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Research Paper

Hypothermic Preconditioning Reverses Tau Ontogenesis in Human Cortical Neurons and is Mimicked by Protein Phosphatase 2A Inhibition

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ABSTRACT

Hypothermia is potently neuroprotective, but the molecular basis of this effect remains obscure. Changes in neuronal tau protein are of interest, since tau becomes hyperphosphorylated in injury-resistant, hypothermic brains. Noting inter-species differences in tau isoforms, we have used functional cortical neurons differentiated from human pluripotent stem cells (hCNs) to interrogate tau modulation during hypothermic preconditioning at clinically-relevant temperatures. Key tau developmental transitions (phosphorylation status and splicing shift) are recapitulated during hCN differentiation and subsequently reversed by mild (32 °C) to moderate (28 °C) cooling — conditions which reduce oxidative and excitotoxic stress-mediated injury in hCNs. Blocking a major tau kinase decreases hCN tau phosphorylation and abrogates hypothermic neuroprotection, whilst inhibition of protein phosphatase 2A mimics cooling-induced tau hyperphosphorylation and protects normothermic hCNs from oxidative stress. These findings indicate a possible role for phospho-tau in hypothermic preconditioning, and suggest that cooling drives human tau towards an earlier ontogenic phenotype whilst increasing neuronal resilience to common neurotoxic insults. This work provides a critical step forward in understanding how we might exploit the neuroprotective benefits of cooling without cooling patients.

1. Introduction

The lack of neuroprotective treatments for acute and chronic brain disorders presents a major challenge to modern medicine. Therapeutic hypothermia is a rare example of a proven neuroprotective intervention — however, it is only practically useful in restricted patient groups (Yenari and Han, 2012; Jacobs et al., 2013; Andrews et al., 2015). Improved mechanistic understanding of neuroprotective hypothermia could reveal novel molecular targets with which to exploit the protective effect of cooling in a wider clinical context. Against this background, a range of experimental systems and studies of hibernating animals have implicated several pathways that might mediate hypothermic neuroprotection (Arendt et al., 2003; Chip et al., 2011; Yenari and Han, 2012; Rzechorzek et al., 2015). A potentially important candidate is microtubule-associated protein tau. Specifically, reversible hyperphosphorylation of tau in hibernating, hypothermic and developing brains – brains which are comparatively resistant to injury – suggests cold-inducible changes in tau might contribute to hypothermic neuroprotection (Mawal-Dewan et al., 1994; Arendt et al., 2003; Planel et al., 2007; Stieler et al., 2011).

Interspecies differences in cellular and molecular biology have impeded translation of neuroprotective strategies from pre-clinical models to man. With regard to tau, a potentially significant species difference lies in the developmentally-regulated expression of its multiple isoforms (Janke et al., 1999). The relative abundance of tau isoforms determines tau function in health and disease (Trojanowski and Lee, 1995), therefore this balance might be altered under conditions that influence neuronal survival, such as hypothermia. Whilst there is little evidence of this effect in in vivo models of hypothermic neuroprotection (Stieler et al., 2011), several core human tau isoforms are absent in the adult rodent brain (Janke et al., 1999). These observations provide a strong

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rationale to explore tau in the context of hypothermic neuroprotection in a physiologically relevant human system.

We recently established an experimental platform of functional hCNs (Bilican et al., 2014; Livesey et al., 2014) and demonstrated its value for exploring molecular mechanisms of hypothermic preconditioning (Rzechorzek et al., 2015). Here we show that hCN differentiation recapitulates the principal features of early human cortical tau development which are subsequently reversed by hypothermia, returning tau transcriptionally and post-translationally to an earlier ontogenic state. Further we confirm that cooling protects these neurons in an injury- and temperature-specific manner (Rzechorzek et al., 2015). Finally we provide evidence that cooling-induced hCN tau hyperphosphorylation results from a disproportionate suppression of protein phosphatase 2A (PP2A) relative to glycogen synthase kinase 3β (GSK3β) (Planel et al., 2007), which is both necessary and sufficient to protect hCNs from oxidative stress. These findings establish a role for PP2A inhibition in hypothermic preconditioning of human neurons and suggest that phospho-tau may participate in this neuroprotective effect.

2. Materials and Methods

2.1. Differentiation of hCNs

hCNs were differentiated from expanded anterior neural precursors (aNPCs) as described elsewhere (Bilican et al., 2014; Livesey et al., 2014; see also Supplemental Materials and Methods). Briefly, upon removal of FGF2, aNPCs (passages 17 to 39) were plated in 12 or 24-well plates (Nunc) at 1×10^5 cells cm^{-2} onto glass coverslips coated with Poly-L-Ornithine (1 in 1000, Sigma), Laminin (1 in 100, Sigma), Fibronectin (10 μg/ml, Sigma) and Reduced growth-factor Matrigel® (1 in 200, BD Biosciences). Differentiating aNPCs were cultured in default media at 3% O_2, 5% CO_2, 37 °C. For KCl stimulation experiments aNPCs were differentiated in Matrigel® (1 in 100)-coated 6-well plates at the same density. Cultures were fed twice weekly until 21 d, after which they were fed every other day. Periodic testing with a PCR-based detection kit (Minerva Biolabs) confirmed that both precursor and differentiated cultures were *Mycoplasma*-free. For developmental characterization, samples were harvested at aNPC stage and 14, 28, 42 and 49 d after plating for differentiation. Electrophysiological characterization of hCNs is described elsewhere (Bilican et al., 2014; James et al., 2014; Livesey et al., 2014).

2.2. Cooling Paradigm

Triplicate plates for each hCN batch were maintained in normal differentiation media. Hypothermia was induced at 5 wk when >90% of cultured cells are differentiated neurons and >95% of these fire action potentials (Bilican et al., 2014). Identical plates were separated and incubated at 28, 32 or 37°C to simulate 'moderate hypothermia', 'mild hypothermia' or 'normothermia' respectively (Rzechorzek et al., 2015). Hypothermic temperatures were selected to simulate suspended animation at the lower limit of cardiac stability and the minimum clinical target temperature for therapeutic hypothermia (Zell and Kurtz, 1985; Yenari and Han, 2012; Andrews et al., 2015). Thermic period was calibrated with a sentinel culture plate. Time zero was set when media in the sentinel plate reached the desired incubation temperature, measured by digital thermometer. Samples for early and late analysis of transcripts were captured for RNA extraction at 3 and 24 h respectively. At 24 h cells were fixed for immunocytochemistry and additional samples lifted for phospho-protein (see Supplemental Materials and Methods). For media additions during the hypothermic period, solutions were pre-warmed to the respective temperatures.

2.3. Electrophysiology

Whole-cell patch-clamp recordings were made from hCNs using an Axon Multiclamp 700B amplifier (Molecular Devices, Union City, CA). Patch electrodes (~4–7 MΩ) were filled with an internal recording solution comprising (in mM): K-gluconate 155, $MgCl_2$ 2, HEPES 10, Na-PiCreatine 10, Mg_2-ATP 2 and Na_3-guanosine triphosphate 0.3, pH 7.3 (300 mOsm). Coverslips containing hCNs were super-fused with an extracellular solution composed of (in mM) NaCl 152, KCl 2.8, HEPES 10, $CaCl_2$ 2, glucose 10, pH 7.3 (320–330 mOsm) using a gravity-feed system at room temperature (20–23 °C). The recording solution was supplemented with glycine (50 μM), picrotoxin (50 μM), strychnine (20 μM), and tetrodotoxin (300 nM). Recordings were made at a holding potential of −74 mV (including liquid junction potential correction). Series resistances (R_s) were generally less than 25 MΩ.

2.4. PP2A Enzyme Activity

PP2A activity was assayed using an Immunoprecipitation Phosphatase Assay Kit (Millipore) according to the manufacturer's instructions, with a few minor adaptations. Briefly, cell pellets from 5 wk old hCNs were thawed on ice, solubilised in cold phosphate extraction buffer (20 mM Imidazole-HCl (Santa Cruz), 2 mM EDTA, 2 mM EGTA, protease inhibitors and 100 μM PMSF) and sonicated for 10 s. After centrifugation (2000 ×*g* for 5 min at 4 °C), supernatants were collected and their protein concentration measured by BCA assay (Pierce). 100 μg of each lysate was incubated (constant rocking for 1 h at 4 °C) with an antibody specific to the active subunit of PP2A (Anti-PP2A, C subunit, clone 1D6) and Protein A agarose slurry in pNPP Ser/Thr Assay Buffer. Agarose beads were washed several times with TBS and Ser/Thr Assay Buffer before the addition of a Threonine Phosphopeptide (K-R-pT-I-R-R, final concentration 750 μM). Identical samples from each cortical batch were incubated for 10 min on a shaking incubator under one of 4 conditions (28, 32 or 37 °C or at 37 °C in the presence of 100 nM of fostriecin (CalBiochem)). After brief centrifugation, triplicate aliquots of each sample were transferred to a 96-well microtitre plate. Malachite Green Phosphate Detection Solution was added to each well and the plate incubated at room temperature for 15 min. Absorbance was measured on a spectrophotometer at 620 nm. Sample readings were compared to a 200–2000 pM Phosphate Standard Curve after subtraction of the blank (negative control) value. The specific PP2A activity (picomoles of phosphate released min^{-1} μg^{-1} protein) was calculated for each sample and its internal negative control (with fostriecin) so that this background 'activity' relating to residual phosphate levels could then be subtracted. Hypothermic sample values were then compared to their respective normothermic controls.

2.5. Statistical Analysis

Pairwise correlations were performed by two-tailed Pearson correlation. All remaining analyses were performed using linear mixed models in Stata SE (Version 9.2, Stata Corp, TX, USA) with random effects for intercept by batch, and where necessary, with random effects for coefficient by concentration or time (Aarts et al., 2014). *N* denotes the number of individual cell lines used and *n* describes the number of independently differentiated batches of hCNs. Unless otherwise stated, data are presented as standardized point estimates (SPE) + standardized estimated standard error (SESE) after normalizing to control values. Control values refer to aNPC, normothermia (37 °C) or untreated cells for differentiation, hypothermia, KCl/FPL stimulation and pharmacological studies respectively. In every case, asterisks denote significance of the test statistic as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0005$.