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COLD STRESS PROTEIN RBM3 RESPONDS TO TEMPERATURE CHANGE IN AN ULTRA-SENSITIVE MANNER IN YOUNG NEURONS

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Abstract—Extremely mild hypothermia to 36.0 °C is not thought to appreciably differ clinically from 37.0 °C. However, it is possible that 36.0 °C stimulates highly sensitive hypothermic signaling mechanism(s) and alters biochemistry. To the best of our knowledge, no such ultra-sensitive pathway/mechanisms have been described. Here we show that cold stress protein RNA binding motif 3 (RBM3) increases in neuron and astrocyte cultures maintained at 33 °C or 36 °C for 24 or 48 h, compared to 37 °C controls. Neurons cultured at 36 °C also had increased global protein synthesis (GPS). Finally, we found that melatonin or fibroblast growth factor 21 (FGF21) augmented RBM3 upregulation in young neurons cooled to 36 °C. Our results show that a 1 °C reduction in temperature can induce pleiotropic biochemical changes by upregulating GPS in neurons which may be mediated by RBM3 and that this process can be pharmacologically mimicked and enhanced with melatonin or FGF21.

Key words: targeted temperature management, RBM3, CIRBP, hypothermia, global protein synthesis, FGF21.

INTRODUCTION

Hypothermia has long been known to have protective or detrimental effects depending on depth and duration. For CNS injury, therapeutic hypothermia (TH) is one of few treatments proven to reduce neurologic impairment (Dietrich, 1992; Lyden et al., 2006; Povlishock and Wei, 2009; Wei et al., 2011). Optimal target temperature remains a matter of debate. Each 1 °C reduction in body temperature decreases cerebral metabolism and blood flow by ~6% (Rosomoff and Holaday, 1954; Wong, 1983). Cooling at or below 35.5 °C (but above deep hypothermia) inversely correlates with greater neuroprotection (Busto et al., 1987; Minamisawa et al., 1990; Welsh et al., 1990; Weinrauch et al., 1992). In contrast, 36 °C is not thought to activate hypothermia regulated neuroprotective mechanisms – although it does cause thermoregulatory vasoconstriction and shivering. Furthermore, 36 °C has been used as normothermic control in a number of brain injury studies (Busto et al., 1987, 1989; Onesti et al., 1991). A recent multicenter clinical trial on cardiac arrest found neurological outcomes were almost identical in patients maintained to 36 °C versus those given classic TH to 33 °C. Fever prevention in both groups has been suggested to explain these surprising outcomes. Cooling to 36 °C is not a trivial intervention to accomplish clinically (Hostler et al., 2010), and thus it is also possible that 36 °C activates as of yet unidentified ultra-sensitive hypothermia regulated protective mechanisms.

Cold shock proteins are up-regulated by cold stress. They include RNA binding motif 3 (RBM3) and cold-inducible RNA binding protein (CIRBP) (Danno et al., 1997; Nishiyama et al., 1997). CIRBP is a novel damage-associated molecular pattern (DAMP) that induces inflammation in mice (Qiang et al., 2013). Ischemic brain injury is reduced in CIRBP KOs (Zhou et al., 2014). In contrast, RBM3 appears to be a potent neuroprotective protein. RBM3 mRNA/protein is increased in hippocampal slices by mild cooling to 33.5 °C (Tong et al., 2013). Knockdown of RBM3 reduces hypothermia mediated neuroprotection *in vitro* (Chip et al., 2011). Furthermore, exposing mice to brief periods of deep hypothermia upregulates RBM3 in the brain and markedly reduces neurodegenerative pathologies caused by prion or Alzheimer's disease (Peretti et al., 2015). Deep cooling-induced neuroprotection is blocked by RBM3 knockdown. Non-CNS cells are also protected by RBM3 (Ferry et al., 2011). A key function of RBM3 is to augment global protein synthesis (GPS), which has been

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Abbreviations: ARA-C, cytosine β-d-arabinofuranoside hydrochloride; CIRBP, cold inducible RNA binding protein; DIV, day *in vitro*; EDTA, ethylenediaminetetraacetic acid; FGF21, fibroblast growth factor 21; GPS, global protein synthesis; HEPES, 4-(2-hydroxyethyl)-1-piperazi neethanesulfonic acid; RBM3, RNA binding motif 3; SUnSET, surface sensing of translation; TH, therapeutic hypothermia; UMH, ultra-mild hypothermia.

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confirmed in neurons *in vitro* as well as in brain *in vivo* (Dresios et al., 2005; Smart et al., 2007; Peretti et al., 2015). It also is a pleiotropic regulator of miRNA and mRNAs (Pilotte et al., 2011; Liu et al., 2013).

Here we tested if RBM3 and/or CIRBP are increased in primary cortical neuron and/or astrocyte cultures cooled to 36 °C versus conventional hypothermia to 33 °C, as compared with normothermia to 37 °C. Surprisingly, 36 °C upregulated RBM3 in young 6d primary neuron cultures but failed to do so in mature 26-d-old neurons. Also, 36 °C for 48 h mildly increased RBM3 in astrocytes. RBM3 upregulation in young neurons cooled to 36 °C was also associated with elevated GPS. Finally, small molecule RBM3 activators have not been reported – such drugs could theoretically augment neurorecovery in brain-injured patients. Fibroblast growth factor 21 (FGF21), melatonin, and liver X receptor (LXR) agonist T0901317 increased RBM3 levels in young neurons cooled to 36 °C but were ineffective in mature neurons. Our findings suggest that even a 1 °C reduction in temperature can induce a bonafide cold shock response. Sensitivity to cold shock-induced RBM3 may be amplified in the developing brain and capable of being pharmacologically manipulated.

EXPERIMENTAL PROCEDURES

Reagents

Puromycin dihydrochloride was purchased from SIGMA (Cat# P9620-10 mL; St. Louis, MO, USA). FGF21 was purchased from R&D Systems (Minneapolis, MN, USA). Melatonin, T0901317, and SRT1720 were purchased from Tocris (Bristol, UK). AZD1080 was purchased from Cayman Chemical (Ann Arbor, MI, USA). *Primary antibodies:* Anti-RBM3 was purchased from Proteintech Group and used at 1:1000 (Cat#14363-1-AP; Chicago, IL, USA). Anti-CIRBP, anti-α-Tubulin, anti-pAKT(Ser473), anti-AKT total, anti-pERK, anti-ERK total, anti-eIF2α(Ser51), anti-eIF2α total were purchased from Cell Signaling Technology(Danvers, MA, USA). Anti-puromycin (12D10) was purchased from EMD Millipore and used at 1:15,000 (Cat#MABE343; Billerica, MA, USA). Goat anti-rabbit secondary antibodies were purchased from Life Technologies (Grand Island, NY, USA).

Animals

Animal studies were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Euthanasia protocols follow recommendations established by the American Medical Veterinary Association Guideline for Euthanasia. Female timed pregnant Sprague–Dawley rats were purchased from Charles River (Wilmington, MA, USA) and granted *ab libitum* accesses to food and water, and maintained on a 12-h light/dark cycle prior to euthanasia to harvest embryos.

Primary cortical neuron culture

Embryos (mixed gender) were isolated from timed pregnant (E16-17) Sprague–Dawley rats. Neuron cultures were performed as previously described by our group (Jackson et al., 2013). Cortices were dissected in ice-cold Hanks balanced salt solution (HBSS; Life Technologies, Grand Island, NY, USA) containing sodium bicarbonate (SIGMA), penicillin–streptomycin (Life technologies), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Life Technologies). Brain tissues were dissected under a Leica M651light microscope (Buffalo Grove, IL, USA), and placed in a 1.5 mL tube containing ~1 mL prepared HBSS. Tissues were minced and transferred to a 15 mL conical tube. Cells were spun 5 min/200 g/4 °C. Supernatant was aspirated, cells resuspended in 2 mL trypsin solution, and incubated 8 min at 37 °C with gentle mixing. Trypsin activity was quenched in 10 mL Neurobasal/B27 (Life Technologies) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Pittsburgh, PA, USA). Cells were transferred into a new 15 mL conical tube and spun 5 min/200 g/4 °C. Supernatant was replaced with 1.5 mL trituration media. Cells were dissociated by 10 passages through a fire-polished glass Pasteur pipette. The cell pellet was resuspended in plating media warmed to 37 °C (Neurobasal Media/B27 supplement prepared + 25 μM L-Glutamic Acid + Pen-Strep). Cell number was quantified by automatic counting on a *Cellometer* (Nexcelom Bioscience, Lawrence, MA, USA). Cells were seeded onto 6-well culture plates coated with poly-D-lysine (density ~1.2–1.5 × 10^6/well). Cultures were maintained by half media replacement every ~3–4 days *in vitro* (DIV). The mitotic inhibitor cytosine β-D-arabinofuranoside hydrochloride (ARA-C; 4 μM final concentration) was added in study 1 experiment (e.g. DIV10–11 cultures) to ensure purity of neuronal biochemistry for initial assessment of temperature treatments. Mitotic inhibitors at low levels are non-toxic but still potentially represent a mild stressor (though necessary to guarantee neuron purity) (Ahlemeyer et al., 2003). Therefore we also confirmed results of temperature treatments in younger DIV6 neurons in the absence of ARA-C (neuron enriched cultures; > 90% neurons). Astrocytes are allowed to proliferate in aged cultures, which are not treated with ARA-C. We observe that astrocytes are necessary for long-term survival of neuron cultures (i.e. DIV26 mature neuron cultures).

Primary astrocyte culture

Pure rat astrocyte cultures were prepared as previously reported by our group (Jackson et al., 2013). In brief, brains were collected from postnatal day 2 SD rat pups. Mixed brain cells were seeded onto T75 culture flasks and maintained in DMEM/F12/10%FBS/Pen-Strep culture media. Astrocytes were repeatedly split and propagated in new T75 flasks until pure. At propagation #5, astrocytes were seeded onto poly-D-lysine coated 6-well plates (~7.5 × 10^4/well) and maintained for an additional ~3 d. At ~60–70% confluency in 6-well plates, astrocytes were given fresh media exchange then temperature treatments were initiated for 24 h and 48 h.

Hypothermia protocols

Neurons were maintained in a 37 °C (normothermic) incubator. Before start of hypothermia, two identical incubators of the same make/model with humidity of ~95% (all in the same room) were programed to 36 °C and 33 °C. Incubators were allowed a minimum of 48 h equilibration time prior to starting hypothermic experiments. In some experiments, as an additional control, a thermometer was placed inside hypothermic incubators as a secondary gauge of temperature. Temperature as assessed by that approach was independently confirmed to never be under target temperature and fluctuate by no more than ~0.5 °C. On DIV of interest, culture plates were assigned to one of the three experimental temperatures for 24 or 48 h. *Drug treatments:* Drugs were prepared in DMSO or PBS (i.e. for FGF21). All drugs were diluted in conditioned culture media and applied for 24 h. Controls received equal amounts of DMSO/PBS without drugs. Primary neurons were harvested for biochemistry at key time points. RBM3 and CIRBP levels were measured by Western blot. Investigators were not blinded to temperature treatment groups.

Analysis of de novo protein synthesis

Surface sensing of translation (SUnSET) is a recently developed non-radioactive method using puromycin incorporation to accurately measure rate of new protein synthesis in cells (Schmidt et al., 2009). We adapted that protocol with a slight modification. Briefly, at the end of temperature treatments (on DIV6), conditioned media were collected by removing 1 mL from each well of six-well plate, for all plates, and set aside. Conditioned media were pooled but separated by the temperature group to control for potential secreted proteins during 48-h treatment. Pooled conditioned media (separated by temperature group) and fresh neurobasal/B27 media (stored at 4 °C) were pre-warmed to 37 °C. Remaining culture media from six-well plates were then aspirated off and replaced with either 1 mL of conditioned media (specific for each temperature) or 1 mL fresh media; fresh neurobasal/B27 media were applied in some neurons to stimulate global protein synthesis. Neurons were incubated for 1 h, media aspirated off, and replaced with ~1.5 mL 37 °C warmed PBS containing 10 ng/mL puromycin. Neurons were incubated for ~30 min, washed once with ice cold PBS (without puromycin), and harvested for biochemistry. Investigators were not blinded to temperature treatment groups.

Western blot

Cells were quickly washed with ice cold PBS and harvested in RIPA buffer containing EDTA, protease inhibitors, and phosphatase inhibitors (Thermo Scientific-PIERCE). Samples were sonicated ~20 s in 0.6 mL conical tubes. Homogenized material was spun 10 min/16,000 g/4 °C, and supernatant was used for downstream SDS–PAGE (whole cell extracts). Protein concentration was determined using the BCA assay (Thermo Scientific-PIERCE). 15–30 μg of protein was mixed with Laemmli loading buffer (BioRad, Hercules, CA, USA). Samples were heated 95 °C/5 min, and loaded on pre-cast TGX 4–15% gradient gels (BioRad). Gels were run at 150 V and proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Transfers were run at 100V for ~1 h 15 min. During the course of investigations GE Healthcare discontinued our preferred PVDF membrane Hybond-P (Cat#RPN2020F). While testing alternative PVDFs, prior to consuming current stock, we inadvertently discovered that RBM3 is difficult to detect using some brands of membrane (Fig. 1). This suggests PVDF selection may be an important variable to study RBM3. All RBM3/CIRBP Western blots in this report used (now discontinued) Hybond-P membrane; we found it displays optimal RBM3 binding/detection characteristics. However, results suggest new Hybond P 0.2 membrane is also a good alternative to detect RBM3 with lower non-specific background. Precision Plus Kaleidoscope protein standards (BioRad) were used for MW estimation. Membranes were washed using 1X Tris-Buffered-Saline (TBS; BioRad) and blocked in TBS-Tween-20 (TBST) containing 7.5% blotting grade milk (TBS-T/milk) for 1 h. Primary antibodies were prepared in TBS-T/7.5%milk and incubated overnight in a 4 °C cooler. Blots were washed with TBS and incubated with secondary antibodies for 2 h. Blots were given final TBS washes, incubated with ECL-2 HRP-detection reagent (Thermo Fisher-PIERECE), and imaged in a dark room. Films were scanned and compiled in Photoshop. Densitometry analyzed by UN-SCAN-IT software (Silk Scientific, Orem, UT, USA).

Statistical analysis

Data were analyzed and graphed using GraphPad PRISM software (GraphPad Software Inc., La Jolla, CA, USA). Multiple comparisons were analyzed by ANOVA and Newman–Keuls Multiple Comparison post hoc analysis. Data were considered significant at $p < .05$ using two-tailed tests. All graphs show mean + SEM. Intrablot group comparisons of densitometry, collected as relative pixel intensity, were standardized for graphing by expressing group differences as values between 0 and 1 on the y axis. In Fig. 4D, α-tubulin normalized densitometry values were transformed to give log(Y) values and used for statistical analysis to correct for non-normality in distribution.

RESULTS

Experimental hypothermic temperatures were tested in pure neuron cultures as outlined (Fig. 2A). The predicted molecular weight of RBM3 and CIRBP is ~17 kDa and ~18 kDa, respectively. Antibodies detected RBM3 and CIRBP proteins in DIV11 neurons cooled to 33 °C for 48 h. Western blots show a

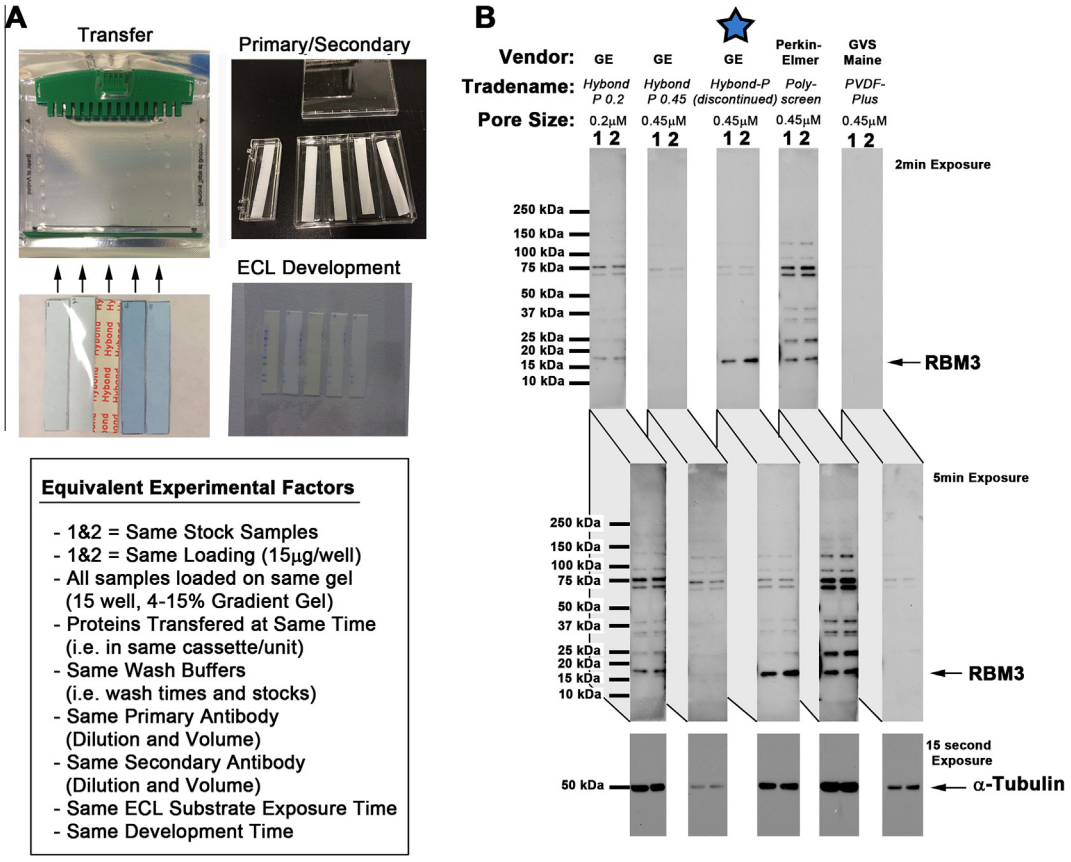

Fig. 1. Comparison of PVDF Membranes Used to Detect RBM3 by Western Blot. (A) Methods used to equalize factors for comparison of PVDF membranes. Neuron homogenates (i.e. samples 1and 2) were loaded onto a 15-well 4–15% SDS gradient gel in five replicates. PVDF membranes were precisely cut to span the width of two lanes and match gel length. Proteins were transferred to all five membranes at the same time (in the same tank/cassette). Membranes were processed using the same volume/time incubation in blocking solution, TBS washes, primary antibody, secondary antibody, and ECL detection reagent. Membranes were put inside a single film holder for equivalent film exposures in a dark room. (B) Western blots show comparison of PVDF membranes to capture and/or detect RBM3.

dominant ~17 kDa RBM3 band, and a fainter ~18 kDa CIRBP band (Fig. 2B, C). We next compared 24-h exposures to either 36 °C or conventional mild hypothermia (33 °C) to induce RBM3/CIRBP in neurons (Fig. 2D). Neuronal RBM3 protein levels were ~twofold higher after 24 h 36 °C, compared to 37 °C normothermic controls (Fig. 2F; $n = 5$/group; ANOVA, $p < 0.0001$). RBM3 levels were highest in the 33 °C group at 24 h ($n = 5$) – although statistically significant this represented only a minor increase above 36 °C RBM3 levels at 24 h (Fig. 2F). By 48-h RBM3 levels in 36 °C and 33 °C groups were ~twofold higher than 37 °C normothermic controls (Fig. 2E, G; $n = 5$/group; ANOVA, $p = 0.0169$). RBM3 levels were not significantly different comparing 36 °C versus conventional 33 °C groups at 48 h (Fig. 2G). CIRBP was barely detectable in primary rat neurons and did not increase with cooling which is consistent with prior studies by others (Fig. 2D, E).

We next tested if temperature treatments alter global protein translation in young DIV6 neurons. Experiments were performed as outlined (Fig. 3A). RBM3 levels increased with 48-h cooling in both 36 °C and 33 °C groups (Fig. 3B–E). In the 36 °C group, RBM3 levels were augmented more if re-warmed for 1 h in fresh neurobasal/B27 media compared to neurons re-warmed in conditioned media (Fig. 3D, E). In young DIV6 neurons, RBM3 levels were highest in the 33 °C group - regardless of 1-h treatment with fresh or conditioned media. Fresh media exchange on cells is assumed to stimulate protein translation by providing renewed nutrients and growth factors but other factors such as altered pH may also be involved. To validate that assumption, de novo GPS was measured by SUnSET in 37 °C treated control neurons given conditioned or 1-h fresh media. As expected, neurons given fresh media had higher levels of puromycin incorporation (i.e. higher GPS). Also, anti-puromycin antibody failed to detect signal in DIV6 neurons that were not treated with puromycin (Fig. 3F). Next we compared if conditioned media versus fresh media affected or unmasked, respectively, the ability of hypothermia treatments to alter protein translation. 36 °C increased protein synthesis in neurons given fresh media but not to the same extent in those given conditioned media (Fig. 3G, H). GPS was reduced in neurons given 33 °C for 48 h. Consistent with that observation, eIF2α (a master regulator of global cap-dependent protein translation)

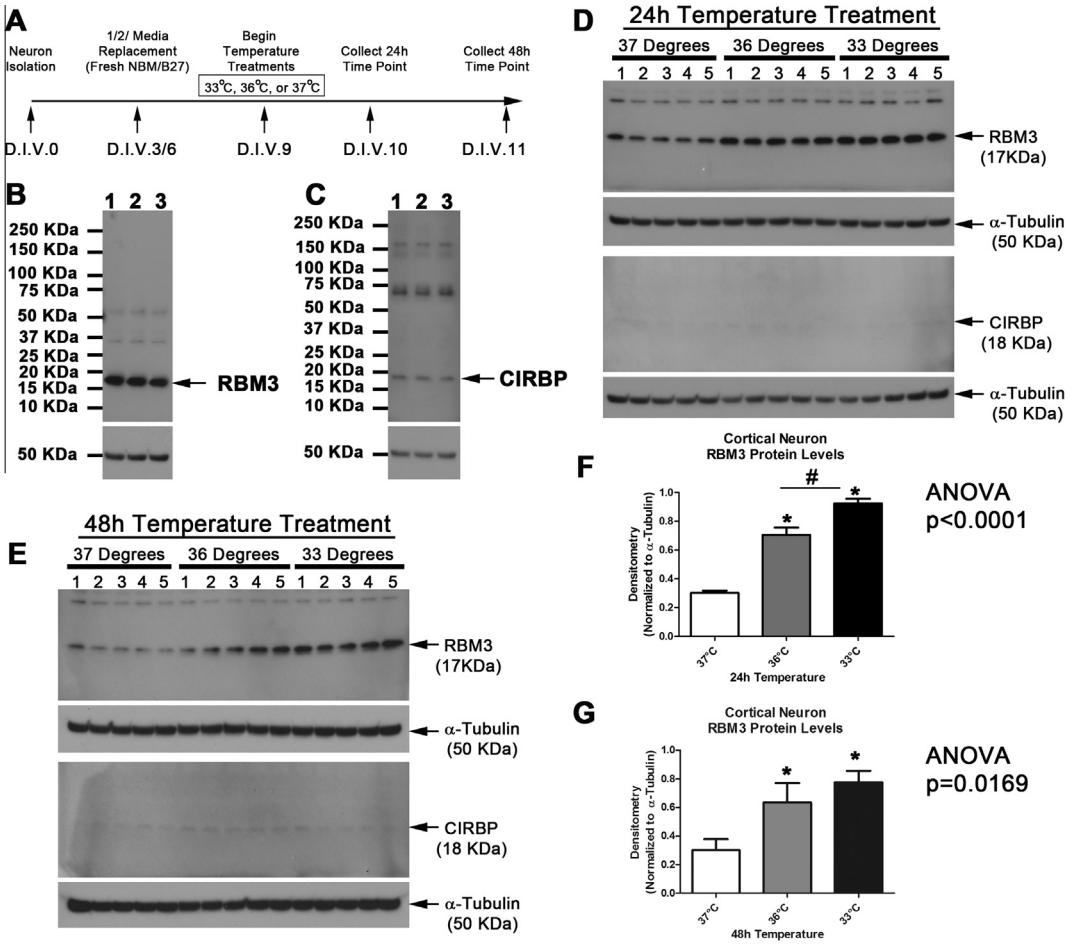

Fig. 2. Mild Hypothermia Increases RBM3 in DIV10–11 Neurons. (A) Timeline of experimental procedures. (B) Western blot showing specificity of antibodies to detect RBM3 and (C) CIRBP in neurons given 33 °C hypothermia for 48 h. (D) Western blot show increased RBM3 but not CIRBP in primary cortical neurons treated 24 h or (E) 48 h with hypothermia. (F) Densitometry of protein changes for RBM3 (n = 5/group) at 24 h and (G) 48 h (n = 5/group). Multiple comparisons were analyzed by a one-way-ANOVA and Newman–Keuls post hoc. Data were significant at $p < .05$. *Indicates post hoc significant difference compared to 37 °C normothermia. #Indicates post hoc significant difference comparing UMH to mild hypothermia. Graphs show mean + SEM.

was inhibited most in 33 °C cooled neurons compared to 36 °C or 37 °C groups (i.e. increased Ser51 phosphorylation; Fig. 3I, J).

We next explored if 36 °C and/or 33 °C induces RBM3 in rat brain astrocytes. Astrocytes were propagated to purity. Approximately 3d after seeding onto 6-well plates, astrocytes were given fresh media exchange and temperature treatments immediately initiated (Fig. 4A). 24-h exposure to hypothermia temperatures did not appear to alter RBM3 levels compared to 37 °C controls (Fig. 4B). RBM3 was significantly increased after 48-h exposure to 36 °C (Fig. 4C, D). However, 33 °C induced much higher levels of RBM3 compared to 36 °C (Fig. 4C, D). Phosphorylation of eIF2α in astrocytes was unaffected by temperature treatments at either time point (Fig. 4B, C).

We next screened several agents for potential RBM3 augmenting effects in young neurons. DMSO (drug dissolving vehicle) did not alter baseline RBM3 levels (data not shown). In young DIV6 neurons, cooling to 36 °C for 24 h was insufficient duration to induce RBM3 (Fig. 5A) (i.e. in contrast RBM3 was mildly increased in DIV6 neurons by 36 °C for 48 h; Fig. 3B). However, addition of FGF21 or melatonin (Mel-T) during the shorter 24-h cooling period augmented RBM3 levels at 36 °C (Fig. 5A). The LXR agonist T090 mildly increased RBM3 levels at both 37 °C and 36 °C. Neither SRT1720 (Sirtuin antagonist) nor AZD1080 (GSK-3β antagonist) increased RBM3 levels at 37 °C and 36 °C. Similarly, 24-h treatment with increasing dose of FGF21, Mel-T, and T090 had minor effects on RBM3 protein expression in DIV7 neurons cultured at 37 °C (Fig. 5B). In contrast, FGF21 increased RBM3 above baseline in a bi-phasic manner when cultured at 36 °C (Fig. 5C). As a positive control we also tested if FGF21 activated AKT/ERK survival signaling at 37 °C as previously reported in neurons (Leng et al., 2015). Paradoxically, AKT/ERK phosphorylation was decreased 24 h later potentially suggesting feedback inhibition (Fig. 5D). Combination therapy of drugs increased

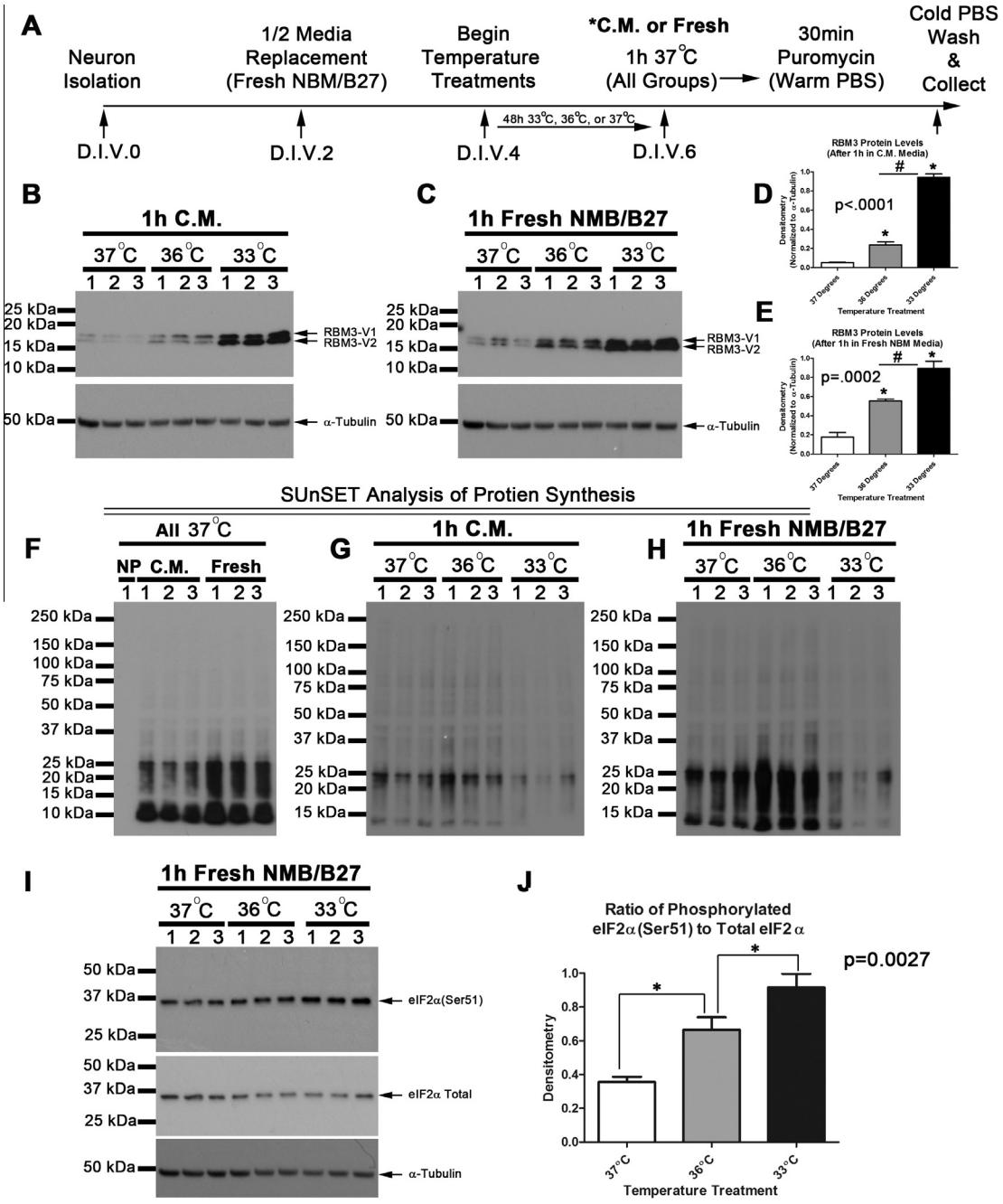

Fig. 3. Mild Hypothermia Increases RBM3 in DIV6 Neurons and Increases Protein Synthesis. (A) Timeline of experimental procedures. (B) 20-ug protein was loaded onto gels. Western blot show increased RBM3 in primary cortical neurons treated 48 h with hypothermia and subjected to 1 h re-warm in conditioned media (C.M.) or (C) fresh neurobasal B27 supplement (NBM/B27). (D) Densitometry of RBM3 protein changes in neurons treated with hypothermia for 48 h (n = 3/group) and then re-warmed in C.M. or (E) Fresh NBM/B27. Multiple comparisons were analyzed by a one-way-ANOVA and Newman–Keuls post hoc. (F) Western blot shows validation of SUnSET and effect of fresh media exchange on protein translation at 37 °C. Negative control (no puromycin; NP) are ARA-C treated DIV6 neuron homogenates. Neuron homogenates incubated with puromycin for 30 min stain positive with anti-puromycin antibody indicating level of de novo protein synthesis. (G) Level of puromycin staining in neurons treated with hypothermia and rewarmed 1 h in C.M. or (H) NBM/B27. (I) Western blot show increased phosphorylation of eIF2α in neurons treated with hypothermia. (J) Densitometry of phosphorylated eIF2α levels, normalized to eIF2α total, in hypothermia treated neurons (n = 3/group). Data were significant at $p < .05$. *Indicates post hoc significant difference compared to 37 °C normothermia. #Indicates post hoc significant difference comparing UMH to mild hypothermia. Graphs show mean + SEM.

RBM3 levels above baseline in DIV7 neurons cooled to 36 °C. CIRBP levels were unaffected by treatments (Fig. 5E). Finally, 48 h to 36 °C alone or with drug combinations failed in induce RBM3 in mature DIV26 neurons. RBM3 was marginally increased in control neurons cooled to 33 °C for 48 h (Fig. 5F).

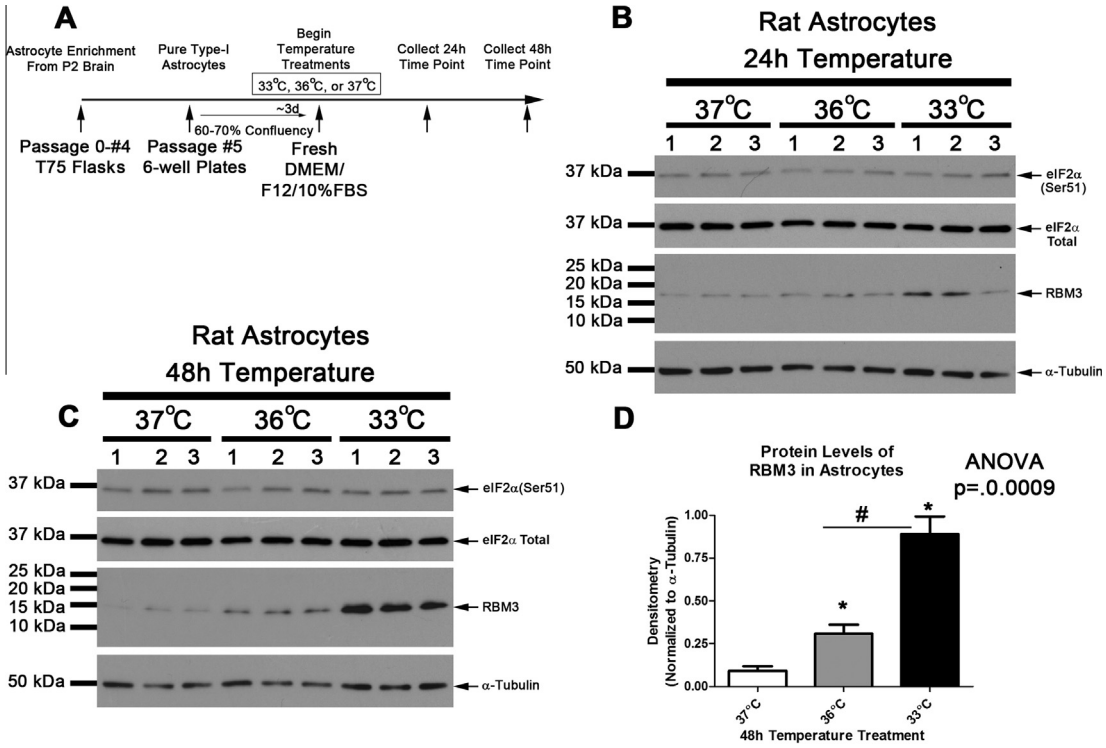

Fig. 4. Mild Hypothermia Increases RBM3 in Rat Astrocytes. (A) Timeline of experimental procedures. Western blots (20 μg/well) showing eIF2α and RBM3 changes in astrocytes given 37 °C, 36 °C, or 33 °C for hypothermia for (B) 24 h and (C) 48 h. (D) Densitometry of protein changes for RBM3 (n = 3/group) at 48 h. Data were transformed to log(Y) for analysis. Multiple comparisons were analyzed by a one-way-ANOVA and Newman–Keuls post hoc. Data were significant at $p < .05$. *Indicates post hoc significant difference compared to 37 °C normothermia. #Indicates post hoc significant difference comparing UMH to mild hypothermia. Graphs show mean + SEM.

DISCUSSION

Young neurons respond to cold stress in an ultra-sensitive manner: implications for temperature management in developmental brain injury

To the best of our knowledge only a single study has tested if extremely mild hypothermia (a temperature reduction of only 2 °C) upregulates cold-stress proteins. In that study, long-term maintenance of embryonic stem cells to 35 °C did not increase mRNA levels of RBM3/CIRBP (Belinsky and Antic, 2013). Here we report in pure primary cortical neurons and astrocytes that either ultra-mild hypothermia (UMH) to 36 °C or traditional clinically used mild hypothermia levels to 33 °C increase protein levels of RBM3. Notably, potentially harmful CIRBP did not increase in neurons.

RBM3 is developmentally regulated. It is abundant in the postnatal brain but low in adults (Pilotte et al., 2009). We anticipated cold stress to induce RBM3 with greater amplitude in young neurons. Consistent with that prediction 36 °C robustly increased RBM3 in young DIV6–DIV11 neurons but not in mature DIV26 cultures. The mechanism(s) regulating inhibition of RBM3 during CNS development are unclear. Neurons in culture spontaneously fire excitatory potentials beginning around DIV7–10 followed by other progressive biochemical changes including alterations in expression of glutamatergic AMPA versus NMDA receptors DIV14–21 (Zona et al., 1994; Lin et al., 2002). Neurons adopt a more stable adult phenotype by DIV25 (Lesuisse and Martin, 2002). We speculate that synaptic activity and other coordinated developmental processes regulate epigenetic changes suppressing protective RBM3 in the adult brain (Martinowich et al., 2003; Fagiolini et al., 2009). Future studies need to test that hypothesis.

Clinically conventional levels of mild TH have greater efficacy in newborns than adults with hypoxic-ischemic brain injury (Shankaran et al., 2005; Nielsen et al., 2013). RBM3 is more easily induced by hypothermia in developing neurons and thus may be an underappreciated component of endogenous neuroprotective responses activated by TH in the young. The American Academy of Pediatrics recently published 2014 recommendations to enhance/extend TH in community hospitals for treatment of neurological injury in neonates. It will be important to understand if RBM3 has a role in brain recovery in that population.

Pharmacological strategies to augment RBM3 upregulation in young and mature neurons

RBM3 signaling is downregulated in the adult brain. To the best of our knowledge only a single study demonstrated methodology to upregulate RBM3 in the adult brain of non-hibernating animals. In that report, deep hypothermia to 16 °C was used in combination with 5-AMP injection to induce a hypometabolic state which mimics conditions in hibernating animals (Peretti et al., 2015). Induction of RBM3 by deep cooling/5-AMP injection is ill-advised in the setting of acute brain

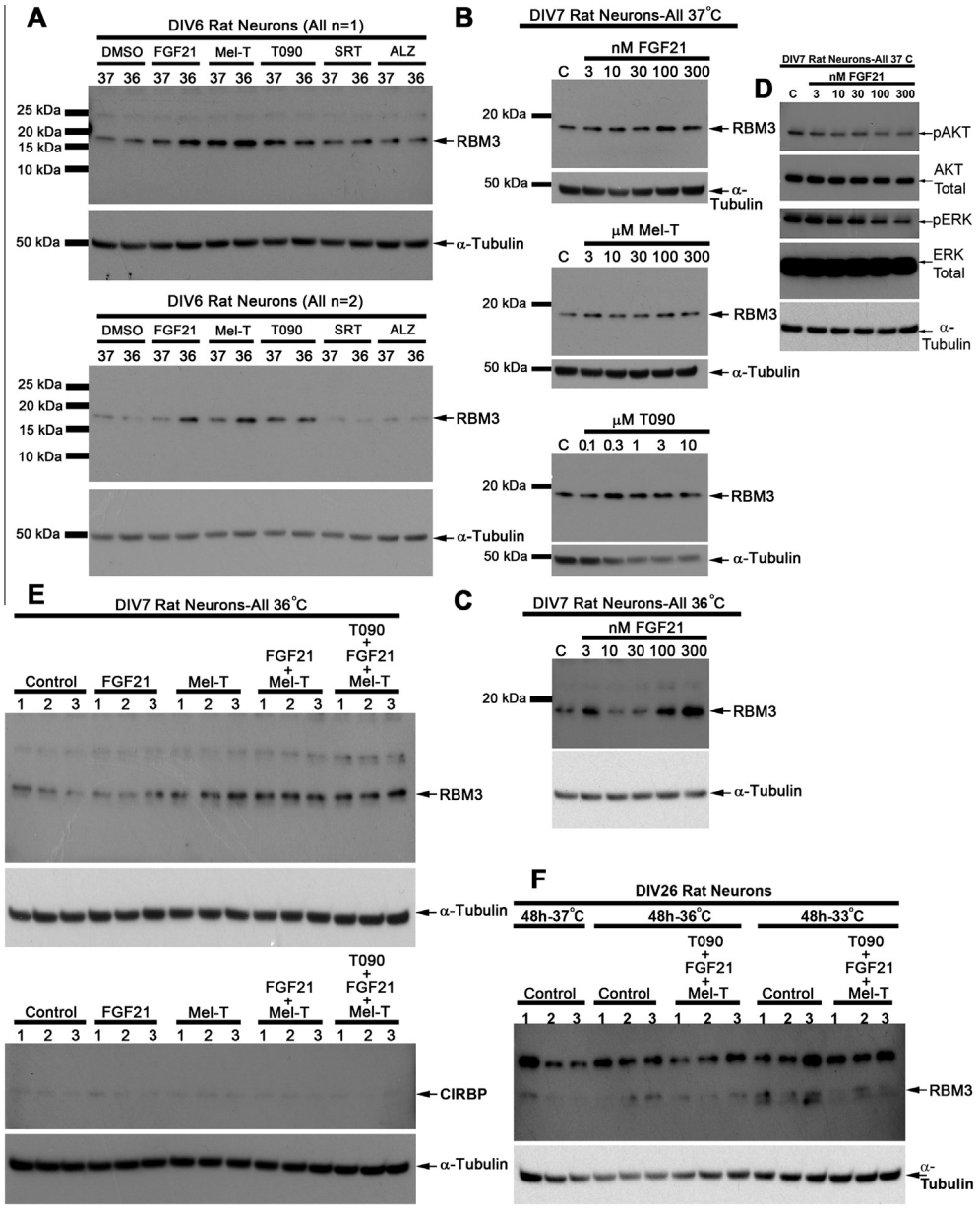

Fig. 5. FGF21 and Melatonin augment RBM3 induction in young neurons. (A) Western blots (n = 2) show effect of 24-h treatment of DIV6 neurons with 5 nM FGF21, 100 μM melatonin, 1 μM T090, 5 μM SRT1720, or 1 μM AZD1080. (B) Western blots show dose effect of 24 h FGF21, melatonin, or T090 at 37 °C on RBM3 levels in DIV7 neurons. (C) Western blot shows dose effect of 24 h FGF21 at 36 °C on RBM3 levels in DIV7 neurons. (D) Western blots show the effect of FGF21 on AKT and ERK activation. (E)Western blots show effect of 24-h treatment combinations (n = 3/group) to induce RBM3 in DIV7 neurons cooled to 36 °C. FGF21 was applied at 50 nM, melatonin 100 μM, and T090 at 1 μM. (F) Western blots show effect of 48 h treatment combinations to induce RBM3 in DIV26 neurons cooled to 36 or 33 °C. FGF21 was applied at 50 nM, melatonin 100 μM, and T090 at 1 μM (n = 3/group).

injury. 5-AMP is well known to chemically induce hypothermia. Unfortunately, its obvious benefits as an easy strategy to lower core body temperature are eclipsed by serious side effects including profound hypotension and hyperglycemia, which together aggravate ischemic brain injury (Zhang et al., 2009). Furthermore, it is well known that deep hypothermia can exacerbate ischemic brain damage whereas mild cooling is protective (Weinrauch et al., 1992). Given those limitations, we attempted to identify drugs which might safely augment RBM3 signaling under mild hypothermic conditions. We tested several agents including the hormones FGF21 and melatonin. Both molecules cause slight body temperature reduction in mammals (Dawson et al., 1996; Inagaki et al., 2007). The LXR agonist T0901317 is a potential caloric restriction (CR) mimetic known to upregulate RBM3 mRNA in mouse liver as measured by microarray (Corton et al., 2004). We also tested Sirtuin agonist SRT1720 – which belongs to a different class of potential CR mimetic drugs (Smith et al., 2009). Finally, we tested the GSK-3β inhibitor AZD1080 (Georgievska et al., 2013). GSK-3β inhibitors activate the developmentally essential Wnt/β-catenin pathway (Castelo-Branco et al., 2004).

FGF21 and melatonin increased hypothermia-induced RBM3 in young neurons cooled to 36 °C but failed to do so in mature neurons or young neurons incubated at 37 °C. T0901317 mildly induced RBM3 in young neurons at both temperatures but failed to do so in mature neurons. SRT1720 and AZD1080 did not affect RBM3 levels in young neurons. Our results suggest that FGF21 and/or melatonin merit pre-clinical evaluation *in vivo* as promoters of RBM3 in the immature injured brain. Of note, melatonin reportedly augments hypothermia (33.5 °C)-induced neuroprotection in a piglet model of perinatal ischemic brain injury (Robertson et al., 2013). It remains to be determined if RBM3 could have contributed to improved outcomes in that study. More work is needed to investigate the mechanism(s) underlying RBM3 induction by those compounds in pre-clinical models of developmental brain injury.

Global protein translation is a target for treatment of brain injury

Though not addressed by this study, we speculate RBM3 is neuroprotective in acute brain injury such as in stroke, cardiac arrest, or traumatic brain injury. A major function of RBM3 is to stimulate GPS (Dresios et al., 2005; Liu et al., 2013) (Smart et al., 2007). Loss of GPS persists in vulnerable CA1 neurons after brain ischemia, for example, and coincides with delayed cell death (Vosler et al., 2012). Interestingly, TH has been shown to reverse aberrant GPS in injured CA1 although to the best of our knowledge the underlying mechanism(s) have not been elucidated (Yamashita et al., 1991; Widmann et al., 1993). As RBM3 is a cold shock-induced protein logic suggests that it is in some way involved in TH-mediated alterations in GPS. Furthermore, increasing RBM3 in the diseased hippocampus is thought to augment GPS which improves synaptic plasticity/sparing *in vivo* (Peretti et al., 2015). Here we show for the first time that cooling neurons to 36 °C is sufficient to increase RBM3 and is associated with enhanced GPS.

Unexpectedly, GPS was not increased by exposure to 33 °C despite higher RBM3 levels. That observation can be explained by overall greater inhibition of GPS in the 33 °C group. Hypothermia is well known to temporally decrease rates of GPS but at the same time upregulate cold stress proteins prior to rewarming. In our experiments, before harvesting neurons, all groups received a 1-h re-warming period to 37 °C in media following 48 h of experimental temperature exposure. The duration of time necessary for GPS to return to normal after cooling in primary neurons has not been reported to the best of our knowledge. It might be that 1-h rewarming was insufficient to allow return of normal translational mechanisms in the 33 °C group, delaying RBM3 from maximally augmenting GPS. In contrast 36 °C, being closer to normothermia, might not alter baseline GPS to the same extent thus facilitate the ability of RBM3 to augment protein translation at that temperature. This interpretation is supported by results on eIF2α. eIF2α is a master regulation of cap-dependent protein synthesis. Phosphorylation at Ser51 is a potent mechanism cells use to shutdown global protein translation (Clemens, 2001). We found eIF2α phosphorylation was greatest in the 33 °C group.

Phosphorylation of eIF2α did not increase in astrocytes cooled to 36 °C or 33 °C for 48 h. Thus, *in vitro*, astrocytes appear more resistant than neurons to translational shutdown by eIF2α inhibition after mild hypothermia. The cause of that resistance is unclear but may relate to differences in biochemical mechanisms regulating energy and metabolism; for instance access to glycogen stores in astrocytes may alter protein translation homeostasis. Alternatively, differences might relate to relative cell culture conditions. Astrocytes were maintained, and temperature treatments initiated, under high serum conditions (i.e. 10% FBS). In contrast, neurons were maintained, and temperature treatments initiated, with serum free neurobasal/B27 supplement. High growth factor support in astrocytes might make them resistant to mild translational inhibition by hypothermia. Nevertheless, RBM3 was potently induced by 33 °C and mildly increased by 36 °C in astrocytes after 48 h. Thus our work confirms 36 °C can increase RBM3 in multiple CNS cell types. Future studies also need to test if RBM3 is differentially induced by cooling in type-1 versus type-2 astrocytes – the former being the majority of cells in monolayer cultures in our studies.

Besides its effect on GPS, RBM3 has other beneficial actions which have been noted. RBM3 knockdown blocks mild TH-induced neuroprotection *in vitro* (Chip et al., 2011). In contrast, RBM3 overexpression prevents death in serum starved cells (Wellmann et al., 2010).

Small temperature shifts may alter disease outcomes

Tiny temperature shifts are increasingly being recognized to influence outcomes in critically ill patients. Recent trials suggest that targeted temperature management (TTM) to 36 °C is equally effective on neurologic outcome and mortality in brain-injured patients compared to conventional TH (Nielsen et al., 2013; Moler et al., 2015). Hyperthermia (such as by fever) exacerbates neuronal death after brain ischemia (Noor et al., 2003). Clinically, it has been shown that increases in 1 °C body temperature in the initial 72 h post hypoxic ischemic period is associated with an over threefold increase in the odds of poor outcome in newborns (Laptook et al., 2008). Prevention of fever is likely a key mechanism of benefit with TTM. Nevertheless it is tempting to speculate that temperatures representing UMH might also increase protective cold shock proteins.

Ultra-mild hypothermia: semantics or biologically relevant?

Ironically, 36 °C has been used as the temperature in the control group in many studies testing traditional levels of TH but our data suggest it may not represent a normothermic control –at least in young (Onesti et al., 1991; Clark et al., 1996). Contrary to concern, classic studies by Busto et al. in adult rodents show that mild hypothermia to 33 °C or 34 °C dramatically improves histological outcomes after global forebrain ischemia compared to 36 °C controls (Busto et al., 1987). Our findings

do not prove RBM3 activation or 36 °C is protective. Rather, results suggest that it may be important to recognize that extremely mild levels of hypothermia may have biological effects in some circumstances. Temperatures slightly below normothermia may activate molecular cold shock mechanisms in the very young – until now thought to be only induced by traditional levels of TH. We believe it is appropriate to consider these extremely mild temperature reductions to represent UMH (i.e. 36–35.6 °C). UMH may have important distinctions from ''mild hypothermia" which technically includes 36 °C but more colloquially implies temperatures proven to induce neuroprotection (35.5–33 °C). Notwithstanding the latter definition, UMH might still be neuroprotective over and above benefits gained by preventing fever after brain injury - depending on the myriad circumstances of the model (such as in neonates) or clinical condition. In summary, here we show for the first time that UMH at 36 °C induces a bonafide cold-stress response in young neurons and astrocytes *in vitro*.

AUTHOR CONTRIBUTIONS

TCJ designed experiments. TCJ and SEK performed experiments. TCJ, MDM, EKJ, RSBC, and PMK contributed to data analysis and interpretation. TCJ, MDM, EKJ, RSBC, and PMK contributed to writing the manuscript.

CONFLICT OF INTEREST/DISCLOSURE

Travis C. Jackson and Patrick M. Kochanek are co-innovators on a submitted provisional patent application titled ''Method to Improve Neurologic Outcomes in Temperature Managed Patients." (Application #62/164,205).

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