for meta-analysis. The following information was collected from each of the selected articles: authors' names and date of publication, study type and randomization, characteristics of patient population [*e.g.*, mean age, sample size, intra-cranial pressure, and Glasgow coma score (GCS) at admission], methods of whole-body cooling (target temperature, duration of hypothermia, and rate of rewarming), interventions in the no cooling group, and mortality rates in the study groups.

1.2 Data evaluation

The collected data were evaluated based on a statistical and a biomedical approach. Statistically, we assessed the randomization of the collected studies based on the authors' statements, Jadad analysis, and detailed evaluation of the randomization protocol described in the study. In the biomedical evaluation, we narrowed the list of eligible studies to those that dealt with the effects of therapeutic whole-body hypothermia compared with no temperature management, therefore, we excluded those articles, in which selective brain cooling (Harris *et al*., 2009; Liu *et al*., 2006; Qiu *et al*., 2006) or antipyretic drugs (Hifumi *et al*., 2016; Maekawa *et al*., 2015; Suehiro *et al*., 2015) were used, or when patients with spontaneous (accidental) hypothermia were involved in the study (Bukur, Kurtovic, *et al*., 2012; Tohme *et al*., 2014). Eligible studies resulting from the statistical or the biomedical refinement or both were compared with the random effect model of meta-analysis for odds

ratio (OR) as effect size. To assess which parameters of the cooling methods have the biggest impact on the outcome of severe TBI, the studies were divided into subgroups based on target cooling temperature, cooling duration, and speed of rewarming. Target cooling temperatures were classified as “moderate” (32-33°C) (Povlishock & Wei, 2009) and “mild” (33-35°C) (Jiang *et al*., 2000; Povlishock & Wei, 2009). Cooling duration was divided into short (24-48h) and long (>48h) subgroups (Jiang *et al*., 2006). According to recent recommendations about the use of very slow (0.1-0.2°C/h) rewarming rates in patients with severe TBI (Polderman, 2009, 2015), the speed of rewarming was divided into 0.25-1°C/h and less than 0.25°C/h, also including passive. Rewarming was passive if the cooling process ensued without the use of any cooling or rewarming device within the next 18-24 hours (Schwab *et al*., 1998; Steiner *et al*., 2001). For quantification, the speed of passive rewarming was considered as 0.06°C/h (Schwab *et al*., 1998; Steiner *et al*., 2001).

1.3 Calculation of the cooling index

The influence of the used combinations of the three cooling parameters together was also assessed on the outcome of severe TBI. When all three parameters, *i.e*., target cooling temperature, cooling duration, and speed of rewarming, were reported in the same study, we calculated the integrated measure of cooling and named it as the cooling index (COIN). The COIN represents the area between the T_b curve of cooled patients and a hypothetical horizontal line corresponding to a normal T_b of 36.5°C (Fig. 2). The following formula was used: $COIN = \Delta T \times t + (\Delta T \times \Delta T/R)/2$, where “ΔT” is the difference between

normal T_b (36.5°C) and the temperature reached at the end of cooling (in °C); ‘‘t’’ is hypothermia duration (in hours); and ‘‘R’’ is the rate of rewarming (in °C/h). The impact of the rewarming rate was incorporated in the COIN as the area of the triangle, which represents the change in T_b during the rewarming phase. It has to be noted that due to the relatively narrow range (0.06-1°C/h) of the rewarming rates used in the analyzed studies, this area had the smallest contribution to the final value of the COIN, while depth and duration of the cooling were more dominant contributors. The COIN corresponds with the integrated measure of the magnitude and duration of therapeutic hypothermia. For example, a similarly high COIN can result from short and deep cooling with rewarming at 0.25°C/h (Fig. 2B), as well as from longer, but milder cooling with slower, passive rewarming (Fig. 2A). A moderate COIN can result from mild and short cooling followed by slow rewarming at ~0.08°C/h (Fig. 2C), while COIN is low if cooling duration is brief and rewarming is faster (1°C/h) even if target cooling temperature is very low (Fig. 2D). For meta-analysis, the included studies were evenly distributed (N = 4) into subgroups with low (<160°C × h), moderate (160-200°C × h), and high (>200°C × h) COIN. Examples for the assessment of the COIN in each of the three subgroups are presented in Figure 2.

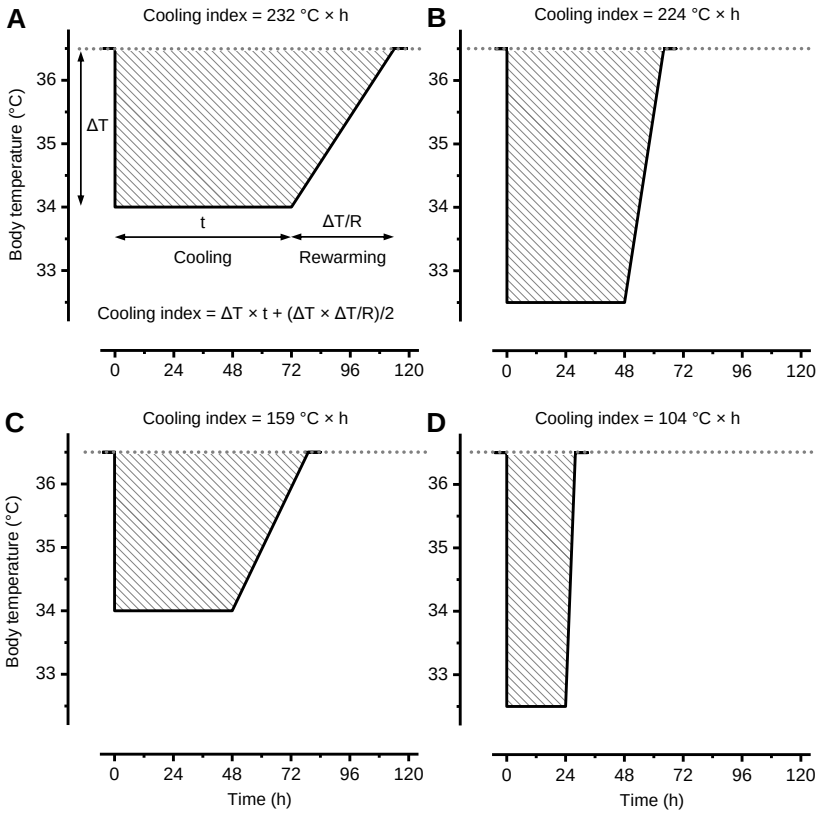

Figure 2 Calculation method of the cooling index (COIN) and examples for high (**A** and **B**), moderate (**C**), and low (**D**) calculated values. The shown examples were calculated from the cooling parameters reported by Smrcka *et al*. (2005) (**A**), Clifton *et al*. (1993) (**B**), Shiozaki *et al*. (2001) (**C**), and Marion *et al*. (1997) (**D**). In all panels, the striped area represents the COIN; the calculated values are indicated. Dotted gray line illustrates normal (not cooled) deep T_b considered as 36.5°C. ΔT, difference between normal T_b and cooling target temperature; t, cooling duration; R, rate of rewarming. See main text for detailed explanation (Olah *et al*., 2018).

From each study, we extracted the targeted cooling parameters (*viz.*, cooling temperature, cooling duration, and speed of rewarming), accounted for any deviations from or adjustments to them, and calculated COIN values (see Table 1 for details).

Table 1. Characteristics of the Patient Groups with Severe Traumatic Brain Injury in the Studies Included in the Meta–Analyses

Study	*Mean age (year)*	*GCS*	*Treatment*	*Number of patients (N)*	*ICP (mmHg)*	*ISS*	*Cooling duration (h)*	*Target temperature (°C)*	*Rewarming rate (°C/h)*	*Cooling index (°C×h)*	*Cooling index subgroup*
Clifton (1993)	29.1	4–7	Cooling	24	14.5	NR	48	32–33	0.25	224	High
			No cooling	22	16.4	NR					
Clifton (2001)	31.5	3–8	Cooling	190	18.1	28 ± 9	48	32.5–34	0.25	177	Moderate
			No cooling	178	17.9	28 ± 8					
Clifton (2011)	28.5	3–8	Cooling	52	NR	30 ± 6	48	33–34	0.25	162	Moderate
			No cooling	45	NR	30 ± 9					
Hashiguchi (2003)	34.1	3–8	Cooling	9	11.1 ± 4.8	30.7 ± 5.6	48	33.5–34.5	~ 0.08	159	Low
			No cooling	8	11.9 ± 4.2	33.0 ± 6.4					
Jiang (2000)	41.4	3–8	Cooling	43	29.6 ± 2.2	NR	72–336	33–35	1	243	High
			No cooling	44	30.3 ± 3.0	NR					
Liu (2015)	NR	3–8	Cooling	110	27.5 ± 16.9	NR	72–288	35–36	< 0.25	–	–
			No cooling	110	26.8 ± 17.5	NR					
Marion (1997)	33.5	3–7	Cooling	40	15.4	NR	24	32–33	1	104	Low
			No cooling	42	19.7	NR					
Polderman (2002)	36.7	3–8	Cooling	64	37.0 ± 20.0	NR	24	32	0.08	–	–
			No cooling	72	< 20.0	NR					
Shiozaki (1993)	35.4	<8	Cooling	16	35.4 ± 12.0	NR	48	33.5–34.5	Passive	172	Moderate
			No cooling	17	36.9 ± 12.1	NR					
Shiozaki (2001)	38.5	3–8	Cooling	45	NR	NR	48	33.5–34.5	~ 0.08	159	Low
			No cooling	46	NR	NR					
Smrcka (2005)	41.0	3–8	Cooling	35	NR	11.8 ± 5	72	34	Passive	232	High
			No cooling	37	NR	17.6 ± 7					
Tang (2017)	41.1	3–8	Cooling	30	NR	NR	48	32–35	0.25	162	Moderate
			No cooling	30	NR	NR					
Zhao (2011)	37.2	3–8	Cooling	40	15.9 ± 4.3	NR	72	32.5–33	Passive	387	High
			No cooling	41	17.1 ± 5.0	NR					
Zhi (2003)	42.5	3–8	Cooling	198	26.9 ± 4.6	NR	24–168	32–35	0.25	138	Low
			No cooling	198	26.6 ± 4.9	NR					

–, not applicable; GCS, Glasgow Coma Scale score; ICP, intracranial pressure; ISS, Injury Severity Score; NR, not reported.

After we originally introduced the COIN in 2018 (Olah *et al*., 2018), the results of a large, multicenter, randomized clinical trial (POLAR) were published, which seemed to contradict the benefits of therapeutic hypothermia in severe TBI (Cooper *et al*., 2018). In order to also account for the results from that trial, in our recent meta-analysis we also included the data from POLAR (Olah, Poto, *et al*., 2021).

1.4 Statistical analysis

The statistical analysis was performed according to the standard methods of meta-analysis. The primary effectiveness outcome was all-cause mortality. OR with 95% confidence intervals (CI) for mortality in the adult patients with severe TBI were calculated in a random-effects model of meta-analysis. Summary effect estimates were stratified by study design and the between-groups effects were assessed.

Publication bias was assessed with funnel plots by using the Duval and Tweedie trim and fill method (Duval & Tweedie, 2000) and the Egger's test (Egger's test values of less than 0.1 were considered as indicators of significant small-study effect). Between-study heterogeneity was tested with Q homogeneity test (p values of less than 0.05 were considered as indicators of significant heterogeneity) and with I^2 statistical test, where I^2 is the proportion of total variation attributable to between-study variability (an I^2 value of more than 50 was considered as indicating considerable heterogeneity). Results of the meta-analyses were depicted as forest plots. All analyses were performed using the Comprehensive Meta-Analysis software (version 3.3; Biostat, Inc., Engelwood, MJ, USA).

2. Experiments in animal models

2.1 Animals

In our animal experiments (Olah, Rumbus, *et al*., 2021), we used 109 adult mice of both sexes. As in our earlier studies (de Oliveira *et al*., 2014; Pozsgai *et al*., 2017), male *Trpa1*$^{-/-}$ and *Trpa1*$^{+/+}$ mice (n = 18 and 14, respectively) and C57BL/6 mice (n = 77) were obtained from the Laboratory Animal Centre at the University of Pécs where they were bred and kept under standard pathogen-free conditions. The mice were housed in standard polycarbonate cages kept in a room with T_a maintained at ~24.5°C and humidity at 30–40%. Light was controlled on a 12 h light-dark cycle (lights on at 5:00 a.m.). Standard rodent chow and tap water were available *ad libitum*. For thermophysiological experiments, mice were extensively habituated to staying inside wire-mesh cylindrical confiners, as described previously (Garami *et al*., 2011). All procedures were conducted under protocols approved by the Institutional Animal Use and Care Committee of the University of Pécs (registration no.: BA02/2000–6/2018, approved on 27 February 2018) and were in accordance with the directives of the National Ethical Council for Animal Research and those of the European Communities Council (86/609/EEC).

2.2 Surgeries

2.2.1. Anesthesia and perioperative care

Mice were anesthetized with intraperitoneal (i.p.) administration of a ketamine-xylazine cocktail (81.7 and 9.3 mg/kg, respectively) and received antibiotic protection intramuscularly (gentamycin, 6 mg/kg). During surgery, mice were heated with a temperature-controlled heating pad (model TMP-5a; Supertech Instruments UK Ltd., London, UK) placed under a surgery board. Each mouse was subjected to one of the surgical procedures described below. The experiments were performed 4–8 days after the surgery.

2.2.2. Intracerebroventricular cannula implantation

For the intracerebroventricular (i.c.v.) substance administration, a 22-G steel guide cannula (Plastics One, Roanoke, VA, USA) was implanted into the right lateral cerebral ventricle using a stereotaxic manipulator (Narishige Scientific Instruments Laboratory, Tokyo, Japan), as described previously (Pakai *et al*., 2018). In brief, after incision of the scalp and removal of the periosteum, two supporting microscrews (Fine Science Tools, Heidelberg, Germany) were driven into the skull and the guide cannula was inserted through a small hole drilled in the skull 0.5 mm posterior from Bregma and 1.0 mm lateral from midline. The tip of the cannula was placed within the right lateral ventricle (2.0 mm

from dura). The cannula was fixed to the supporting microscrews with dental cement and closed by a dummy cannula.

2.2.3. Intraperitoneal catheter implantation

For the i.p. administration of substances, a polyethylene (PE)-50 catheter filled with pyrogen-free saline was implanted into the peritoneal cavity, as described elsewhere (Garami *et al*., 2011; Pakai *et al*., 2018). Through a small midline incision on the abdominal wall, the internal end of the catheter was fixed to the abdominal wall with a suture, while the external end of the catheter was exteriorized at the nape and heat-sealed. The surgical wound was sutured in layers. The catheter was flushed with 0.1 mL of saline on the day after the surgery and every other day thereafter.

2.3 Experimental setups

Thermoregulatory experiments in unanesthetized mice were performed in either the thermocouple thermometry setup or the respirometry thermometry setup. The experiments were conducted at a T_a of 30°C in the thermocouple thermometry setup a T_a of 22°C in the respirometry thermometry setup, which is subneutral for mice in these setups (Pakai *et al*., 2018).

2.3.1. Thermocouple thermometry

The mice were placed in cylindrical confiners and equipped with copper-constantan thermocouples (Omega Engineering, Stamford, CT, USA) to measure colonic temperature (a form of deep T_b). The colonic thermocouple was inserted 3 cm deep beyond the anal sphincter, fixed to the base of the tail with adhesive tape, and plugged into a data logger device (Cole-Palmer, Vernon Hills, IL, USA) connected to a computer. Mice in their confiners were then placed into a temperature-controlled incubator (model MIDI F230S; PL Maschine Ltd., Tarnok, Hungary) set to a T_a of 30°C, which is slightly below the thermoneutral zone for mice in this setup. When the mouse was pre-implanted with an i.c.v. cannula, a needle injector (Plastics One, Roanoke, VA, USA) was fitted into the guide cannula and connected to a PE-50 extension, which was prefilled with a solution of Na_2S or GYY4137 or with saline. The injector needle protruded 1.0 mm beyond the tip of the guide cannula. The extension was passed through a port of the incubator and connected to a 10-µL syringe (model 701N, Hamilton, Reno, NV, USA). When the mouse had an i.p. catheter, it was connected to a PE-50 extension, which was prefilled with the substance of interest and connected to a syringe placed in an infusion pump (model 975; Harvard Apparatus Inc., Holliston, MA, USA). The PE-50 extensions preloaded with the substances were wrapped in aluminum foil in order to prevent the photolytic oxidation of sulfide ions by UV light, which reaction can occur in aqueous sulfide solutions (Linkous *et al*., 2004).

2.3.2. Respirometry thermometry

The respirometry setup was designed to characterize dose dependency of thermal effects and the involvement of metabolic rate inhibition in the thermoregulatory response to Na_2S and GYY4137 in C57BL/6 mice. The mice were equipped with thermocouples and PE-50 extensions the same way as in the experiments in the thermocouple thermometry setup. Then, the mice in their confiners were transferred to a Plexiglas chamber of the four-chamber open-circuit calorimeter integrated system (Oxymax Equal Flow, Columbus Instruments, Columbus, OH, USA). The chambers were sealed, submerged into a temperature-controlled water bath, and continuously ventilated with room air (200 mL/min). The fractional concentration of oxygen was measured in the air entering and exiting the chamber, and the rate of oxygen consumption (VO_2) was calculated according to the manufacturer's instructions using the Oxymax Windows software (version 3.1).

2.3.3. Drugs and drug administration

Na_2S nonahydrate ($Na_2S{\cdot}9H_2O$) was purchased from Sigma-Aldrich (St. Louis, MO, USA). On the day of the experiment, $Na_2S{\cdot}9H_2O$ was freshly dissolved in pyrogen-free saline to achieve final concentrations of Na_2S of 1.5, 5, or 10 mg/mL. For the i.p administration, the working solution (1.5 mg/mL) of Na_2S (or saline) was infused over 4 min (3.3 mL/kg) to deliver Na_2S at 5 mg/kg. For the i.c.v. administration of Na_2S (at doses of 0.5 and 1 mg/kg), the working solutions (5 and 10 mg/mL, respectively) of Na_2S (or saline) were infused (1 µL/min) over a 3 min period.

The slow-releasing H_2S donor GYY4137 was synthesized by our collaborators at the University of Exeter Medical School. On the day of the experiment, GYY4137 was freshly dissolved in saline to make a working solution of 30 mg/mL. By infusing this solution into the lateral ventricle (1 µL/min for 3 min), a total dose of 3 mg/kg of GYY4137 was delivered i.c.v. Control mice were infused with saline.

2.4 Measurement of *Trpa1* mRNA expression

2.4.1. RNAscope in situ hybridization

For RNAscope studies 3 month-old male C57BL/6 mice (n = 4) were perfused as described above using 30 mL PBS followed by 100 mL of 4% paraformaldehyde in Millonig's phosphate buffer. Brains were postfixed for 24 h at room temperature, rinsed in PBS, dehydrated, and embedded in paraffin using standard procedures. 5 µm sections were cut using a sliding microtome (model HM 430; Thermo Fisher Scientific, USA). The RNAscope Multiplex Fluorescent Reagent Kit v2 (ACD, Hayward, CA, USA) was used according to the manufacturer's protocol. In short, sections were heat-treated, deparaffinized, H_2O_2-blocked, boiled, and pretreated with Protease Plus. Subsequently, the sections were hybridized with probes specific to mouse *Trpa1* mRNA and with 3-plex positive and negative control probes. Sequential signal amplification and channel development were performed. Nuclear counterstaining with 4′, 6-diamidino-2-phenylindole (DAPI) was applied and sections were mounted with ProLong Diamond Antifade Mountant for confocal imaging.

As in earlier studies (Ali *et al*., 2020; Morikawa *et al*., 2019), cortical samples were stained for the *ppib* mRNA (red) as positive technical control and the bacterial *dabP* mRNA staining (red) was used as negative technical control. According to the atlas by Paxinos and Franklin (2001) fluorescent images of the medial preoptic area (MPO; +0.14 mm to +0.02 mm from Bregma), dorsomedial hypothalamic area (DMH; −1.58 mm to −1.70 mm from Bregma), as well as the lateral parabrachial nucleus and rostral raphe pallidus (LPB and rRPa; −5.34 mm to −5.40 mm from Bregma for both) were acquired using an Olympus Fluoview FV-1000 laser scanning confocal microscope and Fluoview FV-1000S-IX81 image acquisition software system (Olympus, Tokyo, Japan). The confocal aperture was set to 80 µm. The analog sequential scanning was performed using a 40× objective lens (NA: 0.75). The optical thickness was set to 1 µm and the resolution was 1024 × 1024 pixels. The excitation time was set to 4 µs per pixel. Blue and red virtual colors were selected to depict fluorescent signals of DAPI (nuclear counterstain) and of Cyanine 3 (*Trpa1* mRNA), respectively.

2.5 Data processing and analysis

Data on deep T_b, $\dot{V}O_2$, and blood flow intensity were analyzed through the application of two-way ANOVA. As in our previous studies (Banki *et al*., 2014; Garami, Pakai, *et al*., 2018), ANOVA was followed by the Fisher's LSD post hoc test. Sigmaplot 11.0 (Systat Software, San Jose, CA, USA) software was used for statistical analyses. Differences were considered significant when $p < 0.05$. All data are reported as mean ±SE.

Results

1. The value of therapeutic hypothermia in human patients with severe traumatic brain injury

In a typical case like the one presented in Figure 1, focal lesions are diagnosed with routine imaging techniques, such as CT. The 35-year-old-man with severe TBI due to motor vehicle collision was admitted to the St. Joseph's Hospital and Medical Center in Phoenix, Arizona, United States. The diagnosis of severe TBI was established by cerebral computed tomography (cCT) on admission and based on the presence of pathological cCT scan findings. The primary head injury can initiate a number of pathophysiological processes which will determine the extent and the duration of the damage. These mechanisms involve ischemia, swelling (edema), neurotransmitter release (excitotoxicity), formation of free radicals, ionic flux-mediated damage, metabolic and mitochondrial dysfunction, and neuroinflammatory responses (Finfer & Cohen, 2001; Maas *et al*., 2008; McGinn & Povlishock, 2016). Many of these process are dependent from temperature and can be mitigated at lower temperatures, therefore the question was raised whether therapeutic hypothermia could be beneficial for similar patients. To answer this question, we performed meta-analyses of the data available in scientific literaure (Olah *et al*., 2018; Olah, Poto, *et al*., 2021).

In our first meta-analysis (Olah *et al*., 2018), the literature search identified altogether 709 studies from the PubMed, EMBASE, and Cochrane databases. After enabling filters for human studies and English language and using additional filters (study

types) 321 studies remained, which were screened for title and abstract for inclusion criteria. 273 articles were excluded because of insufficient data reporting or because children were studied. 48 studies were included in qualitative synthesis. A further 21 articles were excluded due to the lack of mortality data. 27 studies were included and pooled for quantitative synthesis (Fig. 3). When we compared the effects of therapeutic hypothermia with no cooling by including all 27 identified studies in the meta-analysis (Fig. 4), we did not find a significant difference in the OR for mortality between the groups. Importantly, the included studies were methodologically quite heterogeneous with regards to both statistical and clinical designs ($Q = 167$, $p < 0.001$; $I^2 = 84$).

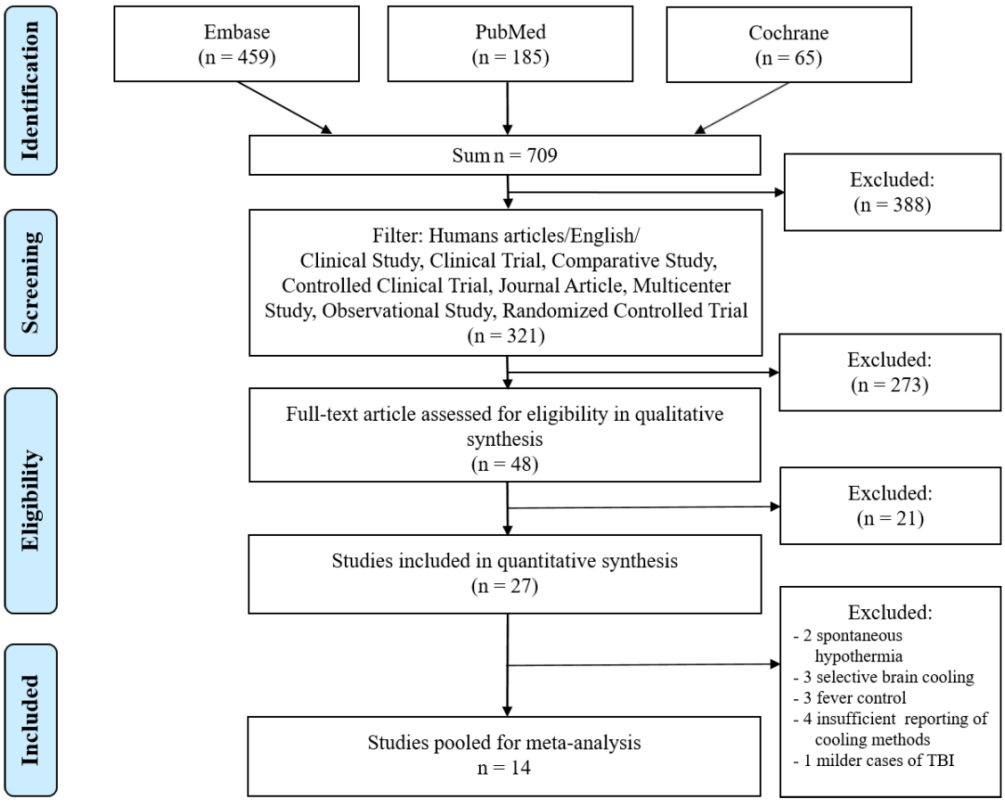

Figure 3 Flow chart of study selection and inclusion in our meta-analysis (Olah *et al*., 2018).

As a statistical approach to reduce heterogeneity, we analyzed separately those studies which were RCTs. In that analysis, therapeutic hypothermia tended to improve the outcome in patients with severe TBI, but the between-study heterogeneity was still reasonably high. To mitigate heterogeneity, we evaluated the studies based on clinical and methodological designs. Our goal was to study the effect of therapeutic hypothermia applied to the whole body of patients with severe TBI, but without spontaneous (accidental) hypothermia and without temperature control. Therefore, 3 trials using selective brain cooling (Harris *et al*., 2009; Liu *et al*., 2006; Qiu *et al*., 2006), 2 articles including cases of spontaneous hypothermia (Bukur, Kurtovic, *et al*., 2012; Tohme *et al*., 2014), and 3 studies applying fever control (Hifumi *et al*., 2016; Maekawa *et al*., 2015; Suehiro *et al*., 2015) had to be excluded from the analysis. Further 4 studies could not be included in the final analyses, because the applied cooling methods were not reported in sufficient details (Balvers *et al*., 2016; Lee *et al*., 2010; Rincon *et al*., 2014; Suehiro *et al*., 2014). In one of the trials, the GCS of the included patients ranged between 3-15 (Andrews *et al*., 2015a), which population also includes mild (GCS = 13-15) and moderate cases of TBI (GCS = 9-12), therefore it had to be excluded from further analysis.

As result of the combined (statistical and physiological) evaluation of the studies identified by our literature search, 14 full-text publications (involving 1,786 adult patients with severe TBI; 896 in the therapeutic hypothermia group and 890 in the no cooling group), were included in the next steps of our analysis. All of them were RCTs, in which the exact cooling methods of the whole body (target temperature, cooling duration, and speed of rewarming) were reported, and the effect of therapeutic hypothermia on mortality

was compared with patients without temperature management in severe TBI. The homogeneity of the studies was verified by Egger's test, Q, and I^2 statistics, which showed no significant difference in inter-study variability (Egger's p = 0.509; Q = 17, p = 0.224; I^2 = 21). Meta-analysis of these studies revealed that therapeutic hypothermia significantly improved the outcome of severe TBI (OR = 0.675; p = 0.004) (Fig. 5).

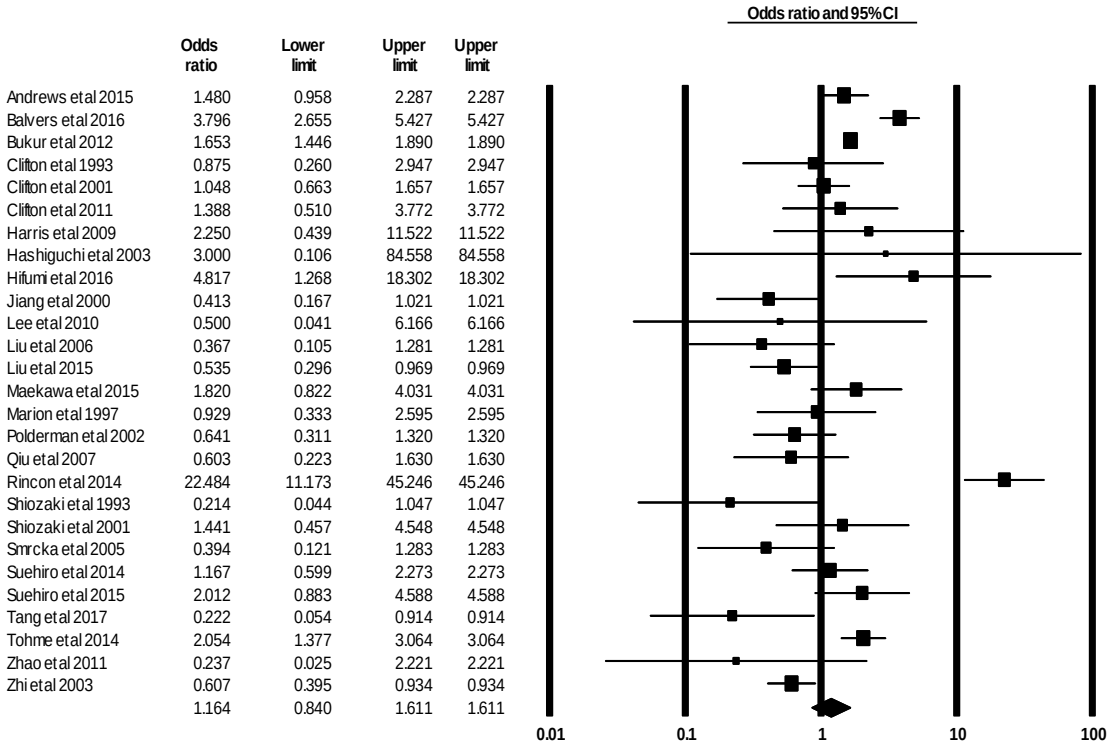

Figure 4 Forest plot of the odds ratios (ORs) for mortality rate between cooled and not cooled groups of patients with severe TBI using random-effects model in 27 trials of all study types (Olah *et al*., 2018). The OR was calculated by dividing the odds of death to survival in the therapeutic hypothermia group with the odds of death to survival in the normothermia group. A ratio <1 indicates that therapeutic hypothermia reduced the odds of death, whereas a ratio >1 indicates increased odds of death in therapeutic hypothermia. Full references to the analyzed studies can be found in the list of references. CI, confidence interval.

Our findings are in line with several clinical trials showing a beneficial effect of therapeutic hypothermia on the outcome of severe TBI (Clifton *et al*., 1993; Jiang *et al*., 2000; Liu *et al*., 2015; Marion *et al*., 1997; Polderman *et al*., 2002; Shiozaki *et al*., 1993; Smrcka *et al*., 2005; Tang *et al*., 2017; Zhao *et al*., 2011; Zhi *et al*., 2003), whereas they contradict other trials which found no or even adverse effect of whole-body cooling in TBI (Clifton *et al*., 2001; Clifton *et al*., 2011; Hashiguchi *et al*., 2003; Shiozaki *et al*., 2001). Differences in statistical, clinical, and methodological design among the studies can be assumed to account for the contradictory results. The highest quality human studies are RCTs, while observational studies and retrospective analyses provide lower level of evidence. Among RCTs the randomization protocol can vary, which results in different levels of statistical bias involved in the trials. Three multi-center clinical trials (Clifton *et al*., 2001; Clifton *et al*., 2011; Shiozaki *et al*., 2001) found either no change or worse mortality rates in the cooled groups of patients with TBI, whereas all of the single-center studies (Clifton *et al*., 1993; Jiang *et al*., 2000; Liu *et al*., 2015; Marion *et al*., 1997; Polderman *et al*., 2002; Shiozaki *et al*., 1993; Smrcka *et al*., 2005; Tang *et al*., 2017; Zhao *et al*., 2011; Zhi *et al*., 2003) showed that therapeutic hypothermia was associated with a lower mortality rate. Differences between results from single-center versus multi-center trials have been also observed earlier with regards to therapeutic hypothermia (Marion & Bullock, 2009).

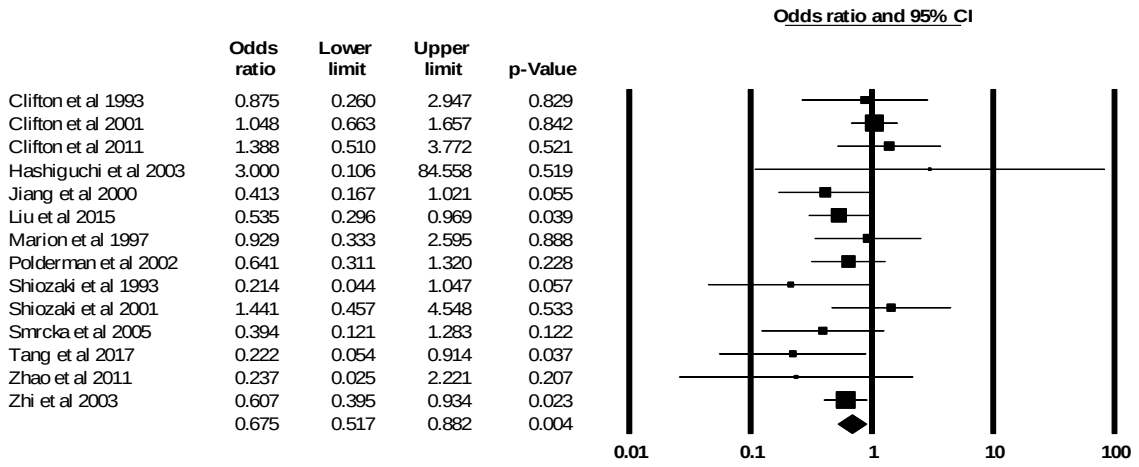

Figure 5 Forest plot of the odds ratios (ORs) for mortality rate between cooled and not cooled groups of patients with severe TBI using random-effects model in statistically, clinically, and methodologically homogenous RCTs (Olah *et al*., 2018). Full references to the analyzed studies can be found in the list of references. CI, confidence interval.

In our next approach, we studied the integrated effect of the cooling parameters on the outcome of the disease. From the cooling parameters reported in the studies with medium and good level of randomization, the COIN was assessed by considering all three variables, *viz.*, target temperature, cooling duration, and speed of rewarming, in the formula (for details, see Methods and Fig. 2A). The reported parameters and the COIN derived from these data are shown in Table 1. By calculating the COIN, we were able to compare the effect of the overall extent of hypothermia among studies which used different cooling parameters in their protocols. The OR (estimated expected average) in the subgroups with low (<160°C × h), moderate (160-200°C × h), and high (>200°C × h) COIN was 0.831 ($p = 0.535$), 0.754 ($p = 0.340$), and 0.470 ($p = 0.035$), respectively (Fig. 6). Importantly, the only significant effect for an OR of less than 1, *i.e.*, when cooling was beneficial compared to no cooling, was observed in the subgroup of studies with high

COIN. These results suggest that in addition to the different independent contribution of each cooling parameter, the integrated measure of the magnitude and duration of therapeutic hypothermia (as indicated by the COIN) can play a decisive role in determining whether the applied cooling protocol will decrease the risk of death in patients with severe TBI.

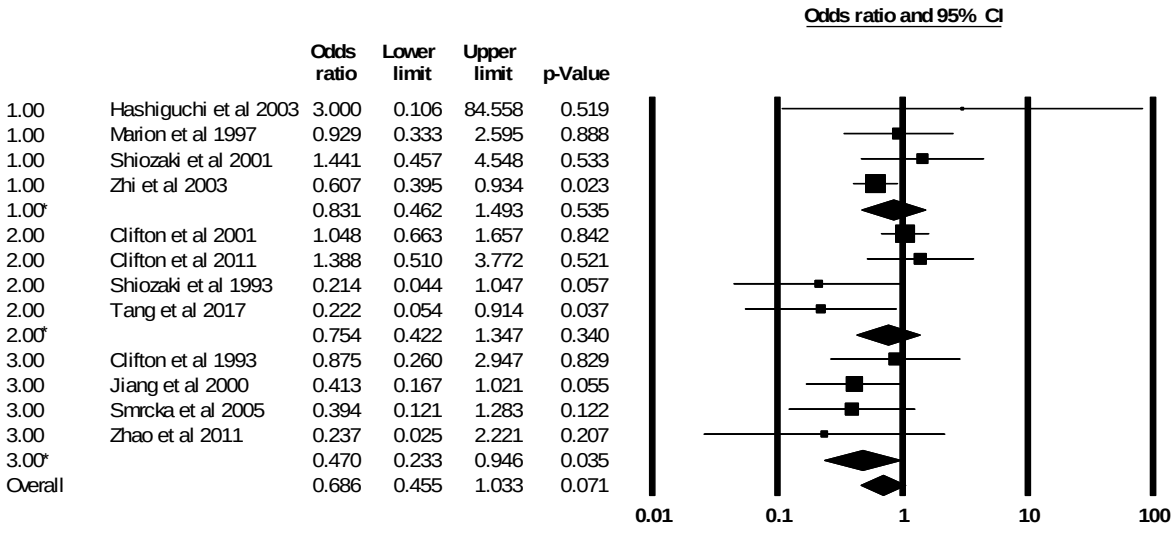

Figure 6 Forest plot of the odds ratios (ORs) for mortality rate between cooled and not cooled groups of patients with severe TBI using random-effects model in RCTs divided into low (<160°C × h; group label 1), moderate (160-200°C × h; group label 2), and high (>200°C × h; group label 3) subgroups based on the cooling index (COIN) (Olah *et al*., 2018). Full references to the analyzed studies can be found in the list of references. CI, confidence interval.

In our later study (Olah, Poto, *et al*., 2021), we extended our analysis based on COIN by including in it the data reported in the POLAR study. The COIN value would have been high in the POLAR study – if the targeted parameters (Table 2) were met. However, the targeted cooling parameters were reached in less than 50% of the patients

receiving therapeutic hypothermia (Table 2). In the hypothermia group, 85 patients (33%) were cooled for less than 48 hours; 27 patients (10%) never reached a deep T_b of 35°C; and 65 patients (27%) never reached 33°C (Cooper *et al*., 2018). Hence, many patients in the POLAR study had a low level of COIN, and the overall level achieved in that study was only moderate (Table 2).

Table 2 Cooling parameters reported in the POLAR study and the calculated cooling index (COIN) in Olah, Poto, *et al*. (2021).

	Cooling duration (h)	***Cooled deep body temperature (°C)***	***Rewarming rate (°C/h)***	***No. of patients [%]***	***COIN (°C × h)***	***COIN subgroup***
Targeted parameters	72≤	33.0±0.5	≤0.25	260 [100]	276	High
Cooling compliance criteria	48≤	≤35	≤0.25	124-125 [48]	77	Low
Sensitivity analysis criteria	~72	≤35	≤0.25	120-121 [46]	112	Low
Overall study parameters	72.2	33-35	≤0.25	124 [48]	193	Moderate

Based on the above, we included the POLAR data in the "Moderate" COIN subgroup of our meta-analysis (Fig. 7). For all data, including POLAR, the OR for death was 0.686 ($p = 0.007$), indicating that, overall, therapeutic hypothermia significantly decreased mortality in severe TBI. However, a significant decrease in OR (indicating a beneficial effect of therapeutic hypothermia) was observed only in the "High" COIN subgroup: 0.470 ($p = 0.013$). The ORs in subgroups with "Low" or "Moderate" cooling intensity were 0.718 ($p = 0.081$) and 0.846 ($p = 0.533$), respectively.

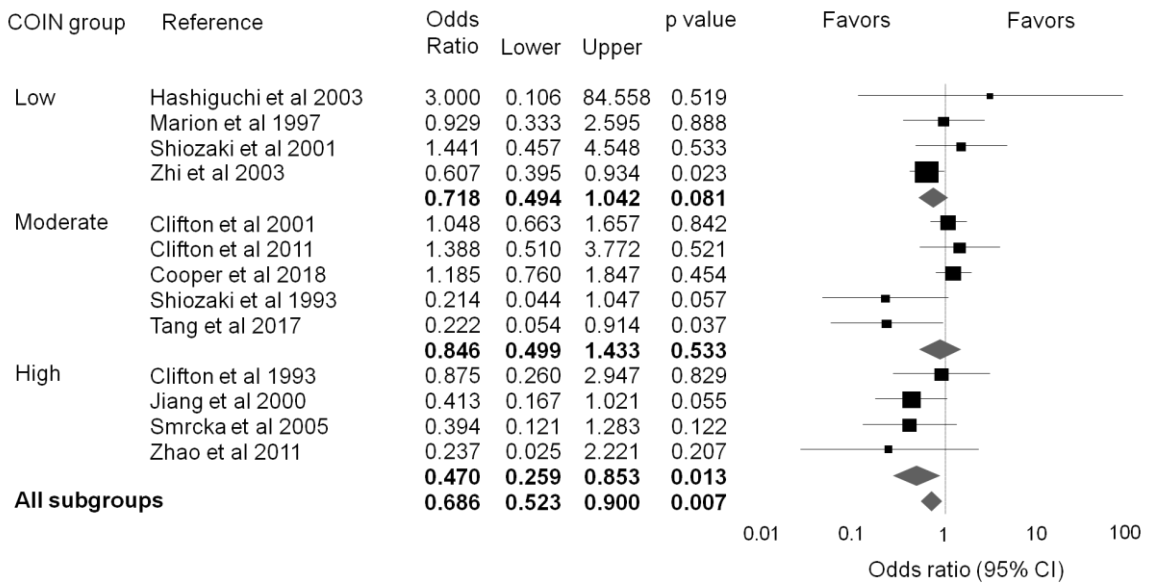

Figure 7 Forest plot of the effects of therapeutic hypothermia on mortality in patients with severe TBI (Olah, Poto, *et al*., 2021). The ORs were compared by using random-effects model in high-quality, RCTs divided into a "Low" (<160°C × h), "Moderate" (160–200°C × h), and "High" (>200°C × h) subgroups based on the cooling index (COIN). Note that the POLAR study (Cooper *et al*., 2018) is included in the "moderate" COIN subgroup (for details, see Table 2). Full references to the analyzed studies can be found in the list of references. CI, confidence interval.

2. The mechanisms of H_2S-induced hypothermia in animal experiments

2.1 Central administration of Na_2S decreases deep body temperature in mice via inhibition of thermogenesis and induction of vasodilation in the skin

First, we studied the thermoregulatory effect of Na_2S, a fast-releasing H_2S donor, administered i.c.v. in C57BL/6 mice by using respirometry thermometry. In response to Na_2S, the mice developed a decrease in deep T_b, which was more pronounced at the higher dose, whereas saline did not cause any effects (Fig. 8).

The hypothermic response to Na_2S developed rapidly at both of the applied doses, and at 20 min it reached the biggest mean decrease of −0.5 ± 0.3°C at 0.5 mg/kg and −0.8 ± 0.3°C at 1 mg/kg ($p = 0.045$ and 0.005, respectively). The effect of the treatment on T_b was significant for both the lower and the higher doses of Na_2S as compared to saline ($p < 0.001$ for both). At the 0.5 mg/kg dose of Na_2S, deep T_b was significantly lower than in saline-treated mice at 20, 170, and 180 min, while at the 1 mg/kg dose the drop in deep T_b was significant between 20–180 min compared to saline.

In the same experiments, we also measured the rate of oxygen consumption (VO_2), which was regarded as an indicator of non-shivering thermogenesis (*i.e.*, one of the principal autonomic thermoeffectors), as in previous studies in mice (Banki *et al*., 2014; Garami *et al*., 2011). Na_2S microinjection (3 μL, i.c.v) also significantly affected oxygen consumption. The Na_2S-induced hypothermia was brought about by a fall in VO_2, which changed with similar dynamics as deep T_b (Fig. 8). Similar to T_b, the effect of the

treatment on VO_2 was significant for both the lower and the higher doses of Na_2S as compared to saline ($p = 0.024$ and $p < 0.001$, respectively). At the 1 mg/kg dose, VO_2 was significantly lower than in saline-treated mice between 20 min and 110 min. In a separate set of experiments, we also checked whether skin vasodilation – to increase heat loss – contributes to H_2S-induced hypothermia and we found that Na_2S administered centrally caused a marked cutaneous vasodilation in the back-skin of the mice, thereby indicating that increased heat dissipation also participates in the hypothermic response to H_2S [for details, see Olah, Rumbus, *et al*. (2021); also included in the appendix].

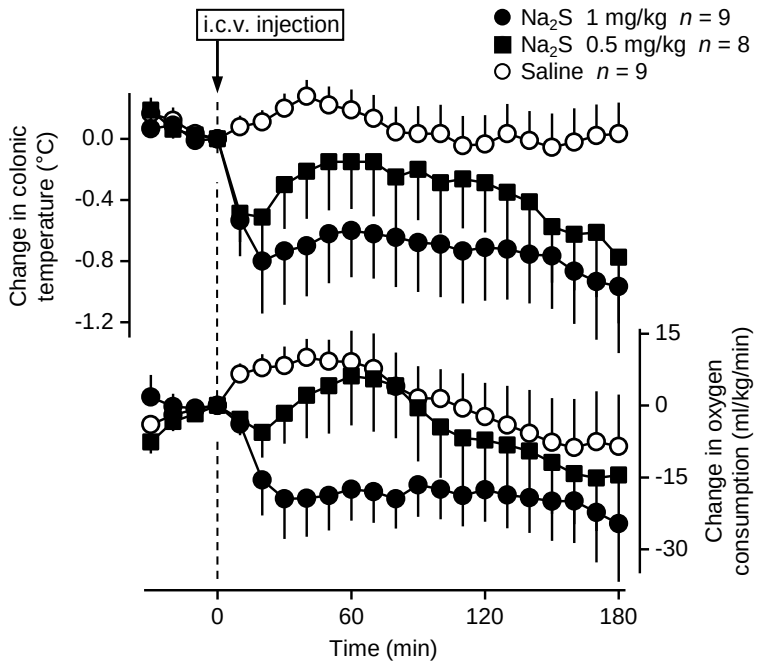

Figure 8 Colonic temperature and oxygen consumption (VO_2) responses of C57BL/6 mice to Na_2S (doses indicated) and saline administered i.c.v. The changes in colonic temperature (a form of deep T_b) are shown in the upper panel, while the changes in VO_2 (an indicator of thermogenesis) are depicted in the lower panel (Olah, Rumbus, *et al*., 2021).

The rapid, dose-dependent development of the hypothermic and hypometabolic response to centrally administered Na_2S suggests that the site of action for the released H_2S is located in the central nervous system. To test whether the hypothermic effect of Na_2S can be triggered from a peripheral site, next we studied the thermoregulatory response to the i.p. administration of a high dose (5 mg/kg) of Na_2S. As expected, i.p. infusion of saline did not have any effect on deep T_b (Fig. 9). In contrast with the i.c.v. administration, when the mice were infused with Na_2S i.p. their deep T_b did not differ significantly from that of saline-treated mice at any time points during the experiment ($p > 0.05$) even though a 10 times higher dose was delivered i.p. than the i.c.v. administered lower dose which caused hypothermia (see Fig. 8).

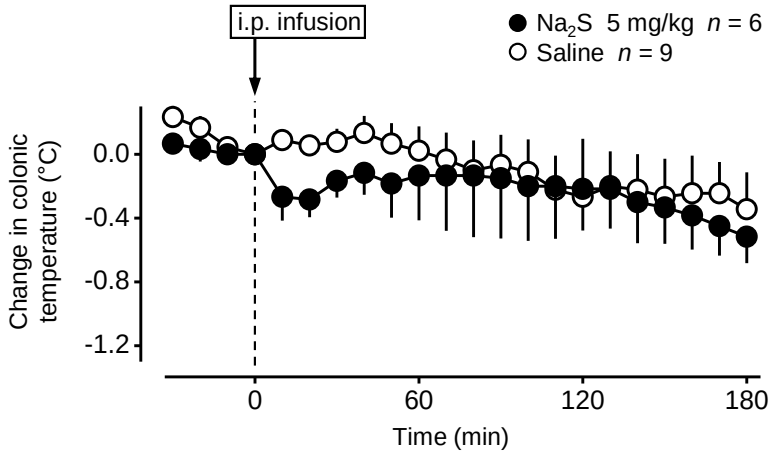

Figure 9 Colonic temperature response of C57BL/6 mice to Na_2S (dose indicated) and saline administered i.p. (Olah, Rumbus, *et al*., 2021).

2.2 Central administration of GYY4137 decreases deep body temperature in mice

We also wanted to investigate whether the observed thermoregulatory effects of Na_2S can be triggered by GYY4137, which is a slow-releasing H_2S donor. When administered i.c.v. in the respirometry thermometry setup, GYY4137 (3 mg/kg) caused a marked hypothermia and hypometabolism as compared to saline treatment (Fig. 10). Between the GYY4137-treated and saline-treated mice, deep T_b differed significantly at 80–100 min ($p < 0.05$) and 130–180 min ($p \leq 0.001$), and the difference in VO_2 was significant at 20–30 min and 160–180 min ($p < 0.05$).

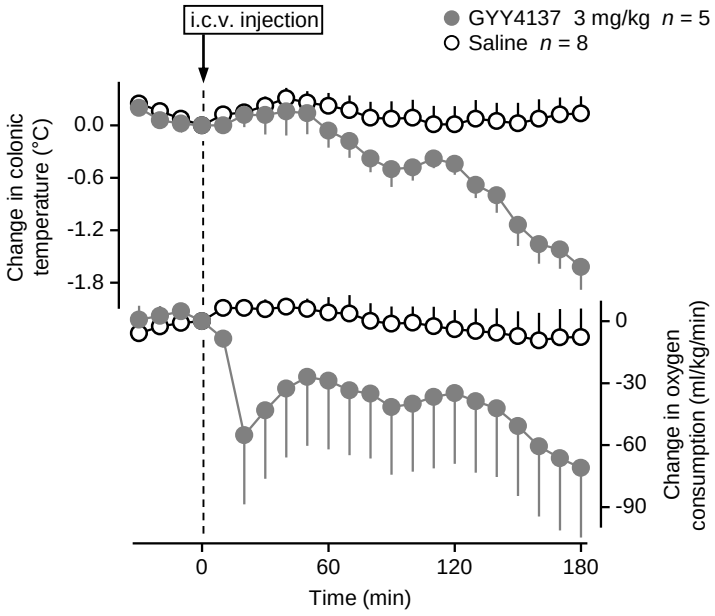

Figure 10 Colonic temperature and VO_2 responses of C57BL/6 mice to GYY4137 (dose indicated) and saline administered i.c.v. (Olah, Rumbus, *et al*., 2021).

2.3 The hypothermic response to Na_2S and GYY4137 is attenuated in the absence of the transient receptor potential ankyrin-1 channel

Previously it has been shown that the TRPA1 channel mediates different effects of H_2S, including nociceptive, inflammatory, vasomotor, and neurophysiological effects [for a review, see Pozsgai, Bátai, *et al*. (2019)], but it has remained unknown whether it contributes to the development of the H_2S-induced hypothermia. Therefore, next, we investigated whether the TRPA1 channel is involved in the thermoregulatory responses to different H_2S donors. For that reason, we used *Trpa1*$^{-/-}$ and *Trpa1*$^{+/+}$ mice. As expected from our experiments in C57BL/6 mice (Fig. 8), the i.c.v. administration of Na_2S (1 mg/kg) caused a sudden, pronounced drop in the colonic temperature (>1.5°C) and VO_2 (>40 mL/kg/min) of the *Trpa1*$^{+/+}$ mice (Fig. 11A). However, in the *Trpa1*$^{-/-}$ mice the hypothermic and hypometabolic effects of the same dose of Na_2S were markedly attenuated ($p < 0.001$ for both parameters). The *Trpa1*$^{+/+}$ mice had significantly lower deep T_b between 30 and 180 min, as well as reduced VO_2 between 60 and 180 min post-Na_2S administration as compared to the *Trpa1*$^{-/-}$ mice.

We also studied the thermoregulatory effect of GYY4137 in *Trpa1*$^{-/-}$ and *Trpa1*$^{+/+}$ mice in the thermocouple thermometry setup (Fig. 11B). The i.c.v. administration of GYY4137 (3 mg/kg) caused a marked fall in the deep T_b of *Trpa1*$^{+/+}$ mice; however, the hypothermic response to the same dose of GYY4137 was significantly attenuated in *Trpa1*$^{-/-}$ mice ($p < 0.001$). The colonic temperature of *Trpa1*$^{-/-}$ mice remained

significantly higher than that of *Trpa1*$^{+/+}$ mice between 20 and 80 min post-GYY4137 administration.

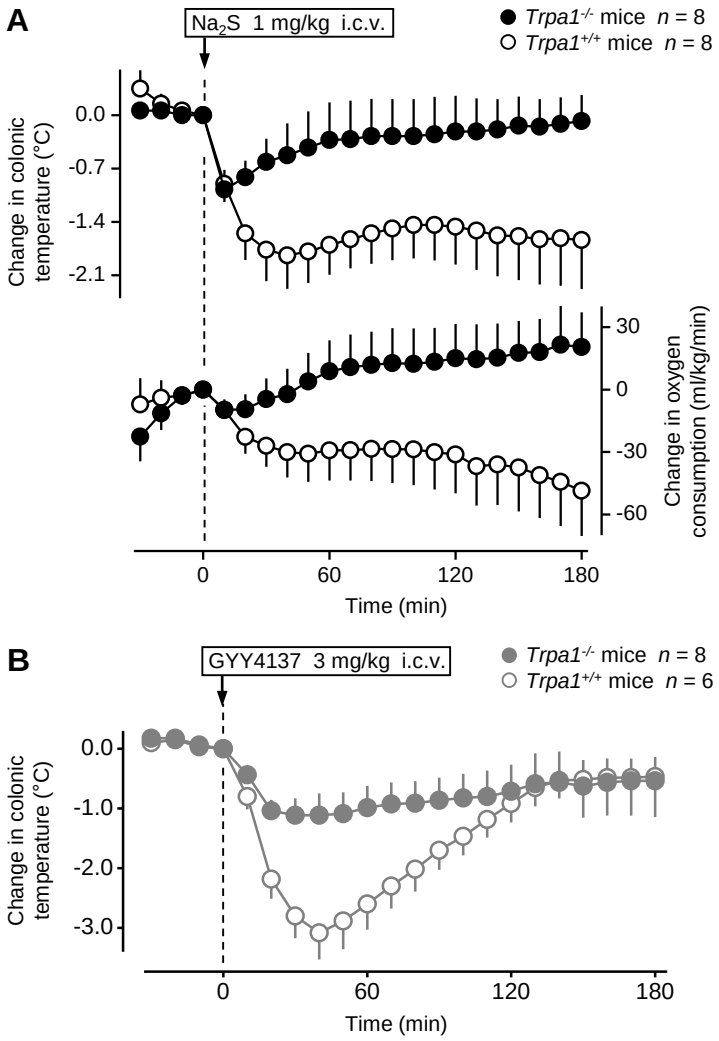

Figure 11 Colonic temperature (upper panel) and oxygen consumption (VO_2; lower panel) responses to Na_2S (A) and colonic temperature responses to GYY4137 (B) administered i.c.v. (doses indicated) in *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice (Olah, Rumbus, *et al*., 2021).

2.4 *Trpa1* mRNA is modestly expressed in brain neurons within autonomic thermoeffector pathways

Our thermophysiological results suggested that the thermoregulatory responses to H_2S donors are triggered from the central nervous system. We, therefore, studied the expression of the TRPA1 channel in thermoregulation-related brain structures. We used RT-qPCR to assess *Trpa1* mRNA in the hypothalamus of the mice, but we did not find any detectable amount, although the expression of *Trpa1* mRNA was clearly present in the trigeminal ganglion, which was used as a positive control [for details, see Olah, Rumbus, *et al*. (2021); also included in the appendix]. Then we used RNAscope in situ hybridization, a highly sensitive method that can detect transcripts as single molecules (Femino *et al*., 1998). We found detectable *Trpa1* mRNA transcripts in all of the studied thermoregulation-related nuclei, *viz.*, in the medial preoptic area, dorsomedial hypothalamic area, lateral parabrachial nucleus, and rostral raphe pallidus, although it should be noted that the number of the *Trpa1* transcripts was low (Fig. 12).

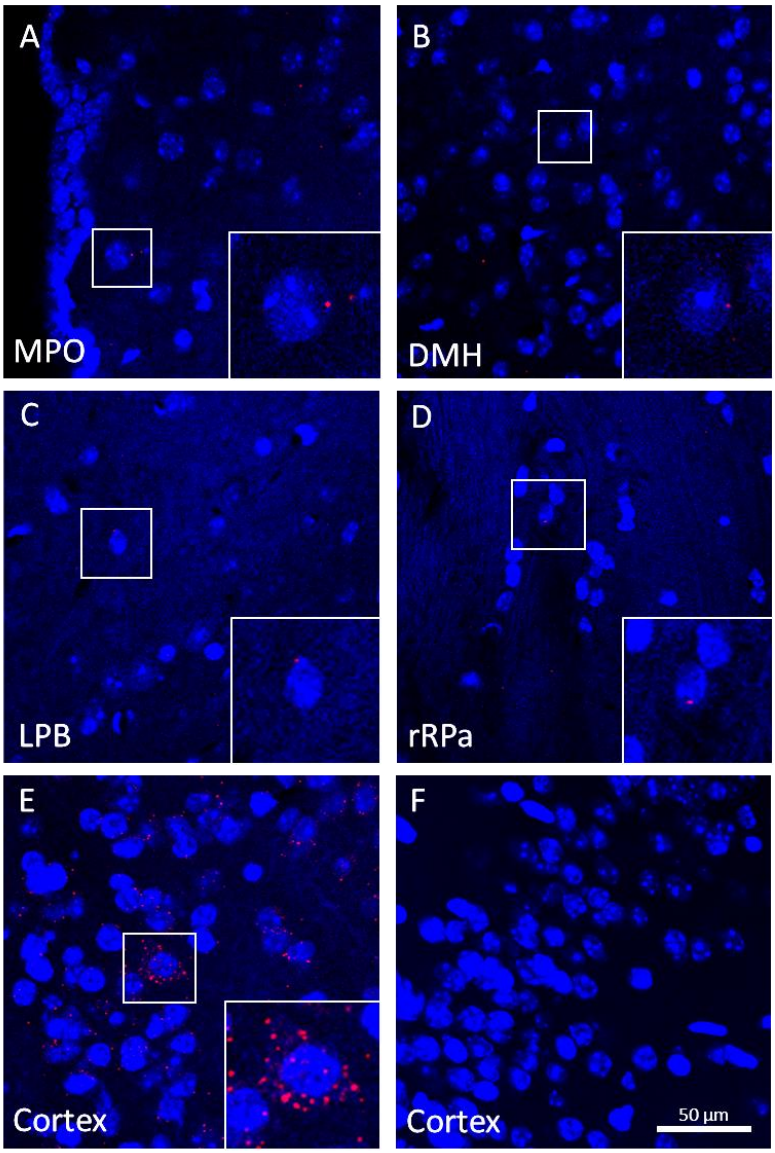

Figure 12 RNAscope in situ hybridization in the mouse brain for *Trpa1* mRNA. Representative confocal images of (**A**) the medial preoptic area (MPO), (**B**) the dorsomedial hypothalamic area (DMH), (**C**) the lateral parabrachial nucleus (LPB), and (**D**) the rostral raphe pallidus nucleus (rRPa). Note the low number of *Trpa1* mRNA transcripts (red) in the areas **A-D** as also shown in higher magnification insets representing the respective boxed areas. Positive control staining for *Ppib* mRNA (red) in the cortex (**E**) and negative control staining for the bacterial *dabP* mRNA (red) in the same cortical area (**F**). Cell nuclei were counterstained with DAPI (blue). Scale bar: 50 µm for all images (Olah, Rumbus, *et al.*, 2021).

Discussion

During the past 20 years there were several clinical trials which compared therapeutic moderate hypothermia (32 to 35°C) and normothermia in patients with severe TBI. In the majority of these trials there was no improvement of the outcome with the use of hypothermia, although there were subgroups of patients that may have benefited from hypothermia. Methodological differences prevented the direct comparison of these studies.

In the present thesis, we showed that whole-body cooling decreases the risk of death in patients with severe TBI by conducting meta-analysis of clinical trials (Olah *et al*., 2018), which were homogenous with regards to statistical, clinical, and methodological designs. With forest plot analysis of the cooling parameters we revealed that deeper and longer cooling and to a lesser extent reasonably fast rewarming are the most important to improve the outcome of severe TBI.

We introduced the COIN to assess the summed effect of the cooling, and showed that therapeutic hypothermia is beneficial in severe TBI only if the COIN is sufficiently high (Olah *et al*., 2018). The benefits of therapeutic hypothermia in severe TBI have been long debated. In 2018, the POLAR study (Cooper *et al*., 2018), a high-quality international trial, appeared to end the debate by showing that therapeutic hypothermia did not improve mortality in severe TBI. However, the POLAR-based recommendation to abandon therapeutic hypothermia was challenged by different authors. We calculated the COIN for the POLAR study and ran a new meta-analysis (Olah, Poto, *et al*., 2021), which included the POLAR data and accounted for the cooling extent.

Our novel "POLARized" COIN analysis indicated that the results of POLAR strengthen our former conclusions about the beneficial effects of therapeutic hypothermia on death rate in severe TBI if COIN is sufficiently high. Although POLAR was a high-quality, international study, deviations from the protocol resulted in smaller extent of cooling (low-to-moderate COIN) than targeted (high COIN) (Table 2), which could mask the benefits of therapeutic hypothermia in the overall cohort. It would be inevitable to separately analyze the outcomes in those patients who fully complied with the targeted cooling criteria (high COIN) and in those patients who did not. Until such result are available from POLAR or other high-quality trial(s), the idea of therapeutic hypothermia in severe TBI should not be abandoned.

The benefits of therapeutic hypothermia were also shown in the recent review by Moore *et al*. (2020). The authors applied the umbrella review methodology to several potentially low-value clinical practices and found that therapeutic hypothermia was the only one with evidence of benefit. However, the POLAR study was not included in any of the systemic reviews analyzed in the umbrella review by Moore *et al*. (2020). When the results of POLAR were translated into treatment guidelines prior to our POLARized meta-analysis (Chesnut *et al*., 2020; Hawryluk *et al*., 2019), the absence of an overall beneficial effect led to the recommendation to decrease the use of therapeutic hypothermia in severe TBI. However, some deviations from the cooling protocol occurred at different POLAR-participating centers and decreased the overall extent of cooling from "high" (targeted) to "moderate" (overall achieved) and even "low" (observed in many patients). This decrease in the COIN was likely to mask the benefits of therapeutic hypothermia in the overall

cohort. Identification of the exact cooling protocol for a specified patient population would be in line with recent paradigms in the treatment of TBI, suggesting the need for targeted management of individuals or subsets of patients to improve the outcome (Sheriff & Hinson, 2015; Stocchetti *et al*., 2017).

In the second part of my work, we studied the mechanisms of H_2S-induced hypothermia in animal experiments (Olah, Rumbus, *et al*., 2021) and we concluded that fast- and slow-releasing H_2S donors cause hypothermia which is mediated by reduced thermogenesis and increased cutaneous vasodilation. The hypothermic and hypometabolic effects are triggered from the central nervous system and both of them are strongly attenuated in the absence of the TRPA1 channel. TRPA1 channels located on hypothalamic neurons within autonomic thermoeffector pathways can be suggested as the molecular targets for the H_2S-induced hypothermia.

Our aim was to find the molecular target responsible for the hypothermic response to H_2S. Temperature-sensitive members of the TRP channel superfamily can function as thermoreceptor elements in the thermoregulation system (Romanovsky, 2018), but nonthermal activation of some of these TRP channels can also occur and contribute to the regulation of deep T_b independently from whether the given channel is a thermosensor or not, as it was discovered in case of TRPV1 (Garami *et al*., 2020; Romanovsky *et al*., 2009). With regards to an interaction between H_2S and thermosensitive TRP channels, strong evidence accumulated until present days for an action of H_2S on the TRPA1 channel in a vast number of different experimental models (Pozsgai, Bátai, *et al*., 2019), but whether TRPA1 also mediates the hypothermic effect of H_2S was unknown until now. In

our work (Olah, Rumbus, *et al*., 2021), we studied the thermoregulatory response to H_2S donors (Na_2S and GYY4137) in the genetic absence of the TRPA1 channel by using *Trpa1*$^{-/-}$ mice. We showed that the hypothermic and the hypometabolic responses are both attenuated in *Trpa1*$^{-/-}$ mice as compared to their *Trpa1*$^{+/+}$ littermates. The contribution of TRPA1 to the thermal effect of the H_2S donors used in our study is in harmony with a previous report about the hypothermic effects of a polysulfide, dimethyl trisulfide, which was also attenuated in *Trpa1*$^{-/-}$ mice (Pozsgai *et al*., 2017). However, polysulfides activate TRPA1 320 times more potently than H_2S (Kimura *et al*., 2013), thus it was crucial to understand whether H_2S delivered by different non-polysulfide donors can evoke TRPA1-mediated hypothermia. Our findings clearly indicate, for the first time to our knowledge, that hypothermia induced by either fast- or slow-releasing H_2S donors is mediated by the TRPA1 channel in unanesthetized mice.

Conclusions

In the present work, we used a novel approach to determine the efficacy of therapeutic hypothermia in TBI. In our meta-analysis, we carefully evaluated all studies identified by literature search based on statistical design, patient inclusion criteria, and the applied cooling protocol, thereby we identified studies which were homogenously designed from three aspects: statistically (randomization), clinically (whole-body cooling versus no temperature management of patients with severe TBI), and methodologically (cooling protocols precisely reported). Then, we conducted meta-analyses of these studies to evaluate the effects of therapeutic hypothermia as well as that of the individual parameters of the cooling protocol on the mortality rate of patients with severe TBI. We introduced the COIN, an integrated measure of therapeutic hypothermia calculated from three different cooling parameters, and studied its relation to mortality in severe TBI. In conclusion, including the POLAR study in our COIN-based meta-analysis suggests that the COIN should be flipped again to settle the dispute on the use of therapeutic hypothermia in severe TBI.

Seminal work by Blackstone *et al*. (2005), has re-established H_2S as a thermoregulatory mediator (Mancardi *et al*., 2009). Findings from the second part of my dissertation are consistent with the concept of H_2S-induced thermoregulatory effects are TRPA1-mediated. Furthermore, central H_2S effects induce hypothermia, in part, through inhibiting thermogenesis. We demonstrated that administration of H_2S-donors, specifically Na_2S and GYY4137, (i) induce hypothermia in a dose-dependent fashion following i.c.v.

administration; (ii) evoke hypothermia with different dynamics; (iii) do not induce significant thermoregulatory effects when delivered i.p.; and (iv) evoke the hypothermic response through the mediation of the TRPA1 channel. In sum, we showed that slow- and fast-releasing H_2S donors induce hypothermia through hypometabolism and cutaneous vasodilation in mice and that the hypothermic effect of H_2S is mediated by TRPA1 channels located in the brain, presumably on hypothalamic neurons within the autonomic thermoeffector pathways. Our findings highlight the importance of central TRPA1-mediated H_2S signaling in the thermoregulation system. In severe forms of systemic inflammation (*e.g.*, septic shock), which is often associated with hypothermia (Garami, Steiner, *et al.*, 2018) and by enhanced production of H_2S (Bhatia & Gaddam, 2021; Whiteman & Winyard, 2011), the interaction between TRPA1 and H_2S can play a crucial role in the development of the response and, as perspective, may serve as a therapeutic target. Furthermore, the H_2S-induced activation of central TRPA1 channels may pave the road to the development of controlled induction of therapeutic hypothermia, but future research is needed to reveal the true thermopharmacological potential of the central TRPA1-H_2S interaction. A postulation that is also supported by Kwiatkoski *et al.*, who reported that increased H_2S induces cryogenic effects during hypoxia (Kwiatkoski *et al.*, 2012).

Future perspectives

Reducing deep T_b is likely to be beneficial in humans and other species when subjected to severe TBI and possibly to other brain-damaging insults. The manner of achieving the decrease in T_b, whether it is physically forced or pharmacologically regulated, may have a profound effect on the therapeutic efficacy of the hypothermia. Pharmacologically induced and controlled hypothermia would seem to be the best method of achieving a therapeutic benefit of hypothermia. Our understanding of regulated hypothermia in large species such as humans is poorly understood. More research on the mechanisms of thermoregulation with special emphasis on the pharmacological possibilities and its control is essential for future use of hypothermia as a therapeutic agent.

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Finally, I cannot be greatful enough to my family for standing beside me, my husband and my four children for encouraging me all the time with patience and love.

Appendix

Publications related to the subject of the thesis

- Number of publications related to the subject of the thesis: 3
- Number of publications not related to the subject of the thesis: 17
- Number of book chapters: 3
- Sum of all impact factors: 37.435
- Sum of impact factors from publications related to the topic of PhD thesis: 14.886
- All citations: 75
- Independent citations: 57

Publications related to the topic of the PhD thesis

Olah, E; Poto, L; Hegyi, P; Szabo, I; Hartmann, P; Solymar, M; Petervari, E; Balasko, M; Habon, T; Rumbus, Z; Tenk, J; Rostas, I; Weinberg, J; Romanovsky, A A; Garami A: Therapeutic whole-body hypothermia reduces death in severe traumatic brain injury if the cooling index is sufficiently high: Meta-analyses of the effect of single cooling parameters and their integrated measure. J Neurotrauma. 2018;35(20):2407-2417. **(IF: 3.754)**

Olah, E; Rumbus, Z; Kormos, V; Tekus, V; Pakai, E; Wilson, HV; Fekete, K; Solymar, M; Kelava, L; Keringer, P; Gaszner, B; Whiteman, M; Keeble, J; Pinter, E; Garami, A: The hypothermic effect of hydrogen sulfide is mediated by the transient receptor potential ankyrin-1 channel in mice. Pharmaceuticals. 2021;14(10): 992. **(IF 5.863)**

Olah, E; Poto, L; Rumbus, Z; Pakai, E; Romanovsky, A A; Hegyi, P; Garami, A: POLAR study revisited: Therapeutic hypothermia in severe brain trauma should not be abandoned. J Neurotrauma. 2021;38(19): 2772-2776. (**IF: 5.269)**

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Horváth, ÁI; Szentes, N; Tékus, V; Payrits, M; Szőke, É; **Oláh, E**; Garami, A; Fliszár-Nyúl, E; Poór, M; Sár, C; Kálai, T; Pál, Sz; Percze, K; Nagyné Scholz, É; Mészáros, T; Tóth, B; Mátyus, P; Helyes, Zs: Proof-of-Concept for the Analgesic Effect and Thermoregulatory Safety of Orally Administered Multi-Target Compound SZV 1287 in Mice: A Novel Drug Candidate for Neuropathic Pain. Biomedicines 2021;9(7): 749, 18 p. (**IF 6.081)**

Keringer, P; Furedi, N; Gaszner, B; Miko, A; Pakai, E; Fekete, K; **Olah, E**; Kelava, L; Romanovsky, AA; Rumbus, Z; Garami, A: The hyperthermic effect of central cholecystokinin is mediated by the cyclooxygenase-2 pathway. American Journal of Physiology-Endocrinology and Metabolism. 2021; Epub ahead of print. **(IF 4.310)**

Rumbus, Z; **Olah, E**; Lamberti, A; Lorincz, A; Fekete, K; Kelava, L; Pakai, E; Garami, A: TR(i)Ps in the realm of thermophysiology In: Rakonczay, Zoltán; Kiss, Lóránd (szerk.) Proceedings of the EFOP-3.6.2-16-2017-00006 (LIVE LONGER) project Szeged, Magyarország: University of Szeged 2020; 99: 51-51.

Ottóffy, M; Mátrai, P; Farkas, N; Hegyi, P; Czopf, L; Márta, K; Garami, A; Balaskó, M; **Pótóné-Oláh, E**; Mikó, A; Rostás, I; Wobbe, B; Habon, T: Uninterrupted or Minimally Interrupted Direct Oral Anticoagulant Therapy is a Safe Alternative to Vitamin K Antagonists in Patients Undergoing Catheter Ablation for Atrial Fibrillation: An Updated Meta-Analysis. J. Clinical Medicine 2020;9(10): 3073. (**IF 4.241**)

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Garami A, Pákai E, Kéringer P, Rumbus Z, Mikó A, Gáspár P, **Oláh E**, Romanovsky A. A: Transient receptor potential vanilloid-1 (TRPV1) channel antagonists as candidates of targeted body temperature modulation – an overview. PPTR 7th International Conference on the Physiology and Pharmacology of Temperature Regulation, Split, October 7-12, 2018

P. Keringer, Z Rumbus, A Miko, A Csenkey, P Gaspar, E Pakai, **E Olah**, N Khidhir, N Furedi, Z Horvath-Szalai, C Zsiboras, M Solymar, E Polyak, B Gaszner, A Garami: Acute effects of saccharin on the energetic homeostasis in rodents. PPTR 7th International Conference on the Physiology and Pharmacology of Temperature Regulation, Split, October 7-12, 2018

Rumbus Z, Jakus P, **Oláh E**, Pákai E, Gáspár P, Kéringer P, Garai J, Loránd T, Garami A: The involvement of the macrophage migration inhibitory factor in lipopolysaccharide-induced fever and hypothermia in mice. PPTR 7th International Conference on the Physiology and Pharmacology of Temperature Regulation, Split, October 7-12, 2018

National oral and poster presentations

Kéringer P, Erdélyi A, **Oláh E**, Tékus V, Solymár M, Pákai E, Rumbus Z, Kemény Á, Gaszner B, Pintér E, Garami A: A tranziens receptor potenciál ankyrin-1 szerepe hidrogén-szulfid által indukált hipotermiában. Magyar Kísérletes és Klinikai Farmakológiai Társaság, Magyar Anatómus Társaság, Magyar Mikrocirkulációs és Vaszkuláris Biológiai Társaság, Magyar Élettani Társaság Közös Vándorgyűlése – FAMÉ 2019, Budapest, 2019. jún. 5-8.

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Rumbus Z, **Oláh E**, Pótó L, Hegyi P, Szabó I, Hartmann P, Solymár M, Petervári E, Balaskó M, Habon T, Tenk J, Rostás I, Weinberg J, Romanovsky AA, Pákai E, Kéringer P, Garami A: A novel measure to assess the efficacy of therapeutic whole-body hypothermia in severe traumatic brain injury: the cooling index. Magyar Idegtudományi Társaság 16. Kongresszusa. 2019. január 17-18. Debrecen

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Therapeutic Whole-Body Hypothermia Reduces Death in Severe Traumatic Brain Injury if the Cooling Index Is Sufficiently High: Meta-Analyses of the Effect of Single Cooling Parameters and Their Integrated Measure

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Abstract

Therapeutic hypothermia was investigated repeatedly as a tool to improve the outcome of severe traumatic brain injury (TBI), but previous clinical trials and meta-analyses found contradictory results. We aimed to determine the effectiveness of therapeutic whole-body hypothermia on the deaths of adult patients with severe TBI by using a novel approach of meta-analysis. We searched the PubMed, EMBASE, and Cochrane Library databases from inception to February 2017. The identified human studies were evaluated regarding statistical, clinical, and methodological designs to ensure interstudy homogeneity. We extracted data on TBI severity, body temperature, death, and cooling parameters; then we calculated the cooling index, an integrated measure of therapeutic hypothermia. Forest plot of all identified studies showed no difference in the outcome of TBI between cooled and not cooled patients, but interstudy heterogeneity was high. On the contrary, by meta-analysis of randomized clinical trials that were homogenous with regard to statistical, clinical designs, and precisely reported the cooling protocol, we showed decreased odds ratio for death in therapeutic hypothermia compared with no cooling. As independent factors, milder and longer cooling, and rewarming at <0.25°C/h were associated with better outcome. Therapeutic hypothermia was beneficial only if the cooling index (measure of combination of cooling parameters) was sufficiently high. We conclude that high methodological and statistical interstudy heterogeneity could underlie the contradictory results obtained in previous studies. By analyzing methodologically homogenous studies, we show that cooling improves the outcome of severe TBI, and this beneficial effect depends on certain cooling parameters and on their integrated measure, the cooling index.

Keywords: induced hypothermia; meta-analysis; mortality; thermoregulation; traumatic brain injury

Introduction

Traumatic brain injury (TBI) is recognized as a significant cause of death and morbidity predominantly in the young population.[1] TBI is estimated to affect 10 million persons annually worldwide, and by 2020 it can be one of the major causes of death and disability, posing a global health and financial burden for the society.[2] Among the leading causes of TBI are motor vehicle accidents in both more and less developed countries, most frequently involving young males.[2,3] Those with mild head injuries may recover fully without any specific treatment, whereas severe injuries are often rapidly fatal or leave survivors with disabilities.[4] Severe TBI constitutes a major health and socioeconomic problem worldwide.[5]

The pathomechanisms underlying severe TBI are complex and often involve focal as well as diffuse changes.[6] Focal lesions include contusional damages of the brain parenchyma, most commonly in the frontal and temporal lobes, as well as disruptions in vasculature, resulting in intracerebral and extracerebral heamatomas.[5,6] In a typical case like the one presented in Supplementary Table S1 (see online supplementary material at ftp.liebertpub.com), focal lesions are diagnosed with routine imaging techniques, such

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as computed tomography (see Supplementary Fig. S1; see online supplementary material at ftp.liebertpub.com). The primary head injury can initiate a number of pathophysiological processes that will determine the extent and the duration of the damage. These mechanisms involve ischemia, swelling (edema), neurotransmitter release (excitotoxicity), formation of free radicals, ionic flux-mediated damage, metabolic and mitochondrial dysfunction, and neuroinflammatory responses.[4–6]

Many of the pathological mechanisms associated with TBI are temperature-sensitive,[7] suggesting that at a lower deep body temperature the adverse processes can be decelerated and neuroprotective effects can be achieved. Therapeutic hypothermia has been investigated as a possible neuroprotective strategy to attenuate the harmful effects of severe TBI. In animal models of TBI, beneficial effects of therapeutic hypothermia have been shown repeatedly,[8–11] but clinical studies provided contradictory results.

The first report of the use of hypothermia in TBI was in 1943,[12] while randomized controlled studies appeared only at the end of the 1990s.[13] Over the last 25 years, numerous clinical trials have been conducted to assess the effects of induced hypothermia in severe TBI.[13–31] Cooling of patients with severe TBI improved the outcome in several of these studies,[14,17,21,23,25,26,29–32] while other trials suggested weak or no evidence for the use of therapeutic hypothermia after TBI.[16,20,27,33–35] It has to be noted that the study design (e.g., randomization), inclusion criteria of patients, and the applied cooling protocol varied substantially among the trials, which differences could have contributed to the contradictory findings in the human studies.

As attempts to ultimately answer the question of whether therapeutic hypothermia improves the outcome of TBI, several meta-analyses have also been performed.[36–42] The different analyses provided contradictory results, however. While about half of them showed that therapeutic hypothermia might be effective in the treatment of patients with TBI and could reduce death,[36,38,39,41] the other half indicated that it did not decrease the mortality rate.[37,40,42] It can be assumed that the high interstudy variability in the statistical and clinical designs of the trials that were included in some of the meta-analyses and the different study selection protocols were responsible for the contradictory results. Indeed, high interstudy heterogeneity was reported in all of the performed meta-analyses so far.[36–42]

In the present study, we used a novel approach to determine the efficacy of therapeutic hypothermia in TBI. In our meta-analysis, we carefully evaluated all studies identified by literature search based on statistical design, patient inclusion criteria, and the applied cooling protocol; thereby, we identified studies that were homogenously designed from three aspects: statistically (randomization), clinically (whole-body cooling vs. no temperature management of patients with severe TBI), and methodologically (cooling protocols precisely reported). Then, we conducted meta-analyses of these studies to evaluate the effects of therapeutic hypothermia as well as that of the individual parameters of the cooling protocol on the mortality rate of patients with severe TBI. We introduced the cooling index, an integrated measure of therapeutic hypothermia calculated from three different cooling parameters, and studied its relation to the mortality rate in severe TBI.

Methods

Our meta-analysis was conducted in accordance with the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis Protocols (Supplementary Table S2; see online supplementary material at ftp.liebertpub.com).[43] The analysis was based on the Participants, Intervention, Comparison, Outcome (PICO) model: in the severe TBI population, we aimed to assess the effect of therapeutic whole-body hypothermia compared with no cooling on the mortality ratio. This meta-analysis has been registered with PROSPERO International Prospective Register of Systematic Reviews (CRD42017056535).

Literature search

A systematic search of the PubMed, EMBASE, and Cochrane Library databases was performed from inception to February 2017 with using the following Medical Subject Headings and search terms: (''hypothermia'' OR ''cooling'') AND (''traumatic brain injury'' OR ''TBI'') AND (''mortality'' OR ''death'' OR ''survival''). Then, the ''human'' filter was selected. We restricted our search to original studies published in English without time period limitations. We included journal articles of the following study types: clinical study, clinical trial, comparative study, controlled clinical trial, controlled study, multi-center study, observational study, and randomized controlled trial.

The search for the articles was conducted separately by two authors (EO and AG), who also assessed study eligibility, and extracted data from the selected studies independently. Disagreements were resolved by consensus with the help of a third author (ZR).

Data extraction

Only those studies were included in the meta-analyses in which the effect of therapeutic hypothermia was compared with no cooling interventions on the mortality ratio of adult patients with severe TBI. Nineteen randomized controlled trials (RCTs)[13,14,16,18,20–31,44–46] and eight articles[15,17,19,34,47–50] of other study types were eligible for meta-analysis. The relevant data were extracted from these studies by two investigators (EO and AG) independently into Microsoft Excel software (Microsoft Corporation, Redmond, WA). The following information was collected from each of the selected articles: authors' names and date of publication, study type and randomization, characteristics of patient population (e.g., mean age, sample size, intracranial pressure [ICP], and Glasgow Coma Scale [GCS] score at admission), methods of whole-body cooling (target temperature, duration of hypothermia, and rate of rewarming), interventions in the no cooling group, and mortality rates in the study groups.

Data evaluation protocols

The collected data were evaluated based on a statistical and a biomedical approach. Statistically, we assessed the randomization of the collected studies based on the authors' statements, Jadad analysis, and detailed evaluation of the randomization protocol described in the study (see Quality assessment). In the biomedical evaluation, we narrowed the list of eligible studies to those that dealt with the effects of therapeutic whole-body hypothermia compared with no temperature management; therefore, we excluded those articles in which selective brain cooling[44,46,51] or fever control with antipyretics[34,47,48] was used, or when patients with spontaneous hypothermia were involved in the study.[49,52] Eligible studies resulting from the statistical or the biomedical refinement or both were compared with the random effect model of meta-analysis for odds ratio (OR) as effect size.

To assess which parameters of the cooling methods have the biggest impact on the outcome of severe TBI, the studies were divided into subgroups based on target cooling temperature, cooling duration, and speed of rewarming. Target cooling temperatures were classified *a priori* as ''moderate'' (32–33°C)[53] and ''mild'' (33–35°C).[29,53] Cooling duration was divided into short (24–48 h) and long (>48 h) subgroups.[54] According to recent recommendations about the use of very slow (0.1–0.2°C/h) rewarming rates in

patients with severe TBI,[55,56] the speed of rewarming was divided into either of two subgroups: 0.25–1°C/h and less than 0.25°C/h, also including passive. Rewarming was determined passive if the cooling process ensued without induction of any cooling or rewarming device within the next 18–24 h.[57,58] For quantification purposes, the speed of passive rewarming was considered to equal 0.06°C/h.[57,58]

In addition to analyzing the effect of each cooling parameter individually, the influence of the used combinations of the three parameters together was also assessed on the outcome of severe TBI. When all three parameters—i.e., target cooling temperature, cooling duration, and speed of rewarming—were reported in the same study, we calculated the integrated measure of cooling and named it as the cooling index. The cooling index represents the area between the body temperature curve of cooled patients and a hypothetical horizontal line corresponding to a normal body temperature of 36.5°C (Supplementary Fig. S2; see online supplementary material at ftp.liebertpub.com). The following formula was used: "cooling index" = $\Delta T \times t + (\Delta T \times \Delta T/R)/2$, where "$\Delta T$" is the magnitude of hypothermia, i.e., the difference between normal (36.5°C) and target cooling temperatures expressed in °C; "t" is duration of the maintenance of hypothermia in hours; and "R" is the rate of rewarming (°C/h); see also Fig. S2A.

The impact of the rewarming rate was incorporated in the cooling index as the area of the triangle that represents the change in body temperature during the rewarming phase. It has to be noted that because of the relatively narrow range (0.06–1°C/h) of the rewarming rates used in the analyzed studies, this area had the smallest contribution to the final value of the cooling index, while depth and duration of the cooling were more dominant contributors.

The cooling index corresponds with the integrated measure of the magnitude and duration of therapeutic hypothermia. For example, a similarly high cooling index can result from short and deep cooling with rewarming at 0.25°C/h (Supplementary Fig. S2B), as well as from longer, but milder cooling with slower, passive rewarming (Supplementary Fig. S2A). A moderate cooling index can result from mild and short cooling followed by slow rewarming at ~0.08°C/h (Supplementary Fig. S2C), while the cooling index is low if cooling duration is brief and rewarming is faster (1°C/h) even if the target cooling temperature is very low (Supplementary Fig. S2D). For meta-analysis, the included studies were evenly distributed ($n = 4$) into subgroups with low (<160°C × h), moderate (160–200°C × h), and high (>200°C × h) cooling index. Examples for the assessment of the cooling index in each of the three subgroups are presented in Supplmentary Figure S2.

In five of the analyzed studies,[14,25,26,29,31] the cooling protocol was changed depending on the ICP. Thus, we also compared whether the outcome of TBI differed between the group of studies that reported adjustments in the cooling protocol based on ICP and the group of studies that did not.

Quality assessment

To evaluate the quality of the included trials, two independent reviewers (EO and ZR) assessed the bias of the included studies according to the Cochrane Handbook.[59] The methodology described for random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, completeness of outcome data, and selective outcome reporting was assessed during the analysis.

Because of the nature of the studies eligible for our meta-analyses, the blinding method could not be assessed. The Jadad score, a five-point quality scale, was used also to analyze the quality of the articles.[60] Further, an additional three-step evaluation of the randomization used in each study was performed, which was more relevant for such studies included in our meta-analysis. The first two questions of this evaluation were similar to those of the Jadad score—viz., whether the use of randomization was stated and whether the randomization procedure was appropriate and reported in the study. In the third step, the level of randomization was determined as good if the use of randomization and its exact protocol was reported in the study. If the use of randomization was stated, but the exact method was not described, the randomization level was assessed as medium, while in cases when the authors did not mention randomization, the level of randomization was considered low. This evaluation was necessary, because there were several studies in which the use of randomization was stated, but the exact method was not described,[21,23,25,27–31] which leaves the statement open for questions.

Statistical analysis

The statistical analysis was performed according to the standard methods of meta-analysis. The primary effectiveness outcome was all-cause death. The OR with 95% confidence intervals (CI) for death in the adult patients with severe TBI were calculated in a random-effects model of meta-analysis. Summary effect estimates were stratified by study design and the between-groups effects were assessed.

Publication bias was assessed with funnel plots by using the Duval and Tweedie trim and fill method[61] and the Egger test (Egger test values of less than 0.1 were considered as indicators of significant small-study effect) (see Supplementary Figs. S6–S9). Between-study heterogeneity was tested with the Q homogeneity test (p values of less than 0.05 were considered as indicators of significant heterogeneity) and with the I^2 statistical test, where I^2 is the proportion of total variation attributable to between-study variability (an I^2 value of more than 50 was considered as indicating considerable heterogeneity). Results of the meta-analyses are depicted as forest plots. All analyses were performed using the Comprehensive Meta-Analysis software (version 3.3; Biostat, Inc., Engelwood, NJ).

Results

Study selection

The flow chart of the study selection is presented in Figure 1. Until February 23, 2017, the literature search identified altogether 709 studies from the PubMed, EMBASE, and Cochrane databases. After enabling filters for human studies and English language and using additional filters (study types), 321 studies remained, which were screened for title and abstract for inclusion criteria. Then 273 articles were excluded because of insufficient data reporting or because children were studied; 48 studies were included in qualitative synthesis. A further 21 articles were excluded because of the lack of death data. Then 27 studies were included and pooled for quantitative synthesis.

When we compared the effects of therapeutic hypothermia with no cooling by including all 27 identified studies in the meta-analysis (Supplementary Fig. S3; see online supplementary material at ftp.liebertpub.com), we did not find a significant difference in the OR for death between the groups. Importantly, the included studies were methodologically quite heterogeneous with regard to both statistical and clinical designs ($Q = 167$, $p < 0.001$; $I^2 = 84$).

As a statistical approach to reduce heterogeneity, we analyzed separately those 19 studies that could be considered as RCTs (Supplementary Fig. S4; see online supplementary material at ftp.liebertpub.com). Therapeutic hypothermia tended to improve the outcome in patients with severe TBI, but the difference did not reach the level of significance (OR = 0.782; $p = 0.075$). Between-study heterogeneity, however, was still reasonably high as indicated by the nearly significant result of the Q homogeneity test ($p = 0.053$). Based on detailed quality assessment of the randomization protocols in the studies, the level of randomization was assessed as good in three

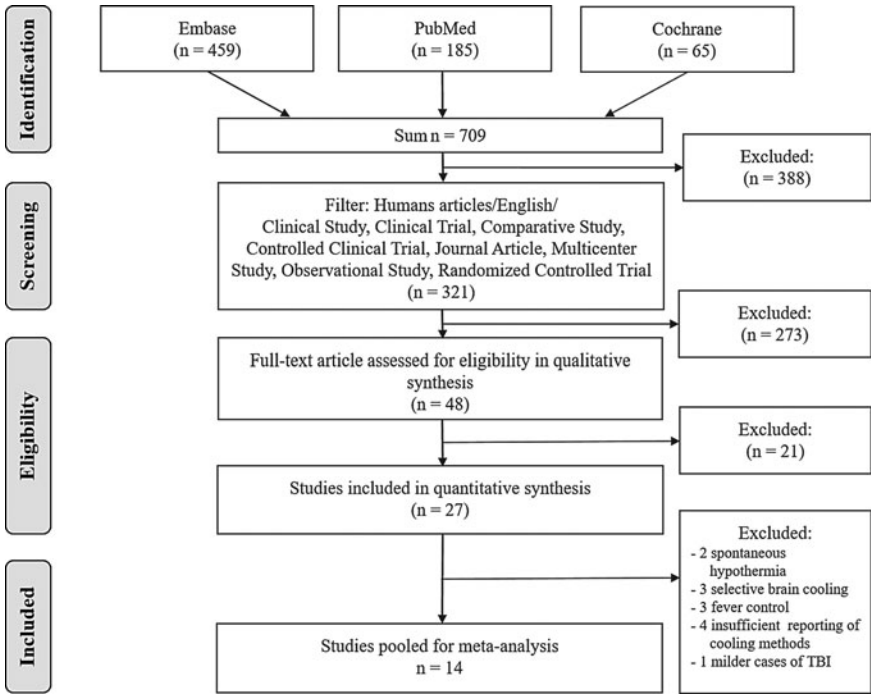

FIG. 1. Flow chart of study selection and inclusion.

studies, medium in nine studies, and low in two studies (Supplementary Table S3; see online supplementary material at ftp.liebertpub.com). The remaining five RCTs were not included in this assessment, because they did not pass the criteria set for homogenous clinical and methodological designs (see below).

The use of therapeutic hypothermia significantly decreased the risk of death in the subgroup of medium randomization level (OR = 0.676; $p = 0.041$), while no significant difference was found between cooling versus no cooling in the other two subgroups (Supplementary Fig. S5; see online supplementary material at ftp.liebertpub.com). When the good and medium subgroups were merged, their pooled OR remained significantly lower than 1 (OR = 0.66, $p = 0.003$), which indicated better outcome in therapeutic hypothermia, but clinically, the interstudy heterogeneity was still considerably high.

We evaluated the studies based on clinical and methodological designs. Our goal was to study the effect of therapeutic hypothermia applied to the whole body of patients with severe TBI, but without spontaneous hypothermia and without temperature control. Therefore, three trials using selective brain cooling,[44,46,51] two articles including cases of spontaneous hypothermia,[49,52] and three studies applying fever control[34,47,48] had to be excluded from the analysis. Further, four studies could not be included in the final analyses, because the applied cooling methods were not reported in sufficient details.[15,18,19,22] In one of the trials, the GCS score of the included patients ranged between 3–15,[16] which sample also includes mild (GCS = 13–15) and moderate cases of TBI (GCS = 9–12); therefore, it also had to be excluded from further analysis (Fig. 1).

As a result of the combined (statistical and physiological) evaluation of the studies identified by our literature search, 14 full-text publications (involving 1786 adult patients with severe TBI; 896 in the therapeutic hypothermia group and 890 in the no cooling group), were included in the next steps of our analysis. The descriptive statistics of the age, GCS score, ICP, and injury severity score in the patient populations from these 14 studies are presented in Table 1. These data reported from a big patient population (N >1700) correspond with the clinical parameters observed in the patient presented in Supplementary Table S1. The publication years of the studies ranged from 1993 to 2017. All of them can be considered as randomized trials, in which the exact cooling methods of the whole body (target temperature, cooling duration, and speed of rewarming) are reported, and the effect of therapeutic hypothermia on death was compared with patients without temperature management in severe TBI.

The homogeneity of the studies was verified by Egger test, Q, and I^2 statistics, which showed no significant difference in interstudy variability (Egger $p = 0.509$; $Q = 17$, $p = 0.224$; $I^2 = 21$). Meta-analysis of these studies revealed that therapeutic hypothermia significantly improved the outcome of severe TBI (OR = 0.675; $p = 0.004$) (Fig. 2).

Next, we studied the different parameters of the cooling protocol—viz., target temperature, cooling duration, and speed of rewarming. Based on target cooling temperatures, four studies used ''moderate'' (equal or less than 33°C) and seven studies ''mild'' (33–35°C) hypothermia, while in three studies, the reported target temperature range overlapped between the moderate and mild groups (Fig. 3). We found that the use of mild therapeutic hypothermia considerably improved the outcome (decreased the risk of death) compared with no cooling (OR = 0.627; $p = 0.050$). No significant differences were found in the moderate hypothermia and the overlapping target temperature groups (Fig. 3).

With regard to cooling duration, in nine studies, short (24–48 h) hypothermia was used, while in five studies, cooling was longer than 48 h (Fig. 4). The effect of long-term hypothermia was beneficial on the mortality rate of patients with severe TBI compared with patients with no temperature management (OR = 0.534; $p < 0.001$), while in the case of short-term hypothermia, the effect was not significant (Fig. 4).

Based on the speed of rewarming, the studies were divided into subgroups using rewarming rates of either 0.25–1°C/h or less than

Table 1. Characteristics of the Patient Groups with Severe Traumatic Brain Injury in the Studies Included in the Meta–Analyses

Study	*Mean age (year)*	*GCS*	*Treatment*	*Number of patients (N)*	*ICP (mmHg)*	*ISS*	*Cooling duration (h)*	*Target temperature (°C)*	*Rewarming rate (°C/h)*	*Cooling index (°C × h)*	*Cooling index subgroup*
Clifton (1993)	29.1	4–7	Cooling	24	14.5	NR	48	32–33	0.25	224	High
			No cooling	22	16.4	NR					
Clifton (2001)	31.5	3–8	Cooling	190	18.1	28 ± 9	48	32.5–34	0.25	177	Moderate
			No cooling	178	17.9	28 ± 8					
Clifton (2011)	28.5	3–8	Cooling	52	NR	30 ± 6	48	33–34	0.25	162	Moderate
			No cooling	45	NR	30 ± 9					
Hashiguchi (2003)	34.1	3–8	Cooling	9	11.1 ± 4.8	30.7 ± 5.6	48	33.5–34.5	~ 0.08	159	Low
			No cooling	8	11.9 ± 4.2	33.0 ± 6.4					
Jiang (2000)	41.4	3–8	Cooling	43	29.6 ± 2.2	NR	72–336	33–35	1	243	High
			No cooling	44	30.3 ± 3.0	NR					
Liu (2015)	NR	3–8	Cooling	110	27.5 ± 16.9	NR	72–288	35–36	< 0.25	–	–
			No cooling	110	26.8 ± 17.5	NR					
Marion (1997)	33.5	3–7	Cooling	40	15.4	NR	24	32–33	1	104	Low
			No cooling	42	19.7	NR					
Polderman (2002)	36.7	3–8	Cooling	64	37.0 ± 20.0	NR	24	32	0.08	–	–
			No cooling	72	< 20.0	NR					
Shiozaki (1993)	35.4	<8	Cooling	16	35.4 ± 12.0	NR	48	33.5–34.5	Passive	172	Moderate
			No cooling	17	36.9 ± 12.1	NR					
Shiozaki (2001)	38.5	3–8	Cooling	45	NR	NR	48	33.5–34.5	~ 0.08	159	Low
			No cooling	46	NR	NR					
Smrcka (2005)	41.0	3–8	Cooling	35	NR	11.8 ± 5	72	34	Passive	232	High
			No cooling	37	NR	17.6 ± 7					
Tang (2017)	41.1	3–8	Cooling	30	NR	NR	48	32–35	0.25	162	Moderate
			No cooling	30	NR	NR					
Zhao (2011)	37.2	3–8	Cooling	40	15.9 ± 4.3	NR	72	32.5–33	Passive	387	High
			No cooling	41	17.1 ± 5.0	NR					
Zhi (2003)	42.5	3–8	Cooling	198	26.9 ± 4.6	NR	24–168	32–35	0.25	138	Low
			No cooling	198	26.6 ± 4.9	NR					

–, not applicable; GCS, Glasgow Coma Scale score; ICP, intracranial pressure; ISS, Injury Severity Score; NR, not reported.

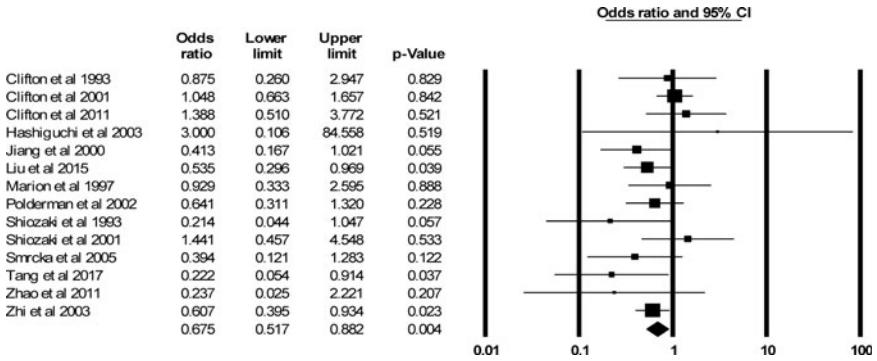

FIG. 2. Forest plot of the odds ratios for mortality rate between cooled and not cooled groups of patients with severe traumatic brain injury using random-effects model in statistically, clinically, and methodologically homogenous randomized controlled trials. CI, confidence interval.

0.25°C/h. We found that therapeutic hypothermia improved the outcome of severe TBI when the rewarming rate was below 0.25°C/h (OR = 0.58; $p = 0.014$), while there was only a tendency for the better outcome when the rewarming rate was ≥0.25°C/h (OR = 0.74; $p = 0.085$) (Fig. 5). These results suggest that certain parameters of the cooling protocol can be associated with more beneficial effects of therapeutic hypothermia on the mortality rate of patients with severe TBI, but how the combination of the different parameters together—i.e., the total extent of cooling—contributes to the outcome could not be established firmly.

In our next approach, we studied the integrated effect of the cooling parameters on the outcome of the disease. From the cooling parameters reported in the studies with medium and good level of randomization (Supplementary Table S3; see online supplementary material at ftp.liebertpub.com), the cooling index was assessed by considering all three variables—viz., target temperature, cooling duration, and speed of rewarming—in the formula (for details, see Methods and Supplementary Fig. S2A; see online supplementary material at ftp.liebertpub.com). The reported parameters and the cooling index derived from these data are shown in Table 1. By calculating the cooling index, we were able to compare the effect of the overall extent of hypothermia among studies that used different cooling parameters in their protocols.

The OR (estimated expected average) in the subgroups with low (<160°C × h), moderate (160–200°C × h), and high (>200°C × h) cooling index was 0.831 ($p = 0.535$), 0.754 ($p = 0.340$), and 0.470 ($p = 0.035$), respectively (Fig. 6). Importantly, the only significant effect for an OR of less than 1—i.e., when cooling was beneficial compared with no cooling—was observed in the subgroup of studies with high cooling index. These results suggest that in addition to the different independent contribution of each cooling parameter, the integrated measure of the magnitude and duration of therapeutic hypothermia (as indicated by the cooling index) can play a decisive role in determining whether the applied cooling protocol will decrease the risk of death in patients with severe TBI.

We also evaluated whether adjustments of the cooling parameters depending on the ICP of the patients impacts the outcome in severe TBI. When we compared the outcome of TBI between the group of studies that adjusted the cooling protocols based on ICP with the group of studies that did not, we found that when ICP was taken into account, the effect of therapeutic hypothermia was beneficial on the mortality rate of patients with severe TBI compared with patients with no temperature management (OR = 0.53; $p < 0.001$), while the beneficial effect was not significant when the cooling protocol was not changed depending on ICP (OR = 0.85; $p = 0.265$) (Fig. 7). These results support the necessity of monitoring ICP and adjusting the cooling parameters depending on ICP during therapeutic hypothermia.

Discussion

In the present study, we show that whole-body cooling decreases the risk of death in patients with severe TBI by conducting a meta-

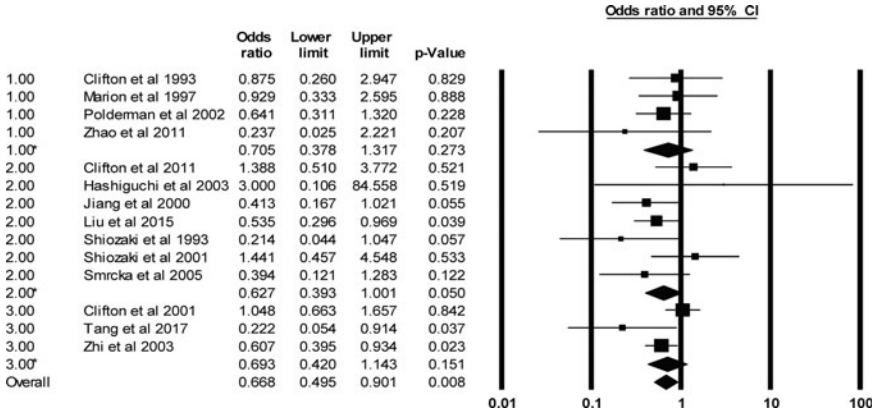

FIG. 3. Forest plot of the odds ratios for mortality rate between cooled and not cooled groups of patients with severe traumatic brain injury using random-effects model in randomized controlled trials divided into moderate (32–33°C; group label 1), mild (33–35°C; group label 2), and overlapping (32–35°C; group label 3) hypothermia subgroups based on target cooling temperature. Lines marked with * denote the mean values of the respective subgroups. CI, confidence interval.

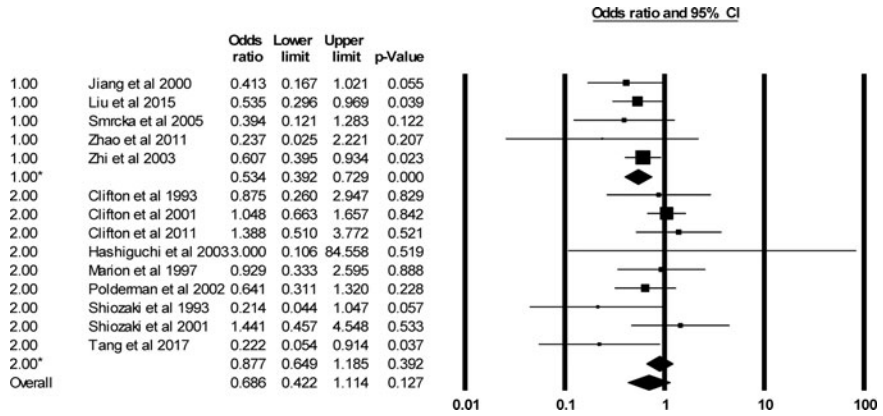

FIG. 4. Forest plot of the odds ratios for mortality rate between cooled and not cooled groups of patients with severe traumatic brain injury using random-effects model in randomized controlled trials divided into long (>48 h; group label 1) and short (24–48 h; group label 2) subgroups based on cooling duration. Lines marked with * denote the mean values of the respective subgroups. CI, confidence interval.

analysis of clinical studies, which were homogenous with regard to statistical, clinical, and methodological designs. By analyzing the individual cooling parameters, we reveal that milder and longer cooling and slower rewarming speed than 0.25°C/h are the most important to improve the outcome of severe TBI. We introduce the cooling index to assess the overall extent of cooling and show that therapeutic hypothermia is beneficial in severe TBI only if the cooling index is sufficiently high.

Our findings are in line with several clinical trials showing a beneficial effect of therapeutic hypothermia on the outcome of severe TBI,[13,14,17,21,23,25,26,29–31] whereas they contradict other trials that found no or even adverse effect of whole-body cooling in TBI.[20,24,27,28] Differences in statistical, clinical, and methodological designs among the studies can be assumed to account for the contradictory results. The highest quality human studies are RCTs, while observational studies and retrospective analyses provide a lower level of evidence. Among RCTs, the randomization protocol can vary (see Supplementary Table S3; see online supplementary material at ftp.liebertpub.com), which results in different levels of statistical bias involved in the trials.

Three multi-center clinical trials[20,27,28] found either no change or worse mortality rates in the cooled groups of patients with TBI, whereas all of the single-center studies[13,14,17,21,23,25,26,29–31] showed that therapeutic hypothermia was associated with a lower mortality rate. Differences between results from single-center versus multi-center trials have been observed earlier with regard to therapeutic hypothermia.[62] Large multi-center trials are usually considered as higher quality studies than single-center trials mostly when pharmacological treatments are investigated; however, when precise execution of the studied intervention is crucial as in the case of therapeutic hypothermia, then the different protocols used in different centers can lead to heterogeneous results, which can mask the differences between the treated and control groups. The study type and randomization level should be taken into account when the findings of a study are evaluated and, especially, when several studies are compared with meta-analysis.

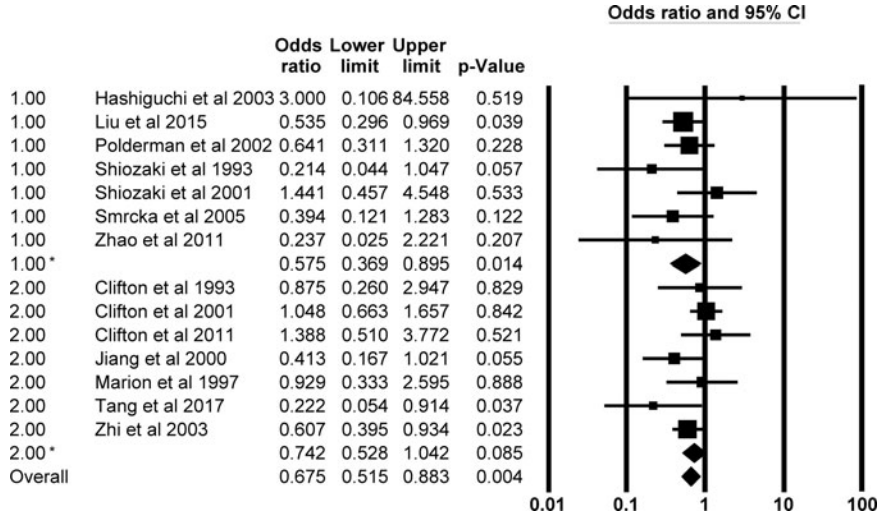

FIG. 5. Forest plot of the odds ratios for mortality rate between cooled and not cooled groups of patients with severe traumatic brain injury using random-effects model in randomized controlled trials divided into two subgroups based on rewarming rate: less than 0.25°C/h (group label 1) and 0.25–1°C/h (group label 2). Lines marked with * denote the mean values of the respective subgroups. CI, confidence interval.

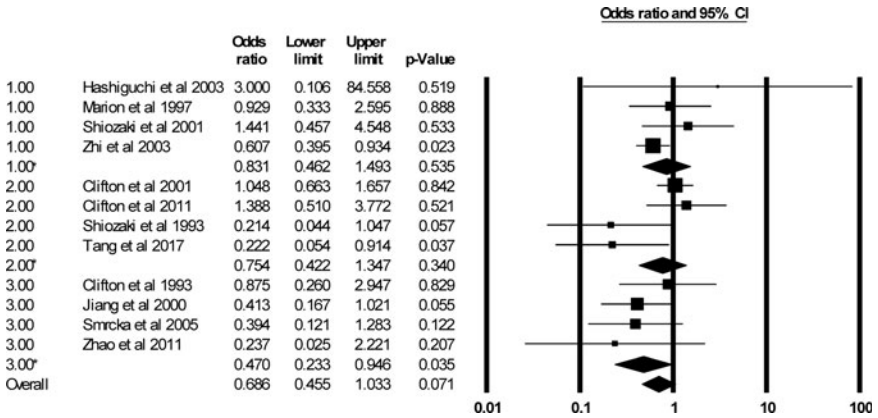

FIG. 6. Forest plot of the odds ratios for mortality rate between cooled and not cooled groups of patients with severe traumatic brain injury using random-effects model in randomized controlled trials divided into low (<160°C × h; group label 1), moderate (160–200°C × h; group label 2), and high (>200°C × h; group label 3) subgroups based on the cooling index. Lines marked with * denote the mean values of the respective subgroups. CI, confidence interval.

At least in some cases, the clinical design of the studies could contribute clearly to mask the effects of therapeutic hypothermia on the outcome. In the study by Andrews and associates,[16] an adverse effect of therapeutic hypothermia on the outcome of TBI was found; however, patients with a GCS score of 3–15 were included in the trial, and subgroup analysis for the different severities of TBI was not performed. Further, it can be assumed that the deleterious effects of cooling on the outcome could be attributable to the different conventional treatments, which were not controlled for between groups.[63]

Clifton and coworkers[30] could not confirm the efficacy of therapeutic hypothermia in TBI, which contradicted the results of an earlier phase 2 trial.[27] Patients with spontaneous hypothermia, however, were also included in the study,[30] which could have influenced the results. As opposed to therapeutic hypothermia when deep body temperature decreases because of forced cooling, spontaneous hypothermia is regarded as a regulated, adaptive mechanism in response to severe forms of a disease (e.g., systemic inflammation),[64] and it is often associated with worse outcome.[65]

In some studies the authors used fever control,[34,47,48] which could also influence the results. Most of the antipyretics (e.g., nonsteroidal anti-inflammatory drugs) do not only reduce fever, but also exert anti-inflammatory and other effects. It has been shown that unlike the use of antipyretics, fever was not associated with death in systemic inflammation,[66] suggesting different biological and clinical implications of fever and antipyretics.[66] Because inflammatory processes are also involved in the pathomechanism of severe TBI,[4–6] the use of antipyretics in the control (no cooling) group could change the outcome of the disease.

Methodologically, studies using selective brain cooling[44,46,51] should be distinguished from those applying whole-body hypothermia, because selective brain cooling is occasionally invasive, and if maintained for several hours, it may induce systemic hypothermia.[67] A critical appraisal of published clinical studies showed that the methods of therapeutic hypothermia were heterogeneous in terms of timing, depth, duration, and rewarming rate.[68] The impact of the different cooling methods on the outcome can be reduced if exclusively those studies are compared, in which the cooling parameters are precisely reported, and the role of the parameters is also analyzed.

Previous meta-analyses of the available data from the clinical trials led to contradictory results regarding the efficacy of

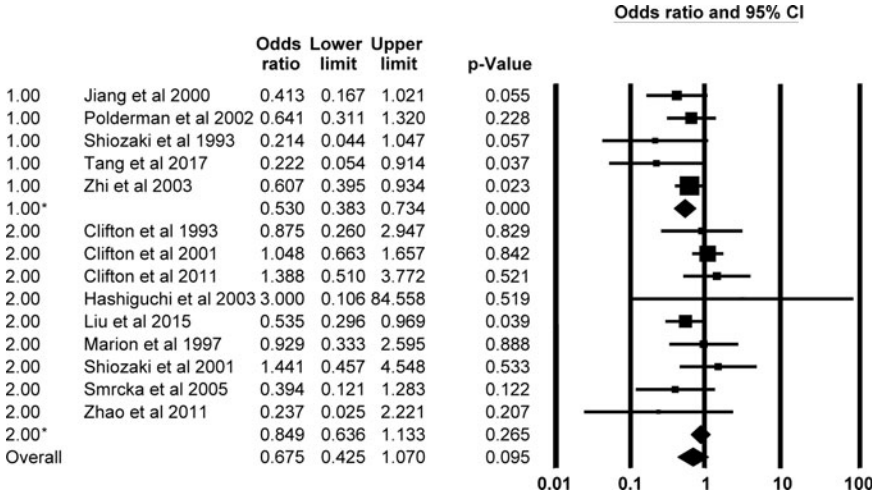

FIG. 7. Forest plot of the odds ratios for mortality rate between cooled and not cooled groups of patients with severe traumatic brain injury using random-effects model in randomized controlled trials divided into two subgroups: cooling protocol adjustments based on intracranial pressure (ICP) (group label 1) and no ICP-based changes in the protocol (group label 2). Lines marked with * denote the mean values of the respective subgroups. CI, confidence interval.

therapeutic hypothermia in TBI.[36–42] It can be assumed that the substantial heterogeneity among the trials included in these meta-analyses could be a crucial factor, which influenced the outcome of the analysis. By using meta-analysis, weighting of the included results can be based only on the deviation reported in a trial. The more uncertain data (greater variability, smaller sample) are taken into account with a lesser weight, but the selection of the studies cannot be accounted for. The novel approach in our meta-analysis is that we extended the conventionally used protocols for study selection by detailed evaluation of the statistical, clinical, and methodological design of the studies. As a result, we identified a group of studies that were homogenous with regard to all three evaluated aspects of the design. By analyzing the results reported in these studies, we found that therapeutic hypothermia improves the outcome of severe TBI and its beneficial effect can be associated with certain cooling parameters (milder and longer cooling, and slower rewarming than 0.25°C/h).

Our results about the importance of slow rewarming are well in agreement with data obtained from experimental animals showing that hypothermia followed by slow rewarming provides maximal neuroprotective effect,[69,70] and further support the current recommendations about the use of very slow (0.1–0.2°C/h) rewarming rates in patients with severe TBI.[55,56] On the contrary, the use of uncontrolled rewarming may potentially offset the benefits of hypothermia, particularly because it may cause rebound intracranial hypertension.[13,30]

Nevertheless, it also has to be noted that because the reported rewarming rates covered only a narrow range, an extended analysis, which would take in account fast, moderate, and slow rewarming of the patients, could not be performed in our study and that the contribution of rewarming to the cooling index was smaller than that of the other two cooling parameters. Importantly, different combinations of the studied parameters can be also advantageous if the total extent of hypothermia (cooling index) is sufficiently high.

A high cooling index ($\sim 230°C \times h$), thereby beneficial effect of therapeutic hypothermia could be achieved by different combinations of the cooling parameters—e.g., by short (48 h) and deep (32.5°C) cooling followed by rewarming at a rate of 0.25°C/h,[30] as well as by longer (72 h) and milder (34°C) cooling followed by slower (0.06°C/h) rewarming[23] (also compare with Supplementary Fig. S2A and B; see online supplementary material at ftp.liebertpub.com). By looking at the single cooling parameters used in the two studies,[23,30] the effect of therapeutic hypothermia on the outcome could seem controversial, because moderate and short cooling[30] with slower rewarming[23] were not beneficial, while milder and longer hypothermia[23] with not so slow rewarming[30] improved the outcome in severe TBI. However, if the integrated measure of the magnitude and the duration of cooling is considered—viz., the cooling index—then a high extent of hypothermia can be demonstrated in both studies,[23,30] resulting in a beneficial effect of whole-body cooling on the outcome of severe TBI as seen in the subgroup with high cooling index (OR = 0.47; Fig. 6).

Because the clinical data of the patient presented in Supplementary Table S1 (see online supplementary material at ftp.liebertpub.com) are similar to the analyzed patient population, it is tempting to speculate that cooling of this patient with the methods specified above could have improved the outcome in his case. Our meta-analysis included data from a total of 1786 adult patients with severe TBI, but because of the nature of this method, we have studied the reported mean in populations of patients, rather than the association in individual subjects. Therefore, precisely designed clinical trials are needed to confirm our results in clinical settings. The design of such trials should include appropriate randomization protocols, targeted population of patients (severe TBI, no pre-existing thermoregulatory disorders, no differences between the study and control groups in medical-surgical treatment), and precise cooling protocols (a combination of parameters with sufficiently high cooling index). By validation of our findings in clinical trials, it would be possible to identify a subpopulation of patients with TBI in which well-controlled therapeutic cooling could improve the outcome.

The addition of further patient characteristics (e.g., ICP, age, comorbidities) and methodological parameters (e.g., initiation time of cooling) to the inclusion criteria of such trial could help to specify accurately the patient population that can benefit the most from precisely conducted therapeutic hypothermia. Increased ICP can be regarded as both an indicator of the actual brain damage and as a cause of additional pathological changes in severe TBI.[55] A strong body of evidence supports that therapeutic hypothermia is a useful tool to decrease ICP in TBI; however, this does not necessarily improve the outcome of the disease.[68] The contradiction between the management of intracranial hypertension and the lack of improvement in the outcome can be caused by inadequate maintenance of the hypothermia-induced lower ICP, caused by the applied cooling protocol. Rebound increases of ICP are more common in patients with TBI if the cooling duration is too short and the rate of rewarming is too fast.[53] To avoid the adverse changes in ICP, five of the analyzed studies reported that the designed cooling protocol was adjusted based on the ICP.[14,25,26,29,31] Here, we showed that in these studies, therapeutic hypothermia significantly improved the outcome in severe TBI compared with those trials that followed the protocol regardless of ICP.[8,13,17,20,23,24,27,28,30] These results highlight the importance of continuous ICP monitoring during therapeutic hypothermia and warrant for the need of adjustments in the cooling protocol based on ICP. Unfortunately, the data about the deviations from the set protocols were not reported in sufficient details in any of the studies, in order to account for the changes in our calculation of the cooling index.

It would also be interesting to study how pharmacological tools can be implemented in the induction of hypothermia in addition to external cooling of the body. Ideal candidates for such drugs could be among the new generation of the antagonists of the transient receptor potential vanilloid-1 channel, which were developed as painkillers, but can cause marked hypothermia.[71] These additional parameters could not be included in the present meta-analysis because of insufficient data availability.

For the same reason, the upper limit of the cooling index—i.e., above which the harmful effect of hypothermia would dominate over the beneficial effects—could not be determined in our analysis. In the included trials, even from which the highest cooling indices were calculated, cooling of the patients improved the outcome.[21,23,29,30] It can be expected that the upper safety limit of the cooling index would need to be established separately for the different study populations. Identification of the exact cooling protocol for a specified patient population would be in line with recent paradigms in the treatment of TBI, suggesting the need for targeted management of individuals or subsets of patients to improve the outcome.[72,73]

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Author Disclosure Statement

No competing financial interests exist.

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Supplementary Data

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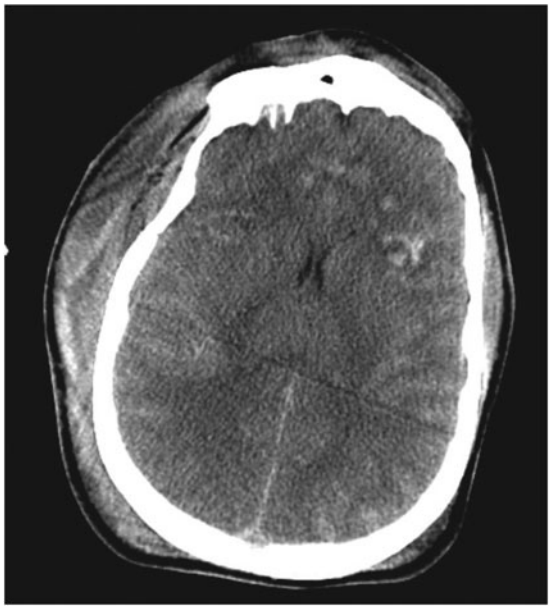
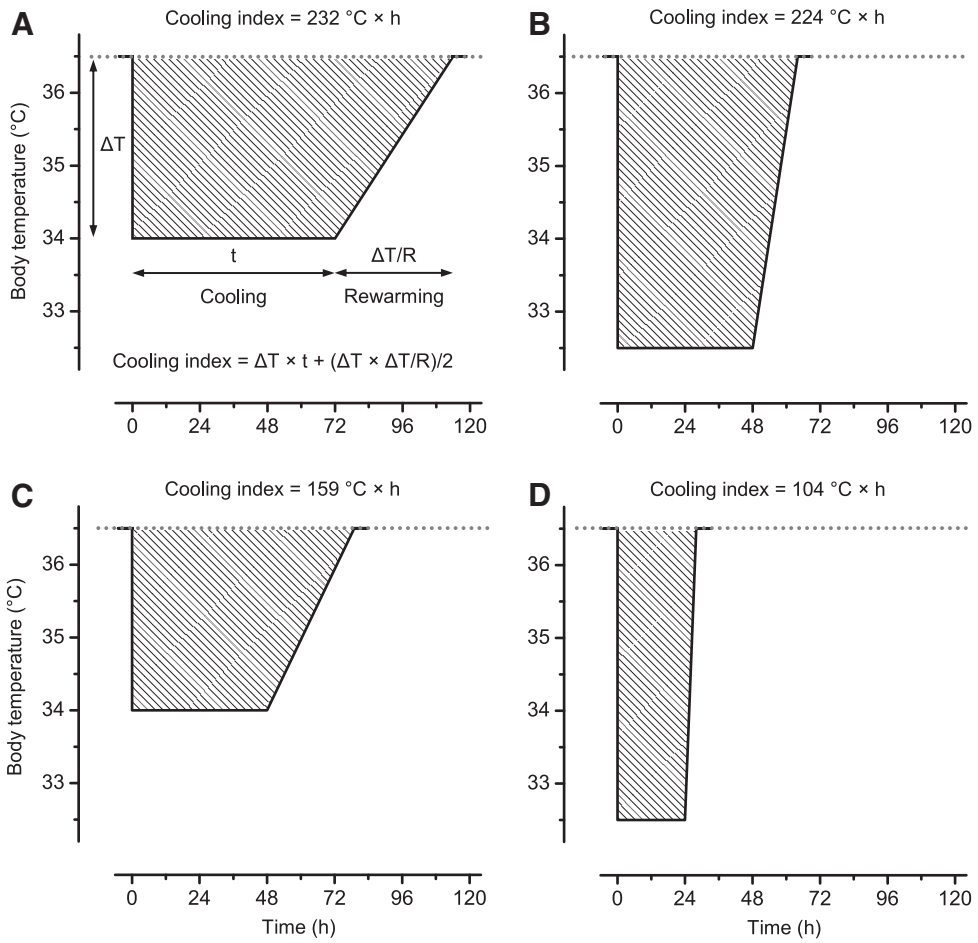
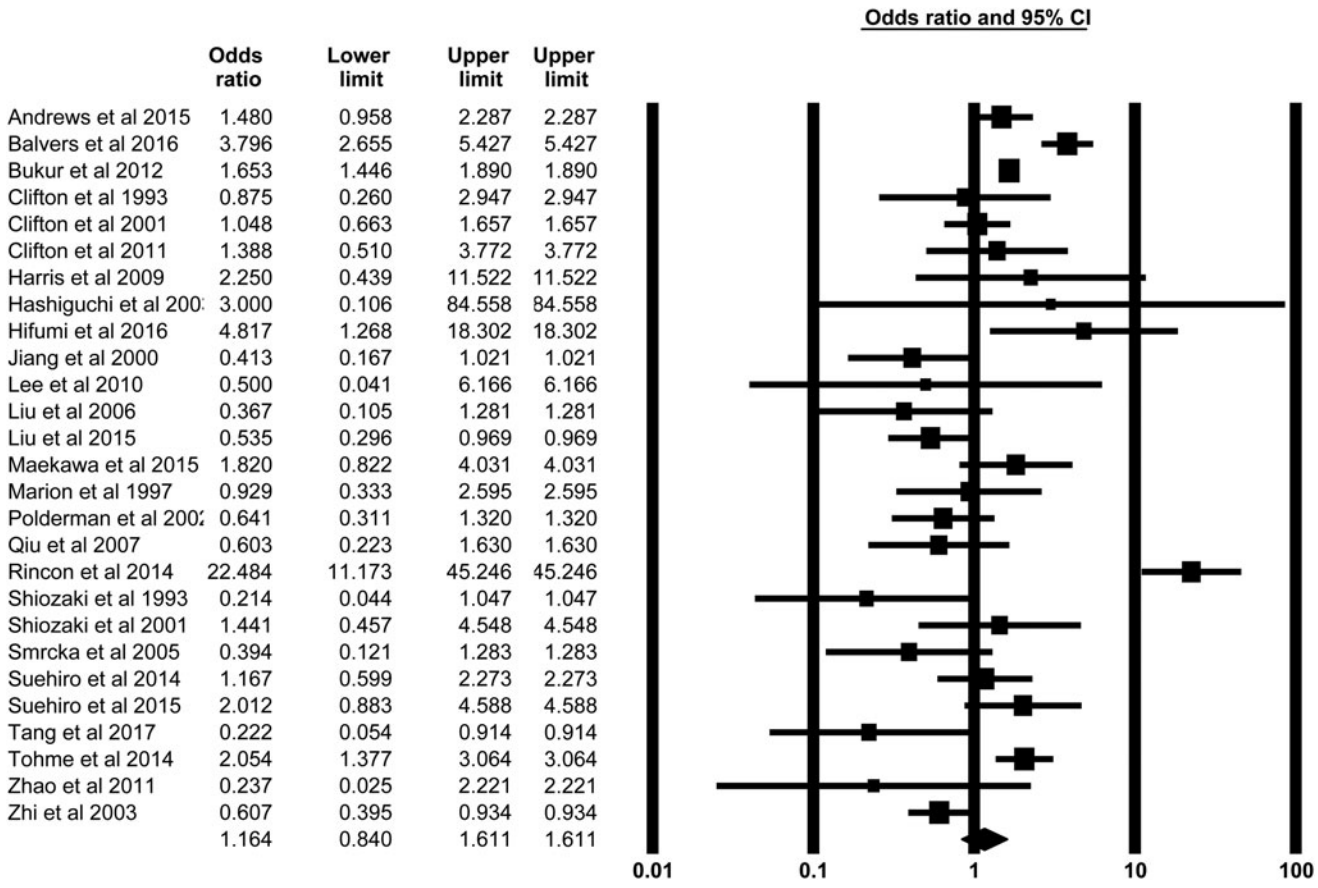


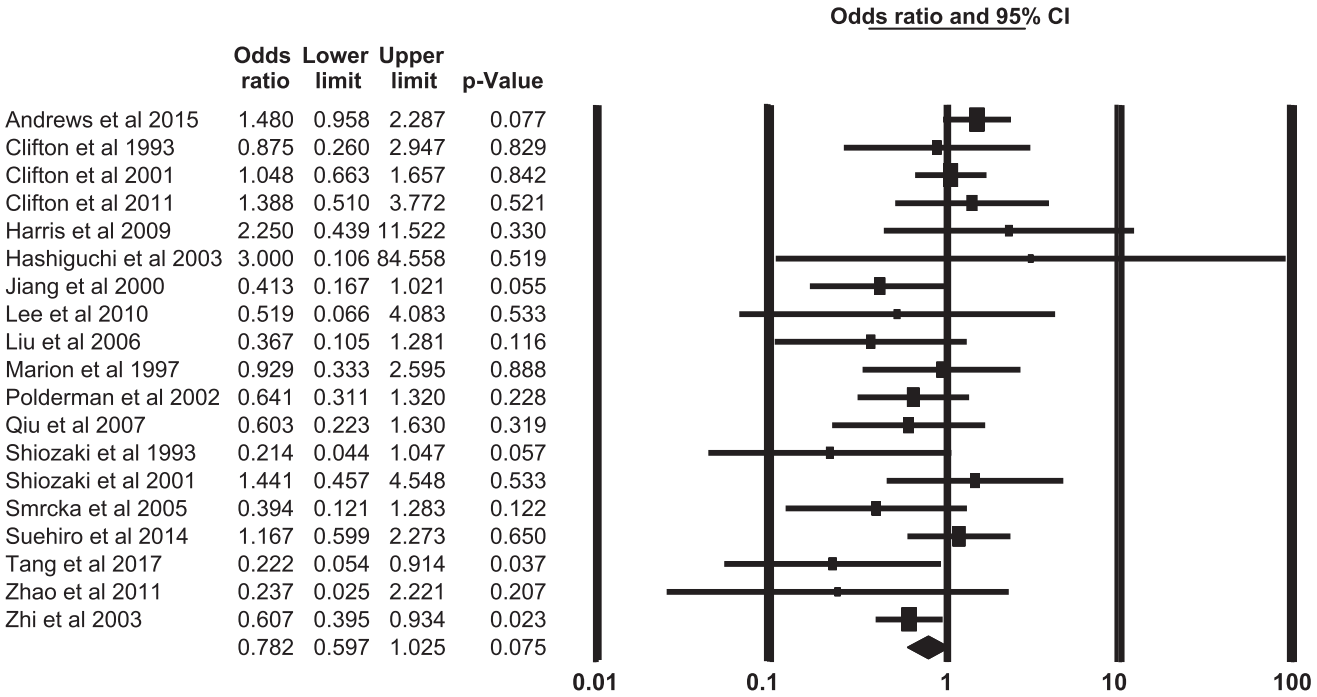
SUPPLEMENTARY FIG. S1. Axial CT image of the patient admitted with severe TBI, obtained at time of arrival to level 1 trauma center. TBI comprised diffuse bilateral subarachnoid hemorrhage, bilateral frontal contusions, small right subdural hematoma, and effacement of basal cisterns secondary to cerebral edema.



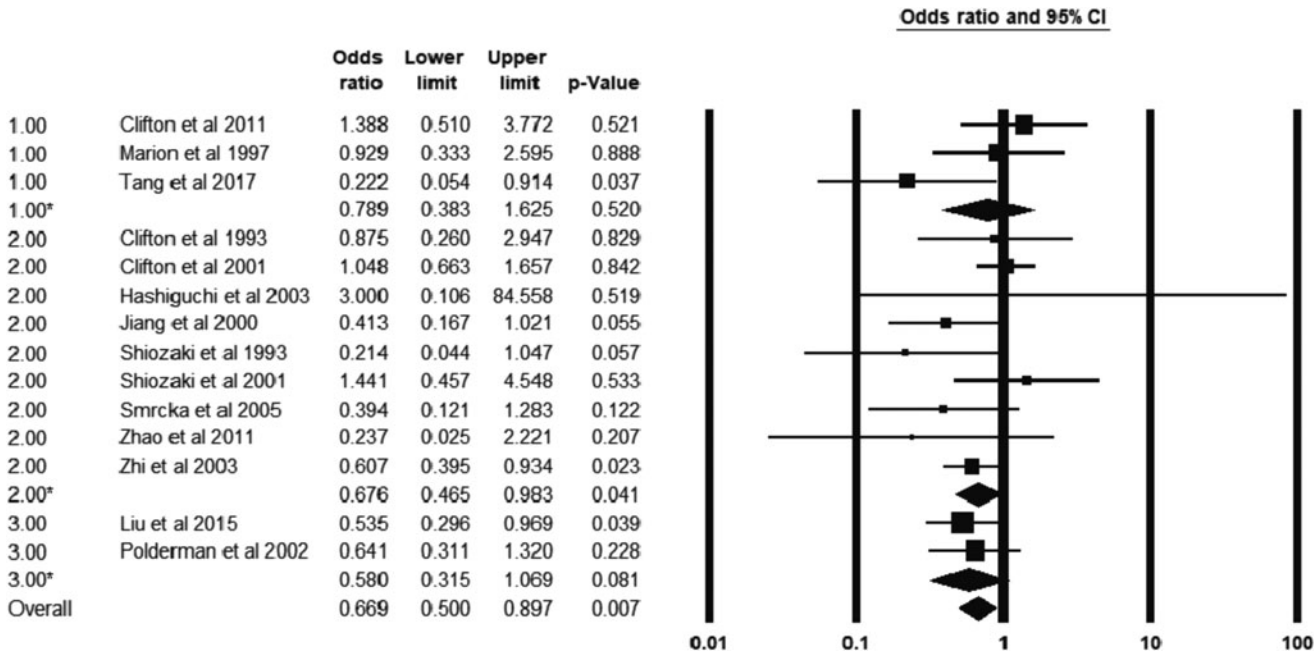
SUPPLEMENTARY FIG. S2. Calculation method of the cooling index and examples for high (**A** and **B**), moderate (**C**), and low (**D**) calculated values. The shown examples were calculated from the cooling parameters reported by Smrcka et al. (2005) (**A**), Clifton et al. (1993) (**B**), Shiozaki et al. (2001) (**C**), and Marion et al. (1997) (**D**). In all panels, the striped area represents the cooling index; the calculated values are indicated. Dotted gray line illustrates normal (not cooled) deep body temperature considered as 36.5°C. ΔT, difference between normal body temperature and cooling target temperature; t, cooling duration; R, rate of rewarming. See main text for detailed explanation.



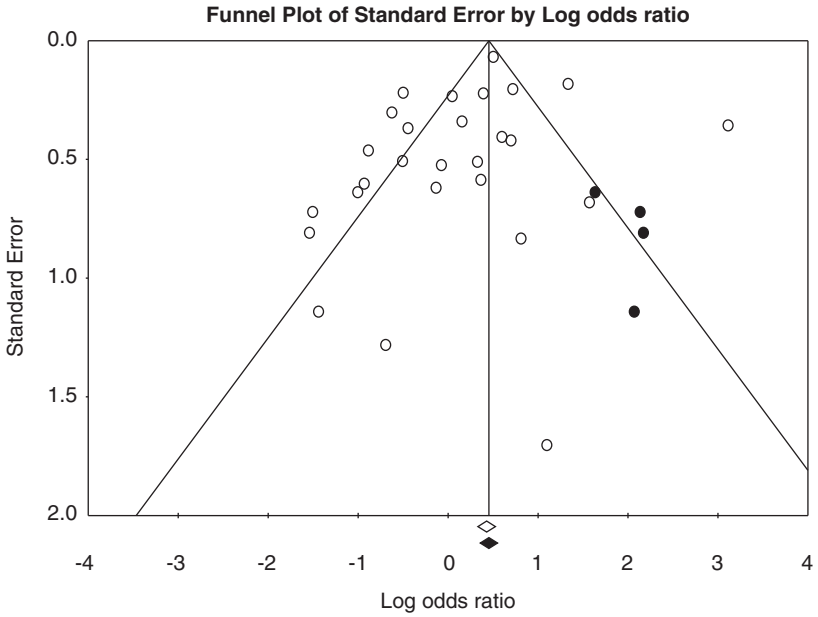
SUPPLEMENTARY FIG. S3. Forest plot of the odds ratios for mortality rate between cooled and not cooled groups of patients with severe TBI using random-effects model in 27 trials of all study types.



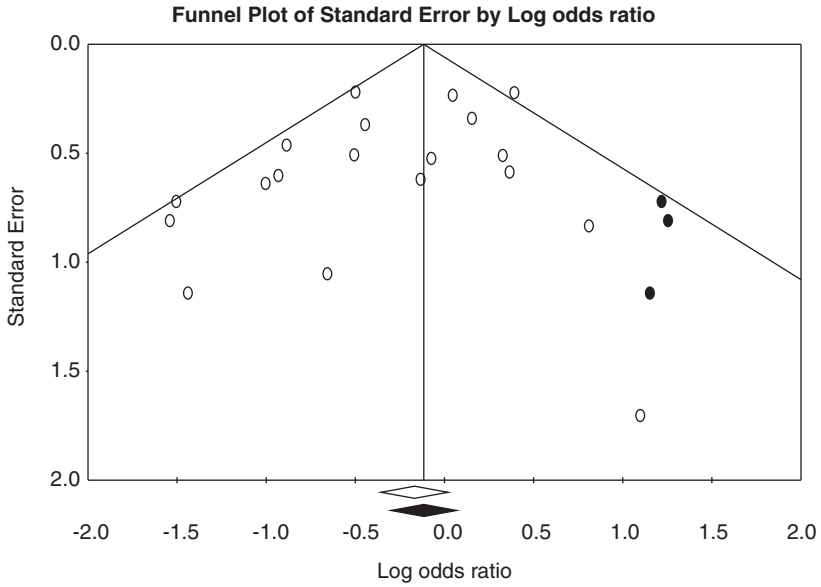
SUPPLEMENTARY FIG. S4. Forest plot of the odds ratios for mortality rate between cooled and not cooled groups of patients with severe TBI using random-effects model in all RCTs.



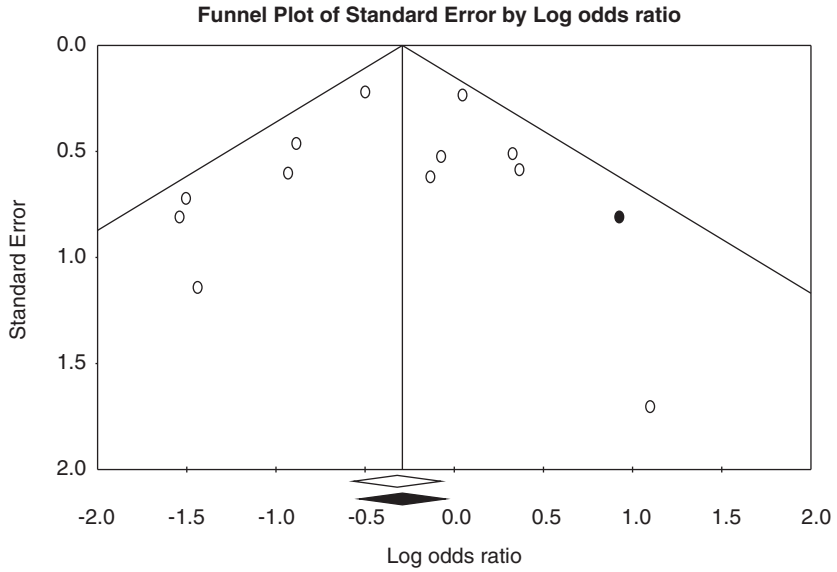
SUPPLEMENTARY FIG. S5. Forest plot of the odds ratios for mortality rate between cooled and not cooled groups of patients with severe TBI in RCT subgroups of good (group label 1), medium (group label 2), and low (group label 3) level of randomization protocols. The lines marked with * denote the mean values of the respective subgroups.



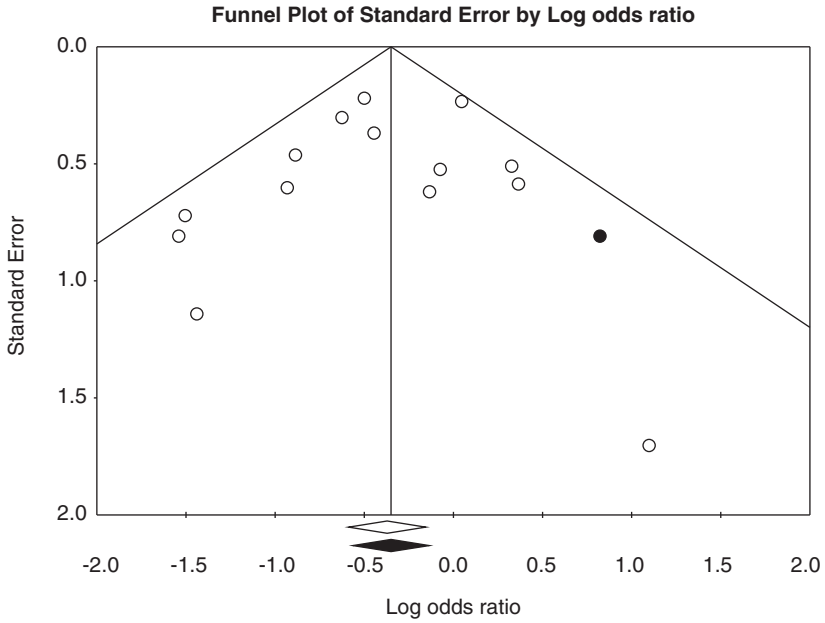
SUPPLEMENTARY FIG. S6. Funnel plot of 27 studies included in Figures S3. Here, and in Figures S7–S9, open circles represent results from studies included in the forest plot, while closed circles indicate studies that appeared to be missing according to the trim and fill method of Duval and Tweedie. Open and closed diamonds represent the average estimated effect size without (open diamond) and with trim and fill correction (closed diamond). Duval and Tweedie corrected value: 1.37 (95% CI, 1.00, 1.88).



SUPPLEMENTARY FIG. S7. Funnel plot of 19 studies included in Figure S4. Duval and Tweedie trim and fill corrected value: 0.86 (95% CI, 0.65, 1.13).



SUPPLEMENTARY FIG. S8. Funnel plot of 14 studies included in Figures 2–5, 7, and S5. Duval and Tweedie trim and fill corrected value: 0.70 (95% CI, 0.53, 0.92); p = 0.509 with Egger test.



SUPPLEMENTARY FIG. S9. Funnel plot of 12 studies included in Figure 6. Duval and Tweedie trim and fill corrected value: 0.73 (95% CI, 0.52, 1.03); p = 0.474 with Egger test.

Supplementary Table S1. Characteristics of a Patient with Severe TBI Admitted to one of Our Centers (St. Joseph's Hospital)

Age (year)	35
Gender	male
Mechanism of Injury	Motor vehicle collision
GCS	3
CSF opening pressure (cm H2O)	6
Injury Severity Score	38
Temperature on admission (°C)	38.4
Peak temperature during hospitalization (°C)	39.1
Description of Brain Injury	Fig S1
Associated Injuries	Bilateral multiple rib fractures, bilateral scapula fractures, bilateral pneumothoraces, T8 and T9 vertebral body fractures, comminuted nasal bone and osseous nasal septal fractures, fracture of right ethmoid air cells, segmental fracture of right lamina paprycea
Hospital Course	Patient underwent orotracheal intubation for depressed mental status, and bilateral chest tube placement for pneumothoraces. Transferred to level 1 trauma center for definitiveevaluation and treatment. ICP monitor and ventriculostomy placed. He was admitted to the Neuro Intensive Care Unit, and underwent continuous EEG and ICP monitoring. He developed increasing ICPs, managed initially with pressor support to augment cerebral perfusion pressure. Barbiturate-induced coma was initiated on hospital day #5 for refractory elevated ICP. On hospital day #7, the patient's family elected to initiate palliative care secondary to poor neurologic prognosis, and patient died on hospital day #7.

SUPPLEMENTARY TABLE S2. PRISMA CHECKLIST

Section/topic	*#*	*Checklist item*	*Reported on page #*
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	1
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	1–2
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	2
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	2
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	2
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	2
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	2, Figure 1
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	2
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	2–3
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	2–3
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	3
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	3
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I2) for each meta-analysis.	2–3
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	3
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	3
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	3; Figure 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	3–4
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	3–4; Table S3

(*continued*)

SUPPLEMENTARY TABLE S2. (CONTINUED)

Section/topic	*#*	*Checklist item*	*Reported on page #*
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	4, 6; Figures 2–7 and S3–S5
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	4, 6; Figures 2–7
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	3–4; Table S3
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	6; Figures 6 and S2
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	6–9
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	7–8
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	9
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	9–10

Supplementary Table S3. Detailed Quality Assessment of the Studies included in the Meta-Analysis According to the Cochrane Handbook and the Jadad Score. In the Own Quality Assessment of the Randomization Protocols, the Level of Randomization was Assessed as Good, Medium and Low

	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Other bias	Jadad score	Own assessment
Clifton 1993	?	+	-	-	?	-	?	2	?
Clifton 2001	?	+	-	-	?	?	?	2	?
Clifton 2011	+	+	-	+	?	?	?	3	+
Hashiguchi 2003	?	?	-	-	?	?	?	1	?
Jiang 2000	?	?	-	-	?	?	?	1	?
Liu 2015	-	-	-	-	?	?	?	1	-
Marion 1997	+	+	-	+	?	?	?	3	+
Polderman 2002	-	-	-	-	?	?	?	1	-
Shiozaki 1993	?	?	-	-	?	-	?	1	?
Shiozaki 2001	?	?	-	-	?	-	?	1	?
Smrcka 2005	?	?	-	-	?	-	?	1	?
Tang 2017	+	+	+	?	?	-	?	4	+
Zhao 2011	?	-	-	-	?	-	?	1	?
Zhi 2003	?	-	-	-	?	-	?	1	?

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SHORT COMMUNICATION CLINICAL STUDIES

POLAR Study Revisited: Therapeutic Hypothermia in Severe Brain Trauma Should Not Be Abandoned

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Abstract

The benefits of therapeutic hypothermia (TH) in severe traumatic brain injury (sTBI) have been long debated. In 2018, the POLAR study, a high-quality international trial, appeared to end the debate by showing that TH did not improve mortality in sTBI. However, the POLAR-based recommendation to abandon TH was challenged by different investigators. In our recent meta-analysis, we introduced the cooling index (COIN) to assess the extent of cooling and showed that TH is beneficial in sTBI, but only when the COIN is sufficiently high. In the present study, we calculated the COIN for the POLAR study and ran a new meta-analysis, which included the POLAR data and accounted for the cooling extent. The POLAR study targeted a high cooling extent (COIN of 276°C × h; calculated for 72 h), but the achieved cooling was much lower (COIN of 193°C × h)—because of deviations from the protocol. When the POLAR data were included in the COIN-based meta-analysis, TH had an overall effect of reducing death (odds rate of 0.686; $p = 0.007$). Among the subgroups with different COIN levels, the only significantly decreased odds rate (i.e., beneficial effect of TH) was observed in the subgroup with high COIN (0.470; $p = 0.013$). We conclude that, because of deviations from the targeted cooling protocol, the overall cooling extent was not sufficiently high in the POLAR study, thus masking the beneficial effects of TH. The current analysis shows that TH is beneficial in sTBI, but only when the COIN is high. Abandoning the use of TH in sTBI may be premature.

Keywords: cooling index; induced hypothermia; meta-analysis; mortality; thermoregulation; traumatic brain injury

Introduction

The usefulness of therapeutic hypothermia (TH) in severe traumatic brain injury (sTBI) has been long debated. In December 2018, the Prophylactic Hypothermia Trial to Lessen Traumatic Brain Injury (POLAR), a multi-center randomized controlled trial, appeared to end the debate by showing that TH did not improve the outcomes, including death rates, in sTBI.[1] However, the POLAR-based recommendation to abandon TH was challenged.[2–4] It was especially difficult to achieve consensus regarding the use of moderate TH (deep body temperature <35°C), given that 24 of the 35 surveyed experts stated that it had a role in the treatment of sTBI with elevated intracranial pressure (Seattle International sTBI Consensus Conference, Survey 3).[5] After several rounds of voting, the routine use of *moderate* TH was not recommended, whereas *mild* TH was recommended, but only as tier-three treatment.[5,6]

In our recent meta-analysis,[7] we introduced the cooling index (COIN) to quantify the extent of TH in sTBI and showed that TH was beneficial only when the COIN was high. Our results suggested that the COIN

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(as a measure of the magnitude of hypothermia) is an important factor in the effectiveness of TH, given that the effect of cooling on neuroprotection can depend on its overall extent determined by depth, duration, and rewarming rate. Could it be that the overall COIN was not sufficiently high in POLAR, thus masking the true potential of TH and limiting its use? To test this hypothesis, we ran a new meta-analysis, which included the POLAR data and accounted for the extent of TH.

Methods

After confirming, through the quality assessment protocol,[7] that the POLAR study met the criteria for high-quality trials (a high level of randomization), we extracted the targeted cooling parameters (*viz.*, cooling temperature, cooling duration, and speed of rewarming), accounted for any deviations from or adjustments to them, and calculated the COIN values (see Table 1 for the formula). Based on the COIN, we included the death rates (assessed as odds ratio [OR]) reported by POLAR in the appropriate subgroup of the COIN-based meta-analysis, as described in details previously,[7] and formally analyzed this new, extended data set, which included data from 13 studies.[1,8–19]

Publication bias was assessed with funnel plots by using the trim-and-fill method[20] and the test by Egger and colleagues[21] (see Supplementary Fig. S1). Egger's test values <0.1 were considered as indicators of a significant small-study effect. Between-study heterogeneity was tested with the Q homogeneity test (p values <0.05 were considered as indicators of significant heterogeneity) and with the I^2 statistical test, where I^2 is the proportion of total variation attributable to between-study variability (an I^2 value >50% was considered as indicating considerable heterogeneity).

Results

The COIN value would have been high in the POLAR study—if the targeted parameters (Table 1) were met. However, the targeted cooling parameters were reached in less than one half of patients receiving TH (Table 1). In the hypothermia group, 85 patients (33%) were cooled for <48 h; 27 patients (10%) never reached a deep body temperature of 35°C; and 65 patients (27%) never reached 33°C.[1] Hence, many patients in the POLAR study had a low level of COIN, and the overall level achieved in that study was only moderate (Table 1).

Based on the above, we included the POLAR data in the ''Moderate'' COIN subgroup of our meta-analysis (Fig. 1). For all data, including POLAR, the OR for death was 0.686 ($p=0.007$), indicating that, overall, TH significantly decreased mortality in sTBI. However, a significant decrease in OR (indicating a beneficial effect of TH) was observed only in the ''High'' COIN subgroup (0.470; $p=0.013$). ORs in subgroups with ''Low'' or ''Moderate'' cooling intensity were 0.718 ($p=0.081$) and 0.846 ($p=0.533$), respectively.

Between-study heterogeneity was relatively small, as indicated by the Q homogeneity test ($Q=19.1$; $p=0.085$) and the I^2 statistical test ($I^2=37.3\%$). Neither of the used assessment methods indicated the presence of publication bias (Supplementary Fig. S1).

Discussion

Including the POLAR results in our COIN-based meta-analysis strengthened our former conclusion that TH has a significant beneficial effect on death rate in sTBI, but only when COIN is sufficiently high. The benefits of TH were also shown in the recent review by Moore and colleagues.[22] The authors applied the umbrella review methodology to several potentially low-value clinical practices and found that TH was the only one with evidence of benefit. However, the POLAR study was not included in any of the systematic reviews analyzed in the umbrella review by Moore and colleagues.[22]

When the results of POLAR were translated into treatment guidelines preceding the present work,[5,6] the absence of an overall beneficial effect led to the recommendation to reduce the use of TH in sTBI. However, some deviations from the cooling protocol occurred at different POLAR-participating centers and decreased the overall extent of cooling from ''High'' (targeted) to ''Moderate'' (overall achieved) and even ''Low'' (observed in many patients). This decrease in the COIN was likely to mask the benefits of TH in the overall cohort. It would be desirable to analyze separately the outcomes in those patients who fully met the targeted cooling criteria and those who did not. Until such results are available from the POLAR group or obtained in other high-quality trial(s), it may be premature to abandon the use of TH in sTBI.

Table 1. Cooling Parameters Reported in the POLAR Study and the Calculated Cooling Index (COIN)

	Cooling duration (h)	*Cooled deep body temperature (°C)*	*Rewarming rate (°C/h)*	*No. of patients [%]*	*COIN[a] (°C×h)*	*COIN subgroup*
Targeted parameters	72≤	33.0±0.5	≤0.25	260 [100]	276	High
Cooling compliance criteria	48≤	≤35	≤0.25	124–125 [48]	77	Low
Sensitivity analysis criteria	~72	≤35	≤0.25	120–121 [46]	112	Low
Overall study parameters	72.2	33–35	≤0.25	124 [48]	193	Moderate

[a]$\text{COIN}=\Delta T\times t+(\Delta T\times \Delta T/R)/2$, where ΔT is the difference between normal deep body temperature (36.5°C) and the temperature reached at the end of cooling (in °C); ''t'' is hypothermia duration (in hours); and ''R'' is the rate of rewarming (in °C/h).

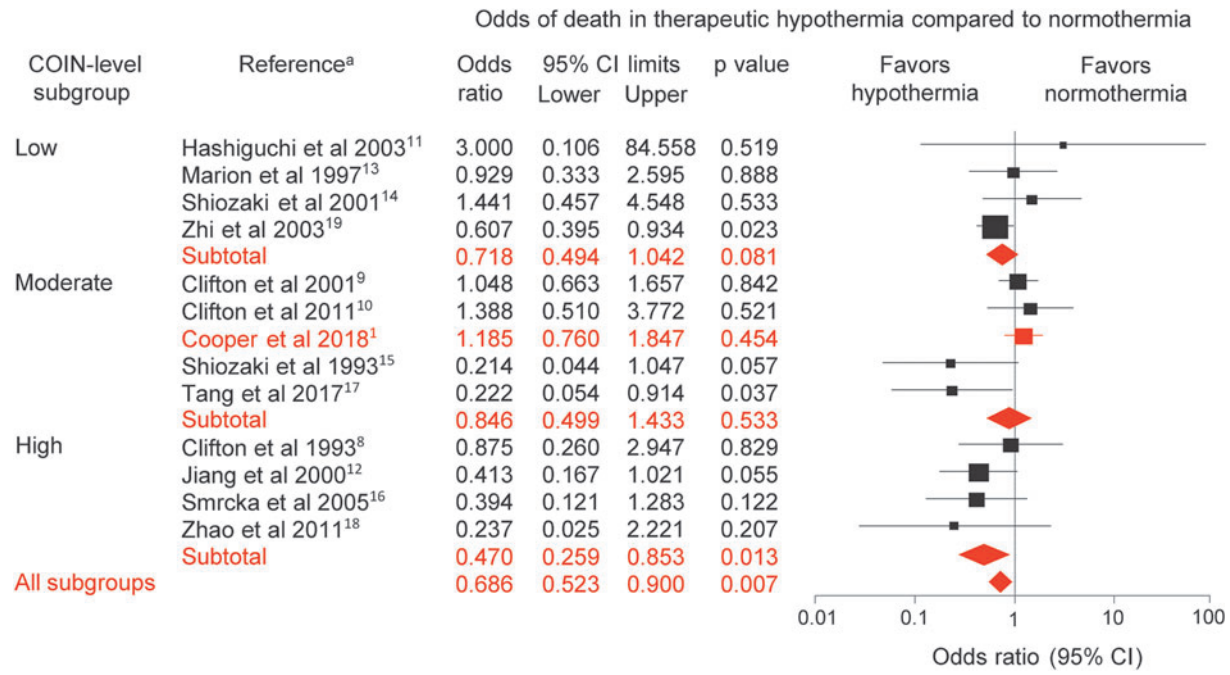

FIG. 1. Forest plot of the effects of therapeutic hypothermia on mortality in patients with severe traumatic brain injury. The odds ratio was calculated by dividing the odds of death to survival in the therapeutic hypothermia group with the odds of death to survival in the normothermia group. A ratio <1 indicates that therapeutic hypothermia reduced the odds of death, whereas a ratio >1 indicates increased odds of death in therapeutic hypothermia. The odds ratios were compared by using a random-effects model in high-quality, randomized controlled trials divided into "Low" (<160°C × h), "Moderate" (160°C–200°C × h), and "High" (>200°C × h) subgroups based on the cooling index (COIN). All new data compared to our previous analysis[7] are highlighted in red. Note that the POLAR study (Cooper and colleagues 2018)[1] is included in the "Moderate" COIN subgroup (for details, see Table 1). [a]Full references to the analyzed studies can be found in the list of references. CI, confidence interval. Color image is available online.

Our meta-analysis included four multi-center randomized controlled trials,[1,9,10,14] whereas the remaining nine studies were single-center randomized controlled trials.[8,11–13,15–19] It should be mentioned that intervention effects for binary outcomes (such as mortality) were shown to be, on average, larger in single-center randomized controlled trials than in multi-center trials.[23] With regard to TH, some differences between results from single-center versus multi-center trials were noticed previously.[24] The differences were explained by the difficulty of keeping all parameters that influence the outcome constant across the centers in multi-center trials. Indeed, several significant intercenter differences (e.g., in disease severity, drug selection, drug doses, and personnel experience) were found in a careful analysis of a multi-center trial.[24]

In our earlier report,[7] we noted that large multi-center trials are often considered to have higher quality than single-center trials when pharmacological treatments are investigated. When a complex intervention is involved, and the precise execution of this intervention is crucial (as in the case of TH), then different protocols used in different centers can lead to heterogeneous results, which can mask the differences between the treated and control groups. It should be also mentioned that the adherence to the TH protocol, including all the surrounding management, can be more closely monitored and controlled in single-center studies than in large multi-center randomized trials.

Given that positive results of single-center trials were occasionally contradicted when tested in multi-center settings, some authors concluded that physicians should apply the findings of single-center trials only after careful evaluation of their methodology.[25] It should be also noted, however, that the only way to avoid publication bias is to base meta-analyses on as complete collections of studies as possible.[26] Publication bias (i.e., the possibility of missing studies) must be always addressed

according to the PRISMA guidelines,[27] which were followed in our study. In our original review protocol (registration no.: CRD42017056535), we aimed at including all available studies in the meta-analysis, without limitations to the study type—in order to achieve the most comprehensive review of the topic and avoid publication bias. This approach produced a heterogeneous set of trials with regard to both clinical and statistical designs. To reduce the heterogeneity, we used a novel approach: We extended the conventional study selection protocols by the detailed evaluation of statistical, clinical, and methodological design aspects.[7]

As a result, we identified a group of 12 studies that were homogenous with regard to all three design aspects. Importantly, the POLAR study also fulfilled the inclusion criteria and did not increase heterogeneity in the present analysis. Moreover, the presence of any sizable publication bias was successfully avoided (Supplementary Fig. S1). Based on the above, we believe that the inclusion of both single-center and multi-center studies in our analysis is justified: This is the only way to conduct the most extensive analysis of the available data while minimizing the risk of publication bias.

Conclusion

In conclusion, including the POLAR study in our COIN-based meta-analysis suggests that the COIN should be flipped again to settle the dispute on the use of TH in sTBI.

Funding Information

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Author Disclosure Statement

No competing financial interests exist.

Supplementary Material

Supplementary Figure S1

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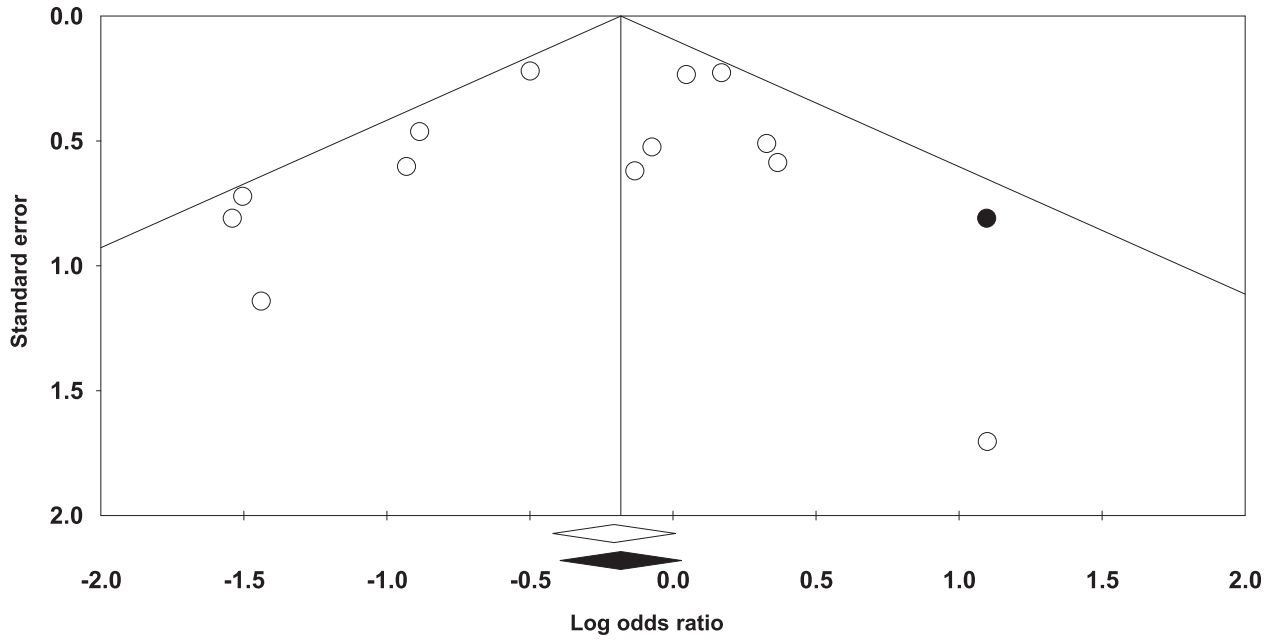
Supplementary material



SUPPLEMENTARY FIG. S1. Funnel plot of 13 studies included in Figure 1.

Open circles represent results from studies included in the forest plot, while closed circles indicate studies that appeared to be missing according to the trim-and-fill method of Duval and Tweedie. Open and closed diamonds represent the average estimated effect size without (open diamond) and with trim-and-fill correction (closed diamond). Duval and Tweedie corrected value: 0.73 (95% CI, 0.52, 1.03); $p = 0.271$ with Egger's test. CI, confidence interval.

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Article

The Hypothermic Effect of Hydrogen Sulfide Is Mediated by the Transient Receptor Potential Ankyrin-1 Channel in Mice

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Abstract: Hydrogen sulfide (H_2S) has been shown in previous studies to cause hypothermia and hypometabolism in mice, and its thermoregulatory effects were subsequently investigated. However, the molecular target through which H_2S triggers its effects on deep body temperature has remained unknown. We investigated the thermoregulatory response to fast-(Na_2S) and slow-releasing (GYY4137) H_2S donors in C57BL/6 mice, and then tested whether their effects depend on the transient receptor potential ankyrin-1 (TRPA1) channel in *Trpa1* knockout (*Trpa1*$^{-/-}$) and wild-type (*Trpa1*$^{+/+}$) mice. Intracerebroventricular administration of Na_2S (0.5–1 mg/kg) caused hypothermia in C57BL/6 mice, which was mediated by cutaneous vasodilation and decreased thermogenesis. In contrast, intraperitoneal administration of Na_2S (5 mg/kg) did not cause any thermoregulatory effect. Central administration of GYY4137 (3 mg/kg) also caused hypothermia and hypometabolism. The hypothermic response to both H_2S donors was significantly ($p < 0.001$) attenuated in *Trpa1*$^{-/-}$ mice compared to their *Trpa1*$^{+/+}$ littermates. *Trpa1* mRNA transcripts could be detected with RNAscope in hypothalamic and other brain neurons within the autonomic thermoeffector pathways. In conclusion, slow- and fast-releasing H_2S donors induce hypothermia through hypometabolism and cutaneous vasodilation in mice that is mediated by TRPA1 channels located in the brain, presumably in hypothalamic neurons within the autonomic thermoeffector pathways.

Keywords: hypothermia; thermoregulation; H_2S; TRPA1; GYY4137

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1. Introduction

Hydrogen sulfide (H_2S) was considered to be an environmental toxin before it was identified as an endogenously produced mediator in the 1940s (for a review, see [1]). At present, H_2S is recognized as an important gasotransmitter, which plays roles in a wide spectrum of physiological processes in the brain as well as in peripheral tissues in health and disease [2,3]. H_2S is synthesized both centrally and peripherally by specific enzymes including cystathionine β-synthase, cystathionine γ-lyase, and 3-mercaptopyruvate sulfurtransferase, but alternative H_2S-producing pathways are also known [3,4]. Endogenously,

H_2S is present within the nM to low μM concentration range and it is catabolized by enzymatic and non-enzymatic processes [5]. In the past decades, the role of H_2S has been implicated in a number of physiological and pathological conditions [2–4], including the regulation of core body temperature (T_b) [6].

In 2005, Blackstone et al. [7] reported that the inhalation of H_2S induces concentration-dependent hypometabolism and hypothermia in mice without causing behavioral or functional damages to the animals. The authors hypothesized that the thermal effect was evoked through the H_2S-induced inhibition of the terminal enzyme complex in the electron transport chain and called the condition as suspended animation-like state [7]. The hypometabolic and hypothermic response to H_2S inhalation was also shown by other authors [8], and similar results were obtained by the administration of the H_2S donor sodium hydrosulfide (NaHS) in anesthetized rats [9] and by infusion of dimethyl trisulfide in conscious mice [10]. However, the thermoregulatory role of H_2S was debated later, when Hemelrijk et al. [11] could not reproduce the H_2S-induced thermal effects and concluded that H_2S exacerbates hypoxia-induced hypometabolism, but H_2S in itself does not decrease metabolic rate and deep T_b. The mediation of hypoxia-induced hypothermia by H_2S was supported by the results of Kwiatkoski et al. [12], showing that production of H_2S increases in the hypothalamus of rats exposed to hypoxia. Further complicating the issue, in larger animals the effects of H_2S on metabolism and T_b remained contradictory, because H_2S-induced hypothermia was shown in pigs by some authors [13,14], whereas other groups did not find an effect on T_b in pigs [15,16] and in sheep [17].

Several mechanisms underlying the thermoregulatory effects of H_2S have been proposed (for review, see [6]), but the molecular site of action and the thermoeffector mechanism underlying H_2S-induced hypothermia has remained unclarified. Peripheral and central inhibition of H_2S-synthetizing enzymes were recently both shown to influence the fever response in rats, but neither of them altered deep T_b in afebrile animals [18,19]. Within the central nervous system, endogeneous H_2S production in the hypothalamus was shown to be involved in the hypothermia associated with endotoxic shock [20] and hypoxia [12]. However, injection of low doses of sodium sulfide (Na_2S), i.e., an H_2S-releasing salt, into the lateral ventricle or the medullary raphe did not cause any significant effect on T_b of euthermic rats [19,21]. With regards to the thermoeffector mechanism, although it is well known that H_2S plays a role in the regulation of the vascular tone [3,6], it is unknown whether skin vasodilation, a principal autonomic heat-dissipating thermoeffector [22], is recruited in the H_2S-induced hypothermia. A plausible molecular target for a thermoregulatory effect could be a sensor that is expressed on neurons located within the thermoeffector pathways, therefore its stimulation can directly lead to a change in deep T_b via modulation of the activity of one or more thermoeffector organs. Interestingly, H_2S has been shown to interact with transient receptor potential (TRP) channels also including temperature-sensitive receptors, e.g., TRP ankyrin-1 (A1) and vanilloid-1 (V1) (for a review, see [23]), which channels are expressed within the neural thermoeffector pathways [22]. The TRPA1 channel can be of crucial importance for the thermal action of H_2S, since TRPA1 channel-mediated effects of sulfide donors and polysulfide were identified in a plethora of experimental models used for the study of pain, inflammation, vasomotor responses, as well as neuronal, urinary, and cardiorespiratory functions.

An extensive list of studies that describeTRPA1-mediated effects of H_2S was recently collected by Pozsgai et al. [24]. However, thermoregulatory effects were not mentioned by the authors. The diverse existence of H_2S-induced TRPA1 activation in different homeostatic processes may suggest that it could also be involved in thermoregulation. In support of this possibility, TRPA1 was shown to be essential in the autonomic thermoregulatory response, particularly in cutaneous vasoconstriction, following cold exposure in mice [25]. Seemingly contradicting this finding is a previous study [26] that found that TRPA1 channels do not play a cold sensor role for autonomic thermoregulation in rodents. However, it is important to note that even if a TRP channel does not play a thermosensor role in the thermoregulation system, its modulation with ligands can still lead to changes of deep

T_b, as was shown in the case of another temperature-sensitive receptor, TRPV1 [27]. In summary, the question of whether and how the TRPA1 channel is involved in H_2S-induced hypothermia has remained unanswered.

In the present study, we have investigated the thermoeffector mechanisms of the hypothermic response to fast- and slow-releasing H_2S donors, then we used *Trpa1* knockout mice to show the contribution of TRPA1 channels to the response. We also studied TRPA1 expression in thermoregulation-related brain nuclei to explore the possible site of action for the hypothermic effect of H_2S.

2. Results

2.1. Central Administration of Na_2S Decreases Deep T_b in Mice via Inhibition of Thermogenesis and Induction of Vasodilation in the Skin

First, we studied the thermoregulatory effect of Na_2S, a fast-releasing H_2S donor [28], administered intracerebroventricularly (i.c.v.) in C57BL/6 mice by using respirometry thermometry (for details, see Experimental Setups in Materials and Methods). In response to Na_2S, the mice developed a decrease in deep T_b, which was more pronounced at the higher dose, whereas saline did not cause any effects (Figure 1).

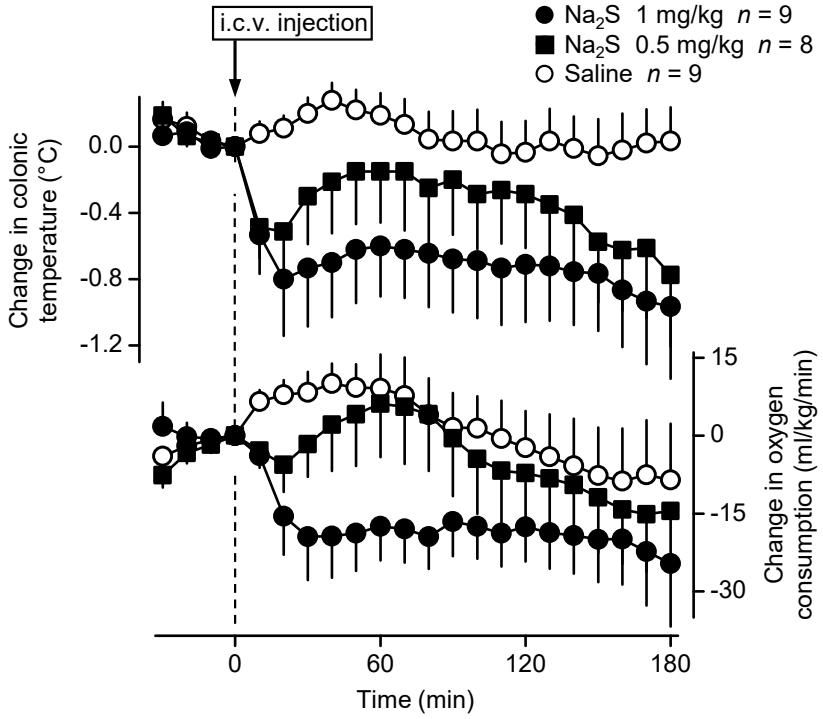

Figure 1. Colonic temperature and oxygen consumption (VO_2) responses of C57BL/6 mice to Na_2S (doses indicated) and saline administered i.c.v. The changes in colonic temperature (a form of deep T_b) are shown in the upper panel, while the changes in VO_2 (an indicator of thermogenesis) are depicted in the lower panel. Here and in Figures 2–5, n is the number of animals in each experimental group.

The hypothermic response to Na_2S developed rapidly at both of the applied doses, and at 20 min it reached the biggest mean decrease of -0.5 ± 0.3 °C at 0.5 mg/kg and -0.8 ± 0.3 °C at 1 mg/kg ($p = 0.045$ and 0.005, respectively). The effect of the treatment on T_b was significant [ANOVA, $F_{(2506)} = 41.158$, $p < 0.001$] and so was the effect of time [ANOVA, $F_{(21,506)} = 1.809$, $p = 0.015$]. The effect was significant for both the lower and the higher doses of Na_2S as compared to saline ($p < 0.001$ for both). At the 0.5 mg/kg dose of Na_2S, deep T_b was significantly lower than in saline-treated mice at 20, 170, and 180 min, while at the 1 mg/kg dose the drop in deep T_b was significant between 20–180 min compared to saline.

In the same experiments, we also measured the rate of oxygen consumption ($\dot{V}O_2$), which was regarded as an indicator of non-shivering thermogenesis (i.e., one of the principal autonomic thermoeffectors), as in previous studies in mice [29,30]. The Na_2S-induced hypothermia was brought about by a fall in $\dot{V}O_2$, which changed with similar dynamics as deep T_b (Figure 1). Similar to T_b, the effect of the treatment on $\dot{V}O_2$ was significant [ANOVA, $F_{(2506)} = 28.860$, $p < 0.001$] and so was the effect of time [ANOVA, $F_{(21,506)} = 1.654$, $p = 0.034$]. The effect was significant for both the lower and the higher doses of Na_2S as compared to saline ($p = 0.024$ and $p < 0.001$, respectively). At the 1 mg/kg dose, $\dot{V}O_2$ was significantly lower than in saline-treated mice between 20 and 110 min. Since the expected effect was hypothermia, these experiments were performed at a subthermoneutral ambient temperature of 22 °C. Mice and rats exhibit cutaneous vasoconstriction in a subneutral environment as also indicated by their low tail-skin temperature [30,31], which did not allow us to study the potential contribution of skin vasodilation to the Na_2S-induced hypothermia in this setup.

In order to determine whether cutaneous blood perfusion is affected by central Na_2S administration, as our next step, we measured changes of skin blood flow intensity on the lumbar back of anesthetized mice with laser speckle contrast imaging. The i.c.v. administration of Na_2S (1 mg/kg) caused a prompt elevation in the cutaneous blood flow intensity, which reached the highest average of 14 ± 6% already at 2 min, then it decreased somewhat but remained higher than in the saline-treated mice throughout the experiment [ANOVA, $F_{(1250)} = 74.081$, $p < 0.001$] (Figure 2). The effect of time was not significant [ANOVA, $F_{(24,250)} = 0.881$, $p = 0.629$]. The blood flow intensity was significantly higher in response to Na_2S compared to saline at 1–4, 6, and 20–24 min.

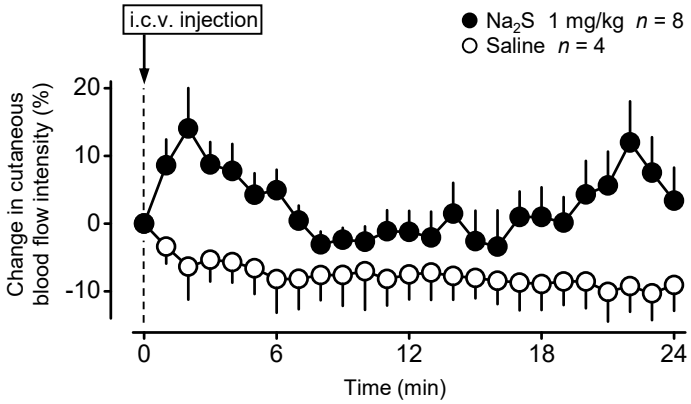

Figure 2. Blood flow intensity changes in the lumbar back-skin of anesthetized C57BL/6 mice in response to Na_2S (dose indicated) and saline administered i.c.v.

The rapid, dose-dependent development of the hypothermic and hypometabolic response to centrally administered Na_2S suggests that the site of action for the released H_2S is located in the central nervous system. To test whether the hypothermic effect of Na_2S can be triggered from a peripheral site, next we studied the thermoregulatory response to the intraperitoneal (i.p.) administration of a high dose (5 mg/kg) of Na_2S. As expected, i.p. infusion of saline did not have any effect on deep T_b (Figure 3). In contrast with the i.c.v. administration, when the mice were infused with Na_2S i.p. their deep T_b did not differ significantly from that of saline-treated mice at any time points during the experiment ($p > 0.05$) even though a 10 times higher dose was delivered i.p. than the i.c.v. administered lower dose which caused hypothermia (see Figure 1). The effect of time was also not significant [ANOVA, $F_{(21,286)} = 1.285$, $p = 0.183$]

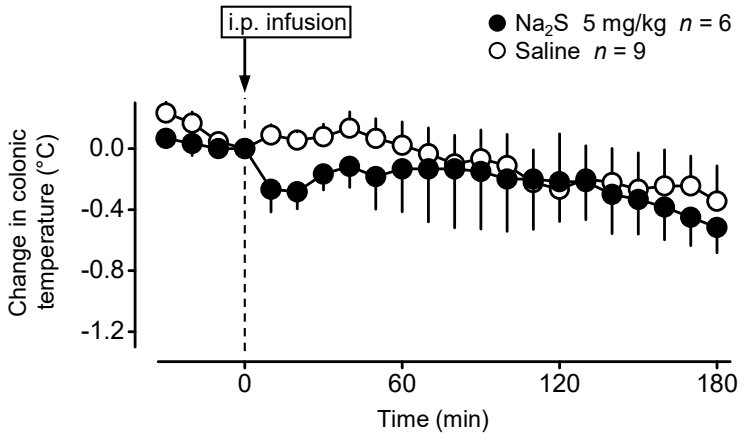

Figure 3. Colonic temperature response of C57BL/6 mice to Na_2S (dose indicated) and saline administered i.p.

2.2. Central Administration of GYY4137 Decreases Deep T_b in Mice

We also wanted to know whether the observed thermoregulatory effects of Na_2S can be triggered by GYY4137, which is a slow-releasing H_2S donor [28]. When administered i.c.v. in the respirometry thermometry setup, GYY4137 (3 mg/kg) caused a marked hypothermia and hypometabolism as compared to saline treatment (Figure 4). In accordance, the effect of treatment was statistically significant on both colonic temperature [ANOVA, $F_{(1242)} = 108.220$, $p < 0.001$] and $\dot{V}O_2$ [ANOVA, $F_{(1220)} = 41.420$, $p < 0.001$]. The effect of time was also significant on colonic temperature [ANOVA, $F_{(21,242)} = 6.815$, $p < 0.001$], but not on $\dot{V}O_2$ [ANOVA, $F_{(21,220)} = 1.019$, $p = 0.441$]. Between the GYY4137-treated and saline-treated mice, deep T_b differed significantly at 80–100 min ($p < 0.05$) and 130–180 min ($p \leq 0.001$), and the difference in $\dot{V}O_2$ was significant at 20–30 min and 160–180 min ($p < 0.05$).

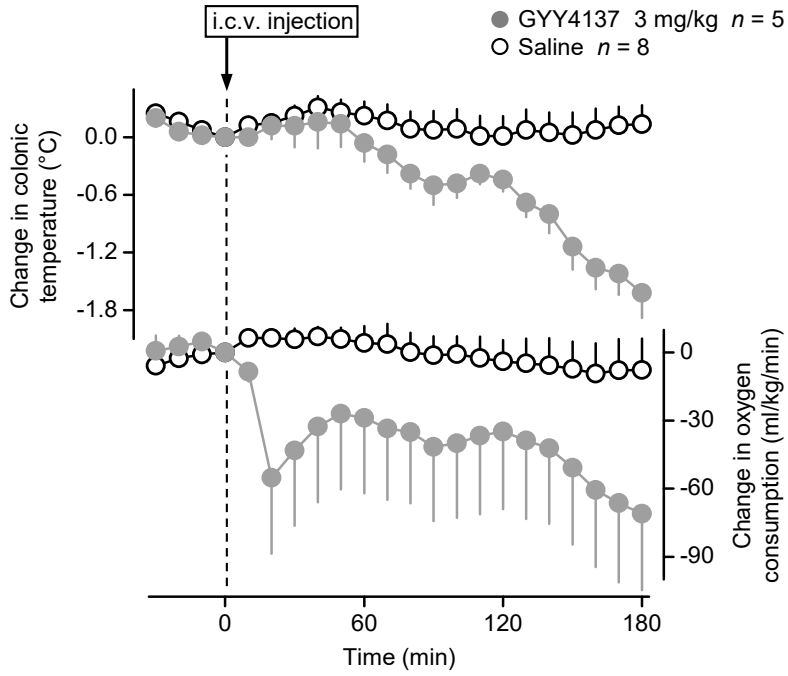

Figure 4. Colonic temperature and $\dot{V}O_2$ responses of C57BL/6 mice to GYY4137 (dose indicated) and saline administered i.c.v. Note that in the bottom graph the number of saline-treated mice is only 7, because in one of the animals the $\dot{V}O_2$ data could not be collected due to technical difficulties.

2.3. The Hypothermic Response to Na_2S and GYY4137 Is Attenuated in the Absence of the TRPA1 Channel

Previously it has been shown that the TRPA1 channel mediates different effects of H_2S, including nociceptive, inflammatory, vasomotor, and neurophysiological effects (for a review, see [24]), but it has remained unknown whether it contributes to the development of the H_2S-induced hypothermia. Therefore, next, we investigated whether the TRPA1 channel is involved in the thermoregulatory responses to different H_2S donors. For that reason, we used mice with (*Trpa1*$^{-/-}$) or without (*Trpa1*$^{+/+}$) a homozygous mutation in the *Trpa1* gene and compared their hypothermic responses. As expected from our experiments in C57BL/6 mice (Figure 1), the i.c.v. administration of Na_2S (1 mg/kg) caused a sudden, pronounced drop in the colonic temperature (>1.5 °C) and VO_2 (>40 mL/kg/min) of the *Trpa1*$^{+/+}$ mice (Figure 5A). However, in the *Trpa1*$^{-/-}$ mice the hypothermic and hypometabolic effects of the same dose of Na_2S were markedly attenuated [ANOVA, $F_{(1308)} = 73.278$, $p < 0.001$ and $F_{(1308)} = 62.496$, $p < 0.001$, respectively, for intergenotype difference]. The effect of time was also significant on colonic temperature [ANOVA, $F_{(21,308)} = 2.535$, $p < 0.001$], but not on VO_2 [ANOVA, $F_{(21,308)} = 0.173$, $p = 1.000$]. The *Trpa1*$^{+/+}$ mice had significantly lower deep T_b between 30 and 180 min, as well as reduced VO_2 between 60 and 180 min post-Na_2S administration as compared to the *Trpa1*$^{-/-}$ mice.

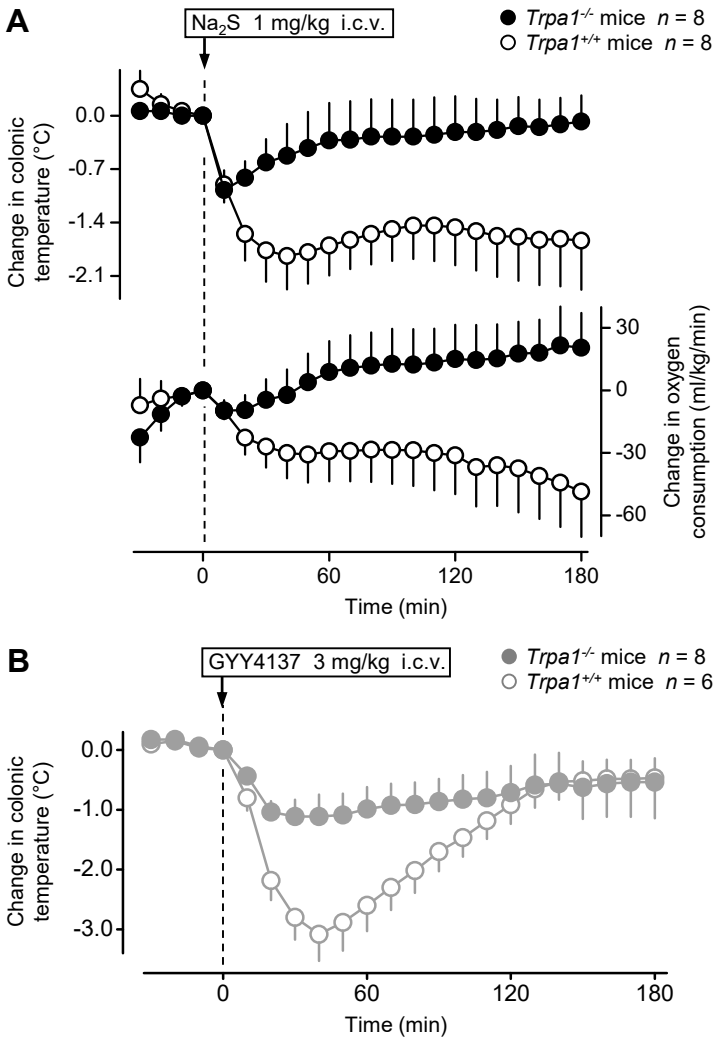

Figure 5. Colonic temperature (upper panel) and VO_2 (lower panel) responses to Na_2S (**A**) and colonic temperature responses to GYY4137 (**B**) administered i.c.v. (doses indicated) in *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice.

We also studied the thermoregulatory effect of GYY4137 in *Trpa1*$^{-/-}$ and *Trpa1*$^{+/+}$ mice in the thermocouple thermometry setup (Figure 5B). The i.c.v. administration of GYY4137 (3 mg/kg) caused a marked fall in the deep T_b of *Trpa1*$^{+/+}$ mice; however, the hypothermic response to the same dose of GYY4137 was significantly attenuated in *Trpa1*$^{-/-}$ mice [ANOVA, $F_{(1264)} = 31.819$, $p < 0.001$]. The effect of time was also significant [ANOVA, $F_{(21,264)} = 8.579$, $p < 0.001$]. The colonic temperature of *Trpa1*$^{-/-}$ mice remained significantly higher than that of *Trpa1*$^{+/+}$ mice between 20 and 80 min post-GYY4137 administration.

2.4. Trpa1 mRNA Is Modestly Expressed in Brain Neurons within Autonomic Thermoeffector Pathways

Our thermophysiological results suggested that the thermoregulatory responses to H_2S donors are triggered from the central nervous system. We, therefore, studied the expression of the TRPA1 channel in thermoregulation-related brain structures. Since the specificity of commercially available antibodies for the TRPA1 protein is debated [32,33], we measured its expression at the mRNA level with two different techniques: real-time-quantitative (RT-q)PCR and RNAscope in situ hybridization (for details, see Measurement of *Trpa1* mRNA Expression in Materials and Methods). Importantly, in both cases, the mice were carefully perfused before the brain sample collection. This step was required in order to avoid the contamination of the samples with blood, which was repeatedly shown to contain a detectable amount of TRPA1 channels [34–36]. First, we used RT-qPCR to assess *Trpa1* mRNA in the hypothalamus of the mice, but we did not find any detectable amount, although the expression of *Trpa1* mRNA was clearly present in the trigeminal ganglion, which was used as a positive control (Figure S1, Supplementary Materials).

Because the turnover of the TRPA1 protein in neurons may not necessitate a high rate of mRNA transcription, then we used RNAscope in situ hybridization, a highly sensitive method that can detect transcripts as single molecules [37]. We found detectable *Trpa1* mRNA transcripts in all of the studied thermoregulation-related nuclei, viz., in the medial preoptic area (Figure 6A), dorsomedial hypothalamic area (Figure 6B), lateral parabrachial nucleus (Figure 6C), and rostral raphe pallidus (Figure 6D), although it should be noted that the number of the *Trpa1* transcripts was low.

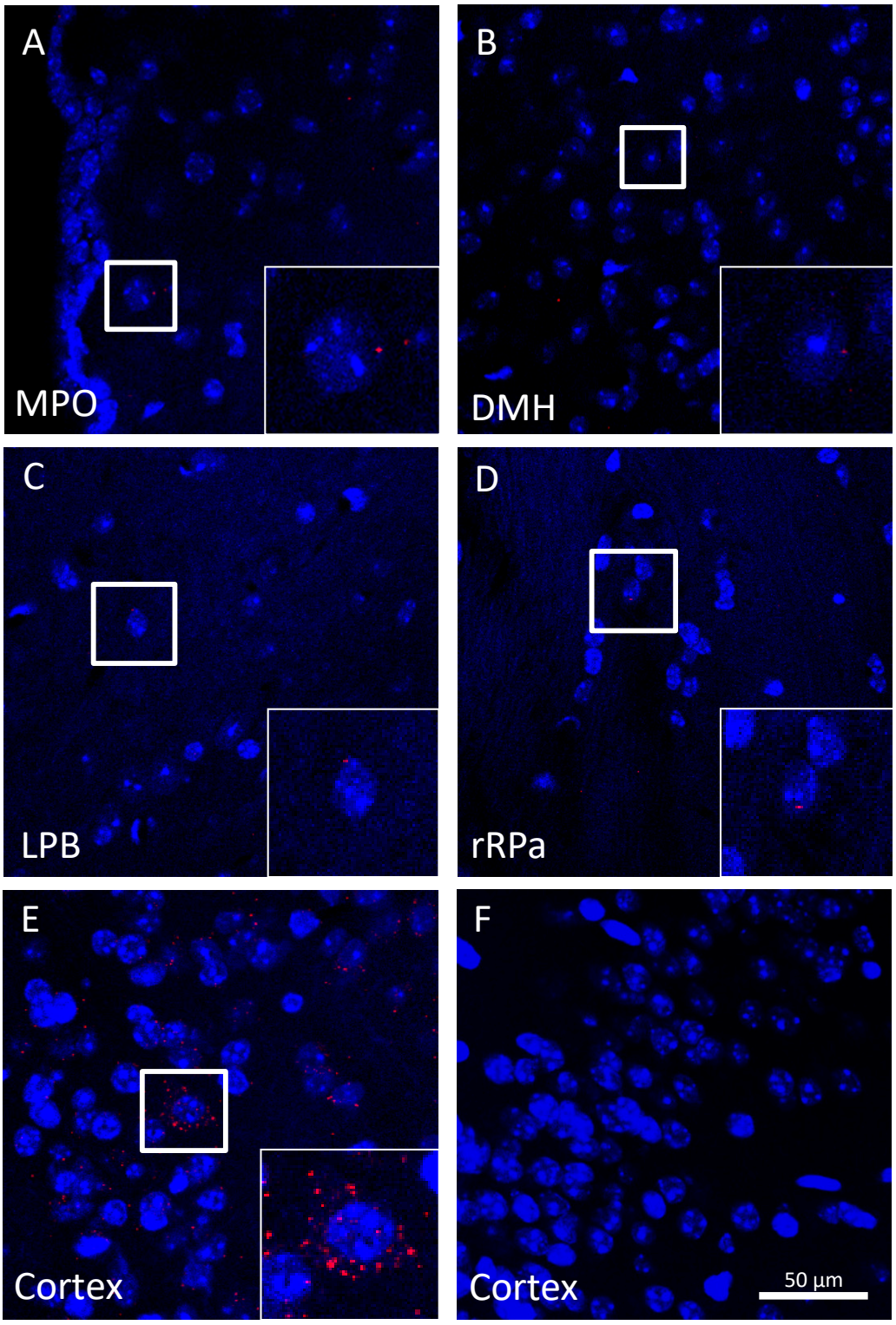

Figure 6. RNAscope in situ hybridization in the mouse brain for *Trpa1* mRNA. Representative confocal images of (**A**) the medial preoptic area (MPO), (**B**) the dorsomedial hypothalamic area (DMH), (**C**) the lateral parabrachial nucleus (LPB), (**D**) the rostral raphe pallidus nucleus (rRPa). Note the low number of *Trpa1* mRNA transcripts (red) in all areas as also shown in higher magnification insets representing the respective boxed areas (**A**–**D**). Positive control staining for *Ppib* mRNA (red) in the cortex (**E**) and negative control staining for the bacterial *dabP* mRNA (red) in the same cortical area (**F**). Cell nuclei were counterstained with DAPI (blue). Scale bar: 50 µm for all images.

3. Discussion

In the present study, we show that fast- and slow-releasing H_2S donors cause hypothermia which is mediated by reduced thermogenesis and increased cutaneous vasodilation. The hypothermic and hypometabolic effects are triggered from the central nervous system and both of them are strongly attenuated in the absence of the TRPA1 channel. TRPA1 channels located on hypothalamic neurons within autonomic thermoeffector pathways can be suggested as the molecular targets for the H_2S-induced hypothermia.

First, we showed that injection of H_2S donors into the lateral ventricle of the brain caused hypothermia and hypometabolism. These effects were more pronounced at higher doses in the case of Na_2S. Our findings are in line with previous reports about the hypothermic response to H_2S inhalation [7,8] and NaHS administration [9], whereas they contradict the results of other authors showing no significant thermoregulatory effect of centrally administered Na_2S [19,21]. An explanation for the contradiction between the results obtained with Na_2S can be that in the previous experiments Na_2S was microinjected at low doses i.c.v. (870–1000 nmol/kg) [19] or into the medullary raphe (0.7–0.8 nmol/kg) [21], whereas in our study the doses were 6.4 and 12.8 µmol/kg and the lower dose of Na_2S had modest thermoregulatory effects. Therefore, it is likely that Na_2S was delivered at subthreshold concentrations to evoke a thermoregulatory effect in earlier studies. In accordance, NaHS also caused hypothermia when infused (to unspecified site) at a rate of 18–72 µmol/kg/h for 4 h [9]. It should be mentioned, however, that after the administration of the donors, H_2S concentrations were not measured in the studies, thus a direct comparison of the H_2S-induced effects at the different doses is not feasible. An alternative reason for the different findings can be that our experiments were performed in mice, whereas in the previous studies rats were used [19,21], which raises the possibility that the thermoregulatory effect of central H_2S differs between mice and rats. Indeed, the lack of the hypothermic effect was reported in larger (non-rodent) animal species by different groups [15–17].

Next, we demonstrated that Na_2S administered in the same way (1 mg/kg i.c.v.) as in the hypothermia experiments also increased the blood perfusion in the skin of the trunk (viz., lumbar back). Cutaneous vasodilation is an autonomic thermoregulatory response, which is recruited to decrease deep T_b (or to prevent its elevation) through increased heat dissipation to the environment [22]. In small rodents, heat-exchange organs with non-hairy skin, e.g., tail and paws, are the predominant body parts for heat transfer between the body and the environment [38]. Our experiments were performed in anesthetized mice placed on a heating pad that was in contact with the ventral surface of the animal including its tail and limbs, therefore, the heating could have interfered with H_2S-induced changes in blood flow in these body parts. On the contrary, the heating had minimal local effects on the vasculature in the skin of the lower back, thus we assessed the effects of H_2S on the blood perfusion in that region. The use of the trunk blood perfusion for the assessment of heat dissipation is also justified by the recent finding that in mice the trunk contributes more to the whole-body heat loss than in rats and it is likely that the largest fraction of total heat loss comes from the body trunk in mice [39]. The involvement of skin vasodilation—in addition to reduced thermogenesis—in H_2S-induced hypothermia indicates that H_2S acts on two distinct efferent thermoeffector pathways or perhaps on neurons that are situated in the common afferent or efferent part of the warmth-sensitive pathways before it divides into separate branches to the different autonomic thermoeffectors. Theoretically, a direct effect of H_2S on the vessels and the brown adipose tissue can be also mentioned, but that scenario is unlikely, since in our experiments the systemic (i.p.) administration of a high dose (10 times higher than what was effective i.c.v.) of Na_2S did not have an effect on deep T_b (Figure 3), thereby indicating a central (intrabrain) site for the thermoregulatory effects.

While Na_2S is considered as a purer donor of H_2S than NaHS, both substances yield H_2S in a bolus instantaneously, which may question the physiological relevance of their effects (reviewed in [40]). In order to circumvent this issue, we also tested the thermoregulatory effects of GYY4137, a very slow-releasing H_2S donor [41], which was used repeatedly to study the true physiological functions of H_2S in a variety of experimental

models, as reviewed by Whiteman et al. [42]. The i.c.v. administration of GYY4137 caused hypothermia and hypometabolism in the mice similar to Na_2S. However, the dynamic of the response was different since the decrease in both $\dot{V}O_2$ and deep T_b developed slower and its extent was more pronounced than in the case of Na_2S. The difference in the dynamics of the hypothermia between GYY4137 and Na_2S is well in harmony with their different capabilities of H_2S generation, because H_2S is released from GYY4137 in a slow and sustained manner, which was shown to evoke slow-onset vasodilatory effects [41].

Last, we wanted to discover the molecular target responsible for the hypothermic response to H_2S. Temperature-sensitive members of the TRP channel superfamily can function as thermoreceptor elements in the thermoregulation system [22], but nonthermal activation of some of these TRP channels can also occur and contribute to the regulation of deep T_b independently from whether the given channel is a thermosensor or not, as it was discovered in case of TRPV1 [43,44]. With regards to an interaction between H_2S and thermosensitive TRP channels, strong evidence accumulated until present days for an action of H_2S on the TRPA1 channel in a vast number of different experimental models [24], but whether TRPA1 also mediates the hypothermic effect of H_2S was unknown until now. In the present work, we studied the thermoregulatory response to H_2S donors (Na_2S and GYY4137) in the genetic absence of the TRPA1 channel by using *Trpa1*$^{-/-}$ mice. We showed that the hypothermic and the hypometabolic responses are both attenuated in *Trpa1*$^{-/-}$ mice as compared to their *Trpa1*$^{+/+}$ littermates. The contribution of TRPA1 to the thermal effect of the H_2S donors used in the present study is in harmony with our previous report about the hypothermic effects of a polysulfide, dimethyl trisulfide, which was also attenuated in *Trpa1*$^{-/-}$ mice [10]. However, polysulfides activate TRPA1 320 times more potently than H_2S [45], thus it was crucial to understand whether H_2S delivered by different non-polysulfide donors can evoke TRPA1-mediated hypothermia. The present findings clearly indicate, for the first time to our knowledge, that hypothermia induced by either fast- or slow-releasing H_2S donors is mediated by the TRPA1 channel in unanesthetized mice.

Since in our experiments we also showed that the hypothermic response is triggered from the central nervous system, then we focused our attention on the expression of the TRPA1 channel in the brain. Because nonspecific binding was shown for different commercially available antibodies against the TRPA1 protein [33,46], we investigated the expression of the channel at the mRNA level in the hypothalamus of the mice, which brain region harbors high number of neurons within the autonomic thermoeffector pathways [47]. We did not detect the presence of *Trpa1* mRNA in the hypothalamus of mice with RT-qPCR. This finding is in accordance with a previous study, in which TRPA1 (formerly also called as ANKTM1) was not detected in the brain of mice [48]; however, it contradicts previous reports showing some TRPA1 expression in the hypothalamus of rats [49,50] and in the brain of mice [51,52]. It must be noted that in our experiments and in the study by Story et al. [48] the mice were perfused before the brain sample collection, whereas the animals were not perfused in any of the other studies [49–52], thus those results were also influenced by *Trpa1* mRNA originating from the components of the blood. In particular, TRPA1 was repeatedly detected in whole blood [34–36] and it is expressed in various peripheral blood leukocytes [53], monocytes [54], and lymphocytes [55,56]. In another study, extremely long exposure times for Northern blots were needed to detect *Trpa1* transcripts in the brain of humans suggesting low-abundance expression [57], but also questioning the sensitivity of the method for the detection of *Trpa1* mRNA. Considerable between-study differences in TRPA1 expression (3.6% vs. 56.5%) were also present in the dorsal root ganglia [48,58], which were presumed to be due to the detection sensitivity of in situ hybridization [59].

RNA integrity can be a critical issue in gene expression studies with qPCR, because fragmented RNA impairs qPCR amplification. The brain is characterized by a higher RNA degradation rate than other tissues [60], which also warrants for the need of more sensitive techniques to study gene expression in brain tissue. In contrast with qPCR, which requires the intact cDNA sequence (from the 5′ of the forward primer to the 3′ end of the reverse primer) for amplification, RNAscope requires the annealing of only 3 pairs of the

20 possible double Z probes to produce a detectable signal. Therefore, shorter mRNA molecules could still be detected by RNAscope probes in brain samples. In accordance with the higher sensitivity, with RNAscope we detected some *Trpa1* mRNA transcripts in all of the studied brain nuclei within the autonomic thermoregulatory pathways. The low-abundance expression of *Trpa1* mRNA in the studied thermoregulatory brain structures may indicate that these neurons play a minor role in the effect of H_2S, but it has to be noted that mRNA expression does not necessarily correlate with the rate of protein translation, since a low mRNA expression can be associated with high protein levels, as shown in different studies [57,61]. Importantly, despite its low mRNA expression, TRPA1 is thought to play critical physiological functions in various tissues [57,61–63]. Similar to TRPA1, another temperature-sensitive TRP channel, TRPV3 was also shown to contribute to neurophysiological functions despite the low abundance of its mRNA in vagal afferent neurons [64]. It should be also mentioned that TRPA1 protein levels can be controlled via regulation of the protein's lifetime by modulation of its ubiquitination status [57]. This may result in the presence of functional TRPA1 proteins on the neurons despite the low abundance of *Trpa1* mRNA. It was shown that de-ubiquitination of TRPA1 by the ubiquitin hydrolase protein CYLD increases the cellular pool of TRPA1 proteins [57] and CYLD expression was detected in different brain regions of mice, also including the hypothalamus [65]. Consequently, it is possible that the TRPA1 channel is expressed to a sufficient extent at the protein level to mediate the effects of H_2S directly from the studied thermoregulation-related neurons. However, it cannot be excluded that TRPA1 channels in other brain structures are the primary sites for the action of H_2S and modulate the activity of thermoeffectors through their projections to neurons within the thermoregulation pathways. In support of that scenario, physiological function for TRPA1 was found in the somatosensory cortex [66], in the hippocampus [63,66], as well as in the supraoptic and solitary nuclei [67,68], which brain regions are also involved in the regulation of deep T_b [47,69,70].

The involvement of TRPA1 was shown in the development of hypothermia in response to diverse stimuli, such as acetaminophen [71], relative hypoxia [72], and thiazoline-related innate fear-eliciting compounds [73]. Although these agents should not be considered as selective activators of TRPA1, the hypothermic response to them was markedly attenuated or completely absent after the pharmacological or genetic blockade of TRPA1 channels yielding to the conclusion that activation of the TRPA1 channel by the applied stimuli mediates the hypothermia [71–73]. In one of these studies, the TRPA1 agonist cinnamaldehyde induced a marked hypothermia in *Trpa1*$^{+/+}$ mice, which was significantly reduced in *Trpa1*$^{-/-}$ mice [71]. These findings support our conclusions about the involvement of an H_2S-induced activation of TRPA1 in the hypothermic response, but future research is warranted to reveal the exact nature of the interaction between H_2S and TRPA1 and its contribution to the hypothermia.

Until the H_2S-TRPA1 interaction in association with the hypothermic response is fully understood, alternative mechanisms of the hypothermic effect must be also stated. H_2S can influence a variety of cellular structures and TRPA1 can be a channel that is important as a downstream mediator in different signal transduction pathways. In particular, the activation or expression of TRPA1 can be modulated by kinases, transcription factors, hormones, and reactive oxygen species, which can be activated by sulfides (for review, see [24]). Interestingly, through their activation in the central nervous system at least some of these signaling pathways can also contribute to a decrease in deep T_b in response to different stimuli, as proposed, for example, for AMP-activated protein kinase [74], p38α mitogen-activated protein kinase [75], and estrogens [76]. Despite the involvement of TRPA1 in the signaling pathways, their activity does not necessarily depend solely on TRPA1, hence the blockade of the channel (e.g., in *Trpa1*$^{-/-}$ mice) may not lead to the inhibition of the whole pathway, which could explain why some (attenuated) hypothermic response to the used sulfide donors was still present in *Trpa1*$^{-/-}$ mice in our experiments.

Whether H_2S triggers hypothermia through the direct or indirect activation of TRPA1 channels remains the subject for future research.

4. Materials and Methods

4.1. Animals

Experiments were performed in 109 adult mice of both sexes. As in our earlier studies [10,26], male *Trpa1*$^{-/-}$ and *Trpa1*$^{+/+}$ mice (n = 18 and 14, respectively) were obtained from the Laboratory Animal Centre of the University of Pecs, where they were bred from breeding pairs generously donated by Dr. Pierangelo Geppetti. Seventy-seven C57BL/6 mice were also obtained from the Laboratory Animal Centre at the University of Pecs where they were bred and kept under standard pathogen-free conditions. The mice were housed in standard polycarbonate cages kept in a room with ambient temperature maintained at 24–25 °C and humidity at 30–40%. The room was on a 12 h light–dark cycle (lights on at 5:00 a.m.). Standard rodent chow and tap water were available ad libitum. At the time of the experiments, the *Trpa1*$^{-/-}$, *Trpa1*$^{+/+}$, and C57BL/6 mice weighed 26 ± 2, 26 ± 3, and 27 ± 4 g, respectively. For thermophysiological experiments, mice were extensively habituated to staying inside wire-mesh cylindrical confiners, as described previously [30]. All procedures were conducted under protocols approved by the Institutional Animal Use and Care Committee of the University of Pecs (registration no.: BA02/2000–6/2018, approved on 27 February 2018) and were in accordance with the directives of the National Ethical Council for Animal Research and those of the European Communities Council (86/609/EEC).

4.2. Surgeries

4.2.1. Anesthesia and Perioperative Care

Mice were anesthetized with i.p. administration of a ketamine-xylazine cocktail (81.7 and 9.3 mg/kg, respectively) and received antibiotic protection intramuscularly (gentamycin, 6 mg/kg). During surgery, mice were heated with a temperature-controlled heating pad (model TMP-5a; Supertech Instruments UK Ltd., London, UK) placed under a surgery board. Each mouse was subjected to one of the surgical procedures described below. The experiments were performed 4–8 days after the surgery.

4.2.2. I.c.v. Cannula Implantation

For the i.c.v. substance administration, A 22-G steel guide cannula (Plastics One, Roanoke, VA, USA) was implanted into the right lateral cerebral ventricle using a stereotaxic manipulator (Narishige Scientific Instruments Laboratory, Tokyo, Japan), as described previously [77]. In brief, after incision of the scalp and removal of the periosteum, two supporting microscrews (Fine Science Tools, Heidelberg, Germany) were driven into the skull and the guide cannula was inserted through a small hole drilled in the skull 0.5 mm posterior from Bregma and 1.0 mm lateral from midline. The tip of the cannula was placed within the right lateral ventricle (2.0 mm from dura). The cannula was fixed to the supporting microscrews with dental cement and closed by a dummy cannula.

4.2.3. I.p. Catheter Implantation

For the i.p. administration of substances, a polyethylene (PE)-50 catheter filled with pyrogen-free saline was implanted into the peritoneal cavity, as described elsewhere [30,77]. Through a small midline incision on the abdominal wall, the internal end of the catheter was fixed to the abdominal wall with a suture, while the external end of the catheter was exteriorized at the nape and heat-sealed. The surgical wound was sutured in layers. The catheter was flushed with 0.1 mL of saline on the day after the surgery and every other day thereafter.

4.3. Experimental Setups

Thermoregulatory experiments in unanesthetized mice were performed in either the thermocouple thermometry setup or the respirometry thermometry setup. The experiments were conducted at an ambient temperature of 30 °C in the thermocouple thermometry setup and an ambient temperature of 22 °C in the respirometry thermometry setup, which is subneutral for mice in these setups [77]. Cutaneous blood flow measurement was performed by laser speckle contrast imaging in anesthetized animals.

4.3.1. Thermocouple Thermometry

The mice were placed in cylindrical confiners and equipped with copper-constantan thermocouples (Omega Engineering, Stamford, CT, USA) to measure colonic temperature (a form of deep T_b). The colonic thermocouple was inserted 3 cm deep beyond the anal sphincter, fixed to the base of the tail with adhesive tape, and plugged into a data logger device (Cole-Palmer, Vernon Hills, IL, USA) connected to a computer. Mice in their confiners were then placed into a temperature-controlled incubator (model MIDI F230S; PL Maschine Ltd., Tarnok, Hungary) set to an ambient temperature of 30 °C, which is slightly below the thermoneutral zone for mice in this setup. When the mouse was pre-implanted with an i.c.v. cannula, a needle injector (Plastics One, Roanoke, VA, USA) was fitted into the guide cannula and connected to a PE-50 extension, which was prefilled with a solution of Na_2S or GYY4137 or with saline. The injector needle protruded 1.0 mm beyond the tip of the guide cannula. The extension was passed through a port of the incubator and connected to a 10-µL syringe (model 701N, Hamilton, Reno, NV, USA). When the mouse had an i.p. catheter, it was connected to a PE-50 extension, which was prefilled with the substance of interest and connected to a syringe placed in an infusion pump (model 975; Harvard Apparatus Inc., Holliston, MA, USA). The PE-50 extensions preloaded with the substances were wrapped in aluminum foil in order to prevent the photolytic oxidation of sulfide ions by UV light, which reaction can occur in aqueous sulfide solutions [78].

4.3.2. Respirometry Thermometry

The mice were equipped with thermocouples and PE-50 extensions the same way as in the experiments in the thermocouple thermometry setup. Then, the mice in their confiners were transferred to a Plexiglas chamber of the four-chamber open-circuit calorimeter integrated system (Oxymax Equal Flow, Columbus Instruments, Columbus, OH, USA). The chambers were sealed, submerged into a temperature-controlled water bath, and continuously ventilated with room air (200 mL/min). The fractional concentration of oxygen was measured in the air entering and exiting the chamber, and the rate of oxygen consumption was calculated according to the manufacturer's instructions using the Oxymax Windows software (version 3.1).

4.3.3. Laser Speckle Contrast Imaging

The mice were anesthetized, then the fur on the lumbar back-skin was clipped and the animals were placed in a ventral position on a heating pad (model TMP-5a; Supertech Instruments UK Ltd., London, UK) to maintain their deep body temperature at 36 °C for the duration of the experiment. A needle injector was fitted into the preimplanted i.c.v. guide cannula and connected to a PE-50 extension, which was prefilled with a solution of Na_2S or with saline. The blood flow intensity on the lumbar back-skin of the mice was measured by a PeriCam PSI system (Perimed AB, Järfälla, Sweden), which applies laser speckle contrast analysis technology. The system measures blood perfusion by recording changes in the speckle pattern as motion blurring in the regions of interests. If there is more movement in the region, e.g., due to higher red blood cell flow, blurring will increase and the speckle contrast will be lower, which correlates with blood flow. The change in blood perfusion (recorded in arbitrary perfusion units) during the experiments was expressed as percentage compared to the baseline value determined at the time of substance administration.

4.3.4. Drugs and Drug Administration

Na_2S nonahydrate ($Na_2S{\cdot}9H_2O$) was purchased from Sigma-Aldrich (St. Louis, MO, USA). On the day of the experiment, $Na_2S{\cdot}9H_2O$ was freshly dissolved in pyrogen-free saline to achieve final concentrations of Na_2S of 1.5, 5, or 10 mg/mL. For the i.p administration, the working solution (1.5 mg/mL) of Na_2S (or saline) was infused over 4 min (3.3 mL/kg) to deliver Na_2S at 5 mg/kg. For the i.c.v. administration of Na_2S (at doses of 0.5 and 1 mg/kg), the working solutions (5 and 10 mg/mL, respectively) of Na_2S (or saline) were infused (1 µL/min) over a 3 min period.

The slow-releasing hydrogen sulfide donor GYY4137 was synthesized at the University of Exeter Medical School, as described elsewhere [41]. On the day of the experiment, GYY4137 was freshly dissolved in saline to make a working solution of 30 mg/mL. By infusing this solution into the lateral ventricle (1 µL/min for 3 min), a total dose of 3 mg/kg of GYY4137 was delivered i.c.v. Control mice were infused with saline.

4.4. Measurement of Trpa1 mRNA Expression

4.4.1. RT-qPCR

Three month-old male C57BL/6 mice ($n = 7$) and *Trpa1*$^{-/-}$ mice ($n = 2$, for negative control) were deeply anesthetized with an overdose of urethane (2.4 mg/kg) and perfused transcardially with 30 mL of phosphate-buffered saline (PBS). Brains and trigeminal ganglia were quickly dissected after decapitation, frozen on dry ice, and stored at −80 °C. Then, brains were sliced using razor blades on a coronal brain matrix (Ted Pella, Redding, CA, USA) to obtain 1 mm thick coronal sections, according to the technique described by Palkovits et al. [79]. A microdissecting tool (Ted Pella, USA) of 1 mm diameter was used to punch the hypothalamus between coronal planes of −0.5 mm to −2.5 mm caudal to Bregma, based on the atlas by Paxinos and Franklin [80]. Eight tissue punches were cut and pooled in order to collect samples representative of the entire hypothalamus of each animal. The microdissection procedure was performed on a dry ice-chilled mat and the punches were immediately snap-frozen in precooled Eppendorf vials on dry ice. Then, the samples were stored at −80 °C until the RNA isolation procedure.

Total RNA from the microdissected mouse brain samples and trigeminal ganglia were extracted with Direct-zol RNA Microprep kit (Zymo Research, Irvine, CA, USA). The concentration and purity of total RNA quality were assessed by a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA, USA). For further analysis, only those RNA samples were used that showed an A260/280 ratio between 1.9 and 2.1 and an A260/A230 ratio higher than 2.0. The RNA samples were treated with DNase I (Zymo Research, USA) to remove genomic DNA. Using Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA), 1 µg RNA was reverse transcribed into cDNA. Applied Biosystems QuantStudio 3 RT PCR System (Thermo Fisher Scientific, USA) was used to perform qPCR experiments, using SensiFast SYBR Lo-ROX Kit (Bioline, Taunton, MA, USA) according to the manufacturer's manual. All qPCR experiments were performed in technical replicates and included a melt curve analysis to ensure the specificity of the signal. Reverse transcriptase minus control showed the lack of genomic DNA contamination. The geometric mean of the reference gene Ct values was determined and *Trpa1* mRNA expression relative to the reference genes beta-actin (*Actb*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was calculated. Primers used to amplify target loci for RT-qPCR are listed in Table 1.

Table 1. Primers used to amplify target loci for RT-qPCR.

Gene Amplified (Mus Musculus)	Nucleotide Sequence of Primer	Primer Type	Product Length in bp	NCBI RefSeq
Trpa1	ATCCAAATAGACCCAGGCACG	sense	101	NM_177781.5
	CAAGCATGTGTCAATGTTTGGTACT	antisense		
Gapdh	TTCACCACCATGGAGAAGGC	sense	237	NM_001289726.1
	GGCATGGACTGTGGTCATGA	antisense		
Actb	CTGTATGCCTCTGGTCGTAC	sense	214	NM_007393.5
	TGATGTCACGCACGATTTCC	antisense		

4.4.2. RNAscope In Situ Hybridization

For RNAscope studies 3 month-old male C57BL/6 mice (n = 4) were perfused as described above using 30 mL PBS followed by 100 mL of 4% paraformaldehyde in Millonig's phosphate buffer. Brains were postfixed for 24 h at room temperature, rinsed in PBS, dehydrated, and embedded in paraffin using standard procedures. 5 µm sections were cut using a sliding microtome (model HM 430; Thermo Fisher Scientific, USA). The RNAscope Multiplex Fluorescent Reagent Kit v2 (ACD, Hayward, CA, USA) was used according to the manufacturer's protocol. In short, sections were heat-treated, deparaffinized, H_2O_2-blocked, boiled, and pretreated with Protease Plus. Subsequently, the sections were hybridized with probes specific to mouse *Trpa1* mRNA and with 3-plex positive and negative control probes. Sequential signal amplification and channel development were performed. Nuclear counterstaining with 4′, 6-diamidino-2-phenylindole (DAPI) was applied and sections were mounted with ProLong Diamond Antifade Mountant for confocal imaging. Probes and applied dilutions of fluorophores are listed in Table 2.

Table 2. RNAscope probes and applied dilutions.

Probes (Mus Musculus)	Catalog Number	Fluorophores	Dilution
Trpa1	400211	TSA Plus Cyanine 3	1:750
3-plex Negative Control Probe	320871	TSA Plus Fluorescein, Cyanine 3 and 5	1:750
3-plex Positive Control Probe	320881	TSA Plus Fluorescein, Cyanine 3 and 5	1:750

In accordance with earlier studies [81,82], cortical samples were stained for the *ppib* mRNA (red) as positive technical control (Figure 5E) and the bacterial *dabP* mRNA staining (red) was used as negative technical control (Figure 5F). According to the atlas by Paxinos and Franklin [80], fluorescent images of the medial preoptic area (+0.14 mm to +0.02 mm from Bregma), dorsomedial hypothalamic area (−1.58 mm to −1.70 mm from Bregma), as well as the lateral parabrachial nucleus and rostral raphe pallidus (−5.34 mm to −5.40 mm from Bregma for both) were acquired using an Olympus Fluoview FV-1000 laser scanning confocal microscope and Fluoview FV-1000S-IX81 image acquisition software system (Olympus, Tokyo, Japan). The confocal aperture was set to 80 µm. The analog sequential scanning was performed using a 40× objective lens (NA:0.75). The optical thickness was set to 1 µm and the resolution was 1024 × 1024 pixels. The excitation time was set to 4 µs per pixel. Blue and red virtual colors were selected to depict fluorescent signals of DAPI (nuclear counterstain) and of Cyanine 3 (*Trpa1* mRNA), respectively.

4.5. Data Processing and Analysis

Data on deep T_b, $\dot{V}O_2$, and blood flow intensity were compared by two-way ANOVA. As in our previous studies [27,29], ANOVA was followed by the Fisher's LSD post hoc test. Sigmaplot 11.0 (Systat Software, San Jose, CA, USA) software was used for statistical analyses. Differences were considered significant when $p < 0.05$. All data are reported as mean ±SE.

5. Conclusions

In conclusion, we show that slow- and fast-releasing H_2S donors induce hypothermia through hypometabolism and cutaneous vasodilation in mice and that the hypothermic effect of H_2S is mediated by TRPA1 channels located in the brain, presumably on hypothalamic neurons within the autonomic thermoeffector pathways. Our findings highlight the importance of central TRPA1-mediated H_2S signaling in the thermoregulation system. In severe forms of systemic inflammation (e.g., septic shock), which is often associated with hypothermia [83] and by enhanced production of H_2S [40,84], the interaction between TRPA1 and H_2S can play a crucial role in the development of the response and, as perspective, may serve as a therapeutic target. Furthermore, the H_2S-induced activation of central TRPA1 channels may pave the road to the development of controlled induction of hypothermia, but future research is needed to reveal the true thermopharmacological potential of the central TRPA1-H_2S interaction in health and disease.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ph14100992/s1, Figure S1: Representative electrophoretogram of the RT-qPCR products.

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Supplementary Material

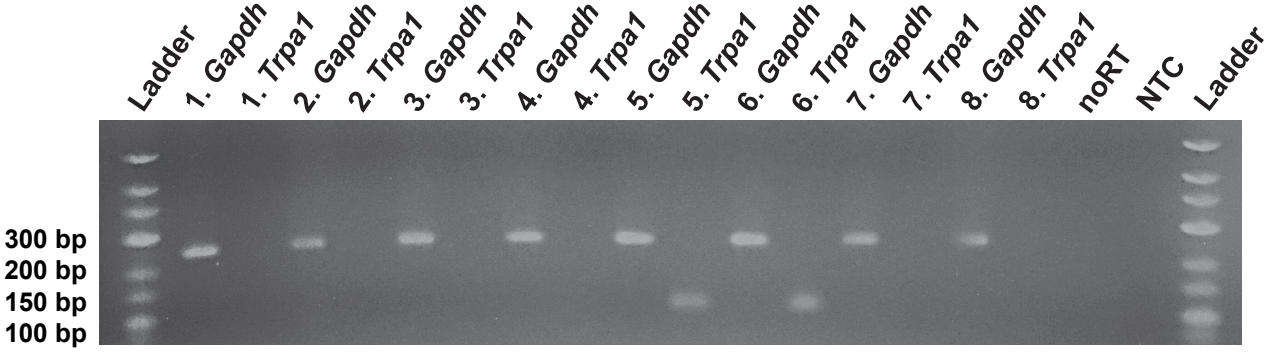

Figure S1. Representative electrophoretogram of the RT-qPCR products. Samples 1-4 represent the expression of the studied genes (*Gapdh* and *Trpa1*) in the hypothalamus of C57BL/6 mice. Samples 5 and 6 represent the expression of the genes in the trigeminal ganglia of C57BL/6 mice (positive controls), while samples 7-8 represent the gene expressions in the trigeminal ganglia of *Trpa1*$^{-/-}$ mice (negative controls). Note that the housekeeping gene *Gapdh* (size: 237 bp) was expressed in all samples, whereas the gene of interest *Trpa1* (size: 101 bp) was detectable only in the trigeminal ganglia of C57BL/6 mice. No reverse transcriptase (noRT) and no template controls (NTC) are shown as technical controls.