BRAIN COMMUNICATIONS

Cold stress protein RBM3 responds to hypothermia and is associated with good stroke outcome

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RNA-binding motif protein 3 is a molecular marker of hypothermia that has proved neuroprotective in neurodegenerative disease models. However, its relationship to the well-recognized therapeutic effect of hypothermia in ischaemic stroke had not been studied. In this work, the expression of RNA-binding motif protein 3 was investigated in ischaemic animal models subjected to systemic and focal brain hypothermia, specifically the effects of RNA-binding motif protein 3 silencing and overexpression on ischaemic lesions. Moreover, the association of RNA-binding motif protein 3 levels with body temperature and clinical outcome was evaluated in two independent cohorts of acute ischaemic stroke patients ($n = 215$); these levels were also determined in a third cohort of 31 patients derived from the phase III EuroHYP-1 trial of therapeutic cooling in ischaemic stroke. The preclinical data confirmed the increase of brain RNA-binding motif protein 3 levels in ischaemic animals subjected to systemic and focal hypothermia; this increase was selectively higher in the cooled hemisphere of animals undergoing focal brain hypothermia, thus confirming the direct effect of hypothermia on RNA-binding motif protein 3 expression, while RNA-binding motif protein 3 up-regulation in ischaemic brain regions led to functional recovery. Clinically, patients with body temperature <37.5°C in the first two cohorts had higher RNA-binding motif protein 3 values at 24 h and good outcome at 3 months post-ischaemic stroke, while RNA-binding motif protein 3 levels in the cooled third cohort tended to exceed those in placebo-treated patients. These results make RNA-binding motif protein 3 a molecular marker associated with the effect of hypothermia in ischaemic stroke and suggest its potential application as a promising protective target.

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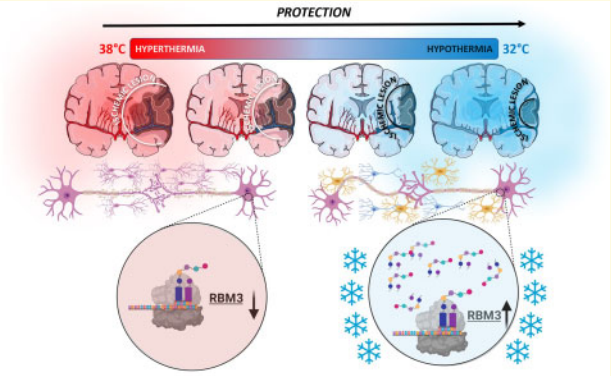
Abbreviations: AAV = adeno-associated virus; CIRP = cold-inducible RNA-binding protein; CSPs = cold shock proteins; FC = focal control; FH = focal hypothermia; GFP = green fluorescent protein; IS = ischaemic stroke; RBM3 = RNA-binding motif protein 3; SC = Systemic normothermic control groups; SH = systemic hypothermia; WB = western blot

Graphical Abstract



Introduction

Hypothermia is one of the most effective neuroprotective treatments in preclinical models of ischaemic stroke (IS) (Castillo *et al.*, 1998; van der Worp *et al.*, 2007; Campos *et al.*, 2012; Vieites-Prado *et al.*, 2016). However, its clinical translation remains hampered by side effects such as shivering, hypotension, arrhythmia and increased risk of pneumonia that usually require sedation or anaesthesia (Darwazeh and Yan, 2013; Geurts *et al.*, 2017). We recently showed that non-invasive focal hypothermia applied to the cerebral ischaemic region decreases the side effects of cooling stress in awake animals while retaining benefits similar to those of systemic hypothermia; unfortunately, clinical translation remains a daunting challenge since the thickness of human skull effectively insulates the brain, thus requiring the use of prolonged skin-damaging cold to achieve targetintracerebral temperatures (Vieites-Prado *et al.*, 2016).

Despite theseclinical limitations, the physiology underpinning the therapeutic effects of hypothermia provides key protective targets for reducing ischaemic lesions (Han *et al.*, 2012). At the same time as hypothermia down-regulates global protein synthesis and cell metabolism, it up-regulates cold shock proteins (CSPs). The two main CSPs described in mammals are cold-inducible RNA-binding protein (CIRP) and RNA-binding motif protein 3 (RBM3). While CIRP is detrimental in enhancing the inflammatory response (Zhou *et al.*, 2014), interest in RBM3 has significantly increased due to its critical role in the protective effect of hypothermia (Zhou *et al.*, 2014; Knott, 2015; Zhu *et al.*, 2016*a*).

RMB3 is a glycine-rich protein (17 kDa) that promotes global protein synthesis at 32°C by accelerating ribosome assembly, stabilizing mRNA and decreasing microRNA expression (Dresios *et al.*, 2005). In perinatal asphyxia models, RBM3 mediates rescue from apoptotic neuronal death during therapeutic cooling (Wellmann *et al.*, 2010). Up-regulated RBM3 expression is associated with hibernation: it helps restore brain activity in awakening animals and protects cells against cold damage (Peretti *et al.*, 2015; Wood, 2015). In mouse models of Alzheimer's disease, RBM3 mediates protective cooling effects by reducing synaptic loss (Knott, 2015; Zhu *et al.*, 2016*a*). More recently, RBM3 has been found to stimulate neuronal differentiation and inhibit hypoxic-ischaemia-induced apoptosis in the two main areas of persistent adult neurogenesis, the subventricular and subgranular zones (Zhu *et al.*, 2019). However, although cooling is a well-recognized therapy in cerebral ischaemia, a role for RBM3 has yet to be sought in this regard. We therefore evaluated the effect of systemic and focal hypothermia on cerebral RBM3 expression in ischaemic animals and analysed the relationship between changes in blood RBM3 levels and body temperature in IS patients.

Materials and methods

Animal handling

All experimental protocols were approved by the local Animal Care Committee according to European Union rules (86/609/CEE, 2003/65/CE and 2010/63/EU). The

manuscript conforms to Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Male 280–330 g Sprague-Dawley rats were housed individually, in stable environmental conditions (23°C, 40% relative humidity, 12-h light-dark cycle), with free access to food and water. Younger 130–150 g rats were used for premature viral injections.

Cerebral ischaemia

Transient focal ischaemia (45 min) was induced by intraluminal occlusion of the middle cerebral artery as previously described (Vieites-Prado *et al.*, 2016; Fernandez-Susavila *et al.*, 2017). Cerebral blood flow was monitored during surgery and evaluated by MRI during occlusion, before hypothermic/normothermic treatment, to confirm uniformity of the initial ischaemic lesion in all animals (detailed protocol in Supplementary material).

MRI

MRI was performed using a 9.4 T system (Bruker BioSpin, Billerica, MA, USA) with 440 mT/m gradients combining a linear birdcage resonator (7 cm diameter) for signal transmission with a 2 × 2 surface coil array for signal detection (details in Supplementary material).

Inducing systemic and focal hypothermia

Systemic hypothermia was induced to a rectal and brain temperature of 32°C for 4 h under 2% sevoflurane anaesthesia animal models using a rectal thermostat-controlled pad (Neos Biotec), as previously described (Vieites-Prado *et al.*, 2016). Animals were then returned to their home cages for spontaneous rewarming. Normothermic systemic controls were also maintained for 4 h at 37°C (rectal and brain temperature). We found differences lower than 2°C between rectal and brain temperature, measured either via non-invasive magnetic resonance thermometry or by means of invasive temperature probes (Vieites-Prado *et al.*, 2016). Based on these results, we selected rectal temperature as a reliable monitoring approach that reflected brain temperature throughout the experimental procedures.

Brain focal hypothermia was induced for 24 h via a metallic spiral tube implanted over the temporal skull and connected to a thermal bath, allowing free movement. The tube was also implanted for 24 h in normothermic focal controls, after which the tube was removed and the animals returned to their home cages. This protocol selectively cools the targeted hemisphere to 32°C by the spiral device without affecting body temperature (Vieites-Prado *et al.*, 2016). Brain RBM3 expression was measured by quantitative polymerase chain reaction (qPCR) and western blot (WB) 3 h after cooling (details in Supplementary material; Supplementary Tables 1 and 2). Animals were randomly assigned to the experimental groups (n = 3) using the GraphPad software randomization tool (www.graphpad.com).

Brain RBM3 silencing and enhancing in ischaemic animals

We evaluated the impact on cerebral ischaemia of silenced or enhanced RBM3 expression using two different constructs of adeno-associated virus (AAV) (Vector Biosystems, PA, USA) and their respective empty vectors: AAV2-CamKII-rat-Rbm3-CamKII-eGFP and AAV2-GFP-U6-rat-Rbm3-shRNA (GenBank access number for RBM3: NM_053696). All animals weighing 130–150 g were injected four times with 2 µl virus in the somatosensory cortex. Stereotaxic injection of 0.2 µl/min was performed using 30 Ga cemented Hamilton syringes (Hamilton, NV, USA) under microinjector control (Stoelting Co., Wood Dale, IL, USA). The needle was left in place for 10 additional minutes to avoid reflux. Animals were divided into four experimental groups: (i) RBM3 overexpression; (ii) RBM3 overexpressing vector without the insert; (iii) RBM3 silencing; and (iv) RBM3 silencing vector without the shRNA sequence. T_2*-weighted MRI was performed after stereotaxic surgery to confirm correct injection positioning or to discard needle-induced haemorrhage. Infected animals were allowed to recover from surgery and express the vectors for 4 weeks before the ischaemic injury was induced. Animals were randomly assigned to the experimental groups ($n = 8$) using the GraphPad software randomization tool (www.graphpad.com).

RBM3 levels in IS patients

RBM3 blood levels were measured in two independent IS cohorts: one from the Clinical Hospital of Santiago de Compostela, and the other from Dr. Josep Trueta University Hospital in Girona, following approval by the respective ethics committees. The study was carried out in accordance with the Declaration of Helsinki of the World Medical Association, fulfilling the criteria of the STROBE Statement (Guidelines for reporting observational studies). Application of the inclusion and exclusion criteria (Supplementary Tables 3 and 4) yielded a total of 215 eligible patients (113 from Santiago and 102 from Girona). RBM3 levels were determined at admission and at 24 h after stroke onset. Body temperature was measured at admission and for the first 24 h. Hyperthermia was defined as an axillary temperature ≥37.5°C and normothermia as <37.5°C (details in Supplementary material).

RBM3 levels in cooled IS patients

Blood samples for RBM3 analysis were obtained from the phase III EuroHYP-1 trial of therapeutic cooling in IS patients (van der Worp *et al.*, 2014). The trial randomized patients to hypothermic treatment plus standard care

or standard care alone (controls). In the hypothermic arm, cooling was started within 6 h of symptom onset and within 90 min of starting thrombolysis (or within 90 min of hospital admission in those not treated with thrombolysis) using 20 ml/kg refrigerated normal saline (4°C) IV over 30–60 min or a pre-specified surface cooling method. Cooling was maintained at 34–35°C for 24 h using a surface or endovascular technique. Thereafter, patients were passively rewarmed at a rate of 0.2 ± 0.1°C/h until rectal or bladder temperature reached 36.0°C, after which the cooling device was disconnected. All patients received treatment for acute IS and secondary prevention according to published guidelines. Pre-defined per-protocol analyses were performed in all patients whose body temperature was maintained at ≤35.0°C for at least 6 h during active cooling. We selected 31 patients (14 treated and 17 controls) for the study based on blood sample availability. Sample collection for biomarker measurement was pre-specified at three time-points: basal (pre-cooling baseline); 26 h (2 h after rewarming) and 72 h after stroke onset.

RBM3 analysis in human blood samples

Serum RBM3 levels were measured using commercial ELISA kits following manufacturer instructions (Biotez RBM3 ELISA, Berlin, Germany).

Statistical analysis

All preclinical data were analysed in GraphPad Prism 5.01 as the mean and standard error of the mean. One- or two-way ANOVA followed by *post hoc* Bonferroni evaluation was used to determine significant difference between multiple groups, with P set at <0.05. Investigators responsible for the assessment of outcomes (infarct size, behaviour and molecular biology analyses) were blinded to treatment groups (coded animals, samples and MRI images). Investigators performing surgeries could not be blinded to the hypothermic treatment (systemic and focal) due to the differences in the experimental procedure. Sample size was established based on our previous study where we have already evaluated the effect of systemic and focal hypothermia in the same experimental ischaemic animal model (Vieites-Prado *et al.*, 2016).

Clinical data were analysed blind in SPSS 21.0 (IBM, USA) as percentages for categorical variables and as the mean ± standard deviation (SD) or median and range (25th to 75th percentiles) for continuous variables depending on their adjustment to normality, assessed by Kolmogorov–Smirnov and Lilliefors tests; correlations were performed using Spearman coefficients. Differences between patients according to outcome group were determined by ANOVA and chi-square tests. Logistic regression analysis was adjusted by the clinically significant variables identified in the bivariate analysis and the results shown as odds ratios (ORs) with 95% confidence intervals (95% CI).

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its Supplementary material.

Results

Temporal profile of brain RBM3 expression in hypothermia

We began by measuring brain RBM3 expression at different time-points in healthy animals subjected to systemic hypothermia or normothermia + anaesthesia. After cooling for 4 h, all animals were sacrificed at 0, 3, 6, 12 or 24 h. *Rbm3* mRNA expression in normothermic conditions after 4 h showed an increase over baseline at 3 h. The increase was greater in cooled animals. The same increasing profile was observed in RBM3 protein levels (Fig. 1A, Supplementary Tables 5 and 6). Based on these findings, RBM3 expression was determined 3 h post-cooling in subsequent study groups. Post-focal hypothermic quantification showed selective increases in *Rbm3* mRNA and RBM3 protein levels in the cooled brain region of healthy animals versus controls. Focal normothermic conditions (24 h with the cooling device without hypothermia in awake conditions) did not significantly affect *Rbm3* mRNA or RBM3 protein expression (Fig. 1B, Supplementary Tables 5 and 6). All animals which passed the surgical inclusion criteria completed the hypothermic experimental procedures.

RBM3 expression in cooled ischaemic animals

Having established the effect of temperature in healthy animals, *Rbm3* mRNA and protein levels were then quantified in ischaemic animals undergoing systemic or focal hypothermia and their respective normothermic controls. MRI data determined using apparent diffusion coefficient maps confirmed baseline lesion volumes between 35% and 45% of the ipsilateral hemisphere in each animal (Fig. 2A), before cooling treatment.

In ischaemic animals undergoing systemic normothermia, *Rbm3* mRNA and RBM3 protein expression tended to increase in both hemispheres compared with healthy controls. This increase was significantly enhanced by systemic hypothermia in both hemispheres (Fig. 2B, Supplementary Tables 3 and 4). In ischaemic animals undergoing focal hypothermia, *Rbm3* mRNA expression increased in the ipsilateral hemisphere ischaemic region where the cooling spiral was located. RBM3 protein

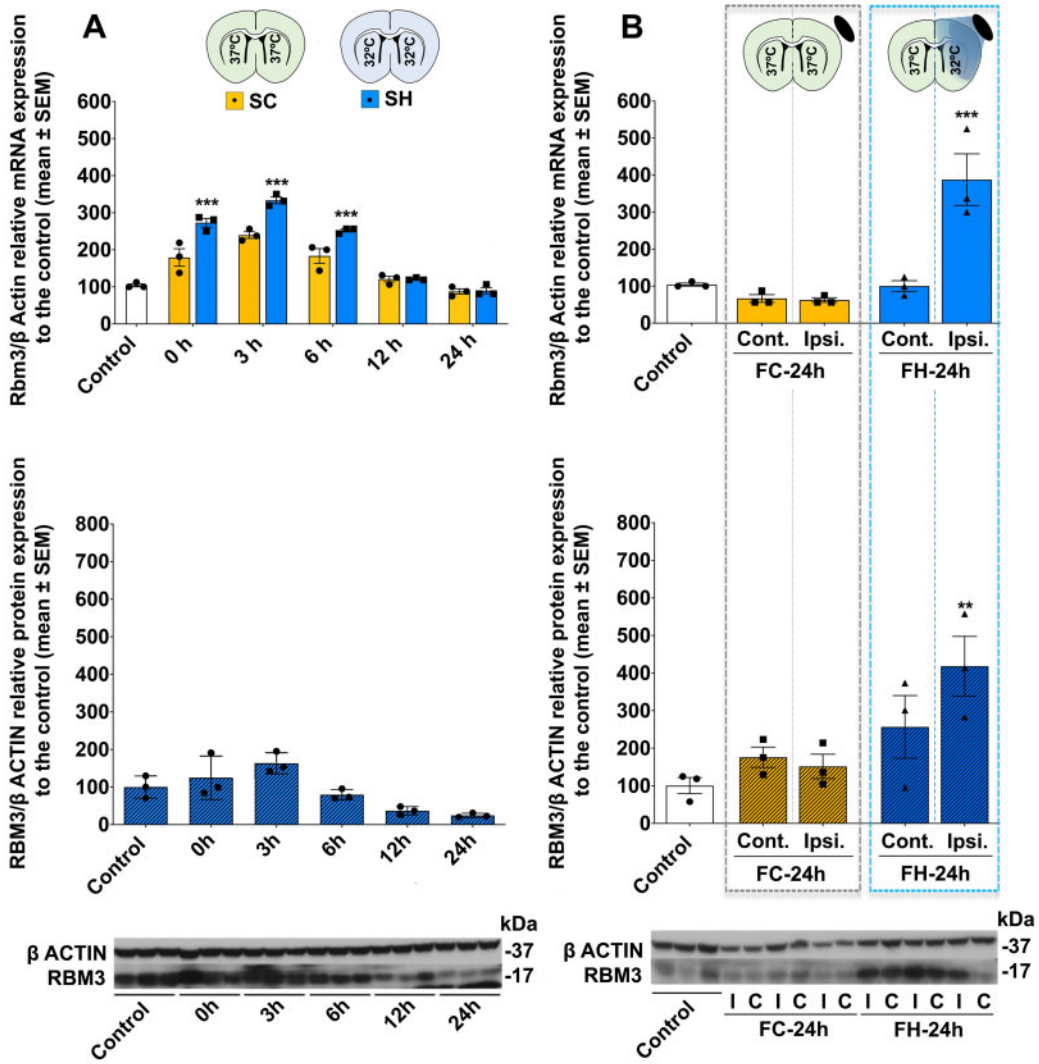


Figure 1 Temporal profile of RBM3 expression after hypothermia. **(A)** *Above*: Temporal brain *Rbm3* mRNA levels after 4 h of SH (target temperature 32°C) or normothermia (target temperature 37°C). *Bottom*: Temporal brain RBM3 protein expression after 4 h of SH. **(B)** *Above*: Brain *Rbm3* mRNA expression in healthy animals under FC conditions for 24 h (FC-24h, target temperature 37°C) or FH for 24 h (FH-24h, target temperature 32°C) followed by 3 h of recovery. *Bottom*: Brain RBM3 protein expression in healthy animals subjected to FC-24h or FH-24h. *Rbm3* mRNA levels were relativized to β-actin then normalized to untreated controls. RBM3 protein expression was relativized to β-actin then normalized to control animal expression. WB bands corresponding to β-actin and RBM3 (37 and 17 kDa, respectively) are shown below. Data are shown as mean ± SEM. $^{**}P < 0.01$ or $^{***}P < 0.001$; using one-way or two-way ANOVA followed by a *post hoc* Bonferroni test ($n = 3$/group). FC = focal hypothermia control group; FH = focal hypothermia group; SC = Systemic normothermia control groups; SH = systemic hypothermia group. Full-size WB gels are available in Supplementary material.

analysis confirmed the focal hypothermia-induced increase in RBM3, although it was also observed in the contralateral region (Fig. 2C, Supplementary Tables 5 and 6).

Analysis of brain RBM3 silencing and enhancing in ischaemic damage

To evaluate the protective effect of RBM3 on infarct volume reduction, we induced artificial expression (up-regulation and down-regulation) in the ipsilateral ischaemic cortical region (before inducing ischaemia) using AAV infection followed by confirmatory MRI. AAV injection selectively infected cortical neurons determined by co-localization with NeuN (neuronal marker) and green fluorescent protein (GFP) as reporter gene (Fig. 3A and B). No GFP co-localization was observed with markers of astrocytes [glial fibrillary acidic protein (GFAP)] or microglia [ionized calcium-binding adaptor molecule 1 (Iba1)]. One month after virus injection, animals underwent cerebral ischaemia to evaluate the effect of RBM3 overexpression or silencing on ischaemic damage. Following the same protocol used for inducing cerebral

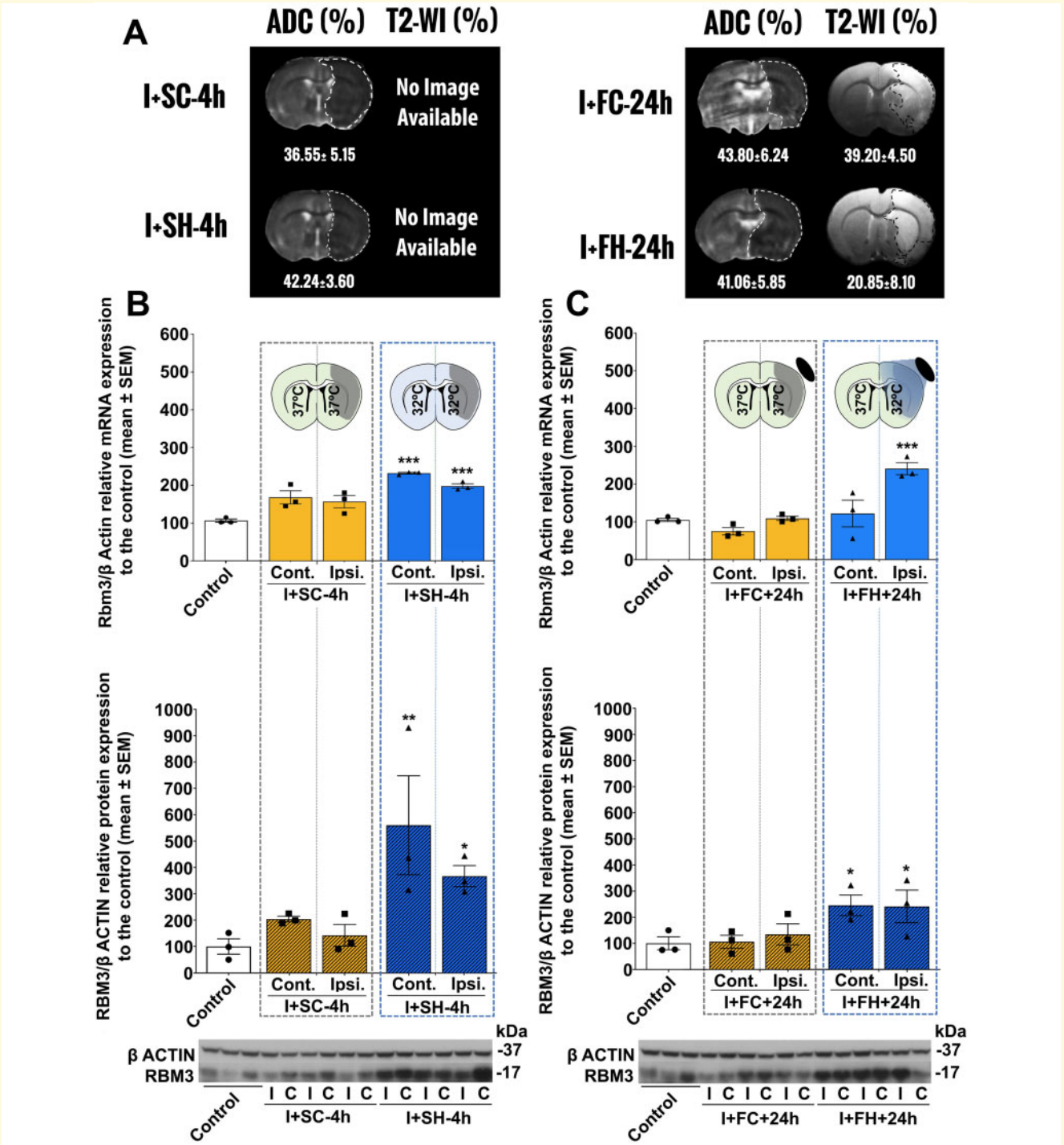


Figure 2 Hypothermia reduces ischaemic lesion volume and enhances RBM3 expression. (**A)** MRI assessments of ischaemic injury. Apparent diffusion coefficient maps were recorded before treatment (during surgical ischaemia induction) and T2 images were acquired after 24 h. Since animals subjected to systemic normothermia or SH were sacrificed 3 h after treatment (7 h after hypothermia induction), no T2 images at 24 h are available for these groups. (**B)** *Above*: Brain *Rbm3* mRNA levels in ischaemic animals subjected to systemic normothermia (I+SC-4h, target temperature 37°C) or SH (I+SH-4h, target temperature 32°C) followed by 3 h of recovery. *Below***:** RBM3 protein expression in animals subjected to I+SC-4h or I+SH-4h, followed by 3 h of recovery. (**C)** *Above*: Brain RBM3 mRNA levels in ischaemic animals subjected to focal normothermia (I+FC-24h, target temperature 37°C) or FH (I+FH-24h, target temperature 32°C) followed by 3 h of recovery; *Below***:** Brain RBM3 protein expression in ischaemic animals subjected to I+FC-24h or I+FH-24h, followed by 3 h of recovery. *Rbm3* mRNA levels were relativized to β-actin then normalized to the expression in untreated controls; RBM3 protein expression was relativized to β-actin then normalized to the expression in controls. WB bands corresponding to β-actin and RBM3 (37 and 17 kDa, respectively) are shown below. Data are shown as mean ± SEM. $^{**}P < 0.01$ or $^{***}P < 0.001$; using one-way or two-way ANOVA followed by a *post hoc* Bonferroni test ($n = 3$/group). FC = focal hypothermia control group; FH = focal hypothermia group; SC = systemic normothermia control groups; SH = systemic hypothermia group. Full-size WB gels are available in Supplementary material.

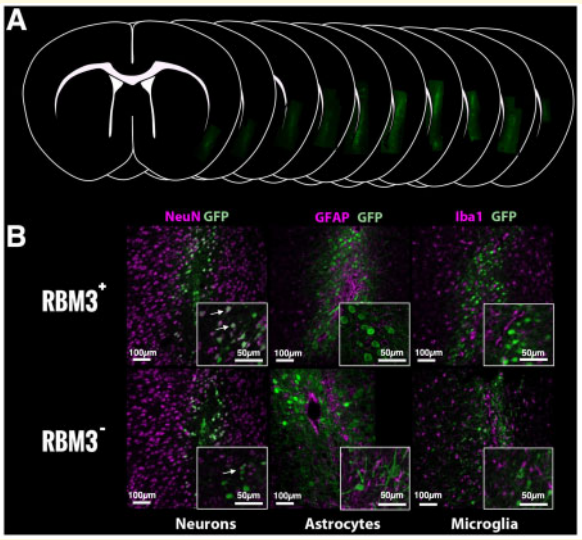


Figure 3 Histological analysis of RBM3 in brain tissue. **(A)** Cortical AAV infection 1 month before ischaemia induction. **(B)** GFP immunofluorescence with neuronal (NeuN), astrocyte (GFAP) and microglial (Iba1) markers. White arrows represent the co-localization observed only for GFP and NeuN.

ischaemia, all animals were scanned by MRI to confirm uniformity of basal infarct volume (Fig. 4A). Analysis of infarct volume 24 h post-ischaemia showed no significant impact on ischaemic damage by local changes in RBM3 expression (Fig. 4B), although rotarod testing revealed functional recovery in animals with up-regulated protein expression (Fig. 4C and D).

Association between blood RBM3 levels, temperature and outcome in IS patients

IS patient characteristics in both cohorts (Supplementary Table 7) showed a negative correlation between basal RBM3 levels and body temperature at admission (Spearman coefficient −0.150, $P = 0.028$) and at 24 h (Spearman coefficient −0.662, $P < 0.0001$). Individual correlation data (Supplementary Table 8) showed that normothermic (<37.5°C) patients had higher RBM3 values at 24 h than hyperthermic (≥37.5°C) patients (Fig. 5A). Interestingly, mean variation between the RBM3 levels determined at admission and 24 h after stroke onset (ΔRBM3) was positive in normothermic patients versus negative in hyperthermic patients (Fig. 5B).

Having observed several variables related to temperature (Supplementary Table 9), we evaluated ORs for the association between RBM3 and maximum temperature at 24 h <37.5°C by performing a logistic regression analysis using the variables identified as clinically and statistically significant in the model. The analysis revealed an independent association between maximum temperature at 24 h <37.5°C and RBM3 levels at 24 h ≥300 pg/ml (obtained from the ROC curve), with an adjusted OR of 15.83 (95% CI: 6.71–37.39; $P < 0.0001$) (Table 1).

We observed a clear relationship between RBM3 levels at 24 h and functional outcome at 3 months measured on the modified Rankin Scale (mRS) (Fig. 5C). In agreement with the trend observed with temperature, mean ΔRBM3 was positive in patients with good outcome versus negative in those with poor outcome (Fig. 5D). In addition, logistic regression analysis revealed that an ΔRBM3 value ≥10 (obtained from the ROC curve) was an independent marker of good functional outcome at 3 months (Table 2), after adjusting for the independent variables associated with poor functional outcome at that time-point (Supplementary Table 10).

Blood RBM3 levels in cooled IS patients

In the 31 patients from the EuroHYP-1 trial (van der Worp *et al.*, 2014), we found no significant differences in RBM3 levels between those treated with hypothermia ($n = 14$) or placebo ($n = 17$), although rewarming levels tended to be higher in those that received active treatment ($P = 0.131$) (Fig. 6A, left). Results were similar ($P = 0.153$) when including only those patients meeting the pre-defined criteria for per-protocol analyses (Fig. 6A, right). We observed the same trend ($P = 0.278$) towards higher RBM3 levels in cooled patients when comparing ΔRBM3 values between baseline and rewarming (2 h after completing the 24-h treatment, i.e. at 26 h) with those in placebo patients (Fig. 6B, left). Results were again similar ($P = 0.601$) in patients meeting the pre-defined criteria for per-protocol analyses (Fig. 6B, right). No significant correlation was found between RBM3 levels and body temperature except for a negative correlation between RBM3 after rewarming (26 h) and temperature at 24 h ($R = -0.414$, $P = 0.029$) (Fig. 6C, left). Separate group analysis revealed similar results in the hypothermic group ($R = -0.565$, $P = 0.44$ for RBM3 after rewarming and body temperature at 24 h) (Fig. 6C, right). No such correlation at these or other time-points was found in the placebo group.

Discussion

RBM3 is an evolutionarily old protein whose relationship to cold stress remained undescribed until 1997 (Danno *et al.*, 1997). Given its novelty and presumably complex role in the cell, its functions are still poorly understood. It has been reported to be involved in ribosome assembly, limitation of the unfolded protein response, interaction with microRNA regulators and cell-cycle regulation (Dresios *et al.*, 2005; Matsuda *et al.*, 2011; Pilotte *et al.*, 2011; Wong *et al.*, 2016; Zhu *et al.*, 2016b). All knowledge to date concerning RBM3 and

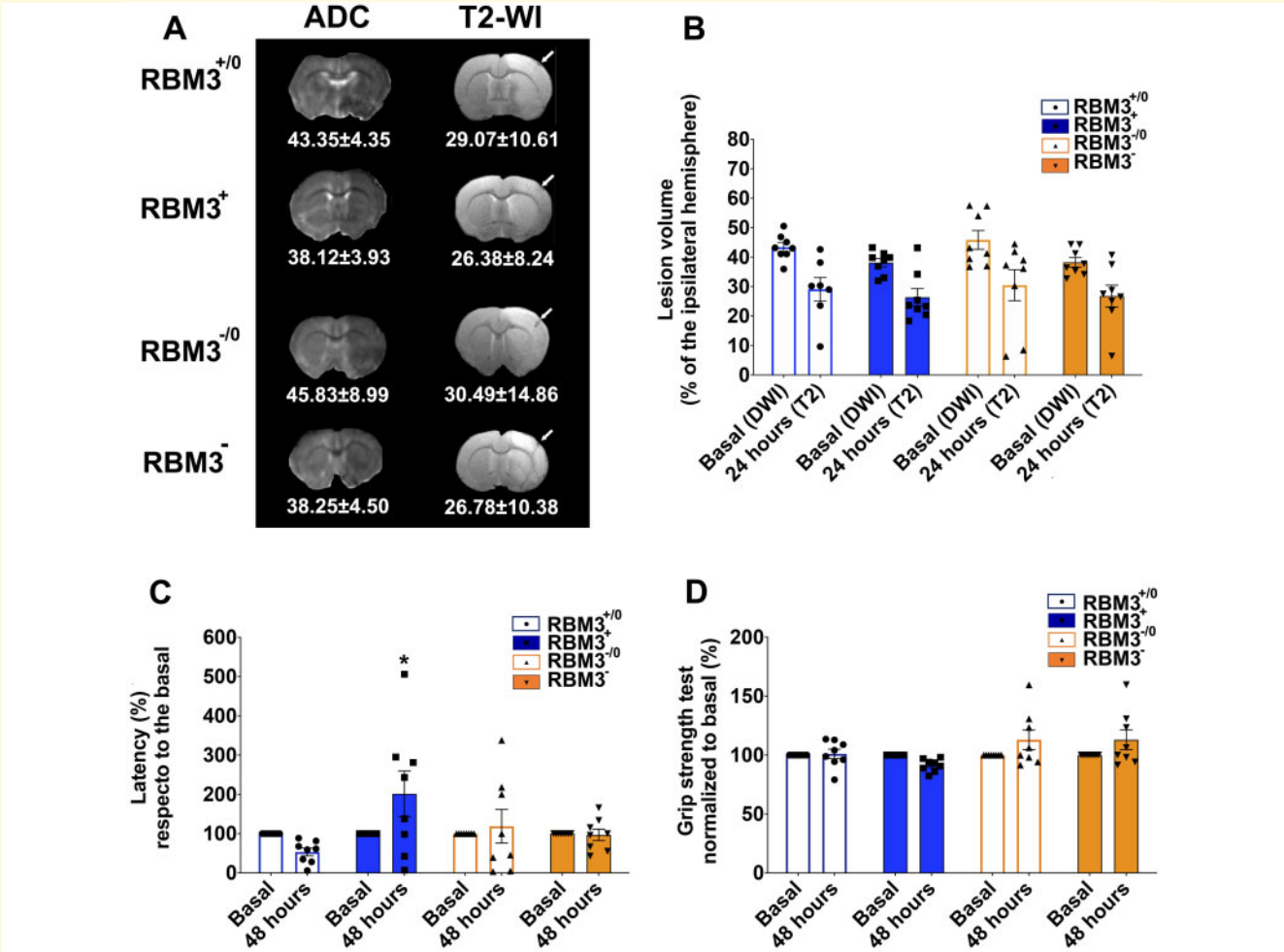


Figure 4 Effect of RBM3 expression on neurological ischaemic damage. **(A)** MRI assessments of ischaemic injury and ischaemic lesion volume in AAV-infected animals. Apparent diffusion coefficient maps were recorded before treatment (during surgical ischaemia induction) and T2 images were acquired after 24 h. White arrows show the needle track after virus injection. **(B)** Representation of the ischaemic injury determined in the four groups studied. Functional deficit in AAV-infected animals determined by grip **(C)** and rotarod tests **(D)** respect to the basal conditions (before ischaemia) and 48 h after ischaemia. RBM3^{+}: animals infected with AAV2-CamKII-rat-Rbm3-CamKII-eGFP used to enhance RBM3 expression; RBM3$^{+/0}$: control group infected with the same empty vector; RBM3^{-}: animals infected with AAV2-GFP-U6-rat-Rbm3-shRNA used to silence RBM3 expression; RBM3$^{-/0}$: control group infected with the same empty vector. Data are shown as mean ± SEM ($n = 8$/group).

hypothermia has been obtained from preclinical *in vitro* and *in vivo* studies. Clinical relevance had not previously been investigated. Our study is the first to demonstrate an association between RBM3 and hypothermia not only in cerebrally lesioned adult rats but also in IS patients.

Our group had already established that both hypothermia protocols used in this study, focal and systemic hypothermia, reduce ischaemic lesion volume in animal models (Vieites-Prado *et al.*, 2016). Focal hypothermia requires maintaining for 24 h to achieve the same protection as that afforded by 4 h of systemic cooling. But while focal hypothermia can be used in awake and freely moving animals, systemic hypothermia must be performed under anaesthesia to avoid hypothermic stress. Using magnetic resonance thermometry, we showed that focal hypothermia exerted a local cooling effect in the ischaemic region where the cooling device was located, whereas systemic hypothermia lowered whole body and brain temperature. The present study complements these earlier findings by showing that the decreases in brain temperature recorded by magnetic resonance thermometry during focal and systemic hypothermia are associated with increased levels of RBM3.

Selective RBM3 expression in the ipsilateral hemisphere where the focal hypothermia device is located appears a direct effect of temperature on cerebral tissue. Diffusion no doubt accounts for the slight increase in RBM3 protein levels observed in the contralateral hemisphere (Figs 1B and 2C).

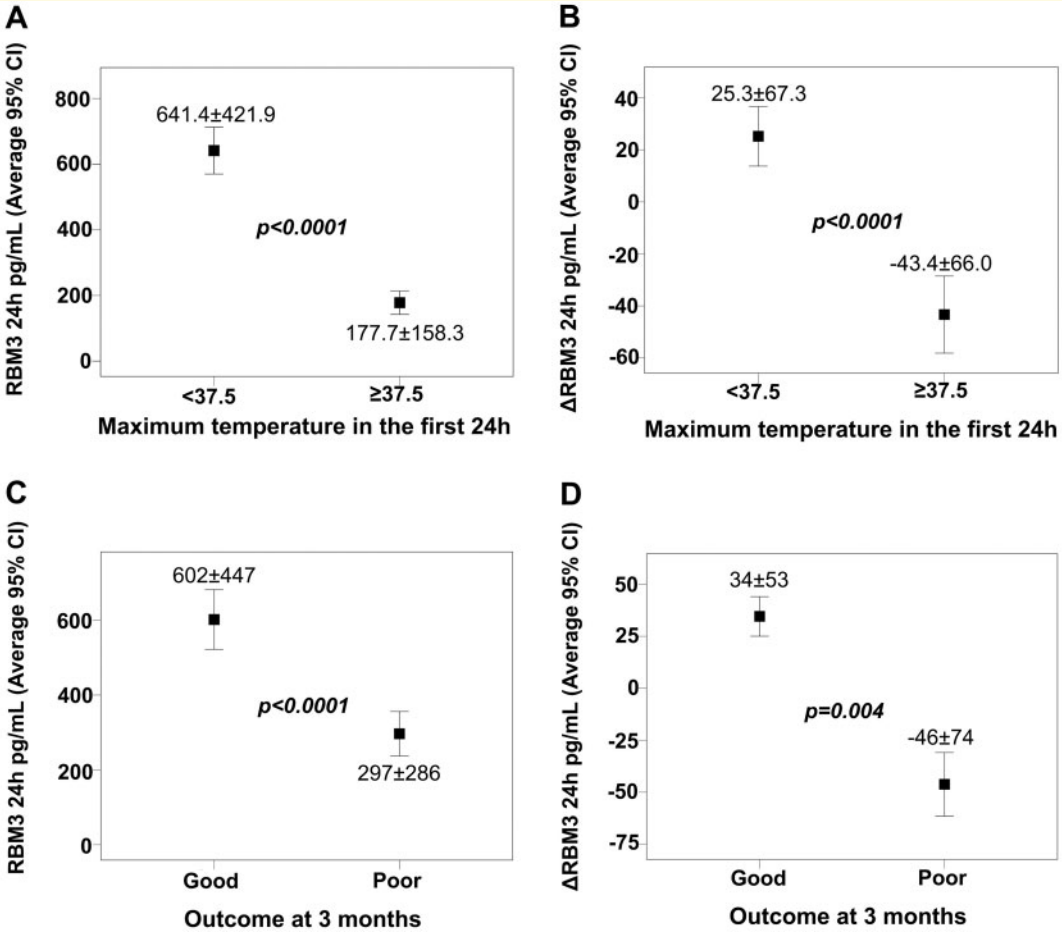


Figure 5 RBM3 is associated with a good outcome and is temperature-dependent. (A) RBM3 levels at 24 h and **(B)** ΔRBM3 in normothermic (<37.5°C) and hyperthermic (≥37.5°C) IS patients. **(C)** RBM3 levels at 24 h and **(D)** ΔRBM3 in IS patients with poor and good clinical outcome at 3 months. ΔRBM3 was defined as % variation of RBM3 at 24 h respect to the basal levels.

Table 1 Multivariate analysis

	OR not adjusted	95% CI	*P*	OR adjusted	95% CI	*P*
Center (cat)	1.32	0.76–2.31	0.325	1.22	0.43–3.50	0.708
Glycaemia	0.99	0.99–0.99	0.002	0.99	0.99–1.00	0.177
Leukocytes	0.82	0.74–0.90	<0.0001	0.79	0.69–0.93	0.004
Fibrinogen	0.99	0.99–0.99	<0.0001	0.99	0.99–1.00	0.070
Systemic fibrinolysis (cat)	2.86	1.53–5.36	0.001	2.81	0.91–8.74	0.074
Endovascular treatment (cat)	0.40	0.17–0.97	0.042	0.49	0.12–1.97	0.317
Previous Rankin	0.76	0.58–0.99	0.040	0.94	0.65–1.37	0.764
NIHSS on admission	0.91	0.87–0.96	<0.0001	0.92	0.86–0.98	0.014
RBM3 at 24 h ≥300 pg/ml	16.11	7.81–33.26	<0.0001	15.83	6.71–37.39	<0.0001

Dependent variable: maximum temperature in the first 24 h <37.5°C, not adjusted and adjusted by clinically relevant variables found in our study.
Cat = categorized; OR = odds ratio.

Additionally, the increased RBM3 observed during systemic normothermia in both healthy and ischaemic animals could be caused by the sevoflurane used during the experimental procedure: sevoflurane mediates cell signalling pathways related to AKT, a protein impacting RBM3 activity and expression (Matchett *et al.*, 2009; Wang *et al.*, 2010). Supporting evidence is that RBM3 did not increase in non-anaesthetized normothermic focal control groups.

The viral infection strategy used in this study to evaluate the effect of enhanced or blocked RBM3 expression on cerebral ischaemic damage has been tested in other models. Thus, cerebral overexpression of RBM3 mediated by lentiviral infection restored structural synaptic

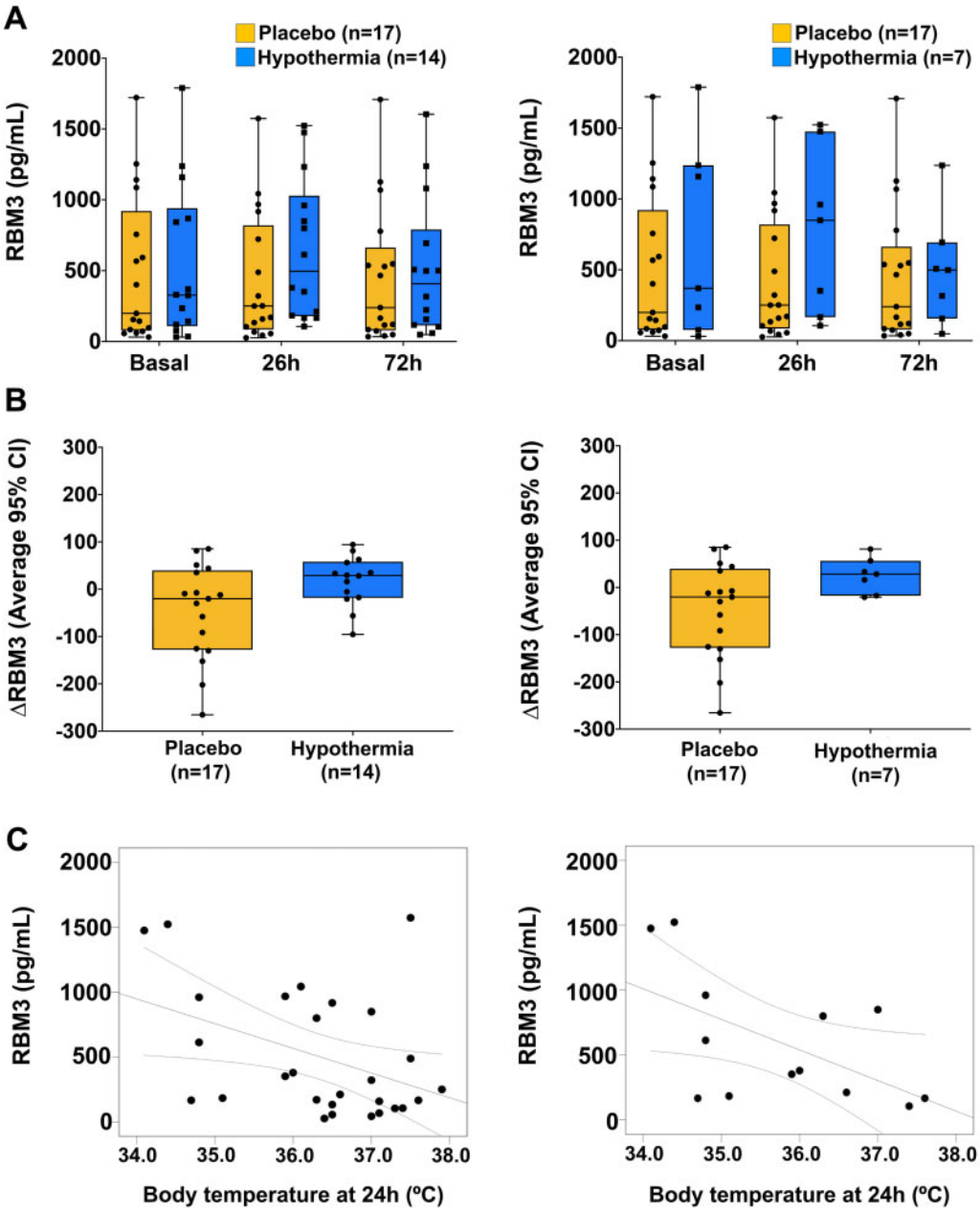


Figure 6 RBM3 levels in patients treated with hypothermia versus placebo. **(A)** RBM3 levels across the pre-specified collection time-points: basal (before cooling), 26 h (2 h after starting rewarming) and 72 h after stroke onset. Left panel includes all 31 patients and the right panel the SH group only, comprising patients fulfilling pre-defined criteria for per-protocol analyses ($n = 7$). **(B)** Boxplots represent the median (interquartile range) of ΔRBM3 values (%) between baseline and rewarming time-points in the SH and placebo arms (left, all cases; right, only patients fulfilling pre-defined criteria for per-protocol analyses). **(C)** Correlation between RBM3 levels at rewarming and body temperature at 24 h in all patients (left) and those treated with SH (right).

plasticity and reversed neuronal deficit in an animal model of Alzheimer's disease (Peretti *et al.*, 2015). In the present study, the viral infection we implanted in the cortical region was successful (based on GFP gene reporter data) in enhancing and inhibiting local RBM3 expression. Although viral tissue infection was optimized in order to maximize GFP expression with minimal tissue damage caused by the needle injection, neither approach significantly impacted ischaemic infarct volume, possibly due to the wide ischaemic lesion compared with the reduced population of infected neurons. However, a trend in functional recovery was observed in animals with up-regulated RBM3.

The analysis of RBM3 levels in two independent cohorts of IS patients showed an association between increasing ΔRBM3 and good outcome at 3 months,

Table 2 Multivariate analysis

	OR not adjusted	95% CI	*P*	OR adjusted	95% CI	*P*
Center (cat)	0.45	0.26–0.78	0.005	0.84	0.25–1.98	0.682
Age	0.97	0.94–0.99	0.030	1.00	0.96–1.05	0.955
Temperature in the first 24 h <37.5°C (cat)	3.86	2.15–6.92	<0.0001	0.61	0.22–1.70	0.341
Cardioembolic versus non-cardioembolic	3.69	1.88–7.24	<0.0001	2.05	0.75–5.58	0.162
Previous Rankin	0.33	0.22–0.51	<0.0001	0.37	0.22–0.64	<0.0001
NIHSS on admission	0.84	0.79–0.89	<0.0001	0.87	0.81–0.95	0.001
ΔRBM3 ≥10%	18.87	9.45–37.68	<0.0001	26.52	9.32–75.44	<0.0001

Dependent variable: Clinical outcome at 3 months, not adjusted and adjusted by clinically relevant variables found in our study.
Cat = categorized; OR = odds ratio.

reflecting a potentially protective role on neuronal tissue. The fact that the association with good prognosis was stronger than with temperature itself suggests the involvement of RBM3 in other neuroprotective mechanisms independent of body temperature. High RBM3 levels have been clinically associated with prolonged overall survival in intestinal-type gastric cancer (Ye *et al.*, 2017), good prognosis in invasive breast cancer (Kang *et al.*, 2018) and colon cancer (Jang *et al.*, 2017), and improved response and survival in metastatic colorectal cancer (Siesing *et al.*, 2017), none of which benefits appear temperature-related.

The blood samples from the IS patients that underwent hypothermic treatment in the EuroHYP-1 trial confirmed the association between RBM3 and cooling. Although the results were not statistically significant, this trend observed in just 17 patients can be considered remarkable. Although a larger sample size might achieve significance, the pool of eligible patients will remain limited until hypothermic treatment becomes established clinical practice. EuroHYP-1 was a pioneering phase III trial but access to multiple samples was limited by the low number of patients enrolled, incompatibility between hypothermic procedures and blood sampling, and the inability of many patients to complete the cooling protocol.

In conclusion, these preclinical and clinical results identify RBM3 as a molecular marker associated with the protective effect of hypothermia in IS and suggest its potential application as a promising protective target.

Supplementary material

Supplementary material is available at *Brain Communications* online.

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Competing interests

The authors report no competing interests.

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