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In the Era of Therapeutic Hypothermia, How Well Do Studies of Perinatal Neuroprotection Control Temperature?

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Perinatal brain injury · Cerebral palsy · Hypoxic-ischemic encephalopathy · Asphyxia · Neuroprotection

Abstract
In the era of therapeutic hypothermia, reliable preclinical studies are integral to successfully identify neuroprotective strategies to further improve outcomes of encephalopathy at term. We reviewed preclinical neuroprotection studies reported between January 2014 and June 2016 to assess the use of effective temperature monitoring and control. As a secondary measure, we examined whether studies addressed other methodological issues such as stage of brain development, sex differences, the timing of the treatment relative to the insult, and the histological and functional endpoints used after hypoxia-ischemia. The extent and duration of temperature monitoring was highly inconsistent. Only a minority of papers monitored core (19/61; 31%) or brain temperature (3/61; 5%). Most (40/45) of the neuroprotectants either were likely to affect thermoregulation or their impact is unknown. In 85% of papers neonatal rodents were used (67% at P7); 51% of papers did not report the sex of the animals or tested the effect of potential neuroprotectants on just one sex. In 76% of studies, treatment was before or immediately after the insult (within the first 2 h), and few studies assessed long-term histological and behavioral outcomes. In conclusion, many recent preclinical neonatal studies cannot exclude the possibility that apparent neuroprotection might be related to drug-induced hypothermia or to other methodological choices. Close monitoring and control of brain temperature during, as well as for many days after, experimental hypoxia-ischemia are now critical to reliably develop new ways to improve neurodevelopmental outcomes after perinatal hypoxic-ischemic encephalopathy.

Introduction

Mild "therapeutic" hypothermia for hypoxic-ischemic encephalopathy (HIE) in near-term and term infants is now well established to improve survival without disability [1]. Current hypothermia protocols are only partially protective, such that nearly half of infants still die or survive with disability despite cooling [2], and so it is vital to identify adjunct therapies to further improve outcomes from HIE [3]. The corollary of the successful clinical translation of therapeutic hypothermia is that we can now be certain that even small changes in brain and/or body

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temperature critically affect outcome in both animals [4–7] and humans [8–10]. Indeed, many apparent neuroprotective effects of various pharmacological agents can be mediated by drug-induced hypothermia, as previously reviewed [11]. Conversely, neuroprotection can be masked by delayed hyperthermia [6].

Neonatal rodents are widely used to model HIE and assess neuroprotective strategies. However, due to their small body mass relative to surface area, lack of subcutaneous fat, naked skin, poor control of peripheral vasculature, and absent shivering [12], the infant rat produces less heat and loses more body heat than adults [13]. These factors make them functionally poikilothermic and particularly susceptible to rapid changes in body and brain temperature during changes in environmental temperature [14]. Further, their brains are relatively small and flat, which can allow selective brain cooling, particularly if blood flow is reduced after HI [11]. Behavioral thermoregulation in neonatal rodents begins to emerge during the 1st week of life, primarily through metabolism of brown adipose tissue and group huddling [15]. However, their thermoregulatory ability is not fully developed until the 3rd week of life [16].

Thus, in this study, we reviewed studies reported between January 2014 and June 2016 to assess the use of effective temperature monitoring and control in preclinical neuroprotection studies in the era of therapeutic hypothermia. As a secondary measure, we examined whether these studies addressed other important methodological issues [17], such as the stage of brain development, sex differences, the timing of the treatment relative to the insult, and the duration of follow-up for histological and functional endpoints after HI.

Analysis Strategy

In this review, a group of researchers focusing on perinatal neuroprotection have examined preclinical studies of perinatal neuroprotection reported from January 1, 2014, to June 30, 2016, for how well these studies control for temperature in near-term or term-equivalent brain injury. The studies were grouped into those that demonstrated improved or unaffected neuropathological or neurodevelopmental outcomes. Studies were further subdivided by species, type of insult used to induce HIE, the treatment used, timing of treatment relative to the insult, extent of temperature monitoring, sex of the subjects, main study outcomes (histological and/or functional), and survival time after the insult based on histological and/or functional assessments. Studies were searched for on PubMed, MEDLINE (OvidSP), and Google Scholar using the following terms: (brain injury OR hypoxia-ischemia OR hypoxia OR ischemia OR cerebral ischemia OR occlusion) AND (neuroprotection) AND (fetal OR neonatal OR postnatal). Other sources used to identify studies included relevant papers and reviews.

Abstracts were initially identified and screened by an investigator from our study group (R.G.) and duplicated by another investigator (J.M.D.). Full-text articles were then obtained using Papers software (version 3.4.6; Labtiva Inc., Cambridge, MA, USA) for review. Relevant studies were selected based on group consensus. Studies were deemed eligible if they presented clear histological and/or functional outcomes from in vivo experiments that investigated neuroprotection using pharmacological interventions or mechanisms of protection via less pragmatic interventions, such as gene deletion, gene inactivation, or conditioning. All pharmacological interventions were considered individually, even in studies that used more than 1 treatment. The class of drug was searched for to assess whether the neuroprotectant had potential to affect thermoregulation. Specifically, the neuroprotectants were searched for on PubMed, MEDLINE (OvidSP), and Google Scholar using the following terms: (*name of relevant drug*) AND (temperature OR vasodilation). The drug was considered *likely* to affect temperature if it had previously been shown to affect thermoregulation or belonged to a family of drugs known to affect body or brain temperature. It was deemed *unlikely* to affect temperature if no effect had previously been reported. *Unknown* indicates no studies have investigated whether the drug affects temperature. Histological outcomes were based on assessment of gray and/or white matter cell survival or death. Functional outcomes were based on assessment of behavior or recovery of brain activity, as assessed using neurophysiology or examination of clinical seizures.

Inclusion criteria were in vivo studies of rodents on postnatal days (P) 7–11, sheep at 0.80–0.85 of gestation and P1–5 neonatal piglets. These developmental ages are often used to study treatments for term infants, although the cortical maturity of laboratory rats at P7 is consistent with the late preterm human infant [18]. We excluded in vitro studies and those that did not meet the age criteria. The methodological quality of the studies was assessed according to whether temperature was controlled, treatment was randomized, and investigators were blinded to the intervention during histological and functional assessments. The results are summarized in Table 1 and Figures 1–4.

Table 1. Preclinical studies of perinatal neuroprotection

Reference	Species	Insult	Treatment (route)	Timing	Temperature, °C	Pathology	Functional outcome	Survival	Sex
Improved neurodevelopmental outcomes									
39	P7 mice	HI: 90 min 8% O_2	*Hydrogen (i.p.)* *HI + vehicle* *HI + hydrogen-rich saline*	Post-Tx: 24, 48, 72 h	Ambient: 36°C, during 2 h recovery	↓ infarct vol, $p < 0.05$ vs. vehicle		3 days	?
40	P1 piglets	HI FiO_2 6%	*HT and argon* HI + HT HI + HT + argon	Post-Tx: 2 h	Core: 33.5°C, continuous monitoring	~67% ↓ cell death, $p < 0.05$ vs. HT	↑ PCr/Pi and ↓ thalamic, white matter Lac/NAA and improved aEEG recovery vs. HT, $p < 0.05$	2 days	♂
41	P1–2 piglets	HI FiO_2 10%	*Cannabidiol (i.p.) and HT* HI + HT + vehicle HI + HT + cannabidiol	Post-Tx: 30 min	Core: 33–34°C, continuous monitoring	↓ cortical neuronal necrosis vs. HT, $p < 0.05$	↑ Lac/NAA and improved aEEG recovery vs. HT, $p < 0.05$	6 h	♂
42	P7 rats	HI: 90 min 8% O_2	*Sevoflurane* HI HI + sevoflurane	Immediate Tx: 0 h	Ambient: 36.5°C, during HI	↓ CA1 and 3 hippocampal neuronal loss, $p < 0.05$ vs. HI	Improved learning and memory, $p < 0.05$ vs. HI	3 weeks	♂/♀
43	P7 rats	HI: 90 min 8% O_2	*Helium* HI He + HI	Pre-Tx: –24 h	Ambient: 37°C, during HI	↓ infarct vol vs. HI, $p < 0.05$	Improved sensorimotor function vs. HI	Pathology: 1 day behavior: 3 weeks	?
44	P10 rats	HI: 3.5 h 8% O_2	*MicroRNA-210 antagonist (i.c.v.)* vehicle + HI miRNA antagonist + HI	Post-Tx: 4 h	Ambient: 37°C, during HI	47% ↓ infarct vol vs. vehicle, $p < 0.05$	Improved spatial learning and sensorimotor function vs. vehicle	Pathology: 2 days behavior: 6 weeks	?
45	P10 mice	HI: 15 min 8% O_2	*DHA (i.p.)* HI + vehicle HI + DHA	Immediate Tx: 0, 1 h	Ambient: 37°C, during HI	49% ↓ in infarct vol, $p < 0.05$ vs. vehicle		1 day	?
45	P10 mice	HI: 15 min 8% O_2	HI + vehicle HI + DHA	Immediate Tx: 0, 1 h	Ambient: 37°C, during HI	32% ↑ in brain tissue vol, $p < 0.05$ vs. vehicle	Improved memory performance, $p < 0.05$ vs. vehicle	9 weeks	?
46	P7 rats	HI: 2 h 8% O_2	*HT and NAC (i.p.)* HI + HT + vehicle HI + HT + NAC	Immediate Tx: 0 h	Core: 36.5–37.5 vs. 33.5–34.5°C during 2 h recovery	↓ infarct vol in females, $p < 0.05$ HT + NAC vs. HT no effect in males	Improved sensorimotor function vs. HT.	Pathology: 2 days behavior: 6 weeks	♂/♀
47	P9 mice	HI: 1 h 10% O_2	*HT* HI + NT HI + HT	Immediate Tx: 0 h	Core: 35 vs. 27° C during 4 h recovery	↑ hippocampal neuronal density and ↓ total neural injury score	Improved sensorimotor function and cognition vs. NT	Pathology: 1 week behavior: 3 weeks	♂/♀
47	P9 mice	HI: 1 h 10% O_2	HI + NT HI + HT	Post-Tx: 2 h	Core: 35 vs. 27°C during 4 h recovery	↑ hippocampal neuronal density and ↓ neural injury score	No effect on behavior	Pathology: 1 week behavior: 5 weeks	♂/♀
48	P7 rats	HI: 2 h 8% O_2	*Dexmedetomidine (i.p.)* HI + vehicle HI + dexmedetomidine	Post-Tx: 30, 60, and 120 min	Ambient: 37°C, during HI		Improved sensorimotor function and learning and memory vs. vehicle	4 weeks	?
49	P9 mice	HI: 50 min 10% O_2	*Atg7 (autophagy-related protein 7)* WT + HI Atg7 KO + HI	Constitutive gene KO	Ambient: 36°C, during HI	~49% ↓ in infarct vol, $p < 0.05$ vs. WT		8 days	♂/♀
50	P7 rats	HI: 2 h 8% O_2	*Isoflurane* HI HI + isoflurane	Immediate Tx: 0 h	Ambient: 37°C, during HI	↑ cortical and thalamic neuronal density vs. HI	Improved learning and memory performance vs. HI	Pathology: 1 week behavior: 5 weeks	♂/♀
50	P7 rats	HI: 2 h 8% O_2	HI HI + isoflurane	Post-Tx: 3 or 6 h	Ambient: 37°C, during HI	↑ cortical and thalamic neuronal density vs. HI	Improved learning and memory performance vs. HI	Pathology: 1 week behavior: 5 weeks	♂/♀
51	P7 rats	HI: 90 min 8% O_2	*Argon* HI + nitrogen with O_2 HI + argon with O_2	Immediate Tx: 0 h	Not specified	47% ↓ in infarct vol, $p < 0.05$ vs. nitrogen		4 weeks	?
52	P7 rats	HI: 2 h 8% O_2	*Taurine (i.p.)* HI + vehicle HI + taurine	Post-Tx: 12, 24, 26, 48 h	Ambient: 37°C, during HI	↓ infarct vol and cell death score		2 days	♂/♀
53	P10 mice	HI: 25 min 8% O_2	*Creatine (dietary suppl.)* HI + normal diet HI + 3% creatine	Post-Tx: 10–70 days	Core: 36°C, during HI	↓ infarct vol in creatine: 17.5 ± 2.5% vs. normal diet: 27.8 ± 4.1%, $p < 0.05$	Improved memory and motor function	10 weeks	♀
54	P1 piglets	HI FiO_2 12%	*HT* HI + NT (38.5° C) HI + HT (35° C) HI + HT (33.5° C) HI + HT (30° C)	Post-Tx: 2 h	Core: 38.5 vs. 35, 33.5, or 30°C, continuous monitoring	↓ white matter apoptosis in 35 and 33.5°C groups vs. NT, $p < 0.05$	None	2 days	♂

Table 1 (continued)

Reference	Species	Insult	Treatment (route)	Timing	Temperature, °C	Pathology	Functional outcome	Survival	Sex
55	P7 rats	HI: 2 h 15 min 8% O_2	*Resveratrol (i.p.)* vehicle + HI resveratrol + HI	Pre-Tx: –10 min	Ambient: 36°C, during HI	~83% ↓ infarct vol, ↓ cortical and hippocampal injury scores and ↑ myelination vs. vehicle, $p < 0.05$	Improved memory performance	Pathology: 1 week behavior: 12 weeks	♂/♀
31	P10 rats	HI: 45 min 8% O_2	*HT* HI + NT HI + 4 h HT	Immediate Tx: 0 h	Core: 31 vs. 36°C during 4 h recovery	Greater ↓ in regional injury in males vs. females $p < 0.05$	None	1 week (8 days)	♂/♀
31	P10 rats	HI: 45 min 8% O_2	HI + NT HI + 4 h HT	Immediate Tx: 0 h	Core: 31 vs. 36°C during 4 h recovery	↓ cortical and hippocampal injury in males; no significant effect of HT in females	HT improved working memory in males but not females	Pathology and behavior 3 weeks (20 days)	♂/♀
56	P10 rats	HI: 2.5 h 8% O_2	*G-CSF (s.c.)* HI + vehicle HI + G-CSF	Post-Tx 1 h	Ambient: 37°C, during HI	34% ↓ infarct vol vs. vehicle, $p < 0.05$	None	1 day	?
57	P7 mice	HI: 100 min 7.5% O_2	*Carvacrol (i.p)* vehicle + HI carvacrol + HI (30 or 50 mg/kg)	Pre-Tx: –30 min	Ambient: 37°C, during HI	↓ infarct vol by 46 and 90% in 30- and 50-mg groups, respectively vs. vehicle, $p < 0.05$	Improved sensorimotor performance in 50 mg/kg vs vehicle, $p < 0.05$	Pathology: 1 day behavior: 1 week	?
58	P10 mice	HI: 25 min 8% O_2	*Creatine monohydrate (dietary suppl.)* HI + normal diet HI + 2% creatine	Post-Tx: 10–105 days	Ambient: 36°C, during HI	No change in infarct vol vs. normal diet, $p < 0.05$	Improved motor function, learning and memory vs. normal diet	Pathology: 15 weeks behavior: 17 weeks (118 days)	♂
59	P7 rats	HI: 2.5 h 8% O_2	*Uridine (i.p.)* HI + vehicle HI + uridine	Immediate Tx: 0 h, 1, and 2 days	Ambient: 37°C, during HI	↓ infarct vol: uridine, 11 ± 1% vs. vehicle, 20 ± 3%, $p < 0.05$	None	3 days	♂/♀
60	P10 mice	HI: 45 min 8% O_2	*HT* HI + NT HI + 4 h HT	Immediate Tx: 0 h	Ambient: 37 vs. 28.5°C during 4 h of recovery	~34% ↑ NeuN+ staining area vs. HI + NT, $p < 0.05$	None	1 week	♂
61	P7 rats	HI: 2.5 h 8% O_2	*Gap26 (Cx43 mimetic peptide) (i.p.)* vehicle + HI Gap26 + HI	Pre-Tx: –1 h	Ambient: 37°C, during HI	↓ infarct vol by ~60% vs. vehicle, $p < 0.05$		2 days	?
61	P7 rats	HI: 2.5 h 8% O_2	*Gap26 (Cx43 mimetic peptide) (i.p.)* HI + vehicle HI + Gap26	Post-Tx: 24 h then daily for 7 days	Ambient: 37°C, during HI	None	Improved motor function and memory vs. vehicle	3 weeks	?
62	P7 rats	HI: 90 min 8% O_2	*HT and Xe* HI + NT HI + 5 h HT HI + 5 h HT + 50% Xe	Post-Tx: 5 h	Core: 36°C, during HI 37 vs. 32°C, during 5 h of recovery	No additive effect of Xe on infarct area vs. HT, $p > 0.05$	HT + Xe improved sensorimotor performance vs. HT; negative correlation between brain injury and motor function females ($r = –0.76$, $p < 0.05$) not males ($p = 0.06$)	Pathology: 10 weeks behavior: 9 weeks	♂/♀
63	P7 rats	HI: 150 min 8% O_2	*Vitexin (5, 7, 4-trihydroxy-flavone-8-glucoside) (i.p.)* HI + vehicle HI + vitexin	Post-Tx: 5 min	Ambient: 37°C, during HI	~64% ↓ infarction volume and ↓ cortical and hippocampal neuronal cell death, $p < 0.05$ vs. vehicle	None	2 days	♂/♀
29	P10–11 rats	HI: 1 h 8% O_2	*HT* HI + NT HI + 4 h HT	Immediate Tx: 0 h	Core: 36–36.5 vs. 32°C, during 4 h of recovery	At week 12: ↓ total and regional damage scores vs. HI + NT, $p < 0.05$	HT improved motor function in moderate but not severely injured subjects vs. NT, $p < 0.05$	Pathology: 12 weeks behavior: 9 weeks	♂/♀
64	P7 rats	HI: 120 min 8% O_2	*NAC (i.p.)* vehicle + HI NAC + HI	Pre-Tx: –30 min, then daily from P8 to P44	Ambient: 36°C, during HI	↑ MBP expression in corpus callosum vs. vehicle, $p < 0.05$	Improved sensorimotor function vs. vehicle	4 weeks	♂
65	P7 rats	HI 3.5 h 8% O_2	*Progesterone (i.p. and s.c.)* HI + vehicle HI + progesterone	Immediate Tx: 0 h i.p. then 2 h and 1, 2, 3, 4, 5, 6, and 7 days s.c.	Ambient: 37°C, during HI	30% ↓ mean tissue loss in HI + progesterone males vs. vehicle (no effect in females)	Behavioral tests showed no benefit of progesterone vs. vehicle, $p > 0.05$	Pathology: 7 weeks behavior: 6 weeks	♂/♀
66	P7 rats	HI: 2 h 8% O_2	*HT* NT during HI HT during HI	During HI	Ambient: 36.1 vs. 32.2°C during 2 h HI, 35.7–37.0°C during recovery	None	Greater improvement in sensory motor and memory performance in HT females vs. HT males vs. NT	10–11 weeks (73–76 days)	♂/♀

Table 1 (continued)

Reference	Species	Insult	Treatment (route)	Timing	Temperature, °C	Pathology	Functional outcome	Survival	Sex
67	P10 mice	HI: 15 min 8% O_2	*Isoflurane* HI + room air HI + isoflurane	Immediate Tx: 0 h	Ambient: 37–37.5°C, during HI	37% ↓ infarct vol vs. room air	Improved memory performance vs. room air	Pathology: 1 day behavior: 8 weeks	?
68	P7–9 mice	HI: 60 min 7.5% O_2	*HPC and K_{ATP} channel blocker (tolbutamide) (i.p.)* HI HPC + tolbutamide + HI HPC + vehicle + HI	Pre-Tx: –2 days	Core: 37°C, during HI and 30 min of recovery	↓ infarct vol in HPC + vehicle: 21.4 ± 3.5% vs. HPC + tolbutamide: 45.3 ± 1.7%, $p < 0.05$; similar infarct vol between HI and HPC + tolbutamide	None	1 day	♂/♀
68	P7–9 mice	HI: 100 min 7.5% O_2	HI HPC + vehicle + HI HPC + tolbutamide + HI	Pre-Tx: 2 days	Core: 37°C, during HI and 30 min of recovery	↓ infarct vol in HPC + vehicle by ~45 and 40% vs. HI and HPC + tolbutamide, respectively, $p < 0.05$	Improved sensorimotor performance in HPC vs. HPC + tolbutamide, $p < 0.05$	Pathology and behavior: 1 week	♂/♀
69	P7 rats	HI: 2.5 h 8% O_2	*HT* HI + NT HI + 3 h HT	Immediate Tx: 0 h	Core: 38 vs. 30°C, during 3 h of recovery	None	Improved neurobehavioral scores vs. NT	3 h	♂
70	P7 rats	HI: 2.5 h 8% O_2	*P5-TAT (i.p.)* vehicle + HI P5-TAT + HI (1, 10, 25, 50 μg/kg)	Pre-Tx: –1 h	Ambient: 37°C, during HI	Dose-dependent ↓ infarct vol. 58% ↓ in 50 μg/kg group, $p < 0.05$	None	2 days	?
70	P7 rats	HI: 2.5 h 8% O_2	HI + vehicle HI + P5-TAT (50 μg/kg/day)	Post-Tx: 1–7 days after HI	Ambient: 37°C, during HI	None	Improved sensorimotor performance vs. vehicle	4 weeks	?
71	P8 mice	HI: 30 min 10% O_2	*NP1 (neuronal pentraxin 1)* WT + HI NP1 KO + HI	Constitutive gene knockout	Ambient: 35°C, during 2 h recovery	↑ cortical, striatal, and hippocampal vol by ~45, 50 and 50%, respectively, $p < 0.05$ vs. WT	None	1 week	?
72	P7 rats	HI: 2 h 8% O_2	*iNOS inhibition (i.c.v.)* vehicle + HI iNOS inhibitor + HI	Pre-Tx: –4 days	Ambient: 37°C, during HI	↑ hippocampal neuronal density by ~67 and 64% in CA1 and CA3, respectively. $p < 0.05$ vs. vehicle	Improved motor function, learning and memory, $p < 0.05$ vs. vehicle	Pathology: 1 week behavior: 3 weeks	♂/♀
73	P7 rats	HI: 150 min 8% O_2	*OMT (oxymatrine) (i.p.)* vehicle + HI OMT + HI	Pre-Tx: –15 min then every 12 h for 2 days post-HI	Ambient: 37°C, during HI	~55% ↓ infarct area and ↓ neuronal cell death vs. vehicle, $p < 0.05$	Improved sensorimotor function and memory vs vehicle, $p < 0.05$	Pathology: 2 days behavior: 7 weeks	♂
74	P9 mice	LPS + HI 20 min 10% O_2	*IDR-1018 (innate defense regulator peptide 1018) (i.p.)* LPS + HI + vehicle LPS + HI + IDR-1018	Post-Tx: 3 h	Not specified	↓ brain injury score in cortex, thalamus hippocampus and striatum and ↓ white matter loss by ~80%, $p < 0.05$ vs. vehicle	None	1 week	♂/♀
75	P7 rats	HI: 2 h 8% O_2	*Sildenafil (i.p.)* HI + vehicle HI + sildenafil citrate	Immediate Tx: 0 h	Not specified	↓ brain tissue loss by 43 ± 15% and ↑ myelination in cingulum and external capsule, $p < 0.05$ vs vehicle	Intermediate improvement in motor performance vs. vehicle	1 week	♂/♀
76	P10 rats	HI: 120 min 8% O_2	*Dexamethasone (i.c.v)* vehicle + HI dexamethasone + HI	Pre-Tx: immediately before HI	Not specified	~57% ↓ infarction size vs. vehicle	None	2 days	♂/♀
77	P9 mice	HI: 50 min 10% O_2	*Q-VD-OPh (broad-spectrum caspase inhibitor) (i.p.)* HI + vehicle HI + Q-VD-OPh	Post-Tx: 12 h then every 24 h for 2 weeks	Ambient: 36°C, during HI	↓ tissue loss (by 31.3%), decreased neuropathological scores in cortex, hippocampus, and thalamus vs. vehicle, $p < 0.05$	Improved sensorimotor function vs. vehicle, $p < 0.05$	Behavior: 6 weeks pathology: 16 weeks	♂
78	P7 rats	HI: 2 h 8% O_2	*nNOS (7-nitroindazole) or iNOS (aminoguanidine) inhibition (i.p.)* vehicle + HI 7-nitroindazole + HI aminoguanidine + HI	Pre-Tx: 30 min	Not specified	Complete and partial reduction in cortical infarct vol in nNOS and iNOS inhibitor groups, respectively, vs. vehicle, $p < 0.05$	None	1 week	♂
78	P7 rats	HI: 2 h 8% O_2	HI + vehicle HI + 7-nitroindazole HI + aminoguanidine	Post-Tx: 3 h	Not specified	Partial reduction in cortical infarct vol in iNOS group vs. nNOS and vehicle, $p < 0.05$	None	1 week	♂
79	Fetal sheep (0.85 GA)	5 × 10 min UCO	*Allopurinol (maternal) (i.v.)* UCO + vehicle UCO + allopurinol	Tx during UCO	Not specified	↓ hippocampal (CA3 and CA4) neuronal damage score vs. vehicle	None	2 days	?

Table 1 (continued)

Reference	Species	Insult	Treatment (route)	Timing	Temperature, °C	Pathology	Functional outcome	Survival	Sex
80	P7 rats	HI: 120 min 8% O_2	*Caffeine citrate (i.p.)* caffeine + HI vehicle + HI	Pre-Tx: immediately before HI then 0, 24, 48, and 72 h	Ambient: 37°C, during HI	↓ cell death in the hippocampus and parietal cortex vs. vehicle, $p < 0.05$	None	1 week	?
81	P7 rats	HI: 2.5 h 8% O_2	*29D7 mAb (tropomyosin related kinase B agonist) (i.c.v.)* vehicle + HI 29D7 (0.1 nmol) + HI 29D7 (0.3 nmol) + HI	Pre-Tx: immediately before HI	Core: 35–37°C, measured intermittently for 7 days post-HI	↓ % area tissue loss in striatum, hippocampus and cortex $p < 0.05$ 0.1 and 0.3 nmol vs. vehicle, $p < 0.05$	None	1 week	♂/♀
81	P7 rats	HI: 2.5 h 8% O_2	vehicle + HI 29D7 (0.3 nmol) + HI	Pre-Tx: immediately before HI	Core: 35–37°C, measured intermittently for 7 days post-HI	↓ % area tissue loss in striatum, hippocampus and cortex by ~72, 77 and 77%, respectively, $p < 0.05$ vs. vehicle	Improved sensory motor function vs. vehicle, $p < 0.05$	5 weeks	♂/♀
82	P7 mice	HI: 30 min 8% O_2	*Cl-amidine (peptidyl-arginine deiminase inhibition) (i.p.)* LPS + HI + vehicle LPS + HI + Cl-amidine	Immediate Tx: 0 h	Ambient: 36°C, during HI	↓ infarct vol in the hippocampus and external capsule by ~62 and 74%, respectively, $p < 0.05$	None	2 days	?
83	P10 mice	HI: 60 min 10% O_2	*HT and sevoflurane* HI + 10% O_2 1 h HI + 3.5% sevoflurane + HT (1 h)	Immediate Tx: 0 h	Core: ~34 vs. 36.5–37.5°C during HI	↓ neuronal damage score in neocortex, striatum, thalamus, and hippocampus vs. HI + 10% O_2, $p < 0.05$	Improved learning and memory vs. HI + 10% O_2, $p < 0.05$	Pathology 1 week behavior: 8 weeks	♂/♀
84	P7 rats	HI: 90 min 8% O_2	*HT and Xe* HI + NT HI + 5 h HT HI + 5 h HT + Xe 50%	Immediate Tx: 0 h	Core: 36°C during HI, 37 vs. 32°C, during 5 h of recovery	↓ infarct area in HI + HT + Xe (19%) vs. HI + HT (37%) and NT (54%), $p < 0.05$	None	1 week	♂/♀
85	P7 rats	HI: 2 h 8% O_2	*IαIP (inter-α inhibitor protein) (i.p.)* vehicle + HI human IαIP + HI	Pre-Tx: immediately before and 24 h after HI	Not specified	Intermediate ↑ cortical and hippocampal tissue vol. vs. vehicle	Improved spatial and nonspatial learning vs. vehicle	Pathology: 22 weeks (157 days) behavior: 11 weeks (80 days)	♂
86	P7 rats	HI: 55 min 8% O_2	*Estetrol (estrogenic steroid) (i.p.)* vehicle + HI estetrol 1, 5, 10, 50 mg/kg/day + HI	Pre-Tx: –3 days	Ambient: 37°C, during HI	↓ infarct area in all treatment groups vs. vehicle, $p < 0.05$	None	1 week	?
86	P7 rats	HI: 55 min 8% O_2	HI + vehicle HI + estetrol 1, 5, 10, 50 mg/kg	Immediate Tx: 0 h	Ambient: 37°C, during HI	↓ infarct area in all treatment groups vs. vehicle	None	1 week	?
87	P8 rats	HI: 45 min 7.7% O_2	*Lithium (i.p.)* HI + vehicle HI + lithium	Post-Tx: 5–19 days	Ambient: 36°C, during HI	↓ tissue vol loss 169 ± 26 vs. 277 ± 27 mm^3 and pathological score, $p < 0.05$ vs. vehicle	Improved motor- and ↓ anxiety-related activity, $p < 0.05$ vs. vehicle	Pathology: 12 weeks behavior: 6 weeks	♂
88	P7 rats	HI: 2 h 8% O_2	*Isoflurane or cyclosporine A (mitochondrial permeability transition pore inhibitor) (i.c.v.)* HI + vehicle HI + isoflurane HI + cyclosporine A	Immediate Tx: 0 h	Ambient: 37°C, during HI	↑ hippocampal and thalamic neuronal density in isoflurane and cyclosporine A groups vs. vehicle, $p < 0.05$		1 week	♂/♀
88	P7 rats	HI: 2 h 8% O_2	HI + vehicle HI + isoflurane HI + cyclosporine A	Immediate Tx: 0 h	Ambient: 37°C, during HI	↑ hippocampal and thalamic neuronal density in isoflurane and cyclosporine A groups vs. vehicle, $p < 0.05$	Similar improvement in spatial learning and memory in isoflurane and cyclosporine A groups vs. vehicle	5 weeks	♂/♀
89	P3–5 piglets	45 min FiO_2 10% then asphyxia 7 min	*HT and HETE-0016 (20-hydroxyeicosatetraeonic acid inhibitor) (i.v.)* Hypox + NT Hypox + HT Hypox + HETE-0016 + HT	Post-Tx: HETE-0016 at 5 min HT at 3 h	Core: 38.5–39.5 vs. 34.0°C, continuous monitoring	↑ neuronal survival in cortex (~22%), putamen (~14%), and thalamus (~28%) with combination therapy, $p < 0.05$ vs. HT	No effect on time to feed after extubation; no significant effect on *n* of clinical seizures, $p < 0.1$	1 week (10 days)	♂

Table 1 (continued)

Reference	Species	Insult	Treatment (route)	Timing	Temperature, °C	Pathology	Functional outcome	Survival	Sex
No improvement in neurodevelopmental outcomes									
90	P7 rats	HI: 150 min 8% O_2	*HT and Xe* NT HT HT + Xe	Immediate Tx: 0 h	Core: 37 vs. 32°C, during 5 h of recovery	No effect of HT or HT + Xe on infarct vol and neuronal loss vs. NT		1 week	♂/♀
55	P7 rats	HI: 2 h 15 min 8% O_2	*Resveratrol (i.p.)* HI + vehicle HI + resveratrol	Immediate Tx: 0 h	Ambient: 36°C, during HI	No effect on infarct vol, neuronal loss or myelination vs. vehicle	None	1 week	♂/♀
91	P9 mice	HI: 50 min 10% O_2	*Osteopontin (i.n./i.p.)* HI + vehicle HI + osteopontin	Immediate and post-Tx: 0, and 3 h and 1–3 days i.n. or 0 h and every 2 days on days 1–15 i.p. or 0 h and every 2 days on days 5–15 i.p.	Ambient: 37°C, during HI	No effect on white (MBP) or gray (MAP2) matter area in any treatment group vs. vehicle; no sex-specific effect	No effect on sensorimotor performance	Pathology: 9 weeks (65 days) behavior: 8 weeks (60 days)	♂/♀
23	Fetal sheep (0.85 GA)	Bilateral carotid artery occlusion 30 min	*HT* ischemia + NT ischemia + HT 3 days ischemia + HT 5 days	Post-Tx: 3 h	Brain: 39.5 vs. 31.3–31.8°C, continuous monitoring	↓ neuronal survival in cortex and dentate gyrus in 5- vs. 3-h HT group, $p < 0.05$	Improved EEG recovery and ↓ brain edema in 3- and 5-day groups, $p < 0.05$ vs. NT	1 week	♂/♀
22	Fetal sheep (0.85 GA)	Bilateral carotid artery occlusion 30 min	*HT and Cx43 (connexin hemichannel blockade) (i.c.v.)* ischemia + NT ischemia + HT ischemia + HT + Cx43	Post-Tx: 3 h	Brain: 39.5 vs. 31.3–32.1°C, continuous monitoring	No additive protection with Cx43 block + HT vs. HT	No additive effect with Cx43 block + HT on EEG recovery or brain edema vs. HT	1 week	♂/♀
92	P1 piglets	45 min FiO_2 6%	*HT (selective head cooling)* Hypox + NT Hypox + HT	Immediate Tx: 0 h	Brain: 38.5 vs. 31°C, continuously for 24 h then daily	No improvement in neuropathology scores in cortical and subcortical gray or white matter	No difference in *n* of electrographic or clinical seizures between groups	3 days	♂/♀
63	P7 rats	HI: 150 min 8% O_2	*Vitexin (5, 7, 4-trihydroxyflavone-8-glucoside) (i.p.)* HI + vehicle HI + vitexin	Post-Tx: 3 h	Ambient: 37°C, during HI	No effect on infarct vol vs. vehicle	None	2 days	♂/♀
93	P7 rats	LPS + HI: 50 min 8% O_2	*HT* LPS + HI + NT LPS + HI + 5 h HT	Immediate Tx: 0 h	Core: 37 vs. 32°C, during 5 h of recovery	No effect on hippocampal neuronal loss vs. NT	None	1 week	♂/♀
94	P3–5 piglets	HI 45 min 10% O_2 then asphyxia 7 min	*HT* HI + NT HI + HT HI + HT slow rewarming HI + HT fast rewarming	Post-Tx: 2 h	Core: 38.5–39.5 vs. 34.0°C, continuous monitoring	↑ cortical neuronal apoptosis in both rewarming groups vs. HT and NT groups, $p < 0.05$	None	1 day	♂
95	P7 rats	LPS + HI 50 min 8% O_2	*HT* LPS + HI + 5 h NT LPS + HI + 5 h HT	Immediate Tx: 0 h	Core: 36°C during HI 37 vs. 32°C during 5 h of recovery	No effect on % brain area loss, HT vs. NT	None	1 week	♂/♀
84	P7 rats	HI: 90 min 8% O_2	HI + NT HI + 5 h HT 35°C HI + 5 h HT 35°C + Xe 20%	Post-Tx: 4 h	Core: 36°C, during HI 37 vs. 32°C, during 5 h of recovery	No effect of delayed combination or HT alone on infarct area vs. NT	None	1 week	♂/♀

aEEG, amplitude-integrated electroencephalogram; DHA, docosahexaenoic acid; GA, gestational age; G-CSF, granulocyte colony stimulating factor; HT, hypothermia; HI, hypoxia-ischemia; Hypox: hypoxia; HPC, hypoxic preconditioning; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MBP, myelin basic protein; NAA, N-acetyl aspartate; NAC, N-acetyl-L-cysteine; NeuN, neuronal nuclear protein; NT, normothermia; nNOS: neuronal nitric oxide synthase; P, postnatal day; P5-TAT, cycline-dependent kinase 5 inhibitor; Tx, treatment; UCO, umbilical cord occlusion; vol, volume; WT, wild type; Xe, xenon.

Studies of Perinatal Neuroprotection

We identified and screened a total of 171 papers. One hundred and ten were excluded due to inappropriate developmental age, ex vivo assessment, or lack of assessment of neurological outcomes. Thus 61 individual papers were included in this analysis (Fig. 1). Studies were further subdivided by treatment regime (i.e., studies that investigated more than 1 timing for treatment relative to the insult) or where pathological and functional outcomes were assessed at different survival times. After

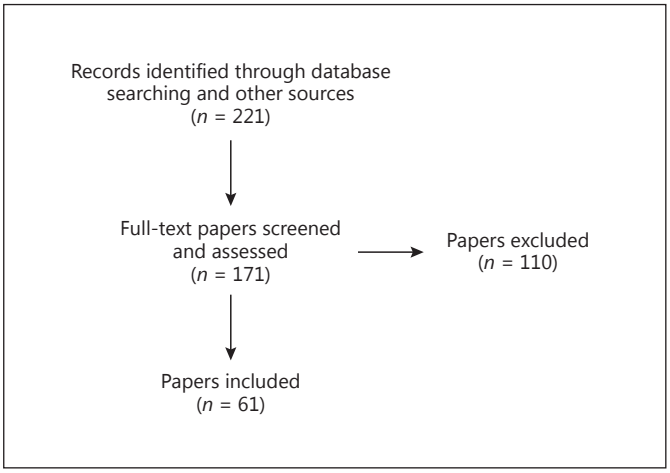

Fig. 1. Flow chart illustrating the number of papers identified through database searching and other relevant sources, the number of full-text articles screened, assessed, and excluded, and the final number of original papers surveyed.

subdividing the studies that used more than 1 survival time for pathological and functional assessments or assessed outcomes after more than 1 treatment time, we identified 75 individual studies. For the purpose of reporting on the treatment regime, timing, outcomes, and survival, we summarized the data based on the individual studies (total $n = 75$). As each of the subdivided studies used the same developmental age at the time of the insult and protocols for temperature control and documentation of sex, the developmental age, type and duration of temperature monitoring, and reporting of sex are summarized based on the number of original papers (total $n = 61$).

The majority of papers 52/61 (85%) studied neonatal rats or mice; 35 out of these 52 (67%) papers studied neonatal rodents at P7, the remainder studied rodents between P8 and P11 (17/52; 32%). These ages are broadly comparable to the late preterm or term human infant at 32–40 weeks [17–19]. All studies used the Rice-Vannucci model of unilateral carotid artery ligation followed by a period of moderate hypoxia [18]. A range of pathological assessments of injury was reported, such as measurements of total and/or regional infarct area or volume or semiquantitative assessments to assign a neuronal injury score. Functional outcomes were assessed using a wide variety of behavioral tests examining memory, sensorimotor function, coordination, or neophobia.

Nine out of 61 papers (15%) studied large animals. Histopathological assessments were mainly performed by quantifying the total number of viable or dying cells within regions susceptible to injury. In term-equivalent fetal sheep, injury was induced using bilateral carotid artery occlusion or repeated umbilical cord occlusion. In the piglet, injury was induced using prolonged isocapnic hypoxia with or without a short (7-min) period of severe asphyxia or bilateral carotid artery occlusion. In both paradigms, injury has been reported in "watershed" zones (areas of white and gray matter that lie within the borders between major arteries where perfusion pressure is least), such as the parasagittal cortex, the dorsal horn of the hippocampus, thalamus, dentate gyrus, cerebellar neocortex, striatum, and parasagittal and periventricular white matter. Functional outcomes were assessed using electroencephalography (EEG) to evaluate recovery of activity and/or electrographic or clinical seizure assessment. In neonatal piglets, clinical seizures were also assessed. In fetal sheep, cortical impedance was used to examine the degree of cytotoxic edema after the insult.

Results

Temperature Monitoring

In 7 of 61 papers, temperature monitoring was not reported in the study design or outcomes. Of these, 6 were in rodents and 1 was in fetal sheep. All of these papers reported neuroprotection (Fig. 1a). Environmental temperature only was reported in 32 papers (all in rodents); 31 out of these 32 papers reported neuroprotection, including 2 papers that tested hypothermia, and 1 paper reported no improvement in histological or functional outcomes. Twenty-eight of these 32 papers reported environmental temperature during HI only (Fig. 2a, 3a).

Core temperature was monitored in 19/61 papers (31%; 14 papers used rodents and 5 papers used piglets; Fig. 2). In rodents, 1/14 papers monitored temperature intermittently for 7 days (Fig. 3), 2 papers monitored core temperature during HI, and 2 papers monitored core temperature during HI for 30 min or 2 h afterwards; all of these papers reported improved outcomes with treatment. Nine papers in rodents monitored core temperature for 3–10 h after HI (Fig. 3c). Of these, 6/9 papers reported neuroprotection at least in some treatment groups.

In all papers using piglets, core temperature was monitored continuously (Fig. 3b). All of these papers focused on hypothermia strategies or adjuvant therapy with hy-

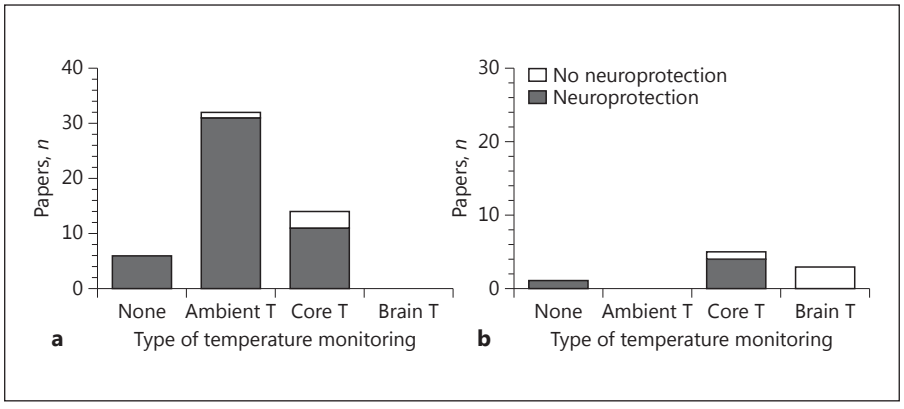

Fig. 2. Number of papers that did not monitor temperature (none) and papers that reported ambient (environmental), core, and brain temperature (T) in rodents (**a**) and large animals (**b**) from 61 papers investigating neuroprotection for term/near-term HIE between January 1, 2014, and June 30, 2016. Open bars show the number of studies that reported no protection. Black bars show the number of studies that reported neuroprotection.

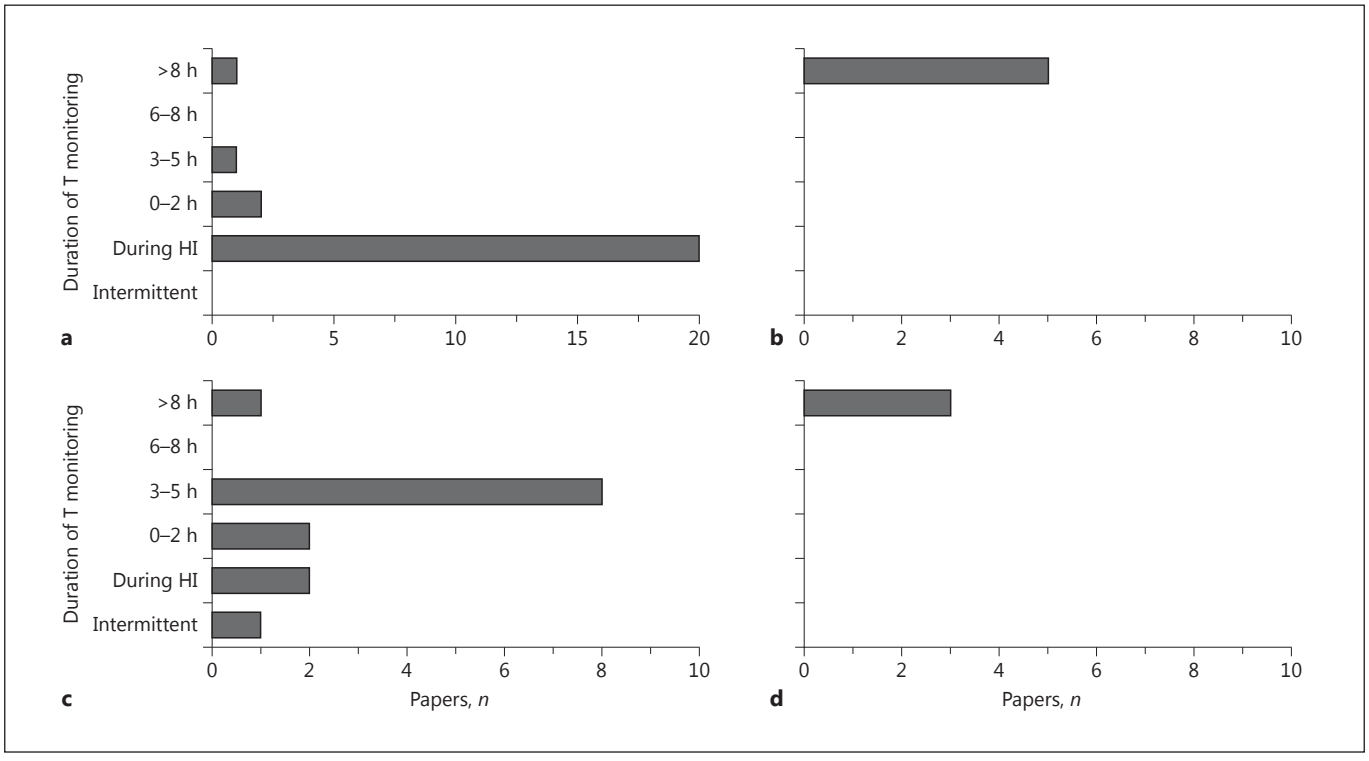

Fig. 3. Number of papers in rodents (n = 39; **a**, **c**) and large animals (n = 7; **b**, **d**) that reported the duration of temperature (T) monitoring: ambient/environmental (**a**) and core temperature (**c**) monitoring in rodents, and core (**b**) and brain temperature (**d**) monitoring in large animals. Total n = 46.

pothermia, and all reported at least partial protection. Brain temperature was monitored in 3/61 papers (5%; 1 in piglets and 2 in fetal sheep; Fig. 3d). In the piglet study, brain temperature was monitored for 24 h and then once daily for 2 days. In 2 papers using fetal sheep, extradural brain temperature was monitored continuously throughout the experiment.

Possibility that Neuroprotectants Affected Thermoregulation

We identified a total of 45 neuroprotectants/protective strategies from the papers reviewed in this study. Twenty treatments were classified as likely to affect thermoregulation, and 5 were considered unlikely to affect temperature control. The effect on thermoregulation was unknown in 20 cases (Fig. 4a).

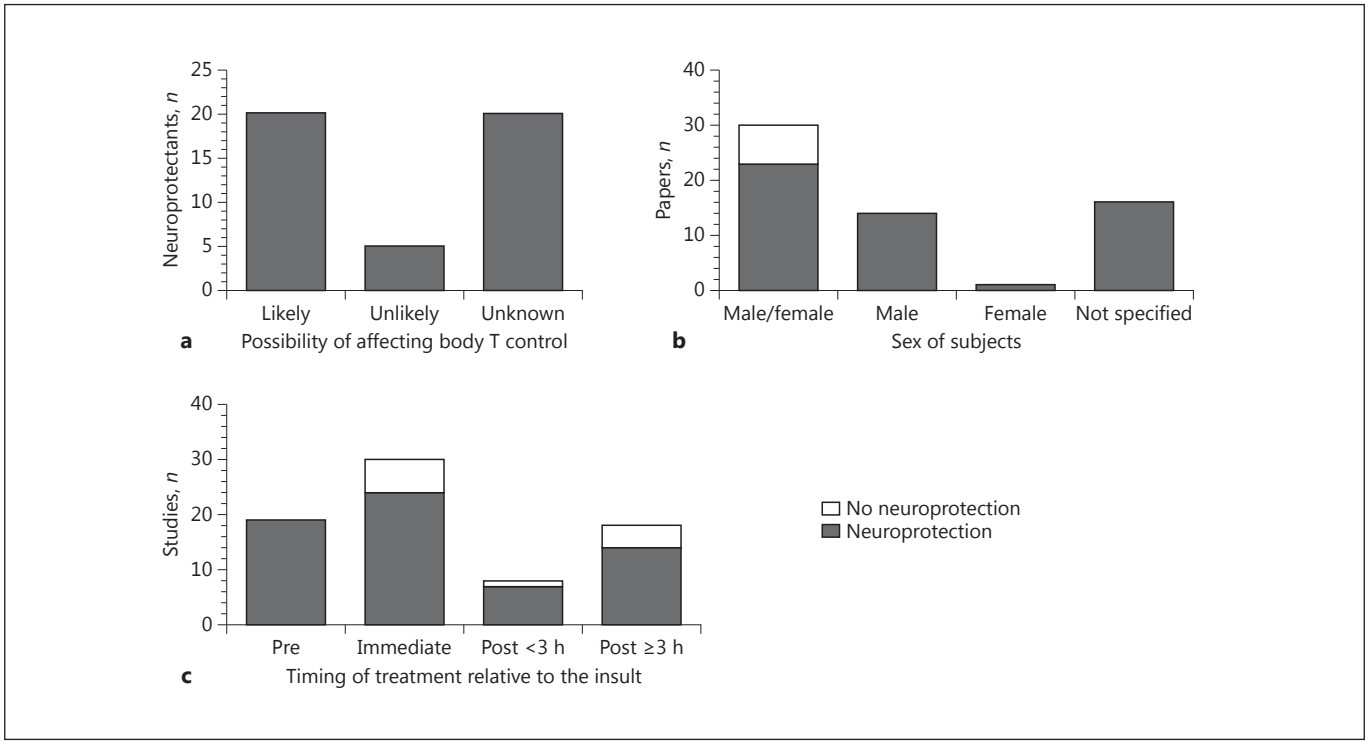

Fig. 4. a Probability of neuroprotectants affecting thermoregulation; 36 neuroprotectants were identified and assessed. *Likely* was defined as a drug previously shown to affect temperature (T) or belonging to a family of drugs reported to affect temperature. *Unlikely* refers to a drug previously shown to have no effect on temperature. *Unknown* indicates no studies have investigated whether the drug/neuroprotective strategy affects temperature. **b** Number of papers that reported no neuroprotection (white bars) or neuroprotection (black bars) in either both sexes, males, or females, or did not report the sex of the subjects studied. **c** Number of studies that reported no neuroprotection (white bars) or neuroprotection (black bars) after treating before, immediately, within less than 3 h, or greater than or equal to 3 h after the insult.

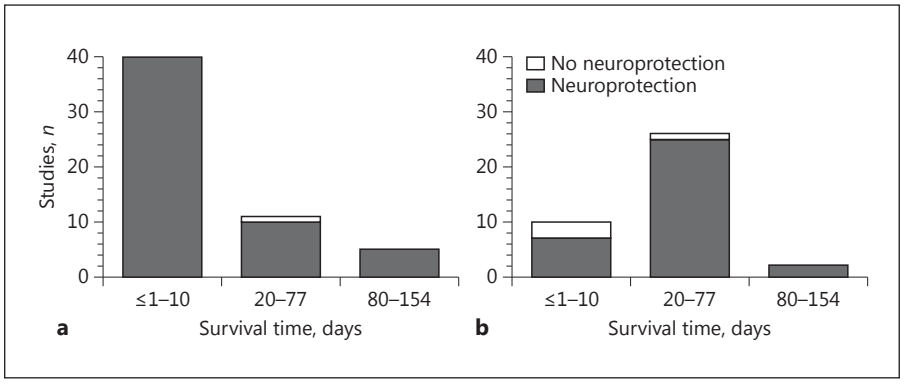

Fig. 5. Number of studies that reported no protection (white bars) or neuroprotection (black bars) after using short (≤1–10 days), medium (20–77 days), or long (80–154 days) survival times for histological (**a**) and behavioral assessment (**b**).

Route of Drug Delivery

The majority of the papers surveyed (52/61; 85%) assessed the effectiveness of therapeutic candidates without hypothermia. Nine out of 61 papers (15%) investigated hypothermia with adjuvant therapies. Twelve out of 61 papers (20%) investigated hypothermia alone. The treatment regimens for candidate drugs were highly variable. Pharmacological interventions were administered in either single or multiple doses. Most of the rodent studies administered candidate treatments subcutaneously or intra-peritoneally ($n = 32$). One rodent study used a combination of intraperitoneal and intra-nasal drug delivery,

and 12 studies delivered candidate drugs in gaseous form during or after HI. Eight studies (7 in rodents and 1 in fetal sheep) used intracerebroventricular drug delivery, 2 studies (both in rodents) delivered the drug as a dietary supplement, 1 study in fetal sheep administered the candidate drug via the maternal circulation, and 1 study in neonatal piglets used intravenous infusion. Two rodent studies used a constitutive gene knockout.

Sex

Thirty of 61 papers (49%) examined the effect of treatment on both sexes (Fig. 4b). Twenty-three studies showed improved neural outcomes, and 7 studies showed no improvement. In a further 14 papers, therapeutic candidates were examined in males, and 1 paper reported outcomes in females only. All reported improved neural outcomes. Finally, in 16 papers, the sex of the subjects was not reported; all of these studies reported neuroprotection.

Timing of Treatment and Neurodevelopmental Outcomes

In total, 64/75 studies demonstrated neuroprotection for HIE. In 19 studies, treatment began before the start of HI (Fig. 4c), including two studies of constitutive gene knockouts (i.e. before HI). In 31 studies, treatment was delivered during or immediately after HI or within 2 h. In just 14 studies, treatment was started 3 h or later after HI. The survival time before histological assessment in 44 studies reporting neuroprotection was less than or equal to 1–10 days (Fig. 5a). In 10 studies, histological outcomes were reported between 20 and 77 days. In 5 studies, outcomes were reported between 80 and 154 days. Five studies did not report histological outcomes.

Thirty-four studies (32 in rodents and 2 in piglets) reported improved functional outcomes. Of these, 7 assessed functional outcomes after less than or equal to 1–10 days, 25 after 20–77 days, and 2 after 80–154 days (Fig. 5b). Interestingly, in 5 studies, histological and functional outcomes were discordant, such that 3 studies reported improved histology with no improvement in function, and 2 studies found improved functional outcomes without improved histology.

Eleven out of 75 perinatal studies reported no neuroprotection with treatment. Seven studies began treatment immediately after HI (or within the first 2 h), and 4 studies delayed treatment for up to 3–4 h after HI (Fig. 4c). In 10 of the 11 studies, histological analysis was performed after relatively short survival times of less than or equal to 1–10 days. One study in rodents examined histological outcomes after approximately 9 weeks (65 days; Fig. 5a). Four studies assessed functional outcomes. Three studies (1 in piglets and 2 in fetal sheep) assessed functional outcomes based on EEG or clinical seizures after less than or equal to 1–10 days, and 1 study in rodents examined behavioral outcomes after 60 days (Fig. 5b).

Discussion

This systematic review shows that the majority of recent studies investigating perinatal neuroprotection for HIE between 2014 and 2016 were performed in rodents, of which only a minority rigorously monitored and controlled body and brain temperatures. Thus, many recent studies cannot exclude the possibility that their findings could have been confounded by unappreciated changes in brain temperature due to drug-induced cooling or environmental conditions.

Carefully conducted studies in neonatal rodents offer many advantages for studies of neuroprotection [17]. They are easy to use and cost effective, which in turn facilitates data collection from large cohorts at multiple time points. Further, rodent studies enable wide application of histological techniques, molecular biology probes, and behavioral assessments, allowing investigators to provide detailed mechanistic and functional insight. However, as highlighted in this systematic review, considerable care is required to be certain that iatrogenic changes in brain temperature do not occur. Large animal studies are expensive, require more laboratory infrastructure relative to studies in smaller animals, and offer limited opportunities for assessing neurodevelopmental outcomes other than neurophysiological monitoring. Nevertheless, they are easier to monitor and have intrinsically greater thermal stability.

The great majority of recent papers used neonatal mice or rats (53/61). Only 14 papers studying rodents measured body temperature at all, and just 9 continuously monitored body temperature for short periods of 3–10 h after HI. No studies reported on temperatures after this early phase of recovery. Given the near-poikilothermic nature of P7 neonatal rats and mice, environmental temperature is often used as a surrogate for body temperatures. However, although most of the studies measured environmental temperature during the insult, few monitored or reported temperature after HI. This is an important consideration since even normal, healthy neonatal rodents often show lower body temperatures in the typical laboratory nesting conditions between P7

and P14 [20]. Core temperature in neonatal rodents will be affected by the amount and composition of nesting material, position in the nest, huddling, distance from the dam, and the time since the last period of suckling. Conversely, the majority of large animal studies continuously monitored and controlled brain and or core temperature throughout the study period. One study did not report temperature in near-term fetal sheep; however, a major advantage of testing potential neuroprotectants in fetal sheep is that body temperature is regulated by the pregnant ewe and, therefore, unless the ewe is febrile, fetal temperatures are highly stable [21–24].

In adult rodents, the use of anesthetics such as halothane, sodium pentobarbital, and isoflurane around the time of the insult have all been shown to induce hypothermia, as previously reviewed [11]. Furthermore, many rodent studies have reported that the beneficial effects of potential neuroprotectants were confounded by hypothermia during the first 6–8 h after the insult [11, 25] and that maintenance of normothermia abolishes neuroprotection [25]. In this study, the majority (40/45; 89%) of the pharmacological interventions being tested over the last 2 years belonged to classes of drugs that are either likely to affect body temperature or whose effect on thermoregulation is unknown. Collectively, these data strongly suggest that significant age-related and drug-induced fluctuations in body temperature, which might affect the severity of neural injury and, therefore, could confound studies of perinatal neuroprotection, may have been missed in the majority of recent studies.

The relative maturity of the species being studied is important for understanding the potential clinical application of the studies. The brain maturation of near-term fetal sheep (0.8–0.85 of gestation) and neonatal piglets is considered to be consistent with term human infants [26, 27]. However, at P7, the rat is closest to the 32- to 36-week-old human infant based on the immaturity of the germinal zone, the limited extent of cortical layering, and reduced density of synapses in the striate cortex [18, 28]. The majority of recent reports of neuroprotection in rodents (64%) studied P7 rats. As recently reviewed, neonatal rats are closest to term equivalent at P10–P14 [17]. The responses to HI at P10 have been carefully characterized [29]. Even at P10–P14, healthy neonatal rodents often show significant spontaneous central hypothermia in the typical laboratory nesting environment [20], and therefore they still require continuous core temperature monitoring and control.

About half (31/61; 51%) of the papers surveyed in this review reported outcomes either for only one sex or did not specify the sex of the subjects used. Preclinical studies have reported sexual dimorphisms in the severity and evolution of injury after HI and in responses to treatment, as previously reviewed [30]. For example, in P7 rats, males exposed to HI had greater deficits in some but not all behavioral tasks compared to females [30]. Similarly, there is some evidence of more variable responses to cooling in female mice [31]. It remains unclear whether there are similar differences in humans [32]. However, these data demonstrate the importance of carefully testing therapeutic agents in both sexes, as recommended by the National Institutes of Health [33].

A key clinical-translation consideration for testing potential neuroprotectants is when to treat. The majority of studies surveyed (57/75; 76%) started intervention either before or immediately after the insult. Scientifically, since the mechanisms of intra-insult treatment are different from those after the insult cell death, efficacy during pretreatment does not necessarily suggest a benefit after the insult. Clinically, because most HI occurs around birth, it is very difficult to identify fetuses who are likely to develop postnatal HIE in advance as the positive predictive value of heart rate monitoring in labor is exceedingly low [34]. It remains a formidable challenge to start any intervention even within the first 6 h after birth [35]. Thus, in most cases, it is very difficult to translate preclinical neuroprotective agents until the window of opportunity *after* HI is known. Collectively, these data support evaluation of delayed treatment after the insult as part of routine preclinical evaluation.

It is still important to test the effectiveness of potential neuroprotectants during normothermia to help understand the mechanism/s by which they induce neuroprotection and to evaluate their potential for use in patients who may not be suitable candidates for therapeutic hypothermia. Nevertheless, given that hypothermia is now standard clinical practice [35, 36], ultimately it is essential to also test the effectiveness of potential drug therapies as adjuvants to therapeutic hypothermia in order to improve current treatment protocols. In this analysis, just 9 out of the 61 papers surveyed (15%) assessed the effectiveness of hypothermia with adjuvant therapies.

Finally, the majority of studies (54/75; 72%) examined relatively short survival times, from ≤1 to 10 days. Less than half of the studies reported functional outcomes after treatment. Shorter survival times provide crucial information about the early outcome of injury and any intervention. Equally, particularly after moderate insults,

injury can continue to evolve over many weeks [37], and behavioral and histological outcomes are sometimes discordant, as noted in this analysis.

This paper examined a sample of preclinical publications investigating neuroprotection between 2014 and 2016. It is not possible to know whether the methodological issues identified in our analysis affected the outcome of the studies. Nevertheless, the findings reported in this study are highly consistent with previous reports of the "adult" literature [38]. If this critical information is not presented in the publications, it is not possible to assess the significance of past and future studies in a meaningful way. Finally, we did not examine papers assessing mechanisms of HIE. It is very likely that these variables are just as important to understand the mechanisms of HIE and, therefore, it would be well worth analyzing them in the future.

Conclusion

The majority of recent studies of perinatal neuroprotection reported limited, or even no, assessment or maintenance of brain or body temperature. In addition, many did not address pragmatic treatment regimes, report or test the sex of the subjects, or assess long-term functional and histological outcomes. It is critical that all future preclinical studies of the mechanisms and treatment of perinatal HIE should carefully control brain and core temperatures during and after HI.

Contributor's Statement

Robert Galinsky, Justin M. Dean, and Alistair J. Gunn conceptualized and designed the study. Robert Galinsky performed the initial search and analysis, and drafted the paper. Christopher A. Lear, Joanne O. Davidson, Simerdeep Dhillon, Guido Wassink, Laura Bennet, and Alistair J. Gunn provided in-depth review of the studies identified in the search and revised the paper. All authors critically reviewed the paper and approved the final version as submitted and agree to be accountable for all aspects of the work.

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Disclosure Statement

The authors have no financial relationships relevant to this article and no conflicts of interest to disclose. The study sponsors had no role in (1) the study design; (2) the collection, analysis, and interpretation of data; (3) the writing of the report; and (4) the decision to submit the paper for publication. No honorarium, grant, or other form of payment was given to anyone to produce the paper.

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