
THE UNIVERSITY
*of* EDINBURGH

# Hypothermic preconditioning in human cortical neurons: coupling neuroprotection to ontogenic reversal of tau.

Nina Marie Rzechorzek

Funded by a Wellcome Trust Integrated Training Fellowship for Veterinarians
(Ref 096409/Z/11/Z)


THE UNIVERSITY *of* EDINBURGH

A thesis submitted for the degree of Doctor of Philosophy at The University of Edinburgh

May 2015

College of Medicine and Veterinary Medicine
University of Edinburgh
**Supervisor: Professor Siddharthan Chandran**
Second supervisor: Professor Jean Manson

# Preface

I declare that the following work, either in part or as a whole, has not been submitted to any other institution for academic merit. The work contained within this thesis is my own, except where the contributions of others have been specified in the acknowledgements as appropriate. The majority of data was generated at the Centres for Clinical Brain Sciences and Regenerative Medicine, University of Edinburgh, Edinburgh, UK. Mass spectrometry data was generated at FingerPrints Proteomics, University of Dundee, Dundee, UK.

**Material within this thesis has been published or submitted for peer review as follows:**

Livesey MR, Bilican B, Qiu J, Rzechorzek NM, Haghi G, Burr K, Hardingham GE, Chandran S, and Wyllie DJ (2014) Maturation of AMPAR composition and the $GABA_AR$ reversal potential in hPSC-derived cortical neurons. *J Neurosci* 34:4070-4075.

Rzechorzek NM, Connick P, Patani R, Selvaraj BT, Chandran S (2015) Hypothermic preconditioning of human cortical neurons requires proteostatic priming. *EBioMedicine In press* DOI: http://dx.doi.org/10.1016/j.ebiom.2015.04.004

Rzechorzek NM, Connick P, Livesey MR, Patani R, Borooah S, Burr K, Story D, Wyllie DJA, Hardingham GE and Chandran S. Neuroprotective hypothermia is mediated by protein phosphatase 2A inhibition and reverses tau ontogenesis in human cortical neurons. *In review*

## Abstract

Hypothermia is potently neuroprotective, but the molecular basis of this effect remains obscure and the practical challenges of cooling have restricted its clinical use. This thesis was borne on the premise that considerable therapeutic potential may lie in a deeper understanding of the neuronal physiology of cooling. Rodent studies indicate that hypothermia can elicit preconditioning wherein a subtoxic stress confers resistance to an otherwise lethal injury. This cooling-induced tolerance requires *de novo* protein synthesis – a fundamental arm of the cold-shock response, for which data in human neurons is lacking. Since cooling protects the human neonatal brain, experiments herein address the molecular effects of clinically-relevant cooling using functional, maturationally-comparable cortical neurons differentiated from human pluripotent stem cells (hCNs). Several core hypothermic phenomena are explored, with particular scrutiny of neuronal tau, since this protein is modified extensively in brains that are resistant to injury. Mild-to-moderate hypothermia produces an archetypal cold-shock response in hCNs and protects them from oxidative and excitotoxic stress. Principal features of human cortical tau development are recapitulated during hCN differentiation, and subsequently reversed by cooling, returning tau transcriptionally and post-translationally to an earlier foetal-like state. These findings provide the first evidence of cold-stress-mediated ontogenic reversal in human neurons. Furthermore, neuroprotective hypothermia induces mild endoplasmic reticulum (ER) stress in hCNs, with subsequent activation of the unfolded protein response (UPR). Reciprocal modulation of both tau phosphorylation and the ER-UPR cascade suggests that cold-induced hyperphosphorylation of tau and ER-hormesis (preconditioning) represent significant

components of hypothermic neuroprotection. Cooling thus modifies proteostatic pathways in a manner that supports neuronal viability. Historically, hypothermic preconditioning has been limited to the acute injury setting, and tau hyperphosphorylation is an established hallmark of chronic neural demise. More recently however, preconditioning has been proposed as a target for neurodegenerative disease and neuroprotective roles of phospho-tau have emerged. To date, hypothermia has protected hCNs against oxidative, excitotoxic and ER stress, all of which have been implicated in traumatic as well as degenerative processes. This 'cross-tolerance' effect places exponential value on the molecular neurobiology of cooling, with the potential to extract multiple therapeutic targets for an unmet need.

## A note of thanks

Opportunity comes in many guises, thus it can be difficult to seize and so very easy to waste. There have been many opportunities and many people that have made this work possible. My first taste of Edinburgh - and research – under the watchful guidance of Bruce, Scott, Gerry and David left me wanting to understand everything: the patient, their problem; its process and its purpose. This led to numerous training opportunities, which (thanks to Tom, Kenny, Roger, Becky, Cambridge and others) fed my addiction to research and supplemented it with travel. 12 years later it was an honour to come back to where I started and (accepting that 'everything' was a little broad) to focus on the most intriguing puzzle of them all – the brain. This particular journey would not have existed without Wellcome Trust support and the foresight of David Argyle to secure opportunities for vets to embrace clinical academia – and long may this continue. Thanks must also go to the teachers and brilliant scientists that have inspired me, and to the friends and family who have backed all of my ideas (however bizarre), even when these drew me further afield. Last but not least to Siddharthan, for giving me the freedom to explore unfamiliar territory and to tease out opportunities from seemingly impossible places. The more I learn about the brain, the more convinced I am that 'keeping a cool head' is very sensible advice. There were times during the last three years when I thought mine would go into meltdown – usually when opportunity dangled just beyond reach. Oddly, doctoral research is a kind of preconditioning; a Nietzschean portal into a career with no guarantees and an ever-expanding sea of questions. But there lies the allure. I am privileged to have the opportunity to keep asking questions and hopefully, one day, build these into opportunities for others.

*For my brother, Neil*

*It matters not how strait the gate, How charged with punishments the scroll,*
*I am the master of my fate: I am the captain of my soul.*
Excerpt from 'Invictus' by William Ernest Henley 1849-1903.

# Table of Contents

## Figures List

## Abbreviations

| | |
|---|---|
| 3R Tau | 3 repeat tau |
| 4R Tau | 4 repeat tau |
| AD | Alzheimer's disease |
| AGD | argyrophilic grain disease |
| ALS | amyotrophic lateral sclerosis |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| AMP | adenosine monophosphate |
| AMPK | AMP-activated protein kinase |
| aNPC | anterior neural precursor |
| AP | alkaline phosphatase |
| AP-1 | activator protein 1 |
| APP | amyloid precursor protein |
| ASF | alternative splicing factor |
| ATF4 | activating transcription factor 4 |
| ATF6 | activating transcription factor 6 |
| cATF6 | cleaved ATF6 |
| fATF6 | full-length ATF6 |
| ATP | adenosine triphosphate |
| BACT | β-actin |
| BBB | blood-brain barrier |
| B2M | β2 microglobulin |
| BCA | bicinchoninic acid |
| BDNF | brain-derived neurotrophic factor |

| | |
|---|---|
| BiP | binding immunoglobulin protein (GRP78, Hsp70 protein 5 or HSPA5) |
| BMR | basal metabolic rate |
| Brn2 | POU class 3 homeobox 2 |
| BSA | bovine serum albumin |
| CBD | corticobasal degeneration |
| CDM | chemically-defined medium |
| cDNA | complementary deoxyribonucleic acid |
| CHO | Chinese hamster ovary |
| CHOP | C/EBP homologous protein |
| CIRBP | cold-inducible RNA binding protein |
| CMT | Charcot-Marie Tooth disease |
| CNS | central nervous system |
| CTE | chronic traumatic encephalopathy |
| Ctip2 | B-cell CLL/lymphoma 11B (zinc finger protein) |
| Cux1 | cut-like homeobox 1 |
| DAPI | 4’,6-diamidino-2-phenylindole |
| D-APV | D(−)-2-Amino-5-phosphonopentanoic acid |
| DDIT3 | DNA damage-inducible transcript 3 (also known as CHOP) |
| DHA | docosahexanoic acid |
| DIV | days *in vitro* |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| DPBS | Dulbecco’s phosphate-buffered saline |
| DS | Down’s syndrome |

| | |
|---|---|
| Dyrk1A | dual-specificity tyrosine phosphorylation-regulated kinase 1A |
| EAA | excitatory amino acid |
| EAAT1 | excitatory amino acid transporter 1 |
| EDTA | ethylenediaminetetraacetic acid |
| EGF | epidermal growth factor |
| EGTA | ethylene glycol tetraacetic acid |
| eIF2α | eukaryotic initiation factor 2α |
| EIF4A2 | eukaryotic translation initiation factor 4A2 |
| Emx2 | empty spiracles homeobox 2 |
| ER | endoplasmic reticulum |
| ERAD | ER-associated degradation |
| ERN1 | endoplasmic reticulum to nucleus signalling 1 |
| ESE | estimated standard error |
| ESI-MS/MS | electrospray ionization mass spectrometry |
| Etv1 | ETS translocation variant 1 |
| FCS | foetal calf serum |
| FGF | fibroblast growth factor |
| FOS | c-Fos |
| Foxg1 | forkhead box protein G1 |
| FTD | frontotemporal dementia |
| FTDP-17 | frontotemporal dementia and parkinsonism linked to chromosome 17 |
| GABA | gamma-aminobutyric acid |
| GADD34 | growth arrest and DNA damage 34 |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |

| | |
|---|---|
| GFAP | glial fibrillary acidic protein |
| GRIN2A | Glutamate (NMDA) receptor subunit epsilon-1 (GluN2A) |
| GRIN2B | Glutamate (NMDA) receptor subunit epsilon-2 (GluN2B) |
| GRP78 | 78 kDa glucose-regulated protein (BiP/HSPA5) |
| GRP94 | 94 kDa glucose-regulated protein |
| GSS | Gerstmann-Sträussler-Scheinker syndrome |
| $H_2O_2$ | hydrogen peroxide |
| hCN | hPS-derived functional excitatory cortical neuron |
| hCNTF | recombinant human ciliary neurotrophic factor |
| HEPES | Tris-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| hES | human embryonic stem cell |
| HES1 | hES line 1 |
| HES2 | hES line 2 |
| HI | hypoxia-ischaemia |
| HPRT1 | hypoxanthine phosphoribosyltransferase 1 |
| hPS | human pluripotent stem cell |
| Hsp70 | heat shock 70 kDa protein |
| HSPA5 | heat shock 70 kDa protein 5 |
| IGF-1 | insulin-like growth factor 1 |
| IMDM | Iscove's Modified Dulbecco's Medium |
| iPS | human induced pluripotent stem cell |
| IPS1 | human iPS line 1 |
| IPS2 | human iPS line 2 |
| IRE1α | inositol requiring enzyme 1α |

| | |
|---|---|
| Irx3 | iroquois homeobox 3 |
| JUN | c-Jun (proto-oncogene) |
| KIRA | kinase-inhibiting RNase attenuators |
| KLF4 | Kruppel-like factor 4 |
| LDH | lactate dehydrogenase |
| MAP2 | microtubule-associated protein 2 |
| MAPT | microtubule-associated protein tau |
| MBP | myelin basic protein |
| MEM | Minimum Essential Medium |
| MiM | Minimal medium |
| MRS | magnetic resonance spectroscopy |
| MYC | c-Myc |
| NA | numerical aperture |
| NEAA | Non essential amino acids |
| NFT | neurofibrillary tangle |
| NPiDC | Niemann-Pick disease type C |
| NMDAR | N-methyl-D-aspartate receptor |
| NMJ | neuromuscular junction |
| NMR | nuclear magnetic resonance |
| Nrf2 | nuclear factor (erythroid-derived 2)-like-2 |
| OCT4 | octamer-binding transcription factor 4 |
| Oct 4 | the protein product of OCT4 |
| Otx 2 | orthodenticle homeobox 2 |
| PAGE | polyacrylamide gel electrophoresis |

| | |
|---|---|
| PBA | 4-phenylbutyric acid |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PD | Parkinson's disease |
| PDC | Parkinsonism-dementia complex |
| PDGF | platelet-derived growth factor |
| PDGFRα | platelet-derived growth factor receptor alpha |
| PE | point estimate |
| PERK | protein kinase R (PKR)-like ER kinase |
| PFA | paraformaldehyde |
| PHF | paired helical filament |
| PI | PERK inhibitor (GSK 2606414) |
| PI3K | Phosphatidylinositol-4,5-bisphosphate 3-kinase |
| PMI | post-mortem interval |
| PMSF | phenylmethanesulfonylfluoride |
| PP2A | protein phosphatase 2A |
| PP2A Bγ | regulatory subunit of PP2A (B gamma) |
| PP2A C | active (catalytic) subunit of PP2A |
| PrP | prion protein |
| PSD-95 | postsynaptic density protein 95 |
| PSP | progressive supranuclear palsy |
| PVDF | polyvinylidene fluoride |
| q-RT-PCR | quantitative reverse transcriptase PCR |
| QA | quinolinic acid |

| | |
|---|---|
| RBM3 | RNA binding motif 3 |
| RBM4 | RNA binding motif 4 |
| RBP | RNA binding protein |
| RD | reverse development |
| REC | Research Ethics Committee |
| REM | rapid eye movement |
| RFU | relative fluorescent units |
| rhGDNF | recombinant human glial-derived neurotrophic factor |
| rhPDGF | recombinant human platelet-derived growth factor |
| RIPA | radioimmunoprecipitation assay |
| RLU | relative luminescent units |
| RMP | resting membrane potential |
| RNA | ribonucleic acid |
| RNP | ribonucleoprotein |
| RNS | reactive nitrogen species |
| ROS | reactive oxygen species |
| RPL29 | ribosomal protein L29 |
| Satb2 | SATB homeobox 2 |
| SCI | spinal cord injury |
| SDS | sodium dodecyl sulphate |
| SEM | standard error of the mean |
| SESE | standardized estimated standard error |
| SGG | salt-glucose-glycine solution |
| SMaRT | spliceosome-mediated RNA *trans*-splicing |

| | |
|---|---|
| SNP | single nucleotide polymorphism |
| SOD | superoxide dismutase |
| Sox1 | sex determining region Y-box 1 |
| SOX2 | sex determining region Y-box 2 |
| Sox2 | the protein product of SOX2 |
| SPE | standardized point estimate |
| Src | proto-oncogene tyrosine kinase |
| SWS | slow wave sleep |
| T3 | triiodothyronine |
| TBI | traumatic brain injury |
| Tbr1 | T-box brain protein 1 |
| Tbr2 | T-box brain protein 2 |
| TBS | Tris-buffered saline |
| TBS-T | TBS-Tween |
| TD | tangle-only dementia |
| TEM | transmission electron microscopy |
| TH | therapeutic hypothermia |
| TIA-1 | T-cell intracytoplasmic antigen 1 |
| Tm | tunicamycin |
| TORC1 | target of rapamycin complex 1 |
| TUDCA | Tauroursodeoxycholic acid |
| UPS | ubiquitin-proteasome system |
| UV | ultraviolet |
| vGlut1 | vesicular glutamate transporter 1 |

| | |
|---|---|
| vGlut2 | vesicular glutamate transporter 2 |
| XBP1 | X-box binding protein 1 |
| XBP1s | spliced XBP1 |

## Acknowledgements

The following are gratefully acknowledged for the provision of materials and technical services: MRC Edinburgh Brain & Tissue Bank and Nusrat Khan, Peault lab, MRC Centre for Regenerative Medicine (human post-mortem brain tissue); David Hay lab, MRC centre for Regenerative Medicine (Glomax luminometer); Steve Mitchell, Electron Microscopy (EM) Facility (Transmission EM sample processing); Andrea Serio, formerly Chandran lab (human pluripotent stem cell (hPS)-derived astroglial progenitors); Steve West, Wellcome Trust Centre for Cell Biology (MYC primers) all University of Edinburgh and, externally, Dougie Lamont, FingerPrints Proteomics, University of Dundee (mass spectrometry), Peter Davies, Albert Einstein College of Medicine of Yeshiva University (PHF-1 antibody). The following, at the University of Edinburgh, are acknowledged for technical advice: Jing Qiu, Centre for Integrative Physiology; Tom Wishart, The Roslin Institute; Zuzana Krejciova, NCJD Unit; Bridget Harris and Peter Andrews, Critical Care Medicine; past and present members of the Chandran lab.

Reprogramming of the IPS1 line was carried out by Shyamanga Borooah. hPS line validation and conversion to neural precursors was performed by Karen Burr. hPS-derived oligodendrocyte progenitors were generated by Dario Magnani. Electrophysiological recordings of NMDA currents in hPS-derived cortical neurons were performed by Matthew R Livesey, who also provided baseline data of N-methyl-D-aspartate receptor (NMDAR) profiling in these cells (Fig.5.12). Stata analysis was performed by Peter Connick who further provided invaluable assistance with statistical modelling and interpretation of nested data.

# Chapter 1: Introduction

## 1.1 Cooling and neuroprotection

Clinical application of neuroprotective hypothermia dates back to 1942 in Philadelphia, where it was successfully used to treat patients with severe head trauma (Fay et al., 1943; Mayer and Sessler, 2005). Over the decades, interest in therapeutic hypothermia (TH) has waxed, waned, and waxed again as complications of deep cooling emerged, and were then superseded by a deluge of studies demonstrating the remarkable efficacy of *mild* hypothermia (Mayer and Sessler, 2005). The current *status quo* is ironically 'luke warm' – i.e. TH has become routine for a select group of patients but otherwise lacks a firm evidence base to support its use in man (Choi et al., 2012; Nielsen et al., 2013). Irrespectively, mild hypothermia remains the single most effective brain-protecting agent known (Barone et al., 1997; Mayer and Sessler, 2005; Dietrich et al., 2009; Choi et al., 2012; Yenari and Han., 2012; Wang et al., 2014), and there is a sense that the true reach of cooling may have been overlooked. Tapping into cellular programmes recruited by TH may unearth the molecular targets needed to reap the benefits of cooling - without cooling the patient.

### 1.1.1 The importance of brain temperature

Human brain temperature itself has been debated, largely because of difficulties in its measurement (Childs et al., 2005; Harris et al., 2012; Harris and Andrews, 2014). The current gold standard requires placement of an intracranial probe, retrieving data from only a single focal point in the brain parenchyma, and typically in patients already undergoing neurosurgery (Mellergard et al., 1990; Childs et al., 2005; Kuo et

al., 2011). With the advent of non-invasive methods such as magnetic resonance spectroscopy (MRS) (Childs et al., 2007; Thrippleton et al., 2013), we can now obtain a global picture of brain temperature in healthy subjects, in real time. As predicted from its high blood flow and rapacious oxidative metabolism (Maier and Chan, 2002; Hlatky and Robertson, 2005), average human brain temperature sits slightly above (0.2-0.8$^{o}$C) that of the core (Childs et al., 2007; Harris, 2010; Kiyatkin, 2010; Wang et al., 2014). However, brain temperature is not fixed; it varies by brain region, and with neural activity, physical exercise, state of consciousness and pathology (Czeisler et al., 1980; Walter et al., 1986; Moser et al., 1993; Hlatky and Robertson, 2005; Kiyatkin, 2010; Harris and Andrews, 2014; Wang et al., 2014). Elevated brain temperature associated with trauma, epilepsy, stroke and drug abuse is well documented and predicts a poorer prognosis (Schiff and Somjen, 1985; Busto et al., 1987; Azzimondi et al., 1995; Reith et al., 1996; Ginsberg and Busto, 1998; Rumana et al., 1998; Henker et al., 1998; Corbett and Thornhill, 2000; Hajat et al., 2000; Kammersgaard et al., 2002; Dietrich et al., 2009; Kiyatkin, 2010; Harris and Andrews, 2014; Schubert et al., 2014; Wang et al., 2014). At the cellular level hyperthermic insults increase resting membrane potential (RMP) and calcium levels, reduce pH and incite oxidative stress (Klose et al., 2008). This supports the rationale for therapeutic cooling - to minimise the cascade of secondary events that perpetuate and extend neuronal damage after an initial insult (Northington et al., 2001a; Northington et al., 2001b; Bramlett and Dietrich, 2004; Weil et al., 2008; McKee et al., 2009; Kim et al., 2013). Such is the importance of brain temperature, that it has been proposed as a driver for hominid evolution and brain function (Kunz and Iliadis, 2007; Harris, 2010; Wang et al., 2014).

### 1.1.2 Mechanisms of hypothermic neuroprotection

The neuroprotection afforded by cooling is multifaceted, as outlined in recent reviews (Mayer and Sessler, 2005; van der Worp et al., 2007; Faridar et al., 2011; Yenari and Han, 2012; Choi et al., 2012; Perman et al., 2014). These protective benefits are well characterized in rodents and other model systems in the context of both spinal cord and brain injury (Busto et al., 1987; Huh et al., 2000; van der Worp et al., 2007; Chip et al., 2011; Ballesteros et al., 2013; Grulova et al., 2013). Undoubtedly, hypothermia reduces cerebral oxygen consumption (Richards et al., 1963; Busija and Leffler, 1987) - but this cannot account entirely for the protective effect of mild cooling, since active cell metabolism continues at 32°C (Fujita, 1999; Ginsberg and Belayev, 2005). In cerebral ischaemia, recognised mechanisms of hypothermic neuroprotection include (1) effects on cerebral blood flow, excitotoxicity and metabolism in the acute phase, followed by (2) effects on apoptosis, inflammation and the blood-brain barrier (BBB) and finally, (3) more delayed effects on neuroglial differentiation and synaptogenesis (Karibe et al., 1994; Zhao et al., 2007; Liu et al., 2008; Silasi and Colbourne, 2011; Yenari and Han, 2012; Xiong et al., 2013). In the context of acute axonal severance, cooling has even diminished the rate of Wallerian degeneration (Sea et al., 1995). Although primary insults in spinal cord injury (SCI), traumatic brain injury (TBI), focal and global cerebral ischaemia are diverse, these disorders share secondary pathways that lead to cell death (Bramlett and Dietrich, 2004). Some studies argue for a straightforward hypothermic downregulation or delay of cellular death programmes (Bossenmeyer-Pourie et al., 2000). Others have shown that cooling elicits a far more complex set of events, not befitting to a simple linear model or anything that resembles a molecular

'off-switch' (Fujita, 1999; Yenari et al., 2008; Lleonart, 2010; Yenari and Han, 2012).

Common secondary neurotoxic mechanisms inhibited by cooling include those arising from oxidative and excitotoxic stress (Maier and Chan, 2002; Ginsberg and Belayev, 2005; Weil et al., 2008; Hardingham and Bading, 2010; Yenari and Han, 2012; Campos et al., 2012). 'Excitotoxicity' describes the neuronal death or dysfunction resulting from overactivation of excitatory neurotransmitter receptors (Olney, 1969; Greenwood and Conolly, 2007). Brain ischaemia and neurotrauma invariably lead to ATP depletion, energy failure, and loss of RMP (Bramlett and Dietrich, 2004; Weil et al., 2008; Khatri and Man, 2013). Consequently, depolarization induces the release and extracellular accumulation of excitatory amino acids (EAAs) – of which glutamate predominates. EEAs can also build up if released from intracellular stores during traumatic and/or necrotic cell death or if clearance mechanisms are compromised (Greenwood and Conolly, 2007). Excess extracellular glutamate leads to inappropriate NMDAR activity, elevated intracellular free $Ca^{2+}$, overwhelmed mitochondrial buffering capacity and disrupted calcium homeostasis (Rothman and Olney, 1986; Choi and Rothman, 1990; Lipton and Rosenberg, 1994; Werner and Engelhard, 2007; Greenwood and Connolly, 2007; Hasel et al., 2014). Excitotoxicity ensues and is an important cause of neuronal death in many acute neurological conditions and neurodegenerative disorders (Lipton and Rosenberg, 1994; Hardingham and Bading, 2010; Hasel et al., 2014). Hypothermia can modify the synthesis, release and reuptake of EAAs (Fig.1.1) and has a U-shaped impact on EAA toxicity (Busto et al., 1989; Huang et al., 1993;

Globus et al., 1995a; Baker et al., 1995; Thoresen et al., 1997; Tymianski et al., 1998; Li et al., 1999; Ginsberg and Belayev, 2005). Interestingly, this reciprocates the original 'bell-shaped curve' depicted by neuronal health in relation to NMDAR activation (Lipton and Nakanishi, 1999; Hardingham and Bading, 2003). More than 90% of excitatory synapses in the mammalian CNS terminate on dendritic spines which are thus highly susceptible to excitotoxic damage (Harris and Kater, 1994; Hasel et al., 2014). In response to hypothermia and other sublethal stresses there is a reversible dendritic swelling and spine loss (Ikegaya et al., 2001; Arendt et al., 2003; Kirov et al., 2004; Roelandse and Matus, 2004; Gisselsson et al., 2005; Yang et al., 2006; Popov et al., 2007; Greenwood and Connolly, 2007). Upon rewarming (or recovery), dendritic varicosities disappear and spines faithfully re-emerge without disruption of synaptic contacts, suggesting that these morphological transitions protect neurons from excitotoxic stress (Ikegaya et al., 2001; Arendt et al., 2003; Hasbani et al., 2001; Kirov et al., 2004; Gisselsson et al., 2005; Yang et al., 2006; Greenwood and Connolly, 2007). Acute excitotoxicity induces similar dendritic beading and spine loss (Greenwood and Connolly, 2007; Hasel et al., 2014). Although its pathological significance is unknown, this 'circuit-breaking' strategy may serve as an early homeostatic defence against excitotoxic insults (Ikegaya et al., 2001). Clearly in the long term, these adaptive features cannot prevent neuronal death.

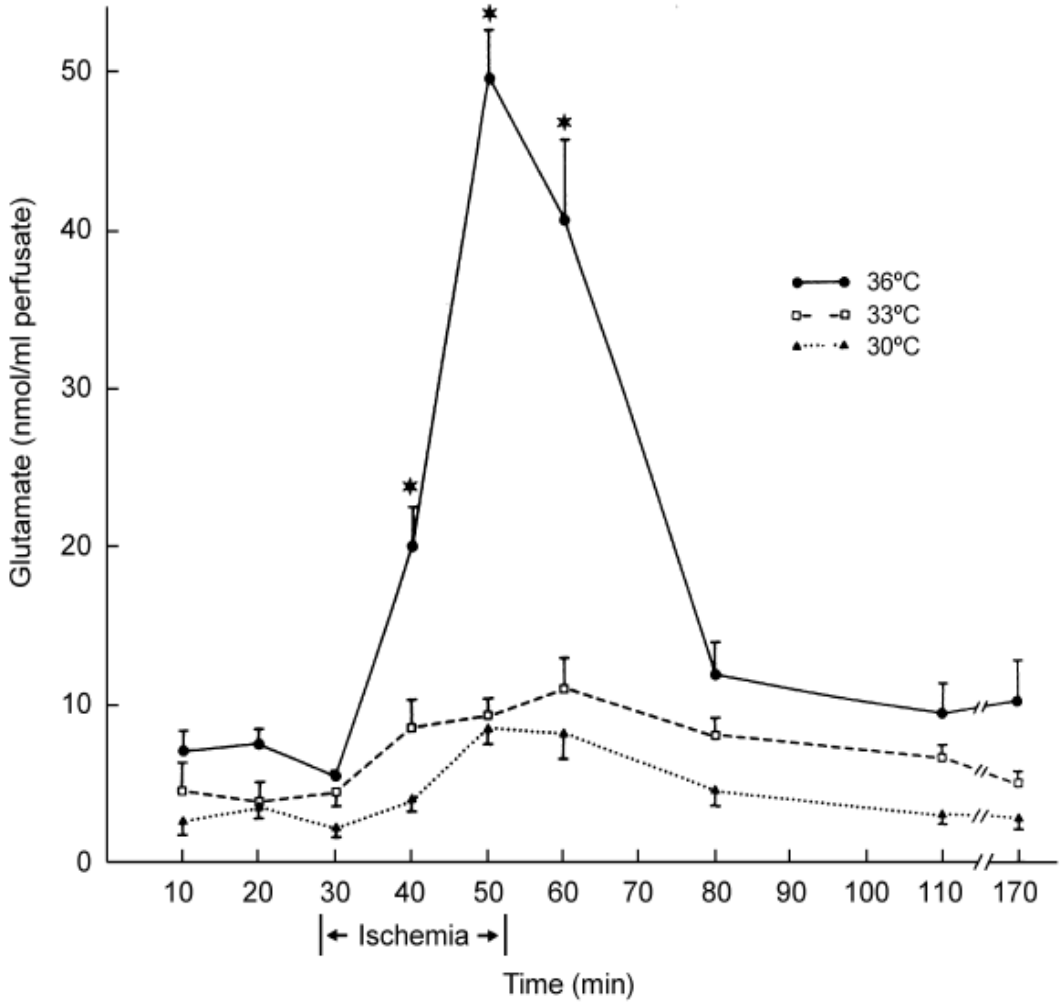


**Figure 1.1 Hypothermia inhibits glutamate release.** Changes in striatal microdialysis perfusate levels of glutamate (nmol/ml) in rats subjected to 20 min global forebrain ischaemia by four-vessel occlusion at 3 different intraischaemic brain temperatures. In normothermic rats (36°C), ischaemia triggered a massive extracellular release of glutamate, whereas in hypothermic animals (33°C or 30°C), glutamate release was almost entirely suppressed. *$p$<0.01. Figure reprinted (with permission) and legend adapted from Busto et al., 1989.

Oxidative stress incurs its damage via free radical oxidation of nucleic acids, proteins and lipids (Maier and Chan, 2002; Bramlett and Dietrich, 2004; Papadia et al., 2008; Kanamaru et al., 2015). Age-associated oxidative stress is an important factor in neurodegenerative disease and results from an excess of reactive oxygen species (ROS), linked to impaired antioxidant capacity (Maier and Chan, 2002; Kanamaru et al., 2015). Protective synaptic activity can enhance antioxidant defences (Papadia et

al., 2008), whilst excitotoxicity-associated mitochondrial impairment exacerbates ROS production (Singh et al., 2003; Hasel et al., 2014). This increase in oxidative species is inhibited by cooling (Globus et al., 1995a; Globus et al., 1995b; Kil et al., 1996; Kumura et al., 1996; Lei et al., 1997, Sakamoto et al., 1997; Si et al., 1997; Thoresen et al., 1997; Guven et al., 2002; Han et al., 2002; Maekawa et al., 2002; Maier and Chan, 2002; Dietrich et al., 2009) and may be critical to the protective reversibility of morphological changes in cooled hippocampal slices (Kirov et al., 2004; Greenwood and Connolly, 2007). Hypothermia also increases activity of the antioxidant enzyme superoxide dismutase (SOD) (Lei et al., 1994; Dekosky et al., 2004). As well as attenuating ROS-mediated neuroinflammation (Han et al., 2012), mild cooling preserves base-excision DNA repair after ischaemia-reperfusion-driven oxidative damage (Mayer et al., 1987; Ji et al., 2007; Luo et al., 2007; Zhao et al., 2007). Depending on insult severity, the final mode of excitotoxic or oxidative neuronal loss may comprise autophagy, apoptosis, necrosis, or necroptosis (programmed necrosis) - mild hypothermia can apparently defeat them all (Colbourne et al., 1999; Phanithi et al., 2000; Yenari et al., 2002; Bramlett and Dietrich, 2004; Degterev et al., 2005; Zhao et al., 2007; Cheng et al., 2013; Neutelings et al., 2013; Nikoletopoulou et al., 2014; Vieira et al., 2014). Ultimately, mechanisms of neurotoxicity and hypothermic protection will be determined by (1) the duration and intensity of the primary insult (Soriano et al., 2006), (2) the vulnerability of specific neuronal populations and different neuronal compartments (Minamisawa et al., 1990; Hosie et al., 2012; Hasel et al., 2014), and (3) the depth and timing of cooling (Tymianski et al., 1998; Bramlett and Dietrich, 2004; Ginsberg and Belayev, 2005; Han et al., 2012).

### 1.1.3 The limits of clinical cooling

Clinical efficacy of TH has been demonstrated in man, for neonatal hypoxic-ischaemic encephalopathy (NHIE), open-heart surgery and acute global cerebral insults after cardiac arrest (The Hypothermia After Cardiac Arrest Study Group, 2002; Bernard et al., 2002; Gluckman et al., 2005; Shankaran et al., 2005; Faridar et al., 2011; Arrich et al., 2012; Jacobs et al., 2013; Perman et al., 2014). Despite this, cooling has failed to translate into wider clinical use for stroke and TBI (Clifton et al., 2001; O'Collins et al., 2006; Hutchison et al., 2008; Harris et al., 2012; Wang et al., 2014). In part, this reflects conflicting or inconclusive trial data (Marion et al., 1997; Clifton et al., 2001; Harris et al., 2012; Nielsen et al., 2013), complicated by adverse effects of whole-body cooling in addition to its practical challenges (application, patient selection and effective timing) (Faridar et al., 2011; Choi et al., 2012; Harris and Andrews, 2014; Perman et al., 2014). Furthermore, much of the cellular and molecular physiology affected by temperature reduction remains unexplored and poorly understood (Chip et al., 2011). In effect, clinical use has been advocated on the evidence base that 'hypothermia can work', rather than addressing 'why'. This strategy risks neglecting mechanisms that might be more easily targeted pharmacologically (Blackstone et al., 2005). It could further yield a poor patient outcome, through as yet unknown deleterious consequences of brain or whole-body cooling. Several neurotoxic pathways are common to both acute and chronic neuronal injury, thus understanding the molecular pathways that mediate cellular responses to hypothermia could reveal novel therapeutic targets for degenerative (Baxter et al., 2014) as well as traumatic neurological conditions.

## 1.2 The cold shock response

### 1.2.1 Physiological and cellular cold-shock

A distinction must be drawn between the 'cold shock response' in physiological terms (at the organism level) and that pertaining to temperature-dependent processes within cells. The former is defined as:

*'a pattern of reflexes driven by cutaneous cold thermoreceptors, and characterised by sympathetically mediated tachycardia, a respiratory gasp, uncontrollable hyperventilation, peripheral vasoconstriction and hypertension'* (Tipton, 1989; Shattock and Tipton, 2012).

Cellular 'cold-shock' is somewhat slower, requiring profound but adaptive molecular changes that equip the cell for endurance at low temperatures (Fujita, 1999). Mammalian cells thus respond to cold-stress by switching from a state of growth and division to one of 'adaptation and survival' (Al-Fageeh et al., 2006). This involves cell-cycle arrest and shut-down of gene transcription and protein translation (Burdon, 1987; Wassman et al., 1998; Sonna et al., 2002; Al-Fageeh et al., 2006; Roobol et al, 2009; Hofman et al., 2012). Conversely, a subset of highly conserved (Derry et al., 1995) 'cold-inducible' RNA chaperones, including RNA binding motif 3 (RBM3) and cold-inducible RNA binding protein (CIRBP) are specifically upregulated in response to hypothermia (25-33$^{o}$C) (Nishiyama et al., 1997a; Nishiyama et al., 1997b; Fujita, 1999; Sonna et al., 2002). These glycine-rich binding proteins perform several important functions under stress conditions, including stabilisation and facilitated translation of essential mRNAs (Burd and Dreyfuss, 1994; Danno et al., 1997; Nishiyama et al., 1997a; Nishiyama et al., 1997b; Fujita, 1999; Phadtare et

al., 1999; Chappell et al., 2001; Sonna et al., 2002; Dresios et al., 2004; Smart et al., 2007; Durandy et al., 2008; Wulhfard, 2009; Pilotte et al., 2009; Lleonart, 2010; Liu et al., 2013). As such, cold-shock proteins have received attention in oncology, since they are associated with proto-oncogenesis through apoptotic inhibition (Kita et al., 2002; Sureban et al., 2008, Sakurai et al., 2006; Saito et al., 2010; Ferry et al., 2011), promotion of an undifferentiated 'stem cell' phenotype (Saito et al., 2010), and increased proliferation (Sureban et al., 2008; Wellmann et al., 2010; Lleonart, 2010; Matsuda et al., 2011; Grupp et al., 2013). Predictably, several of these features might be useful for neuroprotection and repair. Indeed, protective roles for RBM3 and CIRBP have been established in rodent neurons (Chip et al., 2011; Li et al., 2012; Peretti et al., 2015) and CIRBP is upregulated in hypothermic rat cortex (Liu et al., 2010). Although RBM3 and CIRBP transcripts are induced in various human cell lines at 32°C (Danno et al., 2000), there is currently no published data addressing cold-shock protein expression in human neurons.

### 1.2.2 Hypothermic preconditioning

A particularly sparse literature relates to the preconditioning effect of hypothermia (Nishio et al., 2000; Yuan et al., 2004; Stetler et al., 2014). Preconditioning describes the 'delayed tolerance' achieved against an intensively toxic insult by subjecting cells or tissue to a brief period of sublethal stress (Nishio et al., 2000; Rejdak et al., 2001; Soriano et al., 2006; Rodgers et al., 2007; Bell et al., 2011; Stetler et al., 2014). It can be effected by many and varied stimuli, the best known of which is ischaemia (Kato et al., 1991; Kitigawa et al., 1991; Jäättelä, 1999; Bell et al., 2011; Stetler et al., 2014). Such homeostatic priming is critically important at the

level of neural circuitry; extreme hypo- or hyperthermia re-calibrates neuronal circuits so that they can better withstand future temperature shifts (Money et al., 2004; Robertson and Money, 2012). For example, hyperthermic preconditioning at the *Drosophila* neuromuscular junction (NMJ) preserves synaptic transmission and is thought to involve heat shock 70 kDa protein (Hsp70) regulation of intracellular calcium levels (Barclay and Robertson, 2003; Klose et al., 2008). These studies introduce the importance of chaperones in maintaining neuronal homeostasis under stress conditions (Rejdak et al., 2001). Since preconditioning requires *de novo* protein synthesis, it has a relatively slow onset and persists over many days (Kato et al., 1991; Nishio et al., 2000; Klose et al., 2004; Gong and Golic, 2006). An advantage of hypothermia as a preconditioning stimulus over say, hypoxia, heat shock or epileptiform activity, is that it would potentially have a broader therapeutic margin (Nishio et al., 2000; Rejdak et al., 2001; Kapoor et al., 2014). Practically however, there are no instances in which preconditioning of any type could be timed optimally for acute or progressing conditions. Given that cold-shock and hypothermic preconditioning both engage a protective molecular programme (Fujita, 1999; Nishio et al., 2000; Chip et al., 2011), it seems likely that they are part of the same phenomenon or at least share mechanistic features. The question raised is provocative: if we identify the key players in these pathways, might they provide potent neuroprotective drug targets across multiple stages of neuronal compromise?

### 1.2.3 Cold-induced ontogenic reversal

Another possible outcome of cold-stress is reverse development (RD). RD was first described more than a century ago and within the animal kingdom, it is thought to be

unique to *Cnidaria* (Piraino et al., 2004; De Vito et al., 2005; Schmich et al., 2007). RD is an extreme adaptive response to unfavourable environmental conditions and, epigenetically, it can be induced by starvation, temperature or salinity changes (Piraino et al., 2004; Schmich et al. 2007). An alternative (endogenous) route enables RD to proceed through a genetic programme at the onset of senescence or sexual maturity (Piraino et al, 2004; Schmich et al., 2007). These sublethal stresses disturb the structural or functional integrity of one or more cell types including neurons, producing extensive tissue and cell rearrangements (Schmich et al., 2007). Like metamorphosis, RD requires apoptotic removal of redundant cells, a process that may be selectively regulated by cold-shock proteins (Fujita, 1999; Schmich et al., 2007). Whilst such dramatic morphological transitions are inconceivable in the mature mammalian brain, more subtle effects on cell cycle and differentiation state might be accomplished with cooling (Saito et al., 2010; Silasi and Colbourne, 2011). Indeed, in both *C.elegans* and *Drosophila*, stress response pathways can determine developmental fate and are acutely sensitive to temperature shift (Walker et al., 2003). RBM3 and CIRBP expression is developmentally regulated under normothermic conditions and both may play a role in neuronal differentiation (Fujita, 1999; Pilotte et al., 2009; Zeng et al., 2013). Their induction in cooled, mature neurons might thus drive a primordial post-transcriptomic profile. Very mild hypothermia (35°C) can also inhibit hPS differentiation (Belinsky et al., 2013), suggesting that some aspects of cold stress-regulated development are conserved in man. Perhaps a modest response, insufficient to induce trans-or de-differentiation, but enough to retrieve an earlier (and more plastic) ontogenic state could provide a

degree of protection in cooled human neurons (Piraino et al., 2004; Arendt and Bullman, 2013).

## 1.3 Comparative tau biology

### 1.3.1 Tau function and isoform expression

Neuronal axons must be maintained for the lifetime of the organism – and this critically depends on the microtubule cytoskeleton (Prokop, 2013). The microtubule-associated protein tau is encoded by a single gene (*MAPT*) that sits within an inversion polymorphism on human chromosome 17q21.1 (chromosome 11 in the mouse), and consists of 16 exons (Neve et al., 1986; Himmler et al., 1989; Andreadis et al., 1992; Friedhoff et al., 2000; Spires-Jones et al., 2009; Trabzuni et al., 2012). As its namesake suggests, tau is upregulated during neuronal differentiation alongside tubulin (Drubin and Kirschner, 1986); it is abundant in CNS axons, and functions to regulate the dynamics of microtubule assembly (Weingarten et al., 1975; Cleveland et al., 1977a; Cleveland et al., 1977b; Lindwall and Cole, 1984; Binder et al., 1985). Emerging evidence however points to an increasingly pleiotropic nature of tau, with roles in synaptic and nucleic acid homeostasis, signal transduction, neurite outgrowth, establishing neuronal polarity, interaction with the actin cytoskeleton and plasma membrane, anchoring of protein kinases and phosphatases, and regulation of intracellular vesicle transport (Mandelkow and Mandelkow, 1998; Friedhoff et al., 2000; Biernat et al., 2002; Ittner et al., 2010; Mondragon-Rodriguez et al., 2012; Pooler et al., 2012; Pooler et al., 2013; Chesta et al., 2014; Frost et al., 2014). Interspecies differences in cortical complexity (Hill and Walsh, 2005),

cellular and molecular biology have compounded the failure to translate 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, generated by alternative splicing (Goedert et al., 1988; Goedert et al., 1989; Goedert and Jakes, 1989; Janke et al., 1999; McMillan et al., 2008, Fig.1.2 and 1.3). In particular, only tau isofoms containing 4 microtubule binding repeats (4R tau) are expressed in the adult rodent brain and this may result from the lack of a stem loop structure in the intron following exon 10 (Higuchi et al., 2002). Tau isoform expression is also region and cell-specific (McMillan et al., 2008; Trabzuni et al., 2012) and the relative abundance of these isoforms determines tau function in both the normal and pathological brain (Trojanowski and Lee, 1995). Tau isoform balance might thus be subject to change under conditions that influence neuronal survival, such as hypothermia. Whilst there is little evidence for this in *in vivo* models of TH (Stieler et al., 2011), several core human tau isoforms are absent in the adult rodent brain and may be required to evaluate true human tau function and dysfunction (Janke et al., 1999; Andorfer et al., 2003). In addition, the tau haplotypes that have been associated with disease in man do not appear to exist in rodents (Stefansson et al., 2005; Trabzuni et al., 2012). Together these findings provide a strong rationale for exploring tau in the context of TH in a physiologically relevant human system.

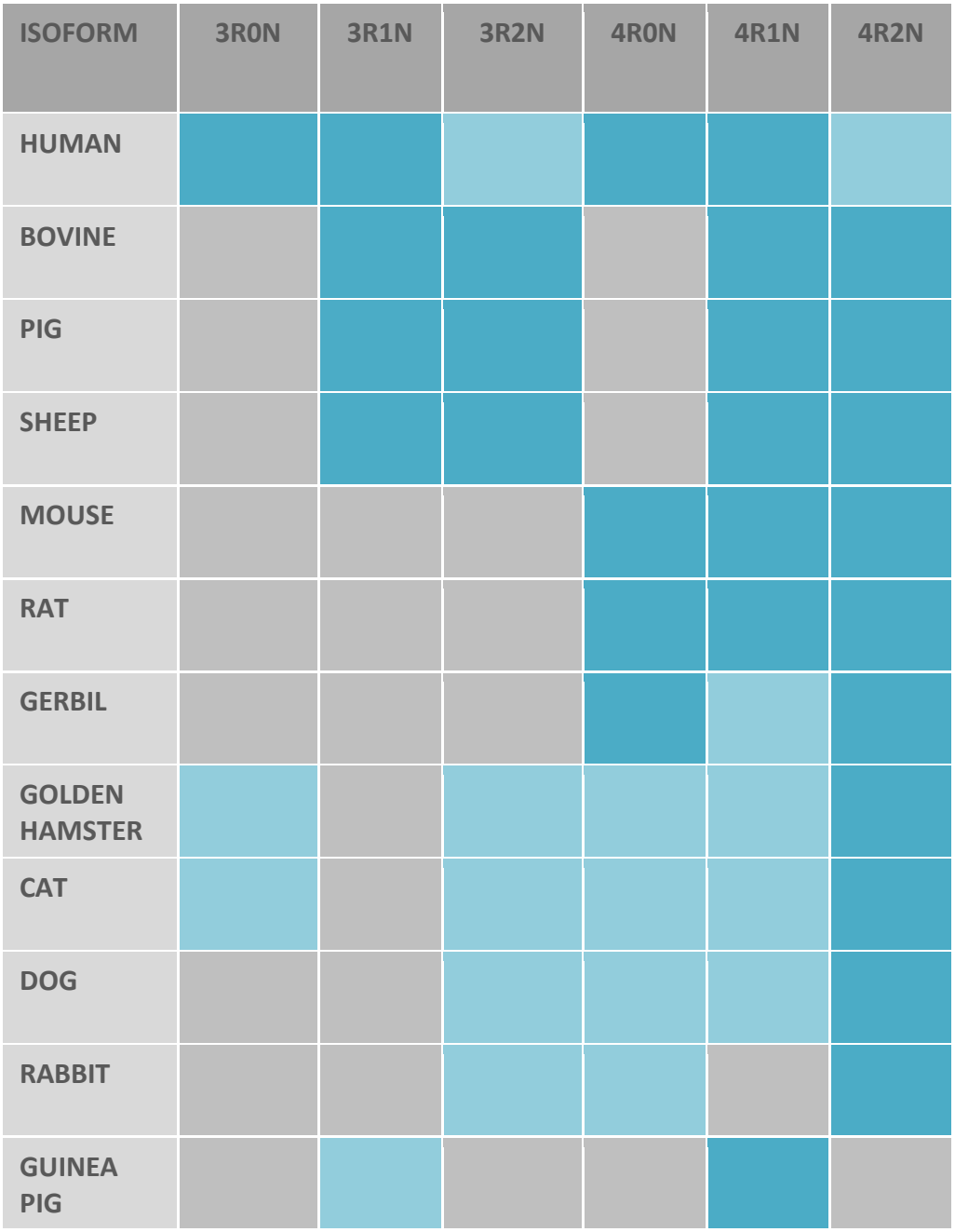


**Figure 1.2 Species-specific expression of tau isoforms in the adult brain.** Expression of tau isoforms in the brain tissue of 12 different mammalian species according to 2D-electrophoresis (adapted from Janke et al., 1999). Note that none of the commonly used laboratory mammals express the full complement of human tau isoforms. Dark aqua = strong isoform expression, light aqua = isoform present, grey = isoform hardly detectable or absent.

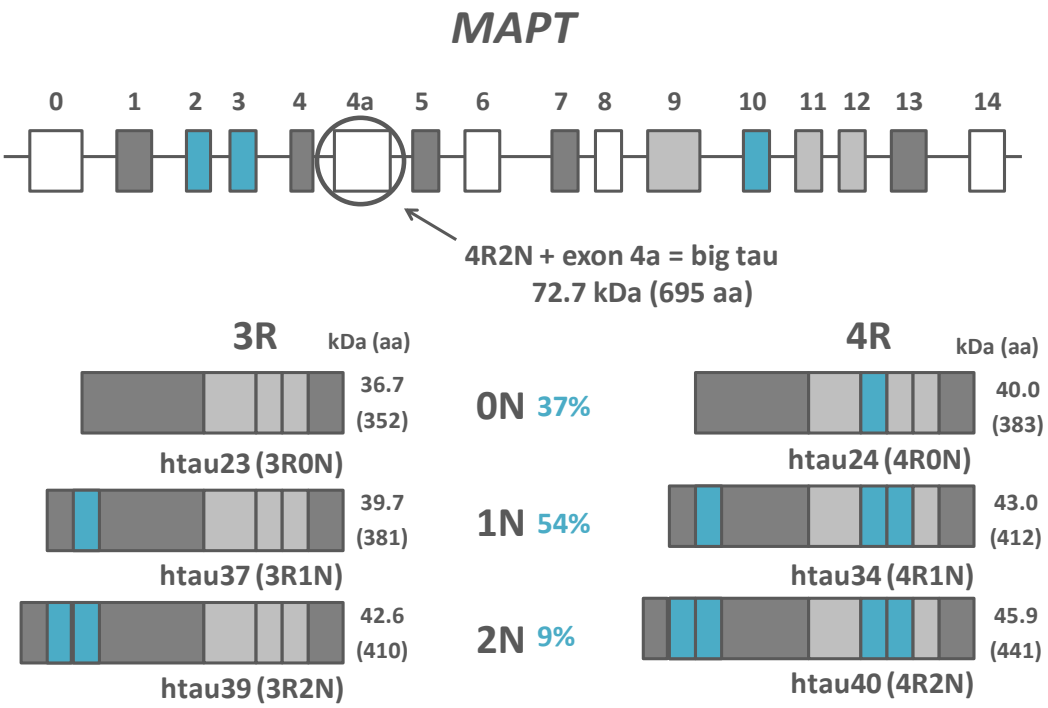


**Figure 1.3 Tau isoforms in the human brain.** Six major tau isoforms are generated in the CNS from a single gene (*MAPT*) by alternative splicing (Goedert et al., 1989; Himmler et al., 1989). Exons 9-12 code for the C-terminal microtubule-binding repeat domains. Splicing is developmentally regulated such that all six isoforms are found in the adult brain, but only the shortest 352aa (htau23, 36.7 kDa, 3R0N or 'foetal tau') isoform is found in the foetal brain (Bramblett et al., 1993; Goedert et al., 1989; 1993). Variable inclusion of exon 10 determines the expression of tau isoforms with either 3 ('3R tau') or 4 ('4R tau') microtubule binding repeats, whereas variable inclusion of exon 2 (with or without exon 3 (Andreadis et al., 1995)) determines the length of the N-terminal extension. Approximately equal proportions of 3R and 4R tau are found in the healthy adult human brain, but the presence of 0, 29 or 58 amino acid N-terminal inserts varies according the percentages shown in aqua. Largely in the peripheral nervous system, inclusion of exon 4a generates a larger isoform known as 'big tau' (Couchie et al., 1992; Goedert et al., 1992). Other minor isoforms have been described (Andreadis, 2006; Mandelkow and Mandelkow, 2012).

### 1.3.2 Tau structure

Tau represents more than 80% of available MAPs (Wang and Liu, 2008). *In vitro*, tau is absolutely required for the assembly of microtubules – a function regulated by the state of tau phosphorylation (Weingarten et al., 1975; Lindwall and Cole, 1984). The entire tau structure has eluded definitive analysis by xray crystallography and magnetic resonance; these and other methods generally report a natively disordered

protein with short elements transiently adopting α-helix or β-sheet conformation (Schweers et al. 1994; Mukrasch et al., 2009; Mandelkow and Mandelkow, 2012; Hyman, 2014) (Fig.1.4). This random, flexible structure is consistent with the high solubility of tau and the fact that its microtubule stabilising activity survives heat, denaturants and acids (Friedhoff et al., 2000). More permanent 'misfolded' conformations of tau are found in the diseased brain (Hyman, 2014). Tau is primarily composed of hydrophilic and charged residues, with an acidic N-terminal region followed by basic domains (Friedhoff et al., 2000). The imperfect microtubule binding repeats of 31-32 amino acids are located in the C-terminal half of the protein – the 'assembly domain' - flanked by proline-rich motifs (Steiner et al., 1990; Higuchi et al., 2002; Mandelkow and Mandelkow, 2012). The positive charge of this region is strongly attracted to the net negative charge of the outer surface of microtubules (Friedhoff et al., 2000). In healthy neurons tau is substoichiometric relative to tubulin (~1 μM versus ~20-40 μM) (Cleveland et al., 1977a; Cleveland et al., 1977b; Hiller and Weber, 1978; Mandelkow and Mandelkow, 2012). Thus *in vivo,* tau readily associates with tubulin but is most concentrated at the distal end of the axon where it facilitates cargo offload (Dixit et al., 2008; Spires-Jones et al., 2009; Amos, 2014). The negatively charged N-terminal 'projection domain' facilitates microtubule spacing and interactions with the plasma membrane (Hirokawa et al., 1988; Brandt, 1996; Chen et al., 1992; Amos, 2014).

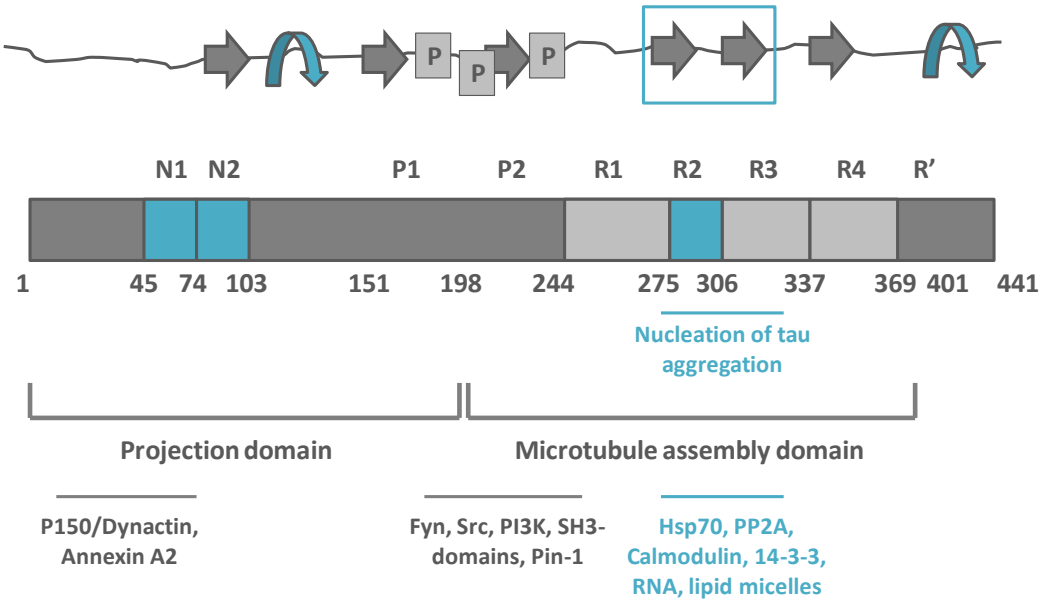


**Figure 1.4 Tau domains and structural components.** Schematic adapted from Mandelkow and Mandelkow (2012) shows the putative secondary structure of tau (top) deduced from nuclear magnetic resonance (NMR) spectroscopy (Mukrasch et al. 2009). Most of the peptide chain is unfolded (dark grey line), with a few short, transient elements of secondary structure (α-helix, aqua; β-strand, dark grey; poly-proline helix, light grey). The region of the two hexapeptide motifs responsible for tau aggregation are highlighted by an aqua rectangle. Tau domain subdivisions are shown in the middle panel (following Gustke et al., 1994). The C-terminal half promotes microtubule assembly whilst the N-terminal half projects out from the microtubule surface. Approximate interaction sites with other proteins are indicated at the bottom. PI3K = Phosphatidylinositol-4,5-bisphosphate 3-kinase. Src = proto-onocogene tyrosine kinase. Hsp70 = heat shock 70 kDa protein.

### 1.3.3 The importance of functional tau

Tau expression is ubiquitous in immature neurons and becomes progressively axonal as they mature (Mandelkow and Mandelkow, 2012). In the chick embryo, tau was found to participate in growth cone collapse – a key event in axonal guidance and retraction (Nakayama et al., 1999). The importance of tau in neuronal development and function is illustrated by tau knockout models. Originally, mice lacking tau were found to have only subtle phenotypes and this was attributed to the partial redundancy of various MAPs within the mammalian brain (Harada et al., 1994;

Dawson et al., 2001; Ke et al., 2012; Bolkan and Kretschmar, 2014). Flies on the other hand express a smaller range of MAPs – ubiquitous reduction of tau in *Drosophila* is lethal, whilst neuron-specific deletion either at an early stage of development or in adulthood leads to progressive neurodegeneration (Bolkan and Kretschmar, 2014). This phenotype can be partially rescued by the transgenic expression of the wild-type human foetal tau isoform 3R0N, indicating functional conservation of this protein from fly to man (Bolkan and Kretschmar, 2014). More recent work in tau knockout mice reveals an age-dependent brain atrophy, cognitive deficits and parkinsonism, indicating a protective role of this protein in the mature brain (Lei et al., 2012; Ma et al., 2014). This loss-of-function phenotype is remarkably similar to patients carrying mutations in the tau gene.

## 1.4 Tau modulation in health and disease

### 1.4.1 Tauopathies

The balance of tau isoforms, their secondary modifications and interactions with binding partners dictates the role of tau in health and disease (Trojanowski and Lee, 1995; Fig.1.4). For example, overexpression of wild-type human 4R tau in mouse neurons produces a severe disruption of axonal transport, axonopathy and neurodegeneration due to excessive binding of 4R tau to microtubules (Spittaels et al., 1999; Wang and Liu, 2008). Tau is also the most commonly misfolded protein in human neurodegenerative disorders (Goedert and Spillantini, 2011). Tauopathies are progressive, neurodegenerative and ultimately fatal diseases defined by the

intracellular accumulation of abnormal filamentous tau (Trojanowski and Lee, 2000; Garcia and Cleveland, 2001). They include Pick's disease, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), fronto-temporal dementia (FTD) and Alzheimer's disease (AD) – in which extracellular deposits of amyloid beta are a co-existing pathognomonic feature (Trojanowski and Lee, 2000). These disorders are classified by the distribution, morphological and biochemical characteristics of the tau inclusions found at post-mortem (Braak and Braak, 1991; Hyman, 2014) (Fig.1.5). For example in AD, neurofibrillary tangles (NFTs) are found in the somatodendritic compartment of affected neurons, whilst neuropil threads (NT) are found in distal dendrites and axons – these protein aggregates are principally composed of tau (Alzheimer, 1907; Brion et al., 1985; Grundke-Iqbal et al., 1986; Kosik et al., 1986; Wood et al., 1986; Lee et al., 1991; Friedhoff et al., 2000; Mandelkow and Mandelkow, 2012; Goedert et al., 2014). Tau pathology is also recognised in a broader spectrum of degenerative disorders, including prion disease, amyotrophic lateral sclerosis (ALS)/Parkinsonism-dementia complex (PDC) and C9ORF72-associated FTD (Spillantini and Goedert, 2013). Furthermore, the characteristic NFTs in AD are likewise typical of chronic traumatic encephalopathy (CTE) (McKee et al., 2009) and trauma is a risk factor for AD, ALS and Parkinson's disease (PD) (Smith et al., 1999; Ikonomovic et al., 2004; McKee et al., 2013). Tau isoform contribution, inclusion type and cell vulnerability vary with disease state (Gasparini et al., 2007; McKee et al., 2009). Hallmark tauopathic features however comprise tau hyperphosphorylation, aberrant folding, aggregation and reduced solubility (Lee et al., 1991; Iqbal et al., 2009; Spillantini and Goedert, 2013).

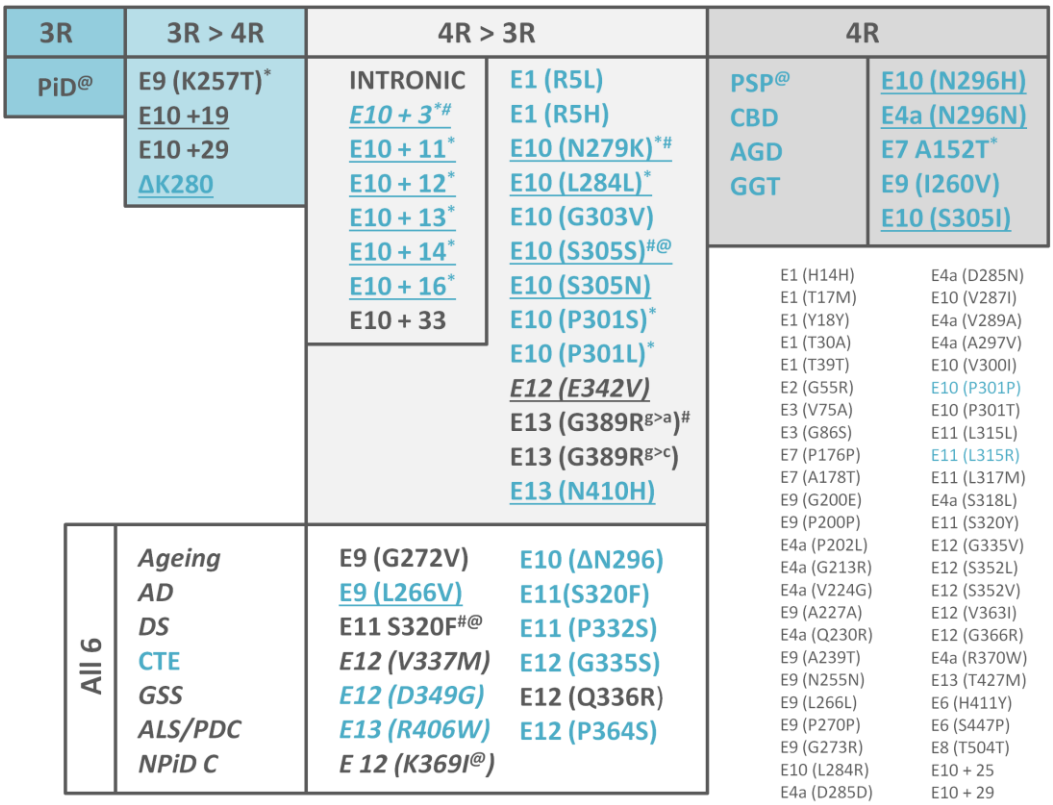


**Figure 1.5 Classification of tauopathies.** Sporadic and familial tau-associated disorders have been classified in the composite table above according to the tau isoforms found within insoluble inclusions in the brain at post-mortem. *MAPT*-associated pathologies are designated an 'E' number corresponding to the exon or intron within which they are located. Mutations are numbered according to the longest human tau isoform. Aqua font depicts pathology affecting both neurons and glia, otherwise tau inclusions are found within neurons only. Italic font depicts PHF conformation; other filamentous forms of tau can include straight[#], twisted[@] and random filaments or twisted ribbons*. Within neurons tau filaments can aggregate into classic NFTs, neuropil threads, dystrophic neurites, neuritic plaques (dystrophic neurites associated with amyloid plaques), Pick bodies. Within glia, filamentous tau appears as tufted astrocytes or coiled bodies within oligodendrocytes. In patients carrying the P364S mutation, striking composite neuronal tau inclusions (CNTI) are seen with 3R isoforms at the core of the lesion and 4R tau at the periphery. Functional mutations either reduce MT assembly or increase filament formation, whilst splicing mutations (underlined) influence the inclusion of exon 10 and thus alter the ratio of 3R:4R tau isoforms. Some mutations have both properties (ΔK280, N296H, N410H). Most mutations are point, missense mutations. Deletion mutations are shown with 'Δ'. Silent mutations are obvious from their unchanged amino acid composition. Mutations listed outwith the table are yet to be fully characterized; most of them are considered to be non-pathogenic or only affect the risk of AD. (G213R, V224G), others are associated with FTD (V75A, G273R, G335V, V363I, G366R). For A239T, the FTD is thought to derive from a mutation in the granulin (*GRN*) gene. A227A is associated with the H2 haplotype and decreased expression of tau. L284R and D285N are associated with PSP. S352L is associated with respiratory failure in youth. V363I and D285N show incomplete penetrance. DS = Down's syndrome, GSS = Gerstmann-Sträussler-Scheinker syndrome, NPiDC = Niemann-Pick disease type C. Table compiled from multiple sources including: Forman et al., 2002; Ingram and Spillantini, 2002; Avila et al., 2004, Sergeant and Buee, 2005; Goedert and Spillantini, 2011; Dujardin et al., 2014, Garcia and Cleveland 2001, Higuchi et al., 2002, Bieniek et al., 2013; King et al., 2013; Di Fonzo et al., 2014; Popovic et al., 2014, http://www.alzforum.org/.

### 1.4.2 Tau mutation

Around 5% of FTD cases result from mutations in *MAPT* (89 mutations have been described to date, some of which are non-pathogenic, see Fig.1.5 and 1.6) (Goedert and Spillantini, 2011). At least 51 of these mutations are sufficient to cause accelerated forms of dementia, described as autosomal dominant FTD and parkinsonism linked to chromosome 17 (FTDP-17) (Wilhemsen et al., 1994; Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998; Goedert et al., 2011 Spillantini and Goedert, 2013). This landmark discovery, alongside disease associations with various tau haplotypes and the absence of amyloid plaques in most sporadic tauopathies (Myers et al., 2007; Iqbal et al., 2009; Wade-Martins, 2012; Trabzuni et al., 2012) (Fig.1.7) underpins the importance of tau in neuronal health and neuropathogenesis**.** Controversy prevails however over the tau species (or intermediate) that might propagate neural demise, impeding development of effective, tau-targeted treatments. To complicate matters, evidence for tau-mediated neuroprotection is growing. For example, Esclaire et al. (1998) observed that the resistance of rat cortical neurons to glutamate-induced apoptosis was directly related to increased tau transcription. Currently, the consensus seems to be that filaments and fibrils are neither necessary nor sufficient for tau-induced toxicity, whilst soluble tau oligomers, promoted by tau cleavage, appear to be most toxic (Bretteville and Planel, 2008; Rocher et al., 2010; Crimins et al., 2011, 2012; Crespo-Biel et al., 2012; Flach et al., 2012; Kopeikina et al., 2012; Cowan and Mudher, 2013; Crimins et al., 2013). Whether tau pathology arises from loss of normal protein function (Cash et al., 2003), a gain of toxic function via oligomerization or tangling (Iqbal et

al., 2009; Feuillette et al., 2010) a combination of these factors or as a bystander effect remains inconclusive – even in FTDP-17 (Bolkan and Kretzschmar, 2014).

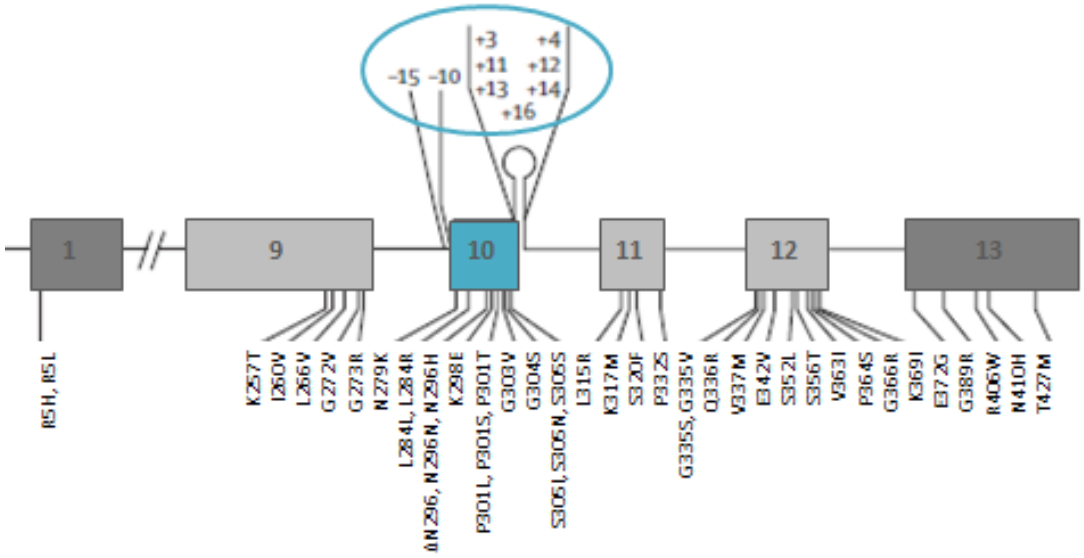


**Figure 1.6 *MAPT* mutations found in cases of FTDP-17.** The schematic is adapted from Spillantini and Goedert (2013) and displays 42 coding region mutations along with 9 intronic mutations flanking exon 10 (highlighted by aqua ring). Some exonic mutations act primarily at the protein level, reducing the ability of tau to bind to microtubules and/or promoting the assembly of tau into filaments. Other exonic mutations and the intronic mutations act at the RNA level and alter the splicing of tau pre-mRNA thus disrupting the balance of 3R to 4R tau isoforms.

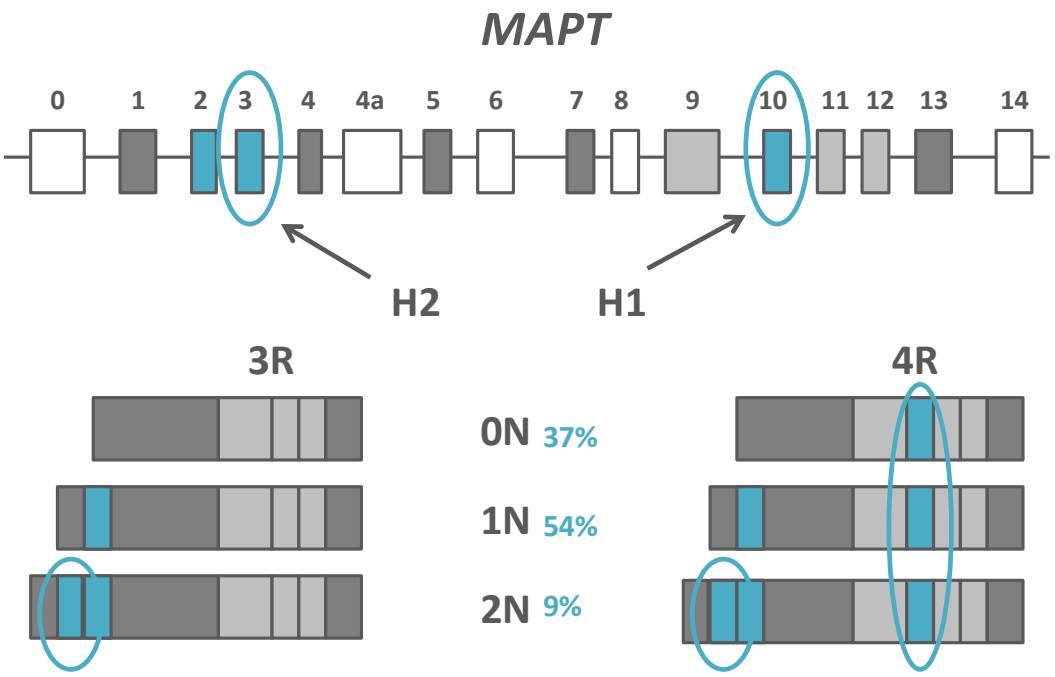


**Figure 1.7 Tau haplotypes.** The *MAPT* locus has two principal genetic haplotypes: the directly oriented H1, and H2, which has an inverted chromosomal sequence of ~ 970 kb (Stefansson et al., 2005; Wade-Martins, 2012). H1 is more common; it associated with increased inclusion of exon 10 (Caffrey et al., 2007) and increased risk of sporadic tauopathy (PSP and CBD, odds ratio, OR ~ 5) and idiopathic Parkinson's disease (PD, OR ~ 1.7) (Wade-Martins, 2012; Spillantini and Goedert, 2013). A single nucleotide polymorphism (SNP) produces the H1 subhaplotype H1c which is strongly associated with PSP (Höglinger et al., 2011). By contrast, the underrepresented H2 is linked to increased expression of exon 3 in grey matter, most prominently in cortical regions (Trabzuni et al., 2012). This is thought to be protective against the formation of tau tangles and decreases the risk of developing PSP, CBD and PD (Caffrey et al., 2008; Trabzuni et al., 2012; Zhong et al., 2012). It is worth noting that the rodent sequence sits in the H2 orientation (Stefansson et al., 2005; Trabzuni et al., 2012) which may explain the lack of spontaneous NFT development in these species.

### 1.4.3 Tau phosphorylation

Tau is a phosphoprotein containing 85 potential phosphorylation sites, largely at serine and threonine residues (Spires-Jones et al., 2009; Buee et al., 2010). Around 45 of these epitopes have been fully elucidated, many of them flanking the microtubule binding domains (Fig.1.8) (Higuchi et al., 2002; Hanger et al., 2009; Iqbal et al., 2009). These sites are variably phosphorylated depending on developmental age, neuronal health and physiological state (Spillantini and Goedert,

2013). A handful of these phospho-epitopes were originally assigned specifically to the diseased brain, however, over the last two decades this list has progressively diminished as equivalent sites have been identified in human foetal or healthy adult biopsy-derived brain samples (Goedert et al., 1993; Bramblett et al., 1993; Brion et al., 1993; Derkinderen et al., 2005; Matsuo et al., 1994; Seubert et al., 1995). What remains is largely a distinction in terms of quantity; tau in the AD brain carries 6-8 moles of phosphate per mole of protein, compared to approximately 4 in normal foetal brain and about 1.9 in normal adult brain (Ksiezak-Reding et al., 1992; Kopke et al., 1993; Sergeant et al., 1995; Mandelkow and Mandelkow, 2012). This hyperphosphorylated tau sequesters normal tau and other MAPs, and the increased phosphorylation state prevents tau binding to microtubules thus leading to microtubule disassembly (Iqbal et al., 1986; Bramblett et al., 1993; Alonso et al., 1996). Hyperphosphorylation also reduces tau turnover, and promotes its misfolding and subsequent aggregation into NFTs (Bancher et al., 1989; Yoshida and Ihara, 1993; Iqbal et al., 2009; Spillantini and Goedert, 2013; Fig.1.9). On the contrary, a collection of studies have shown that phosphorylation can protect tau against PHF formation (Schneider et al., 1999; Friedhoff et al., 2000). Also, by comparison, polyanionic cofactors such as sulphated glycosaminoglycans and RNA can stimulate tau aggregation far more efficiently than phosphorylation (Friedhoff et al., 2000; Mandelkow and Mandelkow, 2012). Thus, whether hyperphosphorylation is required to produce filamentous tau remains to be unequivocally established (Sergeant and Buee, 2005; Cowan and Mudher, 2013; Spillantini and Goedert, 2013). Several other biochemical modifications of tau including proteolytic cleavage, ubiquitination, oxidation, *O*-GlcNac glycosylation, *N*-glycosylation, nitration,

glycation, deamidation, sumoylation and acetylation have been described (Avila et al., 2004; Wang and Liu, 2008; Spires-Jones et al., 2009; Mandelkow and Mandelkow, 2012). Many of these appear to be disease-associated, disease-specific or even disease-stage specific (Wang and Liu, 2008) and may have equivalent or even more significant roles to play than phosphorylation *en route* to tau aggregation.

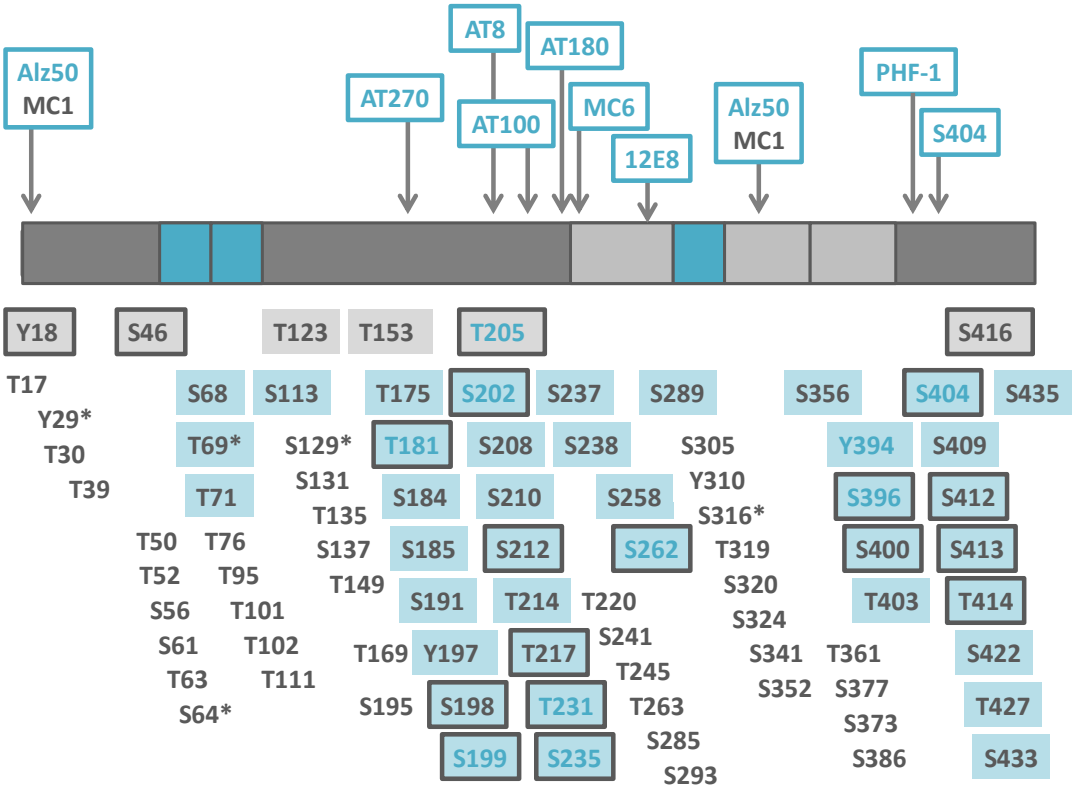


**Figure 1.8. Tau phosphorylation sites.** The longest human tau isoform (4R2N) is depicted together with 85 known phosphorylation sites numbered beneath, adapted from the Hanger lab table (http://cnr.iop.kcl.ac.uk/hangerlab/tautable). These include tyrosine (Y), serine (S) or threonine (T) residues. Filled boxes refer to sites phosphorylated in PHF-tau from Alzheimer's disease brain. Only one of the two closely phosphorylated sites at T414 or S416 are phosphorylated. Light grey filling refers to sites that have been detected by antibody immunoreactivity only, whereas light aqua filling hjghlights sites that have been directly demonstrated by mass spectrometry or Edman degradation. Epitopes outlined in dark grey are also phosphorylated in normal adult brain tau; note that only 2 of 3 closely phosphorylated sites at S412, S413 and T414 are phosphorylated. Epitopes labelled in dark aqua are also known to be phosphorylated in human foetal brain tau (Goedert et al., 1993; Bramblett et al., 1993; Brion et al., 1993; Derkinderen et al., 2005; Matsuo et al., 1994; Seubert et al., 1995). Asterisks denote sites for which a kinase has not yet been identified. Boxes above the peptide sequence refer to the classic complement of antibodies directed against phospho-epitopes to detect PHF-1 tau in pathological samples. Most of these antibodies also detect phospho-tau in human foetal post-mortem brain tissue and human adult biopsy-derived brain (Goedert et al., 1993; Bramblett et al., 1993; Brion et al., 1993; Derkinderen et al., 2005; Matsuo et al., 1994; Seubert et al., 1995). A notable exception is AT100 which is thought to be specific for pathological tau (Matsuo et al., 1994). AT8 recognises tau phosphorylated at both S202/T205 epitopes (Goedert et al., 1995), AT100 = S212/T214 recognises tau phosphorylated at both S212 and T214. AT180 detects tau phosphorylated at both T231 and S235 with weak recognition of phosphorylation at T231 only. AT270 recognises tau phosphorylated at T181 (Shahani et al., 2006). PHF-1 recognises the epitope around S396 and S404 sites. Alz50 supposedly detects a misfolded conformation of tau; the epitope includes amino acids 2-10 and 312-342. MC1 is another conformation-dependent antibody whose reactivity depends on both amino acids 7–9 and 313–322. MC6 recognizes tau phosphorylated at S235 (Hanger et al., 2009). Other than 12E8 and MC1 (which have not been tested), all of these antibodies detect phospho-tau in cultured hCNs (dark aqua labels).

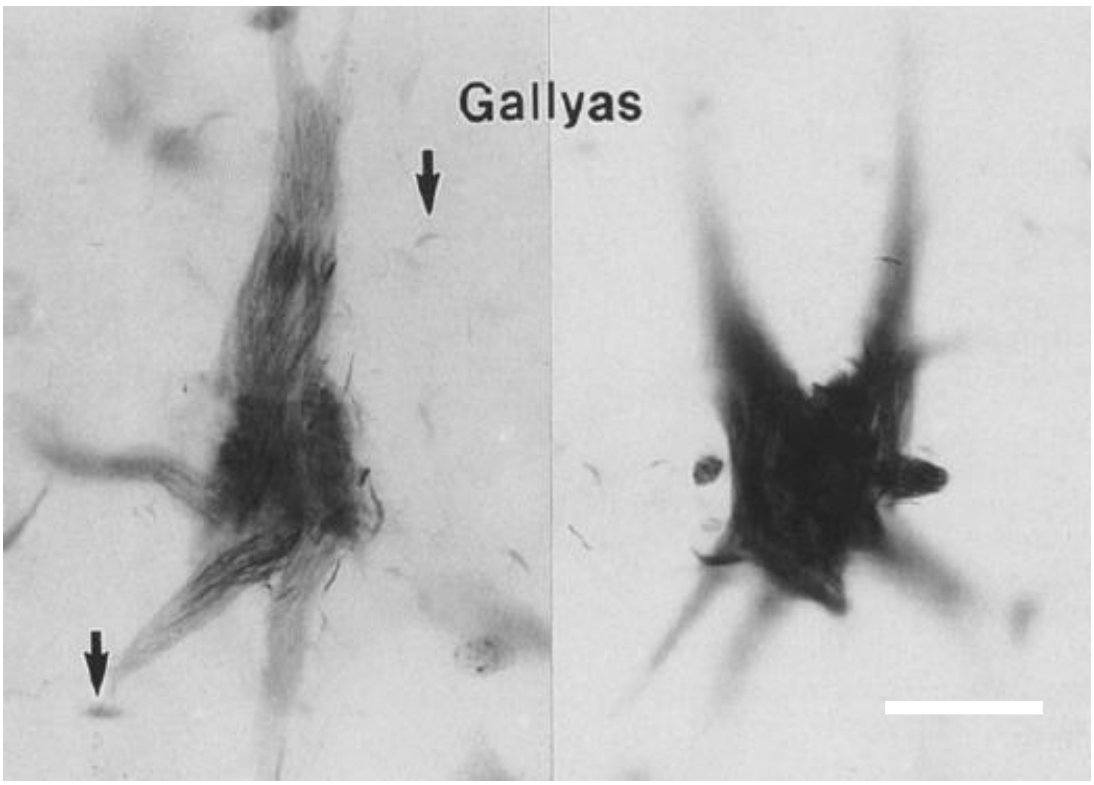


**Figure 1.9 Neurofibrillary tangle pathology in Alzheimer's disease.** Low-power view of a descending transentorhinal layer pre-α neuron immunostained with phospho-tau specific antibody AT8 then de-stained and re-stained with Gallyas silver impregnation for neurofibrillary changes. Densely stained fibrillary bundles represent the transition from an intracellular (left) to an extracellular (right) NFT ('early' ghost tangle), scale bar = 20 µm. The NFT is the most common type of tau aggregate found in neurodegenerative disorders. NFTs are composed of paired helical filaments (PHF) of hyperphosphorylated tau. The image is reprinted (with permission) and legend adapted from Braak and Braak, (1994).

Hyperphosphorylation of brain tau is also observed during development, mitosis, hibernation, hypothermia, anaesthesia, starvation and experimental diabetes mellitus (Mawal-Dewan et al., 1994; Yanagisawa et al., 1999; Planel et al., 2001; Arendt et al., 2003; Ikeda et al., 2007; Wang and Liu, 2008; Ke et al., 2009; Stieler et al., 2011; Chen et al., 2014). The implication is that this facet of tau is functionally significant and likely a prerequisite for neuronal growth, network formation and synaptic integrity. Tau phosphorylation is transiently high in early development and reversibly increases during hibernation and experimental cooling (Goedert et al.,

1993; Mawal-Dewan et al., 1994; Arendt et al., 2003; Planel et al., 2004; Yu et al, 2009; Stieler et al., 2011; Fig.1.10). Increased tau phosphorylation and missorting can also proceed independently of tau aggregation (Eckermann et al., 2007). These discoveries have sequentially eroded the dogma of what constitutes 'pathological tau'. Despite this, the persistence of a hyperphosphorylated state is clearly linked to aggregation; in *Drosophila*, NFT formation requires co-expression of tau kinases alongside human tau (Jackson et al., 2002; Chau et al., 2006). In the human brain, phosphorylation of soluble tau at the AT8 epitope decreases rapidly after death – thus residual AT8 staining reflects the presence of tau inclusions (Braak and Del Tredici, 2011; Matsuo et al., 1994; Goedert et al., 2014). The phosphorylation state of neuronal proteins is strictly regulated by the balanced activity of protein kinases and phosphatases (Fig.1.11) (Chan and Sucher, 2001). Protein phosphatase 2A (PP2A) is a multisubunit enzyme complex forming one of the major serine-threonine phosphatases expressed in the brain (Strack et al., 1998). A large body of evidence converges on the importance of PP2A in determining tau phosphorylation state; PP2A expression increases during development and deteriorates in AD (Gong et al., 1993; Vogelsberg-Ragaglia et al., 2001; Yu et al., 2009), whilst PP2A activity is disproportionately inhibited in the hypothermic brain (Planel et al., 2004). This offers insight into the dynamic modification of tau both in nature and in the context of neural demise.

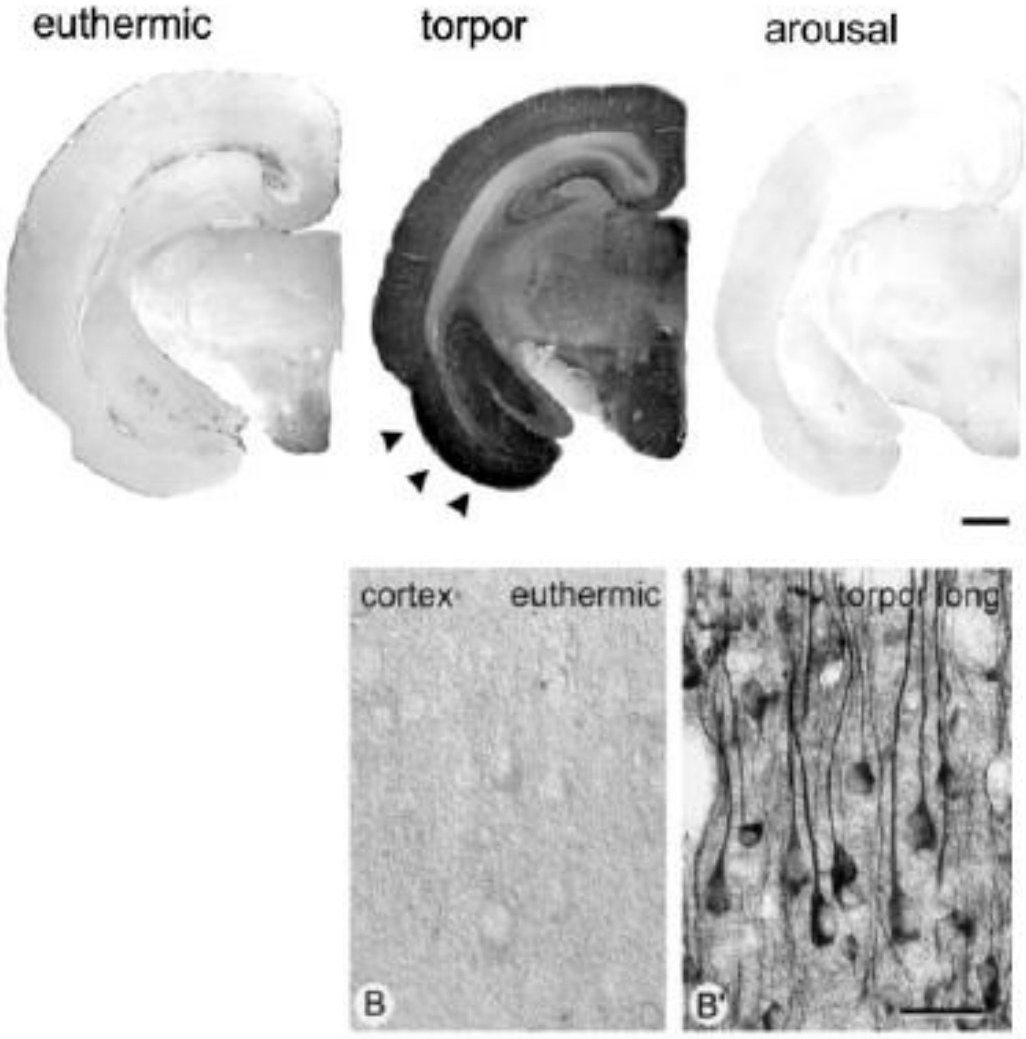


**Figure 1.10 PHF-like tau phosphorylation is physiological and reversible.** Upper panel: immunohistochemical detection of PHF-like phosphorylated tau by the phospho-tau dependent antibody AT8 in the brain of a mammal that undergoes natural hibernation cycles (European ground squirrel, *Spermophilus citellus*). Left = non-hibernating euthermic animal, middle = animal after long torpor, right = animal after long arousal. Torpor results in strong immunoreactivity in the entorhinal cortex (arrowheads), hippocampus, cortex, hypothalamic and epithalamic nuclei which is completely reversed within hours after arousal. Scale bar, 1 mm. Lower panel: PHF-like tau (AT8) in the hippocampus during hibernation (right) as compared to a euthermic animal (left), scale bar = 30 µm. Images reprinted (with permission) and legend adapted from Arendt et al., (2003).

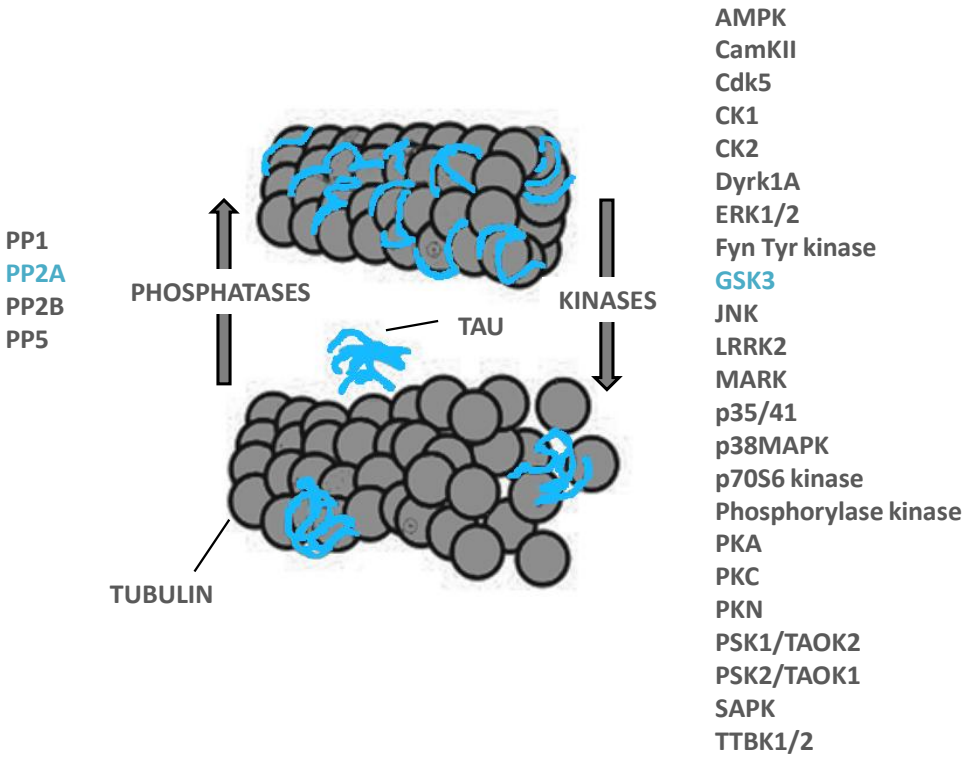


**Figure 1.11 Microtubule dynamics.** Binding of tau to microtubules is regulated by its phosphorylation state. Whilst there are many kinases that phosphorylate tau (major kinases are shown on the right; GSK3 has been most intensively studied), there are relatively few enzymes that dephosphorylate tau (Hanger et al., 2009). Kinases are broadly divided into two groups (Morishima-Kawashima, 1995): (1) proline-directed (e.g. extracellular signal-related kinase, ERK1/2; cyclin-dependent kinase 5, Cdk5) and non-proline-directed (e.g. calcium- and calmodulin-dependent protein kinase II, CamKII; protein kinases A and C, PKA/C; glycogen synthase kinase 3β, GSK3β) (Wang and Liu, 2008). The putative role of ERK1/2 in tau phosphorylation has recently been contested using a mouse model and human neuroblastoma cell line (Noël et al., 2015). PP2A accounts for more than 70 % of phosphatase activity in the brain (Liu et al., 2005). Image entitled 'Tauopathy' authored by Resident Mario (talk) 17:27, 9 January 2012 (UTC) and adapted under a Wikimedia Commons Creative Commons Attribution-Share Alike 3.0 Unported license.

### 1.4.4 'Spread' of tau pathology

Recent emphasis has been placed on the 'prion-like spread' of tau pathology between anatomically-connected regions within the brain (Prusiner, 1984; Clavaguera et al., 2009; Frost et al., 2009; Kim et al., 2010; Guo et al., 2011; de Calignon et al., 2012; Hall and Patuto, 2012; Liu et al., 2012a; Clavaguera et al., 2013). Transmission of tauopathies between human patients has never been demonstrated (Goedert et al.,

2014; Sanders et al., 2014). However, sporadic tau pathology typically advances in a hierarchical and time-dependent manner along functionally-related neuronal tracts. In AD, NFTs originate in layer II of the entorhinal cortex (EC-II) and then appear to progress via the perforant pathway to the hippocampus and neocortex, as categorised by Braak stages I-VI (Hyman et al., 1984; Braak and Braak, 1991). Cell type vulnerability must also play a role since some neurons in this pathway are comparatively resistant to the development of intracellular tau inclusions (Hyman et al., 1984). Later it was shown that brain extracts from mice expressing mutant human tau (P301S) could 'seed' neuronal NFTs in mice expressing wild-type human tau (Clavaguera et al., 2009) and that extracellular tau fibrils can be internalized by cultured neurons, inducing normal tau to form NFT-like inclusions (Frost et al., 2009; Guo and Lee, 2011; Guo and Lee, 2013). These works inspired an elegant set of experiments in which mutant human tau (P301L) was transgenically expressed in a specific set of EC-II neurons in the mouse brain - equivalent to the starting point of AD tau pathology (de Calignon et al., 2012; Liu et al., 2012a). Insoluble, hyperphosphorylated tau aggregates progressed from this area to neurons downstream in the synaptic circuit – neurons that did not express the human transgene. Additional key findings from the study by de Calignon and colleagues (2012) were that human tau co-aggregated with endogenous mouse tau and that early tau pathology in EC-II was associated with age-dependent axonal degeneration, followed by synaptic and neuronal loss as seen in AD. Together, these studies have provided compelling evidence that tau aggregates can 'seed' their own self-propagation, and 'spread' from neuron to neuron through synaptically-linked pathways. They do not however confirm trans-synaptic spread of tau, as claimed by

others using a similar mouse model (Liu et al., 2012a), primary cortical cultures (Pooler et al., 2013) or perhaps more convincingly a microfluidic culture system (Dujardin et al., 2014). Whilst Clavaguera et al. (2009) confirmed NFT formation using ultrastructural analysis and immunoreactivity at AT100 (a phospho-epitope that can only be generated by sequential phosphorylation of tau at particular sites and is considered highly specific for pathological tau (Friedhoff et al., 2000; Higuchi et al., 2002)), de Calignon and co-workers relied on biochemical characterization of tau aggregates using antibodies that were designed to detect hyperphosphorylated or 'pre-tangle' tau (Alz50, PHF-1, AT180) (Friedhoff et al., 2000). Pathologically this pre-tangle stage is distinguished by accumulation and somatodendritic relocalization of tau without PHF formation (Bancher et al., 1989; Bretteville and Planel, 2008). Thus the inclusions observed in the second study could not be described as 'AD-like' NFTs. Interestingly de Calignon et al. (2012) noted an age-dependent decrease in co-expression of the human transgene and abnormal tau protein within cells, whilst Liu et al. (2012) found the opposite. The former group interpreted this as protein-only replication of the 'seed', whilst the latter suggested upregulation of tau transcript in response to loss of normal protein function within aggregates. Neither study considered the possibility that transcriptional and/or translational control may have been impaired by the mutation, or that in the case of de Calignon et al. (2012), tau transcription might be suppressed to counteract the build up of inclusions. Regardless of their differences, these *in vivo* studies all required overexpression of mutant human tau within neurons and it is well established that (1) tau mislocalises, becomes hyperphosphorylated and forms aggregates in a concentration-dependent manner (Eckermann et al., 2007) (overexpression of wild-type murine tau in mice is

sufficient to cause tauopathy (Adams et al., 2009)), (2) tau mRNA expression remains unchanged in AD (Mah et al., 1992), and (3) tau is not mutated in the vast majority of tauopathies (Goedert et al., 2010). Thus there are fundamental concerns with translating findings in rodent models to sporadic human disease, since their physiological relevance is undermined by more than just the species barrier. In addition, most of the transgenic models of human FTDP-17 produce severe spinal cord pathology with a motor neuron phenotype that does not replicate the prominent cortical predilection in human patients (Garcia and Cleveland, 2001). Modelling these rare inherited forms of tauopathy are a particular challenge due to clinicopathological heterogeneity, even between patients carrying the same mutation (Yasuda et al., 2000).

Despite this, the 'prion-like' hypothesis has attracted further endorsement through the inoculation, spreading and passage of human sporadic tauopathy aggregates in mice expressing wild-type human 4R tau (Clavaguera et al., 2013). Critically, using non-transgenic mouse recipients, this study also demonstrated that overexpression of human tau is not required for inclusion formation and spreading since homogenates from AD, PSP, tangle-only dementia (TD) and argyrophilic grain disease (AGD) induced the assembly of mouse tau. This also confirmed the absence of a tauopathic 'species barrier' that is characteristic of classic prion diseases (Hyman, 2014). It was however clear that overexpression of wild-type human tau in the recipient mice promoted the formation of tau inclusions. Aggregate spread was further enhanced by sequence similarity between the 'seed' and recruitable endogenous tau, suggesting a degree of 'strain-specificity' (Colby and Prusiner, 2011). Similar to their earlier

work (Clavaguera et al., 2009), neurodegeneration was not observed within the time course of the study (18 months) (Clavaguera et al., 2013). Intriguingly, the study by de Calignon and colleagues (2012), also reported no evidence of neurodegeneration in association with human/mouse tau co-aggregates (at least not by 24 months). This raises the question of whether this hybrid aggregate retained the 'toxicity' of its founder, or whether it represented an evolved 'strain' with diluted mutant properties. Of paramount importance is that in stark contrast to the characteristic neuronal loss of the original P301S model (Allen et al., 2002), no neurodegeneration was observed in the mice that received P301S extract injections (Clavaguera et al., 2009). As highlighted by the authors, it is possible that the wild-type human tau background delayed the degenerative changes, or that different tau species are responsible for transmission and toxicity (Clavaguera et al., 2009; Clavaguera et al., 2013). An alternative hypothesis is that NFT formation shields the cell from more toxic oligomeric intermediates by rendering them inert (Bretteville and Planel, 2008; Tai et al., 2012; Walther et al., 2015), and that it is *this* protective process which is overwhelmed or defective in the P301S mouse. This is supported by the fact that wild-type human tau seeds are more efficient than P301S human tau seeds at promoting spread of wild-type aggregated mouse tau (Clavaguera et al., 2013). It might also explain the late expression of AD, and that many NFT-bearing neurons remain intact at post-mortem even in the non-demented elderly, having survived these inclusions for several decades (Bancher et al., 1989; Morsch et al., 1999; Castellani et al., 2008). Perhaps the 4$^{th}$ microtubule binding repeat domain, by nature of its extra cysteine residue, enhances a protective aggregating function of tau in the mature brain via the formation of disulfide bridges (Friedhoff et al., 2000) –

this is consistent with *in vitro* data showing that AD phospho-tau preferentially sequesters 4R tau isoforms (Alonso et al., 2001). A mutation within this domain could simultaneously impair the normal microtubule binding and aggregating functions of tau (exposing the cell to soluble mutant species) whilst generating a toxic form of aggregate, thus massively accelerating disease. Accordingly, FTDP-17 mutations that alter tau exon 10 splicing increase the level of 4R tau in both soluble and insoluble fractions – this unifies quite disparate genetic aberrations (Fig.1.5-1.6) into a group that invariably increase the pool of unbound tau (Hong et al., 1998; Garcia and Cleveland, 2001).

### 1.4.5 A protective role for tangled tau?

NFTs and synaptic loss make the best direct correlates with dementia, but NFT numbers far exceed neuronal death (Arriagada et al., 1992; Gomez-Isla et al., 1997; Friedhoff et al., 2000). In the P301S mouse, tau hyperphosphorylation and synapse loss precedes NFT formation (Yoshiyama et al., 2007). Expression of a 'pro-aggregant' tau mutation in mice produced pre-tangles and considerable synaptic loss (40% within 13 months), but no neuronal death (Eckermann et al., 2007). A separation of NFT accumulation and neuronal death was also noted in aged mice transgenic for all wild-type human tau isoforms (htau) and those expressing human P301L tau (Andorfer et al., 2005; Santacruz et al., 2005). Thus, the consistent finding of tau inclusions in tauopathies does not preclude the possibility that these aggregates exert a protective function (Andorfer et al., 2005; Alonso et al., 2006; Wang and Liu, 2008; Cowan and Mudher, 2013). Indeed, formation of tau aggregates can abolish the toxicity of soluble phosphorylated tau (Alonso et al.,

2006; Wang and Liu, 2008). Compared to AD, tissue loss is greater in FTDP-17 and there are 10-fold fewer NFTs - this might explain the accelerated phenotype of these patients (Shiarli et al., 2006; Bretteville and Planel, 2008). Treatment of 3xTg AD mice (expressing tau, APP and presenilin 1 mutations under the murine Thy1.2 promoter) with pyruvate increases the deposition of hyperphosphorylated tau and prevents age-related cognitive decline (Isopi et al., 2014). Another recent study using the rTg4510 mouse that overepxresses P301L confirmed that NFT-bearing neurons were functionally intact, had a stable resting calcium level and were capable of integrating into cortical circuits (Kuchibhotla et al., 2013). Lastly, an unbiased review of human brains aged 1 to 100 years revealed that post-mortem tau-related neuronal changes appeared much earlier than amyloid deposition and often in the absence of amyloid (Braak et al., 2011; Krstic and Knuesel, 2012). This not only weakens the 'amyloid cascade hypothesis' (purporting that NFT pathology is driven by Aβ), it suggests that tau aggregation is a natural corollary to ageing. Perhaps the closest approximation of a mouse model to FTDP-17 has come from the P301L mutation expressed under the mouse prion promoter (Lewis et al., 2000). Whilst this approached a near-physiological level of tau expression and induced age-dependent NFT pathology in the brain, there were 3 main caveats: (1) a strong motor phenotype prevailed alongside behavioural deficits, (2) selective neuronal vulnerability differed from the human condition and (3) the transgene included only the shortest human 4R tau isoform (4R0N) thus ignoring the contribution of exon 2 and 3-contaning mutant 4R tau in human patients (Lewis et al., 2000).

### 1.4.6 The elusive 'toxic tau species'

Conformationally distinct disease-associated 'strains' of tau have since been isolated from human patients with different tauopathies and stably propagated both *in vitro* and *in vivo* (Sanders et al., 2014; Holmes and Diamond, 2014). This infers a core 'prion-like' feature of morphological variants of tau inclusions that may underpin the clinical variability between tauopathies and also between individuals with the same disease, since several conformers can co-exist simultaneously in any given patient (Sanders et al., 2014). If templated spread of pathological tau does indeed occur between synaptically-connected neurons, the molecular identity of this tau species remains mysterious, although it is likely to be soluble and dephosphorylated (Dujardin et al., 2014; Pooler et al., 2013; Goedert et al., 2014). Heat shocked cells release more extracellular tau than normothermic cells (Karch et al., 2012) thus cooling cells down may prevent the spread of putative toxic tau species by increasing their phosphorylation state. Tau is actively secreted into the extracellular space, and can be internalized via heparin sulphate proteoglycans (Holmes et al., 2013), but it is unclear whether it stays in solution during cell-cell transmission or whether it is transported via exosomes (Karch et al., 2012; Pooler et al., 2013; Goedert et al., 2014). It has even been proposed that transmission *in vitro* and *in vivo* may depend on neuronal activity (Pooler et al., 2013; Dujardin et al., 2014; Holmes and Diamond, 2014; Yamada et al., 2014). An activity-related shift in intracellular calcium would increase the release of extracellular tau and thus may be responsible for the increase in CSF tau levels in AD (Karch et al., 2012; Sokolow et al., 2015). Interestingly, FTDP-17 mutations appear to reduce the secretion of cellular tau, prevent its spread to other neurons and more rapidly induce neuronal death (Karch et al., 2012;

Dujardin et al., 2014). This might explain the lack of spreading tau pathology along neural networks in these patients (Dujardin et al., 2014) and may have important implications for the retention of toxic tau species within the cell – especially if their aggregation into insoluble inclusions is impaired as discussed above (Simon et al., 2012). Along a similar vein, Yamada et al. (2011) found that soluble monomeric tau is a normal constituent of brain interstitial fluid (ISF) in mice and is dramatically reduced in the presence of insoluble tau driven by the P301S mutation. They propose an equilibrium between intracellular and extracellular tau that promotes sequestration of ISF tau by intracellular aggregates in tauopathy (Yamada et al., 2011). Although tau is not overexpressed in any tauopathies, increased cytosolic concentrations of tau due to hyperphosphorylation or microtubule loss in a given neuron might be sufficient to cause aggregation – in healthy neurons microtubules are effectively 'chaperones' that prevent tau misfolding (Hall and Patuto, 2012; Mandelkow and Mandelkow, 2012). However, there appears to be no relationship between microtubule loss and PHF-tau in AD, and with a shorter half-life than tubulin, tau should be rapidly degraded upon microtubule disassembly (Drubin et al., 1988; Cash et al., 2003; Lee et al., 2005). Alternatively, if secretion were compromised but translation retained, local tau levels would increase. This imbalance would however require failure or dysfunction of protein homeostasis within the cell, allowing tau production to continue in the face of protein overload. Such mechanisms have been proposed to underly the specific cell-type vulnerability in a number of neurodegenerative disorders and ageing as discussed later (Morimoto and Cuervo, 2009; Koga et al., 2011). Accordingly, caspase or calpain cleavage (thought to mediate Aβ-induced tau toxicity) would create tau fragments both with

and without the N-terminal extension needed to interact with lipid membranes for secretion (Brandt et al., 1995; Johson et al., 1997; Park and Ferreira, 2005; Kim et al., 2010; Lee et al., 2011; Hall and Patuto, 2012; Bright et al., 2014). Fragments lacking the N-terminal extension would be retained intracellularly and may not be sensed by normal proteostatic feedback pathways - thus they might escape degradation and accumulate unchecked (Bright et al., 2014). This would explain the presence of N-terminal fragment tau in CSF (Johson et al., 1997) alongside intracellular NFTs in AD (Kim et al., 2010). It is also supported by a recent study using iPS-derived cortical neurons from AD patients, which secreted only N-terminally truncated tau species (Bright et al., 2014). This extracellular tau induced neuronal hyperactivity and increased Aβ secretion, both of which are thought to enhance secretion of truncated tau resulting in a toxic feed-forward loop (Bright et al., 2014). The late and modest appearance of cleaved tau in NFTs may represent unsuccessful attempts at turnover (Iqbal et al., 2009) or it may provide a vital clue as the true 'toxic species' in AD – perhaps a cytosolic C-terminal fragment that serves as a poor substrate for aggregation. Supporting this hypothesis is the recent finding that relative to controls, a higher percentage of AD pre-synaptic terminals contain truncated tau which can be released during post-mortem depolarization (Sokolow et al., 2015).

Although distinct from other tauopathies in terms of distribution, the NFT stages of CTE progress slowly, and this continues long after the repetitive trauma has ceased (McKee et al, 2009; McKee et al., 2013). The fact that an environmental trigger early in life can manifest in clinical symptoms many years later, suggests that (1)

non-familial tauopathy takes a long time to develop – possibly owing to neural reserve, or a limited capacity for endogenous repair (2) neuronal demise continues after the inciting cause is removed – implying involvement of secondary toxic mechanisms or 'replicative seeding' of a primary injurious factor and (3) protective cellular processes are ultimately outweighed by toxic ones, or they themselves become toxic. Extracellular 'ghost tangles' in late-stage AD are thought to represent the remnants of degenerated NFT-containing neurons, thus eventually, any protective function of this aggregate must be overwhelmed (Lee et al. 2005). Unlike AD, tau inclusions in CTE begin with a patchy, perivascular distribution at the depths of the sulci and are prominent in astrocytes as well as neurons (McKee et al., 2009; McKee et al., 2013). The neuropathological staging is again suggestive of transneuronal spread, although glia may also be involved in propagating tau pathology, as suggested in other disorders (McKee et al., 2013; Spillantini and Goedert, 2013 ). Since CTE is frequently associated with ALS, PD and AD, and NFTs partially co-localise with TAR DNA binding protein (TDP-43) deposits, it has even been suggested that NFTs may 'cross-seed' the aggregation of other neurodegeneration-associated proteins (Morales et al., 2010; McKee et al., 2013). Nevertheless, the neuropathological overlap between classic neurodegenerative disorders and CTE suggests that environmental factors in early life may be important in regulating disease progression.

## 1.5 Hypothermic tau and neuroprotection

Given that most neurons in AD are lost independently of abnormal tau phosphorylation, it is reasonable to conclude that neither tau nor tau phosphorylation

*per se* is toxic (Lee et al., 2005). How then might physiological changes in this protein offer neuroprotection? A number of recent reviews discuss the putative protective roles of tau, focussing chiefly on tau aggregates and hyperphosphorylated tau (Arendt, 2004; Lee et al., 2005; Wang and Liu, 2008; Bretteville and Planel, 2008; Buee et al., 2010; Morris et al., 2011; Arendt and Bullmann, 2013). What follows is a selective synopsis of evidence underpinning a role for tau modulation in neuroprotective hypothermia.

### 1.5.1 Cold-induced cytoskeletal plasticity

Coupled with reduced metabolism and protein synthesis, mammalian cells undergo cytoskeletal changes in response to cold (Stapulionis et al., 1997; Al-Fageeh et al., 2006). In a microarray study of 3T3 cells, a temperature shift from 37°C to 32°C altered the expression of many cytoskeletal genes (Beer et al., 2003) and several other studies have shown reversible disassembly of the microtubular network with cooling (Roth, 1967; Tilney and Porter, 1967; Rodriguez Echandia and Piezzi, 1968; Weisenberg, 1972; Stapulionis et al., 1997; Fujita, 1999). Repolymerization of microtubules observed in the toad sciatic nerve during re-warming is independent of cellular integrity – it does not require new protein synthesis (Rodriguez Echandia and Piezzi, 1968). Highly conserved morphological changes in the neuronal cytoskeleton thus occur rapidly and passively with temperature shift, and do not serve as a useful readout of neuronal health. They may however play a critical role in dictating neuronal survival during cooling and recovery. $Ca^{2+}$ influx during intense excitatory stimulation, though tempered by hypothermia, would augment microtubule disassembly (Weisenberg, 1972; Tymianski et al., 1998). It follows that an

undesirable situation might exist in the acutely injured brain, where temperature is raised (favouring microtubule stability) and dendrites may be unable to retract, leaving them more exposed to highly toxic substances (excitotoxins, ROS and inflammatory components). This lack of cytoskeletal plasticity or 'deficient dynamism' might be a converging feature of neurons harbouring FTDP-17 mutations that increase the relative expression of 4R tau. Such neurons would not only be predisposed to injury, they would be less able to respond physically to a toxic milieu.

### 1.5.2 Tau and hibernation

Intuitively then, retaining plasticity is an effective strategy for avoiding stressful insults - and nowhere is this more obvious than in the hibernating brain (Arendt, 2004; Boerema et al., 2012; Peretti et al., 2015). Hibernation is an overwintering behaviour observed in a variety of species across several mammalian orders that includes a radical decrease in basal metabolic rate (BMR), body temperature, cardiorespiratory parameters and neuronal activity (Wang, 1978; Daan et al., 1991; Arendt and Bullman, 2013). These reversible changes minimize energy expenditure under challenging environmental conditions (Krauchi and Deboer, 2010). Despite a dramatic cerebral ischaemia followed by rapid reperfusion, most animals emerge from torpor neurologically intact (Hindle and Martin, 2013). In ground squirrels, contacts between mossy fibres and hippocampal pyramidal neurons undergo a striking regression during hypothermic torpor followed by re-afferentation during euthermic arousal (Popov et al., 1992; Popov and Bocharova, 1992; Popov et al., 2007; Ruediger et al., 2007). This involves both dendritic spine transformation and temporary synaptic loss (Arendt and Bullmann, 2013). Associated changes in the

cortical transcriptome are indicative of remodelling and synaptic plasticity (Schwartz et al., 2013), whilst the forebrain proteome reveals a temperature-dependent modulation of cytoskeletal proteins and their regulators (Hindle and Martin, 2013). This periodic denervation and re-innervation is rapid; dendrite extension during arousal occurs much faster than that observed during neuronal development or environmental enrichment (Dave et al., 2012). Importantly, these ultrastructural and morphological changes are directly paralleled by fully reversible phosphorylation of synaptic membrane proteins and tau (Shchipakina et al., 1995; Arendt et al., 2003; Hartig et al., 2007; Su et al., 2008). Tau thus becomes hyperphosphorylated during torpor in a 'PHF-like' manner throughout the entorhinal cortex, hippocampus and isocortical areas - but without forming fibrils (Arendt et al., 2003; Su et al., 2008; Arendt and Bullmann, 2013). This enhanced phosphorylation depends primarily on the drop in body temperature and involves inhibition of PP2A activity (Su et al., 2008). Hence, chemical inhibition of protein phosphatases is sufficient to remodel dendritic spines and reduce synaptic number in rat hippocampal cultures (Malchiodi-Albedi et al., 1997). At the transcript level at least, tau splicing also appears to be shifted in favour of 3R tau isoforms (Stieler et al., 2011). The hibernating brain is also highly resistant to injury, placing this 'foetal-like' tau at the very heart of a neuroprotective adaptation (Ihara, 2001; Zhou et al., 2001; Arendt, 2004). Whether these changes simultaneously prevent proteolytic cleavage of tau and reduce its microtubule binding capacity remains to be unequivocally demonstrated (Litersky and Johnson, 1992; Arendt, 2004; Su et al., 2008). However, a hypothermia-induced preservation of tau dynamic function during '*vita minima*' is an attractive postulate (Arendt, 2004; Su et al., 2008). It would appear that arousal-induced re-connection

is faithful, explaining why torpid neuronal phenomena do not confer residual cognitive deficits, and that mechanisms (potentially recruiting phospho-tau) must exist to orchestrate this complex regenerative process (Arendt, 2004). Although the brain clearly tolerates hibernation (in some cases to near-freezing temperatures), there are short-term effects on recent (but not remote) memory that are replicable in laboratory models of hypothermia (Arendt and Bullmann, 2013).

Perplexingly, the parts of the brain 'switched off' during hibernation are those which retain the highest neuroplasticity in adulthood – an obligatory feature for the evolution of higher cognitive function (Rapoport, 1999b). In AD, both the course of neurofibrillary degeneration and decline in mental capacity progress along an inverse hierarchy of cortical connectivity (Pearson et al., 1985; Arendt, 2004). In this 'last in-first out' model, the latest-maturing regions are least myelinated and structurally most plastic (Flechsig, 1920; Brun and England, 1981; Kapfhammer and Schwab, 1994; Arendt et al., 1995a,b,c; 1998a). This plasticity comes at a price; it imparts vulnerability to pathological changes and its failure may give rise to neurodegeneration as seen in AD (Arendt, 2004; Peretti et al., 2015). In this slowly manifesting disorder, aberrant sprouting precedes tangle formation and neuronal death (Ihara, 1988; Arendt and Bruckner, 1992; Su et al., 1993). Hypometabolic states occur even in pre-symptomatic stages and they predict cognitive decline (Stieler et al., 2011). Early adaptive and reversible down-regulation of synaptic contacts turns into marked and irreversible synaptic loss (Rapoport, 1999a). By contrast, the brain of a 'perfect hibernator' (in which torpor is interrupted by frequent inter-bout euthermic arousals (Drew et al., 2001)) offers an elegant solution to

cerebral metabolic compromise through repeated synaptic regeneration. However, Alz50-positive pre-tangles are detected in the brain of the American black bear (*Ursus americanus*) – an 'imperfect' but obligatory hibernator that undergoes prolonged, continuous periods of torpor (Stieler et al., 2011). Thus, the reversibility of torpid tau modulation may be limited, reflecting the higher temperature at which it must operate (and its extended duration) in larger mammals and/or the differing profile of tau isoforms and their site-specific phosphorylation in the adult brain (Janke et al., 1999; Su et al., 2008; Stieler et al., 2011; Tøien et al., 2011; Leon-Espinosa et al., 2013). The development of NFT pathology in aged bears supports this hypothesis (Cork et al., 1988; Stieler et al., 2011), but (as discussed previously) this is not proof of a maladaptive response.

### 1.5.3 Adaptive neuronal plasiticity

Even though the human sleep-wake cycle involves circadian fluctuations in brain temperature (Landolt et al., 1995; Baker et al., 2001; Krauchi and Deboer, 2010; Archer et al., 2014) its analogy to hibernation is questionable; sleep is required in rodents during arousals from torpor - which are metabolically expensive and exceed the BMR (Daan et al., 1991; Su et al., 2008; Avila et al., 2012; Krauchi and Deboer, 2010; Boerema et al., 2012). Likewise in man, sleep can be thought of as a recurrent plastic state for remodelling neural circuits and is an active process (Wang et al., 2011). Indeed, sleep has been likened to an 'abridged version' of neurodevelopment, rehearsing the activity-dependent pruning and refining of synaptic contacts that must occur after an increase in synaptic density (Wang et al., 2011). The early-onset sleep disorders noted in AD and other neurodegenerative diseases could thus derive from,

as well as exacerbate, synaptopathy (Gemignani et al., 2005; Gagnon et al., 2006; De Cock et al., 2007; Compta et al., 2009; Fotuhi et al., 2009; Wang et al., 2011). The importance of tau to sleep quality is evident in tau knockout mice which demonstrate similar sleep disruptions to AD patients including reduced slow wave sleep (SWS) time (Cantero et al., 2010). Rapid eye movement (REM) sleep deprivation also produces region-specific changes in rat brain tau, most notably in the hippocampus (Rodriguez-Vazquez et al., 2012). Unsurprisingly, the neurogenic properties of melatonin are mediated through cytoskeletal rearrangements; through its influence on kinase-phosphatase balance this hormone can attenuate tau hyperphosphorylation and may have therapeutic potential in AD (Wang and Wang, 2006). Moreover, sleep quantity is highest in newborn mammals where, like tau, sleep is critical for maturation of neuronal networks (Wang et al., 2011). These studies further support a role for tau in adaptive neuroplasticity-associated neuroprotection in hibernating mammals. The repeatable nature of tau changes during torpor followed by their resolution also lends itself to the concept of preconditioning. Biochemically, the brains of hibernators have been likened to a 'pre-conditioned state', associated with reprogramming of the cytotoxic cellular response (Stenzel-Poore et al., 2003; Dave et al., 2012). However, ischaemia tolerance pathways are chronically active in euthermic hibernators which circumvents the need for a sublethal stimulus (Dave et al., 2012). In summary, studies of the hibernation cycle confirm that reversible tau hyperphosphorylation is a fundamental characteristic of the hypometabolic state rather than a sentinel of impending pathology (Stieler et al., 2011).

### 1.5.4 Modelling hypothermic tau modulation

Hypothermia-induced tau hyperphosphorylation has been replicated in a multitude of model systems from cell cultures to brain slices and live rodents – including those carrying tau mutations (Bretteville et al., 2012; Maurin et al., 2014; Ahmadian-Attari et al., 2015). Cooling has even been proposed as a tool to screen for tau kinase and protease inhibitors and as a standard positive control for tau hyperphosphorylation (Bretteville et al., 2012; Nikkel et al., 2012; Petry et al., 2014). Moreover, hypothermia is routinely used to improve the viability of cultivated organotypic brain slices, even those harvested from P301S mice (Mewes et al., 2012). The hibernation-associated spine regression, changes in post-synaptic density and reversible tau hyperphosphorylation can thus be mimicked using cooling paradigms in laboratory rodents and brain slices (Arendt and Bullmann, 2013; Peretti et al., 2015). Tau phosphorylation is exquisitely sensitive to temperature, roughly increasing by 80% for every degree Celsius drop below 37°C in mice (Planel et al., 2007a; Papon et al., 2011; El Khoury et al., 2014). However, the problem with exploring tau-mediated hypothermic protection in animal models of neuronal injury is that anaesthesia may confound the data (Planel et al., 2007a; El Khoury et al., 2014). In the critical care setting, patients are not anaesthetised prior to neuronal injury and anaesthetics not only induce hypothermia, they have direct effects on tau phosphorylation state (Ikeda et al., 2007; Planel et al., 2007a; Whittington et al., 2011; Chen et al., 2014). This has raised concern about a potential link with post-operative cognitive dysfunction in geriatric medicine (Baranov et al., 2009; Planel et al., 2009; Spires-Jones et al., 2009; Run et al., 2010; Tan et al., 2010; Hudson and Hemmings, 2011; Papon et al., 2011; Dong et al., 2012; Menuet et al., 2012; Xu et al., 2012; Hussain et al., 2014). Tau

hyperphosphorylation may be responsible for anaesthetic interference of neuronal polarity in developing cortical neurons, the age-dependent impact of propofol on dendritic spine density in the rat cortex and propofol-induced reorganization of the neuronal cytoskeleton (Oscarsson et al., 2001; Briner et al., 2011; Mintz et al., 2012). Moreover, it might explain the acute neuroprotective benefits of propofol in the context of hyperthermia, oxygen-glucose deprivation and ischaemia (Yamaguchi et al., 1999; Adembri et al., 2006; Iijima et al., 2006; Zhao et al., 2009). The relationship of brain temperature to anaesthesia is however not straightforward, since some compounds increase intracranial temperature relative to body temperature (Mellergard, 1992). Arguably this might worsen cognitive outcome in individuals that are already compromised - potentially manifesting in delirium (Poljak et al., 2014). In turn, poor temperature control can complicate the investigation of tau phosphorylation in other contexts (El Khoury et al., 2014). Co-morbid synergy has been proposed to link diabetes mellitus and AD wherein insulin-mediated hypothermia leads to tau hyperphosphorylation and NFT deposition (Planel et al; 2004; Planel et al., 2007b; Ke et al., 2009; El Khoury et al., 2014). These and other studies support the idea that endocrine and thermo-dysregulation serve as age-associated risk factors for dementia (Avila and Diaz-Nido, 2004; El Khoury et al., 2014; Maurin et al., 2014). However, it is important to note that direct and indirect routes to hypothermia may produce tau hyperphosphorylation via distinct mechanisms that could influence whether the outcome is beneficial or deleterious (Planel et al., 2004; Schubert et al., 2004; Avila and Diaz-Nido, 2004)

Post-translational modifications of several proteins have been reported under hypothermic conditions and linked to neuroprotective effects (Han et al., 2012). Mild, neuroprotective hypothermia reduced NMDAR phosphorylation and oxidative carbonyl modification of cytosolic proteins in a piglet model of hypoxia-ischaemia (HI) (Mueller-Burke et al., 2008). The authors hypothesised that soluble cytoskeletal proteins such as tau were the target of carbonyl modification during HI and that cooling renders them resistant to this change. Hyperphosphorylation – by far the most widely explored tau modification at low temperatures – releases tau from microtubules and is the likely mechanism for hypothermic microtubule disassembly (Higuchi et al., 2002). This microtubule dynamism may be highly neuroprotective, as suggested by the resistance of tau knockout hippocampal neurons to Aβ-induced neurodegeneration (Rapoport et al., 2002). Microtubules invade dendritic spines on an activity-dependent basis and may be critically involved in memory (Hu et al., 2008; Gu and Zheng, 2009; Fanara et al., 2010). Furthermore, it is now accepted that tau directly plays a role at the synapse under both physiological and pathological conditions (Hoover et al., 2010; Ittner et al., 2010; Chen et al., 2012b; Crimins et al., 2013). The presence of both tau and PP2A within synaptosomal fractions was described some years ago (Yamamoto et al., 1990), and more recently high levels of tau have been reported in 75% of normal and AD synapses (Sokolow et al., 2015). Excitatory synaptic activation induces translocation of dendritic tau into the post-synaptic density (PSD) and this response is disrupted by exposure to Aβ oligomers (Frandemiche et al., 2014). Oligomeric and hyperphosphorylated tau co-localises with Aβ in synapses of both AD patients and mouse models of this disease (Fein et al., 2008; Takahashi et al., 2010; Henkins et al., 2012; Tai et al., 2012; Tai et al.,

2014) and injection of pre-filamentous tau oligomers into wild-type mice reduces hippocampal spine density (Lasagna-Reeves et al., 2011). Other studies in tau transgsenic mice have likewise implicated soluble rather than fibrillar tau in synaptic dysfunction, spine loss and impairment of neuronal circuitry (Rocher et al. 2010; Fox et al., 2011; Crimins et al., 2013). Amyloidogenic and missorted tau can clearly precipitate dendritic spine and synaptic loss (Eckermann et al., 2007; Zempel and Mandelkow, 2011) and mutant tau can produce pre-synaptic pathology (Harris et al., 2012). However these changes were not originally found to cause neurodegeneration or cognitive deficits in mice (Eckermann, 2007; Harris et al., 2012). This may have been because the hyperphosphorylation of tau and its relocation into dendritic spines is a very early change that precedes the loss of neurons, and even synapses (Hoover et al., 2010). Compensatory increases in synapses in unaffected neurons may mask cognitive change and artificially buffer total synapse numbers in the initial stages of disease (Kopeikina et al., 2012). This is evident in the rTg4510 cortex where neurons undergo structural and functional compensation in response to degeneration (Crimins et al., 2011, 2012, 2013). From a recent study of human post- mortem AD tissue, it is apparent that the presence of AT8 or PHF-1 positive tau is not necessarily associated with the loss of dendritic spines, even in the intermediate stages of neurofibrillary pathology (Merino-Serrais et al., 2013). This is consistent with the notion that tau hyperphosphorylation is not immediately toxic and may be reversed prior to the development of tangles. In P301L mice, redistributed tau does impair cognition but this may be due to disrupted recruitment of glutamate receptors in intact synapses (Hoover et al., 2010). Using the same transgenic model, Hunsberger et al. (2014) found an increase in glutamate release and concomitant decrease in

glutamate clearance mechanisms that manifested in hippocampal hyperexcitability. Such a mechanism might explain the apparently critical role for tau in epilepsy (Holth et al., 2013). More detailed analysis of the 'pro-aggregation' mutant mice revealed neuronal loss as well as NMDAR-dependent LTP and memory impairment in line with NFT formation (Mocanu et al., 2008; Sydow et al., 2011). After turning off the transgene, memory and LTP recovered, despite the persistence of aggregates, suggesting that expression of amyloidogenic tau rather than aggregates themselves disrupted cognition (Sydow et al., 2011). Further support for tau involvement in plasticity comes from its interaction with other dendritic spine components including F-actin (Arendt and Bullmann, 2013). Moreover, tau was found to be necessary for BDNF-induced spine remodelling in rat hippocampal cultures (Chen et al., 2012b). The association of tau with neuronal membranes is both phosphorylation- and fyn tyrosine kinase-dependent (Pooler et al., 2012). Interaction of tau with fyn and PSD95 is regulated by selective phosphorylation of tau in response to NMDAR activation, which may represent a tau-mediated homeostatic feature that prevents 'over excitation' (Mondragon-Rodriguez et al., 2012). However, in the presence of Aβ or reduced PP2A activity, this cooperation of tau with glutamate receptors could become the very conduit of neurotoxicity (Roberson et al., 2007; Ittner et al., 2010; Ittner et al., 2011; Ke et al., 2012; Larson et al., 2012; Lee et al., 2012; Sun et al., 2012; Kamat et al., 2013; Nakanishi et al., 2013; Miller et al., 2014). Under physiological conditions, Arendt and Bullmann (2013) have proposed that by targeting the protein to subsynaptic sites, phosphorylation enables tau to act as a 'master switch' regulating NMDAR-driven synaptic gain. Such a mechanism may be permissive for torpor entry, through reducing the metabolic demands of the brain

(Boerema et al., 2012); its persistence early in the course of AD may be an attempt to counter excitatory-inhibitory imbalance (Morris et al., 2011; Arendt and Bullmann, 2013). Together these studies indicate that early and subtle effects of tau modulation at the level of the cell, or the neuronal network may be clinically very important, and potentially tractable to therapy (Morris et al., 2011).

### 1.5.5 Phospho-tau-mediated neuroprotection

In cultured rodent neurons, glutamate-mediated $Ca^{2+}$ influx can modulate dendritic outgrowth, synaptogenesis and degeneration – it also increases tau mRNA expression and produces NFT-like tau phosphorylation (Mattson et al., 1988; Mattson et al., 1989; Mattson, 1990; Esclaire et al., 1998). PHFs can also be observed in cultured human foetal spinal cord neurons exposed to high concentrations of glutamate (De Boni and McLachlan, 1985). Several other excitotoxins including NMDA and quinolinic acid (QA) can produce similar hyperphosphorylation of tau in human neurons and QA can even induce dendritic beading (Rahman et al., 2013). This beading is associated with NFTs in AD and typically features disrupted microtubules (Greenwood and Connolly, 2007). Mattson (1990) correlated ubiquitin and phospho-tau immuoreactivity with degenerative morphology in hippocampal neurons that were susceptible to glutamate toxicity. However, he also noted that post-translational changes in tau occurred very rapidly at subtoxic doses of glutamate in neurons that showed no signs of degeneration; phospho-tau immunoreactivity was highest in the distal axon – the compartment least susceptible to excitotoxic injury (Mattson, 1990). In the study by Rahman and colleagues (2013), the effect of QA on tau was associated with a decrease in PP2A expression and activity, and was

completely abrogated by memantine – a non-competitive NMDAR channel blocker that at low doses selectively blocks extrasynaptic NMDARs and has potential disease-modifying benefits in AD (Reisberg et al., 2003; Li et al., 2004; Hardingham and Bading, 2010). Further studies linking tau phosphorylation to neuroprotection include the exposure of primary rat cortical cultures to NMDA, serum deprivation and brefeldin-A; neurons expressing AT8 were more resistant to induced apoptosis than those expressing dephosphorylated tau (Lesort et al., 1997; Yardin et al., 1998). Others have found that PP2A-mediated tau dephosphorylation is required for apoptotic execution and that phospho-tau mediates early apoptotic escape in the brain, thus enabling a slower, degenerative neuronal death (Mills et al., 1998; Li et al., 2007; Wang and Liu, 2008; Wang et al., 2014). This phospho-tau mechanism might underlie the relative absence of classical apoptotic features in AD and other tauopathies (Wang and Liu, 2008). Accordingly, overexpression of GSK3β increased tau phosphorylation but prolonged survival in P301L mice (Crespo-Biel et al., 2014). Tau phopshorylation has also been proposed as a protective response to oxidative stress; oxidative damage is one of the earliest events in AD and has an inverse relationship with progressing NFT formation (Nunomura et al., 2001; Smith et al., 2002; Lee et al., 2005; Castellani et al., 2008; Wang and Liu, 2008; Bonda et al., 2011; Povatello et al., 2013). Oxidative stress also enhances tau phosphorylation and embryonic neurons that survive oxidant exposure have relatively high phospho-tau immunoreactivity (Gomez-Ramos et al., 2003; Lee et al, 2005; Wang and Liu, 2008). These findings are difficult to align with previous literature linking tau phosphorylation to neuronal death (Cowan and Mudher, 2013); it might be that distinct tau phosphorylation events mediate protection and toxicity (Povellato et al.,

2013). Together, the literature suggests that in the earliest stages of excitotoxic or oxidative stress, tau phosphorylation increases in susceptible neurons. If the insult is sublethal, phospho-tau may be involved in preconditioning this cell to tolerate further insults. If the insult is too great, the effect of tau is overridden and the cell degenerates. Degeneration over a protracted period would enable irreversible modification of tau into insoluble forms that, in a compromised cell, would be non-degradable.

### 1.5.6 Modelling human tau physiology

Currently, there is no *in vivo* model system that permits the study of wild-type amyloid plaques alongside wild-type NFTs (Clavaguera et al., 2013). No naturally-evolving counterpart to AD has yet been fully described elsewhere in the animal kingdom, despite overlapping clinicopathogenesis for other neurodegnerative disorders between man and other mammalian species (Iqbal et al., 2009; Darusman et al., 2014). The latest '3D *in vitro* model of AD' (Choi et al., 2014) typifies the aspiration to replicate neuropathological mechanisms in human cell culture systems. Beside the fact that these systems cannot faithfully express key symptomatic features of dementia, they have almost exclusively focused on reproducing post-mortem observations, often by overexpressing the proteins of interest (Choi et al., 2014). Moreover, these studies frequently lack quantitative assessment of tau modulation and any physiological relevance of such findings in terms of neuronal and glial functional properties (Shi et al., 2012a; Choi et al., 2014). The exploitation of hPS technology to better approximate human neuroglial biology is both rational and worthwhile. However, despite the large body of evidence supporting an early and

clinically-relevant role for tau and other proteopathic oligomers in synaptic dysfunction (Warmus et al., 2014), there remains an unfortunate preoccupation with generating inclusions (Choi et al., 2014 ). The same criticism could be made of recent *in vivo* models (Clavaguera et al., 2009; de Calignon et al., 2012; Liu et al., 2012a; van Eersel et al., 2015; Boluda et al., 2015). What is missing is real-time analysis of the basic functionality of these disease-associated proteins in human brain cells. What is needed is a methodical approach to understanding human tau biology.

## 1.6 ER stress, the UPR and tau proteostasis

### 1.6.1 Mechanisms of proteostasis

Protein aggregation is the most unifying pathological feature of adult-onset neurodegeneration and is intimately linked to age-related dysfunction of cellular quality-control pathways and protein homeostasis or 'proteostasis' (Holmes and Diamond, 2014). In eukaryotic cells, proteome integrity is continually monitored and maintained by an elaborate network of molecular chaperones and protein degradation factors (Chen et al., 2011). Depending on the severity of protein misfolding, there are 3 strategies that can refold, degrade or sequester misfolded polypeptides (Powers et al., 2009). Although these parallel mechanisms may be spatially separated, they are interlinked by a core set of chaperones that 'triage' the aberrant protein and guide it to the appropriate pathway (Chen et al., 2011a). These strategies each carry advantages and relative risks (Fig.1.12); failure of one or more pathways will thus perturb proteostasis and mobilize cellular stress responses such as the UPR (Chen et al., 2011b). Overexpression of chaperones, activation of

autophagy and/or the proteasome have all mitigated the symptoms of disease-related protein aggregates, confirming that toxic aggregates impair quality control pathways required for proteostasis (Chen et al., 2011).

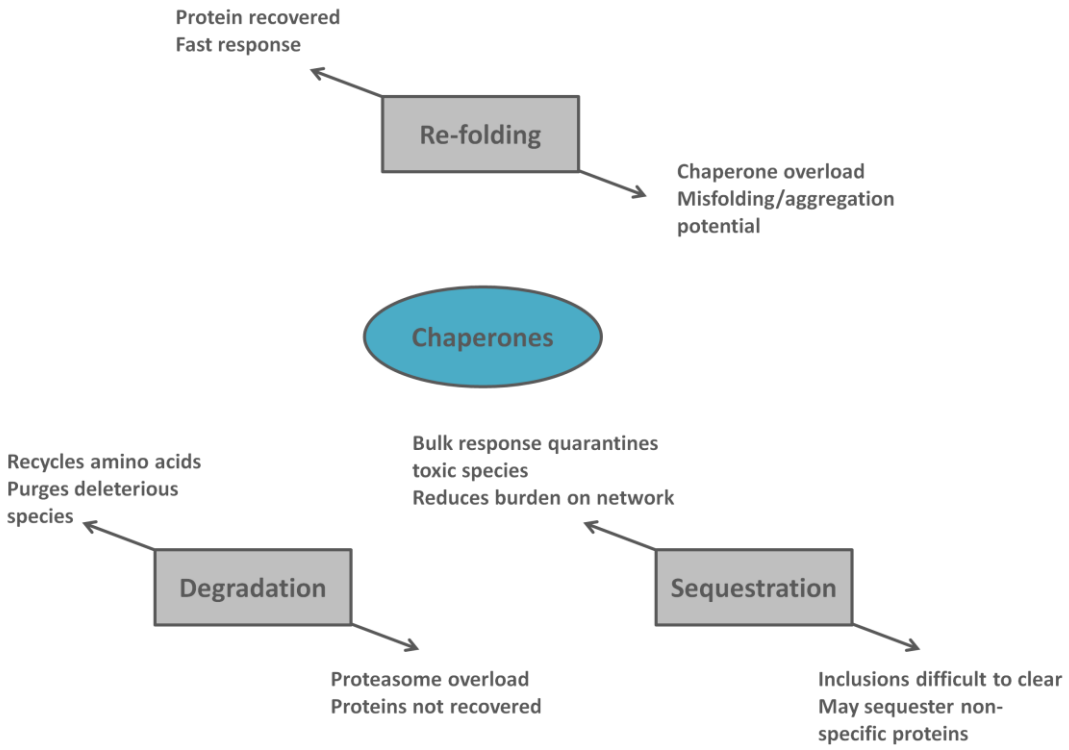


**Figure 1.12 Cellular strategies for proteostasis.** Each strategy presents advantages and drawbacks. Misfolded proteins can be refolded, degraded, or chaperoned to distinct quality control compartments that sequester potentially toxic species. Molecular chaperones ensure the system remains balanced. A failing in any of these strategies disrupts proteostasis and impairs cell viability. Figure and legend adapted from Chen et al. 2012.

### 1.6.2 ER stress and hyperphosphorylated tau

Accumulation of misfolded proteins initially triggers the induction of heat shock proteins (Hsps) – these molecular chaperones can assist in refolding the protein and thus restoring its normal biological function, whilst preventing its aggregation (Dou et al., 2003; Richter-Landsberg and Goldbaum, 2003). If the misfolding events are irreversible, these chaperones target the substrate either to the ubiquitin-proteasome

system (UPS) (for soluble species) or the autophagy-lysosomal pathway (for aggregates) (Chen et al., 2011). Together these represent the major routes for clearance of damaged, misfolded and aggregation-prone proteins (Schwartz and Ciechanover, 1999; Wang and Liu, 2008). These degradation pathways are functionally related, with impairment of one inducing compensatory activation of another (Chen et al., 2011). For example, secretory proteins that fail to fold in the ER can be retrotranslocated into the cytosol for UPS degradation – this is termed ER-associated degradation (ERAD) (Wong and Cuervo, 2012). Inhibition of the UPS blocks retrotranslocation and leads to a back-up of unfolded proteins in the ER and thus ER stress (Wong and Cuervo, 2012). Since the proteolytic core of the proteasome can be clogged up by PHF-tau, ERAD impairment has been suggested as a means by which accumulated tau leads to UPR activation (Keck et al., 2003; Abisambra et al., 2013). Lastly, as an ATP-dependent proteolytic system the UPS is energetically demanding, thus under conditions of metabolic compromise, this pathway may become malfunctional (Hoglinger et al., 2003; Jellinger, 2010). Conceivably, this may be one route through which hypothermia might trigger ER stress .

The ER is important for the synthesis, folding and post-translational modification of transmembrane and secreted proteins (Jellinger, 2010). Factors that perturb ER function and contribute to ER stress include increased protein synthesis, the expression of mutant or misfolded proteins, misfolding rates that exceed chaperone capacity, disturbances in calcium homeostasis or redox balance, and nutrient/glucose deprivation (Kaufman et al., 2002; Tabas, 2011). ER stress contributes to a number

of neurological disorders including AD, PD, cerebral ischaemia and acute and chronic neuronal injury after TBI (Larner et al., 2006, Begum et al., 2014). The UPR (or ER stress response) is directed by 3 ER membrane-associated signal transducers: inositol requiring enzyme 1α (Ire1α), protein kinase R (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (Malhotra and Kaufman, 2011). Under basal conditions, these transducers are held in an inactive state by their interaction with Hsp70 protein 5 (otherwise known as binding immunoglobulin protein or 78 kDa glucose-regulated protein (BiP/GRP78)) – BiP is the principal sensor of ER stress (Bertolotti et al., 2000). Upon binding to the exposed hydrophobic domains of misfolded proteins within the ER lumen, BiP releases the 3 proximal transducers, thus triggering a tripartite signalling cascade (Malhotra and Kaufman, 2011). The UPR has several outcomes that are coordinated to restore proteostasis including reduced translation, mRNA degradation, transcriptional upregulation of chaperone genes and (if required after prolonged or excessive ER stress), programmed cell death (Taylor and Dillin, 2011). The downstream effectors of the UPR are summarized in Fig.1.13 and are discussed in further detail in Chapter 6. The ability to mount this response as well as the heat shock response varies according to cell type (even neuronal subtype) and declines with age (Cleveland et al., 2001; Batulan et al., 2003; Mayer and Bukau, 2005; Taylor et al., 2007; Ben-Zvi et al., 2009; Hashimoto-Torii et al., 2014).

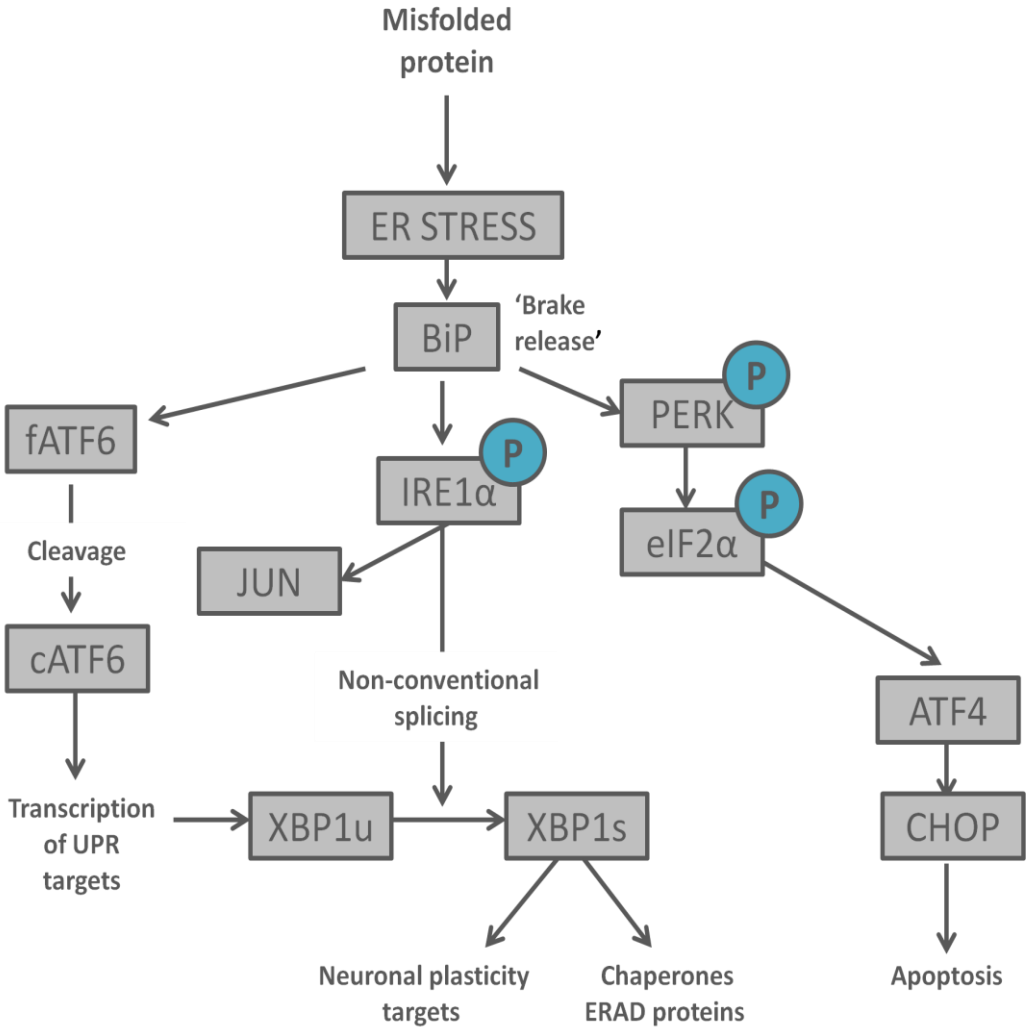


**Figure 1.13 ER stress and the UPR.** Misfolded proteins are detected by the ER which triggers the 3 main pathways of the UPR. BiP = binding immunoglobulin protein, fATF6 = full length activating transcription factor 6, cATF = cleaved ATF6, IRE1α = inositol requiring enzyme 1α, JUN = c-Jun (proto-oncogene), XBP1u = unspliced x-box binding protein-1, XBP1s = spliced XBP1, PERK = protein kinase R (PKR)-like ER kinase, eIF2α = eukaryotic initiation factor 2α, ATF4 = activating transcription factor 4, CHOP = C/EBP homologous protein (otherwise known as DNA damage-inducible transcript 3, DDIT3), ERAD = ER-associated degradation. 'P' symbols within aqua circles represent phosphorylated versions of each element.

As an unfolded soluble protein, tau may be degraded by the core (20S) of the proteasome, operating independently of ubiquitin (David et al., 2002; Wang and Liu, 2008). In its unbound state, tau directly associates with Hsp70 and 90, which promote tau solubility and microtubule binding and reduce tau phosphorylation (Dou

et al., 2003). Beyond this, there is debate over whether tau is degraded by proteasomal or autophagic pathways – perhaps it may both, depending on its conformational state (Pritchard et al., 2011; Mandelkow and Mandelkow, 2012). Certainly the repeat domain of tau contains motifs that could target it for autophagy (Wang et al., 2009). However in AD, hyperphosphorylation acts as a recognition signal for the carboxyl terminus of heat-shock cognate (Hsc)70-interacting protein (CHIP) (a ubiquitin E3 ligase) that together with Hsp70 targets tau to the proteasome (Petrucelli et al., 2004; Sahara et al., 2005; Shimura et al., 2004; Dickey et al., 2007; Wang and Liu, 2008; Mandelkow and Mandelkow, 2012). The Hsp70-CHIP complex binds to the same tau motif (VQIVYK) that is responsible for β-sheet propensity and aggregation and is important for the regulation of tau turnover (Petrucelli et al., 2004; Sarkar et al, 2008; Mandelkow and Mandelkow, 2012). This appears to be protective, since ubiquitination by CHIP can rescue cells from tau-induced cell death (Wang and Liu, 2008). However, the Hsp70-CHIP-tau interaction is vulnerable to ER stress in tauopathies and the capacity of the UPS to handle phosphorylated tau may be further dictated by the specific kinases involved in tau phosphorylation (Blard et al., 2006; Sakagami et al., 2013). Indeed, the presence of polyubiquitinated tau within NFTs suggests that this aggregated tau accumulates due to a deficit in proteasome activity (Braak and Braak, 1991; Keller et al., 2000; Goldbaum et al., 2003; Cripps et al., 2006; Williams et al., 2006; Wang and Liu, 2008). This is also true of the synaptic compartment in AD, where hyperphosphorylated tau oligomers are associated with increased ubiquitinated substrates and proteasome components (Tai et al., 2012). *In vitro*, PHF-tau binds to and inhibits the proteasome, suggesting that hyperphosphorylated and aggregated tau

accelerates its own production (Keck et al., 2003; Wang and Liu, 2008). Such a mechanism may underlie the feed-forward cycle described by Ho et al. (2012) when they inhibited PP2A in rat cortical neurons, and it could also explain the 'trans-synaptic' spread of tau pathology if toxic soluble species transduced a cycle of ER stress into adjacent cells (Stoveken, 2013). Moreover, substrates or UPS components can translocate into and out of the synapse in an activity-dependent manner and the sequestration of ubiquitin by multiple protein aggregates may thus impair the housekeeping function of the UPS within the synapse (Bingol and Schuman, 2006; Tai and Schuman, 2008; Gillingwater and Wishart, 2013). At this point, it is worth noting that enhanced re-synthesis of ubiquitin may contribute to hypothermic neuroprotection – this is consistent with the finding that cooling blocked protein ubiquitination in the PSD of the rat brain following hypoxic asphyxia (Yamashita et al., 1991; Capani et al., 2009). Furthermore, in transgenic mice, overexpression of Hsp70 causes a reduction in tau (Petrucelli et al., 2004; Iqbal et al., 2009), there is an inverse relationship between levels of CHIP, Hsp70/90 and aggregated tau in AD brains, (Dou et al., 2003; Sahara et al., 2005) and in human neuroglioma cells, CHIP can rescue the pathological effects of tau overexpression (Saidi et al., 2014). Together, these studies suggest that a deficit in first-order protein maintenance exists in tauopathy.

### 1.6.3 UPR activation in tauopathy

Evidence for a link between tau accumulation and the UPR comes from several studies showing that pre-tangle neurons and glia from tauopathies such as AD, PSP and FTD have increased levels of phospho-PERK (Hoozemans et al., 2009; Nijholt

et al., 2012; Halliday and Mallucci, 2014). In addition, a genome-wide association study identified a single nucleotide polymorphism within intron 2 of the PERK gene as a risk factor for PSP (Hoglinger et al., 2011). Moreover, sustained ER stress with UPR activation was associated with an increase in phosphorylated tau in a rat model of TBI (Begum et al., 2014). The induction of ER stress by exposure of cells to Aβ oligomers is $Ca^{2+}$-mediated and correlates with the induction of tau phosphorylation (Resende et al., 2008). ER stress can also promote tau hyperphosphorylation through BiP-mediated enhancement of GSK3β binding to tau, (Fu et al., 2010; Liu et al., 2012b). However, BiP maintenance may contribute significantly to neuronal survival - BiP protected rat hippocampal neurons against excitotoxicity and apoptosis via suppression of oxidative stress and stabilization of calcium homeostasis (Yu et al., 1999). Furthermore, the metabolic stress-induced activation of the UPR seen in torpor increases tau phosphorylation – and this is thought to be part of an adaptive response (van der Harg et al., 2014). During normal metabolism a fraction of endogenous human tau is actively released from cells via a non-conventional secretory pathway that is calcium dependent but does not involve the ER (Karch et al., 2012). However, missorted hyperphosphorylated tau associates with the dendritically located rough ER and may lead to protracted ER stress and neural demise (Kim et al., 2008; Conde and Caceres, 2009; Iqbal et al., 2009). On the contrary, hyperphosphorylated tau was shown to attenuate ER-stress-induced apoptosis through upregulation of the UPR (Liu et al., 2012c). Interestingly, the phospho-tau inducing compound DMSO was also shown to increase Hsp70 and decrease NFκB expression in a rat model of haemorrhagic shock (Bini et al., 2008). With regard to tauopathies, it has been proposed that the UPR is activated at the pre-

tangle stage and could initially be protective, but if persistent, would promote further tau hyperphosphorylation and neurodegeneration (Kohler et al., 2014). A challenging question is whether the UPR acts up- or downstream of tau pathology (Hoozemans and Scheper, 2012) – and yet the loop-like nature of proteostatic responses renders this question obsolete. Together the literature suggests that phospho-tau resides at the intersection of neuronal survival and death pathways and is thus an ideal candidate to participate in hypothermic preconditioning.

### 1.6.4 Proteostasis under hypothermic conditions

Protein synthesis is the most energy-consuming process in the cell, and the high metabolic rate of the neuron places substantial burden on protein synthesis machinery (Buttgereit and Brand, 1995; Bottley et al., 2010; Hofman et al., 2012). Since cold-shock reduces metabolic turnover, translational suppression is a prerequisite for survival under hypothermic conditions (Roobol et al., 2009; Hofman et al., 2012). In mammalian cells this occurs via several partially-redundant pathways (Hofman et al., 2012). Passive mechanisms include enzymatic and mitochondrial inhibition along with a fall in ATP levels (Hofman et al., 2012). Active mechanisms involve regulation of adenosine monophosphate-activated protein kinase (AMPK), target of rapamycin complex 1 (TORC1) and PERK-dependent phosphorylation of eukaryotic initiation factor 2α (eIF2α) (Hofman et al., 2012). The last of these suggests activation of the UPR, which might derive from ER stress triggered by cooling (Harding et al., 1999; Hofman et al., 2012; Mollereau, 2015; Rzechorzek et al., 2015). In the context of ischaemic preconditioning, the primary stress needs to be sufficient to induce Hsps (Kitigawa et al., 1991). It

follows then that to elicit preconditioning, hypothermia should also upregulate these chaperones. Cold-shock can certainly induce unfolding, dissociation and inactivation of cellular proteins (King and Weber 1986; Liu et al., 1994; Fujita et al., 1999). Thus, counter-intuitively, the cold-shock response in human cells also upregulates Hsps and a specific increase in Hsp70 may contribute to the neuroprotective effect of hypothermia (Jones and Inouye, 1994, Fujita, 1999; Terao et al., 2009). Accordingly, restoration of BiP is one mechanism by which hypothermia can protect the ischaemic brain (Aoki et al., 2001; Shintani et al., 2010). Deep hypothermia is also known to disrupt the cell secretory pathway (Saraste et al., 1986) which would result in misfolded or unfolded proteins in the ER (Kim et al., 2008; Begum et al., 2012).

### 1.6.5 The UPR as a therapeutic target

Several animal models of neurodegeneration display upregulation of ER stress markers including rTg4510 mice, mice expressing 5 AD linked mutations (5x FAD), mutant SOD1 mice and mutant Huntingtin mice (Halliday and Mallucci, 2014). UPR activation in tauopathies occurs at an early stage of neurofibrillary pathology; it is intimately connected with the accumulation of soluble phosphoryated tau before inclusions form and is independent of Aβ, since it occurs in PSP, PiD and FTDP-17 (Nijholt et al., 2012; Hoozemas and Scheper, 2012; Stutzbach et al., 2013). It also appears to be unrelated to ageing since it arises in relatively young FTDP-17 patients (Nijholt et al., 2012; Ferreiro and Pereira, 2012). Sustained UPR activation can exacerbate tau phosphorylation and neurodegeneration (Hoozemans et al., 2009). Unsurprisingly therefore, various disease models have shown that alleviating ER

stress can be neuroprotective (Begum et al., 2014). Salubrinal - a specific inhibitor of eIF2α phosphatases – increases phospho-eIF2α levels, thereby decreasing new protein synthesis and reducing protein load into the ER (Boyce et al., 2005; Drexler, 2009). This compound can protect against oxidative and excitotoxic stress in cultured neurons and temporarily renders motor neurons resistant to hypoxia-induced injury (Sokka et al., 2007; Lewerenz and Maher, 2008; Zhu et al., 2008). Salubrinal can also ameliorate the cellular response to Aβ and has a profound positive effect on cell tolerance to cold storage (Lewerenz and Maher, 2008; Corwin et al., 2014). Even in the context of chronic ER stress induced by α-synuclein aggregates, salubrinal can prevent accumulation of toxic α-synuclein oligomers (Colla et al., 2012). However, the same compound exacerbated neurotoxicity and reduced survival in prion diseased mice (Moreno et al., 2012). This is because misfolded prion protein (PrP) causes persistent UPR-mediated translational repression of global protein synthesis, leading to the loss of key synaptic proteins and thus neurodegeneration (Moreno et al., 2012). Using this prion disease model, Moreno et al. (2012, 2013) showed that PERK inhibition or overexpression of growth arrest and DNA damage 34 (GADD34) could promote translational recovery by reducing phospho-eIF2α levels. This not only prevented neurodegeneration, it improved clinical outcome later in the disease course despite continued accumulation of misfolded PrP. The authors suggested that similar benefits might be achievable in other proteinopathies and indeed, inhibiting eIF2α phosphorylation has rescued TDP-43 toxicity in ALS disease models and synaptic plasticity in the APP/Presenilin 1 AD model (Kim et al., 2014; Ma et al., 2013). PERK haploinsufficiency also rescued memory deficits and cholinergic neurodegeneration in 5 x FAD mice and

pharmacologically reversing the effects of eIF2α phosphorylation improved spatial and fear-associated learning (Sidrauski et al., 2013; Devi and Ohno, 2014). This is consistent with the literature reporting that stimuli which increase synaptic strength simultaneously decrease the phosphorylation of eIF2α (Halliday and Mallucci, 2014). Notably, a study in mice suggested that a decrease in eIF2α phosphorylation was critical for gene induction leading to long term synaptic changes required for the formation of memories (Costa-Mattioli et al., 2007). Interestingly, whilst ER stress markers are prominent in AD, activated PERK pathway components are not seen in human prion disease unless neurofibrillary pathology is present, suggesting a critical role for tau in UPR induction (Unterberger et al. 2006). Although GADD34-mediated reactivation of translation can be beneficial, if too aggressive, it will lead to C/EBP homologous protein (CHOP)-induced cell death. This was demonstrated in a mouse model of Charcot-Marie Tooth disease (CMT) where restricting GADD34 activity improved myelination of peripheral nerves (D'Antonio et al., 2013). Similarly, inhibiting eIF2α dephosphorylation with guanabenz improved motor performance, attenuated motor neuron loss and improved lifespan in a mouse model of familial ALS (Jiang et al., 2014). The latest generation of GADD34 inhibitors, Sephin 1, recently demonstrated therapeutic potential in mouse models of both CMT and ALS in a single study (Das et al., 2015). Lastly, the compound described by Sidrauski et al. (2013) reduced cell viability in the face of chronic ER stress. Therefore, the correlation of GADD34 immunostaining with ischaemic damage in the human hippocampus after cardiac arrest could equally represent a pro-survival or pro-death response (White et al., 2004). These paradoxical results highlight the delicate balance that must be achieved by the UPR in order to safeguard proteostasis.

Although misfolded protein aggregates are central to a range of debilitating disorders, the sequestration of toxic intermediates into insoluble inclusions can reduce their toxicity and even reverse proteasomal dysfunction (Arrasate et al., 2004; Bodner et al., 2006). Indeed, the concentration of soluble misfolded proteins to enhance their refolding or degradation may be more treacherous, given that aggregation is largely concentration-dependent (Eckermann et al., 2007; Chen et al., 2011). Despite the cognitive advantages achieved *in vivo* through UPR manipulation (Halliday and Mallucci, 2014), it is almost inconceivable that simply perpetuating protein load on the one hand, or inhibiting protein synthesis on the other would be beneficial in the long term. Either of these approaches might be improved by simultaneously targeting chaperones, such as BiP (Morris et al., 1997; Jin et al., 2000; Moreno et al., 2013). Strictly speaking, chaperones 'neutralize' the toxic intermediate rather than actually preventing inclusion formation (Chen et al., 2011). For example, BiP binds to mutant PrP and targets it to the proteasome for degradation (Jin et al., 2000). Overexpressing Hsp70 suppresses the toxicity associated with Aβ, tau, α-synuclein, SOD1 and poly-Q expanded Huntingtin (Muchowksi and Wacker, 2005). This chaperone can also protect neurons and astrocytes from experimental stroke and stroke-like insults through a variety of mechanisms including suppression of apoptosis and microglial activation (Yenari et al., 2005). This is consistent with an earlier report showing that pharmacological activation of the heat shock response could delay disease progression in ALS mice (Kieran et al., 2004). In a *Drosophila* model of tauopathy, the chaperone nicotinamide mononucleotide (NAD) synthase nicotinamide mononucleotide adenylyltransferase (NMNAT) interacted with hyperphosphorylated tau, promoted

the ubiquitination and proteasome-mediated clearance of toxic tau oligomers and suppressed age-dependent neurodegeneration (Ali et al., 2012). Enhancing chaperone function early in disease, prior to UPR activation may present an even more effective and readily translatable strategy. Kudo et al. (2008) showed that pre-treatment with a BiP inducer could mitigate ER-stress-induced apoptosis in neuroblastoma cells and reduce infarction area in a mouse model of focal cerebral ischaemia. As discussed previously, the cytoskeleton participates in activity-dependent processes underlying synaptic plasticity and is one of the earliest and most sensitive targets of thermal stress (Dalle-Donne et al., 2001; Klose et al., 2004). *Hyper*thermic preconditioning increases the upper temperature limit of nervous system operation and involves an acquired synaptic thermotolerance mediated by interactions of the cytoskeleton with Hsps (Klose et al., 2004). In *Drosophila* motorneurons this thermotolerance can be enhanced with overexpression of Hsp70 (Xiao et al., 2006). Similarly, in the rat brain, Hsp70 is rapidly recruited to the synapse in response to heat shock (Bechtold et al., 2000). Proteasome inhibition can induce thermotolerance via the induction of Hsps and ER chaperones (Bush et al., 1997), thus the effect of phospho-tau on the proteasome should elicit a similar response. In a rat model of ischaemia-reperfusion, pre-cooling increased BiP expression and reduced CHOP expression and apoptosis (Liu et al., 2013). It follows then that hypothermic preconditioning could bring about an adaptive UPR by increasing tau phopshorylation. As will be discussed further in Chapter 6, the UPR itself can be harnessed to precondition the ER and favour cell survival (Tabas, 2011). Overall, these studies have established the UPR as a potentially valuable target

across a wide spectrum of neurological disorders - and it is thus worthy of further investigation in the context of neuronal tau and thermal stress.

## 1.7 Hypothesis and statement of aims

Against this background, several hypotheses were derived:

(1) That mild-to-moderate cooling would protect human cortical neurons against common neurotoxins including oxidative and excitotoxic stress

(2) That mild-to-moderate cooling would elicit a classic cold-shock response in human cortical neurons

(3) That human cortical neuronal tau protein development would be recapitulated *in vitro* and subsequently reversed by cooling

(4) That hypothermic protection of human neurons would be mediated in part by cooling-induced changes in tau in concert with proteostatic pathways.

The aims of this thesis were as follows:

(1) To establish a human *in vitro* model of TH using hCNs

(2) To characterize the cold-shock response in hCNs

(3) To determine the effect of cooling on microtubule-associated protein tau in hCNs

(4) To explore potential mechanisms of hypothermic preconditioning in hCNs that might incorporate tau and the UPR.

# Chapter 2: Materials and methods

## 2.1 Human brain tissue

Human post-mortem brain samples were included as controls for tau and cold-shock protein expression; these are developmentally-regulated proteins in the human cortex (Miller et al., 2014 and The Allen Institute for Brain Science). Tissue was obtained under full ethical/Institutional Review Board approval of the University of Edinburgh. Written informed consent was obtained for each sample. Adult frontal cortical samples (3 healthy controls, aged 17, 44 and 75 y and 3 Alzheimer's disease patients, aged 60, 61 and 81 y, Braak stages 5-6, post-mortem interval (PMI) 1-4 d) were provided by the MRC Edinburgh Brain & Tissue Bank as fresh-frozen tissue blocks (stored at -80 °C). Human foetal brain samples were procured after elective surgical abortion (PMI 4-48 h), with full ethics permission of the NHS Lothian Research Ethics Committee (REC 08/S1101/1). These samples included intact cerebral hemispheres (gestational age 14 to 19 w) which, immediately after retrieval at post-mortem, were placed into 5-20 % foetal calf serum (FCS) in phosphate-buffered saline (PBS) with 1% Pencillin-Streptomycin (Invitrogen) and transferred at 4°C prior to further processing.

## 2.2 Cell culture

### 2.2.1 Generation of iPS lines

Human iPS lines (IPS1 and IPS2, derived from the fibroblasts of healthy female control donors at 55 y and 40 y respectively) were reprogrammed in-house, after

obtaining written informed consent from each participant and ethics permission from the NHS Lothian Research Ethics Committee (REC/10/S1103/10). Induction and characterization of these clones was performed as described previously (Bilican et al., 2012; Bilican et al., 2014; Livesey et al., 2014) with minor adjustments. Briefly, this included reprogramming of fibroblasts by Sendai (Life Technologies) (IPS1) or retroviral (Vectalys) (IPS2) transduction introducing vectors expressing coding sequences for the Yamanaka reprogramming factors (octamer-binding transcription factor 4, OCT4; c-Myc, MYC; sex determining region Y-box 2, SOX2; Kruppel-like factor 4, KLF4) (Takahashi and Yamanaka, 2006). Human embryonic stem cell (hES)-like colonies were selected and expanded. Validation of iPS lines was performed by confirmation of pluripotency (Fig.2.1), demonstration of transgene silencing with upregulation of endogenous transcription factors and normal clonal karyotyping. There is conflicting data regarding the influence of donor age and epigenetic memory on reprogramming efficiency, differentiation potential and replicative senescence of iPS lines (reviewed in Rohani et al., 2014). Whilst the data below demonstrates a similar acquisition of pluripotency between the two iPS lines generated, more detailed analysis would be required to rule out potential donor-related epigenetic factors on pluripotency and the neurons differentiated from iPS clones. To minimise these effects, it was important to ensure that the control donors were of similar age and that conversion was performed within a short range of passage numbers. A similar approach was taken when differentiating hCNs from explandible neural precursors; no obvious passage number effects were noted within the ranges used (see below).

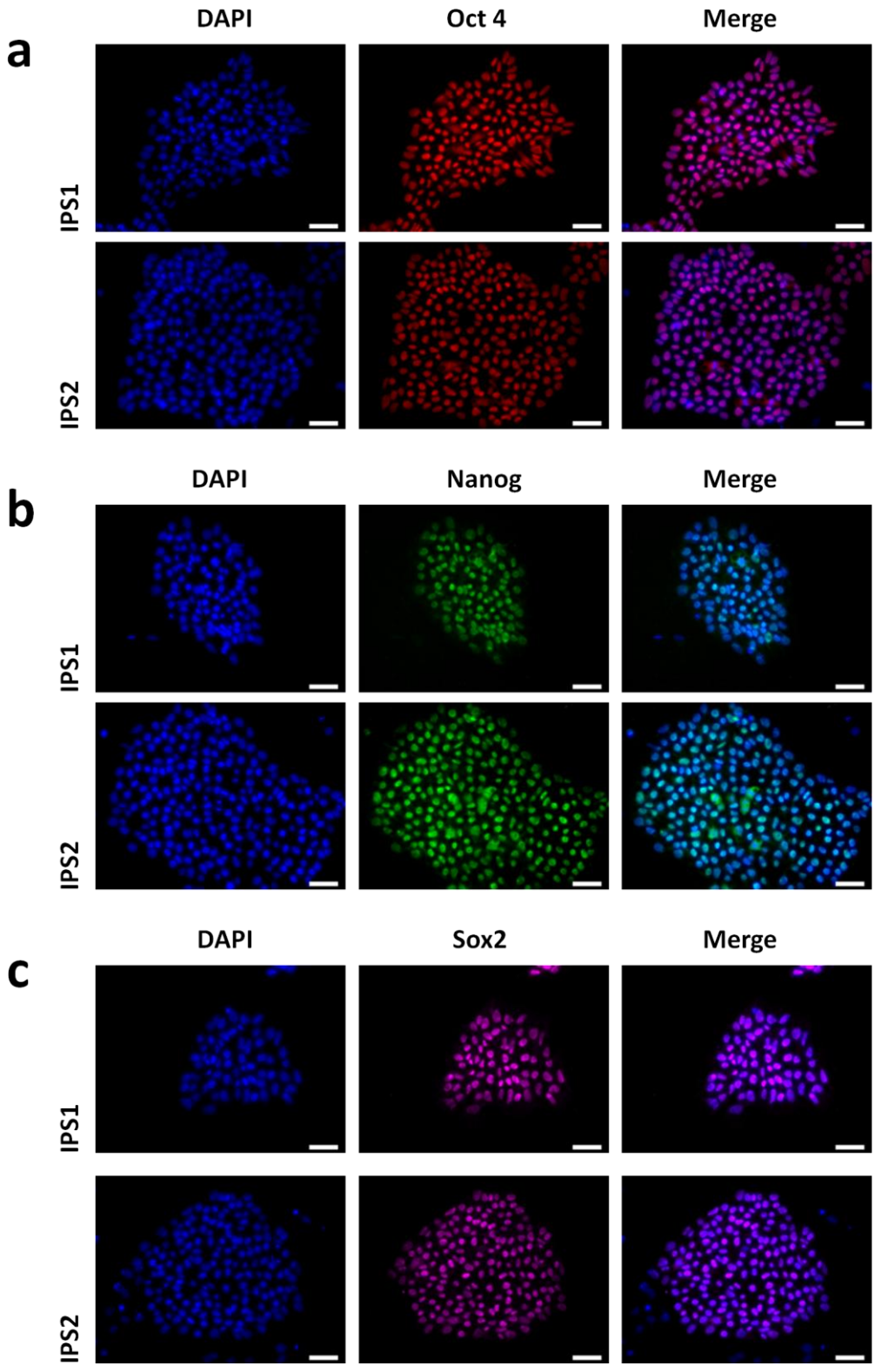


**Figure 2.1 Immunocytochemical validation of iPS lines.** hES-like colonies from IPS1 and IPS2 were stained for pluripotency markers. Representative flurorescent micrographs show results for Oct 4, Nanog and Sox2, scale bar = 50 μm.

### 2.2.2 Neural conversion of hPS lines

hES lines (H9, female, WiCell, Madison, WI and Shef 4, male, UK Stem Cell Bank, designated HES1 and HES2 respectively) were obtained under full ethical/Institutional Review Board approval of the University of Edinburgh. All hPS lines were maintained on CF-1 irradiated mouse embryonic fibroblasts, with Advanced DMEM/F12, 20% Knockout Serum Replacement, 10 ng/ml fibroblast growth factor-basic (FGF2), 1 mM L-glutamine, 100 μM 2-mercaptoethanol and 1 % Penicillin-Streptomycin. hPS colonies underwent neural conversion in suspension at 20 % $O_2$ under feeder-free conditions as described previously (Bilican et al., 2014). Briefly, colonies cultured in chemically-defined medium (CDM) were lifted in a 1:1 Dispase/Collagenase mix and suspended in neuralisation medium with continuous shaking to enable conversion to neuroectoderm (formation of embryoid bodies followed by neurospheres). After 7 d, spheres were gradually switched to neural rosette medium to encourage rosette development over another 7-14 d. Converted spheres were then plated onto 1 in 100 Laminin (Sigma)-coated Nunc plates for 2-3 d prior to mechanical isolation of rosettes and long-term expansion as anterior neural precursors (aNPCs; Bilican et al., 2014; Livesey et al., 2014).

### 2.2.3 Expansion of aNPCs

Cryopreservation, maintenance and expansion of aNPCs was performed using aNPC expansion medium and freezing medium as described (Bilican et al., 2014), with the exception that thawed aNPCs were plated onto 1 in 100 Laminin (Sigma) for the first two passages. Thereafter, aNPCs were routinely maintained on 1 in 100 Matrigel®-coated Nunc plates. For immunocytochemical analysis of aNPC markers (Fig.2.2),

cells were plated as described below, but in plating medium containing FGF2 (Peprotech). Cells were fixed at 100% confluence.

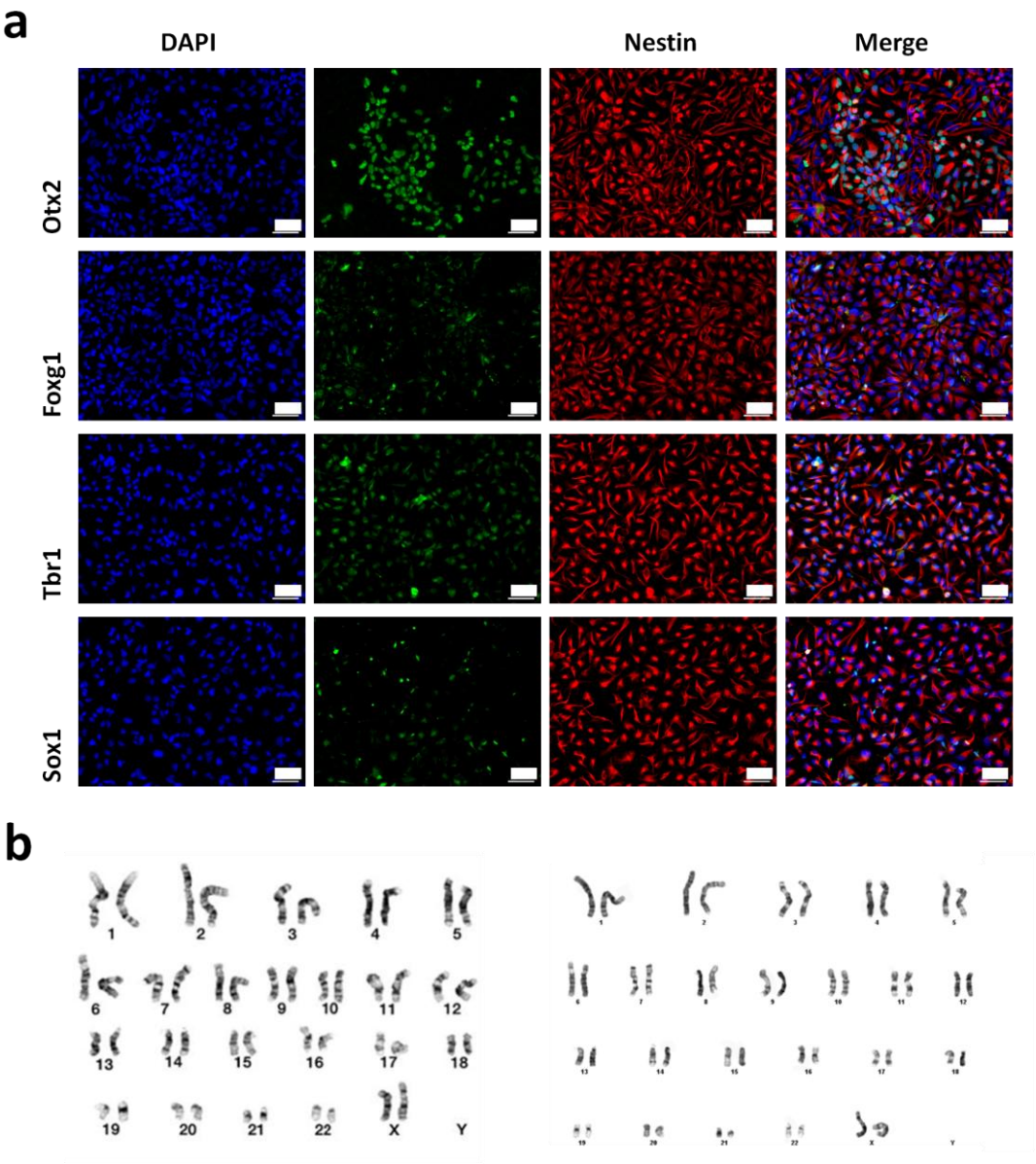


**Figure 2.2 Validation of aNPCs. (a)** Representative fluorescent micrographs (HES1) demonstrate co-expression of NPC marker Nestin and mosaic expression of various early dorsal telencephalic markers in green, cells counter-stained with DAPI, scale bar = 50 µm. **(b)** Left: karyograph showing G-banding of HES1-derived aNPCs (HES1) at passage 25 confirming that a normal karyotype is retained (original image published in Bilican et al., 2014). Right: normal karyograph of IPS1-derived aNPCs.

### 2.2.4 Differentiation of aNPCs into hCNs

hCNs were differentiated from aNPCs as described previously (Bilican et al., 2014; Livesey et al., 2014), with minor alterations. Briefly, confluent aNPCs were lifted in aNPC plating medium (passages 17 to 39) and plated in 12 or 24-well plates (Nunc) at approximately 1 x $10^5$ 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 more mature cultures required feeding every other day. For hypothermia experiments incubation temperature was the only environmental adjustment. Periodic testing using 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.

### 2.2.5 hPS-derived glia

hPS-derived astroglial progenitors efficiently differentiate into a highly pure (>90 %) monolayer of functionally mature astrocytes within 14 to 21 d (see details in Serio et al., 2013). Briefly, astroglial progenitors (derived from IPS2) were expanded in maintenance medium (Advanced DMEM F12, 1 % Anti-Anti-Antibiotic Antimycotic, 1 % Glutamax, 1 % N2, 0.1 % B27, 10 ng/ml FGF2, 20 ng/ml epidermal growth factor (EGF, R&D Systems) in six-well Nunc plates coated in 1 in

80 Matrigel® and passaged at 60 % confluence as required. Progenitors were maintained at 37°C, 5 % $CO_2$, 20 % $O_2$. For differentiation, progenitors (passages 7 to 14) were lifted with Accutase® and plated at $10^6$ per well in six well plates. Differentiation media comprised Neurobasal Medium containing 1 % Anti-Anti-Antibiotic Antimycotic, 1 % Glutamax, 1 % Minimum Essential Medium Non essential amino acids (MEM NEAA), 0.2 % B27, 0.1 % recombinant human ciliary neurotrophic factor (hCNTF, R&D Systems). Astroglia were harvested at 0, 2 and 5 w after removal of FGF and EGF. For immunocytochemistry, astroglia were lifted with TrypLE™ Express Dissociation Reagent and re-plated at 20,000 cells per 13 mm coverslip (pre-coated with Poly-L-Ornithine and 1 in 80 Matrigel®) in 24-well Nunc plates. Cells were incubated for 3 d at 37°C, 5 % $CO_2$, 3 % $O_2$ prior to fixing.

### 2.2.6 Primary culture

Mixed neuronal cultures (*n*=4) and neurospheres (*n*=1) were isolated from post-mortem human foetal brains (gestation 14, 15, 16 and 19 w), adapting published protocols (Busciglio et al., 1995; Chandran et al., 1998; Deshpande et al., 2008; Pelsman et al., 2003; Fig.2.3 and 2.4). Briefly, cerebral hemispheres were gently freed from their meninges at 4°C. After sectioning cortical tissue for protein extraction, dorsal and ventral telencephalic regions (for immediate plating and neurosphere expansion respectively) were roughly minced and incubated at 37°C for 20 min with Trypsin- ethylenediaminetetraacetic acid (EDTA) (0.25 %). Samples were mechanically dissociated into a single-cell suspension using gentle passage by glass pipette and spun at 2500 rpm for 2.5 min. Pellets were resuspended in primary plating medium containing Trypsin Inhibitor (0.05 %, Sigma) and DNase (0.001 %,

Worthington) and incubated at 37°C for 5 min. After one wash, cells were either plated into coated 24-well Nunc plates (dorsal telencephalic cells) at ~1 x $10^5$ cells $cm^{-2}$ in plating medium or seeded into T75 flasks (ventral telencephalic cells) at 2 x $10^5$ per ml in neurosphere medium. Cultures were incubated at 20 % $O_2$, 37°C, 5 % $CO_2$. After 4 d, plates were washed once and FCS was removed for maintenance (to bias the culture towards a neuronal phenotype). Thereafter, cultures were fed every 3-5 d by 50 % media changes and terminated after 3 w. Samples from plates were harvested for RNA, protein or immunocytochemistry as described below. T75 flasks were gently agitated daily and supplemented after 4 d with recombinant human platelet-derived growth factor (rhPDGF-AA, 20 ng/ml, R&D Systems), insulin-like growth factor 1 (IGF-1, 10 ng/ml) and triiodothyronine (T3, Sigma). Neurospheres were mechanically passaged every 28 d by manual chopping. Mixed glial cultures were periodically prepared by dissociating spheres into a single cell suspension before removal of mitogens and plating in 24-well plates. Mixed glial cultures were terminated after 3 w. Coverslips were stained at various time points for astroglial and oligodendroglial markers.

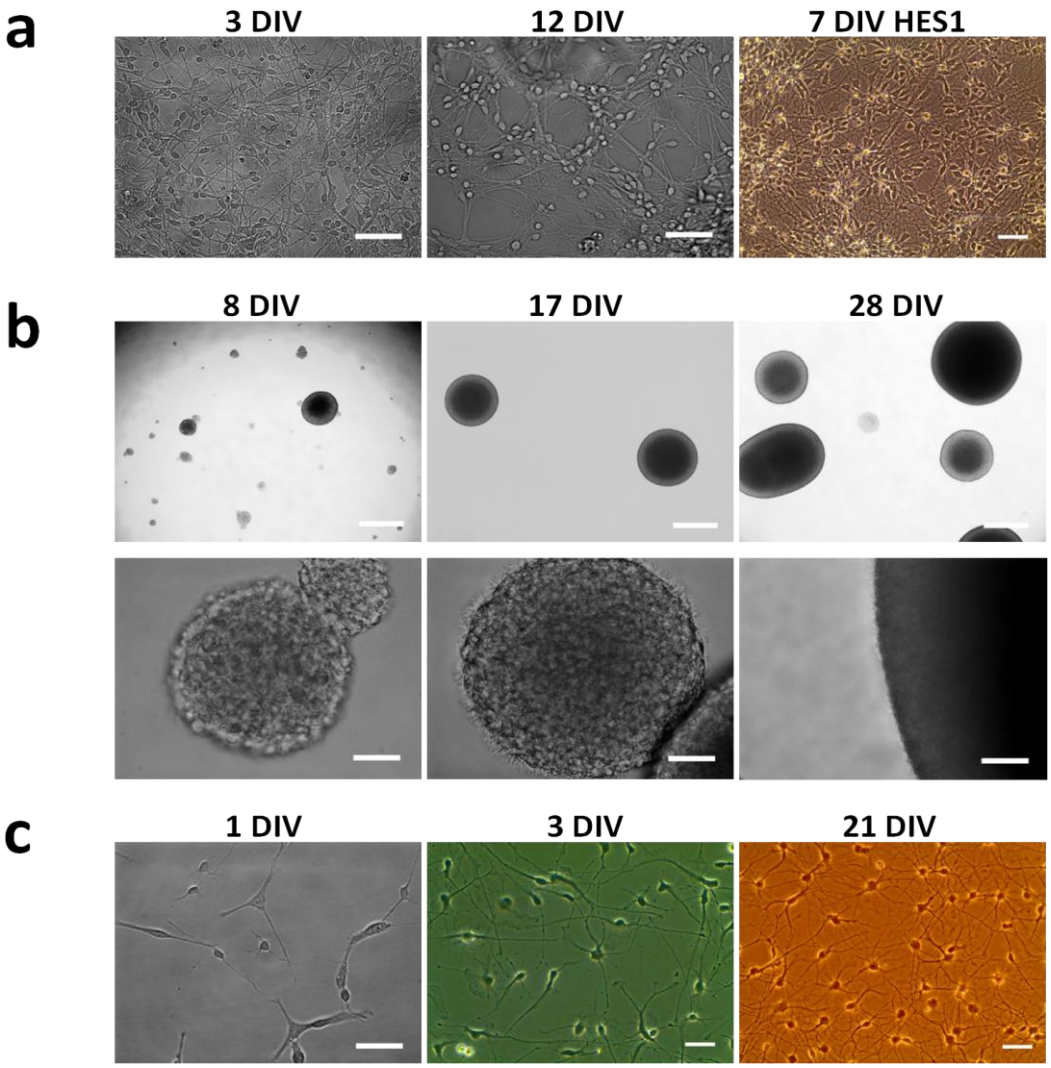


**Figure 2.3 Primary human cultures. (a)** Phase contrast micrographs of early differentiating primary cortical neurons (left and middle; 14 w foetus) as compared to HES1-derived hCNs (right), scale bar = 50 µm. **(b)** Phase contrast micrographs of expanding primary neurospheres (19 w foetus), upper scale bar = 500 µm, lower scale bar = 50 µm. **(c)** Phase contrast micrographs of primary differentiating mixed glia (19 w foetus), scale bar = 50 µm.

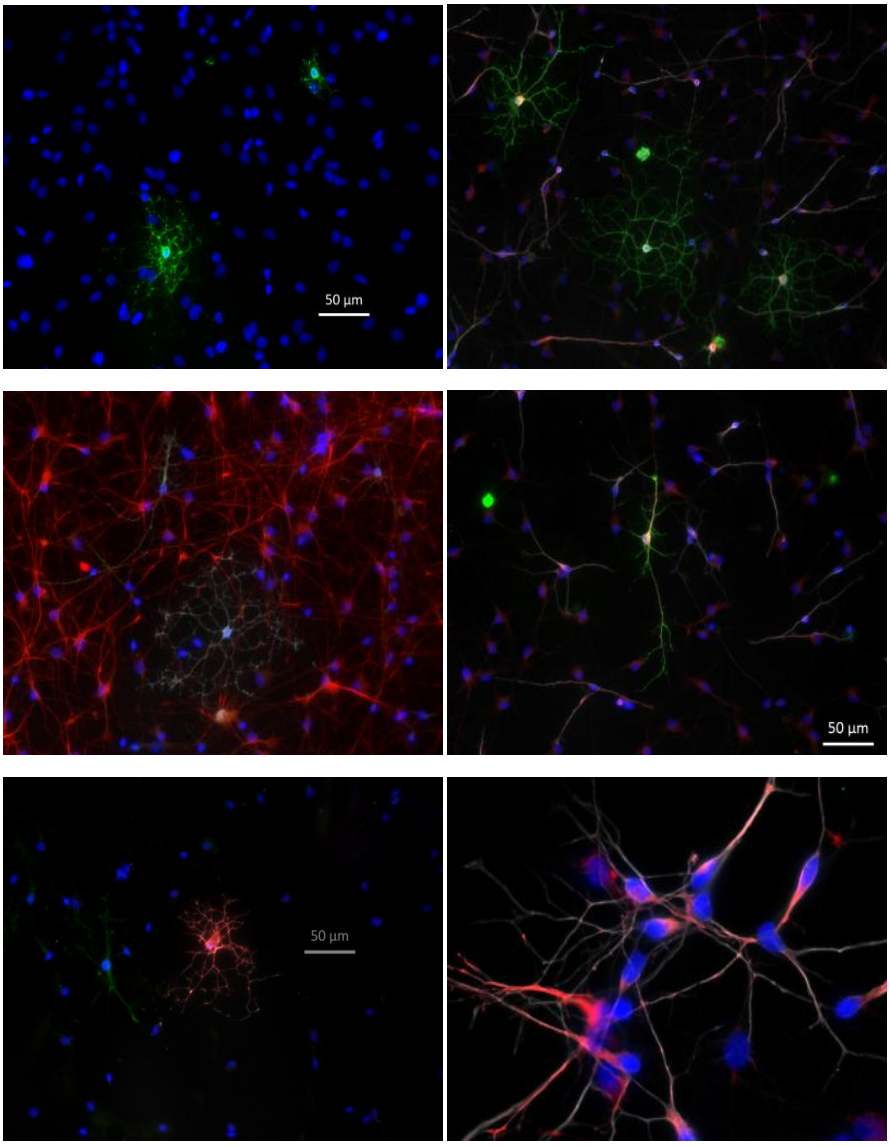


**Figure 2.4 Primary human glia and neurons.** Fluorescent micrographs of primary mixed cultures (19 w foetus) stained for (in order from top left to bottom right): O4 (green), 3DIV; O4 (green), βIII-tubulin (white) and 3R tau (red), 10 DIV; O4 (white), GFAP (red) and total tau (green), 10 DIV; O4 (green), βIII-tubulin (white) and 3R tau (red), 25 DIV; O4 (white), PDGF receptor α (PDGFRα, green) and myelin basic protein (MBP, red), 25 DIV; βIII-tubulin (white) and 3R tau (red), 25 DIV. All cells were counterstained with DAPI (blue).

## 2.3 Cooling paradigm

For hypothermia experiments triplicate plates for each hCN batch were generated and maintained in normal differentiation media. Hypothermia was induced at 5 w, prior to significant upregulation of glial transcripts (see Fig.3.1) and when >90 % of aNPCs have differentiated into hCNs, with >95 % of these neurons firing action potentials (Belinsky et al., 2013; Bilican et al., 2014; Livesey et al., 2014). At this time point, triplicate plates were separated and cultured at 28, 32 or 37°C to simulate 'moderate hypothermia', 'mild hypothermia' or 'normothermia' respectively (Fig.2.5). These temperatures were selected to simulate the clinically-targeted temperatures oftherapeutic hypothermia (32-35°C) and suspended animation (28°C) (Choi et al., 2012; Yenari and Han, 2012), as well as depths of hypothermia that have been shown to induce a cold-shock response and neuroprotection in other model systems (Nishiyama et al., 1997b; Danno et al., 2000; Chappell et al., 2001; Saito et al., 2010; Wellmann et al., 2010; Chip et al., 2011; Ferry et al., 2011; Kaneko and Kibayashi, 2012; Li et al., 2012; Sumitomo et al., 2012; Tong et al., 2013). Thermic period was calibrated with a sentinel culture plate containing the same media composition and volume as experimental plates. Time zero was set when the media in the sentinel plate reached the desired incubation temperature, measured by digital thermometer (typically within 1 h). RNA samples for early and late assessment of transcripts were lifted at 3 and 24 h respectively. After 24 h additional cells were fixed for immunocytochemistry and harvested for protein. For any media additions that were necessary during the hypothermic period (e.g. for stressor application), solutions were warmed to the respective temperatures before adding to the cultures.

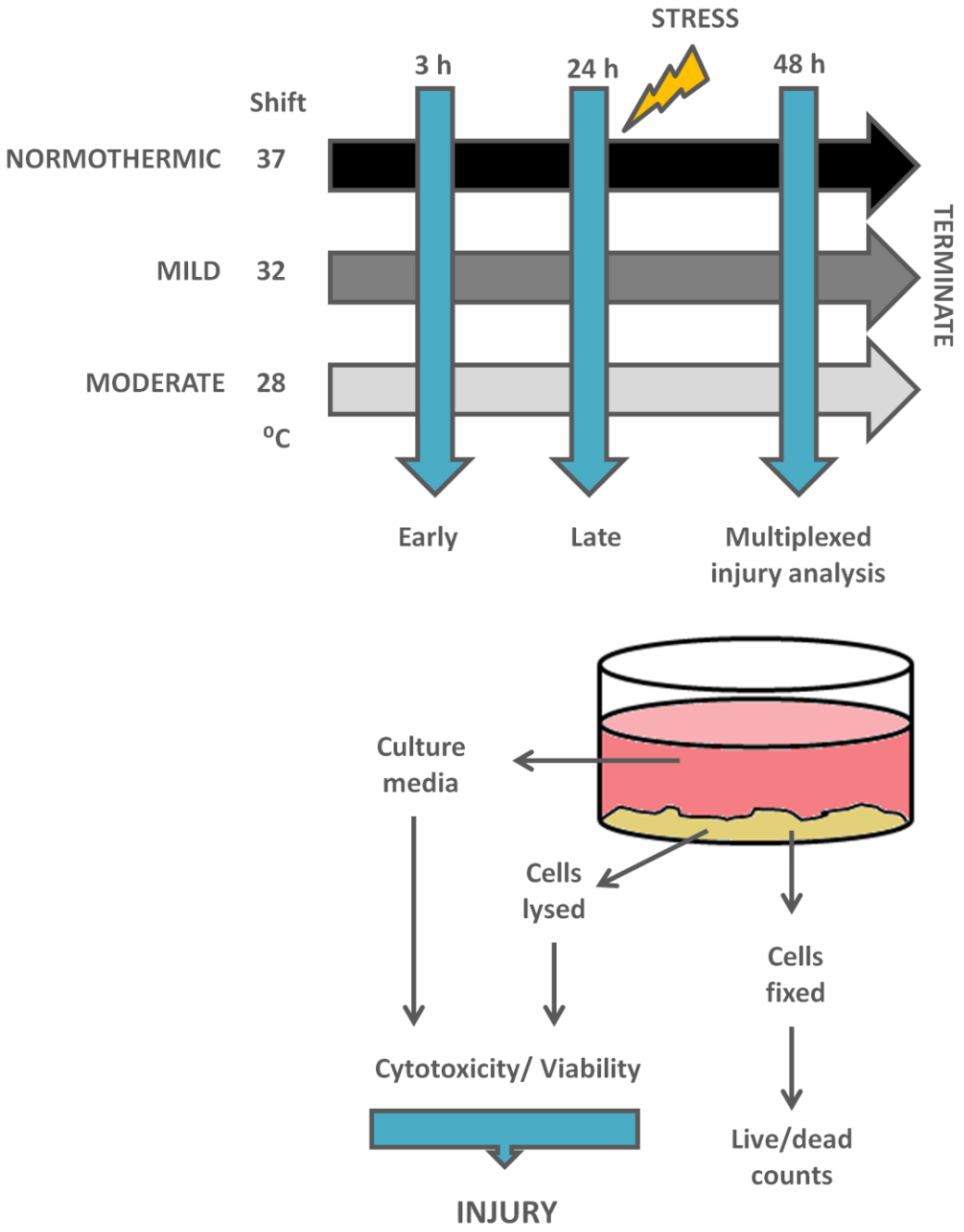


**Figure 2.5 Cooling and stress protocol.** Sampling for transcript and protein analysis was carried out at early (3 h) and/or late (24 h) timepoints after the start of temperature shift. For injury experiments stressors were applied after 24 h of cooling for a duration of 24 h after which a multiplexed injury analysis was performed, providing paired viability and cytotoxicity data for each cell culture well. For each culture plate, some coverslips were instead fixed for immunocytochemistry to perform confirmatory cell death counts (100 µM $H_2O_2$ and 30 µM glutamate conditions only).

For staining of hCNs, hPS-derived astroglia and primary human neurons and astroglia, every step was carried out at room temperature. Cells were fixed with 2 % (astroglia) or 4 % (hCNs) paraformaldehyde (PFA) for 10 min and permeabilized with 0.1 % Triton X for 10 min. Cells were blocked in 3 % goat serum (Dako) for 45 min, then incubated with primary antibodies (see below) overnight at 4°C. Coverslips were washed and all subsequent steps were performed in the dark. Secondary antibodies (Alexa Fluor® 488, 555 ± 647 diluted at 1 in 2000) were applied for 20 min, followed by washing and counter-staining with DAPI (0.2 µg/ml, Sigma®) for 3 min. Primary (see Table M1) and secondary antibodies were diluted in 3 % goat serum. Primary oligodendroglia were stained live for cell surface markers prior to fixing. Primary antibodies were diluted in culture medium containing 20 % FCS and cultures were incubated at 37°C for 1 h. Subsequent steps were performed at room temperature in the dark. Media was removed and cells were fixed, washed and the secondary antibody applied for 30 min. After washing and permeabilization in 0.2 % Triton X, primary antibody application for intracellular epitopes and subsequent steps were the same as for other cell types. Negative control coverslips received no primary antibody. Coverslips were mounted with FluorSave™ Reagent (Calbiochem) and slides were stored a 4°C in the dark prior to imaging. All fluorescent images were acquired at room temperature using ZEN 2012 software (Zeiss). Widefield fluorescent micrographs were captured with a Zeiss Axiovert 200 microscope fitted with a High Resolution Microscopy Camera (AxioCam Mrm), using the following objective lenses: Plan-Apochromat 20 X, numerical aperture (NA) 0.8, Plan-Apochromat 63 X oil, NA 1.4. Confocal micrographs were generated with a Zeiss LSM 710 Confocal inverted Microscope

using a Plan-Apochromat 63 X oil objective, NA 1.4. Fluorescent images were processed and scale bars were added using ZEN lite 2012 (Zeiss). Phase contrast images of live cells were acquired with an EVOS® XL core Cell Imaging System, using an EVOS® LWD achromatic phase contrast 40 X objective, NA 0.65. Cell counts were performed blind to the temperature variable. Representative fluorescent and phase images were edited in Photoshop CS6 (Adobe). Where necessary, brightness and contrast adjustments were applied to all pixels equally.

## 2.5 Sample harvesting for RNA and protein

hCN samples for RNA were incubated with Accutase® (Sigma) for 5 min, lifted with Advanced DMEM F12, spun at 2500 rpm for 2.5 min, resuspended in Dulbecco's PBS and centrifuged at 3000 rpm for 5 min at 4°C. Samples for protein were lifted from plates in ice-cold Tris-buffered saline (TBS) containing protease inhibitors (cOmplete ULTRA, Roche and 100 μM phenylmethanesulfonylfluoride (PMSF), Fluka Biochimika), and where necessary, phosphatase inhibitors (phosSTOP, Roche). RNA and protein pellets were snap-frozen and stored at -80°C prior to further analysis. Astroglial RNA samples were lifted with TrypLE™ Express, incubating for 15 min at 37°C, protein samples were lifted as for hCNs. Human post-mortem adult and foetal cortical samples for protein were divided into 200-300 mg pieces at 4°C, snap-frozen and stored at -80°C prior to extraction.

## 2.6 Transcript analysis

### 2.6.1 Validation of normalization factors

Reference targets were determined using a combination of geNorm (qbase+, Biogazelle) and NormFinder (Excel) analysis (Vandesompele et al., 2002). Six candidate targets were assessed for their stability with respect to the variables under test (differentiation from aNPC to 6 w post-plating, stimulation with KCl, exposure to hypothermia) (Fig.2.6). Candidates included β-actin (BACT), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribosomal protein L29 (RPL29), eukaryotic translation initiation factor 4A2 (EIF4A2), hypoxanthine phosphoribosyltransferase 1 (HPRT1) and β2 microglobulin (B2M). Analyses were applied to the expression data of 12 samples (extracted from 4 independent batches of hCNs (HES1) at 2, 4 and 7 w post-plating), 10 samples (extracted from 4 batches (HES1) and 1 batch (IPS1) with and without stimulation) and 5 samples (extracted from 1 batch (HES1) after 0, 3, 6, 12, and 24 h culture at 32°C), to derive the most stable targets for each experiment. geNorm analysis was also used to determine the optimal number of reference targets for each condition, determined by the geNorm V value. The following reference targets were selected: for hCN differentiation, RPL29, GAPDH, BACT; for KCl stimulation, RPL29, GAPDH, EIF4A2 and for hypothermia, EIF4A2 and GAPDH.

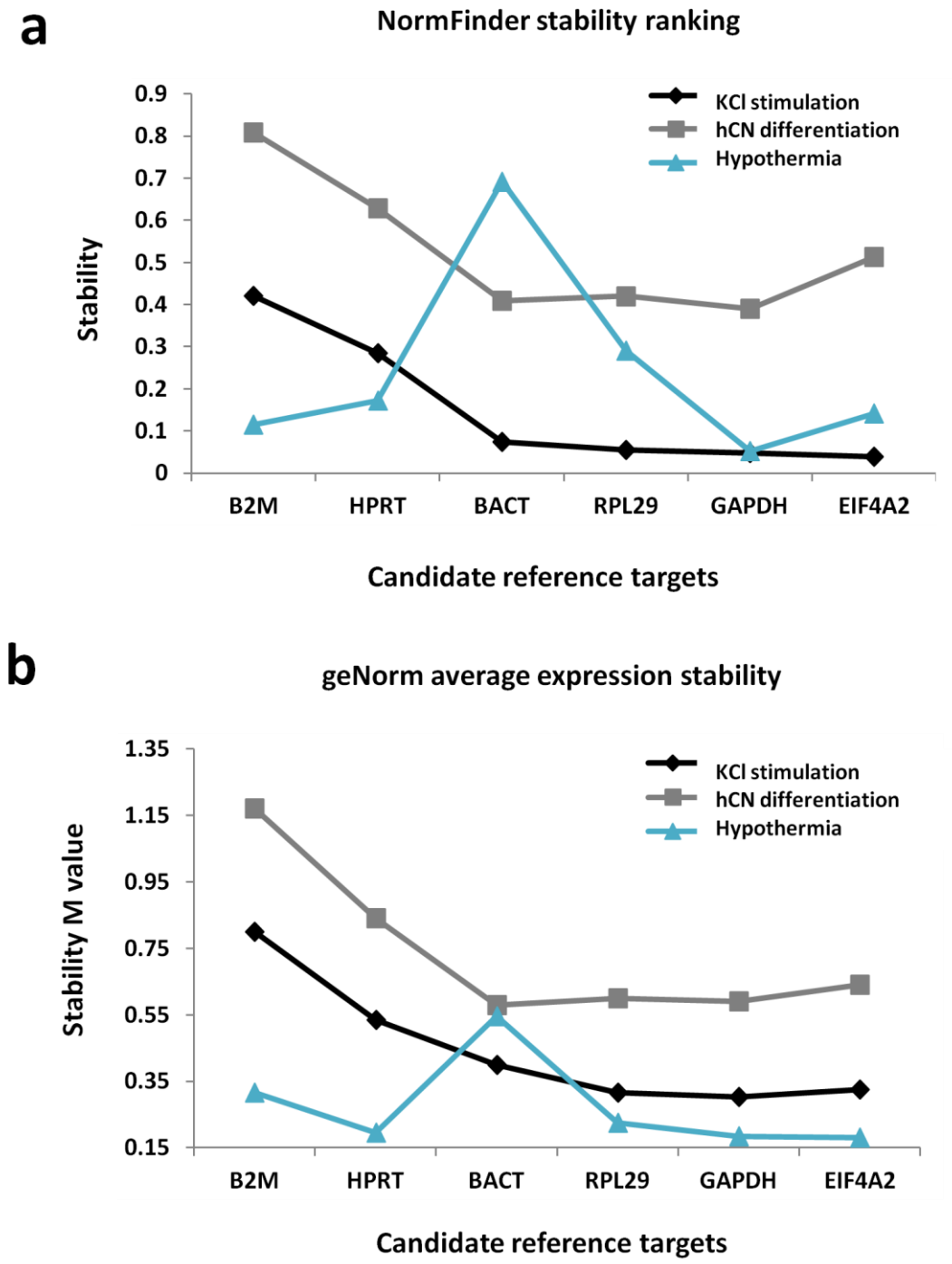


**Figure 2.6 Establishing normalization factors for q-RT-PCR.** NormFinder **(a)** and geNorm **(b)** stability results are presented for six candidate reference targets under the conditions indicated. Note that the lower the stability value, the more stable the target. Whilst this analysis suggested that BACT was unstable with respect to temperature change, this was not evident by Western blot for which BACT has previously been used as a loading control in the context of hypothermia (Chip et al., 2011).

### 2.6.2 Quantitative reverse transcriptase PCR

For q-RT-PCR, RNA was extracted using the Qiagen RNeasy® Mini Kit, according to the manufacturer's instructions, followed by DNA removal using the Ambion® RNA clean up kit (Invitrogen). cDNA was synthesised from 250-500 ng RNA using the DyNAmo™ cDNA Synthesis Kit (Thermoscientific) using 'no reverse transcriptase' reactions as negative controls. For human adult and foetal comparisons, cDNA was synthesised in triplicate from 500 ng commercially prepared RNA (Human Foetal Brain Total RNA, pooled from 59 spontaneously aborted human foetuses, gestation 20-33 w, and Adult Human Brain Cerebral Cortex Total RNA, pooled from 5 human adults (age 20-44 y), both from Clontech). PCR reactions were performed in triplicate using a BioRad CFX96™ Real-Time PCR Detection System and 'no template' reactions were included as negative controls. Cycle conditions were: 95°C for 7 min, 95°C for 10 s, X°C for 30 s, repeated 40-44 times, where X = the optimized annealing temperature for each primer pair. Primer specificity was confirmed by gel electrophoresis. Primer sequences are listed in Table M2.

### 2.6.3 Qualitative RT-PCR

cDNA was prepared as above. PCR reactions were performed using Quick-Load® Taq 2X Master Mix (New England Biolabs) in a BioRad C1000 Thermal Cycler. Cycle conditions are described in Duff et al., (2000). β-actin was included as a housekeeping target. RT-PCR products were resolved on 1.9 % agarose gels run at 110 V for 25 min. Human tau exon spanning primer sequences were as follows (Andorfer et al., 2003): exons 1-5 forward TGAACCAGGATGGCTGAGC and

reverse TTGTCATCGCTTCCAGTCC; exons 9-11 forward CTCCAAAATCAGGGGATCGC and reverse CCTTGCTCAGGTCAACTG.

## 2.7 Activity-dependent gene regulation

An in-house quality control protocol to test hCN functionality by gene expression changes in response to KCl stimulation was conducted as described previously (Bilican et al., 2014) with a few minor adjustments. Briefly, 12 h prior to stimulation, 5 w old hCNs were switched to an antioxidant- and glutamate-free minimal medium (MiM; Gupta et al., 2013). Cultures were then stimulated with 50 mM KCl in the presence of L-type voltage-gated calcium channel agonist FPL64176 (5 μM, Tocris) and N-methyl-D-aspartate receptor (NMDAR) blocker MK-801 (10 μM, Sigma). Control wells received no KCl. Samples were harvested for RNA at 2 and 4 h post-stimulation. Induction of FOS and brain-derived neurotrophic factor (BDNF) Exon IV transcripts was analysed by q-RT-PCR. For each cortical batch, expression data was normalized to the geometric mean of 3 stimulation-stable reference targets and compared to matched, untreated controls.

## 2.8 Biochemistry

### 2.8.1 Soluble protein extraction and dephosphorylation

Extraction of soluble protein was performed using a modified version of the protocol by Ishihara et al. (1999). Cell pellets were homogenized in 2 vol. (v/w) radioimmunoprecipitation assay (RIPA) buffer (50mM Tris pH8, 150mM NaCl, 1%

Triton-X 100, 5mM EDTA, 0.5% Na.deoxycholate (w/v), 0.1% sodium dodecyl sulphate (SDS)) containing protease inhibitors and incubated on ice for 30 min. Tissue samples required homogenization in a Precyllys®24 lysis and homogenization unit (2 x 15 s at 5000 rpm). Lysates were then centrifuged for 20 min at 50,000 x *g*, at 4°C in a Beckman ultracentrifuge. To isolate nuclear and membrane-bound proteins, RIPA-insoluble pellets were further extracted for 20 min in high-detergent RIPA buffer containing 2 % SDS followed by a repeat ultracentrifugation. For phospho-protein analysis only, phosphatase inhibitors were included at each extraction step. Protein concentration was measured by bicinchoninic acid (BCA) assay (Pierce), with absorbance read at 584 nm, according to the manufacturer's instructions. To resolve tau isoforms, paired samples were incubated for 2 h at 37°C with or without 0.04 $U\mu g^{-1}$ alkaline phosphatase (AP) from *E.coli* (Sigma) in AP buffer (50 mM Tris pH 8.5, 50 mM NaCl, 1 mM $MgCl_2$) prior to Western blot analysis.

### 2.8.2 Western blot analysis

Protein samples were boiled at 95-99°C for 10 min in 2X Laemmli buffer or in NuPAGE® LS Sample Buffer with NuPAGE® Reducing Agent prior to loading onto gels (10-20µg per well, depending on experiment). Samples were run against a protein ladder (BioRad Precision Plus Protein™ All Blue Standards) by SDS-polyacrylamide gel electrophoresis (PAGE) at 100-110 V in pre-cast gels (either 4-20 % gradient or 10 % gels, Thermoscientific) in BupH™ Tris-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-SDS Running Buffer (Thermoscientific) using the XCell Sure Lock™ Gel system (Invitrogen). Gels for tau isoform

resolution included a human recombinant tau ladder (rPeptide) containing equal quantities of the 6 major adult brain tau isoforms. Human adult and foetal cortical samples and hPS-derived astroglial samples were included as controls where appropriate. Proteins were subsequently transferred onto Immobilon®-FL polyvinylidene fluoride (PVDF) membranes (Millipore) for 1 h at 15 mA in Tris-Glycine transfer buffer and blocked for 45 min at room temperature with Odyssey™ Blocking Buffer (LI-COR® Biosciences). Membranes were incubated overnight at 4°C with primary antibodies (Table M3). After several washes with TBS-Tween®20 (0.001 %) (TBS-T) membranes were probed for 1 h at room temperature with Fluorescent conjugated secondary antibodies (IRDye®680RD Goat (polyclonal) Anti-Rabbit IgG (H+L) and IRDye® 800CW Goat (polyclonal) Anti-Mouse IgG (H+L), LI-COR® Biosciences). After further washes blots were exposed for 10 min per channel (700 nm and 800 nm) on a LI-COR® Odyssey Fc Dual-Mode Imaging System, with band intensities quantified in Image Studio, after subtraction of background fluorescence. Where necessary, membranes were immediately reprobed or stripped with NewBlot™ PVDF Stripping Buffer (LI-COR® Biosciences) for 20 min. Stripping efficacy was confirmed by re-imaging on the LI-COR® Odyssey Fc prior to re-probing with primary and secondary antibodies. For quantification, samples were run in triplicate to obtain an average intensity reading for each protein target for each independent hCN batch (after normalizing to loading control, or, in the case of phospho epitopes, after normalizing to total target expression).

## 2.9 Stress assays and multiplexed injury analysis

Injury experiments were performed using hCNs at 5 w after plating (passages 22 to 30). 12 h prior to induction of hypothermia, hCNs intended for oxidative stress experiments were switched to MiM (Gupta et al., 2013). With regards excitotoxic stress, preliminary experiments demonstrated that hCNs had a high tolerance to exogenous glutamate (Fig.2.7). For excitotoxicity experiments, pre-incubation in MiM thus commenced 5 d prior to induction of hypothermia (6 d prior to glutamate stress) to acclimatise endogenous glutamate uptake capacity. During this extended pre-incubation, media was supplemented once with L-alanine-L-Glutamine (Glutamax, Invitrogen) to avoid compensatory neuronal upregulation of glutamine synthetase (Chen and Herrup, 2012). After 24 h at respective temperatures, stressors were applied as follows: (a) $H_2O_2$ (Sigma) at 0, 50, 100 or 200 μM to induce oxidative stress or (b) L-glutamic acid (Sigma) at 1, 3, 10, 30 or 100 μM to induce excitotoxic stress in the presence or absence of NMDAR antagonists (i) 100 μM D(−)-2-Amino-5-phosphonopentanoic acid (D-APV, Sigma) or (ii) 10 μM ifenprodil (Sigma). Stressors and antagonists were diluted in MiM and applied via 50 % media replacement. Control wells received MiM with vehicle only. After a further 24 h at the respective temperatures, culture media was extracted from each well for the cytotoxicity assay and cells then lysed for the viability assay. Cytotoxicity was determined using the CytoTox-One™ Homogenous Membrane Integrity Assay (Promega), which determines lactate dehydrogenase (LDH) release from damaged cells, read fluorometrically on a plate reader with excitation at 560 and emission at 590 nm. Viability was quantified using the CellTiter-Glo® Luminescent Cell

Viability Assay (Promega), measuring adenosine triphosphate (ATP) produced by metabolically active cells, with luminescence measured on a luminometer (Promega Glomax). Both assays were performed according to the manufacturer's instructions, but adapted for 24-well plates. These assays are compatible for multiplexed analysis and thus two values (viability and cytotoxicity) were obtained from each well of neurons. 'Injury' was expressed as the ratio 'cytotoxicity/viability' in relative fluorescent units (RFU) divided by relative luminescent units (RLU), thereby removing any potential effect of inter-well variation in cell number. Fluorescence and luminescence readings were taken in triplicate and averaged for each condition, after subtracting mean values obtained for MiM only (cytotoxicity) and no cell control (luminescence). To confirm cellular identity and to obtain a direct quantification of cell death, wells for each batch and at each temperature condition were fixed for immunostaining with antibodies for βIII-tubulin, glial fibrillary acidic protein (GFAP) and 4',6-diamidino-2-phenylindole (DAPI). Assessment of nuclear morphology and cell counts were performed blind to the temperature variable. Cell death was defined by either nuclear fragmentation or nuclear condensation with the absence of stained cellular processes.

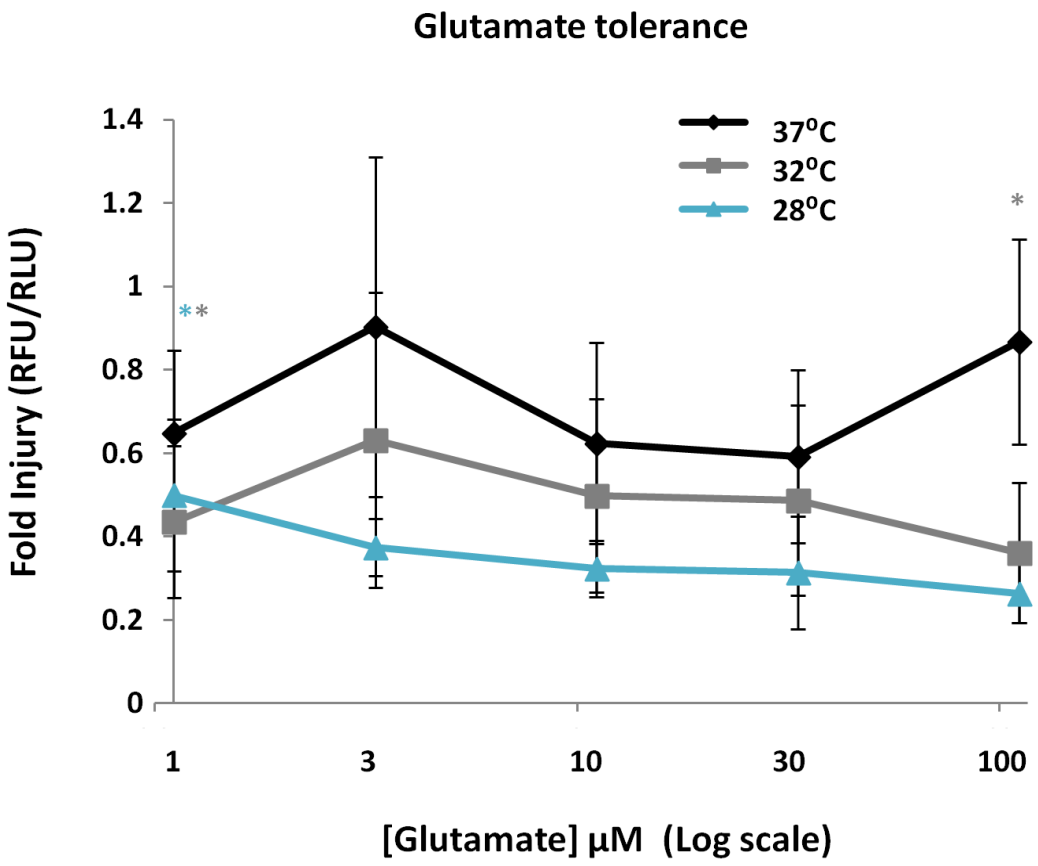


**Figure 2.7 Baseline glutamate tolerance in hCNs.** hCNs (*N* = 1, *n* = 3; HES1) were switched to glutamate-free MiM 12 h prior to temperature shift. After a further 24 h, glutamate was applied at the concentrations indicated for 24 h followed by multiplexed injury analysis. Note reduction in injury at lower temperatures at some concentrations of glutamate, but absence of a clear dose response at any temperature. Data are presented as mean fold injury relative to untreated, normothermic hCNs ± standard error of the mean (SEM). Asterisks refer to significant differences of mildly hypothermic (grey asterisks) or moderately hypothermic (aquaasterisks) cultures compared to normothermic cultures as determined by paired, two-tailed t-test.

## 2.10 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.

## 2.11 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 (Fig.2.8). Briefly, cell pellets from 5 w old hCNs were thawed on ice, solubilised in cold phosphate extraction buffer (20mM Imidazole-HCl (Santa Cruz), 2 mM EDTA, 2 mM ethylene glycol tetraacetic acid (EGTA), protease inhibitors and 100 μM PMSF) and sonicated for 10 s. After centrifugation (2000 *x 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 then 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), to provide specific inhibition of PP2A (Walsh et al., 1997). 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 blank and negative control (fostriecin) values. The specific PP2A activity (picomoles of phosphate released $min^{-1}\mu g^{-1}$ protein) was calculated for each sample. Hypothermic sample values were then compared to their respective normothermic controls.

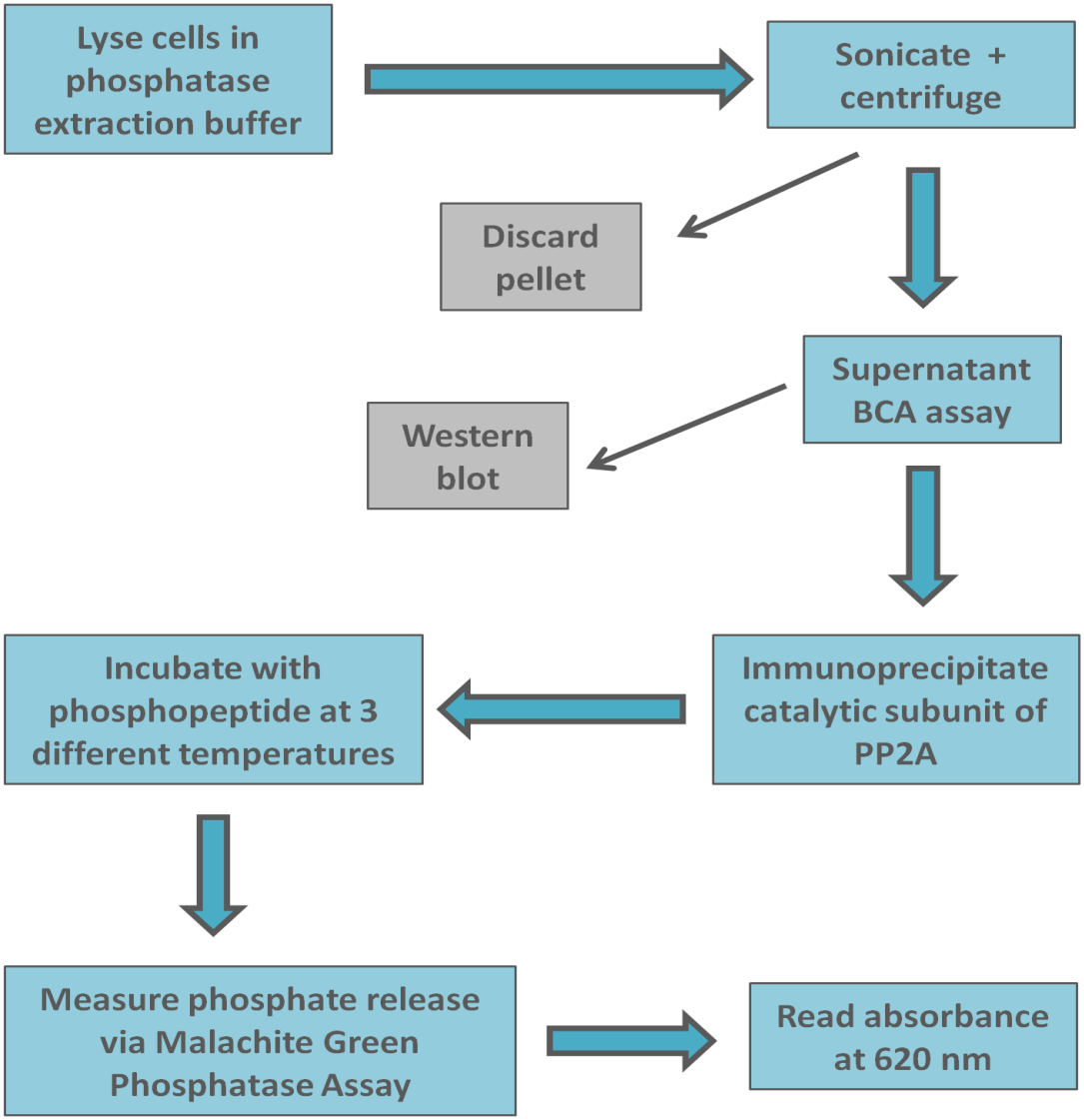


**Figure 2.8 Schematic of PP2A enzyme activity measurement.** After protein extraction, the cell pellet is discarded. The remaining supernatant can be used for standard immunoblot analysis or immunopreciptation of PP2A.

## 2.12 X-box binding protein 1 (XBP1) splicing assay

RNA was harvested from hCNs cultured for 24 h at 28, 32 or 37°C (normothermic control). As a positive control for XBP1 splicing, normothermic hCNs were treated for 24 h with Tm at 0.3 µg/ml to induce ER stress. q-RT-PCR was performed as

above. Conventional RT-PCR was performed using Quick-Load® Taq 2X Master Mix (New England Biolabs) and PCR reactions were performed in a BioRad C1000 Thermal Cycler (cycle conditions: 95°C 300 s, 95°C 30 s, 60°C 30 s, 72°C 30 s, repeated 32 times). GAPDH was included as a housekeeping target and MYC was included to confirm immediate early gene expression in response to cellular stress (cooling and Tm treatment). RT-PCR products were resolved on 2.5 % agarose gels run at 120 V for 33 min.

## 2.13 Electron microscopy

For ultrastructural analysis hCNs were cultured on Thermanox™ coverslips. At various time-points during differentiation, cultures were washed with ice-cold DPBS (Invitrogen) and fixed with 3% glutaraldehyde. Sample processing included osmication, followed by embedding monolayers in Araldite epoxy resin, cutting 60 nm sections and mounting onto Formvar/carbon-coated, copper slot grids. Sections were stained with uranyl acetate and lead citrate prior to imaging on a Philips CM120 Biotwin electron microscope.

## 2.14 Protein mass spectrometry

RIPA-soluble, cytosolic protein samples extracted from a 19 w gestation human foetal cortex and 6 w hCNs (HES1) were separated by 10 % SDS-PAGE. 1D gel sections corresponding to approximate molecular weights 30-75 kDa for each sample were processed in a laminar flow cabinet, using in-gel trypsin digestion, in order to

minimise keratin contamination. The digests were then extracted prior to analysis with nanoLC electrospray ionization (ESI)-MS/MS (Reverse Phase chromatography) using an Ultimate 3000 nano LC (Thermoscientific) coupled to an LTQ Orbitrap XL (Thermoscientific). The resultant data was processed using Proteome Discoverer (Version 1.4) using the Mascot search engine (Version 2.3.2). A Mascot generic file (mgf) was generated and used to search local IPI human peptide databases using a Mascot Server for Protein Identification.

## 2.15 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 dose or time. These multilevel statistical methods were employed to best accommodate nested data (Aarts et al., 2014). Nesting existed at multiple levels within the experiments described below, including nesting by culture batch, cell line, temperature, time, injurious stressor and compound exposure. In each case, *N* denotes the number of individual cell lines used (all derived from wild-type cells) and *n* describes the total number of independently differentiated batches of hCNs, pooled from one or more cell lines and used as the statistical *n* for each experiment (the number of independent observations). 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$^{o}$C) or untreated

cells for differentiation, hypothermia, KCl/FPL stimulation and pharmacological studies respectively. Additional controls are described in the relevant figure legend as needed. Where appropriate, asterisks denote significance of the test statistic as follows: *$p$<0.05, **$p$<0.01,***$p$<0.001,****$p$<0.0005.

## 2.16 Media and supplements

Media components were purchased from Invitrogen unless otherwise stated. CDM comprised 50 % Iscove's Modified Dulbecco's Medium (IMDM), 50 % Advanced DMEM F12 Reduced Serum Medium, 5 mg/ml bovine serum albumin (BSA; Europa), 1 % CD Lipid 100X, 4 % Monothioglycerol (Sigma), 0.07 % Insulin (Roche), 0.05 % Transferrin (Roche) and 0.1 % Penicillin-Streptomycin. Neuralisation medium included CDM supplemented with 0.2 % N-Acetyl cysteine, 0.1 % Activin Inhibitor (R&D Systems) and 100 μM LDN193189 (Stratech). Neural rosette medium comprised Advanced DMEM F12 Reduced Serum Medium, 1 % Anti-Anti Antibiotic-Antimycotic, 1 % Glutamax, 1 % N2, 0.5 % B27 and 2.5 ng/ml FGF2. aNPC expansion medium contained Advanced DMEM F12 Reduced Serum Medium with 1 % Anti-Anti Antibiotic-Antimycotic, 1 % Glutamax, 1 % N2, 0.1 % B27 and 10 ng/ml FGF2. For freezing aNPCs, this medium was supplemented with 10 % DMSO (Sigma). aNPC plating medium comprised Advanced DMEM F12 Reduced Serum Medium with 1% Anti-Anti Antibiotic-Antimycotic, 1 % Glutamax, 0.5 % N2, 1 % B27, 2 μg/ml Heparin (Sigma) and 2.5 ng/ml FGF2. Default medium comprised Advanced DMEM F12 Reduced Serum Medium with 1 % Anti-Anti Antibiotic-Antimycotic, 0.5 % Glutamax, 0.5 % N2, 0.5 % B27 and 2 μg/ml Heparin

(Sigma). For aNPC differentiation, Forskolin (10 µM, Tocris) was added from 7-21 d, and from 21 d onwards, media was supplemented with BDNF and recombinant human glial-derived neurotrophic factor (rhGDNF) (both at 5 ng/ml, Tocris). From 28 d onwards media was further supplemented with human insulin-like growth factor-1 (IGF-1, 10 ng/ml, Peprotech). Primary plating medium comprised Advanced DMEM F12, 2 % B27, 1 % FCS, 1 % Anti-Anti Antibiotic-Antimycotic and 1 % Glutamax. Neurosphere medium contained Advanced DMEM F12, 2 % B27, 1 % Anti-Anti Antibiotic-Antimycotic, 1 % Glutamax, 20 ng/ml FGF2 and 4 µg/ml Heparin. MiM (Gupta et al., 2013) for KCl stimulation and injury experiments comprised 90 % salt-glucose-glycine solution (SGG) (Bading et al., 1993) with 10 % Minimal Eagle's Medium (+Earle's, -Glutamine) and 0.5 % Penicillin-Streptomycin. SGG contains 10 mM HEPES (pH 7.4), 114 mM NaCl, 26.1 mM $NaHCO_3$, 5.3 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCal_2$, 30 mM glucose, 1 mM glycine, 0.5 mM sodium pyruvate and 0.001 % phenol red (Sigma). For oxidative and excitotoxic stress $H_2O_2$ and L-Glutamic acid were applied for a duration of 24 h, after 24 h of temperature shift. Other media additives were applied from the start of the temperature shift (the preconditioning phase) by 50 % media replacement at the following concentrations: tunicamycin 0.3 µg/ml (Sigma), GSK2606414 500 nM (Calbiochem), fostriecin 100 nM (Calbiochem), TCS 2002 15 µM (Tocris). After 24 h, $H_2O_2$ was applied as above for the 24 h injury phase (compounds were thus diluted by 50 %). In preliminary experiments to evaluate their baseline toxicity, each of these compounds were applied to hCNs at a range of doses under normothermic conditions, in the absence of $H_2O_2$. hCNs were switched to MiM 12 h prior to a 24 h exposure, followed by multiplexed injury analysis.

Concentrations which sat within the effective dose range of each compound, but had no toxic effect under basal conditions were selected for the final oxidative injury experiments.

**Table M1. Primary antibodies used for immunocytochemistry**

| Antibody target | Host species/type | Dilution | Source |
|---|---|---|---|
| **Brn2 C-20** | Goat polyclonal | 1 in 400 | Santa Cruz |
| **βIII tubulin** | Mouse monoclonal | 1 in 1000 | Sigma |
| **CIRBP** | Rabbit polyclonal | 1 in 200 | Pierce |
| **Cux 1 (CUTL-1)** | Mouse monoclonal | 1 in 500 | Abnova |
| **GFAP** | Mouse Cy3 conjugate | 1 in 500 | Sigma |
| **hNanog** | Goat polyclonal | 1 in 100 | R&D Systems |
| **hOct3/4 (C-10)** | Mouse monoclonal | 1 in 100 | Santa Cruz |
| **hnRNP A1 (4B10)** | Mouse monoclonal | 1 in 500 | Santa Cruz |
| **hSox2** | Goat polyclonal | 1 in 200 | R&D Systems |
| **MAP2** | Rabbit polyclonal | 1 in 1000 | Abcam |
| **MAP2 Clone HM2** | Mouse monoclonal | 1 in 1000 | Sigma |
| **MBP** | Rat monoclonal | 1 in 50 | Abcam |
| **O4** | Mouse monoclonal | 1 in 300 | R&D Systems |
| **Pan-tau** | Rabbit polyclonal | 1 in 200 | Dako |
| **PDGFRα** | Rabbit polyclonal | 1 in 200 | Cell Signalling |
| **PP2A C subunit (Clone 1D6)** | Mouse monoclonal | 1 in 250 | Millipore |
| **PSD-95 Clone K28/43** | Mouse monoclonal | 1 in 500 | UC Davis/NIH NeuroMab Facility |
| **RBM3** | Rabbit monoclonal | 1 in 1000 | Abcam |
| **RBM4** | Rabbit polyclonal | 1 in 500 | Pierce |
| **RD3 (Clone 8E6/C11) 3R tau** | Mouse monoclonal | 1 in 100 | Millipore |
| **Satb2** | Mouse monoclonal | 1 in 100 | Abcam |
| **Sox2** | Mouse monoclonal | 1 in 100 | Millipore |
| **Synaptophysin** | Mouse monoclonal | 1 in 250 | Millipore |
| **TIA-1** | Mouse monoclonal | 1 in 500 | Abcam |
| **4R tau (Clone 5F9)** | Mouse monoclonal | 1 in 50 | Covance |

## Table M2. Primer sequences used for q-RT-PCR

| Gene target | Forward primer | Reverse primer |
|---|---|---|
| **3R TAU**[a] | TTGCTCAGGTCAACTGGTTTGTA | ACTGAGAACCTGAAGCACCA |
| **4R TAU**[a] | TGCAGATAATTAATAAGAAGCTGGA | GTGTTTGATATTATCCTTTGAGC |
| **ATF4**[b] | TCAAACCTCATGGGTTCTCC | GTGTCATCCAACGTGGTCAG |
| **BACT**[c] | GTTACAGGAAGTCCCTTGCCATCC | CACCTCCCCTGTGTGGACTTGGG |
| **BDNF Exon IV**[c] | TCCACTATCAATAATTTAACT | AAACTCCCACACTCTATT |
| **CIRBP** | TAGAGGAGGAGGGGACCGAG | TCACTGTAGCCACCACTCTG |
| **CTIP2** | TCCAGCTACATTTGCACAACA | GCTCCAGGTAGATGCGGAAG |
| **CUX1** | CCAAGCCGAAACCATAGCTCT | AGGCTCTGAACCTTATGCTCA |
| **DDIT3**[d] | ACCAAGGGAGAACCAGGAAACG | TCACCATTCGGTCAATCAGAGC |
| **EIF4A2** | GAAGCCTTCCGCTATTCAGCA | CTTGGGTCTCCTTGAACTCAATC |
| **EMX2**[e] | GCTTCTAAGGCTGGAACACG | CCAGCTTCTGCCTTTTGAAC |
| **ERN1**[d] | TGGGTAAAAAGCAGGACATCTGG | GCATAGTCAAAGTAGGTGGCATTCC |
| **ETV1**[e] | CTGCCTGCAGTCAAGAACAG | AGGGCCTCATTCCCACT |
| **FOS**[c] | CTACCACTCACCCGCAGACT | AGGTCCGTGCAGAAGTCCT |
| **GADD34** | CGACTGCAAAGGCGGC | CAGGAAATGGACAGTGACCTTC |
| **GAPDH**[f] | GAGTCCACTGGCGTCTTCAC | ATGACGAACATGGGGGCAT |
| **GFAP**[g] | ATCGAGAAGGTTCGCTTCCTG | TGTTGGCGGTGAGTTGATCG |
| **GRIN 2A** | TGGCCTCACCGGGTATGATT | CAATGCCGTCCCTCACTCTC |
| **GRIN 2B** | GTCCCTGGACGATGGAGAT | CAGTCAGCCCTACTGAGTT |
| **GRP94** | TTGGTGTCGGTTTCTATTCC | GCTGGGTATCGTTGTTGTG |
| **HPRT1**[h] | AATTATGGACAGGACTGAACGTCTTG | TCCAGCAGGTCAGCAAAGAATTTATAG |
| **HSPA5 (HSP70/BiP)**[i] | CATCACGCCGTCCTATGTCG | CGTCAAAGACCGTGTTCTCG |
| **IRX3**[e] | CCGTATGGCCAGTACCAGTT | ATAAGCGTTTCCCTCCTCGT |
| **JUN**[j] | TCGACATGGAGTCCCAGGA | GGCGATTCTCTCCAGCTTCC |
| **MAPT (Total tau)**[k] | CCAAGTGTGGCTCATTAGGCA | CCAATCTTCGACTGGACTCTGT |
| **MYC** | CCAGGCTTAGATGTGGCTCT | CTCTGACCTTTTGCCAGGAG |
| **NESTIN**[l] | GGCGCACCTCAAGATGTCC | CTTGGGGTCCTGAAAGCTG |
| **PSD-95**[m] | ACAAGCGGATCACAGAGGAG | CAGATGTAGGGGCCTGAGAG |
| **RBM3**[n] | CTTCAGCAGTTTCGGACCTA | ACCATCCAGAGACTCTCCGT |
| **RBM4** | AAGACAAGACGGCAGCTGAG | GTGCAGGTGGGACTGATGTT |
| **REELIN** | ACATCTACAAGTGTTCAGGCATC | TGGTTACCAAACTGGTGGTCA |
| **RPL29** | CAGTCCCGAAAATGGCACAGA | GGCTTTACGAGGGCCTTGATA |
| **S100β**[g] | TGGCCCTCATCGACGTTTTC | CAGTGTTTCCATGACTTTCTCCA |
| **TBR1**[e] | ATGGGCAGATGGTGGTTTTA | GACGGCGATGAACTGAGTCT |
| **TBR2**[e] | CACCGCCACCAAACTGAGAT | CGAACACATTGTAGTGGGCAG |
| **VGLUT1**[e] | GAAACTCATGAACCCCCTCA | GGGAGATGAGCAGCAGGTAG |
| **VGLUT2**[e] | ATTCCATCAGCAGCCAGAGT | TTGCTCCATATCCCATGACA |
| **XBP1**[o] | TTACGAGAGAAAACTCATGGCC | GGGTCCAAGTTGTCCAGAATGC |
| **XBP1s**[p] | TGCTGAGTCCGCAGCAGGTG | GCTGGCAGGCTCTGGGGAAG |

[a](Deshpande et al., 2008), [b](Li et al, 2014) [c](Bilican et al., 2014), [d](Lin et al., 2007), [e](Espuny-Camacho et al., 2013), [f](Caradec et al., 2010), [g](Serio et al., 2013) [h](Ahn et al., 2008), [i](Harvard primer bank), [j](University of Twente), [k](Zhao et al., 2014), [l](Lee et al., 2013), [m](Hibaoui et al., 2014), [n](Wellmann et al., 2004), [o](Samali et al., 2010), [p](van Schadewijk et al., 2012).

**Table M3. Primary antibodies used for immunoblotting**

| Antibody target | Host species/type | Dilution | Source |
|---|---|---|---|
| **4R tau (Clone 5F9)** | Mouse monoclonal | 1 in 250 | Covance |
| **ATF6** | Mouse monoclonal | 1 in 100 | Abcam |
| **AT8** | Mouse monoclonal | 1 in 1000 | Pierce |
| **AT100** | Mouse monoclonal | 1 in 1000 | Pierce |
| **AT180** | Mouse monoclonal | 1 in 1000 | Pierce |
| **AT270** | Mouse monoclonal | 1 in 1000 | Pierce |
| **β-actin (Clone AC-74)** | Mouse monoclonal | 1 in 20,000 | Sigma |
| **Bax** | Rabbit monoclonal | 1 in 2500 | Abcam |
| **BiP (GRP78)** | Rabbit monoclonal | 1 in 1000 | Abcam |
| **CIRBP** | Rabbit polyclonal | 1 in 500 | Proteintech |
| **eIF2α** | Mouse monoclonal | 1 in 1000 | Abcam |
| **p-eIF2α** | Rabbit monoclonal | 1 in 100 | Cell Signalling |
| **GAPDH** | Mouse monoclonal | 1 in 10,000 | Calbiochem |
| **GFAP** | Rabbit polyclonal | 1 in 500 | Dako |
| **GSK3β total** | Rabbit monoclonal | 1 in 1000 | Cell Signalling |
| **GSK3β pS9** | Rabbit polyclonal | 1 in 1000 | Cell Signalling |
| **GSK3β pY216** | Rabbit polyclonal | 1 in 100 | Abcam |
| **hnRNP A1 (4B10)** | Mouse monoclonal | 1 in 1000 | Santa Cruz |
| **MAP2** | Rabbit polyclonal | 1 in 2000 | Abcam |
| **Pan-tau** | Rabbit polyclonal | 1 in 10,000 | Dako |
| **PERK** | Rabbit monoclonal | 1 in 100 | Cell Signalling |
| **PHF-1 (pS396/S404)** | Mouse monoclonal | 1 in 500 | Peter Davies |
| **PP2A C subunit (Clone 1D6)** | Mouse monoclonal | 1 in 500 | Millipore |
| **pS404 (phospho-tau)** | Rabbit monoclonal | 1 in 1000 | Abcam |
| **RD3 (Clone 8E6/C11) 3R tau** | Mouse monoclonal | 1 in 2000 | Millipore |
| **RBM3** | Rabbit monoclonal | 1 in 100 | Abcam |
| **RBM4** | Rabbit polyclonal | 1 in 500 | Proteintech |

# Chapter 3: Hypothermic neuroprotection in hCNs

## 3.1 Introduction

Several groups have described *in vitro* generation of cortical neurons from hPS lines (Johnson et al., 2007; Zeng et al., 2010; Shi et al., 2012b; Espuny-Camacho et al., 2013; Kadoshima et al., 2013; Bilican et al., 2014). Recently, the Chandran group established a platform of cryo-preservable hPS-derived aNPCs that can be expanded long-term at physiological normoxia (3% $O_2$), circumventing the need for *de novo* neuralization (Stacpoole et al., 2011; Bilican et al., 2014). These aNPCs retain forebrain identity for many passages and, upon removal of exogenous mitogens, they differentiate into deep- and superficial-layer cortical excitatory neurons (hCNs) exhibiting activity-dependent gene regulation, with putative functional synapses (Bilican et al., 2014; Livesey et al., 2014). hCNs display a temporal electrophysiological maturation profile, including transitions in both AMPA receptor subunit composition and $GABA_A$ receptor reversal potential (Livesey et al., 2014). Functional assessment of the subunit composition of inhibitory ionotropic $GABA_A$ and glycine receptors in hCNs has also been reported (James et al., 2014).

Owing to their large metabolic requirements and relatively low antioxidant levels, cortical neurons are highly susceptible to oxidative stress (Papadia et al., 2008; Weil et al., 2008). These neurons are also vulnerable to glutamate–mediated excitotoxicity (Hardingham and Bading, 2010) which is an important contributor to neuronal death in several disease states and TBI (Werner and Engelhard, 2007; Weil et al., 2008; Campos et al., 2012). Oxidative stress and excitotoxcity manifest during

the subacute phase of cerebral ischaemia, and both are inhibited by cooling in animal models of TH (Ginsberg and Belayev, 2005; Yenari and Han, 2012). However, *in vivo* cooling paradigms (particularly in human clinical trials) have suffered from inadequate brain temperature monitoring (Mellergard et al., 1990; Childs et al., 2005; Kuo et al., 2011). Regional differences in brain temperature also exist, fluctuating with neuronal activity and disease state (Wang et al., 2014). Antonic et al. (2014) recently described mild hypothermic protection of hPS-derived neurons *in vitro*, but this study did not address excitotoxicity – one of the principal neurotoxic mechanisms in the brain. This study was also limited to a single cell line, from which relatively immature (11 DIV) and mixed cultures were derived with no demonstration of functional neuronal capacity. The primary aim of the experiments that follow was to establish a more robust and clinically-relevant *in vitro* model of TH using hCNs. Cortical neurons were considered the most appropriate neuronal subtype for this study given (1) their vulnerability to both acute and chronic injury states, (2) their established 'default' differentiation from hPS lines (demonstrated reproducibly by several groups worldwide (see Bilican et al., 2014) – this is not the case for other neuronal subtypes such as hippocampal neurons for which few publications exist (Yu et al., 2014)) and (3) their use in key publications that have modelled glutamate toxicity, cooling-induced neuroprotection and cold-shock protein induction(Choi et al., 1987; Murphy and Baraban, 1990; Chip et al., 2011; Jantzie et al., 2013; Lee et al., 2014; Sato-Numata et al., 2014, Xu et al., 2014).

## 3.2 Results

### 3.2.1 Validation of hCN generation and tolerance to cooling

The derivation and electrophysiological characterization of hCNs was published recently together with quantification of cortical layer-specific markers at 5 w of differentiation (Bilican et al., 2014; Livesey et al., 2014; James et al., 2014). Here, core elements of the differentiation protocol are replicated and extended to show that hCNs express an enriched cortical transcript profile (*N*=2; *n*=5; Fig.3.1) and display axonal-dendritic polarization (Fig.3.2a). Consistent with previous findings of miniature excitatory post-synaptic currents (Bilican et al., 2014) evidence of pre- and post-synaptic features (from 25 DIV) and putative spine morphology (from 35 DIV) can be observed in hCNs using transmission electron microscopy (TEM) (Fig.3.3a). During the first 6 w, cultures rapidly transform from a dense, nestin-positive aNPC population to a highly enriched βIII-tubulin- (86.1 ± 0.94 %), and MAP2 (95.0 ± 0.12 %)-positive population. Alongside the expression of various cortical layer markers (Fig.3.2b), a small percentage (<10 %) of GFAP-positive glia (*N*≥3; *n*≥5; Fig.3.2c) and rapid activity-dependent gene regulation (*N*=2; *n*=6; Fig.3.3b), these data confirm the regional specification, purity and functionality of the model system under test. To investigate whether hypothermia is protective of hCN cultures, 2 temperatures were selected (28°C, 32°C) and compared to control cultures at 37°C. After 24 h of temperature shift, neuronal viability was determined by immunocytochemical quantification of βIII-tubulin-positive cells containing intact nuclei (*N*=3; *n*=8; Fig.3.4a). The proportion of viable neurons was comparable at each temperature confirming that cooling alone was well tolerated.

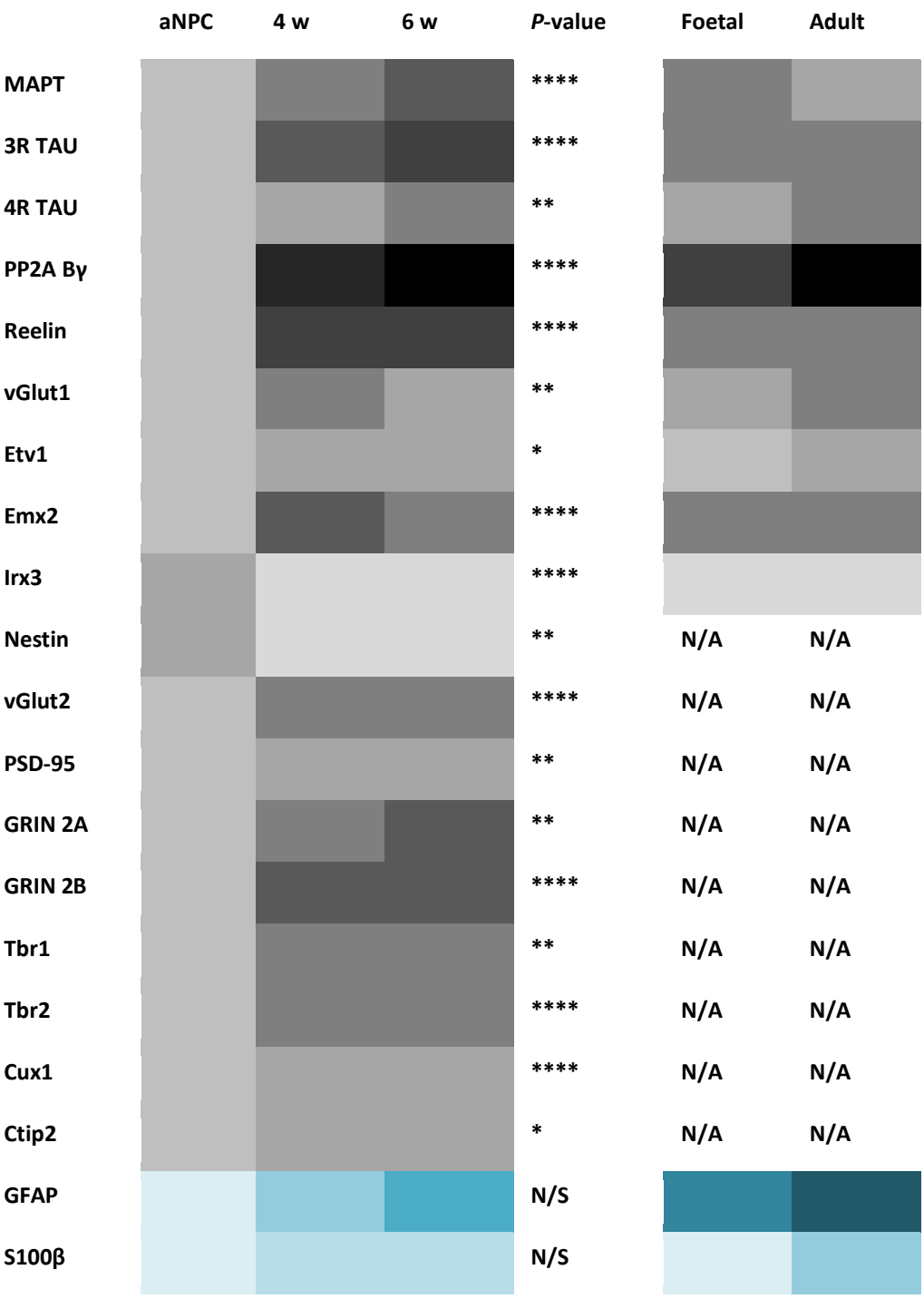


**Figure 3.1 Transcript analysis of hCNs.** A 'heat map' of fold changes in hCN transcript expression relative to aNPC level; asterisks denote statistical significance at 4 w (*N*=2; *n*=5). Note upregulation of deep- and superficial-layer cortical (Tbr1, Ctip2; cut-like homeobox 1, Cux1) and excitatory (vesicular glutamate transporter 1 and2; vGlut1, vGlut2) transcripts alongside glial transcripts (GFAP and S100β) with concomitant downregulation of precursor (Nestin) and diencephalon (iroquois homeobox 3; Irx3) markers. Glial transcripts did not increase significantly until 6 w. Some transcripts were assessed in cDNA synthesised from commercially sourced human RNA (colour change refers to fold differences in adult cortex relative to foetal whole brain). All transcripts were normalized to the geometric mean of 3 developmentally-stable reference targets. N/S = non-significant, N/A = not assessed.

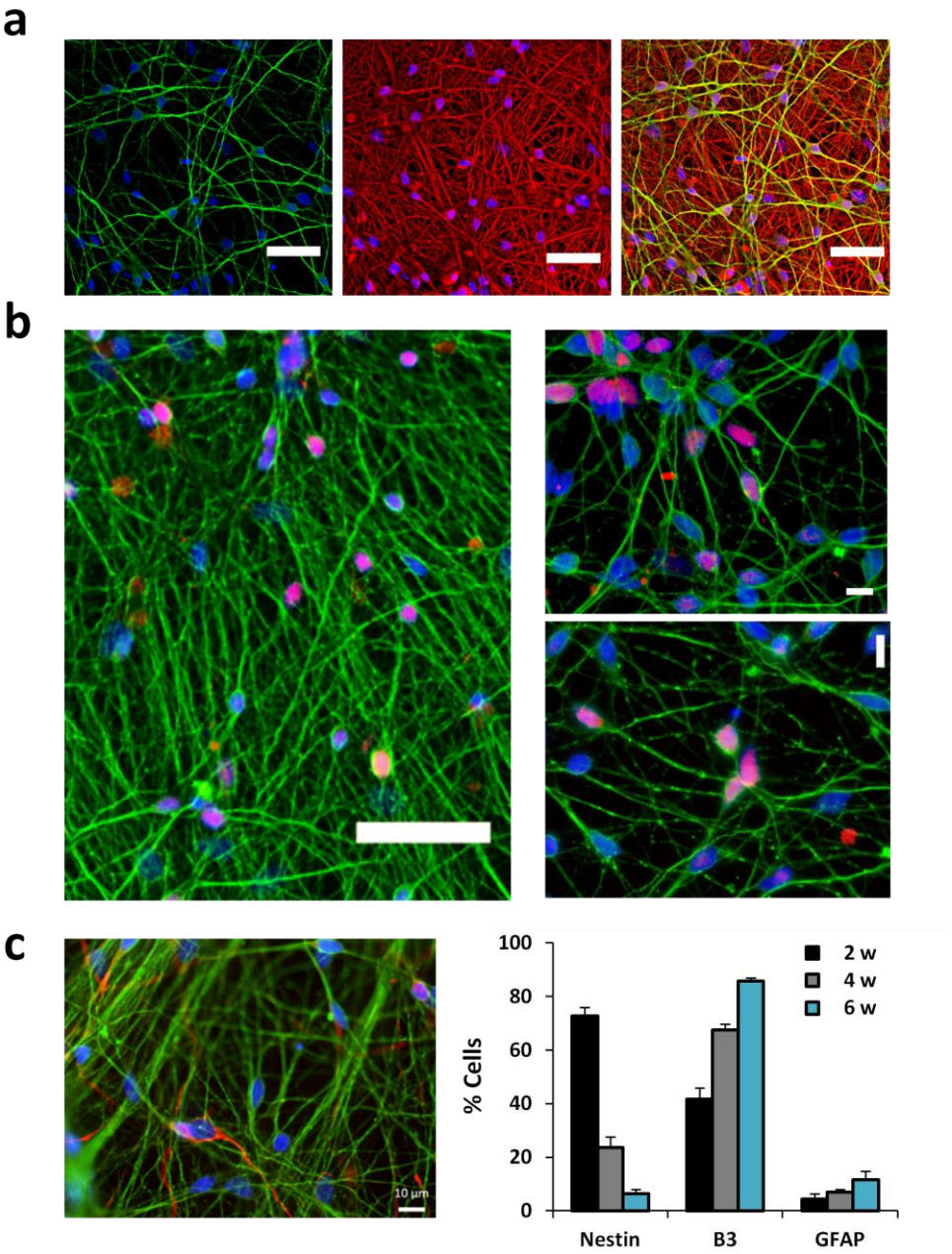


**Figure 3.2 Immunocytochemical validation of hCNs. (a)** Confocal fluorescent micrographs of hCNs developing axonal-dendritic polarity (HES1, 6 w; left, Map2 in green; middle, βIII-tubulin in red; right, merged image), scale bar = 50 µm. **(b)** Widefield fluorescent micrographs of differentiating hCNs co-expressing neuronal marker βIII-tubulin (green) with nuclear markers representative of various cortical layers (left, SATB homeobox 2, Satb2, IPS1 at 6 w, scale bar = 50 µm; right upper, POU class 3 homeobox 2, Brn2, HES1 at 2 w, scale bar = 10 µm; right lower Cux1, HES1 at 2 w, scale bar = 10 µm). **(c)** Left: widefield fluorescent micrograph of hCN culture a 4 w showing highly enriched population of βIII-tubulin expressing neurons (green) and a small proportion of GFAP-expressing glia (red), scale bar = 10 µm. Right: mean % + SEM of DAPI-stained cells expressing cell phenotype-specific markers at various stages of differentiation ($N \geq 3$; $n \geq 5$; data published in Livesey et al., 2014). Note decrease in precursor marker Nestin alongside increase in βIII-tubulin with time in culture. All cells were counterstained with DAPI (blue).

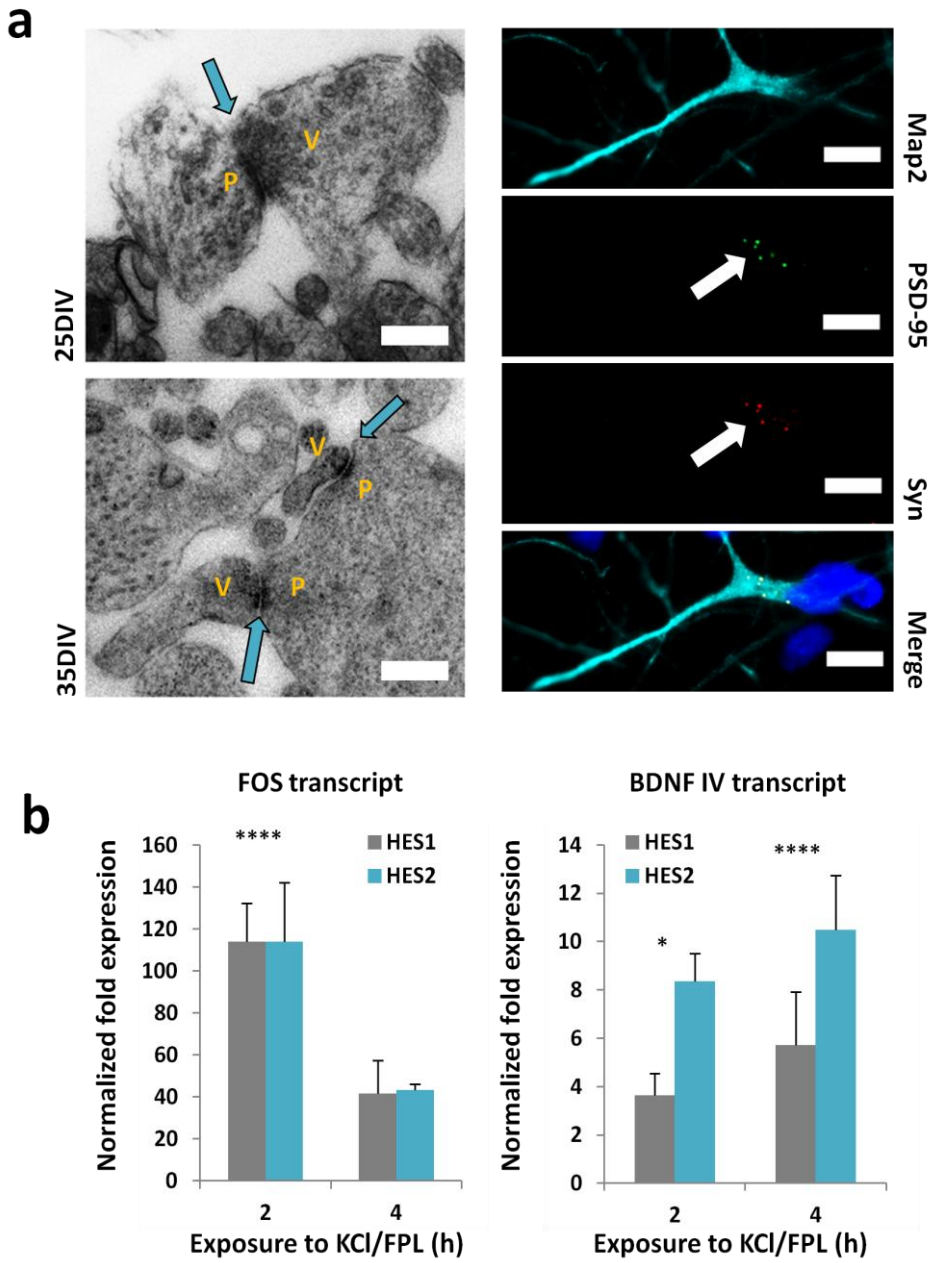


**Figure 3.3 Functional analysis of hCNs. (a)** Left: transmission electron micrographs of hCNs (upper, scale bar = 0.2 µm, lower, scale bar = 0.5 µm). Presynaptic vesicles (V), post-synaptic density (P), red arrows indicate putative synaptic clefts. Right: widefield fluorescent micrographs showing co-localisation of pre-(synaptophysin, Syn) and post-(PSD-95) synaptic markers on MAP2-positive hCNs at 2 w post-plating, white arrows indicate putative synaptic puncta, cells counterstained with DAPI, scale bar = 10 µm. **(b)** Activity-dependent gene regulation in hCNs (*N*=2; *n*=6) as determined by q-RT-PCR showing transcript responses (left, FOS; right, BDNF Exon IV) to KCl stimulation in the presence of FPL64176 and MK-801 (Bilican et al., 2014). Expression was normalized to the geometric mean of 3 stimulation-stable reference targets. Raw data has been split by cell line (*n*=3 batches for each) and is presented as normalized mean fold change over matched untreated control wells. Multilevel statistical analysis was performed on pooled data (see Methods) and showed that FOS induction peaked at 2 h (*N*=2; *n*=6; *P*<0.0005, range 61.9 to 157.6) whilst BDNF Exon IV peaked at 4 h (*N*=2; *n*=6; *P*<0.0005, range 1.45 to 12.9) post-stimulation. Asterisks denote significant increase relative to untreated control based on pooled data. Note that the cell lines responded functionally in a similar manner.

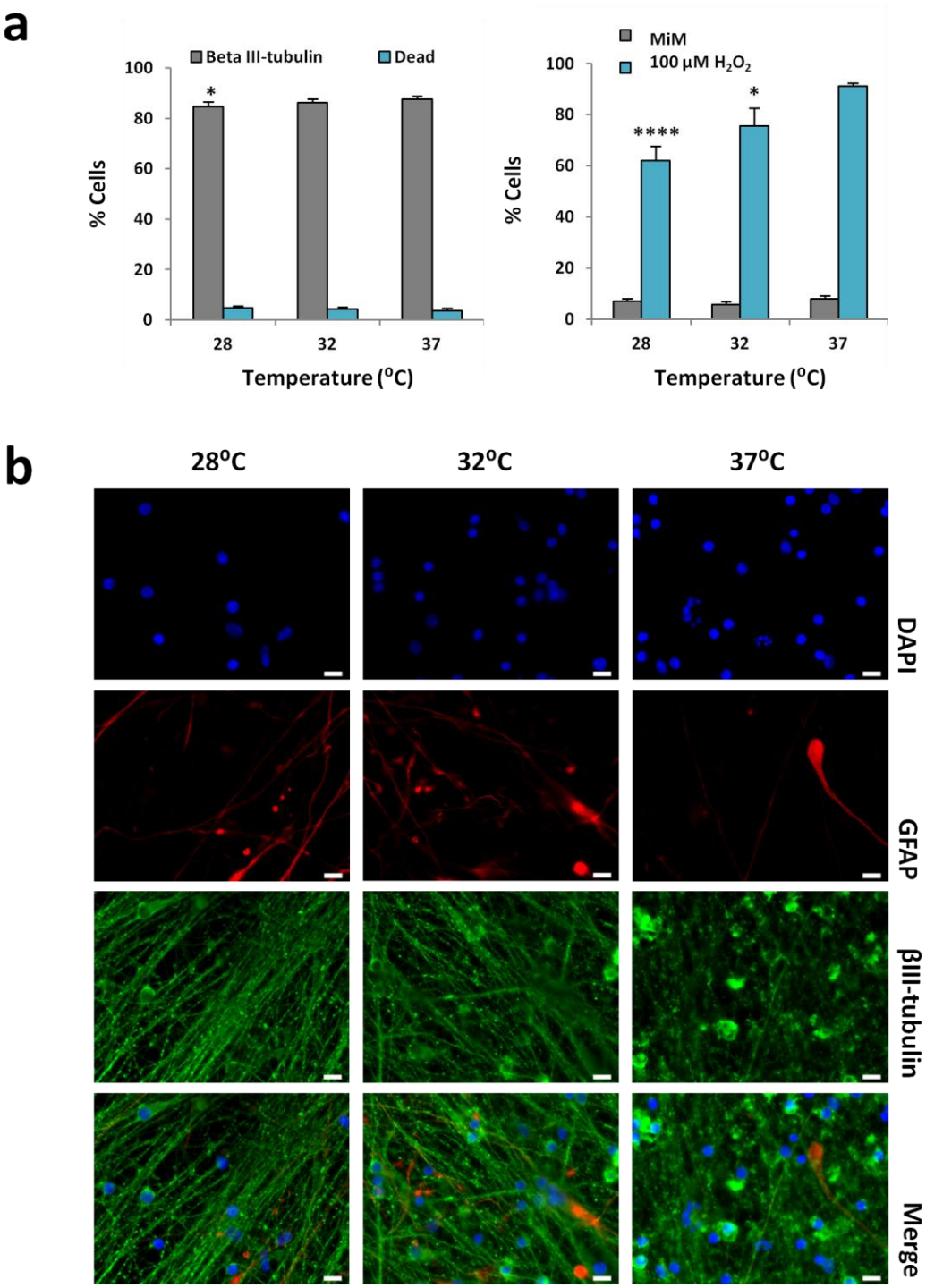


**Figure 3.4 hCN death in response to cooling and $H_2O_2$ (a)** Left: cell counts of hCNs showing little to no effect of 24 h cooling on viability or the proportion of βIII-tubulin-positive cells (*N*=3; *n*=8; *P*<0.05). Right: cell death counts in response to 24 h oxidative stress after cooling (*N*=3; *n*=8). Note that neurons incubated in MiM alone were unaffected by incubation temperature. Counts are presented as mean % + SEM of DAPI-stained cells. **(b)** Representative widefield fluorescent micrographs of hCNs after 24 h incubation with 100 µM $H_2O_2$, scale bar = 10 µm. Cells were co-stained for neuronal (βIII-tubulin), astrocytic (GFAP) and nuclear (DAPI) markers. Note small number of GFAP-positive cells at each temperature, even at 37°C – a temperature at which $H_2O_2$ was highly neurotoxic.

### 3.2.2 Oxidative stress

To avoid a confounding cellular stress by re-warming, hCNs were pre-cooled for 24 h and then maintained at their respective temperatures throughout exposure to stressors (Lleonart, 2010; Chip et al., 2011; Neutelings et al., 2013). The combined cooling and stress paradigm was summarized in Fig.2.4. 24 h treatment with 100 μM $H_2O_2$ produced temperature-dependent injury according to cell death counts (*N*=3; *n*=8) which reported a 14.6 % reduction in death at 32°C (*P*=0.013), and a 29.2 % reduction in death at 28°C (*P*<0.0005) (Fig.3.4a). The protective effect of cooling was clearly evident by immunocytochemistry (Fig.3.4b) and live imaging (Fig.3.5a). Multiplexed analysis (Fig.3.5b-c) showed that oxidative injury was [$H_2O_2$]-dependent and again that hypothermia was neuroprotective (*N*=2; *n*=9); injury in response to 100 μM $H_2O_2$ treatment was reduced by 36 % at 32°C (19.9 % versus 31.3 % at 37°C, *P*=0.046) and 78 % at 28°C (6.9 % versus 31.3 %, *P*<0.0005).

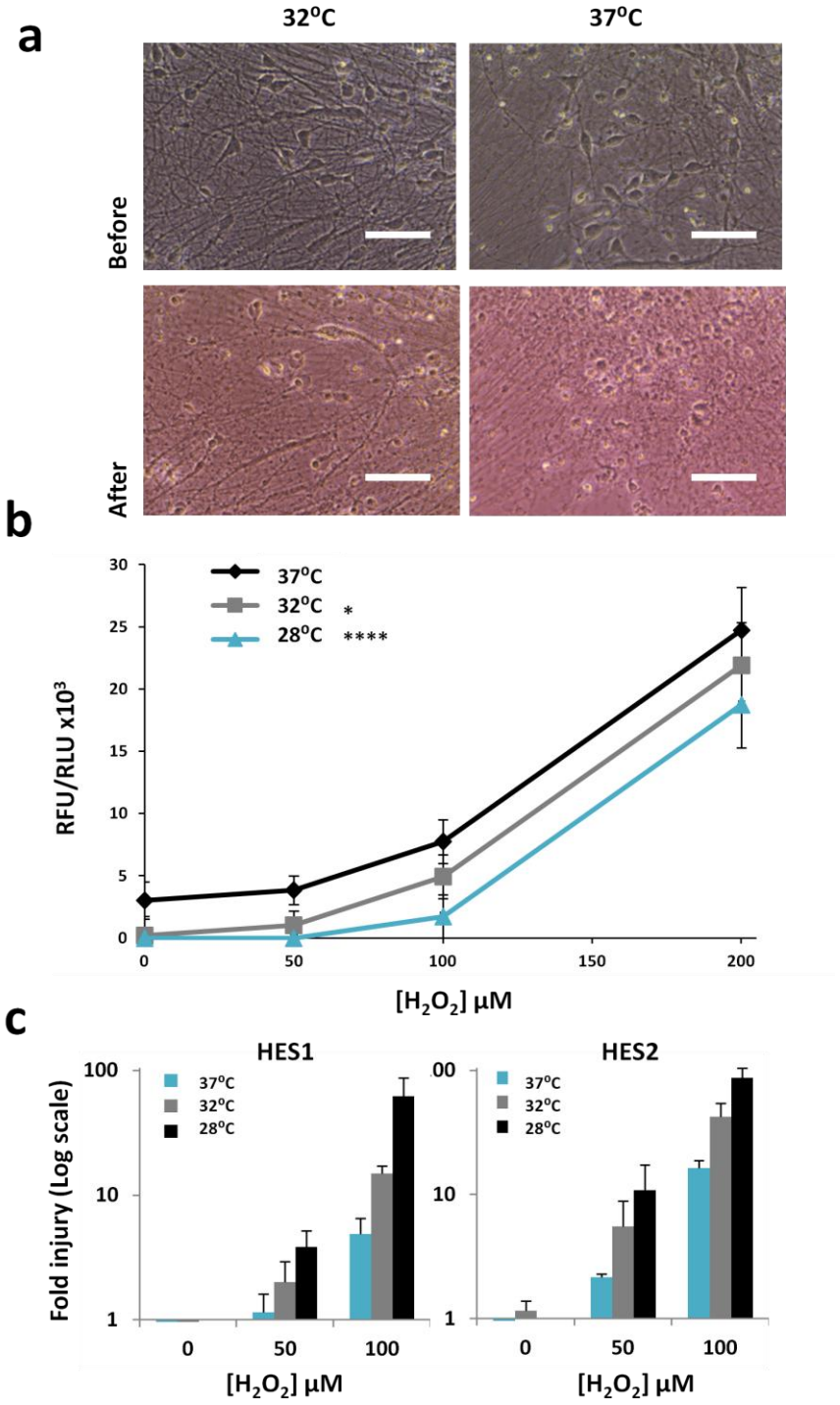


**Figure 3.5 Hypothermia protects hCNs from oxidative stress-mediated injury. (a)** Phase contrast micrographs of hCNs taken before and after incubation for 24 h with 100 µM $H_2O_2$ at the indicated temperatures, scale bar = 20 µm. **(b)** Multiplexed injury analysis with dose response curves for $H_2O_2$ (*N*=2; *n*=9). 200 µM $H_2O_2$ produced maximal injury at 37°C when compared to positive control (cell lysis with Triton-X). Injury data are presented as point estimates (PE) ± estimated standard error (ESE) relative to normothermic control. **(c)** Raw data from **(b)** has been split by cell line (*n*=5 batches for HES1 and *n*=4 batches for HES2) and is presented as mean fold injury (RFU/RLU) relative to untreated control at 37°C + SEM. Note that each cell line responds to $H_2O_2$ and hypothermia in a similar manner, thus justifying pooling of data from these cell lines for statistical analysis.

### 3.2.3 Excitotoxic stress

To determine whether cooling protected hCNs against a different type of neuronal injury the effect of hypothermia on glutamate–mediated excitotoxicity was examined (Gupta et al., 2013). Given that 93 % of neurons in hCN cultures are electrophysiologically active and exhibit an excitatory profile (Bilican et al., 2014; Livesey et al., 2014), the prediction was that they would be susceptible to glutamate toxicity. Preliminary experiments however suggested an almost complete tolerance of 5 w hCNs to glutamate (Fig.2.6). Previous work had shown that hCNs express the glutamate uptake transporter vGlut1 (Bilican et al., 2014), which is upregulated in response to exogenous glutamate and directly controls pre-synaptic glutamate release (Murphy and Baraban, 1990; Wilson et al., 2005). Since hCN culture medium contained relatively high glutamate levels, it was considered that long-term culture in these conditions might upregulate neuronal vGlut1 and thus increase hCN buffering capacity in response to exogenous glutamate. Cells were thus switched to MiM for an extended period prior to stress application, to allow sufficient time for downregulation of glutamate buffering mechanisms. This proved essential in revealing glutamate excitotoxicity in hCNs, sufficient to demonstrate a neuroprotective effect of hypothermia. Although the cell death produced by 30 μM glutamate was much less than that caused by 100 μM $H_2O_2$, cooling was still protective (Fig.3.6). According to absolute counts ($N$=3; $n$=8), cell death was reduced at 32°C (20.5 % versus 30.1 % death at 37°C, $P$=0.001), but not at 28°C (28.8 % death, $P$=0.654), suggesting temperature-specific hypothermic neuroprotection. Multiplexed injury analysis (Fig.3.7b-c) confirmed that glutamate excitotoxicity increased with glutamate concentration in hCNs ($N$=2; $n$=9), but it also

revealed a temperature-dependent protective effect of cooling. Cell injury following exposure to 30 μM glutamate was reduced by 25 % at 32°C and 56 % at 28°C (51.4 % and 29.9 % injury respectively versus 68.6 % injury at 37°C, $P<0.0005$ for both comparisons).

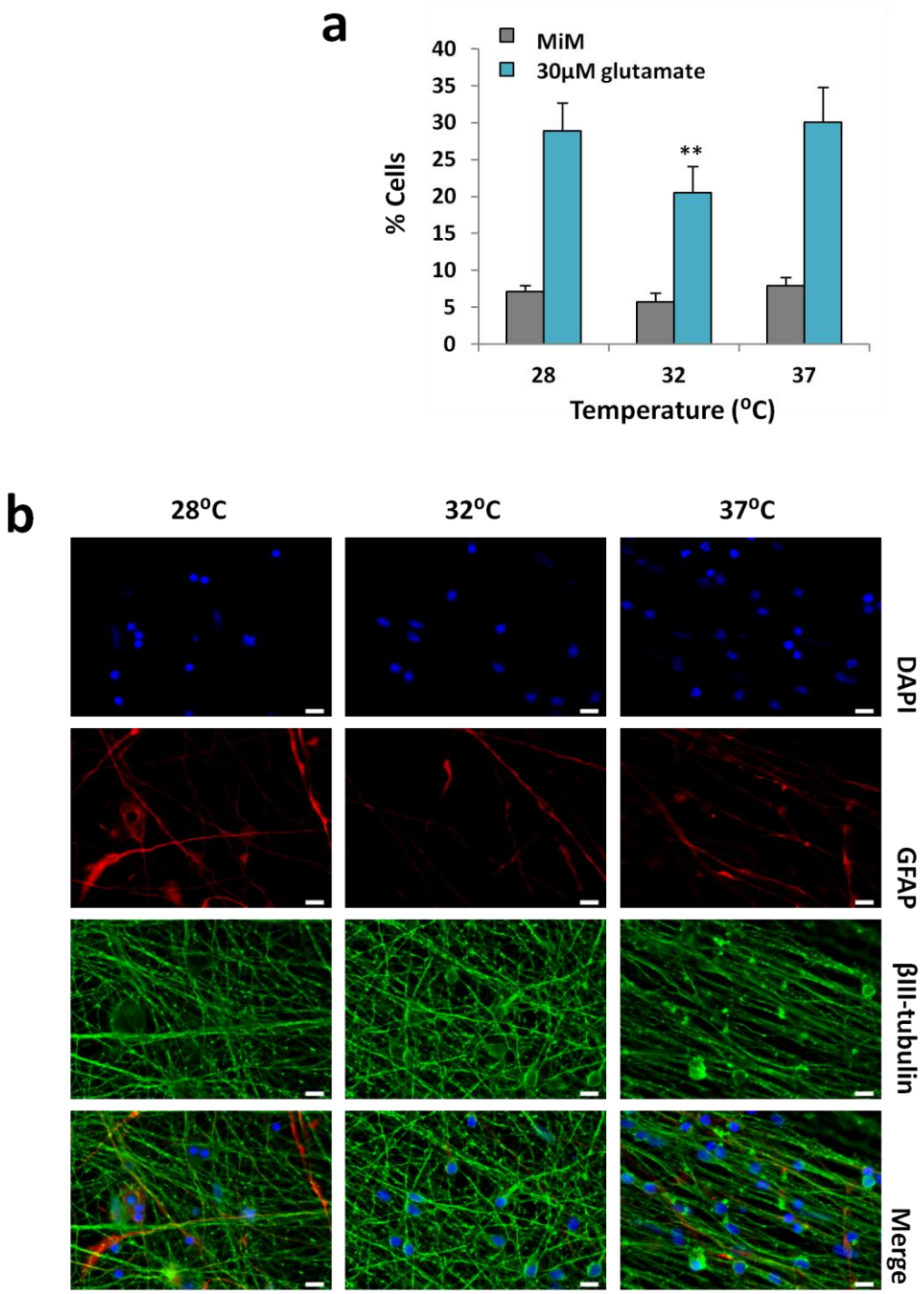


**Figure 3.6 hCN death in response to glutamate.** **(a)** Cell death counts in response to 30 µM glutamate are presented as mean % + SEM of DAPI-stained cells, showing a 31.8 % reduction in cell death at 32°C (*N*=3; *n*=8; *P*=0.001). **(b)** Representative widefield fluorescent micrographs of hCNs after 24 h incubation with 30 µM glutamate, scale bar = 10 µm.

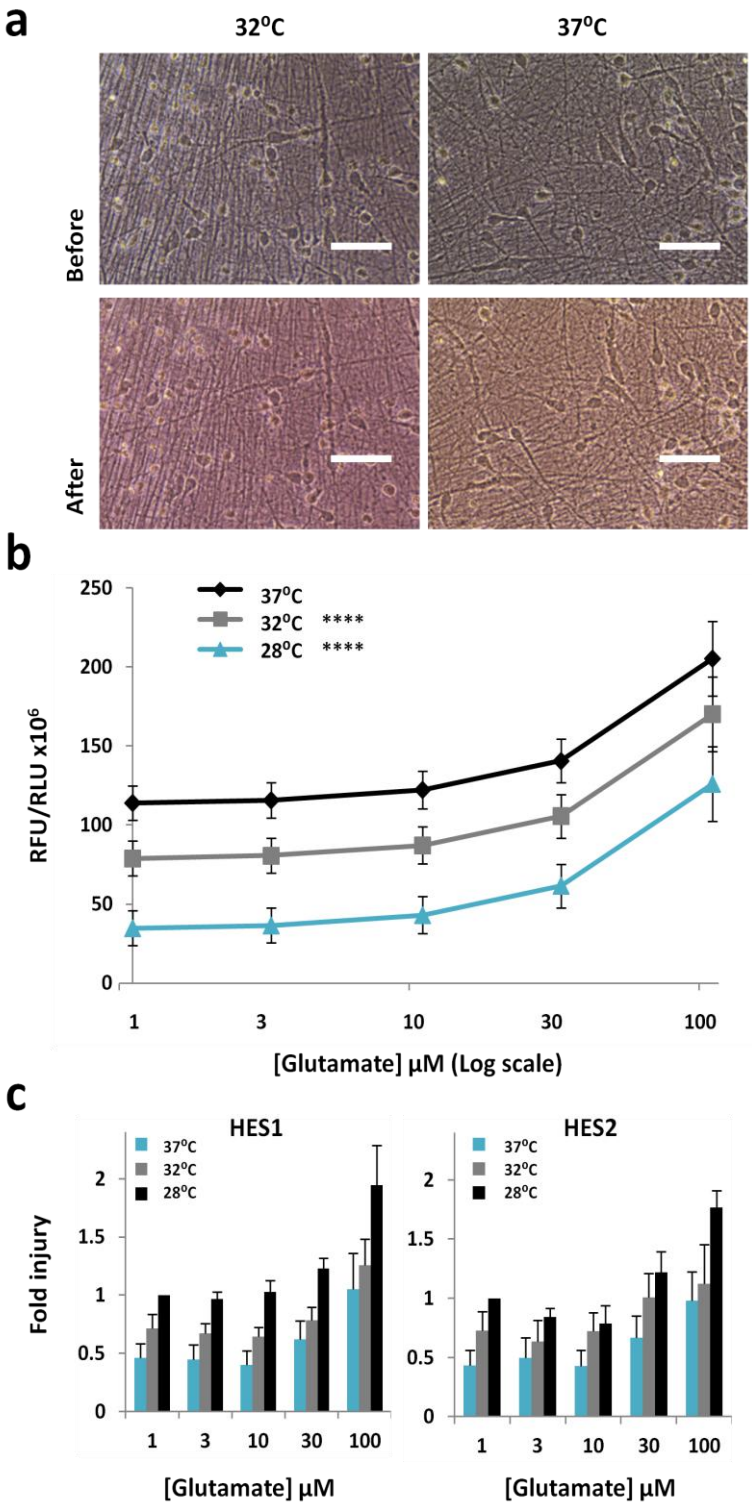


**Figure 3.7 Mild hypothermia protects hCNs from excitotoxic injury.** **(a)** Phase contrast micrographs of hCNs before and after treatment with 30 µM glutamate and incubated at the temperatures indicated, scale bar = 20 µm. **(b)** Dose response curves for excitotoxic injury (*N*=2; *n*=9). Note that maximal injury is not achieved with 100 µM glutamate, suggesting residual glutamate tolerance at baseline. **(c)** Raw data from **(b)** has been split by cell line (*n*=5 batches for HES1 and *n*=4 batches for HES2) and is presented as mean fold injury (RFU/RLU) + SEM relative to untreated control at 37°C. Note that each cell line responds to glutamate and hypothermia in a similar manner, thus justifying pooling of data from these cell lines for statistical analysis.

A role for NMDARs in glutamate-mediated excitotoxicity was recently demonstrated in hPS-derived neurons (Gupta et al., 2013). To verify the physiological relevance of the excitotoxic response in hCNs, NMDAR antagonists were applied during glutamate exposure period (*N*=2; *n*=9). Both D-APV and ifenprodil completely abrogated hCN excitotoxicity ($P<0.0005$; Fig.3.8a), confirming that glutamate injury was mediated predominantly through NMDARs composed of GluN1/GluN2B subunits (NR2B) at each temperature of interest. This result was consistent with q-RT-PCR analysis of subunit transcripts during hCN differentiation (*N*=2; *n*=5; Fig.3.8b) and baseline electrophysiological characterization of 5 w hCNs showing an 80 % reduction in NMDA-mediated current in the presence of ifenprodil (Fig.3.8c). Interestingly, at 32°C, ifenprodil block slightly increased cellular injury at 1 μM glutamate ($P=0.001$).

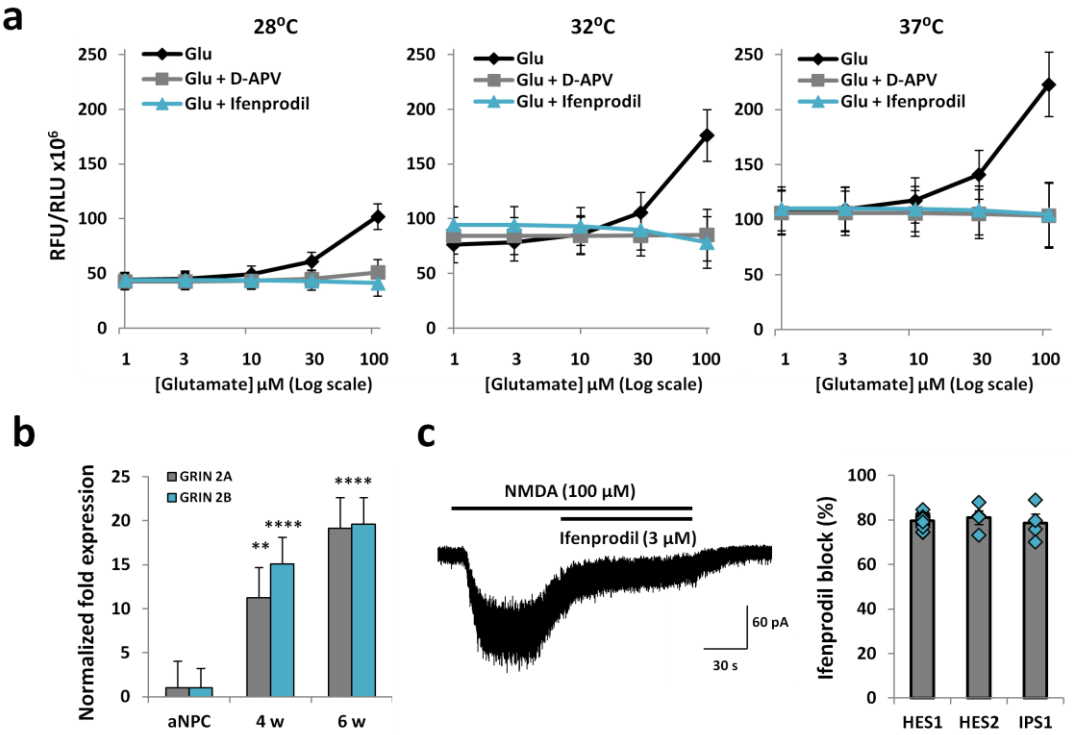


**Figure 3.8 Glutamate injury is NMDAR-dependent. (a)** Glutamate injury curves in the presence of specific (ifenprodil) and non-specific (D-APV) NMDAR blockers (*N*=2; *n*=9). Note shift of each curve downwards with decreasing temperature and a shift upwards at 32°C in the presence of 1 µM glutamate and ifenprodil (*N*=2; *n*=9; *P*=0.001), consistent with a protective effect of low exogenous glutamate (Hardingham et al., 2010). Both blockers prevented the toxic effect of high glutamate, confirming that excitotoxicity was GluN2B-mediated at each temperature. **(b)** q-RT-PCR analysis of subunit-specific NMDAR transcripts during hCN differentiation relative to aNPC expression (*N*=2; *n*=5; GRIN 2A = GluN2A, GRIN 2B = GluN2B, GluN2A at 4 w *P*=0.003, other increases *P*<0.0005). A significant increase was also observed between 4 w and 6 w for the GluN2A transcript (*N*=2; *n*=5; *P*=0.022). Transcript data was normalized to the geometric mean of 3 differentiation-stable reference targets. **(c)** Representative current trace (left) of 5 w hCNs in response to NMDA (100 µM) in the presence of glycine (50 µM) at a holding potential of -74 mV. Application of 3 µM ifenprodil produces approximately 80% block of NMDA-mediated current, confirming that it is largely mediated by GluN1/GluN2B-containing NMDARs. The mean % NMDA receptor block ± SEM was equivalent across all cell lines investigated (right, HES1 *n*=14 cells from 4 independent batches, HES2 *n*=4 cells from 1 batch, IPS1 *n*=4 cells from 1 batch). Injury data are presented as PE ± ESE relative to normothermic control.

## 3.3 Discussion

In respect to the first aim of this thesis, the results above establish an *in vitro* model of TH using hCNs. Initial findings validated previous work in these cultures (Bilican et al., 2014; Livesey et al., 2014) and provided complementary data demonstrating that key developmental changes *in vitro* reflect transitions seen in the developing human cortex (Livesey et al., 2014). Mild-to-moderate cooling protects hCNs from both oxidative and excitotoxic injury. Hypothesis (1) is thus accepted. Intriguingly, hypothermic neuroprotection in these in neurons appears to operate in a temperature- and stress-specific manner, suggesting that mild cooling in particular is beneficial in the context of NR2B-mediated excitotoxicity.

The first *in vitro* demonstration of hypothermic protection of human neurons used dorsal root ganglia and entailed a profound temperature shift to 15-20$^{o}$C (Reyes et al., 2006). This deep hypothermia protocol using adult spinal neurons cannot be reliably compared to TH in hCNs. It did nonetheless demonstrate a synergistic benefit of combining cooling with another protective agent - a strategy advocated by others (Green et al., 1995; Dietrich et al., 1995; Dietrich et al., 1999; Pazos et al., 1999; Adachi et al., 2001; Gao et al., 2014). There are also fundamental differences between the design of hCN experiments and recent work by Antonic et al. (2014) including neuronal regional specification and purity, numbers of cell lines used, temperature and types of stressor applied. Some of the limitations described by the authors have been addressed here including neuronal functional maturity and normalization for cell number. Antonic et al. also demonstrated a benefit of inducing

hypothermia within 6 h post-injury – a point of relevance to acute cerebral insults. Since the focus of this thesis lies in the molecular pathways underpinning hypothermic neuroprotection, post-insult hypothermic induction and the effects of re-warming were not addressed. Whilst they found no protective benefit of hypothermia in the face of oxygen deprivation alone, Antonic et al., did not specify the relative hypoxia achieved compared to their 'normoxic' cultures. Since hCNs are generated and maintained at 3% $O_2$, results relating to oxidative stress are not directly comparable. Interestingly, the small percentage of GFAP-positive astroglia in hCN cultures succumbed to high doses of $H_2O_2$, but demonstrated an increased resilience to oxidative stress relative to hCNs (Fig.3.4b). This is consistent with the higher antioxidant capacity of astrocytes relative to neurons (Sebastià et al., 2004; Papadia et al., 2008). Although the hCN platform reproducibly generates an enriched population of deep- and superficial-layer cortical neurons (Fig.3.1; Bilican et al., 2014), cellular elements that are absent from this system (including other glial and neuronal sybtypes) would likely impact upon the global response to injury and cooling *in vivo*. Thus, although the findings above mirror those seen in animal models and in the clinic, the results are not directly translatable to the entire human cerebral cortex.

One anomaly of the injury data above is that hypothermic protection from absolute glutamate-mediated cell death appeared temperature-specific, whereas that from glutamate-induced cellular injury (proportion dead divided by proportion live) was temperature-dependent. Separating cytotoxicity and viability data (Fig.3.9), reveals that although there was a temperature-dependent decrease in LDH release with

hypothermia, ATP production was similar at both hypothermic temperatures. That cell death counts were lowest at 32°C implies a discrepancy between these readouts. One interpretation is that moderate hypothermia affected lipid bilayer structure (Sonna et al., 2002; Al-Fageeh and Smales, 2006; Wang et al., 2014) in such a way as to falsely reduce LDH release. However, viability may have also been underestimated at 28°C if this temperature affected enzymatic production of ATP (Rodriguez et al., 2012). Despite metabolic suppression, one study found that hypothermia actually increased cellular ATP concentration in Chinese hamster ovary (CHO) cells, indicating more efficient consumption of glucose (Fogolin et al., 2004). If this were the case in cooled hCNs, it would falsely increase the viability component of the multiplexed analysis - and the magnitude of this effect might be temperature-dependent. An alternative explanation is that cell death at 28°C was so rapid that LDH (with a half life of 9 h) partially dissipated before the 24 h measurement. Another confounding factor might be increased or residual precursor proliferation at 32 and 37°C, but complete cell cycle arrest at 28°C which has been observed in primary cultures (Matijasevic et al., 1998). Given these technical difficulties, the injury data is considered to be complementary to, but distinct from quantification of cell death by counts. Overall, the findings above concur with other literature in that short periods (4 – 48 h) of hypothermia have negligible effect on basal viability, but dramatically reduce cell injury in the face of oxidative stress (Yang et al., 2006; Yenari and Han, 2012; Antonic et al., 2014). Cell death counts in response to glutamate mirror the 'U-shaped' curve described by Tymianski et al (1998) in mouse cortical cultures - although the nadir of this curve sits around 10°C higher in hCNs.

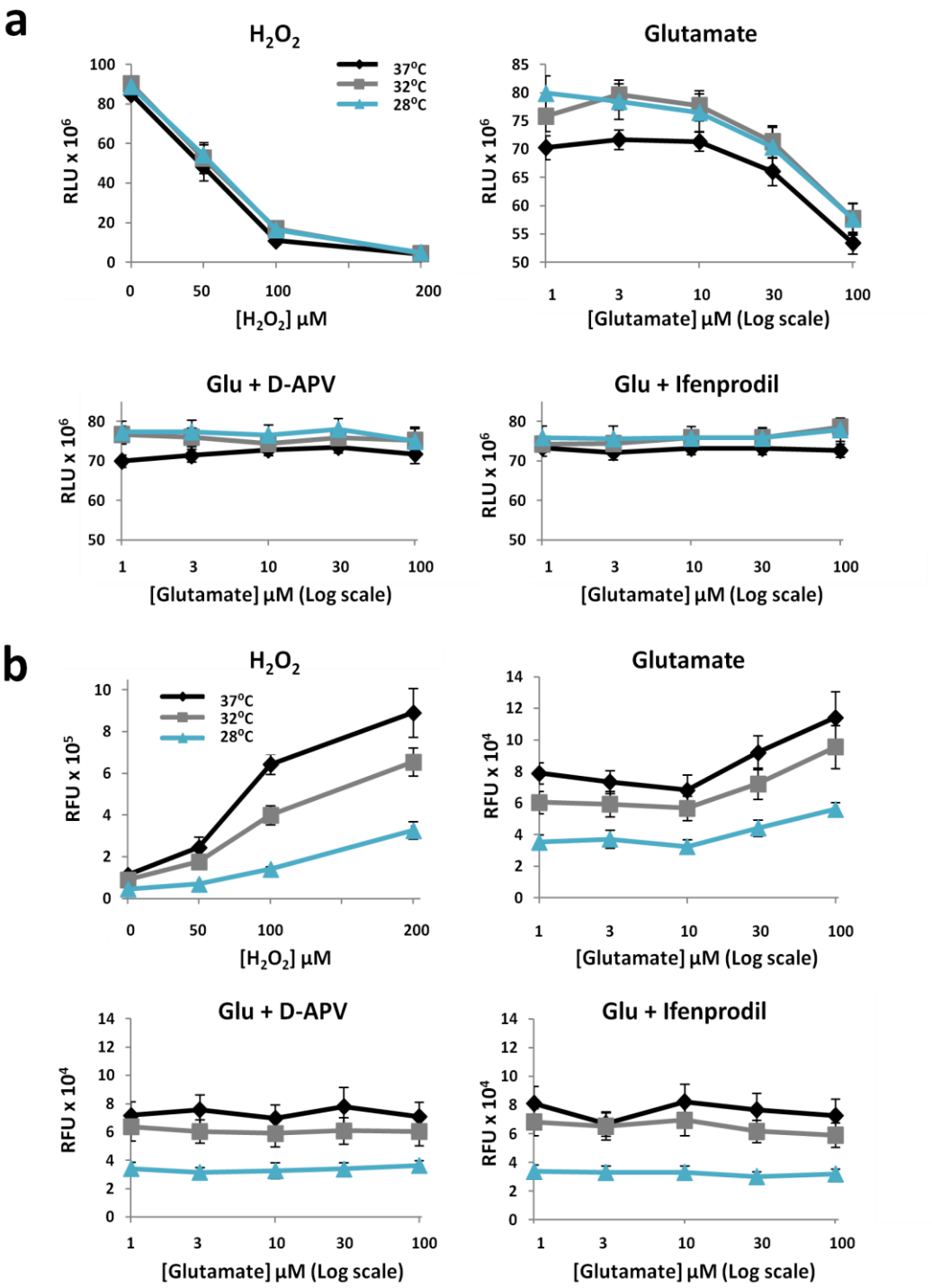


**Figure 3.9 Cytotoxicity and viability of hCNs.** Injury data (*N*=2; *n*=9)has been resolved into its separate components: **(a)** viability measured as ATP production in RLU, **(b)** cytotoxicity measured as LDH release in RFU. Note 10-fold difference in magnitude of LDH release between $H_2O_2$ and glutamate treatment. Raw data are presented as mean ± SEM. The use of LDH release to assess cytotoxicity remains controversial (Chip et al., 2011) although it has reproducibly detected both necrotic and apoptotic neuronal death (Koh et al., 1987). In the context of neuroprotective hypothermia, LDH release is arguably superior to MTT analysis because it may be less vulnerable to biases incurred by temperature shift (Chip et al., 2011).

At the synaptic cleft, one released vesicle can increase local glutamate concentration to as high at 1 mM (Danbolt, 2001). Thus, at first glance, the glutamate concentration range used above might appear conservative. The objective was to simulate physiological levels of extracellular glutamate which (in the intact brain) sit at nano- to micro-molar concentrations due to rapid clearance by neuronal and astrocytic glutamate transporters (Khatri and Man, 2013). The availability of these transporters in hCNs is evident by their punctuate vGlut1 staining (Bilican et al., 2014), their basal resilience to extracellular glutamate (Fig.2.7), and the increase in vGlut1 and vGlut2 mRNA expression during hCN differentiation (Fig.3.1) (Ito et al., 2013). Enriched hPS-derived astrocytic monolayers can efficiently take up glutamate and they express the relevant transporter (excitatory amino acid transporter 1, EAAT1) (Serio et al., 2013). However, astrocytes comprise less than 10% of hCN cultures (Livesey et al., 2014) and their close interaction with neurons at 'tripartite' synapses *in vivo* were unlikely to be replicated under these conditions (Perez-Alvarez and Araque, 2013). It was also important to consider the increased susceptibility of cortical neurons to glutamate *in vitro* (Choi et al., 1987) and the potential relevance of this work to chronic disorders in which extracellular glutamate concentrations might not reach the highest levels seen in acute injury. Therefore, although extending the glutamate dose range in future experiments may yield informative data, that presented above fulfils the aim of this thesis. The low glial content of 5 w hCN cultures does not preclude the possibility that astrocytic glutamate uptake (Serio et al., 2013) buffered the effect of glutamate on neurons. However, GFAP-positive cells also succumbed to 30 μM glutamate exposure (Fig.3.4b) and regardless of temperature, an extended pre-incubation in MiM was required to reveal a neurotoxic

effect. This implies that glial factors had a negligible impact on hypothermic neuroprotection in hCNs. Confusingly, immature cortical neurons have been described as being comparatively resistant, or more vulnerable, to glutamate toxicity than their mature counterparts (Choi et al., 1987; Murphy and Baraban, 1990; Jantzie et al., 2013). Among other developmentally regulated factors, this changeable sensitivity is attributed to temporal variation in NMDAR subunits (Brewer et al., 2007; Jantzie et al., 2013). However the time- and spatial distribution of receptors containing these subunits in the brain (as well as their functional significance) is far from clear cut (Hardingham and Bading, 2010). Basal glutamate tolerance in hCN cultures may simply reflect an immature glutamate receptor profile (Brewer et al., 2007); on the contrary, this very profile may underpin the excitotoxicity observed (Jantzie et al., 2013). The only safe conclusion from these experiments is that glutamate injury in hCNs is GluN2B-mediated, and can be reduced by hypothermia.

Physiological brain temperature fluctuations of 1-3°C have been reported in various animal models (Moser et al., 1993; Wang et al., 2014). Temperature shifts of this size can radically alter neural function since most (if not all) neurophysiological properties are temperature-dependent (Kiyatkin, 2010). The hypothermic temperatures applied to hCNs would thus be expected to affect RMP, action potential dynamics, conduction velocity, refractory period and synaptic transmission (Hodgkin and Katz, 1949; Hodgkin et al., 1952; Ritchie and Straub, 1956; Frankenhaeuser and Moore, 1963; Paintal, 1964; Katz and Miledi, 1965; Davis et al., 1975; Lowitzsch et al., 1977; Ludin and Beyeler, 1977; Westerfield et al., 1978; Borg, 1980; Schiff and Somjen, 1985; Thompson et al., 1985; Louis and Hotson, 1986; Denys, 1991; Moser

et al., 1993; Burke et al., 1999; Masino and Dunwiddie, 1999; Volgushev et al., 2000; Kiernan et al., 2001; Rutkove, 2001; Lee et al., 2005; Kiyatkin, 2010; Wang et al., 2014). Indeed, moderate-to-deep cooling reversibly inactivates neural activity and has been used in laboratory animals to study cortical function (Coomber et al., 2011). Several of these phenomena could explain hypothermic neuroprotection in hCNs, although the slow rate of cooling in this system may have offset any putative impact on excitability (Kiernan et al., 2001; Moldovan and Krarup, 2004). Temperature effects can also vary considerably by species; the squid axon ceases to conduct at the optimal temperature for mammalian nerve conduction (Hodgkin and Katz, 1949). Hypothermia has been shown to affect both pre- (Yang et al., 2005) and post-synaptic physiology (Katz and Miledi, 1965; Thompson et al., 1985; Volgushev et al., 2000), presenting a challenge to interpretation of electrophysiological recordings made at room temperature (Hardingham and Larkman, 1998). Cooling can also stabilise brain potassium homeostasis (Rodriguez et al., 2012) and a related mechanism is thought to underlie the preconditioning effect of heat shock - which promotes clearance of extracellular potassium (Rodgers et al., 2007). Using the squid giant axon, Hodgkin and colleagues (1952) found that the rate at which ionic current changed with time had a temperature coefficient ($Q_{10}$) approximating to 3. Conceivably, in hCNs, hypothermia may have impacted on numerous ionic factors in addition to passive membrane properties (Frankenhaeuser and Moore, 1963; Louis and Hotson, 1986; Schwarz, 1986; Schwarz and Eikhof, 1987; Kiyatkin, 2010; Wang et al., 2014). Finally, cooling results in depolarization (Moldovan and Krarup, 2004). In the anaesthetized rat, this places barrel cortex pyramidal neurons in an 'up state' at 28°C relative to physiological normothermia (36°C), which paradoxically

reduces their excitability (Sachdev et al., 2004; Kalmbach and Waters, 2012). Wang et al. (2014) have implicated this phenomenon in cooling-induced neuroprotection – a plausible hypothesis in the face of excitotoxic stress. Testing this theory would nevertheless require measurable network activity – a feature which remains to be demonstrated both in hCNs, and more generally in hPS-derived neuronal systems.

The 'bell-shaped curve' of neuronal responses to NMDAR activity has since been updated to a model in which the relative stimulation of synaptic- versus extrasynaptically located NMDARs determines the nature of the outcome (protective versus toxic) (Hardingham et al., 2002; Papadia et al., 2008; Okamoto et al., 2009; Hardingham and Bading, 2010). Temperature-insensitive components of NMDAR-mediated excitotoxicity can be demonstrated in mouse cortical cultures using prolonged glutamate exposure (Tymianski et al., 1998). Potentially, this could relate to a temperature-resistant stimulation of extrasynaptic NMDARs (Hardingham et al., 2002; Hardingham and Bading, 2010) that may evade cooling-induced changes in dendritic morphology (Greenwood and Connolly, 2007). It must be emphasised that at present, evidence of dendritic spine architecture in normothermic hCNs is limited. One study of post-ischaemic cooling in gerbils indicated that electrophysiological properties could remain stable under mild hypothermic conditions (Dong et al., 2001). In hCNs however, 32°C provided protection against NR2B-mediated glutamate toxicity which may reflect a temperature-specific effect on synaptic transmission. Without direct evidence that excitotoxicity was synaptically-mediated in hCNs, such a conclusion is merely speculative; indeed an inhibitory effect at extrasynaptic NMDARs might be more likely given the predominant NR1/NR2B

phenotype of immature extrasynaptic NMDARs, the subunit composition of hCN NMDARs, and the beneficial effect of cooling in this system (Tovar and Westbrook, 1999; Hardingham et al., 2002; Hardingham and Bading, 2010). In addition, the majority of synaptic NMDARs can be remarkably dynamic within the neuronal membrane, interchanging between synaptic and extrasynaptic pools (Tovar and Westbrook 2002). Since cooling affects membrane composition, the location, anchorage and accessibility of these receptors might be altered by temperature shift (Sonna et al., 2002; Tovar and Westbrook, 2002). Further and more detailed studies are required to assess basal synaptic function in hCNs, and how this is modified by hypothermia.

The effect of ifenprodil on excitotoxic injury in hCNs is interesting in light of its pharmacological properties. Ifenprodil, a non-competitive NR2B antagonist, produces an activation-dependent block, whilst simultaneously increasing receptor affinity for glutamate site agonists (Kew et al., 1996). Ifenprodil thus potentiates the effect of low [glutamate] but blocks the effect of very high [glutamate]. The beneficial blocking effect at 100 μM glutamate was evident at each temperature in hCNs, however, at 32°C, ifenprodil block over the 1-3 μM range appeared to increase neuronal injury compared to glutamate alone. Explanations might be that mild hypothermia (i) shifts the threshold at which exogenous glutamate concentrations become protective or toxic, (ii) alters the binding properties of ifenprodil such that it binds non-activated receptors with higher affinity, (iii) reduces the impact of ifenprodil on agonist binding properties or (iv) induces downregulation of NMDARs. In this regard, (i) and (iv) seem most likely, given the effect of

hypothermia on the glutamate curve in the absence of blockers. Nonetheless, the shape of the ifenprodil curve at 32°C suggests a protective effect of low exogenous glutamate, consistent with earlier studies (Hardingham et al., 1997; Hardingham, 2009; Hardingham and Bading, 2010). Intuitively, it would seem appropriate for hypothermia to increase neuronal tolerance to glutamate, if only to dampen the energy consuming process of excitatory transmission when metabolic turnover is low.

Glutamate is the most prevalent excitatory neurotransmitter in the vertebrate CNS and is a major instigator of secondary neuronal injury (Fonnum, 1984; Weil et al., 2008). It must be borne in mind however that other glutamate receptors (e.g. AMPA) and non-glutamatergic mechanisms contribute to excitotoxicity and also to hypothermic protection (Lyeth et al., 1993; Newell et al., 1995; Tymianski et al., 1998; Li et al., 1999; Ginsberg and Beyalev, 2005). Similarly, other ROS and reactive nitrogen species (RNS) participate in the milieu of free radicals that create havoc during neurotrauma, ischaemia and degeneration (Dietrich et al., 2009; Rodriguez et al., 2012). CNS glia (astrocytes, oligodendrocytes and microglia) as well as other neuronal subtypes are also vulnerable to excitotoxic injury and play fundamental roles in regulating both excitotoxic and oxidative stress *in vivo* (Weil et al., 2008; Dietrich et al., 2009). The reductionist hCN system employed above could not address these effects, although the procedural and analytical techniques could be applied to test the impact of cooling on other stressors and hPS-derived cellular components in future work.

In conclusion, these experiments provide the first demonstration that hypothermia protects human cortical neurons *in vitro* from glutamate-mediated excitotoxicity. Together with the oxidative stress results, these findings emphasise the potency of cooling to protect neurons from multiple, clinically-relevant neurotoxic insults.

# Chapter 4: The cold shock response in hCNs

## 4.1 Introduction

Having established that cooling protects hCNs from common neurotoxic stressors, the next step was to dissect the molecular pathways that are modified by hypothermia in these cultures to identify potential mediators of its protective effect. Since hypothermia is a recognised physiological stressor (Sonna et al., 2002), both general and more specific, temperature-dependent stress responses were anticipated. Hypothermic induction of cold-inducible proteins RBM3 and CIRBP has been demonstrated in rodent hippocampal slices (Tong et al., 2013) and human cell lines *in vitro* (Wellmann et al., 2010). These markers have also been intensely studied in human tumors, including astrocytoma, in which their proto-oncogenic roles are of primary interest (Lleonart, 2010; Zhang et al., 2013). To date, the expression of RBM3 and CIRBP in cultured human neurons in response to cooling remains untested. Given the high sequence homology between human and rodent RBM3 and CIRBP (Wellmann et al., 2004), it was hypothesised that cooled hCNs would respond in a similar manner to hypothermic rodent neurons.

## 4.2 Results

### 4.2.1 Immediate early markers

Evidence for a general physiological stress response in cooled hCNs was sought using q-RT-PCR analysis. Immediate early transcripts (FOS and JUN) were

measured from RNA samples harvested at both early and late time points during the cooling period (*N*=3; *n*=14). Hypothermic induction of these transcripts was time- and temperature-dependent; FOS increased at 28°C (3 h $P<0.0005$, 24 h $P=0.002$) whilst JUN increased early at both hypothermic temperatures (32°C $P=0.01$, 28°C $P=0.021$) and later, at 28°C ($P<0.0005$) (Fig.4.1a).

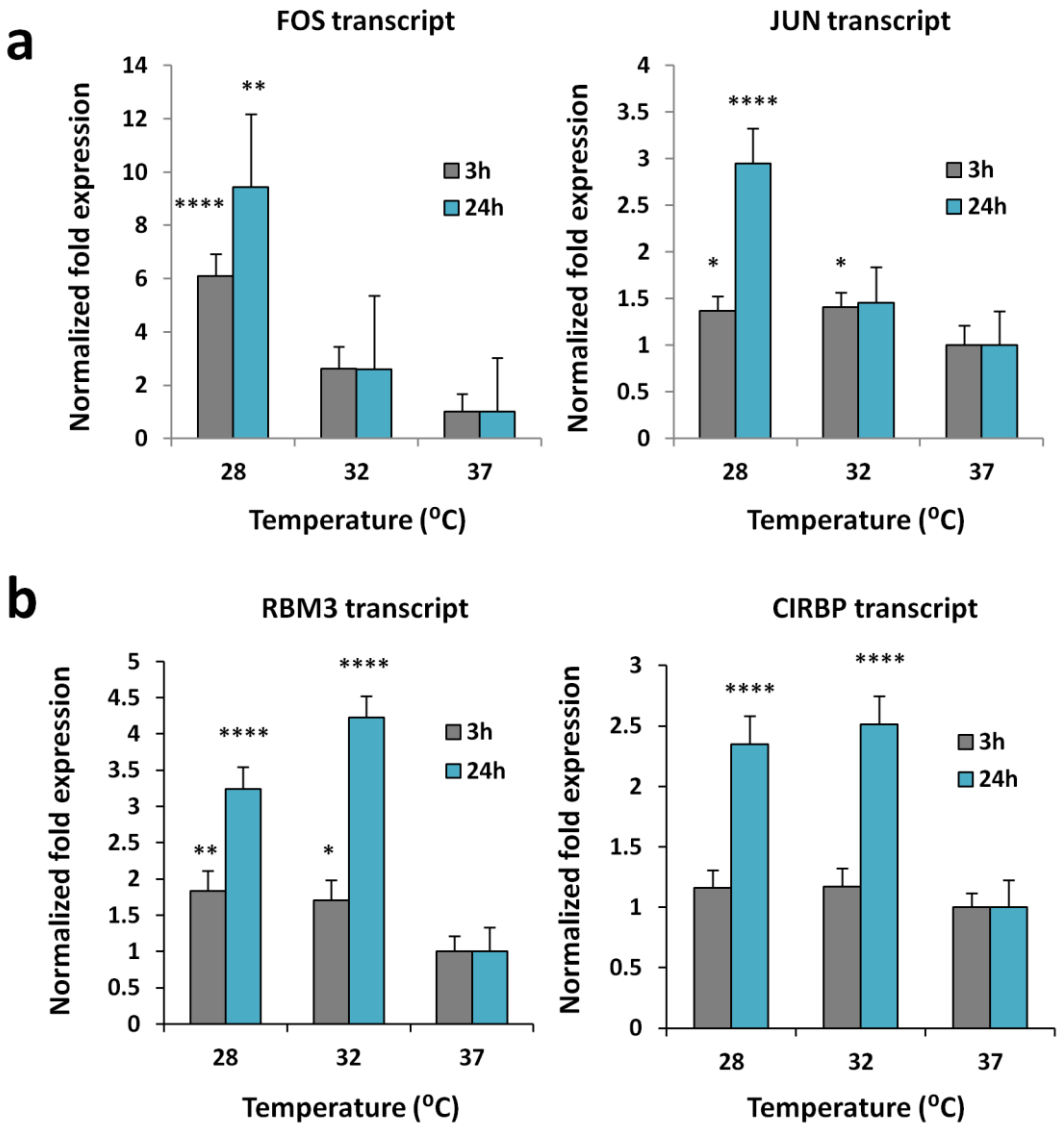


**Figure 4.1 hCN transcripts in response to cooling.** q-RT-PCR data from 5 w hCNs after 3 and 24 h incubation at the temperatures indicated (*N*=3; *n*=14). For each time point, transcript data was first normalized to the geometric mean of two temperature-stable reference targets (GAPDH and EIF4A2) and then to 37°C control. **(a)** Immediate early genes. **(b)** Cold-shock transcripts.

### 4.2.2 Cold-inducible markers

Transcript changes in cold-shock markers were assessed in independently differentiated batches of hCNs (*N*=3; *n*=14) after 3 and 24 h incubation at the 3

temperatures of interest (Fig.4.1b). After 3 h, there was a significant 1.7-fold increase in RBM3 transcript at 32°C $P$=0.011, and a 1.8-fold increase at 28°C $P$=0.003, relative to normothermic control. This rose further to 4.2–fold and 3.2–fold respectively at 24 h $P$<0.0005. The CIRBP transcript response occurred later, but robustly at 24 h at both hypothermic temperatures (2.5-fold increase at 32°C and 2.4–fold increase at 28°C $P$<0.0005). Late RBM3 and CIRBP transcript expression correlated significantly at each temperature (Fig.4.2; Pearson correlation at 37°C $P$=0.001, 32°C $P$=0.012, 28°C $P$<0.0005), suggesting co-regulation in response to hypothermia.

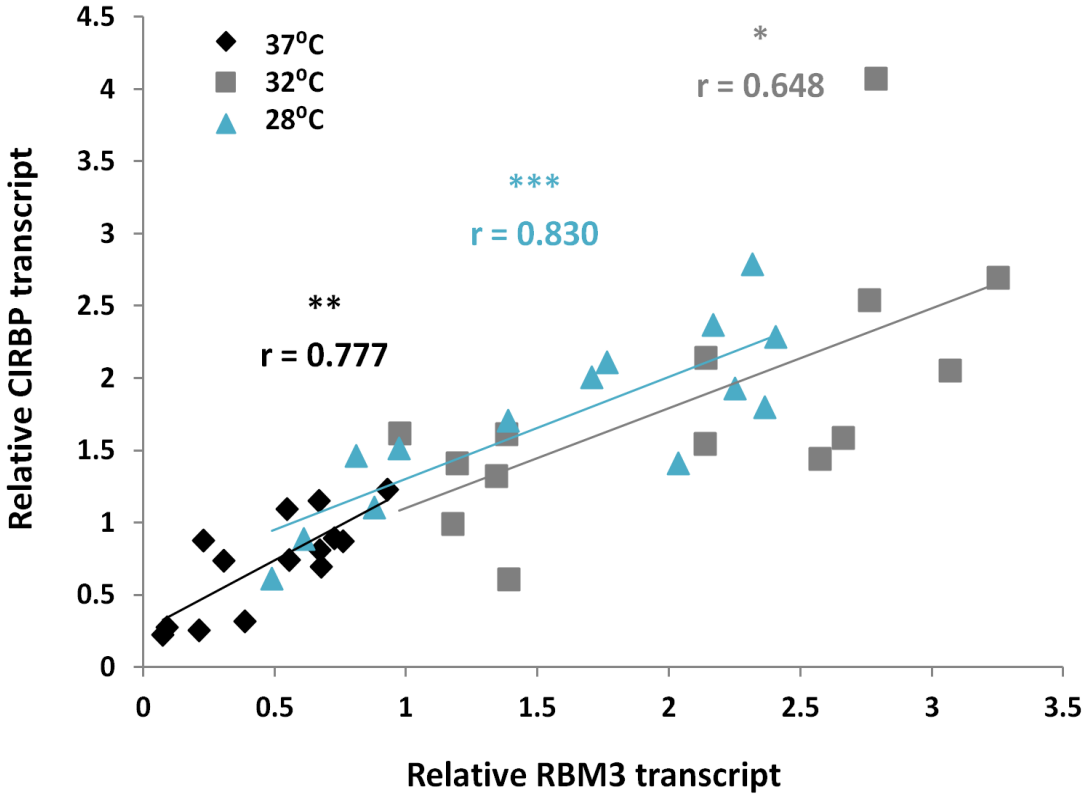


**Figure 4.2 Relative transcript expression in hypothermic hCNs.** Significant two-tailed Pearson correlation of RBM3 and CIRBP transcript expression after 24 h at each temperature ($N$=3; $n$=14).

At each temperature, after 24 h, both RBM3 and CIRBP displayed a nuclear expression pattern on immunocytochemistry (Fig.4.3 and 4.4). There were significant increases in both markers at both hypothermic temperatures according to cell counts ($N$=3; $n$=6; RBM3, 32$^0$C $P$=0.001, 28$^0$C $P$=0.039; CIRBP, 32$^0$C $P$<0.0005, 28$^0$C $P$<0.0005; Fig.4.5a). CIRBP and RBM3 are highly expressed in human foetal brain (Danno et al., 1997) and thus post-mortem foetal samples were included as a positive control for Western blot analysis. When quantified ($N$=3; $n\geq4$; Fig.4.5b), RBM3 expression was greatest at 28°C ($P$=0.002) whilst CIRBP expression peaked at 32°C ($P$<0.0005). Fractionation of cytosolic (RIPA-soluble) and nuclear (high detergent) cell fractions confirmed the subcellular location of both cold-inducible proteins and their responses to cooling (Fig.4.6).

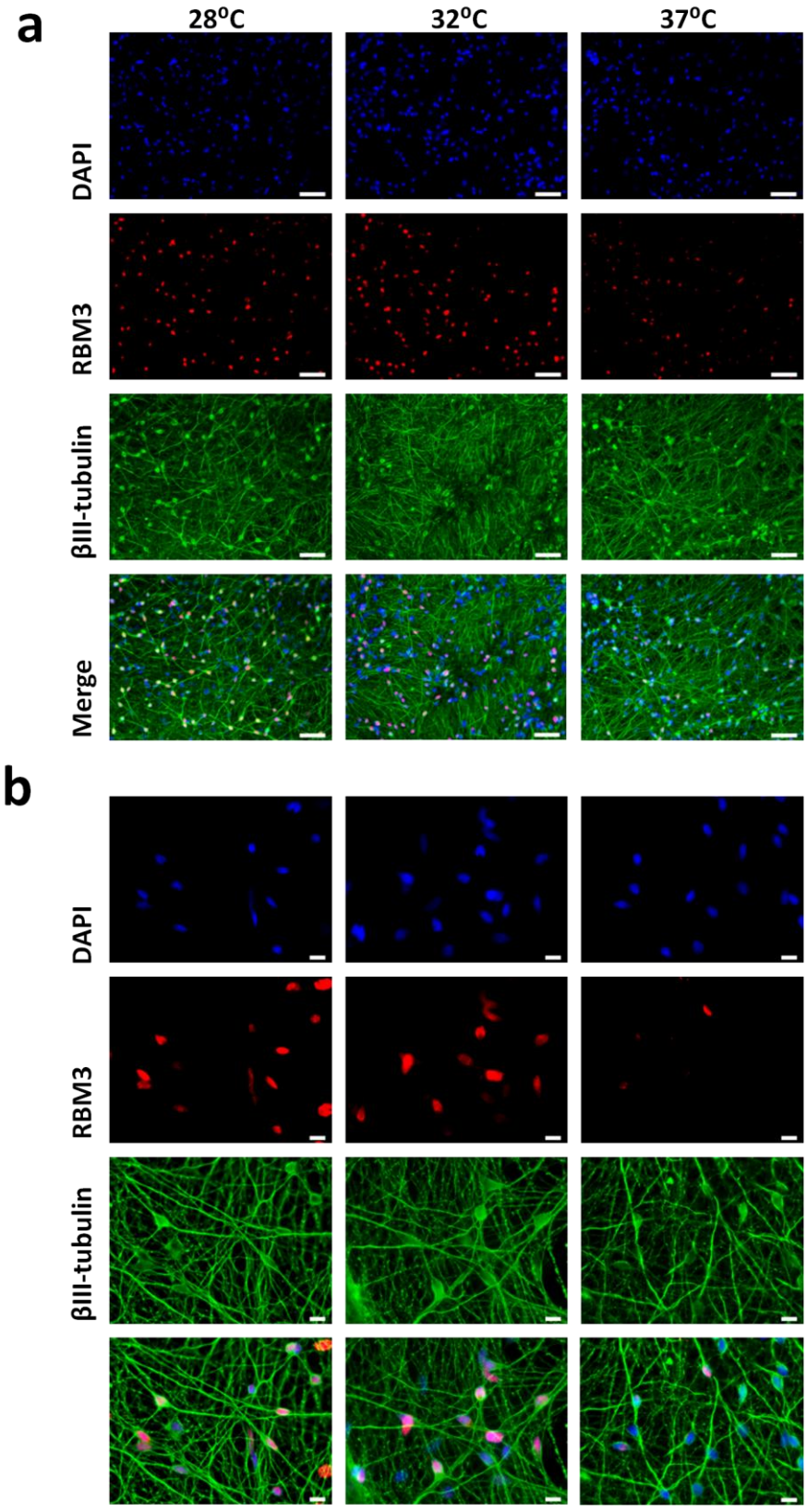


**Figure 4.3 RBM3 expression in hCNs.** Representative widefield fluorescent micrographs of hCNs co-stained for nuclear and neuronal markers (DAPI and βIII-tubulin respectively) and RBM3 after incubation for 24 h at the indicated temperatures, **(a)** scale bar = 50 µm, **(b)** scale bar = 10 µm.

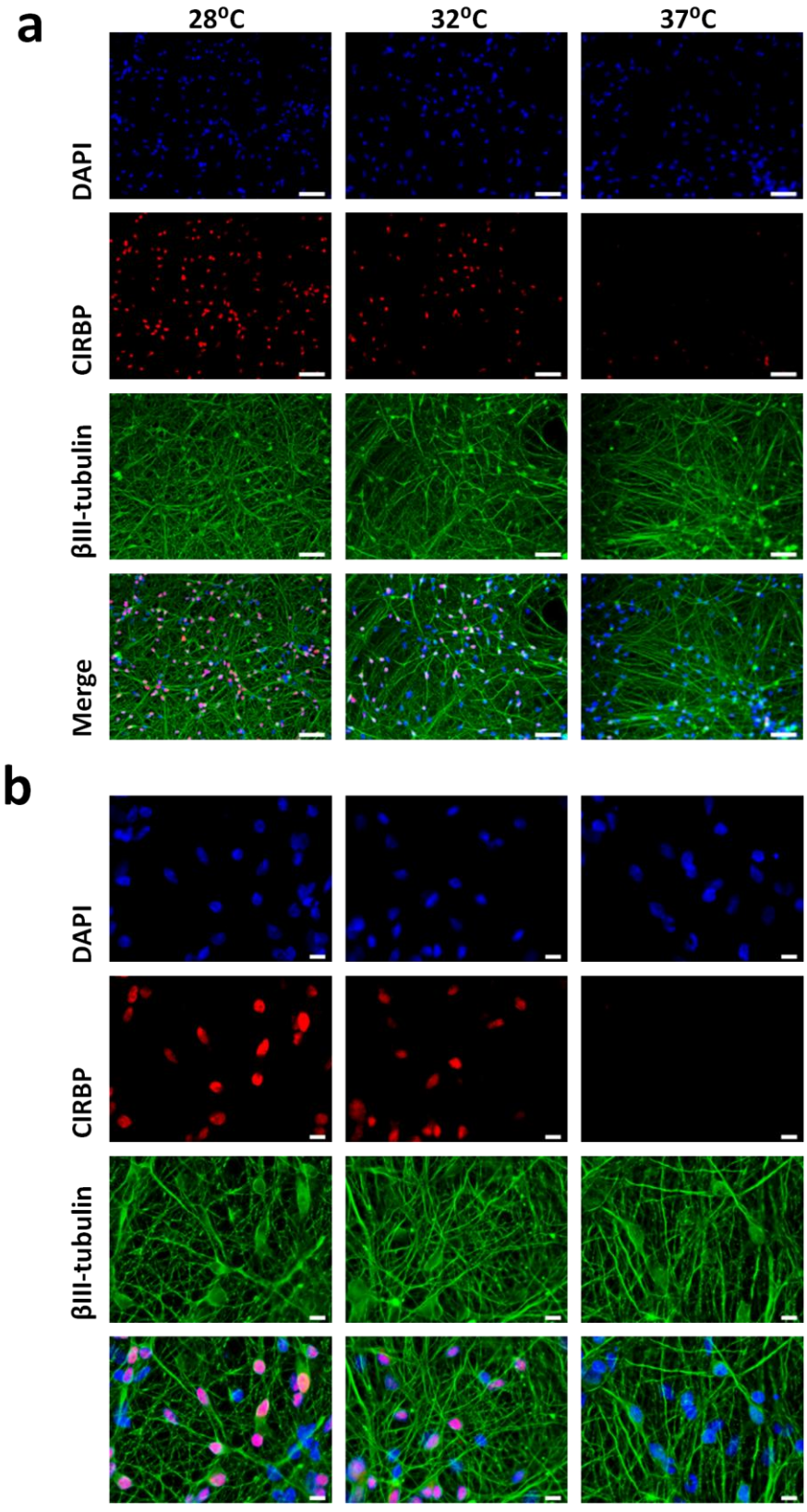


**Figure 4.4 CIRBP expression in hCNs.** Representative widefield fluorescent micrographs of hCNs co-stained for nuclear and neuronal markers (DAPI and βIII-tubulin respectively) and CIRRBP after incubation for 24 h at the indicated temperatures, **(a)** scale bar = 50 µm, **(b)** scale bar = 10 µm.

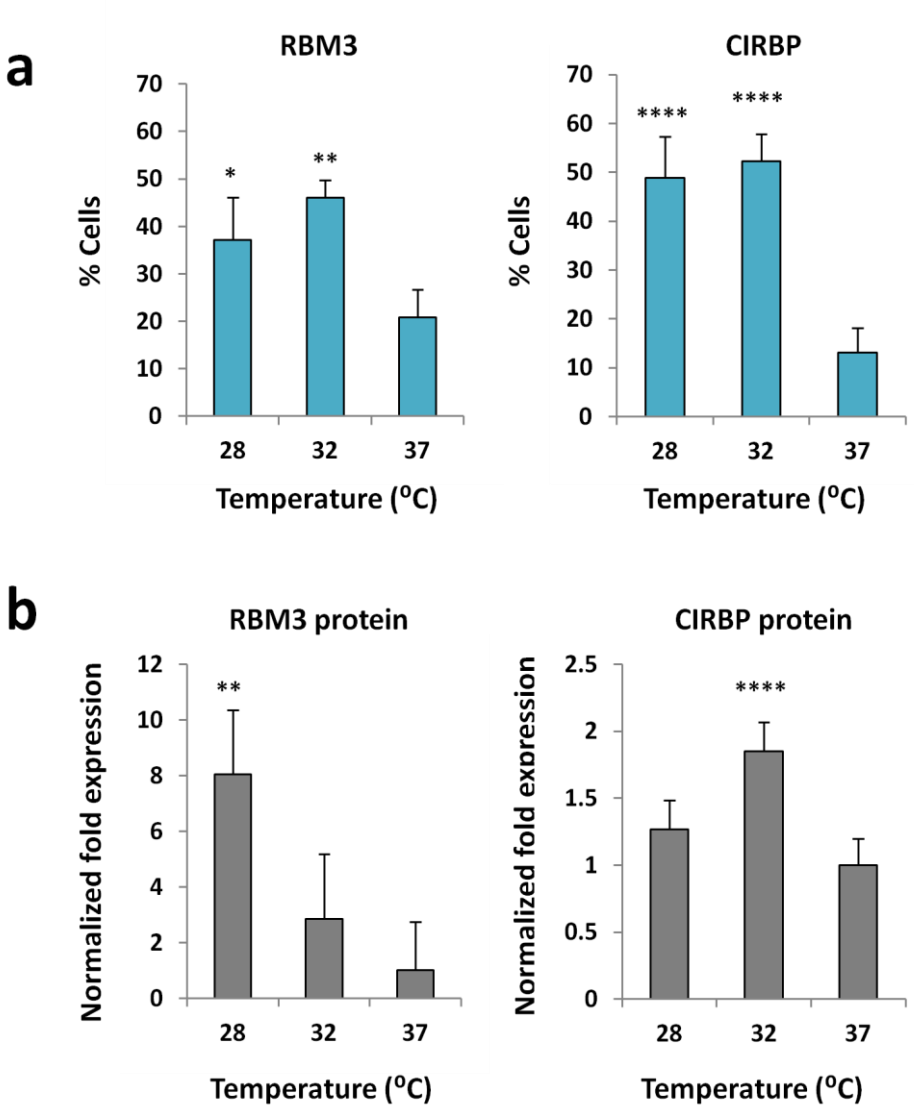


**Figure 4.5 A nuclear cold-shock response in hCNs.** **(a)** Cell counts for cold-shock protein-positive nuclei after 24 h incubation at the respective temperatures (*N*=3; *n*=6). Cell count data is presented as mean % of DAPI-stained nuclei + SEM. **(b)** Quantitative Western analysis of cold-shock markers in hCNs (N=3; n≥4), normalized to loading control and then normothermic control.

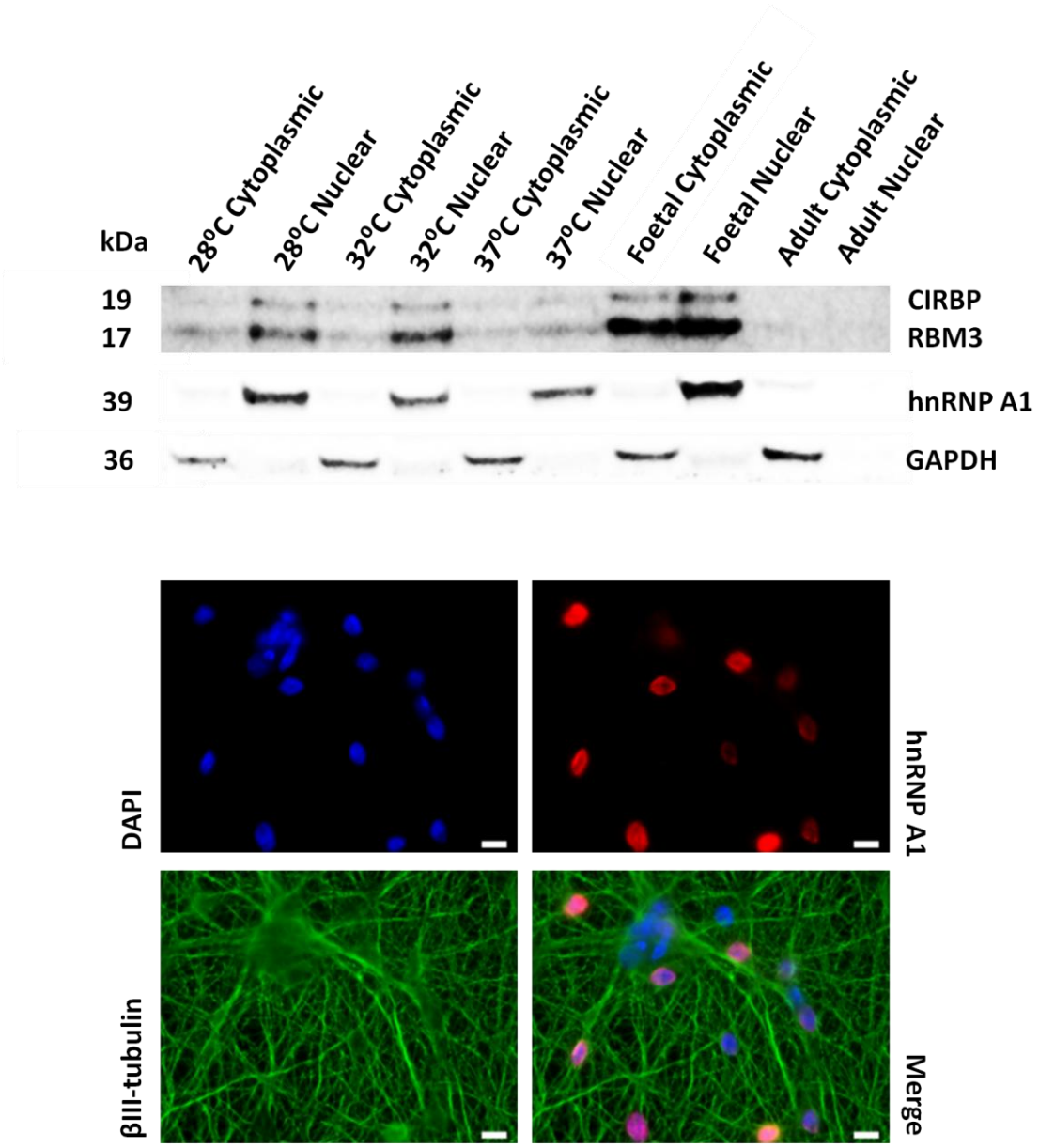


**Figure 4.6 Cold-shock protein expression in hypothermic hCNs.** **(a)** Representative Western blots showing subcellular expression of RBM3 and CIRBP at each temperature, compared with human foetal (16 w) and adult (17 y) cortex. Fractionation of cytosolic and nuclear compartments is depicted by differential expression of loading controls (GAPDH and heterogenous nuclear ribonucleoprotein (hnRNP) A1 respectively). **(b)** Representative widefield fluorescent micrographs confirm subcellular location of hnRNP A1 in 5 w hCNs; top left, DAPI; top right, hnRNP A1; bottom left, βIII-tubulin; bottom right, merged; scale bar = 10 µm. Whilst hnRNP A1 is structurally related to RBM3 and CIRBP, its expression is considered to be stable under mild hypothermic conditions (Danno et al., 1997).

## 4.3 Discussion

In respect to the second aim of this thesis, the archetypal response to cooling has been successfully characterized in hCNs. These cultures respond robustly to hypothermia with upregulation of key stress transcripts including FOS and JUN, predominantly at 28°C. Mild-to-moderate cold-induction of RBM3 and CIRBP was anticipated and subsequently confirmed using molecular and biochemical techniques. Hypothesis (2) is thus accepted.

FOS and JUN unite as a heterodimer within the activator protein 1 (AP-1) complex which binds to DNA as a transcription factor, regulating gene expression in response to various cellular stimuli (Chiu et al., 1988; Angel and Karin, 1991). Since JUN is intronless and AP-1 activation does not require *do novo* protein synthesis, the response can occur within minutes (Angel and Karin, 1991). Synaptic NMDAR activity can enhance AP-1 driven induction of neuroprotective pro-antioxidant genes including sulfiredoxin (Papadia et al., 2008). Immediate early gene induction thus provides an efficient cellular defence against oxidative stress (Soriano et al., 2009). These immediate early genes are rapidly upregulated during heat shock exposure of HeLa cells and other human cell lines (Sonna et al., 2002), and FOS in particular is upregulated in an activity-dependent fashion in hCNs (Fig.3.3). Induction of immediate early genes during ischaemia-reperfusion is biphasic and also occurs faster in the presence of hypothermia, which may enhance neuronal recovery (Kamme et al., 1995). Akaji et al. (2003) found that FOS expression was increased within 3 h during reperfusion at 30°C compared to 40°C, whilst JUN was apparently unaffected by hypothermia. Unfortunately, very few studies have addressed

immediate early gene expression in the context of cooling alone, making it difficult to delineate the effects of hypothermia and reperfusion. In a rat model of cryogenic brain injury, Katano et al. (1997) observed an increase in both FOS and JUN transcripts, peaking within 1 h and returning to baseline with 24 h. In cooled hCNs, RNA samples were harvested at 3 h and 24 h in an attempt to capture both immediate and putative late phase responses. Interestingly, very little (if any) change was noted in either transcript at the early time point – only a 6-fold increase in FOS was observed at 28°C (Fig.4.1a). This was in stark contrast to the effect of hCN depolarization at 37°C which produced an approximate 100-fold induction in FOS transcript within 2 h, decaying to around 30-fold after 4 h (Fig.3.3). Based on these results, it is possible that the early peak was missed, although they may simply reflect the relatively benign nature of the cooling paradigm implemented. Nevertheless, and at odds with the rat data (Katano et al., 1997), higher fold-inductions of FOS and JUN (9- and 3-fold respectively) were noted at 24 h, but only with moderate hypothermia (Fig.4.1a). Overall these results suggest that hCN cooling gave rise to a modest and rather sluggish immediate early gene expression, which increased both with duration of cold exposure and depth of hypothermia. This level of induction is unlikely to be activity-dependent, but it may have contributed to the hypothermic protection of hCNs against oxidative stress (Soriano et al., 2009). Finally, it is worth mentioning that although cJun:cFos heterodimers have optimal DNA binding activity and potent transactivation capacity at 37°C, cJun:cJun homodimers can exist at temperatures below this and may be active within the cell (Smeal et al., 1989; Angel and Karin, 1991). FOS mRNA also has a faster turnover rate than JUN, thus whilst heterodimers likely initiated the AP-1 response to cooling,

cJun homodimers may have sustained this response throughout the hypothermic period (Angel and Karin, 1991).

In the developing mouse cortex, RBM3 is highly expressed in the early post-natal period, then decreases with maturity, residing largely in adult neural stem cell niches (Chip et al., 2011). Dynamic developmental regulation of RBM3 has also been noted in rat brain (Pilotte et al., 2009) and is echoed among other RBPs (Markus et al., 2009). The subtle protein-level changes in response to hypothermia in hCNs, whilst suggestive of ontogenic reversal, may also reflect some degree of culture immaturity at 37°C. Note however that, in the study by Chip et al. (2011), both juvenile and adult neurons demonstrated RBM3 upregulation at 32°C. Other possibilities include build up of ROS and other metabolites or temperature-variable oxygen solubility (Schiff and Somjen, 1985; Ohsaka et al., 2002; Al-Fageeh et al., 2006; Lleonart, 2010) which may have incurred a 'relative' and unavoidable stress at baseline. Finally the transcript analysis suggested that RBM3 had a more acute onset in response to hypothermia than CIRBP which is consistent with the differing induction kinetics of these cold-shock markers reported previously (Spriggs et al., 2010). In 3T3 cells, maximal expression of CIRBP occurred between 6-24 h of exposure to 32°C (Nishiyama et al., 1997b), although peak protein expression in cooled hCNs may have been missed by sampling at 24 h.

Unlike RBM3, CIRBP is ubiquitously expressed in many tissues that are not exposed to cold (Artero-Castro et al., 2009; De Leeuw et al., 2007; Brochu et al., 2013) and it responds to other stresses including hypoxia, ultraviolet (UV) light, cisplatin and

arsenite (Brochu et al., 2013). The subcellular location of CIRBP is specific to the stressor applied; the protein remains nuclear under hypothermic conditions but translocates to the cytoplasm under UVC, and to stress granules in the presence of oxidative stress, osmotic stress, ER stress and arsenite (Yang et al., 2001; Yang et al., 2006; De Leeuw et al., 2007; Yang et al., 2010; Brochu et al., 2013). The nuclear expression of CIRBP at each temperature in hCNs is consistent with previous literature, and indicates that ROS levels in the culture media were relatively stable across this temperature range. However, although a cytoplasmic function for CIRBP has been linked to preservation of specific mRNAs required for survival, its specific role within the nuclear compartment is yet to be determined (Brochu et al., 2013). Of direct relevance to neuroprotection, CIRBP can antagonize the effects of oxidative damage thus permitting bypass of senescence (Artero-Castro et al., 2009; Lleonart, 2010) which might explain hypothermic protection from $H_2O_2$ stress in hCNs. However, hypothermia, CIRBP and RBM3 also regulate circadian gene expression (Nishiyama et al., 1998; Vallone et al., 2006; Morf et al., 2012; Li et al., 2010; Liu et al., 2013; Archer et al., 2014; Lopez et al., 2014; Costa et al., 2015). Because most mammalian cells contain autonomous circadian clocks (Bell-Pedersen et al., 2005), timing of hypothermia induction in these experiments could have affected entrainment of, or indeed been subjected to endogenous clock mechanisms. In the absence of a master clock, autonomous clocks *in vitro* might be entrained to the feeding cycle or brief exposure to environmental oxygen during cell feeding (Archer et al., 2014). Culture temperature was carefully controlled and monitored during cooling which was commenced at the same time in the 24 h cycle, and with respect to cell feeding for all cortical batches. For these reasons, interference from

autonomous ‘cortical clocks’ is unlikely to have affected temperature-dependent changes in CIRBP or RBM3.

Several studies have addressed the temperature-dependent kinetics of synaptic transmission (Katz and Miledi, 1965; Thompson et al., 1985; Hardingham et al., 1998; Masino and Dunwiddie, 1999; Volgushev et al., 2000; Lee et al., 2005; Yang et al., 2005; Kiyatkin, 2010; Wang et al., 2014) but few have considered whether cold-inducible pathways have a subacute influence over synaptic mechanisms. In nature, reversible regression of synaptic contacts during torpor have been linked with synaptic protection in hibernating mammals (Arendt et al., 2003). Similar observations have been made in laboratory models of hypothermia and rewarming, as discussed in the introduction. This suggests that temporary, hypothermia-mediated synaptic plasticity may be ultimately protective (Arendt et al., 2003). If cold-inducible RBPs were also involved in this process, they might be attractive candidates for euthermic manipulation in disease states involving aberrant synaptic development or permanent synaptic loss. Accordingly, RBM3 is upregulated in hibernating and experimentally-cooled brains (Williams et al., 2005; Peretti et al., 2015). Involvement of RBPs in both NMDA- and metabotropic glutamate receptor (mGluR)-dependent synaptic plasticity has already been demonstrated (for review see Ule and Darnell, 2006). Fragile X mental retardation protein (FMRP), a notable example, is proposed to play a key role in synaptic development by locally inhibiting translation of cytoskeletal elements (Brown et al., 2001; Lu et al., 2004; Zhang and Broadie, 2005). It has also been proposed that RBM3 may regulate local dendritic translation, in a manner opposing to FMRP and involving translation initiation

factors including eIF2α (Smart et al., 2007). Overexpression of RBM3 also preserves RMP in staurosporine-stressed myoblasts (Ferry et al., 2011) – hypothermic induction of this protein might thus prevent excitotoxicity under ischaemic conditions. Interestingly, RBM3 is an NMDA-responsive gene (Hsu et al., 2005) and NMDA stimulation of primary cortical neurons in turn causes post-translational modification of RBM3 (Smart et al., 2007). If CIRBP were also subject to such regulation, it might participate in the thioredoxin-mediated antioxidant effect of synaptic NMDAR activity (Papadia et al., 2008). In line with this hypothesis, CIRBP has been shown to regulate thioredoxin expression in human colon carcinoma cells (Yang et al., 2006).

In summary, these experiments have provided the first exploration of cold-shock protein induction in human neurons, establishing that hCNs respond in a predictable fashion to cooling. hCNs thus provide a suitable model with which to further explore the molecular basis of hypothermic preconditioning.

# Chapter 5: Hypothermic modulation of human tau

## 5.1 Introduction

Human brain tau development involves an initial rise in total tau protein, commencing with expression of a highly phosphorylated foetal tau isoform containing 3 microtubule binding repeat domains (3R tau) and no N-terminal extensions (Tau 352 also known as 3R0N, Fig.1.3) (Goedert et al., 1989a). Post-natally alternative splicing of *MAPT* generates further isoforms with variable N-terminal lengths and 4 microtubule binding repeats (4R tau) – a feature which increases microtubule binding capacity (Goedert et al., 1989a; Goedert and Jakes, 1990; Mandelkow and Mandelkow, 2012). The result is a profile of 6 major tau isoforms in the adult brain with approximately equal levels of 3R and 4R tau (Goedert et al., 1989b; Goedert and Jakes, 1990). Concomitantly, tau becomes progressively dephosphorylated, which increases its affinity for microtubules (Gong et al., 2000; Mandelkow and Mandelkow, 2012) and is attributed to increased expression of PP2A – the principal tau phosphatase in the human brain (Yu et al., 2009, Fig.1.7). This transition, both in tau isoform ratio and tau phosphorylation status, reflects the need for early developmental plasticity, followed by maturational stability as neuronal networks develop and synaptic contacts are formed (Arendt and Bullmann, 2013).

A few studies have addressed human *in vitro* tau development and/or pathology (Busciglio et al., 1995; Deshpande et al., 2006; Deshpande et al., 2008; Iovino et al., 2010; Shi et al., 2012a; Israel et al., 2012; Iovino et al., 2013; Fong et al., 2013;

Mertens et al., 2013; Choi et al., 2014; Moore et al., 2015). These have provided useful data on the effects of tau mutation (Iovino et al., 2010; Iovino et al., 2013; Fong et al., 2013), tau overexpression (Mertens et al., 2013) or specific disease states (Busciglio et al., 1995; Shi et al., 2012a; Israel et al., 2012; Choi et al., 2014; Moore et al., 2015), but have been limited in terms of source material (Busciglio et al., 1995; Deshpande et al., 2008; Moore et al., 2015), regional non-specificity of differentiated neurons (Iovino et al., 2010; Israel et al., 2012; Iovino et al., 2013; Fong et al., 2013; Mertens et al., 2013) and demonstration of functional maturity (Deshpande et al., 2008; Iovino et al., 2010; Iovino et al., 2013; Fong et al., 2013; Mertens et al., 2013; Moore et al., 2015). hPS-derived systems also enable high throughput investigation of real-time human biology under physiological protein expression - an application that has been poorly exploited with respect to tau.

Brain tau protein in rodent models and hibernating mammals becomes increasingly and reversibly phosphorylated with decreasing temperature (Mawal-Dewan et al., 1994; Arendt et al., 2003; Stieler et al., 2011). The nature of this phosphorylation has been likened to that seen in neurodegenerative disease (Planel et al., 2004; Stieler et al., 2011), but also to the developing brain (Mawal-Dewan et al., 1994), suggesting that hypothermia exerts an increase in tau plasticity, by recapitulating a 'foetal-like' state. A disproportionate inhibition of tau phosphatase activity (relative to tau kinase activity) is thought to underlie this hypothermic increase in tau phosphorylation (Planel et al., 2004). The primary objective of the following experiments was to examine whether hypothermia could produce changes in hCN tau phosphorylation status that have been described in other systems, including human neuroblastoma lines (Bretteville et al., 2012). In addition it was hypothesised that the human tau

isoform ratio might shift in favour of 3R tau under hypothermic conditions, thus contributing to reacquisition of an earlier ontogenic phenotype.

## 5.2 Results

### 5.2.1 Tau isoforms during differentiation and hypothermic challenge

To determine whether hCN differentiation reflects normal developmental changes in tau, *in vitro* tau development was examined from aNPC stage up to 7 w of differentiation. Early human tau development was recapitulated at transcript level (*N*=2; *n*=5; Fig.5.1a). There were significant increases in total, 3R and 4R tau expression (fold changes at 6 w relative to aNPC were 30.2 ± 5.48, 36.6 ± 6.99 and 9.38 ± 1.34 respectively; 4R tau at 4 w $P$=0.005, other increases $P$<0.0005; Fig.5.1a). There was also a shift in the 3R:4R ratio between 4 w and 7 w (*N*=1; *n*=5; $P$<0.0005), mimicking the transition from human foetal to adult brain (Fig.5.1b). To determine whether hypothermia modifies tau dynamics, tau isoform ratio was evaluated in 5 w hCNs (by which point 4R tau transcript could also be visualized by qualitative RT-PCR; Fig.5.1c), following hypothermic incubation for 24 h (*N*=3; *n*=14). Total tau transcript expression remained stable across temperatures, however an increase in the 3R:4R tau ratio was observed with cooling, most notably at 32°C ($P$<0.0005), but also at 28°C ($P$=0.012; Fig.5.2a). This shift in isoform ratio reflected a significant increase in 3R tau transcript at both 32°C ($P$=0.007) and 28°C ($P$=0.001), and a reduction in 4R tau transcript at 32°C ($P$=0.009, Fig.5.2a-b). Pearson correlations supported a tendency towards dissociation of 3R and 4R tau transcripts at 32°C (28°C $P$<0.0005, 32°C $P$=0.012, 37°C $P$<0.0005; Fig.5.3).

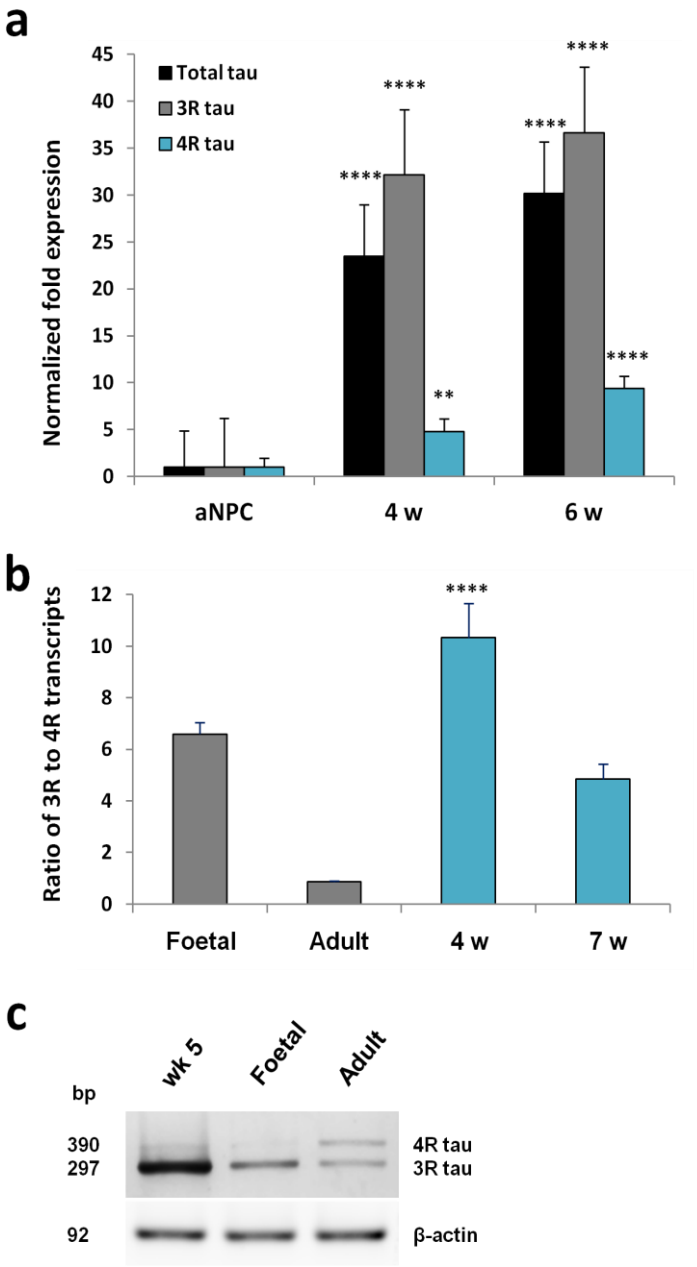


**Figure 5.1 Tau transcripts in differentiating hCNs. (a)** q-RT-PCR analysis of total tau and different tau isoform transcripts from aNPC stage to 6 w. Asterisks denote statistically significant difference relative to aNPC level (*N*=2; *n*=5). **(b)** Shift in 3R:4R tau isoform ratio between 4 w and 7 w mimics transition from human foetal to adult brain (triplicate cDNA synthesised from commercially pooled RNA). Transcript data was normalized to the geometric mean of 3 differentiation-stable reference targets then presented as SPE + SESE relative to aNPC expression (for hCNs) or mean of triplicates + SEM (for pooled human brain). Asterisks denote statistically significant difference between ratio at 4 w compared to ratio at 7w (*N*=1; *n*=5; *P*<0.0005). **(c)** Qualitative RT-PCR analysis of 5 w hCNs using primers that span tau exons 9-11, thus amplifying transcripts with or without tau exon 10 (4R or 3R tau respectively). Note roughly equal expression of 3R and 4R tau species in human adult brain and predominance of 3R tau species in both hCNs and human foetal brain. Note that the ratio of 3R to 4R expression in these hCNs approximates to 8:1, which is consistent with that predicted from the q-RT-PCR results in **(b)**. β-actin serves as a housekeeping target.

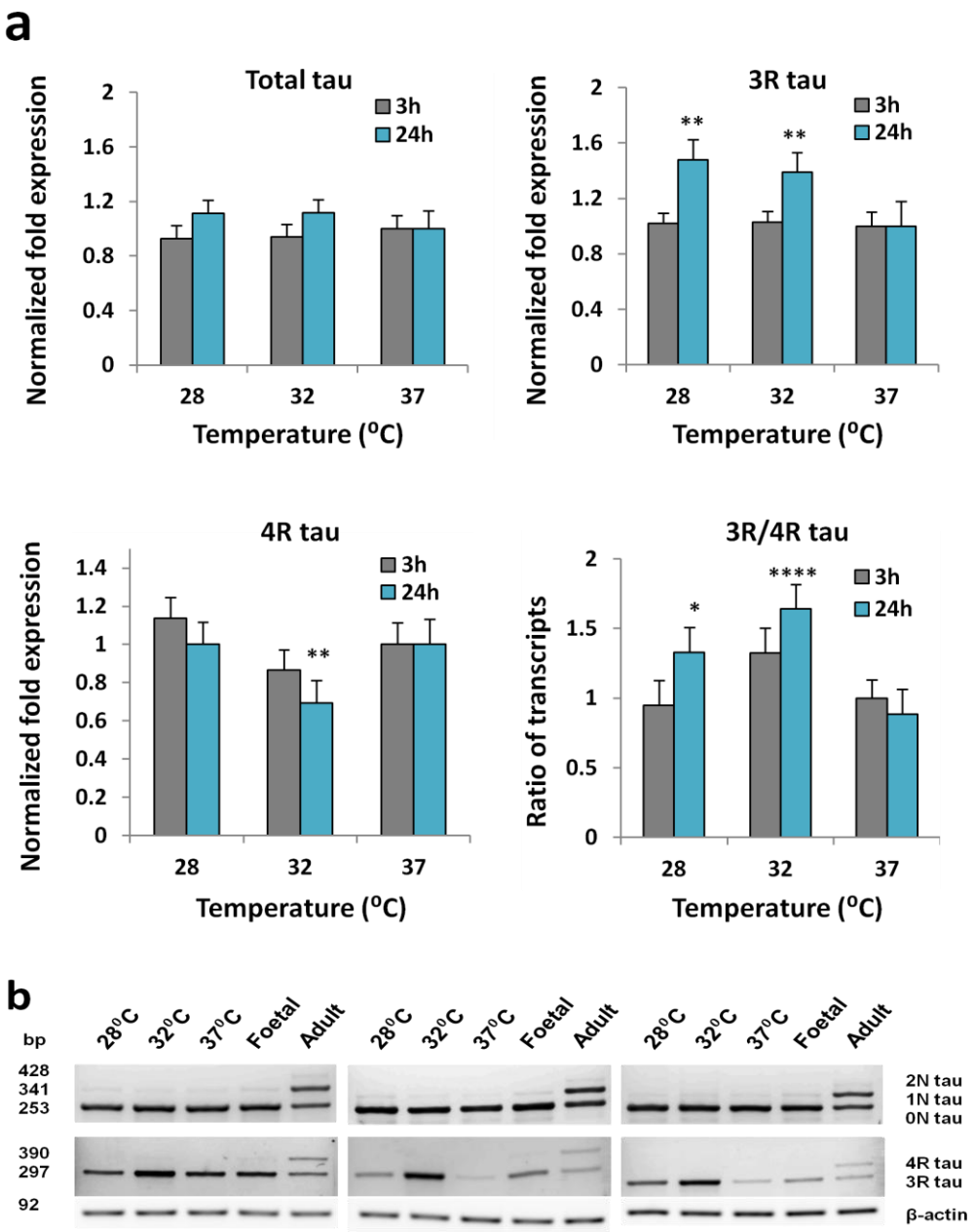


**Figure 5.2 Tau transcripts in hypothermic hCNs. (a)** q-RT-PCR analysis of transcripts in 5 w hCNs after 24 h temperature shift showing no significant change for total tau but a significant increase in 3R tau at both hypothermic temperatures (N=*3*; *n*=14). Note also a late decrease in 4R tau expression. At 24 h this produces a shift in the tau isoform ratio. **(b)** Qualitative RT-PCR products generated with tau exon spanning primers from 3 independent batches of hCNs (left to right; HES1 *n*=2; HES2 *n*=1) after 24 h culture at 3 different temperatures, as compared to human foetal and adult brain. Upper panel shows products obtained with primers that span tau exons 1 to 5, thus amplifying transcripts of variable N-terminal length. Note predominant expression of 0N transcripts in both hCNs and foetal brain with low expression of 1N isoforms, and approximately equal expression of 0N and 1N transcripts in adult brain with low expression of 2N species. No obvious effects of temperature shift on N-terminal length are noted. Lower panel shows products obtained with primers that span tau exons 9-11. There is a clear increase in 3R transcript expression at hypothermic temperatures, consistent with q-RT-PCR results. This increase saturates the signal, thus making it difficult to visualise the very low expression of 4R tau in hCNs at any temperature (compare with Fig.5.1c).

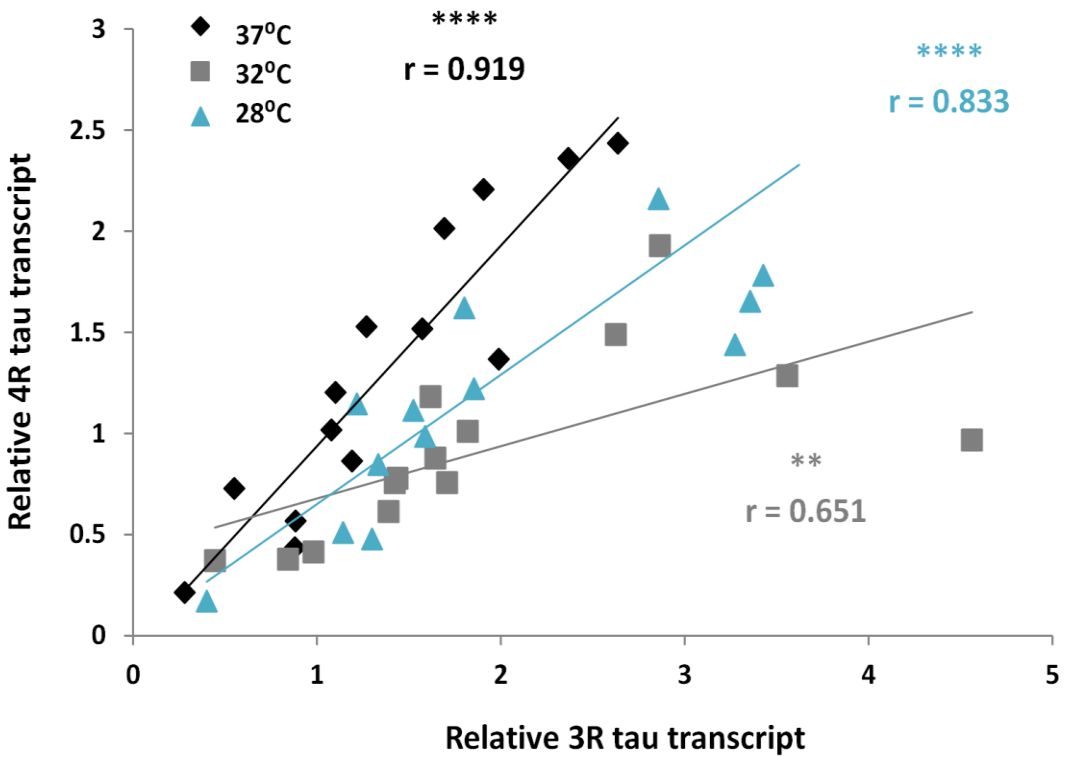


**Figure 5.3 Dissociation of tau isoform transcripts in hypothermic hCNs.** Two-tailed Pearson correlation illustrates tendency towards dissociation of relationship between 3R and 4R tau transcripts at 32°C (*N*=3; *n*=14). Transcript data was normalized to the geometric mean of 2 temperature-stable reference targets then to normothermic control.

At protein level, immunocytochemistry confirmed high enrichment of tau-expressing neurons in hCN cultures; total tau increased during differentiation (*N*=3; *n*≥4; *P*<0.0005) and was predominantly of the 3R isoform type (Fig.5.4, 5.5 and 5.6a). 4R tau was detectable from 4 w but was restricted to the cell soma and dendrites (Fig.5.5). According to Western blot analysis, tau was not detected in neural precursors (Fig.5.6b). Dephosphorylation of soluble cell lysates prior to SDS-PAGE produced a clear shift in electrophoretic mobility and resolved the tau signal at 4 w and 6 w into a single band, corresponding to the foetal isoform 3R0N (Goedert et al., 1989b) (Fig.5.6b). 4R tau was detectable at low levels by 7 w (Fig.5.6c) and mass

spectrometry of gel sections in the 30-75 kDa range identified tau-specific peptides in cytosolic extracts from both a 6 w HES1-derived culture sample and 19 w human foetal cortex (Fig.5.7). Importantly, tau was not detected in negative control samples (primary human and hiPS-derived GFAP-positive astroglia) but was identified immunocytochemically in both neurons and oligodendroglia (Lopresti et al., 1995) within mixed primary cultures derived from human foetal cortex (19 w; Fig.5.8). With cooling, quantitative Western blot analysis confirmed an increase in hCN 3R tau protein expression at 28°C ($N$=3; $n$=6; $P$=0.011), alongside a hypothermic decrease in electrophoretic mobility, but no detectable change in tau solubility (Fig.5.9a-b). Quantitative immunostaining revealed a 35.1 % reduction in 4R tau-positive cells at 32°C ($N$=2; $n$=4; $P$=0.017; Fig.5.9b-c).

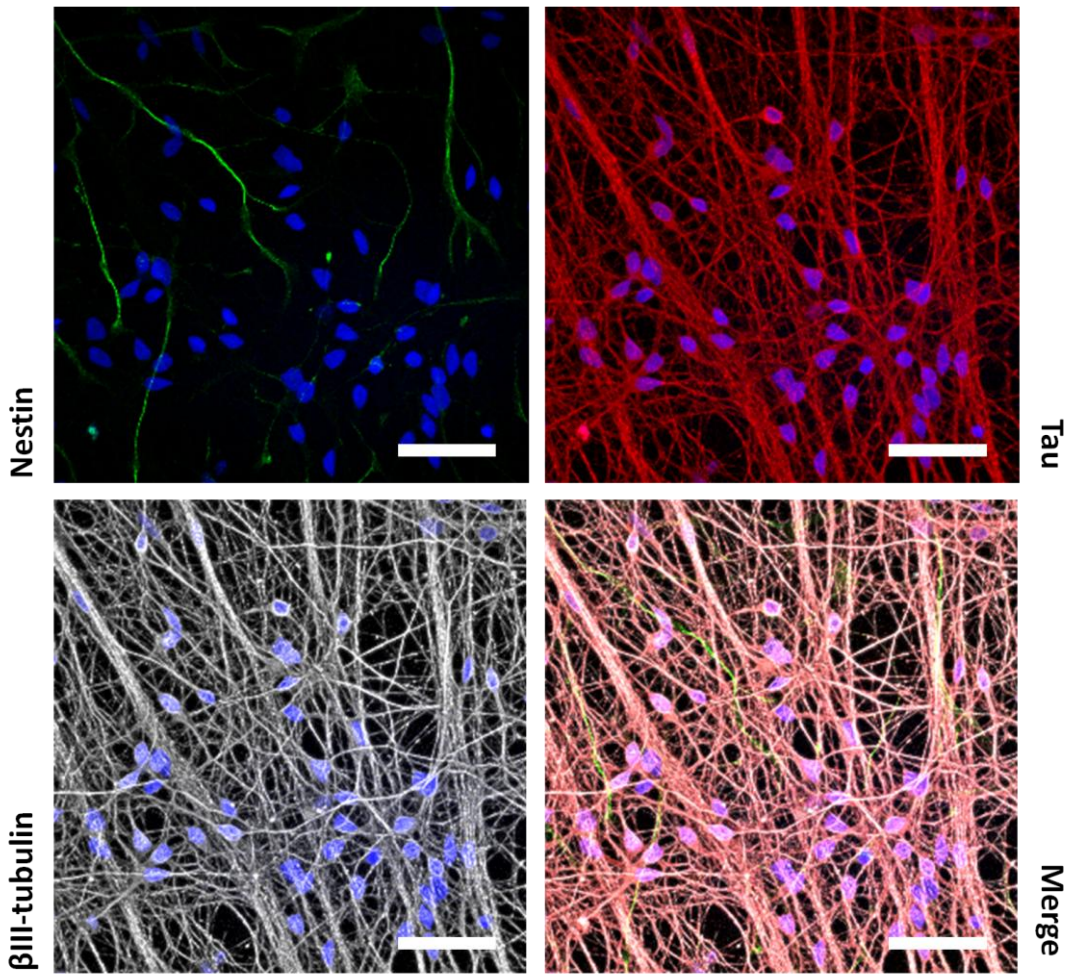


**Figure 5.4 Co-expression of tau and βIII-tubulin in hCNs.** Confocal fluorescent micrographs of hCNs (HES1) at 4 w showing co-expression of total tau (red, top right), βIII-tubulin (white, bottom left) and merged image (bottom right). Note that few Nestin-positive cells (green, top left) remain at this stage of differentiation. Cells co-stained for DAPI, scale bar = 50 µm.

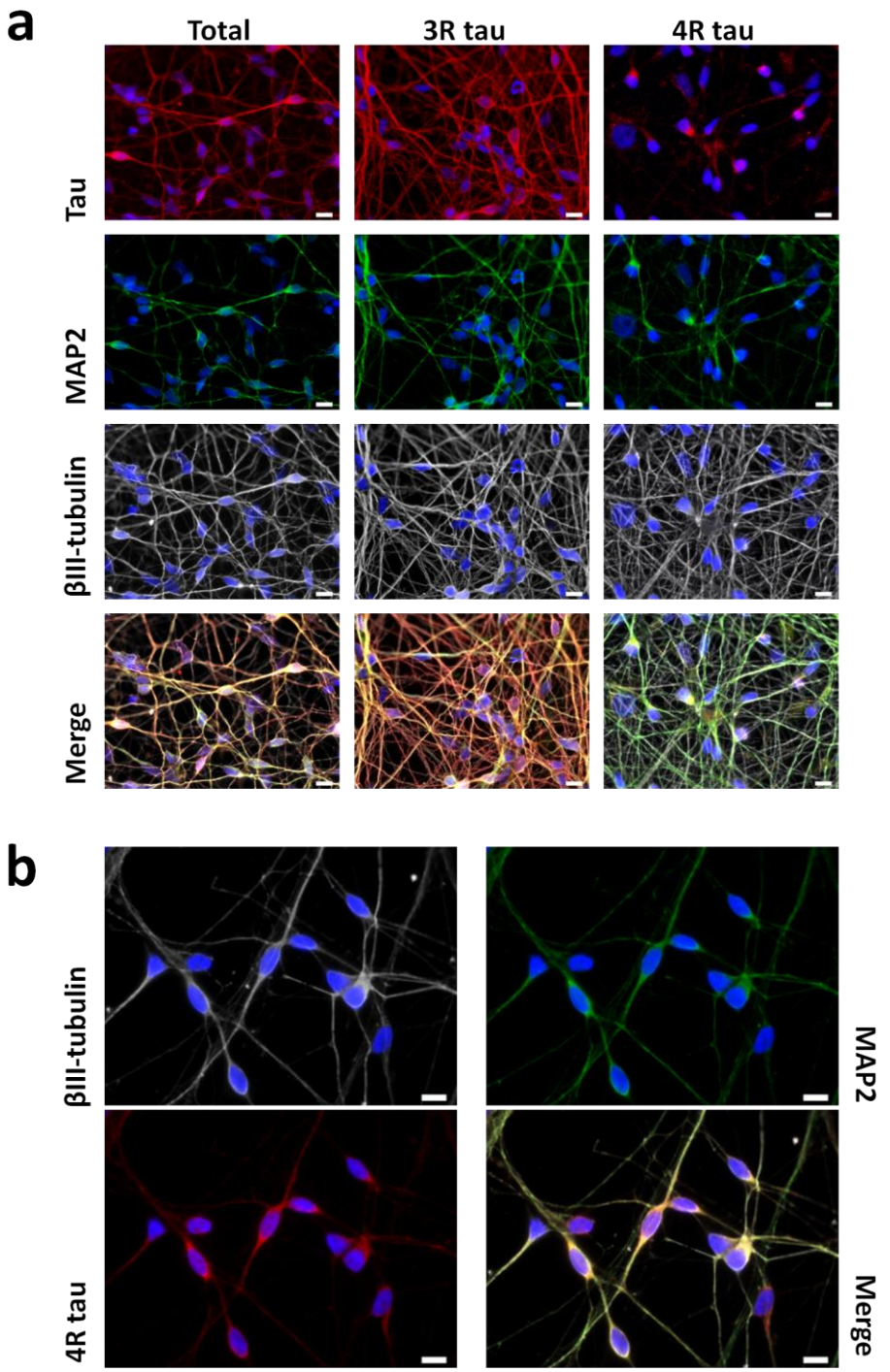


**Figure 5.5 Tau isoform expression in hCNs.** **(a)** Representative widefield fluorescent micrographs of hCNs (IPS1) at 4 w, co-stained for DAPI, neuronal markers (microtubule-associated protein 2 (MAP2) and βIII-tubulin) and tau (pan-tau, 3R- or 4R-specific). Somatic-axonal distribution of 3R and total tau reflects developing polarity, scale bar = 10 µm. **(b)** Representative widefield fluorescent micrographs of hCNs at 53 DIV showing more extensive 4R tau staining (bottom left) with a similar distribution to MAP2 (top right) in βIII-tubulin- (top left) positive neurons. Merged image is shown (bottom right), all cells co-stained with DAPI, scale bar = 10 µm.

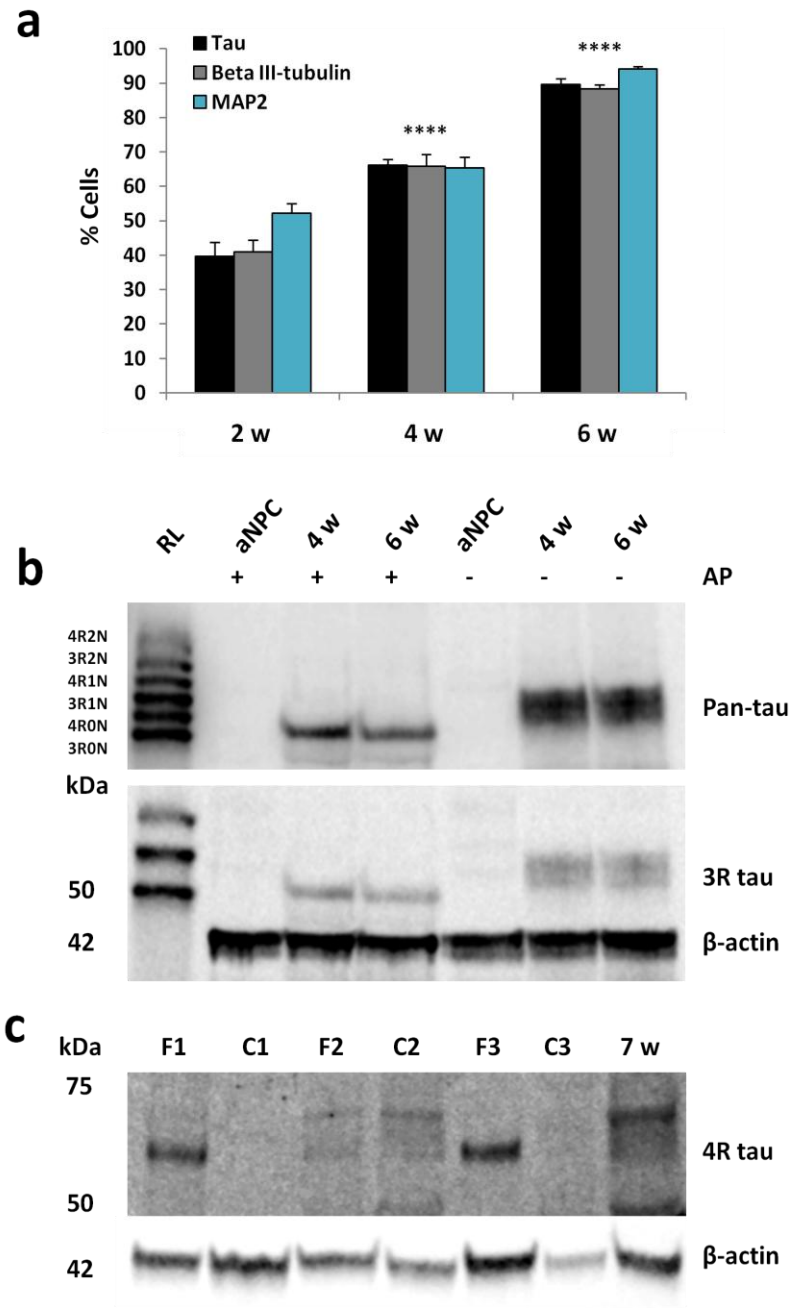


**Figure 5.6 Human cortical tau expression. (a)** Immunocytochemical quantification of hCN protein expression, asterisks denote significant changes in each marker relative to 2 w post-plating (*N*=3; *n*≥4). Cell counts are presented as mean + SEM. **(b)** Western blot of soluble tau during hCN differentiation, run by 10% SDS-PAGE with (+) or without (-) prior dephosphorylation with AP. Blot was probed with pan-tau antibody (upper image) recognising all tau isoforms irrespective of phosphorylation status. Positive control includes recombinant human tau protein ladder (RT) containing 6 human brain tau isoforms. Membrane was re-probed with 3R tau-specific antibody RD3 (lower image). β-actin = loading control. 'Pan-tau' antibody recognises all isoforms of tau irrespective of phosphorylation status. **(c)** Comparison of soluble 4R tau expression in mature hCNs (7 w) and human foetal cortex (F1, F2, F3 at 14, 15 and 16 wk gestation respectively) and primary cultures derived from these samples (C1, C2, C3 respectively). Note signal in both hCN and some foetal samples.

**Tau 352 (3R0N)**

MAEPRQEFEVMEDHAGTYGLGDRKDQGGYTMHQDQEGDTDAGLKAEEAGIGDTPSLEDEAAGHVTQARMVS
KSKDGTGSDDKKAKGADGKTKIATPRGAAPPGQKGQANATRIPAKTPPAPKTPPSSGEPPKSGDRSGYSSPGSPG
TPGSRSRTPSLPTPPTREPKKVAVVRTPPKSPSSAKSRLQTAPVPMPDLKNVKSKIGSTENLKHQPGGGKVQIVYKP
VDLSKVTSKCGSLGNIHHKPGGGQVEVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDH
GAEIVYKSPVVSGDTSPRHLSNVSSTGSIDMVDSPQLATLADEVSASLAKQGL

**Tau 383 (4R0N)**

MAEPRQEFEVMEDHAGTYGLGDRKDQGGYTMHQDQEGDTDAGLKAEEAGIGDTPSLEDEAAGHVTQARMVS
KSKDGTGSDDKKAKGADGKTKIATPRGAAPPGQKGQANATRIPAKTPPAPKTPPSSGEPPKSGDRSGYSSPGSPG
TPGSRSRTPSLPTPPTREPKKVAVVRTPPKSPSSAKSRLQTAPVPMPDLKNVKSKIGSTENLKHQPGGGKVQIINKK
LDLSNVQSKCGSKDNIKHVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQVEVKSEKLDFKDRVQSKIGSL
DNITHVPGGGNKKIETHKLTFRENAKAKTDHGAEIVYKSPVVSGDTSPRHLSNVSSTGSIDMVDSPQLATLADEVS
ASLAKQGL

**Tau 381 (3R1N)**

MAEPRQEFEVMEDHAGTYGLGDRKDQGGYTMHQDQEGDTDAGLKESPLQTPTEDGSEEPGSETSDAKSTPTAE
AEEAGIGDTPSLEDEAAGHVTQARMVSKSKDGTGSDDKKAKGADGKTKIATPRGAAPPGQKGQANATRIPAKTP
PAPKTPPSSGEPPKSGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKKVAVVRTPPKSPSSAKSRLQTAPVPMPDL
KNVKSKIGSTENLKHQPGGGKVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQVEVKSEKLDFKDRVQSKIGSLDNIT
HVPGGGNKKIETHKLTFRENAKAKTDHGAEIVYKSPVVSGDTSPRHLSNVSSTGSIDMVDSPQLATLADEVSASLA
KQGL

**Tau 412 (4R1N)**

MAEPRQEFEVMEDHAGTYGLGDRKDQGGYTMHQDQEGDTDAGLKESPLQTPTEDGSEEPGSETSDAKSTPTAE
AEEAGIGDTPSLEDEAAGHVTQARMVSKSKDGTGSDDKKAKGADGKTKIATPRGAAPPGQKGQANATRIPAKTP
PAPKTPPSSGEPPKSGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKKVAVVRTPPKSPSSAKSRLQTAPVPMPDL
KNVKSKIGSTENLKHQPGGGKVQIINKKLDLSNVQSKCGSKDNIKHVPGGGSVQIVYKPVDLSKVTSKCGSLGNIH
HKPGGGQVEVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDHGAEIVYKSPVVSGDTSP
RHLSNVSSTGSIDMVDSPQLATLADEVSASLAKQGL

**Tau 410 (3R2N)**

MAEPRQEFEVMEDHAGTYGLGDRKDQGGYTMHQDQEGDTDAGLKESPLQTPTEDGSEEPGSETSDAKSTPTAE
DVTAPLVDEGAPGKQAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSKSKDGTGSDDKKAKGAD
GKTKIATPRGAAPPGQKGQANATRIPAKTPPAPKTPPSSGEPPKSGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREP
KKVAVVRTPPKSPSSAKSRLQTAPVPMPDLKNVKSKIGSTENLKHQPGGGKVQIVYKPVDLSKVTSKCGSLGNIHH
KPGGGQVEVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDHGAEIVYKSPVVSGDTSPR
HLSNVSSTGSIDMVDSPQLATLADEVSASLAKQGL

**Tau 441 (4R2N)**

MAEPRQEFEVMEDHAGTYGLGDRKDQGGYTMHQDQEGDTDAGLKESPLQTPTEDGSEEPGSETSDAKSTPTAE
DVTAPLVDEGAPGKQAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARMVSKSKDGTGSDDKKAKGAD
GKTKIATPRGAAPPGQKGQANATRIPAKTPPAPKTPPSSGEPPKSGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREP
KKVAVVRTPPKSPSSAKSRLQTAPVPMPDLKNVKSKIGSTENLKHQPGGGKVQIINKKLDLSNVQSKCGSKDNIKH
VPGGGSVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQVEVKSEKLDFKDRVQSKIGSLDNITHVPGGGNKKIETHK
LTFRENAKAKTDHGAEIVYKSPVVSGDTSPRHLSNVSSTGSIDMVDSPQLATLADEVSASLAKQGL

**Figure 5.7 Human cortical mass spectrometry.** Entire peptide sequences of the 6 major adult human brain tau isoforms. Orange underlined sequence depicts tryptically digested peptide detected in soluble cytosolic protein extracts from both 19 w post-mortem human foetal brain and 6 w hCN sample (HES1) as determined by mass spectrometry. Grey underlined sequence denotes a second tau-specific peptide identified in foetal brain sample only. Note that both highlighted sequences are common to all tau isoforms.

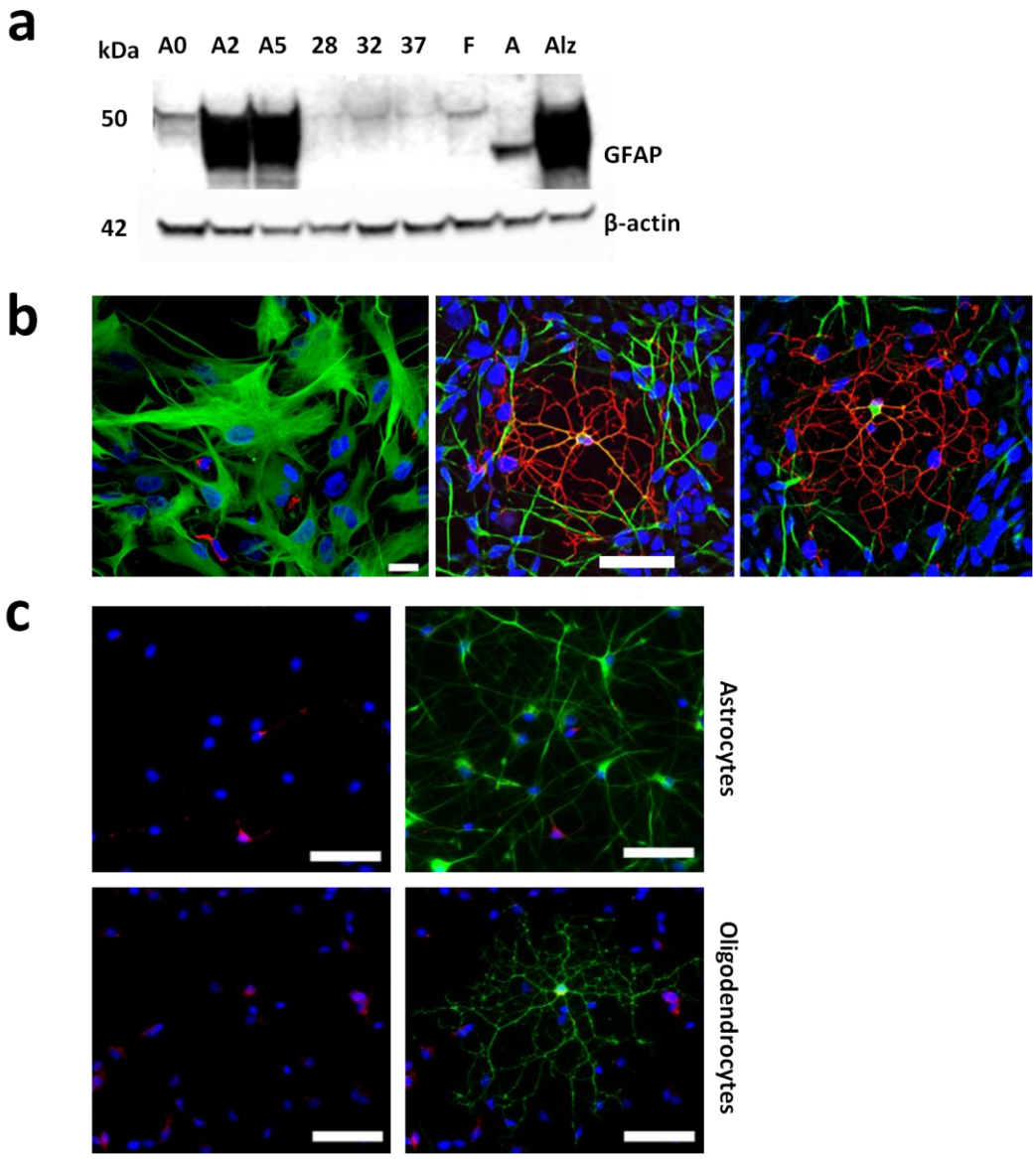


**Figure 5.8 Human glial tau expression. (a)** GFAP expression in hPS-derived astroglial progenitors (A0) and mature astroglia at 2 w and 5 w post-plating (A2, A5) compared to 5 w hCNs at 3 different temperatures (28, 32 and 37$^{o}$C respectively) and human post-mortem cortical samples (foetal, F; adult control, A; Alz, Alzheimer's disease patient). Note low GFAP expression in hCNs and F, and high expression in Alz, reflecting the inflammatory astrocytic reactivity of this disease. **(b)** Confocal fluorescent micrographs of hPS-derived astrocytes (left, IPS2), positive for GFAP (green) but negative for tau (red), scale bar = 10 µm. Confocal fluorescent micrographs of hPS-derived oligodendrocytes (middle and right) stained for O4 (red) and either pan-tau (green, middle) or 3R tau (RD3, green, right), scale bar = 50 µm. Note presence of 3R tau in cell soma and major processes. **(c)** Widefield fluorescent micrographs of astrocytes (top) and oligodendroglia (bottom) in mixed primary cultures derived from 19 w post-mortem foetal ventral telencephalon. Note somatic 3R tau (RD3, red) expression in O4-positive (green) oligodendroglia but absence of total tau expression (red) in GFAP-positive (green) astroglia, scale bar = 50 µm.

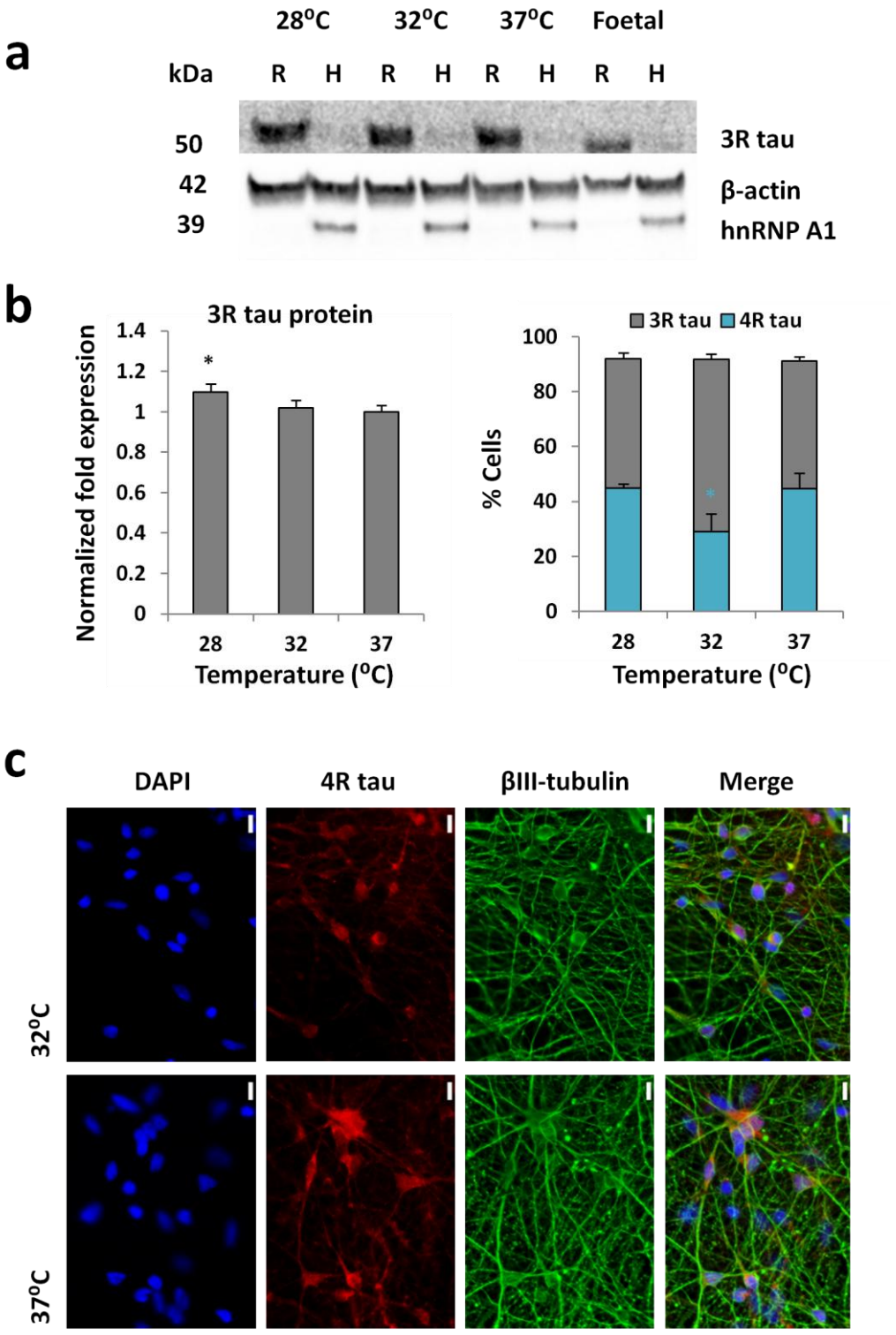


**Figure 5.9 Tau isoform expression in hypothermic hCNs.** **(a)** Western blot depicts upward mobility shift of 3R tau in hypothermic cultures. 3R tau solubility was unaffected (3R tau found in RIPA-soluble fraction (R) but absent from high detergent fraction (H); β-actin and hnRNP A1 serve as cytosolic and nuclear loading controls respectively. **(b)** Western blot quantification (left) confirmed that 3R tau expression increased slightly at 28°C when normalized to total tau (*N*=3; *n*=6). Cell counts (right) show a reduction in 4R tau expression at 32°C (*N*=2; *n*=4). Cell counts are presented as mean % of DAPI-stained cells + SEM. **(c)** Widefield fluorescent micrographs depicting a reduction in 4R tau expression at 32°C, merged images show co-staining for DAPI and βIII-tubulin, scale bar = 10 µm.

### 5.2.3 Tau splicing regulator RBM4

Given the differential effect of hypothermia on tau isoform transcripts, an effect of cooling on tau splicing regulators seemed inevitable. One such factor, RNA binding motif 4 (RBM4), is expressed at high levels in foetal brain and is also present in adult cerebral cortex and hippocampus (Bernert et al., 2002; Kar et al., 2006). RBM4 promotes the inclusion of tau exon 10, thus resulting in more 4R tau transcript (Kar et al., 2006). Accordingly, disruption of foetal RBM4 expression has been associated with Down's syndrome (Bernert et al., 2002) - a syndrome resulting in early-onset 3R-dominant tauopathy (Shi et al., 2008). Considering earlier findings, it was expected that RBM4 expression might be reduced at 32°C. Surprisingly, mild hypothermia had no effect on RBM4 transcript and moderate hypothermia actually produced a small increase in RBM4 mRNA after 24 h ($P$=0.01, $N$=3; $n$=14; Fig.5.10a). Interestingly this fold change was similar to that seen between human foetal and adult brain. Fractionated Western blot analysis showed that RBM4 expression was barely detectable in human adult cortex but was highly expressed in the nuclear fraction (co-localising with hnRNP A1) of human foetal cortex (Fig.5.10b). In hCNs, RBM4 was present in both cellular compartments at each temperature with a slight increase in total expression at 28°C ($N$=3; $n$=6; $P$=0.046; Fig.5.10b).

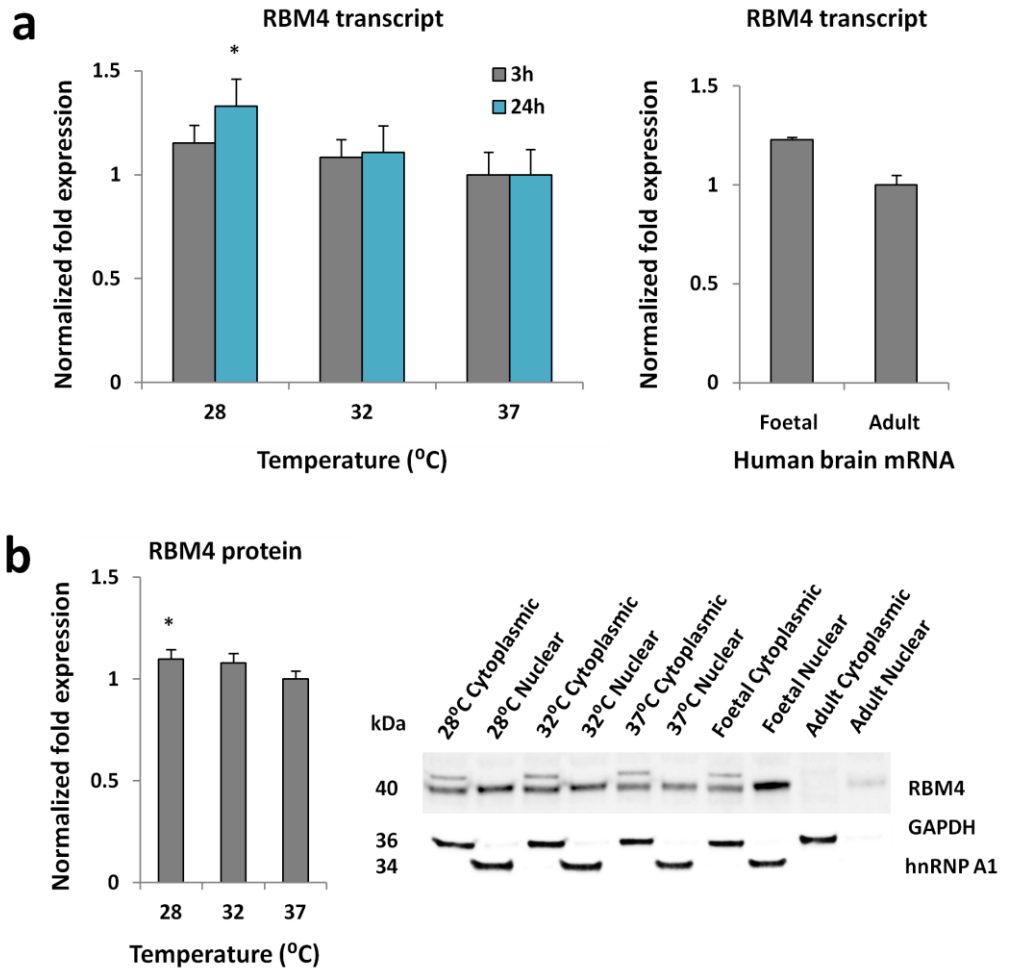


**Figure 5.10 Human cortical RBM4 expression.** **(a)** q-RT-PCR analysis of RBM4 transcripts (left) showing significant increase after 24 h at 28°C (*N*=3; *n*=14). This trend mimics the reverse developmental transition from human foetal to adult brain (right). Transcript data for pooled human brain samples is presented as mean + SEM. **(b)** Quantification by Western blot (left) of total RBM4 expression in 5 w hCNs (*N*=3; *n*=6). Total expression was normalized to GAPDH and then normothermic control. Representative Western blot (right) showing subcellular expression of RBM4 at 3 culture temperatures, as compared to human foetal (19 w) and adult (17 y) cortex. hnRNP A1 and GAPDH serve as compartment-specific loading controls.

With immunocytochemistry there was an increase in the number of RBM4-positive nuclei at both hypothermic temperatures (32°C $P<0.0005$, 28°C $P=0.008$, $N=3$; $n=8$; Fig.5.11 and 5.12). However, cooling also resulted in a greater proportion of hCNs containing RBM4-positive somatic puncta (32°C $P=0.031$, 28°C $P=0.037$;

Fig.5.12b). RBM4 can undergo nucleo-cytoplasmic shuttling in response to cell stress (Lai et al., 2003; Lin et al., 2007a) and under these conditions can be found localised to stress granules. Some of the RBM4-positive somatic puncta in hCNs co-stained with the stress granule marker T-cell intracytoplasmic antigen 1 (TIA-1, Fig.5.12c), consistent with a re-localisation of RBM4 into stress granules in response to cooling (Lin et al., 2007a; Hofman et al., 2012). In summary, the response of RBM4 to hypothermic stress in human neurons is a novel finding that manifests in a change in quantifiable RBM4 protein expression and subcellular redistribution.

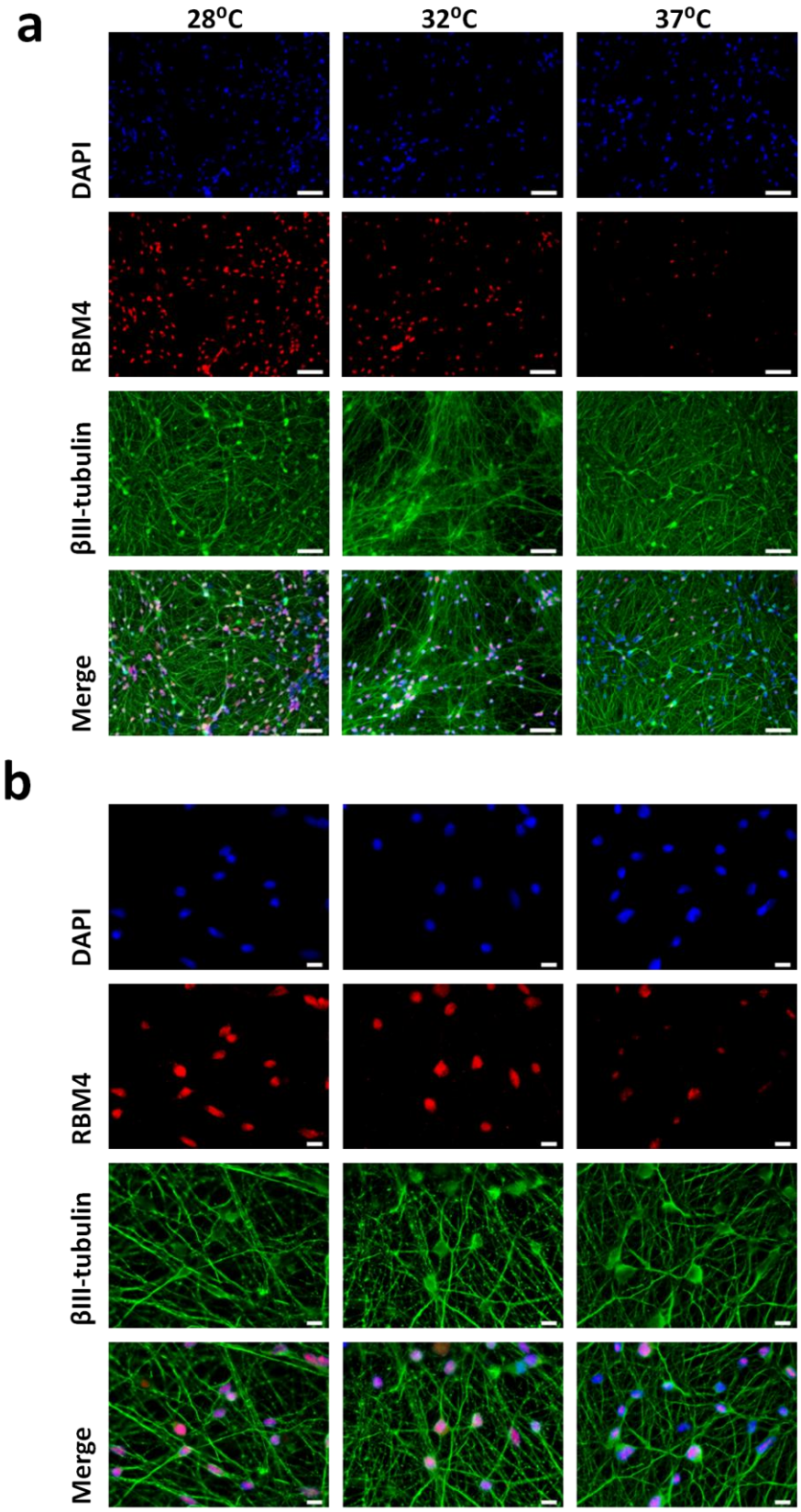


**Figure 5.11 RBM4 expression in hypothermic hCNs.** Representative widefield fluorescent micrographs of hCNs co-stained for nuclear and neuronal markers (DAPI and βIII-tubulin respectively) and RBM4 after incubation for 24 h at the indicated temperatures, **(a)** scale bar = 50 µm, **(b)** scale bar = 10 µm.

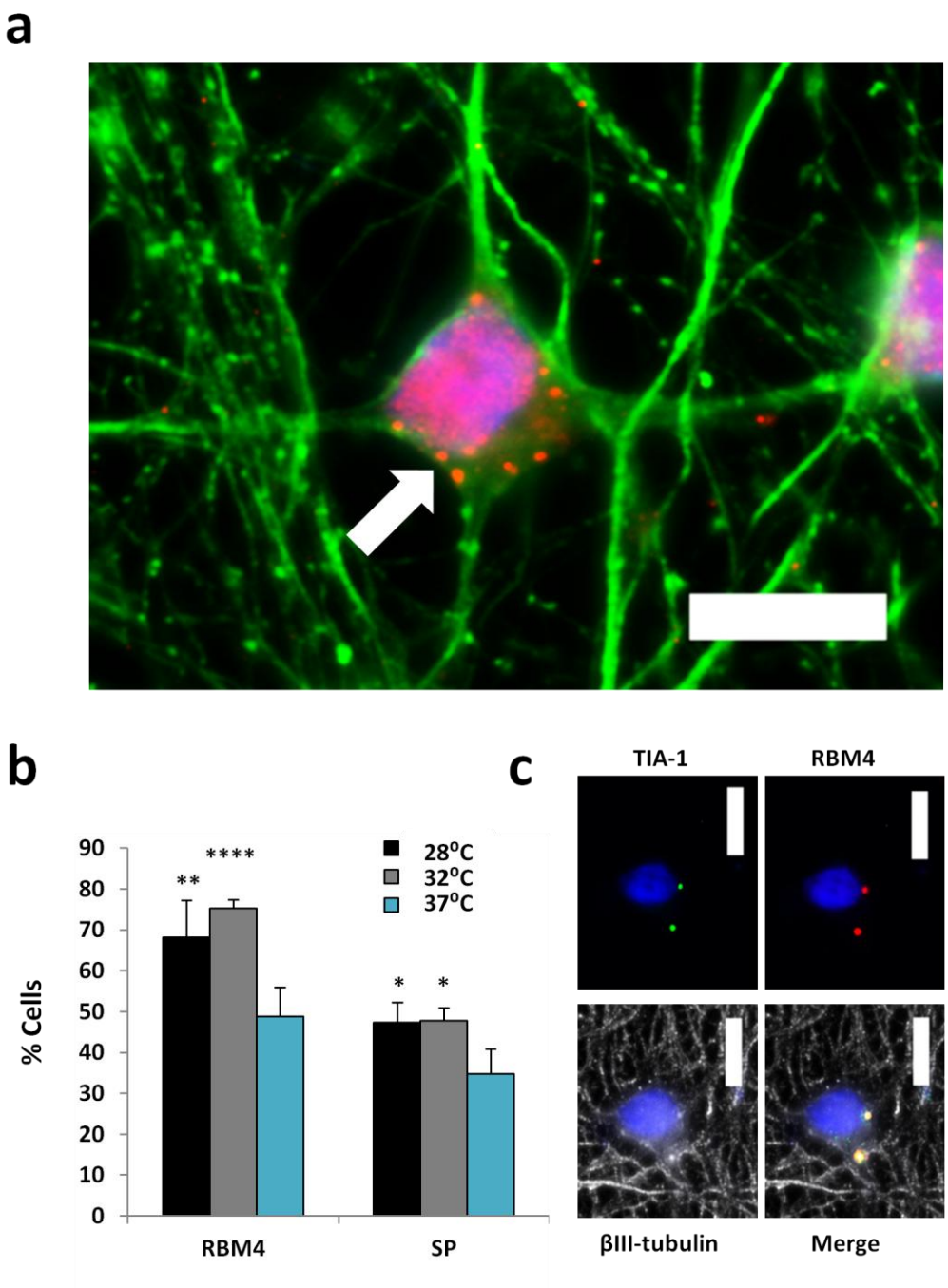


**Figure 5.12 Hypothermia redistributes RBM4 in hCNs.** **(a)** Enlarged central portion of merged 32°C image from Fig.5.9b showing somatic RBM4-positive puncta (white arrow), scale bar = 10 µm. **(b)** Cell counts showing a significant increase in the percentage of RBM4-positive nuclei with hypothermia and an increase in the proportion of cells with RBM4-positive somatic puncta (SP) (*N*=3; *n*=8). Cell count data is presented as mean % of DAPI-stained cells + SEM. **(c)** Widefield fluorescent micrographs of hypothermic hCN showing RBM4-positive somatic puncta that co-stain for stress granule marker TIA-1, scale bar = 10 µm.

### 5.2.3 Tau phosphorylation during differentiation and hypothermic challenge

As has been described for human foetal brain (Goedert et al., 1993), tau phosphorylation in 4 w hCNs was more similar to Alzheimer's disease cortex (Braak stage 6) than to normal adult cortex (age 17 y) at post-mortem, and was seen even at epitopes that were classically assigned as 'disease-specific' (Grundke-Iqbal et al., 1986) (Fig.5.13a-b). There was a decrease in soluble tau phosphorylation (at all epitopes tested) between 4 w and 6 w (Fig.5.13a-b), suggesting some maturation of tau phospho-status. Tau phosphorylated at AT8 and PHF-1 epitopes was noted in hCNs, human foetal brain and Alzheimer cortex, but was completely absent from normal adult cortex (Fig.5.13b). Even at later time points, hCN tau was more phosphorylated than that extracted from human adult and foetal cortical tissue samples (Fig.5.13c-d). Comparison of phosphorylation status in post-mortem samples and hCNs could however be confounded by post-mortem interval (PMI), during which residual PP2A activity can take effect (Matsuo et al., 1994). In support of this, tau phosphorylation was reduced in foetal human post-mortem cortices relative to primary cultures derived from these samples, which exhibited a similar tau electrophoretic profile to 7 w hCNs (Fig.5.13d). Despite a considerable PMI, tau extracted from adult human pathological samples remained hyperphosphorylated (Fig.5.13c), likely due to reduced PP2A expression (Vogelsberg-Ragaglia et al., 2001; Fig.5.17b) or shielding of phospho-epitopes within multimeric tau species.

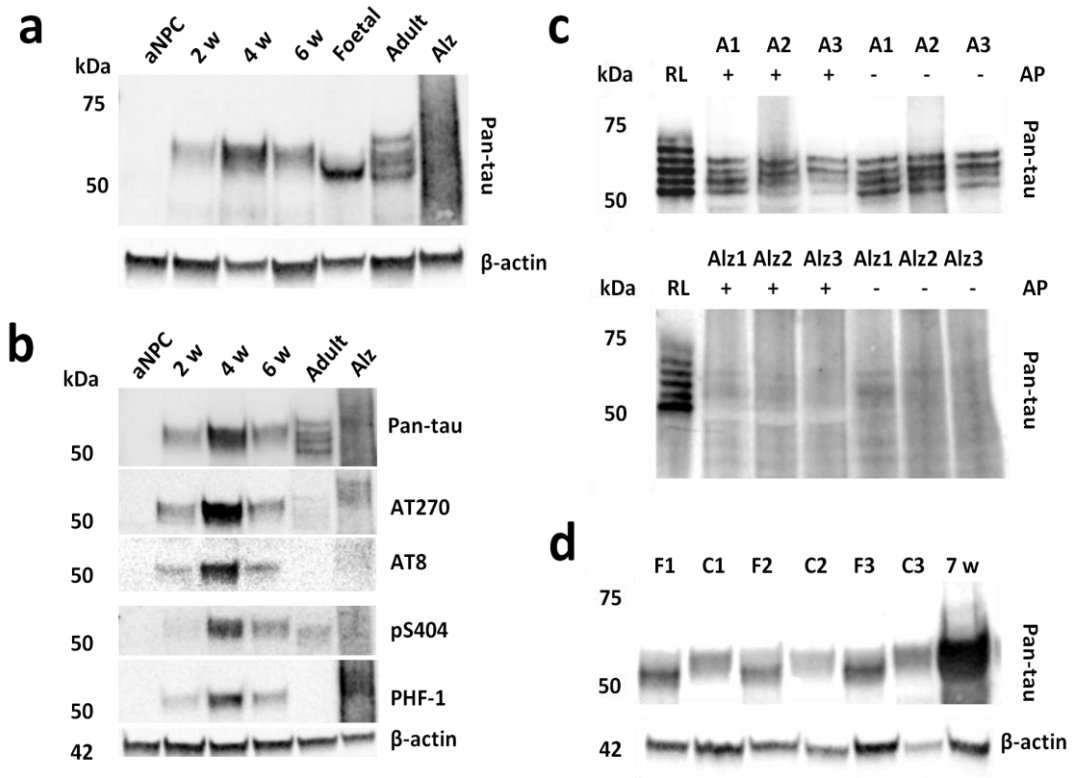


**Figure 5.13 Phospho-tau expression in hCNs.** **(a)** Soluble phospho-tau expression during hCN differentiation. The blot shows total tau in aNPCs and hCNs at various points during differentiation (2, 4 and 6 w post-plating) as compared to a foetal cortical sample at 19 w gestation, adult control cortex, and Alzheimer's disease cortex (Alz). Note expression in 4 w hCNs is greater than at 6 w and in foetal and adult samples but is similar to Alz. Foetal sample sits at much lower molecular weight than hCN samples even in the absence of AP treatment, suggesting lower phosphorylation status. **(b)** Western blots probed with antibodies specific for tau phospho-epitopes; AT8 (S202/T205), AT270 (T181), pS404 and PHF-1 (S396/S404). Note decrease in signal at every tested epitope between 4 w and 6 w of hCN differentiation, weak signal in normal adult human cortex (age 17 y) and prominent signal in Alzheimer's disease (Alz)-affected cortex (age 60 y, Braak stage 6). **(c)** Soluble tau expression in human post-mortem cortical tissue samples. Upper blot: 3 independent adult control samples (A1, A2, A3 ages 17, 44 and 75 y respectively) - note presence of multiple tau isoforms and absence of tau mobility shift upon treatment with AP. RL = recombinant human tau ladder. Lower blot: 3 independent Alzheimer disease samples (Alz1, Alz2, Alz3 ages 60, 61 and 81 y and Braak stages 6, 5 and 6 respectively), note tau 'smears' indicating tau oligomerization with reduced solubility compared to control samples, also unaffected by AP treatment. **(d)** The membrane from Fig.5.4c was originally probed for pan-tau before being stripped and reprobed for 4R tau. Blot image here shows comparison of soluble tau expression in mature hCNs and human foetal cortex. Upper blot: 3 independent foetal cortical samples (F1, F2, F3 at 14, 15 and 16 w gestation respectively) and primary cultures derived from these samples (C1, C2, C3 respectively). 7 w = hCNs at 7 w post-plating. Note mobility shift between tissue and culture samples, indicative that PMI allows continued dephosphorylation of tissue samples prior to processing. Note that 7 w hCN tau aligns with primary culture tau. 'Pan-tau' antibody recognises all isoforms of tau irrespective of phosphorylation status.

Post-translational effects of cooling were first assessed using immunocytochemistry (Fig.5.14) and quantitative Western blot analysis (Fig.5.15) of tau phosphorylation at the AT8 epitope (pSer202/pThr205). AT8 is a key site that is heavily phosphorylated in the foetal and diseased adult human brain, as well as hypothermic rodent models (Planel et al., 2007a). At 24 h there was a temperature-dependent increase in AT8 expression with cooling according to both Western analysis ($N$=3; $n$=6; 32°C $P$=0.004, 28°C $P$<0.0005; Fig.5.15a-b) and cell counts ($N$=3; $n$=6; 32°C $P$=0.008, 28°C $P$<0.001; Fig.5.15b). A similar increase in phosphorylation was seen at other pivotal phospho-tau epitopes typically associated with pathological tau ($N$=3; $n$=6; PHF-1 $P$<0.05, AT180 $P$<0.01, AT270 $P$<0.0005; Fig.5.16a) including AT100 ($N$=3; $n$=5; $P$=0.001; Fig.5.16b).

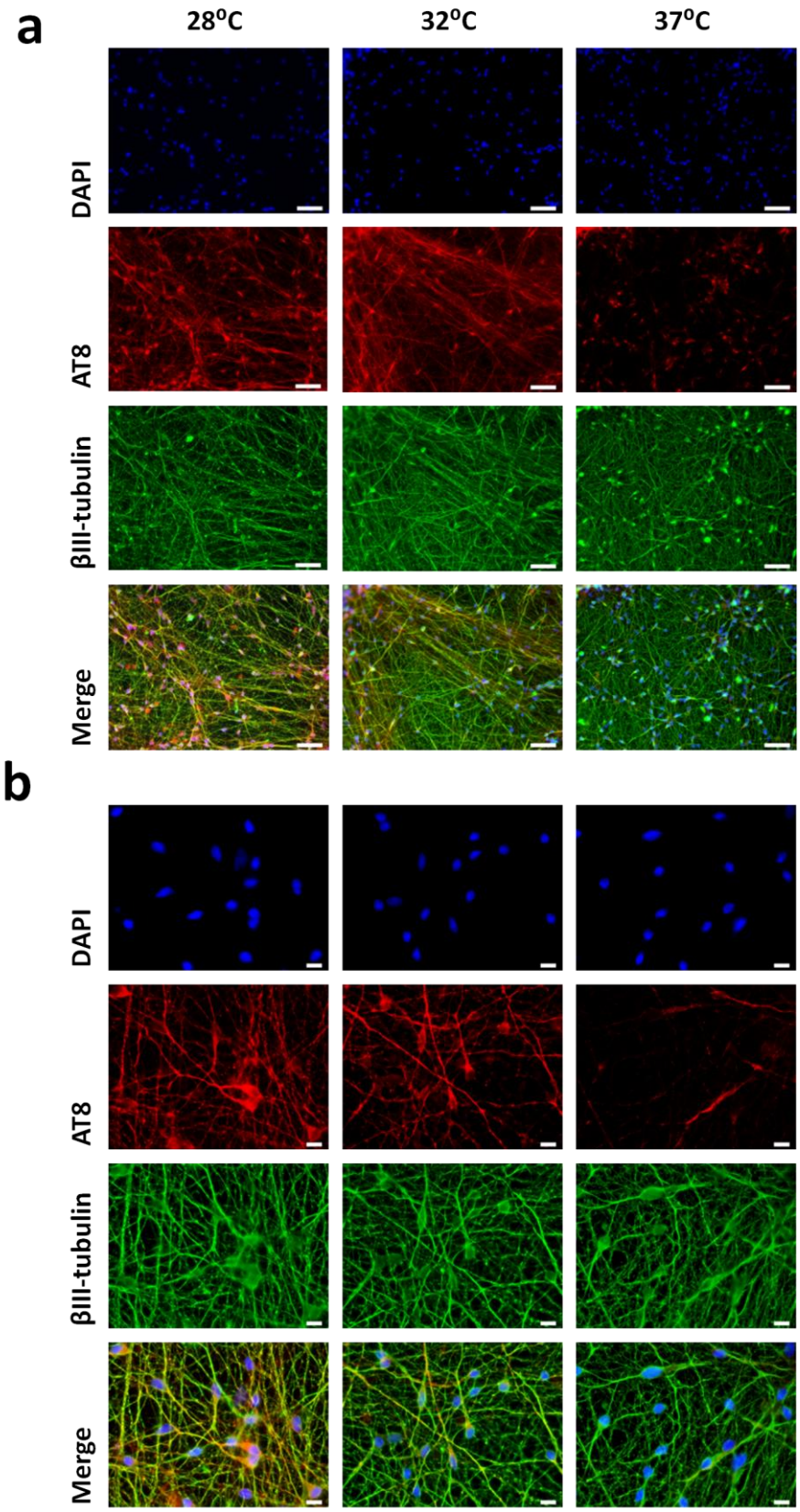


**Figure 5.14 AT8 expression in hypothermic hCNs.** Representative widefield fluorescent micrographs of hCNs co-stained for nuclear and neuronal markers (DAPI and βIII-tubulin respectively) and AT8 after incubation for 24 h at the indicated temperatures, **(a)** scale bar = 50 µm, **(b)** scale bar = 10 µm.

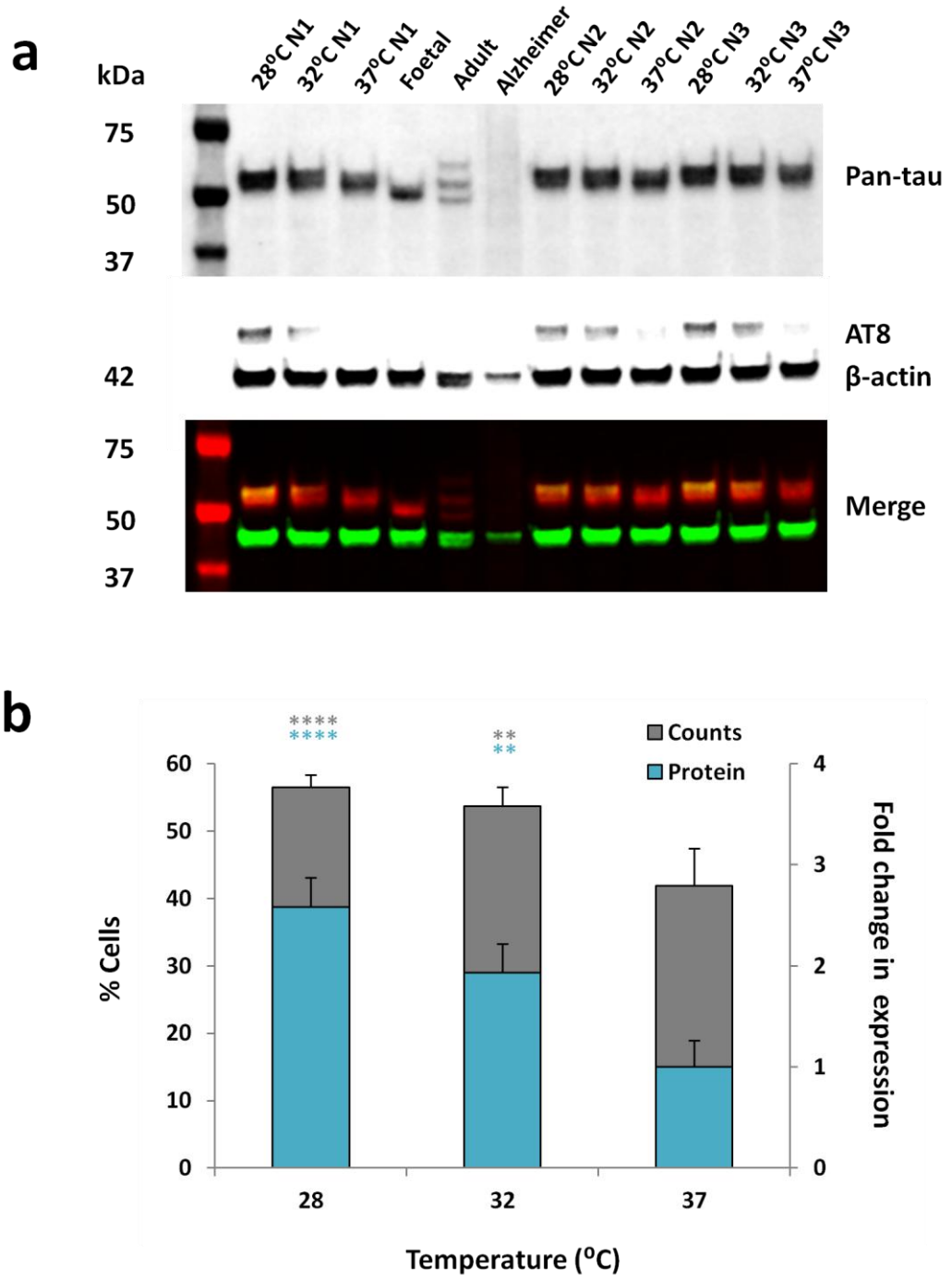


**Figure 5.15 Hypothermia increases AT8 phosphorylation in hCNs.** **(a)** Western blot showing increased phospho-tau (AT8) with hypothermia. β-actin = loading control, for multiplexed image AT8 and β-actin were imaged in the 800 nm channel (green) and total tau in the 700 nm channel (red). As evidenced by the loading control, the Alzheimer sample was loaded at a lower total protein concentration to prevent oversaturation of the AT8 signal, ensuring that hCN AT8 signals could be quantified. This proved to be unnecessary for the soluble fraction due to post-mortem delay, as addressed in the discussion. **(b)** Western blot quantification of AT8 expression, normalized to total tau and quantification by cell count (mean % of DAPI-stained cells + SEM). Significant increases by immunoblot and immunocytochemistry were noted after 24 h (*N*=3; *n*=6).

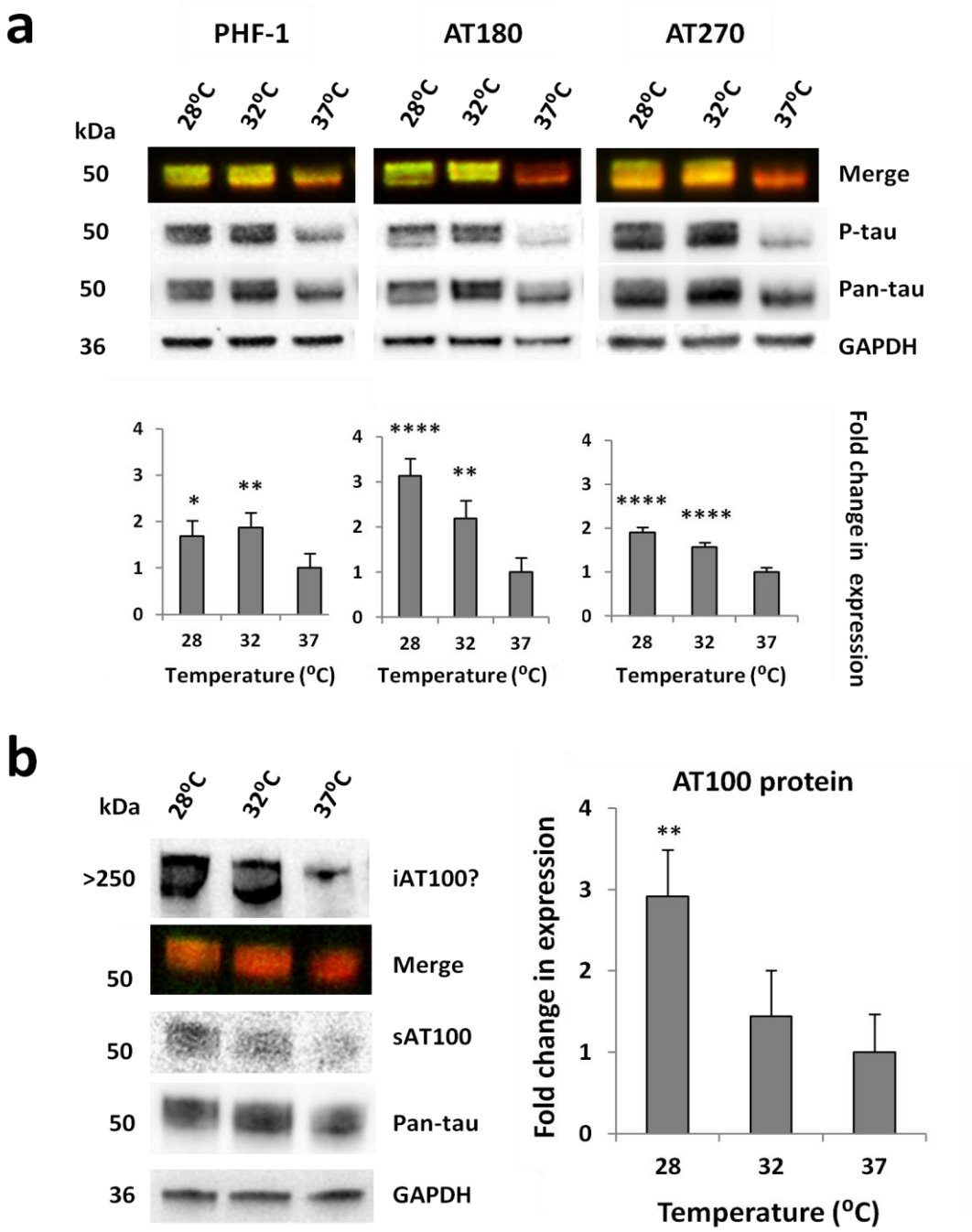


**Figure 5.16 Hypothermic phosphorylation at other tau epitopes. (a)** Immunoblots depicting significant increases in tau phosphorylation at 3 other epitopes with cooling and associated quantification (*N*=3; *n*=6). Phospho-tau expression was first normalized to total tau, then 37°C control. **(b)** Western blot (left) and associated quantification of soluble tau (right; *N*=3; *n*=5) phosphorylated at the 'disease-associated' epitope AT100 (S212/T214), normalized to total tau expression (HES2). Note that soluble (sAT100) expression was much less than other phospho-epitopes and, unlike other phospho-tau antibodies, AT100 detected products of very high molecular weight (largely in hypothermic samples) that may have represented insoluble protein (iAT100) that did not run through the gel. The insoluble protein fraction was not specifically examined.

### 5.2.4 Tau phosphatases and kinases in hCNs

As might be predicted from the literature (Yu et al., 2009), and from the decreasing tau phosphorylation state during hCN differentiation, there was a significant increase in PP2A-Bγ transcript expression compared to aNPC levels (*N*=2; *n*=5; *P*<0.0005; Fig.5.17a). This echoed the transition seen between human foetal and adult brain. With temperature shift, no difference was seen in PP2A-Bγ transcript (*N*=3; *n*=14; Fig.5.17a) or in protein levels of the active subunit of PP2A (PP2A-C) (*N*=3; *n*=6; Fig.5.17b). Others have shown in rodent models that whilst kinase activity reduces in a linear order with temperature reduction, PP2A activity decreases exponentially over the same temperature range (Planel et al., 2004). This bias has been proposed as the principal cause for increased tau phosphorylation under hypothermic conditions (Planel et al., 2004). To evaluate phosphatase activity, PP2A-C was immunoprecipitated from 5 w hCNs and the ability of this isolate to release phosphate from a threonine phosphopeptide (K-R-pT-I-R-R) at the 3 temperatures of interest was measured over a 10 min period (Fig.2.8). Phosphate release was compared to a phosphate standard curve. As an additional negative control, a sample of isolated PP2A-C from each hCN batch was simultaneously incubated at 37°C in the presence of 100 nM fostriecin (a membrane-permeable specific inhibitor of PP2A (Walsh et al., 1997; Douglas et al., 2001), to determine and subtract background phosphate levels within the test lysates. Both mild and moderate hypothermia produced a >60 % reduction in PP2A-C activity (*N*=2; *n*=8; 32°C *P*=0.041, 28°C *P*=0.034; Fig.5.17c). *In vitro*, GSK3β is the most efficient kinase at phosphorylating recombinant tau (Wang et al., 2007). Although tau kinase activity was not directly assessed in hCNs, quantitative Western blot analysis showed that the effect of

hypothermia on total GSK3β or its deactivated form (pS9) was negligible (*N*=3; *n*=4; Fig.5.17d). Cooling had no effect on the expression of the activated form (pY216) of GSK3β (*N*=3; *n*=6; Fig.5.17d).

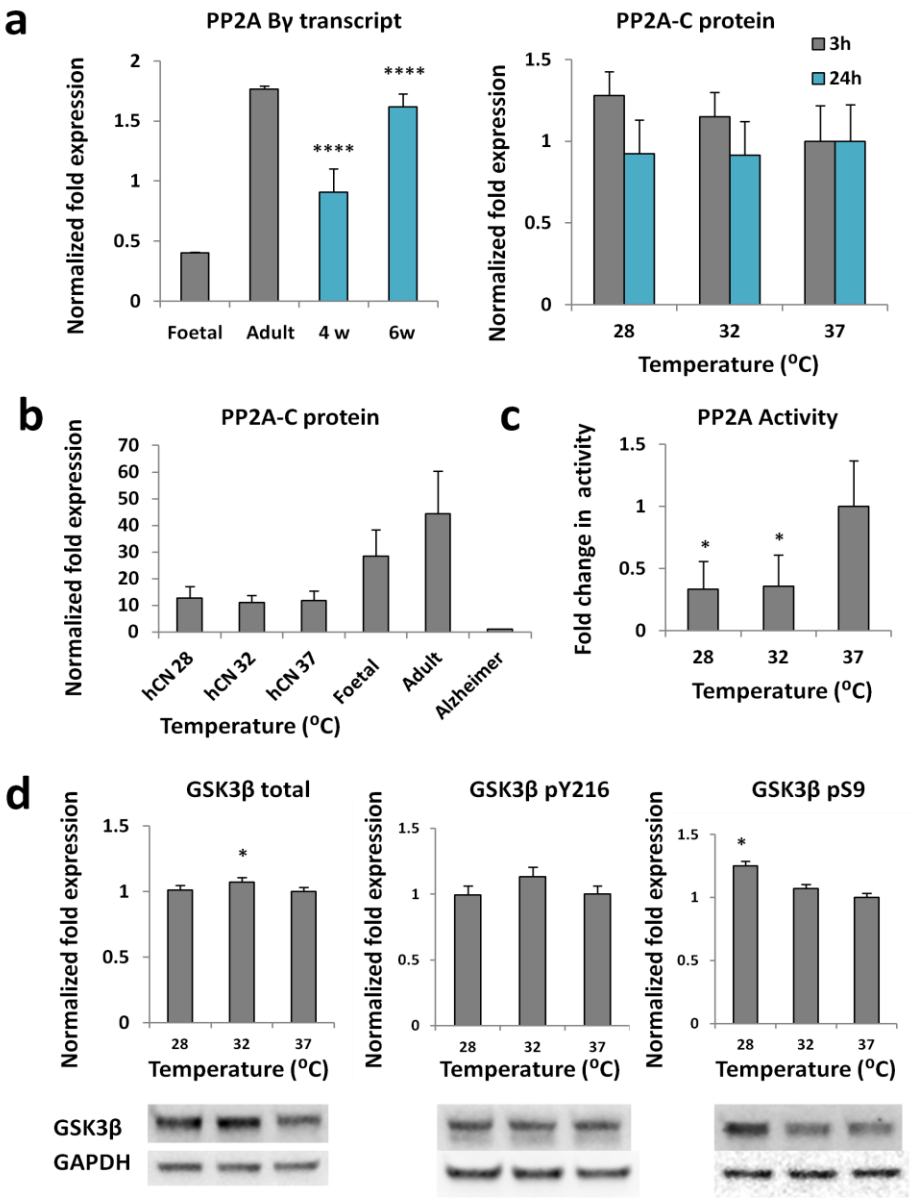


**Figure 5.17 Tau kinases and phosphatases in hypothermic hCNs. (a)** PP2A Bγ transcript changes in hCNs (*N*=2; *n*=5; asterisks denote significant difference relative to aNPC level) echo human brain development. q-RT-PCR analysis of PP2A Bγ transcript in 5 w hCNs (right) showing no significant change after 24 h temperature shift (*N*=3; *n*=14). Transcript data was normalized to the geometric mean of 2 temperature-stable reference targets. **(b)** Western blot quantification of PP2A-C. Note lack of effect of hypothermia (*N*=3; *n*=6), prominent expression in normal human cortex (foetal and adult positive controls) and reduced expression in Alzheimer's disease affected cortex (negative control). **(c)** Hypothermic reduction of PP2A-C activity in hCNs (picomoles phosphate released min-1µg-1 protein; *N*=2; *n*=8). Enzyme activity is presented as mean fold change relative to normothermic control + SESE, after subtraction of background phosphate in the presence of fostriecin (see Methods section 2.11 and Fig.2.8). **(d)** Quantification by Western blot of total, activated (phosphorylated at Y216) and deactivated (phosphorylated at S9) GSK3β expression at 3 temperatures (*N*=3; *n*≥4). Total GSK3β expression was normalized to GAPDH, phosphorylated GSK3β was normalized to total GSK3β. GSK3β phosphorylates tau and is recognized as an apoptosis inducer. Note increase in the deactivated form at 28°C (*P*=0.018). Representative blots for each epitope are provided, with GAPDH as a loading control beneath.

## 5.3 Discussion

The hCN platform has been used to address tau biology in response to hypothermia, showing for the first time that human neurons in culture exhibit tau modifications in a manner similar to other systems (Planel et al., 2007a; Bretteville et al., 2012). The third aim of this thesis has thus been met. Cooling reversed key aspects of the tau developmental profile that had proceeded during cortical differentiation, namely tau isoform ratio and tau phosphorylation status. Importantly, this increased phosphorylation state was associated with impaired functional activity of PP2A in hCNs, without any apparent change in tau solubility. These results are consistent with a cooling-induced reversal of tau ontogenesis, without the oligomerization that is typically assigned to 'pathological tau' (Spillantini and Goedert, 2013). Furthermore, temperature shift altered the expression of the tau splicing regulator, RBM4, also known as LARK (Kar et al., 2006; Markus and Morris, 2006). Together these findings support the hypothesis that one aspect of hypothermic neuroprotection might involve recapitulation of early ontogenic tau plasticity (Arendt et al., 2003). Hypothesis (3) is thus accepted, with the caveat that full reversal from an 'adult-like' tau status cannot currently be demonstrated biochemically in hCNs, due to the absence of more mature tau isoforms.

At transcript, protein and post-translational levels, tau modifications during differentiation of hCNs at 3% $O_2$ recapitulated tau development of the human cortex *in vivo*. Whilst mass spectrometry identified a tau-specific peptide within hCN extracts, this peptide is common to all brain tau isoforms (Fig.5.7), thus the exact tau species could not be determined by this method. However, combined with

molecular, biochemical and imaging data it is highly probable that 3R0N was present from 2 w post-plating, with other isoforms including 4R0N appearing later. This 4R tau emerged by 4 w, coinciding with electron microscopic observations of synaptic ultrastructure (Fig.3.3a), but it was barely detectable by Western blot until 3 w later (Fig.5.6c). Together with the subcellular location of 3R tau (Fig.5.5a), these findings support an early developmental phenotype at the point of cooling (5 w). This is consistent with the developing electrophysiological properties of hCNs (Bilican et al., 2014; Livesey et al., 2014; James et al., 2014). Human corticogenesis proceeds over 70-100 days *in vivo* (Caviness et al., 1995), thus in the culture environment (bereft of full glial support, inhibitory neuronal input or maternally-derived hormonal factors), a mature tau profile is perhaps unrealistic within 7 w. An unresolved challenge is to derive a protocol that efficiently and reproducibly generates human neurons with a mature tau profile – the current hCN system is clearly limited with respect to protein-level analysis of tau isoforms. This is further complicated by sparse availability of reliable 4R tau-specific antibodies. One possible explanation for the discrepancy between the immunocytochemical and biochemical readouts for 4R tau protein in hCNs is that this target resided in a relatively insoluble fraction that was ineffectively extracted prior to SDS-PAGE. Despite these caveats, the data presented above confirms that hCNs can be used to study aspects of developing human tau physiology in a clinically-relevant context. Indeed, an immature profile may be particularly desirable for modelling TH – an intervention most widely used in practice to treat hypoxic ischaemic neonatal encephalopathy (Yenari and Han 2012; Jacobs et al., 2013).

The cooling-induced shift in the ratio of tau isoform transcripts at 32°C is of interest given that this temperature provided the most convincing protection against NR2B-mediated glutamate toxicity and reduced 4R tau protein expression. Stress granules accumulate under conditions where translation is limited, thus they would be expected to form in cooled hCNs (Ramaswami et al., 2013). Based on the preliminary observation that hypothermia altered RBM4 expression and subcellular location, it is tempting to speculate that hypothermic shifts in tau isoform ratio may be mediated indirectly by modification or sequestration of RBM4 or other tau splicing regulators such as fused in sarcoma (FUS), serine/arginine-rich splicing factor 2 (SRp30b), dual-specificity tyrosine(Y)-phosphorylation regulated kinase 1A (Dyrk1A) or neuro-oncological ventral antigen 1 (Nova 1) (Andreadis, 2005; Qian et al., 2011; Andreadis, 2012; Orozco et al., 2012). Indeed, a similar mechanism may operate in Down's syndrome whereby the trisomy-driven increase in Dyrk1A has a knock-on effect on the phosphorylation of alternative splicing factor (ASF), causing its sequestration into nuclear speckles where it cannot promote tau exon 10 inclusion (Shi et al., 2008). Interestingly, the ribonucleoprotein hnRNP K, which belongs to the Nova family of RBPs, is abundantly expressed in neurons and plays a role in axogenesis via post-transcriptional regulation of cytoskeletal elements including tau (Liu et al., 2011). Curiously, several other transcripts that contain hnRNP K binding sequences include PP2A, Fyn and amyloid precursor protein (APP) (Liu et al., 2011), all of which are known tau interactors. This implies a role for RBPs in tau modulation, something which might be exploited in the context of neuroprotection or neurodegeneration. Indeed, RNP aggregates form in many neurodegenerative diseases including C9ORF72-related ALS/FTD, Huntington's disease and some

sporadic AD cases (Ramaswami et al., 2013; Moschner et al., 2014). Several RNPs can also alter the pattern of tau isoforms and induce neurite sprouting (Moschner et al., 2014). Tau exon 10 splice site mutations that increase the inclusion of exon 10 (and thus 4R to 3R ratio) produce a predominantly NFT pathology (Hutton et al, 1998). In Down's syndrome foetal brain Dyrk1A expression is enhanced whereas RBM4 expression is reduced (Bernert et al., 2002; Shi et al., 2008) producing a relative increase in 3R tau. These patients suffer from early onset Alzheimer-like dementia (Wisniewski et al., 1985), supporting a role for RBPs and splicing regulation in tauopathy (Markus and Morris, 2009). Results in cooled hCNs indicate that tau isoform balance may be of real (and immediate) significance to the hypothermic response of neurons and their ability to withstand excitotoxic insults. If confirmed, this would argue for isoform-specific roles of tau in synaptic homeostasis and would reinforce the need for human modelling of neuronal injury, acknowledging the species differences in brain tau isoforms (Janke et al., 1999).

Tau mobility shift post-AP treatment (Fig.5.6b) demonstrated that hCN tau was more phosphorylated than tau extracted from normal adult human cortex and even 19 w human foetal cortex (Fig.5.13). This does not place hCN tau at a higher phosphorylation state than foetal tau *in vivo* – since post-mortem tau phosphorylation in these experiments was clearly affected by PMI. Even if PP2A expression in foetal brain and hCNs was equivalent, PMI would enable some dephosphorylation of soluble tau. The PMI effect was demonstrated directly, by comparing foetal cortical tau to tau extracted from primary cultures derived from these samples – where protein extraction in the presence of phosphatase inhibitors was performed

immediately at culture termination (Fig.5.13d). Conversely, Alzheimer tau remained highly phosphorylated despite PMI. This may reflect reduced PP2A activity in pathological samples (Vogelsberg-Ragaglia et al., 2001) or sequestration of phospho-tau into the insoluble fraction, which was not analysed. It must be remembered that a significant proportion of tau in Alzheimer's disease is both hyperphosphorylated and aggregated into NFTs (Grundke-Iqbal et al., 1986), potentially masking phosphoepitopes from enzymatic activity. At first glance, the AT8 signal in Alzheimer samples on Western blot (Fig.5.13b and 5.15a) might appear to contradict the literature. Note however that only soluble tau was assessed and AT8 is one of the earliest epitopes to become dephosphorylated during PMI, with PHF-1, AT100 and AT270 being affected with slower kinetics (Matsuo et al., 1994; Gartner et al., 1998). This explains the high signal for PHF-1 and AT270 relative to AT8 in pathological samples. The phosphorylation state of foetal cortical samples was less than expected in the context of previous work, however the study of Bramblett et al. (1993) included foetal samples with a PMI of 10-12 h, whereas the average PMI in the experiments above was 24 h. The detection of tau phosphorylated at the AT100 epitope (Fig.5.16b) may reflect the early development of filamentous tau or tau aggregates – interestingly this did appear to increase with cooling in both the soluble and 'insoluble' fractions on Western blot. However, in normal post-mortem rat brain the AT100 antibody picks up a nuclear-specific tau species that is not detectable on a Western blot of soluble protein (Gartner et al., 1998). The standard extraction protocol used for hCNs (0.1% SDS) applied a homogenization procedure that would be expected to pull out a small proportion of nuclear protein. Thus the blot in Fig.5.16b likely reflects minor 'leakage' of nuclear AT100-positive tau into the

cytosolic fraction, with the remainder sitting at high molecular weight in the 'insoluble' fraction. Overall, hypothermia pushed hCN tau towards an earlier post-translational foetal-like state. Tau thus became hyperphosphorylated with a significant reduction in PP2A activity at low temperatures. This implicates hypothermic modulation of PP2A as a key determinant of increased hCN tau phosphorylation, outweighing hypothermic inactivation of tau kinases (Fig.5.17d), as described in other systems (Bretteville et al., 2012).

Reduced 4R tau alongside increased tau phosphorylation might confer a more plastic cytoarchitecture – a likely prerequisite for repair or reorganisation, and a potential candidate for maintaining synaptic health. Indeed, a bias towards juvenile MAPs has been proposed to confer an increase in cytoskeletal plasticity that protects neurons from toxic Aβ (Rapoport et al., 2002). In the adult brain, granular cells of the dentate gyrus express only 3R0N tau transcript (Goedert et al., 1989) and this isoform is expressed transiently during adult neurogenesis (Bullman et al., 2007). This indicates an 'on-demand' reacquisition of early neuronal plasticity that may be reflected in cooled hCNs (Goode and Feinstein, 1994; Wang and Liu, 2008). Moreover, a negative feedback loop has been described involving physiological tau phosphorylation in response to NMDAR activation and subsequent prevention of 'overexcitation' (Mondragon-Rodriguez, 2012). Protein phosphorylation is an established mechanism for regulating NMDAR function, which in turn plays a leading role in the induction of synaptic plasticity via LTP and LTD (Chan and Sucher, 2001). The balance between kinase and phosphatase activity is homeostatically modulated by NMDAR activity because stimulation deactivates PP2A leading to increased phosphorylation of the receptor and LTP (Bliss and

Collingridge, 1993; Mulkey et al., 1993; Soderling and Derkach, 2000; Chan and Sucher, 2001). Since cooling has a similar effect on kinase-phosphatase balance it may promote LTP and thus safeguard synaptic integrity. As discussed in the introduction, the hypothermia-induced increase in hCN tau phosphorylation may have contributed to the protection of cooled neurons against oxidative stress observed in Chapter 3 (Castellani et al., 2008). 4R tau reduction might also be essential when phosphorylation state is high, minimising available sites for excessive phosphorylation and subsequent aggregation. A mismatch between reduced PP2A activity and tau isoform shift, as might occur in hypometabolic states under euthermia (or in 'imperfect' hibernators) (Vogelsberg-Ragaglia et al., 2001; Tøien et al., 2011), could thus be severely cytotoxic. Finally, the compartmental distribution of tau within the neuron may affect its exposure to different populations of kinases and phosphatases (Julien et al., 2012). The order in which tau epitopes become phosphorylated or dephosphorylated might thus differ considerably in dendrites versus axons and this could also be affected by the extent of polarity in developing hCN cultures.

In conclusion, the results presented in this chapter establish that hCNs provide a suitable platform with which to study human tau development and physiology, highlighting aspects of hypothermic tau modulation that can only be addressed in a human system.

# Chapter 6: Hypothermic neuronal preconditioning

## 6.1 Introduction

Preconditioning occurs when a transient, subtoxic cellular stress confers resistance to what would otherwise be a lethal injury (Bell et al., 2011; Stetler et al., 2014). This phenomenon has been described in response to various stressors in many species and in multiple organs including the human brain (Dirnagl et al., 2003). Studies in rodents indicate that cold stress can also produce this state of neuronal adaptation, suggesting that 'hypothermic preconditioning' may be the key to hypothermic neuroprotection (Yuan et al., 2004). Hypothermia can thus precondition neurons to withstand toxic insults (Yuan et al., 2004; Stetler et al., 2014). This adaptive priming requires *de novo* protein synthesis and may derive from the cellular stress response to cooling (Nishio et al., 2000), for which data in human neurons is lacking. Conversely, there is evidence that inadequate stress responses (and thus impaired preconditioning) may underlie several neurodegenerative disorders (Bruening et al., 1999; Sonna et al., 2002; Hetz and Mollereau., 2014; Texel and Mattson, 2011). Clearly, understanding the molecular mechanisms by which cold-inducible phenomena deliver preconditioning will widen their therapeutic potential. In the following experiments, the hCN model of TH was used to further explore the molecular consequences of cooling and its putative neuroprotective mechanism in relation to preconditioning.

## 6.2 Results

### 6.2.1 Endoplasmic reticulum stress and the UPR

Preliminary transcript analysis in cooled hCNs highlighted upregulation of the immediate early marker JUN (Fig.4.1a). Given that cold-shock can induce protein unfolding (Fujita, 1999) and activate PERK and JUN (both of which are components of the UPR), it was considered that cooling might trigger ER stress - the principal driver of UPR activity (Walter and Ron, 2011). This was tested using tunicamycin (Tm) as a positive control for ER stress (Tm inhibits N-linked glycosylation in the ER) (Rutkowski et al., 2006; Lin et al., 2007b). Further transcript analysis ($N$=3; $n$=22) showed a temperature-dependent induction of ER stress marker BiP; 32°C $P$=0.006; 28°C $P$<0.0005; Fig.6.1a), and the magnitude of this induction was much less than that produced by 24 h treatment with Tm ($N$=3; $n$=8; $P$=0.004, Fig.6.1a). At the protein level, BiP expression showed an increasing trend with cooling ($N$=2; $n$=3; 28°C $P$=0.051; Fig.6.1b). Transcription of another key ER chaperone, 94 kDa glucose-regulated protein (GRP94) responded robustly to Tm but not cooling ($N$=3; $n$=7; 32°C $P$=0.432; 28°C $P$=0.473; Tm $P$<0.0005; Fig.6.1c) (Walter and Ron, 2011).

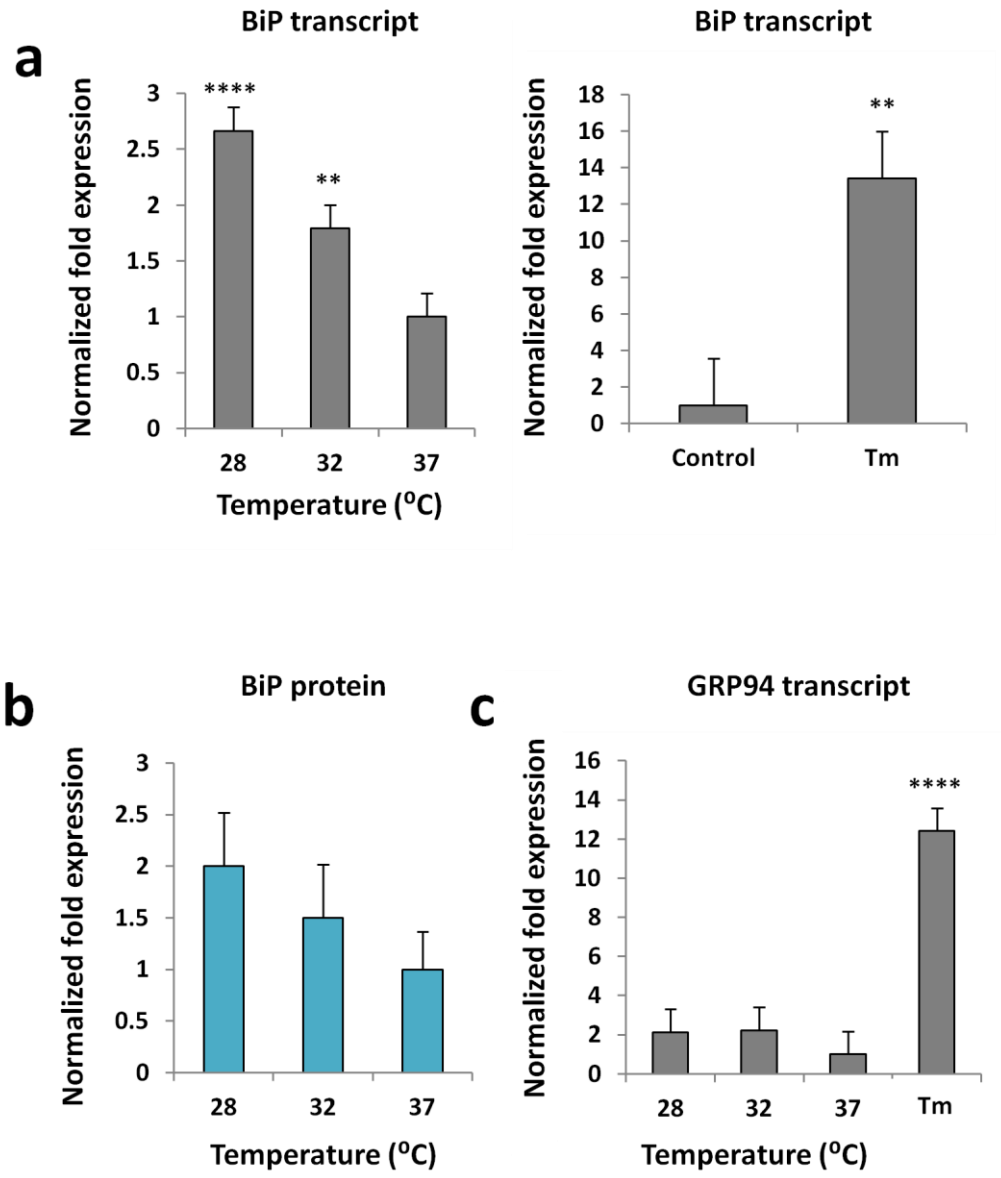


**Figure 6.1 Mild ER stress in hypothermic hCNs.** **(a)** BiP transcripts after 24 h cooling (left; *N*=3; *n*=22) or Tm treatment (right; *N*=3; *n*=8). **(b)** Total BiP protein expression after 24 h temperature shift (*N*=2; *n*=3), normalized to loading control and then normothermic control. A representative blot image for BiP is provided in Fig.6.4 **(c)** GRP94 transcripts after 24 h temperature shift or Tm treatment (*N*=3; *n*=7).

ER stress simultaneously triggers the 3 main branches of the UPR – the evolutionarily oldest branch promotes phosphorylation and activation of Ire1α (Lin et al., 2007b; Tabas, 2011). Activated Ire1α splices an intron from the mRNA of XBP1 and spliced XBP1 (XBP1s) activates transcription of several ER-stress target genes,

whilst the unspliced form represses them (Yoshida et al., 2001; Tabas, 2011; Hetz and Mollereau, 2014). After 24 h incubation, there was a significant upregulation of Ire1α transcript in hCNs at 28 and 32°C relative to 37°C ($N$=3; $n$=14; $P$<0.01; Fig.6.2a). There was also a significant increase in stress-responsive transcripts MYC ($N$=2; $n$=5; $P$<0.01) and unspliced XPB1 (XBP1u, $N$=2; $n$=7; $P$<0.01) as determined by conventional RT-PCR (Fig.6.2b). According to q-RT-PCR and gel analysis there was a mild but measurable increase in XBP1s transcript after cooling ($N$=3; $n$=22; 28°C $P$=0.003; Fig.6.3a-b), again to a lesser extent than that elicited by Tm ($N$=3; $n$=8; $P$<0.0005; Fig.6.3a-b). A second branch of the UPR involving cleavage of activating transcription factor 6 (ATF6) was also induced at 28°C (Hetz and Mollereau, 2014) (Fig.6.4). Within the third pathway, total PERK expression decreased slightly after 24 h cooling ($N$=3; $n$=5; $P$<0.05) and eIF2α was inactive at this time point according to biochemical analysis of its phosphorylated form (Fig.6.5a) (Rutkowski et al., 2006). There was however a significant increase in their downstream targets, activating transcription factor 4 (ATF4), DNA damage-inducible transcript 3 (DDIT3, or CHOP) and GADD34 (also known as protein phosphatase 1 regulatory subunit 15A) at hypothermic temperatures (Fig.6.5b-c and 6.6). The CHOP response was considerably less than that produced by Tm, whereas the ATF4 and GADD34 responses at 28°C were similar to Tm ($N$=3; $n$=7; ATF4; 32°C $P$=0.215; 28°C $P$<0.0005; Tm $P$<0.0005; GADD34; 32°C $P$<0.0005; 28°C $P$<0.0005; Tm $P$<0.0005; Fig.6.5b-c).

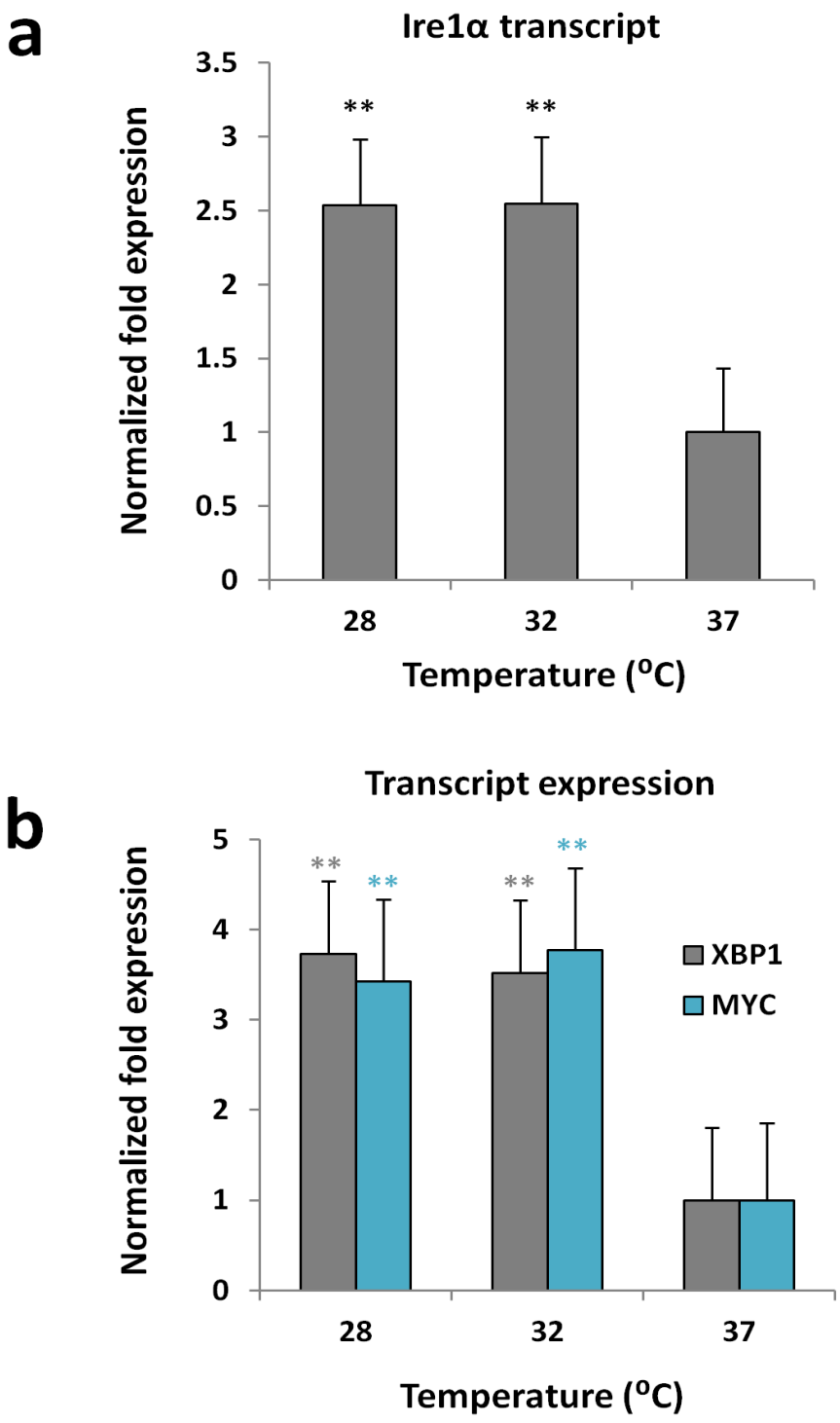


**Figure 6.2 UPR activation in hypothermic hCNs. (a)** Induction of IRE1α transcript analysed by q-RT-PCR (*N*=3; *n*=14). **(b)** Conventional RT-PCR analysis (gel band intensity) of transcripts (normalized to GAPDH then normothermic control), with significant increases for MYC (*N*=2; *n*=5) and unspliced XBP1 (*N*=2; *n*=7).

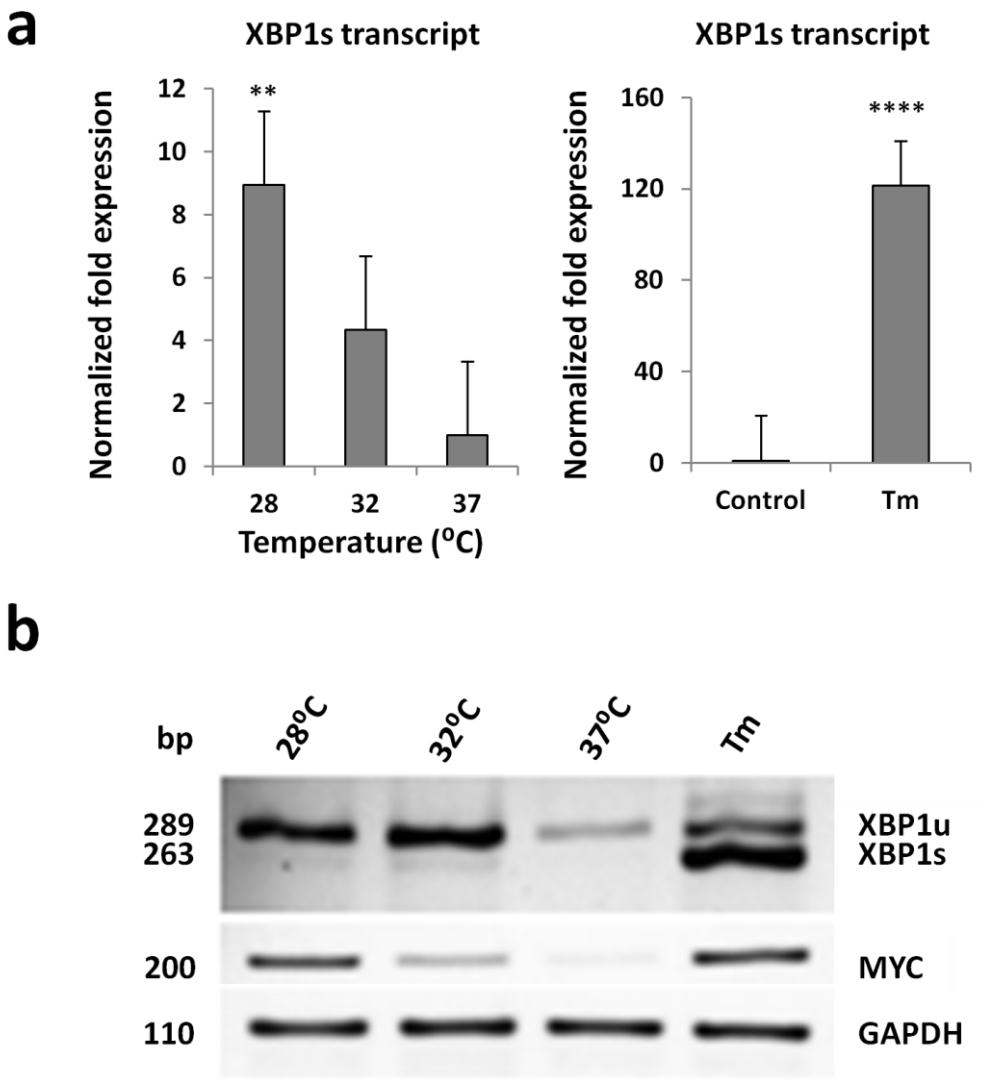


**Figure 6.3 Mild XBP1 splicing in hypothermic hCNs. (a)** q-RT-PCR analysis of XBP1s transcript after cooling (left; *N*=3; *n*=22) or Tm-treatment (right; *N*=3; *n*=8). **(b)** Gel images of RT-PCR products. Faint bands at 263 bp confirm mild splicing of XBP1 in hypothermic hCNs relative to negative (37°C) and positive (Tm-treated) controls. GAPDH = reference target**.**

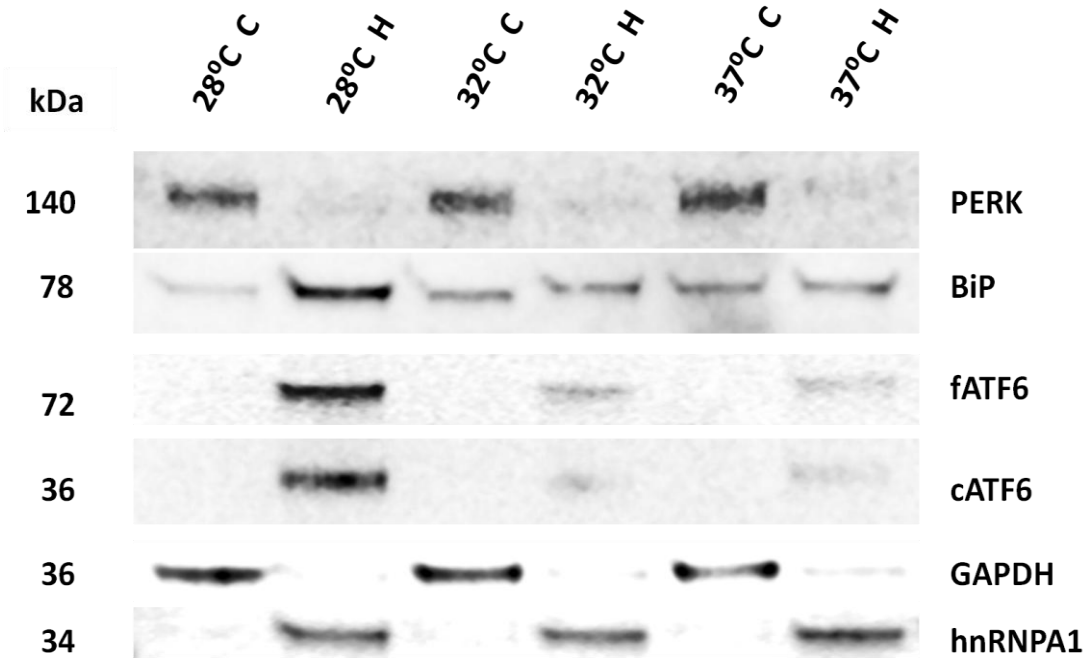


**Figure 6.4 UPR protein products in hypothermic hCNs.** Immunoblots of fractionated lysates (C=cytoplasmic, H=high-detergent) from hCNs after 24 h temperature shift. Note increased BiP, full length (fATF6), and cleaved (cATF6) sitting in the high detergent fraction at 28°C. This is consistent with nuclear translocation of cATF6 and upregulation of its target transcripts (BiP and unspliced XBP1). Note also mild reduction in total PERK expression with cooling (quantified in Fig.6.5).

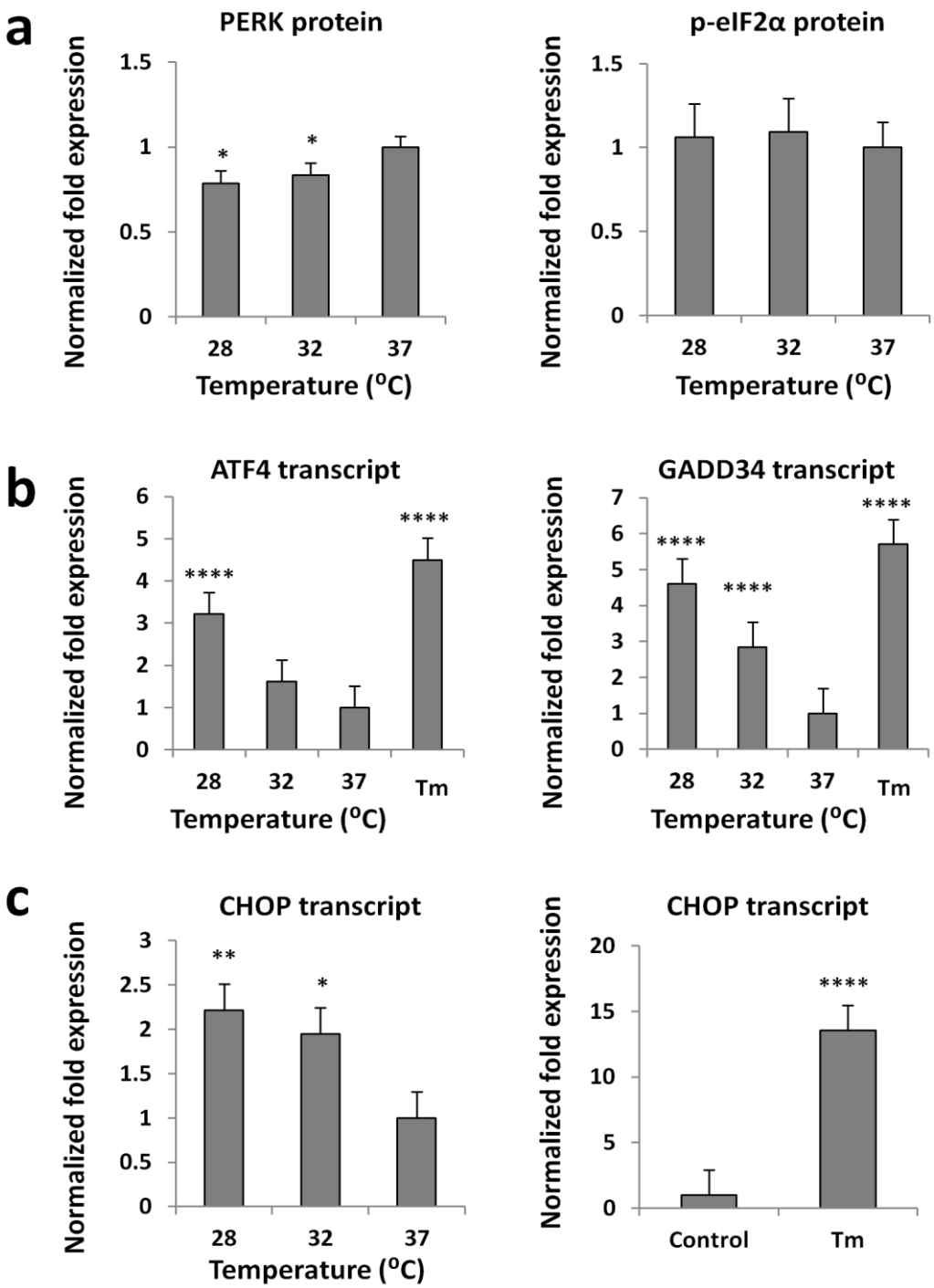


**Figure 6.5 PERK branch activation in hypothermic hCNs.** **(a)** Quantitative Western analysis of total PERK expression (left) and phospho-eIF2α (right) after 24 h of temperature shift (*N*=3; *n*=5). Corresponding PERK immuoblot is shown in Fig.6.2. **(b)** qRT-PCR analysis (*N*=3; *n*=7;) of ATF4 transcripts (left) and GADD34 transcripts (right) after 24 h cooling or Tm treatment. **(c)** qRT-PCR analysis of CHOP transcripts after 24 h cooling (left; *N*=3; *n*=22;; 32°C *P*=0.011; 28°C *P*=0.001) or Tm treatment (right; *N*=3; *n*=8; *P*<0.0005).

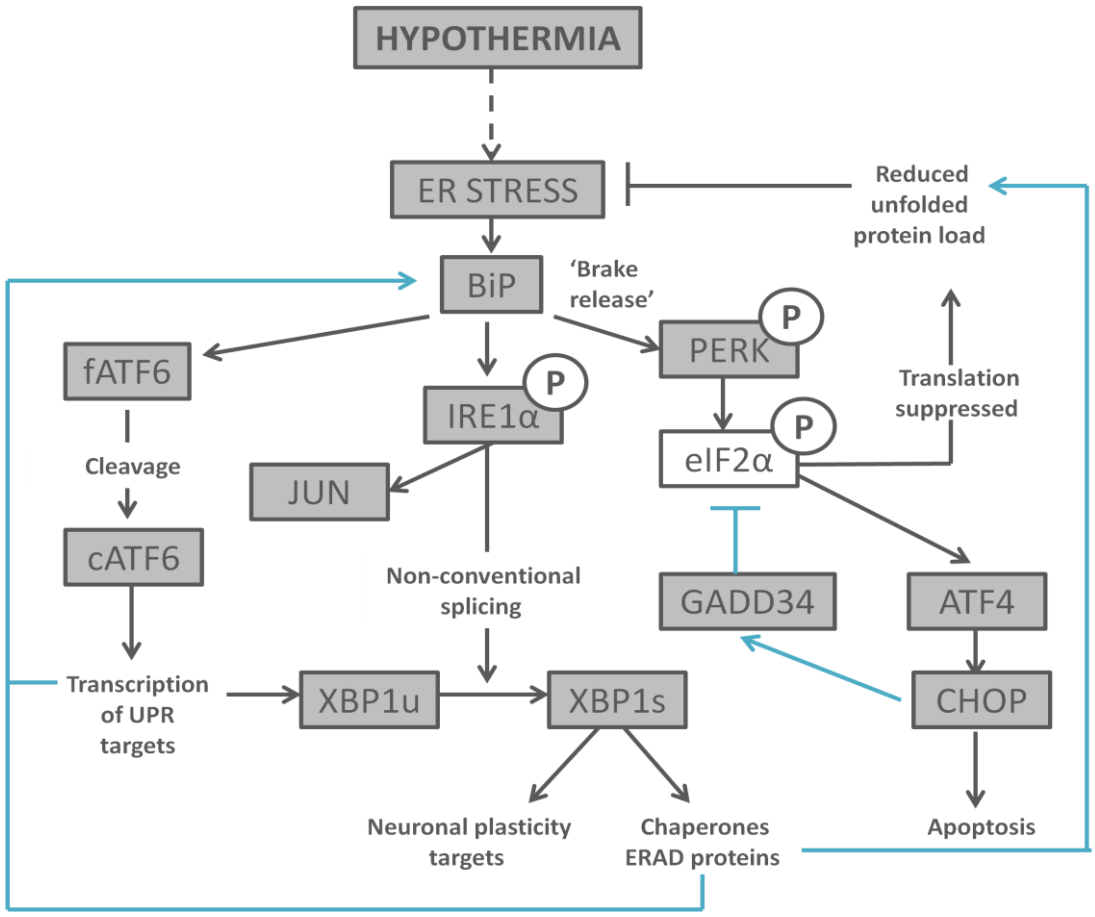


**Figure 6.6 Hypothermic hCNs upregulate all UPR pathways.** The principal branches of the UPR are depicted as in Fig.1.11, but with known regulatory feedback pathways added. Grey-filled boxes denote components induced at transcript and/or protein level in hCNs after 24 h cooling. Phospho-PERK and phospho-IRE1α were not assessed. Dotted black arrow denotes unknown mechanism by which cooling elicits ER stress in hCNs although protein unfolding has been described in response to deep cooling (4°C, King and Weber, 1986). Orange connectors indicate elements of hormesis that resolve the UPR and increase ER resilience to stress by upregulating chaperones and ERAD proteins.

### 6.2.2 The UPR and ER-hormesis

Mild ER stress leading to UPR activation inhibits apoptosis (Fouillet et al., 2012) and may pre-condition neurons to resist more stressful insults – an effect termed ER-hormesis (Mendes et al., 2009; Fouillet et al., 2012). To determine whether ER preconditioning could also account for hypothermic neuroprotection of hCNs they were treated with compounds that modify the ER-UPR cascade, during the pre-

incubation phase of cooling. The PERK inhibitor GSK2606414 was used to block the third branch of the UPR, whilst Tm was used to induce moderate ER stress. Initially, multiplexed injury analyses were run using dose response curves in normothermic hCNs to select concentrations for each compound that were non-toxic at baseline (without $H_2O_2$ exposure; GSK2606414 *N*=3; *n*=5; Fig.6.7a; Tm *N*=3; *n*=8; Fig.6.8a). Multiplexed injury analysis was subsequently applied to hCNs that had been exposed to oxidative stress (increasing concentrations of $H_2O_2$), with or without pre-incubation for 24 h at 28°C. Again, moderate hypothermia was protective of hCNs (Fig.6.7b and 6.8b). However, PERK inhibition increased hCN injury at both 37°C and 28°C (*P*=0.016 and *P*<0.0005 respectively, *N*=3; *n*=3; Fig.6.7b) and abrogated the protective effect of cooling at all but the highest concentration of $H_2O_2$ (28°C remained protective only at 200 μM $H_2O_2$, *P*=0.023). Tm also exacerbated oxidative stress-mediated injury under normothermic conditions (*N*=3; *n*=7; *P*<0.0005), but this augmentative effect was attenuated by pre-conditioning at 28°C (*P*=0.062, Fig.6.8b), thus directly demonstrating hypothermic induction of ER-hormesis.

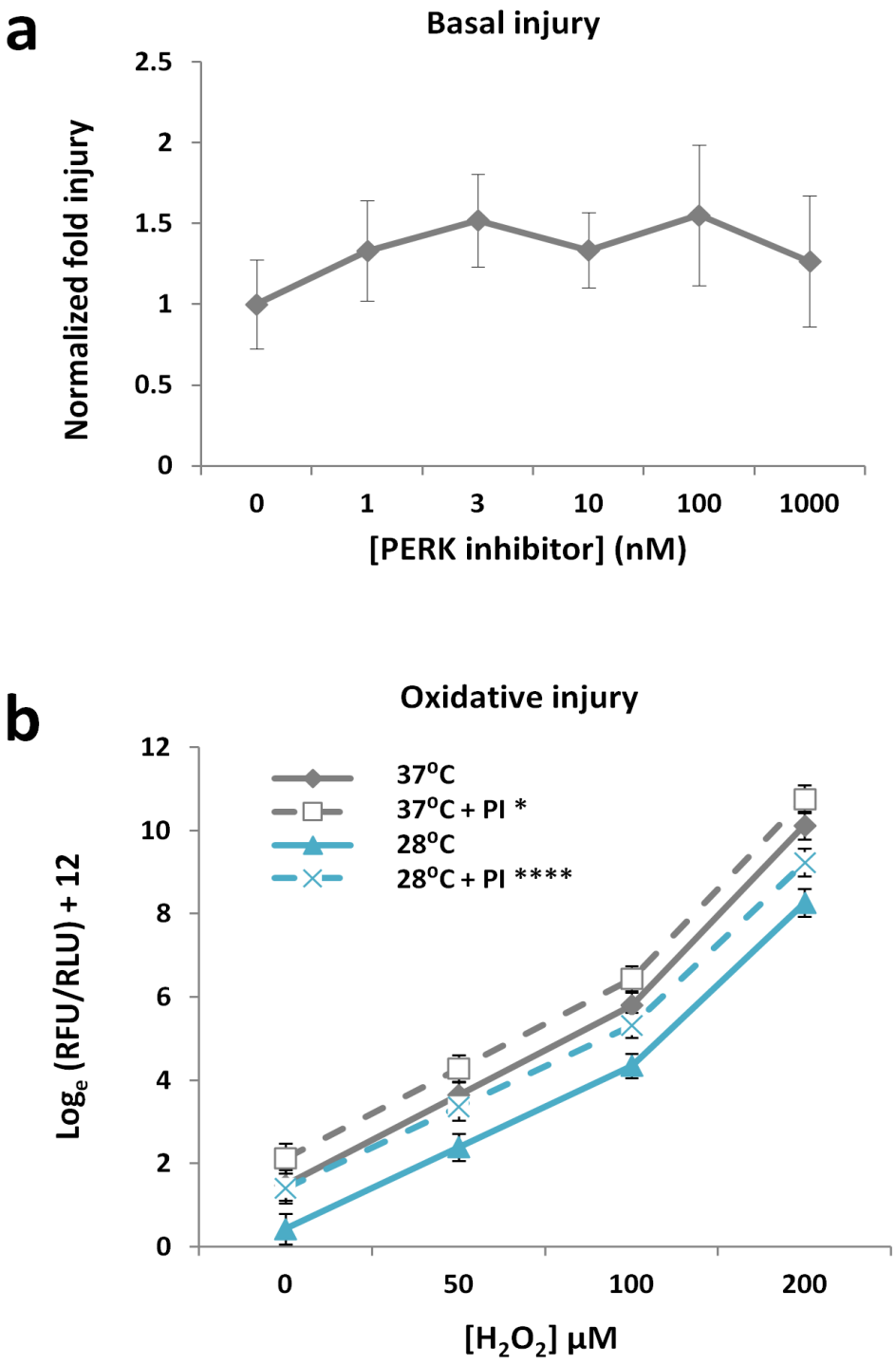


**Figure 6.7 The UPR is required for hypothermic preconditioning in hCNs. (a)** Independent batches of 5 w hCNs were treated with the various concentrations of PERK inhibitor (GSK 2606414) indicated for 24 h in MiM under normothermic conditions. Multiplexed injury analysis was then performed. Injury ratio (RFU/RLU) data was normalized to untreated control and is presented as mean ± SEM. No significant change in injury was noted at any of these concentrations of PERK inhibitor (*N*=3; *n*=5). **(b)** Oxidative stress-mediated injury in the presence of PERK inhibitor (PI; *N*=3; *n*=3)). Note log scale of y-axis.

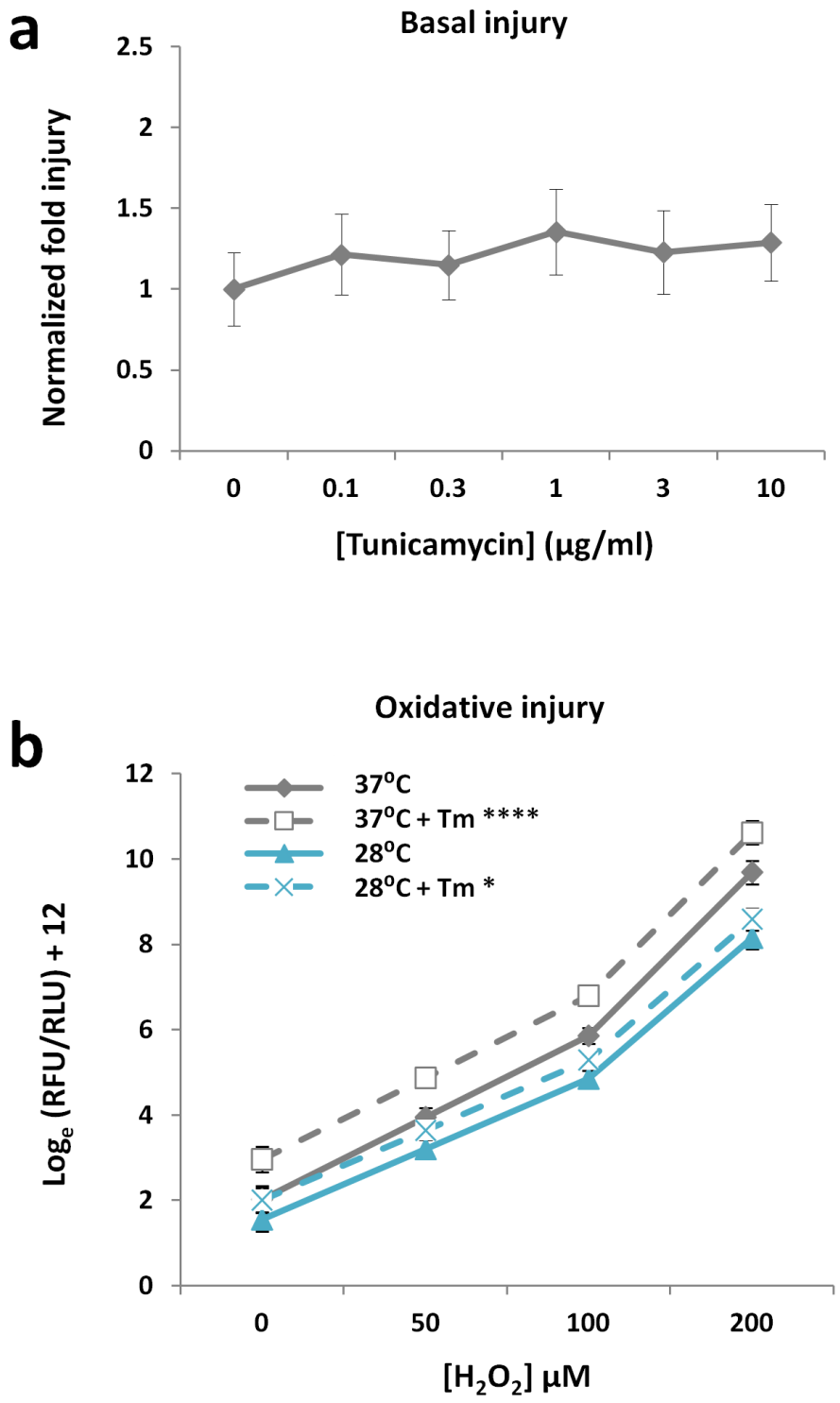


**Figure 6.8 ER preconditioning in hypothermic hCNs.** **(a)** Independent batches of 5 w hCNs were treated with the various concentrations of Tm indicated for 24 h in MiM under normothermic conditions. Multiplexed injury analysis was then performed. Injury ratio (RFU/RLU) data was normalized to untreated control and is presented as mean ± SEM. No significant change in injury was noted at any of these concentrations of Tm (*N*=3; *n*=8). **(b)** $H_2O_2$ injury in the presence of Tm (*N*=3; *n*=7). Note log scale of y-axis.

Quite how cooling precipitates ER stress in hCNs is currently unknown, but likely involves several factors (Rzechorzek et al., 2015). One candidate considered here is hyperphosphorylated tau which is a prominent feature of hypothermic brains and may offer short-term protection by promoting UPR-mediated apoptotic escape (Hetz and Mollereau, 2014; Liu et al., 2012c; Stieler et al., 2011; Wang et al., 2014). Since hypothermia robustly increased tau phosphorylation in hCNs, it was postulated that this might contribute to its neuroprotective effect. The first step was to determine whether neuroprotection could be achieved by inhibiting PP2A. Fostriecin (a membrane permeable compound (Douglas et al., 2001) was applied to normothermic hCNs for 24 h, after which cells were immunostained, harvested for protein extraction or subjected to oxidative stress. Fostriecin treatment produced a visible increase in axonal AT8 immunoreactivity (Fig.6.9) and a concentration-dependent increase in phospho-tau signal on Western blot (Fig.6.10). At a concentration which effectively increased tau phosphorylation, but did not cause any neurotoxicity at baseline ($N$=3; $n$=8; Fig.6.10 and 6.11a), fostriecin produced a reduction in hCN injury in response to $H_2O_2$ ($N$=3; $n$=6; $P$=0.008; Fig.6.11b). Culture at 28°C predictably reduced oxidative stress-mediated injury at each concentration of $H_2O_2$ ($P$<0.0005), however the addition of fostriecin conferred no further benefit ($P$=0.907; Fig.6.11b). Conversely, treatment with the brain-permeable kinase inhibitor TCS 2002 (highly specific for GSK3β (Saitoh et al., 2009)) produced a concentration-dependent decrease in phospho-tau signal as well as a prominent shift in electrophoretic mobility on Western blot (Fig.6.10). Addition of an effective dose of TCS 2002 (causing hCN tau dephosphorylation but no toxicity at baseline, $N$=3; $n$=9; Fig.6.10 and 6.12a) increased hCN injury in response to $H_2O_2$ under both

normothermic and hypothermic conditions (*P*=0.002 and *P*=0.001 respectively, *N*=3; *n*=4; Fig.6.12b). TCS 2002 abrogated the protective effect of moderate hypothermia at 50 μM $H_2O_2$; there was no significant difference between injury in untreated normothermic cultures and hypothermic cultures treated with TCS 2002 (*P*=0.348). Finally, immunostaining of hCNs for activated caspase-3 and AT8 showed that these markers were mutually exclusive, suggesting that phospho-tau positive neurons were indeed resistant to apoptosis (Li et al., 2007) (Fig.6.13a).

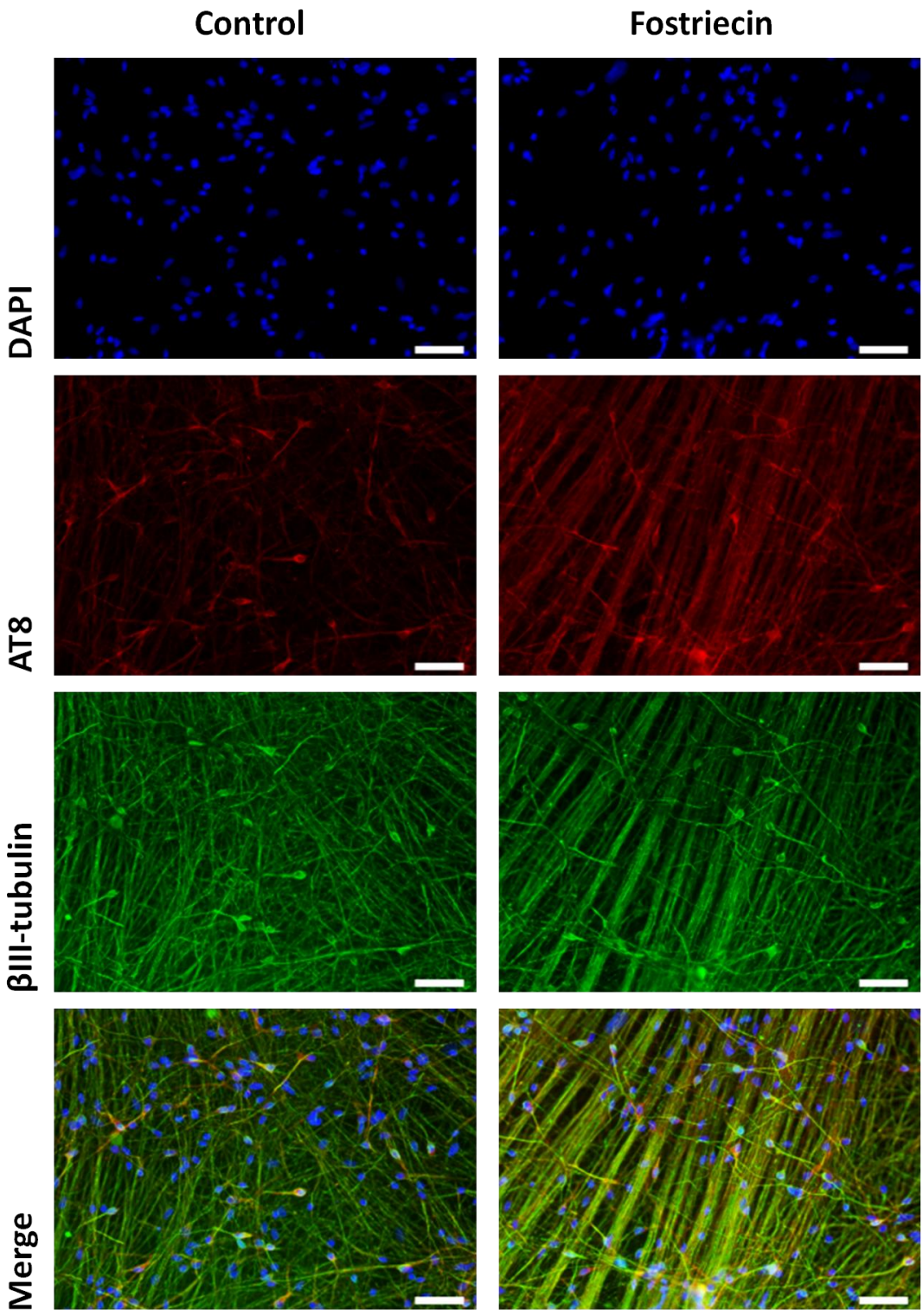


**Figure 6.9 Fostriecin mimics cooling-induced tau phosphorylation in hCNs.** Inhibiting PP2A in normothermic hCNs increases tau phosphorylation as shown by enhanced AT8 immunostaining in the presence of 100 nM fostriecin, scale bar = 50 µm.

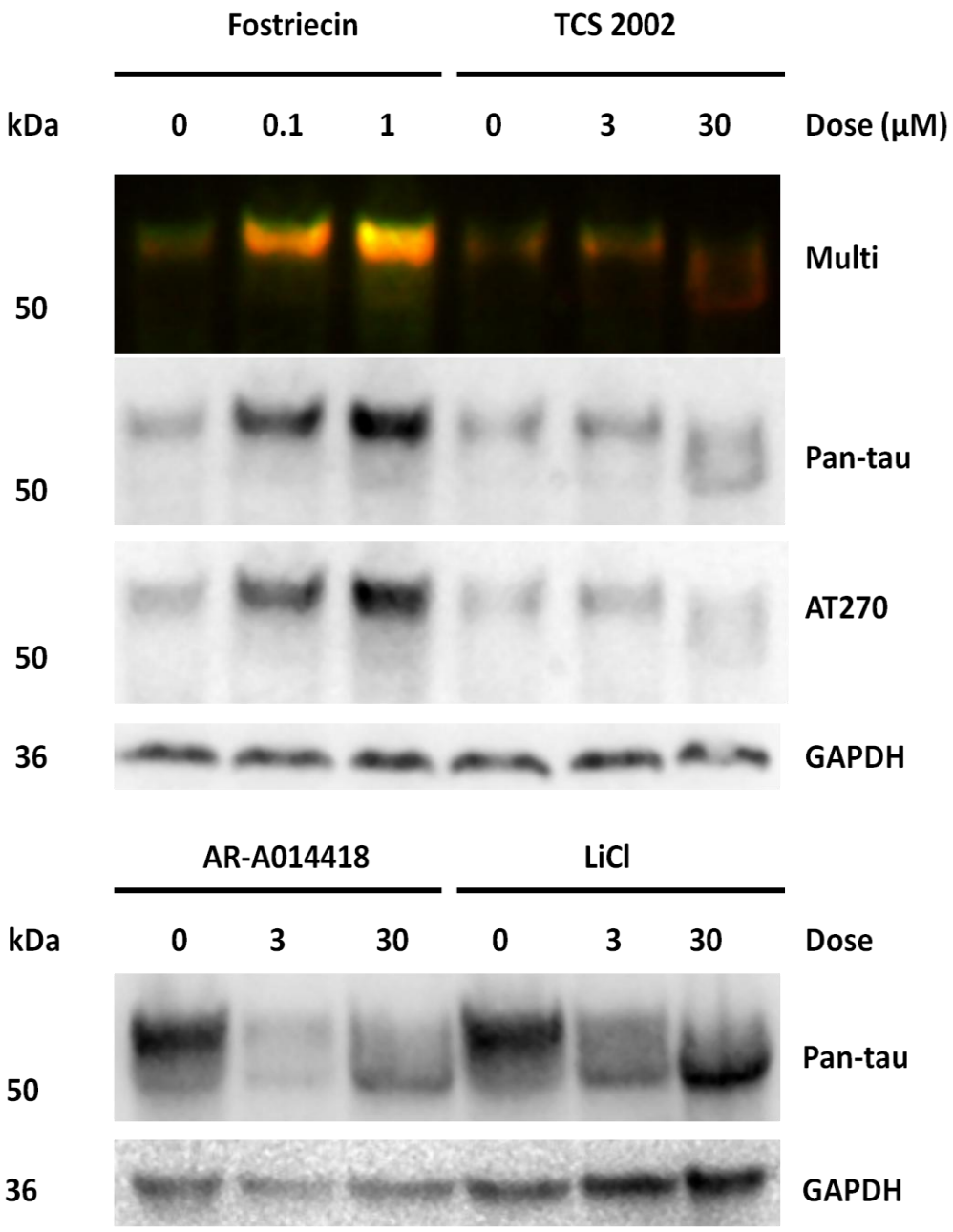


**Figure 6.10 Reciprocal manipulation of tau phosphorylation in hCNs.** Upper panel: fostriecin produces a concentration-dependent increase in AT270 and pan-tau signals on Western blot with decreased electrophoretic mobility. Tau kinase (GSK3β) inhibitor TCS 2002 decreases tau phosphorylation in a concentration-dependent manner and at high concentrations resolves the protein into its dephosphorylated isoforms of lower molecular weight. Lower panel: commonly used GSK3 inhibitors have an effect on normothermic tau, comparable to that of TCS 2002 (Bretteville et al., 2012). AR-A014418 concentrations are in μM, those for LiCl are in mM. AR-A014418 inhibits tau phosphorylation at a GSK3-specific site (S396) in an ATP-competitive manner (Bhat et al., 2003). LiCl is a less specific but potent inhibitor of GSK3 (Zhang et al., 2003). The pan-tau antibody recognises phosphorylated and non-phosphorylated tau, GAPDH =loading control.

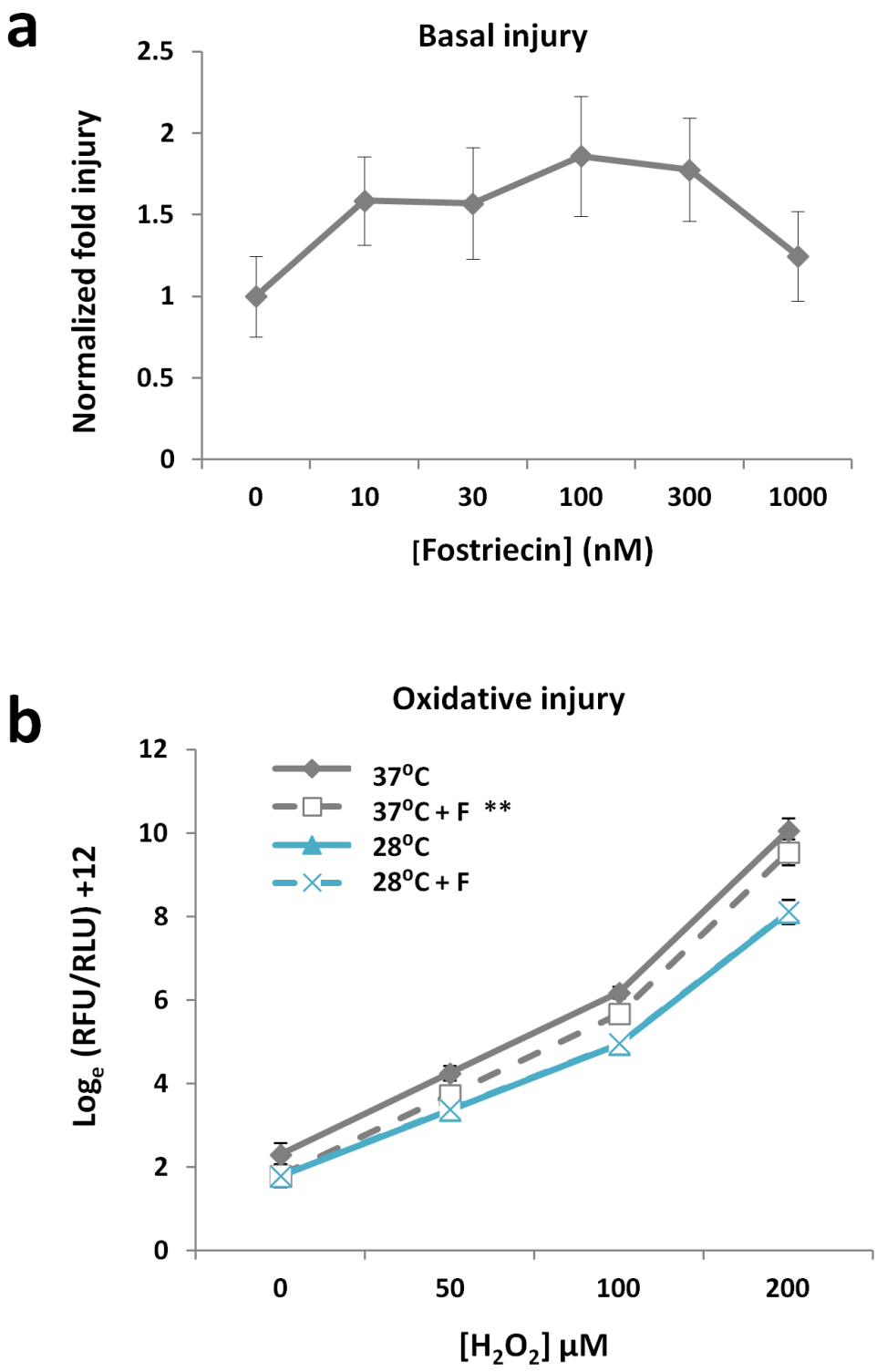


**Figure 6.11 Increasing tau phosphorylation protects normothermic hCNs from oxidative stress. (a)** Independent batches of 5 w hCNs were treated with the concentration range of fostriecin indicated for 24 h in MiM under normothermic conditions. Multiplexed injury analysis was then performed. Injury ratio (RFU/RLU) data was normalized to untreated control and is presented as mean ± SEM. No significant change in injury was noted at any of these concentrations of fostriecin (*N*=3; *n*=8). **(b)** Fostriecin reduces $H_2O_2$-mediated injury but has no effect on injury when hCNs are incubated at 28°C (*N*=3; *n*=6). Note log scale of y-axis.

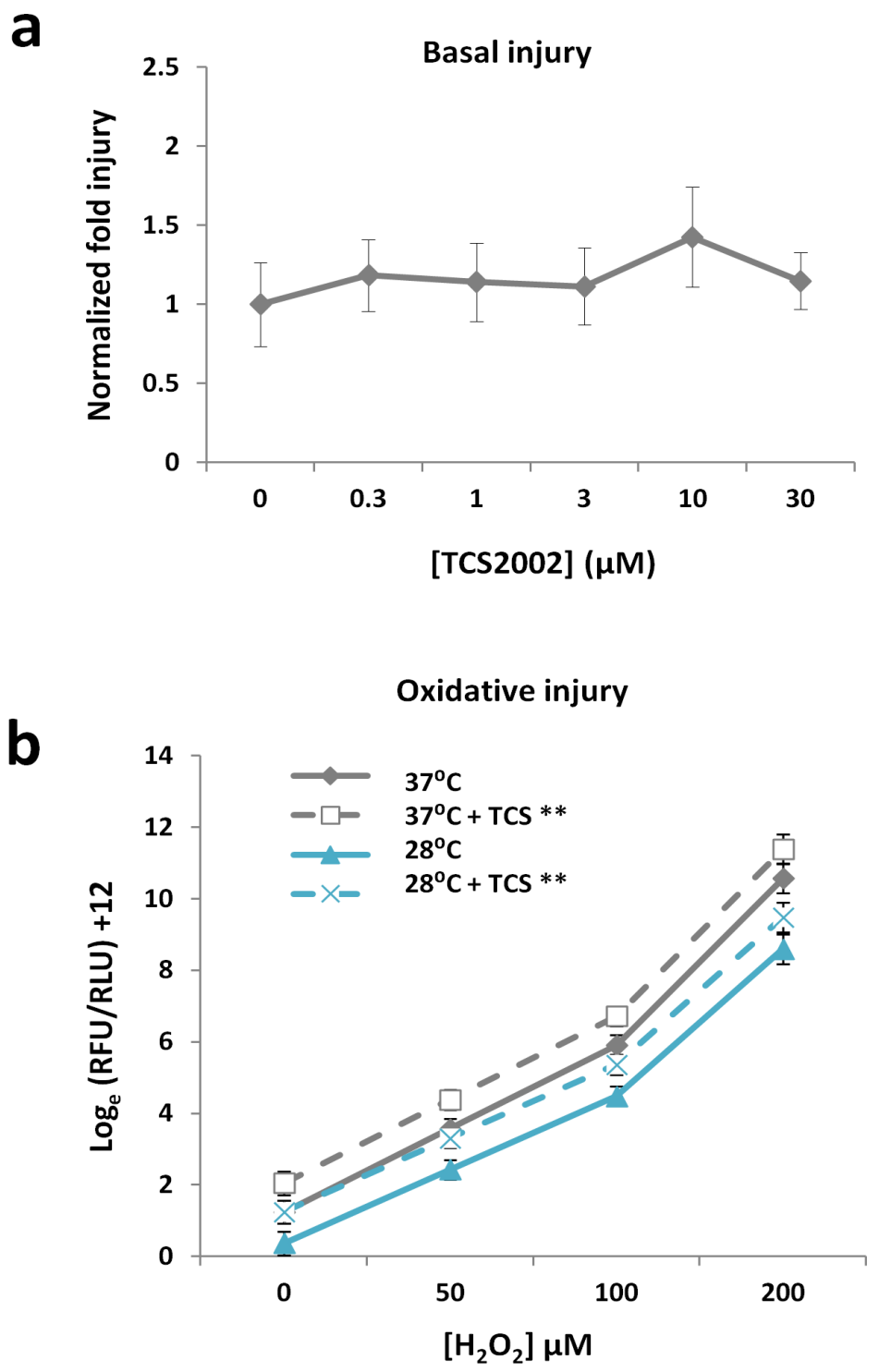


**Figure 6.12 Inhibiting tau phosphorylation abrogates hypothermic preconditioning in hCNs. (a)** Independent batches of 5 w hCNs were treated with the concentration range of TCS 2002 indicated for 24 h in MiM under normothermic conditions. Multiplexed injury analysis was then performed. Injury ratio (RFU/RLU) data was normalized to untreated control and is presented as mean ± SEM. No significant change in injury was noted at any of these concentrations of TCS 2002 (*N*=3; *n*=9). **(b)** TCS 2002 increases $H_2O_2$-mediated injury at 37°C and 28°C (*N*=3; *n*=4). Note log scale of y-axis.

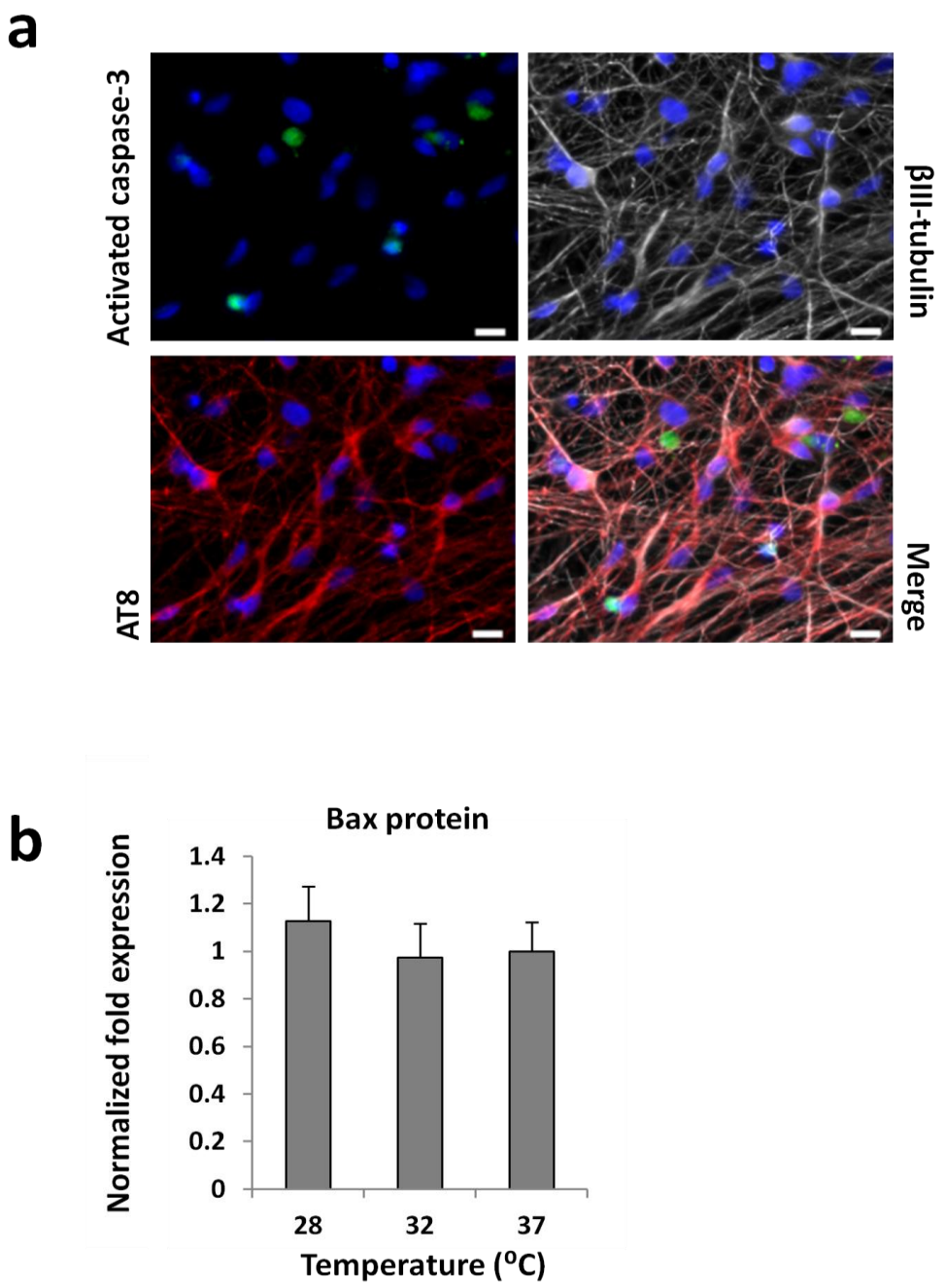


**Figure 6.13 Apoptotic escape of hypothermic hCNs.** **(a)** Widefield fluorescent micrographs of hCNs cultured at 28°C showing mutually exclusive staining of apoptotic marker activated caspase-3 (green, top left) and phospho-tau (AT8, red, bottom left). Cells were co-stained for DAPI (blue) and neuronal marker βIII-tubulin (white, top right). Merged image is shown (bottom right), scale bar = 10 µm. **(b)** Quantitative Western analysis of apoptotic regulator Bax. There was no significant change in Bax protein expression in response to cooling (*N*=3; *n*=6).

## Discussion

These experiments establish several molecular effects of TH in hCNs, modifying proteostatic pathways in a manner that supports neuronal viability. Specifically, cooling triggers mild ER stress in hCNs, sufficient to activate all branches of the UPR. Full hypothermic protection of hCNs against oxidative stress requires PP2A inhibition and an intact UPR in order to prime the ER against intensively toxic insults. In combination with imaging data, these results suggest that both phospho-tau and UPR-mediated ER-hormesis play a role in hypothermic preconditioning. The final aim of this thesis has thus been met, with acceptance of hypothesis (4).

Surmounting the chronic, UPR-mediated translational repression that accompanies protein misfolding might prove useful in neurodegenerative disease (Moreno et al., 2013). However, for acute injury, mildly enhancing the UPR can rescue neurons from programmed cell death and instigate adaptive preconditioning that defends the ER from further insults (Hetz and Mollereau, 2014). PERK inhibition worsened hCN injury in response to $H_2O_2$ and abrogated the protective effect of cooling. A functional UPR is thus essential for hypothermic preconditioning of hCNs against an oxidative challenge. Enhanced injury with PERK inhibition at 37°C may reflect a constitutive proteostatic function of the UPR in long-term culture – potentially through buffering oxidative processes (Cullinan et al., 2003). It might also explain why hypothermic induction of some PERK branch-specific components was not observed, though these may be undetectable biochemically in the context of mild ER stress (Rutkowski et al., 2006). These components can also act independently of each other (Krishnamoorthy et al., 2014). Equally, since PERK branch activity is

subject to homeostatic autoregulation by phosphatases (Lin et al., 2007b), hypothermic induction of GADD34 potentially resolved eIF2α activation by 24 h (Fig.6.5b and Fig.6.6) (Ma and Hendershot, 2003). eIF2α phosphorylation does occur under deep hypothermic conditions (10°C) and contributes to the global suppression of protein translation in mammalian cell lines (Roobol et al., 2009; Hofman et al., 2012). This inhibits translation initiation and stalled pre-initiation complexes accumulate in SGs, along with several RBPs (Kedersha et al., 2002; Hofman et al., 2012). The fact that SGs were observed in cooled hCNs (in association with RBM4 and TIA-1) supports the notion that mild hypothermia is sufficient to activate eIF2α in human neurons.

During an adaptive stress response the three UPR branches undergo considerable cross-talk and complex homeostatic self-regulation (Fig.6.6). The 24 h 'snapshot' taken here cannot convey the dynamism of this response in hCNs but it intimately links UPR activation to neuronal preconditioning, since this was the point at which exogenous stressors were applied (Rzechorzek et al., 2015). Whilst XBP1 splicing was mild at 28°C compared to Tm treatment, Ire1α activity might differ kinetically in response to Tm and hypothermia, thus comparing these treatments at 24 h may have under- or overestimated the effect of cooling on this pathway (Lin et al., 2007b). Irrespectively, the increases in both unspliced XBP1 and XBP1s mRNA (Fig.6.2b and 6.3) strongly suggest that Ire1α and ATF6 were active within the cooling period (Hetz and Mollereau, 2014). BiP and GRP94 are key chaperones that respond to ER stress and regulate protein folding and BiP is the most commonly used marker for UPR activation (Rutkowski et al., 2006; Walter and Ron, 2011). Enhancing BiP is

neuroprotective and has been linked to hypothermia (Aoki et al., 2001; Terao et al., 2009; Liu et al., 2013), whilst BiP deficiency has been implicated in neurodegeneration (Endres and Reinhart, 2013; Hetz and Mollereau, 2014). GRP94 suppresses oxidative stress-mediated neuronal death and stabilizes calcium homeostasis in the ER (Bando et al., 2003). Moreover, the increase in BiP transcript after 24 h is consistent with activation of ATF6 and splicing of XBP1 prior to the oxidative injury assay (Walter and Ron, 2011; Hetz and Mollereau, 2014) (Fig.6.1; a subset of batches ($N$=3; $n$=14) demonstrated no BiP induction after 3 h of hypothermia). The relative magnitude of the XBP1, XBP1s and BiP findings in combination indicates an adaptive UPR profile. The protective roles of Hsps in hypothermic preconditioning are currently unknown (Shintani and Terao, 2012), but enhancing these chaperones improves outcome in models of ischaemic injury (Marber et al., 1995; Zheng et al., 2008) and ALS (Kieran et al., 2004), potentially at the level of the synapse (Ge et al., 2008). The lack of GRP94 induction by cooling may reflect a short half-life, or the rather selective nature of this chaperone, whose client list is much shorter than that of BiP and contains several inflammatory proteins that would likely be suppressed under hypothermic conditions (Marzec et al., 2012). In particular, GRP94 is not induced at high temperatures (Marzec et al., 2012). Mild CHOP induction (Fig.6.5c) is supportive of ATF6 activation since this transcription factor can induce CHOP (Tabas, 2011). CHOP induction could also signify the duration limit of protective cooling; alternatively its knock-on GADD34-mediated negative feedback may be fundamental to hypothermic preconditioning (Fig.6.6, Halterman et al., 2010). Accordingly, others have highlighted the protective role of CHOP in neuronal systems (Chen et al., 2012; Engel et al., 2013). Moreover, it is

possible that hypothermia may have blocked CHOP-induced apoptosis by buffering cytoplasmic calcium (Tabas et al., 2011). In support of this, cooling did not increase levels of the pro-apoptotic marker Bcl-2 associated X protein (Bax), as shown elsewhere (Yenari et al., 2002) (Fig.6.13b). Although this protein has been variably affected by hypothermia in previous studies (Shintani and Terao, 2012), others have observed increased expression of anti-apoptotic proteins in hippocampal neurons at 33°C (Zhang et al., 2001). Prolonged ER stress leads to sequential activation then deactivation of Ire1α, ATF6 and PERK pathways respectively – this might explain the bias of UPR components towards the PERK arm after 24 h cooling (Tabas, 2011). Nevertheless, transcript analysis of hCNs captured distinctive patterns of UPR responses resulting from cooling and Tm. BiP, GRP94, XBP1s and CHOP dramatically increased under Tm, whereas ATF4 and GADD34 induction were comparable between the two conditions. Therefore, in contrast to models described elsewhere (Rutkowski et al., 2006), the negative regulation of eIF2α appears to take precedence over unloading the ER in cooled human neurons (Rzechorzek et al., 2015). This relief of translational repression may confer tolerance to a prolonged hypothermic state (Moreno et al., 2013; Peretti et al., 2015).

In the clinic, 'preconditioning' is typically ascribed to a transient mild stress followed by a recovery interval (Nishio et al., 2000; Stetler et al., 2014). Here the term is applied in its broadest sense – i.e. a subtoxic cellular stress that can lead to a protective state (Stetler et al., 2014) in order to account for the proteostatic priming observed during the pre-incubation phase of cooling. This definition circumvents the need for a re-warming phase which would confound analysis of oxidative injury by

inducing relative hyperthermic and hypoxic stresses (Liu et al., 1994; Lleonart, 2010; Chip et al., 2011; Neutelings et al., 2013). Hypothermic preconditioning may reconcile apparently conflicting data describing UPR modulation in neuronal health; first that ER stress can elicit UPR-mediated hormesis (Mendes et al., 2009; Fouillet et al., 2012), second that circumventing UPR-mediated translational repression promotes long-term survival (Moreno et al., 2013), and third that inhibiting eIF2α phosphatases resolves ER stress (Saxena et al., 2009; Kiskinis et al., 2014). This highlights the importance of fine-tuning the entire network, rather than adjusting a single pathway or component (Rzechorzek et al., 2015). A combined approach has been proposed for ALS (Kiskinis et al., 2014) and cooling has recently demonstrated some benefit in an *in vivo* model of spastic paraplegia (Baxter et al., 2014). Ultimately, disease stage and neuronal subtype would determine whether enhanced or prophylactic preconditioning could be useful in the context of neurodegeneration (Saxena et al., 2009). Indeed, Peretti *et al.* (2015) observed that neurodegenerative synaptic loss could be partially rescued through cooling-induced enhancement of RBM3 expression - but the benefit diminished if treatment was applied later in the disease course. This temporal dependency might be explained if hypothermia-mediated proteostatic priming were elicited prior to the build-up of a significant aggregated protein load (Rzechorzek et al., 2015). Whether the hypothermic preconditioning described here may engage a cytoprotective mechanism that is synergistic with the preservation of synaptic plasticity in currently unknown (Peretti et al., 2015).

Finally, hypothermic neuroprotection was impaired at 28°C and mimicked at 37°C using compounds that reciprocally modify tau phosphorylation. Earlier, it was noted that hypothermia had negligible impact on GSK3β expression despite a dramatic inhibitory effect on PP2A activity (Fig.5.17). In support of this, TCS 2002 (but not fostriecin) impacted upon hCN injury at 28°C, suggesting a residual GSK3β (but not PP2A) activity at this temperature. Injury was unchanged in the presence of fostriecin under hypothermic conditions. This highlights the potency of cooling-induced PP2A suppression and suggests that tau phosphorylation was already saturated at this temperature. Concurrently, inhibiting tau phosphorylation had the opposite effect to fostriecin, preventing the protection that would have otherwise been achieved at 28°C. These results provide evidence that PP2A inhibition is both necessary and sufficient to protect hCNs from oxidative stress, confirming that the major human tau phosphatase participates in the neuroprotective effect of hypothermia. In Alzheimer's disease hyperphosphorylated tau mislocalises to the somato-dendritic compartment and can be found associated with the rough ER (Iqbal et al., 2009). Since increased tau phosphorylation is conducive to neuroprotection in hCNs, and can attenuate ER-stress-induced apoptosis through upregulation of the UPR (Liu et al., 2012c), it may trigger this cascade during neuronal cooling, potentially through inhibiting ER-associated degradation (ERAD) (Keck et al., 2003; Abisambra et al., 2013). This is consistent with the hypothesis that tau modulation may serve a protective function early in the course of neurodegenerative disease, perhaps by inciting ER-hormesis. Since the cooling paradigm above can be used to titrate UPR activation, it represents a simple method to address subtle effects

dictating adaptive versus maladaptive outcomes of this cascade in human neurons (Rzechorzek et al., 2015).

In summary, this work establishes a role for the UPR in hypothermia-driven neuronal preconditioning (Rzechorzek et al., 2015). Mild ER stress and UPR activation are critical features of the hypothermic response and contribute to its protective effect. Impaired stress responses underlie several neurodegenerative disorders (Hetz and Mollereau, 2014), and although hypothermic preconditioning originates from the traumatic setting, preconditioning in general is a proposed target for neurodegenerative disease (Stetler et al., 2014). Further dissection of these pathways might thus reveal potent therapeutic targets for both acute and chronic brain injury.

# Chapter 7: Discussion

## 7.1 Review of key findings and future directions

This thesis presents several novel elements of neurocryobiology within the context of hypothermic neuroprotection, including the first demonstration of a cold-shock response in human neurons. With regards to tau, hypothermia induced changes at transcript, protein and post-translational levels in hCNs, consistent with reversal of the maturational transition in tau status observed during hCN differentiation. Using a human neuronal system has thus revealed that tau responses to cooling extend to alternative splicing of *MAPT,* in a manner that complements an increase in tau phosphorylation. This may be an essential feature of hypothermic tau in human neurons that simultaneously permits cytoskeletal plasticity, whilst retaining tau solubility. Critically, culture conditions which alter transcriptional and post-translational features of tau also deliver human cortical neuroprotection in an injury- and temperature-specific manner. Association of mild hypothermia, RBP induction, tau isoform regulation (Fig.7.1) and protection from NR2B-mediated excitotoxic cell death points to a convergence of these pathways in predicting neuronal survival. Since temperature can influence biomolecular and electrophysiological properties, including cell membrane fluidity (Sonna et al., 2002) and Nernst potential (Kandel et al., 2000), dissecting the immediate physical effects of hypothermia from its downstream molecular effects will be essential to deciphering their relative importance in neuronal health.

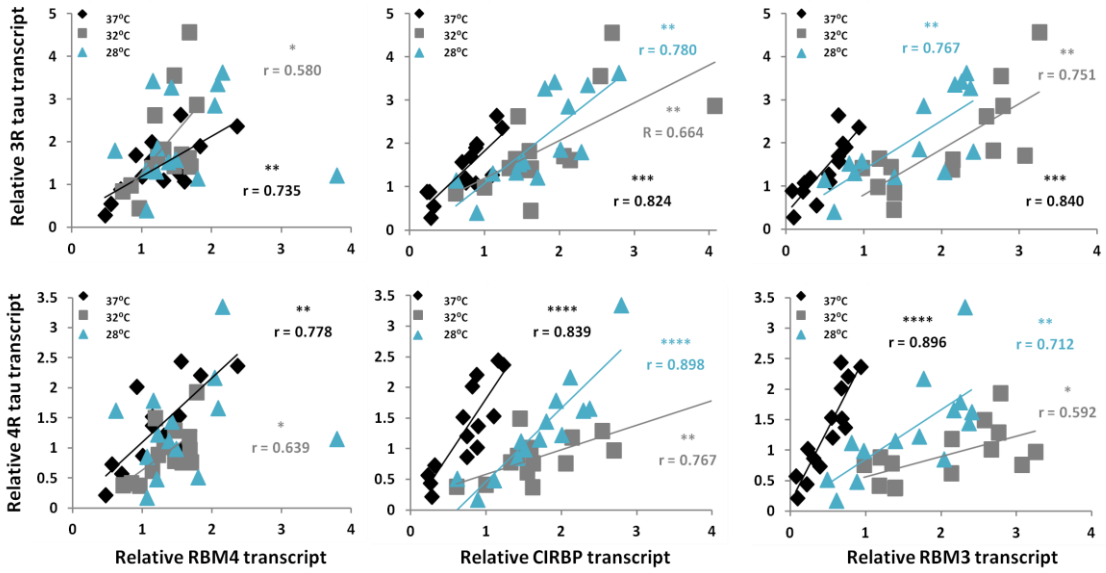


**Figure 7.1 RBP and tau correlates in hypothermic hCNs.** Two-tailed Pearson correlations of RBP and tau isoform transcripts. Note close relationship between RBPs and 4R tau under normothermic conditions, and this relationship is highly significant for CIRBP and RBM3. In these cases, the greatest correlative dissociation occurs at 32°C, suggesting independent regulation of tau exon 10 splicing at this temperature (CIRBP 37°C $P<0.0005$, 32°C $P=0.001$, 28°C $P<0.0005$; RBM3 37°C $P<0.0005$, 32°C $P=0.026$, 28°C $P=0.004$). Note loss of association between RBM4 and 4R tau with decreasing temperature, suggesting that RBM4-regulated inclusion of tau exon 10 is impaired under these conditions.

As for all biochemical processes within living cells, cerebral processes are highly temperature-dependent (Rodriguez et al., 2012; Wang et al., 2014). However, different indices of axonal excitability have variable thermal sensitivity, which also depends on neuronal subtype and state of health (Davis et al., 1976; Burke et al., 1999; Kiernan et al., 2001; Howells et al., 2013). A significant proportion of multiple sclerosis patients report that their symptoms are intensified by increased temperature, referred to as Uhthoff's phenomenon (Guthrie and Nelson, 1995). This is thought to be associated with a reversible conduction block of demyelinated fibres under hyperthermic conditions - although this specific mechanism has been contested (Rasminsky, 1973; Schauf and Davis, 1974; Guthrie and Nelson, 1995). More

recently, it was shown that hyperthermia reduces inward rectification in healthy human subjects, and that motor and sensory axons were differentially sensitive to this effect (Howells et al., 2013). This explains why patients with acute febrile infections have disrupted neuromuscular transmission (Friman et al., 1977). The overall result of hyperthermia is a reduced safety margin for action potential generation and propagation, especially in axons that are already compromised (Howells et al., 2013).

The brain is a metabolically demanding organ that produces considerable heat in the final stages of ATP hydrolysis (0.66 $Jmin^{-1}g^{-1}$ brain tissue) (Yablonskiy et al., 2000; Wang et al., 2014). Despite being one of the most heat-sensitive tissues, the brain is remarkably tolerant to cooling and can endure temperatures that approximate to the lower limit of survival (Yang et al., 2006; Stieler et al., 2011; Wang et al., 2014). Nevertheless, hypothermia can profoundly influence neuronal biology, structure and function. Through a dramatic reduction in neuronal activity, hibernators can save up to 90 % of the energy that would otherwise be required under basal conditions (Arendt and Bullman, 2013). Electrophysiological studies in both hibernating and non-hibernating species show that gradual cooling of the brain produces a biphasic response in neuronal activity, with hyperexcitability followed by transmission block (Arendt and Bullman, 2013). This initial hyperactivity is reminiscent of that seen in the pre-symptomatic stages of AD (Stargardt et al., 2015), but is probably distinct from the 'burst suppression' observed by electroencephalogram during deep hypothermia (Brandon Westover et al., 2015). Predictably, a cooling-induced block impairs cognitive function; in humans, approximately 70 % of memory encoding is lost at a core temperature of 34-35°C – however recall of previously learned

information is untouched (Coleshaw et al., 1983). Not only are these neuroplastic changes reversible, they appear to confer an advantage (Peretti et al., 2015) – the strengthening of cognitive function during sleep is testament that repeated cycles of cooling and re-warming are good for the brain (Wang et al., 2011). Cooling also prolongs and changes the shape of the action potential (see Fig.7.2) and drugs that mimic this effect have been proposed to overcome conduction block in demyelinated nerves (Sherratt et al., 1980).

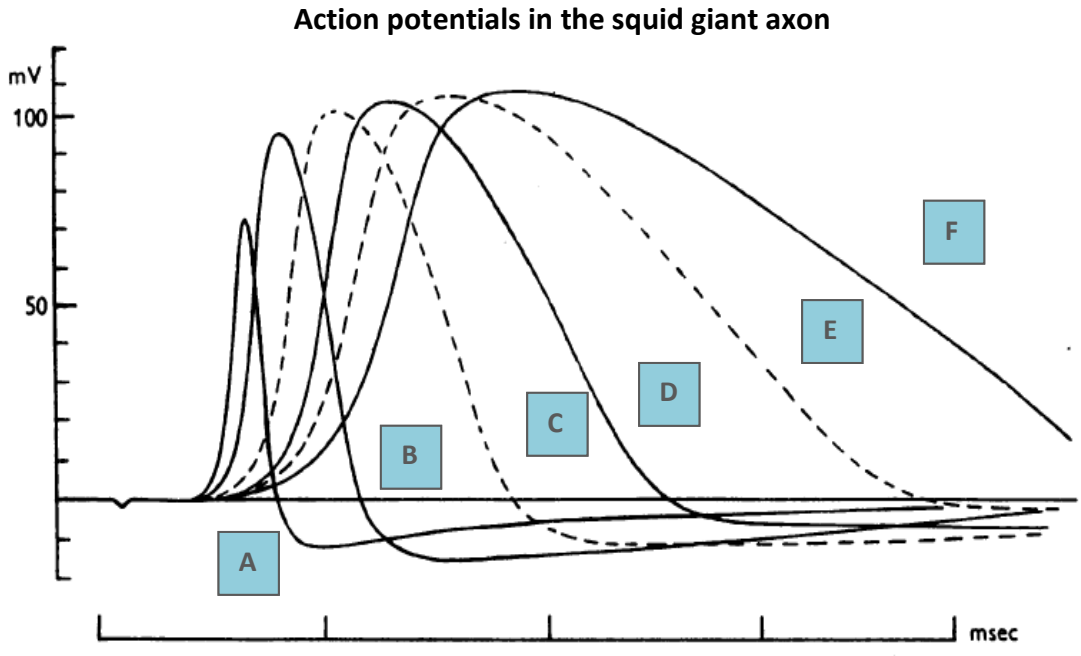


**Figure 7.2 The effect of temperature on the action potential.** Action potentials were recorded in the squid giant axon using an internal recording electrode. Superimposed tracings are shown of 6 spikes at temperatures labelled A-F as follows: 32.5, 20.2, 13.3, 9.8, 6.3, and 3.6°C. With cooling, the wave of the action potential was slowed, but the amplitude was not altered greatly until above 20°C, after which it was reduced in height. The shift of these curves suggests that the rate of fall of the action potential has a higher temperature coefficient than the rate of the rise. The resting potential was almost independent of temperature between 2 and 20°C, above which it diminished. All temperature effects were reversible as long as the temperature did not exceed 35°C. The lowest temperature at which a recording could be made was 1°C. Figure and legend adapted from Hodgkin and Katz (1949) with permission.

The thermosensitivity of the axonal membrane potential can vary depending on prior thermal experience, and desensitization itself is temperature-sensitive (Janssen, 1992;

Money et al., 2004; Klose et al., 2004). Unsurprisingly, cold-shock can also have these preconditioning effects at the level of neural transmission. However, in contrast to the transient stimulus required for preconditioning, chronic cold-stress exposure can sensitize neurons to further stimuli; this chronic stress-induced enhancement of neuronal activity has been implicated in human mood and anxiety disorders (Jedema and Grace, 2003). Thus, several pharmacological targets might emerge from testing the thermal properties of human neurons derived from both healthy individuals, and those with neurological disease. Whilst a few studies have addressed the effects of temperature on human peripheral nerve activity (Rutkove et al., 2001), none have directly analysed these effects in human cortical neurons. Acknowledging that this is extremely difficult in live patients, the hCN system provides a useful platform to explore changes in electrophysiological parameters as temperature increases or decreases. A more detailed discussion of thermal influences on neurophysiological readouts is beyond the scope of this thesis, but is covered in depth elsewhere (Janssen, 1992).

Inasmuch as temperature effects have clinical value, they can also hamper objective measurements of neural function in practice and may yet overturn established fundamental principles of neurobiology (Denys, 1991; Rutkove, 2001). For example, network activity is affected by cooling (Kalmbach and Waters, 2012) and temperature-related effects have critically undermined interpretation of learning-induced changes in field potential in freely moving animals (Moser et al., 1993). Likewise, intra- and inter-subject variability in limb temperature is a frequent cause of misdiagnosis in clinical electromyography (Denys, 1991). This presents a 'Catch-

22'-like conundrum (Heller, 1961); we know that temperature determines electrophysiological properties (Kiyatkin et al, 2010), but how do we truly isolate them when the defining feature of a neuron (its function) itself produces heat? Current-clamp and voltage-clamp techniques are somewhat extraneous in the absence of a 'thermal clamp'. Recording neural activity at room temperature is, to all intents a purposes, an attempt to create such a clamp. However, this cannot eliminate smaller temperature fluctuations occurring during nerve stimulation or as a result of uncoupling protein activity within axon terminals (Wang et al., 2014). Neither does it represent the physiological thermal properties of mammalian cells *in vivo*. When applying Nernst or Goldman-Hodgkin-Katz functions, terms are usually simplified by fixing absolute temperature (Kandel et al., 2000). For *in vitro* studies this equates to room temperature, whilst for *in vivo* extrapolation 37°C is often used to simulate core human body temperature. These assumptions are clearly inadequate for modelling neural function in the dynamic healthy brain (McNaughton et al., 1994), let alone one compromised by injury. Indeed, the effects of temperature on neural conduction can change considerably in the diseased state (see Table D1) (Rasminsky, 1973; Rutkove et al., 2001). The expansion of the stem cell field is an opportunity to revisit earlier works of eminent physiologists who first described the functional properties of the neuron, and re-apply these methods to human CNS neurons and glia under conditions of temperature shift.

**Table D1 Effects of temperature on neurophysiology in the disease state** (adapted from Rutkove et al., 2001; CMAP = compound motor action potential).

| Disease | Effect of temperature |
|---|---|
| **Axonal loss** | |
| CMAP amplitude | Increased sensitivity to cooling |
| Abundance of fibrillation potentials | Decrease with muscle cooling |
| Fasciculation potential amplitude | Increase with muscle cooling |
| Fasciculation potential frequency | Likely decrease with neuronal cooling |
| **Demyelinating lesions** | |
| Conduction velocity | Reduced effect of temperature |
| Conduction block | Increases with heating |
| **NMJ disorders** | |
| Myasthenia gravis | Improvement in decrement with cooling |
| Lambert-Eaton myasthenic syndrome | Baseline amplitude increases with cooling |

Advances in stem cell technology have transformed our ability to track certain aspects of injury and disease in a reductionist setting. The opportunity to derive multiple CNS cell types from patients with neurodegenerative, psychiatric and neurodevelopmental disorders is already offering powerful insight into molecular mechanisms of these disorders and potentially new routes to treatment (Bilican et al., 2012, Donnelly et al., 2013; Serio et al., 2013; Brennand et al., 2014; Hibaoui et al., 2014; Kiskinis et al., 2014; Devlin et al., 2015). It is clear that the dramatic changes observed in human post-mortem samples do not reflect the earlier and more subtle changes leading to these events - particularly at the level of neuronal function (Devlin et al., 2015). Thus there is a need to explore these functional impairments in a disease context. As mentioned previously, these functional impairments can extend to the most fundamental and highly conserved stress responses within the cell – those

that safeguard proteostasis. Validating these pathways in healthy human cells is critical: first it gives us a baseline for comparing proteostatic responses across various cell types and second, it identifies molecular cascades that might be harnessed for therapeutic benefit in disease. For example, motor neurons demonstrate a high threshold for induction of heat shock (Batulan et al., 2003; Taylor et al., 2007) which partly explains selective vulnerability of this cell population in ALS (Bruening et al., 1999). It would be useful to determine whether ALS patient-derived motor neurons respond poorly to hypothermia and whether this abrogates the protective effect of cooling. On the other hand, enhancing the stress response in these cells may promote their survival (Bruening et al., 1999). Thus, by stimulating ER hormesis, hypothermia might precondition these neurons to endure chronic protein misfolding, or else lower the threshold for a beneficial stress response against other toxins such as glutamate (Bruening et al., 1999; Cleveland and Rothstein, 2001). The cooling paradigm outlined in this thesis could thus be applied to a whole range of patient-derived cell lines, and targeted to various stages of the disease process. Because of the 'cross-tolerance' conferred by cold-stress, the objective would be to define the optimal therapeutic window for preconditioning against multiple types of neuronal injury. The influence of cooling on excitotoxicity opens up this approach to disorders where neural conduction, transmission and plasticity are deficient in the absence of protein misfolding. These might include multiple sclerosis, schizophrenia and autism spectrum disorders. Peretti et al. (2015) have provided the proof of concept that at least one element of the cold-shock response can be harnessed to rescue neuroplasticity. The relationship of synaptic strength and eIF2α phosphorylation (Halliday and Mallucci, 2014) together with the findings

presented above suggest that the UPR may also have a role to play in temperature-regulated synaptic function. Indeed, the differential effect of hypothermia on specific types of memory, may be borne out through a modulation of phospho-eIF2α (Costa-Mattioli et al., 2007). Whether there are additional effects on synapse-specific chaperones (as shown for Hsc70 at elevated temperatures) is yet to be determined (Tobaben et al., 2001; Gong and Golic, 2006).

It is well-established that hypothermia induces tau hyperphosphorylation, akin to that seen in early-stage Alzheimer's disease (Bretteville et al., 2012). The experiments in Chapter 5 confirmed this phenomenon in hCNs (the first demonstration in human neurons), and extended its effect to a protein-level shift in tau isoform ratio, consistent with RD of tau. In the context of previous studies (Keck et al., 2003; Iqbal et al., 2009; Liu et al., 2012b,2012c; Abisambra et al., 2013) and parallel findings (Chapter 6), the derived postulate is that this 'foetal-like' tau is intimately involved in cooling-induced ER-hormesis (Fig.7.3). This appears to contradict the findings of van der Harg et al. (2014) who proposed that UPR activation in the brain during torpor was upstream of tau hyperphosphorylation and was a specific outcome of a hypometabolic state - *not* passively related to temperature drop. Importantly they found that simply cooling human neuroblastoma cells did *not* activate the UPR. This conflict may relate to specific differences in experimental technique (e.g. cooling duration and depth, and several UPR targets analysed in hCNs were omitted thus potentially reducing sensitivity of UPR detection in neuroblastoma cells). However, a more likely explanation is the use of an immortal cell line. Unlike post-mitotic neurons, tumour cells are highly resistant to physiological stress – and their inherent

upregulation of molecular chaperones such as BiP allows them to evade chemotherapy (Abdel Malek et al., 2014; Cerezo et al., 2015). As suggested in Chapter 6, van der Harg et al. (2014) also concluded that tau hyperphosphorylation might impair ERAD (Abisambra et al., 2013), but they linked this to perpetuation of a maladaptive UPR in tauopathies, rather than a hormetic stimulus that might offer neuroprotection. Whilst alluring, there are several caveats to their hypothesis: (1) temperature drop produces a rapid increase in phosphorylation at many tau epitopes via PP2A inhibition (Planel et al., 2007a; Papon et al., 2011; El Khoury et al., 2014) - chronologically this post-translational change should happen in advance of any modification effected via the UPR and is supported by the fact that (2) the abnormal hyperphosphorylation of tau in AD makes tau more resistant to degradation by activated neutral proteases, calpains and the UPS (Iqbal et al., 2009) which would lead to ERAD impairment and then ER stress, (3) tau phosphorylation occurs to a much greater extent in cooled hCNs than in Tm-treated neuroblastoma cells suggesting that ER stress is a relatively weak stimulus for phosphorylation of tau, and (4) tangle-bearing neurons in tauopathies survive this state for many decades (Morsch et al., 1999), suggesting that the UPR activation seen alongside early tau hyperphosphorylation in AD brains imparts some resistance to protein misfolding and neuronal death (Hoozemans et al., 2009; Nijholt et al., 2012). In AD, the ER has been highlighted as a regulatory site involved in neurodegeneration via perturbed Ca homeostasis and oxidative stress (Mattson and Guo, 1997). Although this has largely been attributed to aberrant APP and presenilin processing, it could also result indirectly from functional loss of tau. In murine neuroblastoma cells, tau overepxession caused pronounced contraction of the ER so that it no longer reached

the cell periphery and its density and branching decreased, consistent with the idea that transport along microtubules was perturbed (Ebneth et al., 1998). This agrees with previous studies suggesting that PHF-tau disrupts the trafficking of various cellular organelles including mitochondria (Lee et al., 2005; Wang and Liu, 2008). Similarly, Shay and Gonatas (1973) noted clefts in the rough ER of cat spinal cord motor neurons and dendrites after 1-2 h of deep hypothermia (15°C). Whether cooling also precipitates ER stress via changes in ER morphology or distribution in hCNs remains to be determined and will require detailed ultrastructural analysis by electron microscopy. Irrespectively, once the UPR is underway, hyperphosphorylated tau could feature at several points in the cycle, operating both upstream and downstream of ER stress (Hoozemans and Scheper, 2012). Halliday and Mallucci (2014) present an interesting theory to explain the paradoxical effects of salubrinal in different neurodegenerative disease models: that a 'pure' ER stress triggered by protein aggregates within the ER (mutant SOD1, mutant α-synuclein) is ameliorated by promoting translational suppression, whereas the 'nonspecific proteostatic dysregulation' caused by cytoplasmic aggregates (tau, PrP) benefits more from blocking chronic UPR activation. This hypothesis is contestable because even if a 'pure' ER stress existed, it would be sufficiently created by impairing ERAD (Abisambra et al., 2013).

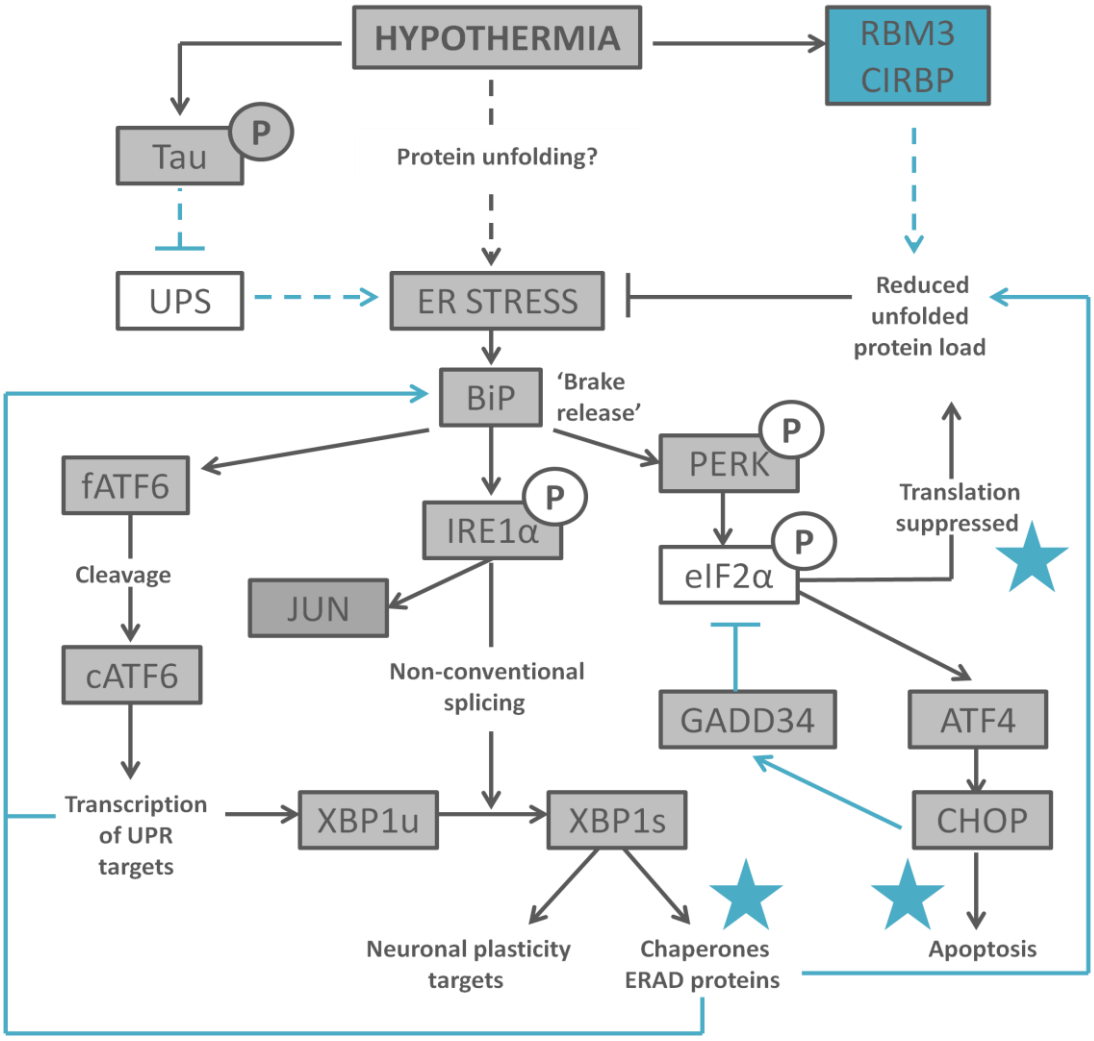


**Figure 7.3 A proposed model for hypothermia-induced ER-hormesis and preconditioning of the UPR in hCNs.** The schematic from Fig.6.6 has been replicated but modified to indicate the putative enrolment of phospho-tau (which may impair ERAD by inhibiting the proteasome (Keck et al.,2003; Abisambra et al., 2013)) and potential points of cooperative preconditioning supported by cold-shock proteins (aqua stars). Specifically, these chaperones might (1) ensure essential mRNAs escape translational suppression, (2) promote apoptotic inhibition and/or (3) be further upregulated by XBP1s. Cooling-induced protein unfolding could be a primary trigger for ER stress (King and Weber, 1986; Fujita, 1999), however this is less likely at the mild hypothermic temperatures used in the hCN cooling paradigm.

van der Harg et al. (2014) also argue that hibernation in tropical species is proof that a temperature drop is not required for UPR activation and subsequent tau phosphorylation (Dausmann et al., 2004; Stieler et al., 2011). Notwithstanding that

the extreme 'ectothermic' behaviour of torpid lemurs (Dausmann et al., 2004) may be unique amongst hibernating mammals, the body temperature measurements in these primates were calibrated to intraperitoneal temperature which may be wildly different from brain temperature (the difficulties of accurately assessing brain temperature have already been discussed in the introduction). Finally, the authors claim that hypothermia impairs metabolic stress-induced UPR activation because they observed that PERK phosphorylation was abolished at 26°C (van der Harg et al., 2014). Since the whole UPR cascade was not explored under their cooling paradigm, it is impossible to draw this conclusion – rather it suggests (as in hCNs) that certain components of the UPR were held in an adaptive hormetic state at this temperature via negative feedback (Fig.7.3). Ultimately van der Harg et al. (2014) maintain that the initiating factor is hypometabolic UPR induction leading to tau hyperphosphorylation – they propose that this might conserve energy associated with axonal transport. This fits well with the spatial predilection of tau pathology in metabolically demanding brain regions in AD, and the abnormal glucose metabolism in these patients (Janson et al., 2004; van der Harg et al., 2014). However, whilst a hypometabolic state clearly increases tau phosphorylation, this is typically coordinated with reduced body temperature in 'perfect hibernators' (Drew et al., 2001). Therefore, the brain of an AD patient might resemble more closely that of a larger mammalian hibernator such as *Ursus americanus*, where metabolic suppression (25 %) is sufficient to modify phospho-tau, but hypothermia is perhaps too mild or slow to exert other neuroprotective changes (such as a shift in tau isoform ratio, dendritic morphogenesis or synaptic plasticity) (Vogelsberg-Ragaglia et al., 2001; Tøien et al., 2011; Stieler et al., 2011; Dave et al., 2012). Since 3R tau

isoforms are more readily secreted by cells than 4R tau isoforms, it might be that the increased 4R:3R ratio in the mature brain renders the cell more susceptible to retention of the protein and thus aggregation (Karch et al., 2012). Although many of the genes overexpressed during hibernation are involved in neuroprotection (Chen et al., 2008), it is unclear which of these are upregulated due to metabolic suppression and which are induced as a response to hypothermic stress. It would be of interest to know whether the ultrastructural synaptic changes observed in ground squirrels also occurred in black bears, and whether the NFT pathology observed in this species correlates with any cognitive decline (Cork et al., 1988). Nonetheless, protein synthesis is more energetically demanding than axonal transport, thus UPR-mediated translational suppression would be expected in neurodegenerative disease, particularly in the presence of misfolded proteins (Hofman et al., 2012; Moreno et al., 2012). Provocatively, by recruiting UPR-driven ER-hormesis, cooling would build proteostatic resistance at a lower energy cost (Fogolin et al., 2004).

Against a vast backdrop of literature covering the cellular response to heat-shock, responses to cooling are relatively unexplored (Fujita, 1999; Phadtare et al., 1999; Sonna et al., 2002; Al-Fageeh et al., 2006). To date around 20 genes have been reported to be induced by moderate hypothermia, compared to more than 100 upregulated by heat stress (Sonna et al., 2002). The Hsps previously reported to be upregulated by cooling were observed only during re-warming and did not include BiP (Sonna et al., 2002), thus the observed BiP induction in cooled hCNs may be an entirely novel finding. RNASeq analysis of hCNs cultured at the 3 temperatures of interest is pending, and may reveal additional cold-specific targets. Mammalian cells

can still proliferate at 32°C, below which proliferative capacity is greatly reduced – however cells can survive for extended periods at much lower temperatures and cryopreservation techniques rely on this cold tolerance (Fujita, 1999; Al-Fageeh et al., 2006). Such remarkable adaptation to cold stress is exploited by industry; cultivating mammalian cells at subphysiological temperatures improves cell viability, enhances recombinant protein production and increases the efficiency of glucose conversion to ATP (Fujita, 1999; Fogolin et al., 2004; Al-Fageeh et al., 2006). This improved metabolic efficiency could be of real benefit in neurodegenerative disorders, particularly within the dendritic compartment (Hasel et al., 2014) and when supportive glial functions are lost or diverted to a 'reactive' phenotype. Despite the potency of hypothermic neuroprotection, cooling must still be regarded as physiological stressor which, depending on its intensity, can trigger a stress response (as evidenced in this thesis), apoptosis or even necrosis (Sonna et al., 2002).

Recently a link has been found between CIRBP expression and NF-κB signalling which suggests an inflammatory role for this cold-shock protein and further implicates it in UPR mechanisms associated with ALS (Brochu et al., 2013; Prell et al., 2014). CIRBP has also been identified as a damage-associated molecular pattern molecule that is secreted by macrophages in response to hypoxia or lipopolysaccharide (LPS) and promotes inflammatory responses in haemorrhagic shock and sepsis (Qiang et al., 2013). Since they derive from the same lineage, it would be interesting to test whether brain-resident macrophages (the microglia) can also secrete this protein and whether it plays a role in neuroinflammation. Of note, Hsp70 expression in microglia appears to play an NF-κB-dependent anti-

inflammatory role in acute ischaemic injury (Zheng et al., 2008). An obvious limitation of the hCN system is the lack of full glial support, interneurons and inflammatory mechanisms that are paramount to most neurological disorders and are so profoundly affected by hypothermia (Weil et al., 2008; Yenari and Han, 2012). Future work will exploit advances in the differentiation of other brain cell types from hPS lines to address the effect of hypothermia in each of these alone and in combination with hCNs. Last year, a US patent was obtained by The Fenstein Institute for Medical Research to use CIRBP inhibition as a means to treat human inflammatory conditions. This highlights the increasing interest in cold-shock proteins as clinical targets for neuroinflammatory and even neuropsychiatric disorders (Zhou et al., 2014; Costa et al., 2015). A recent study exploring forced desynchrony of sleep pattern in human subjects suggested that a resultant decrease in the amplitude of circadian fluctuations in body temperature was associated with transcriptomic changes in RBM3 and CIRBP (Archer et al., 2014). Since these RBPs also regulate circadian gene expression, this raises the possibility that they critically balance thermoregulatory, endocrine and neuroplasticity cycles – all of which may go awry in dementia (Avila and Diaz-Nido, 2004; El Khoury et al., 2014; Maurin et al., 2014; Peretti et al., 2015).

Although RBM3 exhibits many characteristics of a proto-oncogene (Lleonart et al., 2010), its expression has also been associated with improved prognosis in several studies of malignancy (Jogi et al., 2009; Ehlen et al., 2010; Zeng et al., 2013). Such discrepancy may result from a disease-stage-specific expression of this protein. Zeng et al. (2013) found this RBP upregulated in primary prostate cancer tissue but

downregulated in metastatic samples. This suggests a role for RBM3 in disease initiation, followed by an inhibitory function in disease progression (Zeng et al., 2013). In the brain it points to a critical developmental window in which RBM3 levels are coupled to translational demand; high nuclear expression enables rapid neural proliferation and differentiation, after which modest somatodentritic expression supports synaptic plasticity (Smart et al., 2007; Pilotte et al., 2009; Peretti et al., 2015). It is possible that cooling re-opens this window whilst boosting local translation in a compartment that is highly vulnerable to environmental challenge (Smart et al., 2007). However, the idea that RBM3 might be useful in the context of neurodegeneration is not new; Kita et al. (2002) found that overexpressing this protein inhibited apoptosis in PC12 cells transfected with mutant Huntingtin. Okamato et al (2009) reported than synaptic NMDAR activity-driven inclusions were neuroprotective in an *in vitro* model of Huntingdon's disease, whereas exrasynaptic NMDAR activation reduced inclusion formation and rendered neurons more susceptible to glutamate-induced cell death. Since Huntington's disease is associated with increased extrasynaptic NMDAR activity and excitotoxicity (Hardingham et al., 2002; Fan and Raymond, 2007; Milnerwood et al., 2010; Hasel et al., 2014), the resistance of cooled hCNs to glutamate, alongside RBM3-mediated rescue of neuroplasticity in mice is particularly thought-provoking (Peretti et al., 2015). Interestingly, there was an apparent increase in the ratio of GluN2A to GluN2B transcripts in cooled hCNs, which according to preliminary statistical analysis was significant (28°C $P$=0.014; 32°C $P$=0.0005; data not shown). Although several NMDAR subunits (including GluN2A and GluN2B) are downregulated in rat hippocampus during heat exposure (Le Grevès et al., 1997), their expression in cold-

shocked neurons has not been assessed. Given its granular, dendritic expression (Smart et al., 2007), it would be of interest to explore whether RBM3 alters the relative expression of EAA receptor subunits under cooled conditions, and whether this confers resistance to excitotoxicity.

Neuronal polarity during differentiation is partially determined by local translation of specific mRNA molecules as dendrites and axons emerge (Atlas et al., 2007). It is thus unsurprising that several RBPs interact with tau, since these can regulate the spatial and temporal translation of tau mRNA in a rapidly changing environment (Atlas et al., 2007; Moschner et al., 2014). Since RBM3 promotes translation in dendrites (Smart et al., 2007), it will be important to determine whether this RBP contributes to tau isoform switch under hypothermic conditions, and further if this might drive cooling-induced dendritic morphogenesis (Greenwood and Connolly, 2007). Similar mechanisms may be needed for the advancement of growth cones during repair. The application of individual-nucleotide resolution cross-linking and immunoprecipitation (iCLIP) (Modic et al., 2013) would determine whether cold-shock RBPs interact with tau-isoform-specific pre-mRNAs and thus influence 3R:4R isoform ratio under cooled conditions. Furthermore, RNA reprogramming using spliceosome-mediated RNA *trans*-splicing (SMaRT) could be used to modify tau splicing effects under hypothermic conditions to establish whether these contribute to the protective effect observed (Buee et al., 2010). Preferential phosphatase inhibition under hypothermic conditions may also have an indirect effect on tau splicing through enhanced Dyrk1A activity and thence ASF phosphorylation leading to a relative increase in 3R tau (Shi et al., 2008). Whether cooling may impact more

generally on ‘ribostasis’ in addition to proteostasis remains to be determined, although an interplay between RBP mutation and protein misfolding certainly features in neurodegenerative disease (Ramaswami et al., 2013). In this context, the splicing factor SFPQ is worthy of further investigation, given its nucelo-cytoplasmic redistribution in AD, PiD and a cell culture model of P301L tauopathy (Ke et al., 2012). The dendritic spine effects of cooling can be observed *in vitro* using mature neurons where cooling-induced spine loss halts synaptic transmission (Kirov et al., 2004). Further development of the hCN system alongside super-resolution techniques will permit analysis of these dynamic effects both in dendritic spines, and at the level of the synapse. It may however be necessary to cool to much lower temperatures in order to observe morphological changes (Yang et al., 2006). Combining these approaches with live cell imaging will allow real-time exploration of cooling-mediated protection in human neurons.

Hofman et al. (2012) identified SG formation as one of the parallel strategies used by COS7 cells to inhibit protein synthesis and ensure survival under cold conditions (10°C). These findings are in agreement with those observed in hCNs under milder hypothermic temperatures (see Chapter 5). Again, caution must be taken when translating findings from immortal cultures to hCNs, but the fact that SGs did not form in COS7 cells at 30 or 20°C (Hofman et al., 2012) supports the concept of an increased resistance of these cells to physiological stress. In several immortal cell lines, De Leeuw et al. (2007) observed redistribution of nuclear CIRBP into SGs in response to oxidative and ER stress. CIRBP-positive SGs were not obvious at mild-to-moderate hypothermic temperatures in hCNs (Fig.4.4) and there was no

appreciable increase in cytoplasmic expression of this protein (Fig.4.6). However, the granular accumulation of CIRBP is thought to occur via a TIA-1 independent mechanism, and thus might not be detectable using the methods employed in hCNs (De Leeuw et al., 2007). Nevertheless, this does not rule out a contribution of CIRBP to hypothermic protection of hCNs against oxidative stress, as demonstrated in rat cortical neurons treated with $H_2O_2$ (Li et al., 2012). Conceivably, cold-shock proteins may temper activation of the PERK pathway, thereby relieving translational repression of critical mRNAs, whilst limiting CHOP-mediated apoptosis (Dresios et al., 2005; Saito et al., 2010). Of note, RBM3 can inhibit the DNA damage response and might thus modify CHOP responses to DNA damage in stressed, hypothermic neurons (Sureban et al., 2008). However, CHOP has also exhibited protective functions in the face of oxidative stress and impaired memory performance (Chen et al., 2012a; Cano et al., 2014) and it appears to be required for neuronal survival after seizures (Engel et al., 2013). Clearly, a moderated level of CHOP expression would be needed for hormesis and this explains why the increase in CHOP in cooled hCNs was not associated with cell death. Even this small bias in the outputs of proximal UPR transducers would have a critical impact on physiology, because of the strength of negative feedback within the circuit (Tabas, 2011). Ultimately the role of cold-shock proteins in hypothermic preconditioning requires molecular validation, since specific chemical inhibitors of RBM3 and CIRBP are not commercially available. Using a lentiviral-mediated siRNA approach, preliminary experiments suggest that RBM3 is required for hypothermic protection of hCNs against oxidative stress (data not shown). Since chemical chaperones can enhance the adaptive capacity of the ER

(Ozcan et al., 2006), the prediction is that cold-shock proteins participate in cooling-induced ER-hormesis.

It would be remiss to overlook the profound similarities between hypothermia-induced cellular transitions and those observed during oncogenesis; both use a molecular language that favours survival. The power of this language underlies the 'cross-tolerance' of TH (Parsell and Lindquist, 1993; Sonna et al., 2002; Rutkowski et al., 2006; Stetler et al., 2014), but also the highly resistive nature of some tumours. Decoding the crosstalk may deliver potently neuroprotective agents on the one hand, and novel cancer targets on the other (Kim et al., 2008; Martin et al., 2013; Mollereau, 2013). For instance, chemotherapy-resistant cancer cells often express high levels of Hsps and their malignancy may be reduced by selectively diminishing their capacity to mount a stress response (Sonna et al., 2002; Mayer and Bukau, 2005; Chakrabarti et al., 2011; Martin et al., 2013). In the field of oncology, a useful adversary to 'cross-tolerance' is 'collateral sensitivity' in which acquisition of a drug-resistant phenotype confers sensitivity to another stressor, such as hypothermia (Cerezo et al., 2015). Arguably, hypothermic preconditioning could render neurons vulnerable to other disease-associated processes such as necrotic cell death (Burattini et al., 2010) or tumorigenesis. Indeed, cold-shock proteins have been associated with drug-resistant cancers (Lleonart et al., 2010; Zhang et al., 2013). Thus, converting cold-inducible phenomena into treatments for chronic conditions must consider this potential drawback (Peretti et al., 2015), given that many of these patients will be at an increased age-associated risk for neoplastic disease. The impairment of proteostatic responses in neurodegenerative conditions does however confirm a

deficit that needs addressing (Kim et al., 2008; Koga et al., 2011) – the early decrease in translocation of ER proteins in P301S mice shows how compounding this deficit might accelerate disease (Yoshiyama et al., 2007).

Whilst hypothermia has largely neuroprotective benefits, anaesthetics have exhibited short-term protective and long-term neurodegenerative effects (Berns et al., 2009) or even no effect at all (Feiner et al., 2005). This may reflect the divergent mechanisms by which these treatments alter tau phosphorylation; cooling and anaesthesia-induced hypothermia have their primary effect via PP2A inhibition (Planel et al., 2007a), whereas ether modulates tau kinases and propofol increases PP2A activity (Kingston et al., 2006; Ikeda et al., 2007). NMDAR stimulation decreases PP2A activity which in turn enhances NMDA currents (Wang et al., 1994; Chan and Sucher, 2001). This NMDAR-PP2A interaction has been proposed to signal the recent history of NMDAR activation and may thus help regulate synaptic plasticity and dendritic spine growth (Chan and Sucher, 2001). By stimulating PP2A activity and thus inhibiting NMDAR phosphorylation, propofol inhibits LTP – although this may also require propofol-induced neuritic retraction (Takamatsu et al., 2005; Kingston et al., 2006; Feng et al., 2007; Turina et al., 2008). On the one hand this may be responsible for propofol-induced deficits in memory, but in the context of excitotoxicity, it may provide neuroprotection by suppressing intracellular calcium influx or by shielding synapses (Velly et al., 2003; Grasshoff and Gillessen, 2005; Feng et al., 2007; Bickler et al., 2012). Also, whilst cooling can induce microtubule disassembly (Fujita, 1999), pentobarbital anaesthesia leaves microtubules intact even though it hyperphosphorylates tau in transgenic mice expressing all human tau

isoforms (Planel et al., 2008). This is thought to be mediated by selective removal of 3R tau, leaving 4R isoforms attached to the stabilized microtubules (Planel et al., 2008). Notably, anaesthetics can also act as a preconditioning stimulus (Wei and Xie, 2009; Wang et al., 2012). The final experiments in Chapter 6 specifically demonstrated that PP2A inhibition was partially responsible for the neuroprotective effect of hypothermia in hCNs. PP2A is ubiquitously expressed and plays a role in many fundamental cellular processes from DNA replication to cell growth, signal transduction and apoptosis (Chan and Sucher, 2001). Therefore, the inhibition of this major brain phosphatase could contribute to hypothermic neuroprotection via several mechanisms irrespective of tau modulation. The potential role of tau kinases is even less clear – in one study of hibernating ground squirrels, the change in GSK3β activity with cooling was biphasic, commencing with an increase in activity (Su et al., 2008). Conditional knockdown or mutagenesis of cooling-associated tau phospho-epitopes (Hoover et al., 2010) at various temperatures would confirm whether increased tau phosphorylation has a specific role in the neuroprotective effect of cooling.

The molecular mechanisms underlying hypothermic neuroprotection are ill-defined, although candidate pathways are implicated in other model systems and within the cyclical adaptation of some mammalian brains to hibernation (Arendt et al., 2003; Chip et al., 2011; Stieler et al., 2011; Yenari and Han, 2012; Tong et al., 2013). The synaptic plasticity of hibernation has been linked to modification of tau, in a manner that closely resembles hypothermic transitions in hCNs (Arendt et al., 2003). This re-acquisition of foetal-like tau could offer multiple benefits such as potentiation of

hCN tolerance to glutamate (Mondragon-Rodgriguez et al., 2012). Direct effects of cooling on metabolic rate and synaptic transmission have been described previously, although indirect mechanisms as described above are clearly also important. Both kinase-phosphatase asymmetry and ER-hormesis represent significant components of hypothermic preconditioning of human neurons and are essential to defend hCNs against an oxidative challenge. In the context of previous literature, certain predictions can be made from observations in these cooled neurons. RBM3 and CIRBP might inhibit pathways leading to programmed cell death, whilst promoting translation of core survival machinery and biasing the proteome towards a 'undifferentiated' or proliferative phenotype (Saito et al., 2010). Meanwhile, RD of key structural elements such as tau would not only increase neuronal resilience, but also prime neurons for repair. This would include a dramatic shift in post-translational properties that, at hypothermic temperatures, could trigger only an adaptive UPR: apoptotic outcomes would be overridden by inhibited synthesis of apoptotic proteins and activation of cold-shock chaperones. Like other UPR components such as AT4 (Halliday and Mallucci, 2014), these chaperones can escape cooling-induced suppression. CIRBP probably achieves this through transcriptional upregulation (the CIRBP promoter contains a cold-response element), whilst the RBM3 mRNA 5' leader sequence contains an internal ribosome entry site that would improve the efficiency of its translation in a cap-independent manner (Chappell et al., 2001; Sonna et al., 2002). These RBPs would then go on to stabilise essential transcripts needed for neuronal survival. Via GADD34 feedback and BiP upregulation cooling-induced ER-hormesis would ensure the continued synthesis and appropriate folding of critical proteins, whilst the otherwise global hypothermic

suppression of transcription and translation would prevent ER overload. Although definitive studies are needed to address the specific contribution of each of these phenomena to neuroprotection, their synchronous effects could substantially enhance the therapeutic potency of cooling.

The comparison drawn between cooling, hibernation and neurodegeneration raises questions about the true role of tau in neuronal and glial pathology. Tau hyperphosphorylation is similar in dividing and degenerating neurons suggesting that this increased tau phosphorylation state may induce ectopic cell cycle re-entry and activate regenerative signalling (Illenberger et al., 1998; Wen et al., 2004; Andorfer et al., 2005; Khurana et al., 2006; McShea et al., 2007; Wang and Liu, 2008; Frost et al., 2014). This response might seem *de rigeur* in the face of neuronal loss, but it is considered too rudimentary to be effective (Wang and Liu, 2008). Moreover, such aberrant mitosis is thought to precede NFT formation in AD (Wen et al., 2004). It may however be fortuitous if the division is asymmetric and has a fixed polarity; protein aggregates can be partitioned into the newest progeny cell, thus rescuing the longer-lived daughter from cellular stress and apoptosis (Rujano et al., 2006; Fuentealba et al., 2008; Singhvi and Garriga, 2009; Tyedmers et al., 2010; Chen et al., 2012a). Such a clearance mechanism has been demonstrated for Huntingtin aggresomes in HEK293 cells (Rujano et al., 2006). This highly conserved alternative to autophagy could explain a build up of aggregates in the brain before any measurable neuronal loss (Rujano et al., 2006). Interestingly, proteins which are specifically targeted for proteasomal degradation such as GSK3 appear to be preferentially inherited by one mitotic daughter during somatic cell division in a

microtubule-dependent manner (Fuentealba et al., 2008). It is reasonable to consider that cell cycling aspects of 'tauopathy' are merely unguided attempts at neuronal repair (Wang and Liu, 2008) – although an alternative hypothesis is that newborn cells are generated for the deposition of tangles, or to compensate for tangle-bearing neurons. The 'propagation' of tangle material by cell division is unlikely to be frequent enough in the mature brain to explain the 'prion-like' pattern of NFT spread, but it raises the possibility that certain neurons are sacrificed as garbage repositories so that others may be tangle-free. In aged htau mice, markers of cell cycle re-entry do not exist in neuronal populations that bear NFTs – many of which appear morphologically 'healthy' (Andorfer et al., 2005). The specific markers expressed suggest that cells are halted at G1/S and do not proceed to G2/M phase, implying that mitosis is incomplete (Andorfer et al., 2005). This is consistent with the concept that NFTs afford some protective function, but it is also reminiscent of the cell cycle arrest that occurs during cold-shock (Fujita, 1999; Lleonart et al., 2010). Changes in microtubule function, as a result of early tau hyperphosphorylation may prevent cell cycle progression in a proportion of neurons that do not have the capacity to compartmentalize tau oligomers into insoluble aggregates (Andorfer et al., 2005). Others have hypothesised that post-mitotic neurons redirect molecular pathways of the cell cycle towards maintaining synaptic plasticity and that synaptic loss in AD is misinterpreted as a loss of contact inhibition, thus triggering reactivation of the cell cycle (Arendt and Bruckner, 2007). Conceivably, a reduced capacity to upregulate RBM3 in neurodegeneration (Peretti et al., 2015) may facilitate this inappropriate divergence of plasticity mechanisms towards an archaic but stilted ontogenic programme. Since cerebral ischaemia can

bring about cell cycle re-entry and Alzheimer-like tau phosphorylation, it seems likely that several types of physiological stress (including hypothermia) produce a similar response (Wen et al., 2004). More recently, it was shown that pathological tau induces oxidative stress-mediated heterochromatin relaxation leading to a shift in the global genetic programme that promotes neuronal dedifferentiation (Frost et al., 2014). This effect may be compounded by loss of function of dephosphorylated tau that normally accumulates in neuronal nuclei in response to oxidative and heat stress and protects DNA from damage (Sultan et al., 2010). Together with the 'stem-like' features promoted by CIRBP expression (Saito et al., 2010), these findings add to the weight of evidence that tau modulation and cold-shock protein induction during cooling cooperate to trigger RD.

In response to cooling, hCNs displayed all the hallmarks of an adaptive, preconditioning UPR response: mild ER stress and activation of all 3 ER-stress transducers, a low level of CHOP induction that was insufficient to effect apoptosis, absence of detectable levels of phospho-eIF2α, and residual expression of key ER chaperones (Rutkowski et al., 2006; Tabas, 2011; Rzechorzek et al., 2015). UPR-mediated preconditioning has already shown therapeutic promise in a model of PD (Valdes et al., 2014), however, preconditioning critically depends on the duration and intensity of the primary stress (Klose et al., 2004; Tabas, 2011). Hence these parameters, in addition to the rate of onset of hypothermic effects likely dictated the fate of cooled hCNs. What has not been addressed here is the reversibility of these effects and the period over which they would remain protective – this is part of

ongoing work. Indeed in human cells, proteasome-mediated turnover of GADD34 is critical for the orderly synthesis and folding of proteins in the ER and prolonged GADD34 function may actually lead to proteotoxicity (Brush et al, 2008). Another recent study has highlighted the detrimental effects of chronic (2 w) cold water stress *in vivo* which precipitated Alzheimer-like pathology and associated cognitive deficits in the rat (Ahmadian-Attari et al., 2015). In the clinic TH is necessarily transient, and must be tapered with extreme care to minimise the risks of re-warming (Choi et al., 2012).

Proteasome inhibitors (such as MG-132), alternative inducers of ER stress (such as thapsigargin which, unlike Tm, blocks calcium reuptake into the ER lumen and does not require protein synthesis to take effect (Rutkowski et al., 2006)) and chemical chaperones (such as 4-phenylbutyric acid (PBA) and tauroursodeoxycholic acid (TUDCA) that effectively block the whole UPR) will help to determine the proportional contribution of ER-hormesis to hypothermic neuroprotection (Paschen et al., 2003; Ozcan et al., 2006; Engin and Hotamisligil, 2010; Begum et al., 2014; van der Harg et al., 2014). In addition to salubrinal and guanabenz, valproate is a widely prescribed drug used to treat bipolar disorder and epilepsy (Hoozemans and Scheper, 2012). Valproate can increase the levels of BiP and GRP94 and might be a useful experimental modulator of proteostatic priming in cooled hCNs (Bown et al., 2000). This drug can also inhibit GSK3 activity (Kim et al., 2005; Hoozemans and Scheper, 2012) and could help to tease out phospho-tau-UPR interactions in this system. GSK3 inhibition can reduce CHOP activation, shifting the cell towards survival – a mechanism that could potentially manifest under hypothermic conditions

(Meares et al., 2011). Finally, more specific modulators of UPR components such as Ire1α kinase-inhibiting RNase attenuators (KIRAs) that selectively enhance XBP1 splicing are emerging and will promote future delineation of branch-dependent effects (Han et al., 2009; Engin and Hotamisligil, 2010; Bouchecareilh et al., 2011). In particular, the Ire1α-JUN pathway is required for activation of autophagy after ER stress (Hoozemans and Scheper, 2012) and thus could play a significant role in hypothermic preconditioning of patient-derived neurons against protein aggregates. Another extension of UPR-driven benefits beyond the ER might include PERK-mediated upregulation of antioxidant responses via phosphorylation of nuclear factor (erythroid-derived 2)-like-2 (Nrf2) (Cullinan et al., 2003; Hoozemans and Scheper, 2012). The Nrf2 pathway is more active in astrocytes than neurons (Kraft et al., 2004; Baxter, 2011), but can protect neurons if overexpressed in a cell-autonomous manner or if upregulated in astrocytes (Soriano et al., 2008; Vargas et al., 2008; Chen et al., 2009; Calkins et al., 2010; Baxter, 2011). Such a mechanism reversed astrocytic neurotoxicty in the SOD1 mouse model of ALS (Vargas et al., 2008). Interaction of the UPR with these other stress pathways may be important to the 'cross-tolerance' induced by cooling. PERK-mediated Nrf2 activation may have specifically contributed to hCN defences against oxidative stress by a cell-autonomous or indeed non-cell autonomous mechanism (given the small proportion of GFAP-positive cells in hCN cultures).

## 7.2 Concluding statement

Historically, increased tau phosphorylation has been associated with neural demise. By contrast, the data above indicates that real-time tau transitions under hypothermia

promote neuronal survival. In addition, cold-stress upregulates key chaperone-driven pathways in a manner that favours cytoprotective outputs of the UPR. Whilst hypothermia has been shown to activate a beneficial Hsp response in mesenchymal stem cells (Stolzing et al., 2006), PERK activation in COS7 cells at 10°C (Hofman et al., 2012) and consolidation of the UPR in torpid hamsters (van der Harg et al., 2014), this thesis provides the first demonstration of a cold-stress induced UPR cascade in cells (Rzechorzek et al., 2015). Furthermore, as far as the author is aware, this is the first report of cooling-induced ER-hormesis in any biological system. Future work will establish which of these cold-stress-induced changes are required for hCN protection, how they might interact and whether they can be targeted for therapeutic gain. As others have intimated (Lee et al., 2005), therapeutic strategies aimed at reducing tau phosphorylation may inadvertently undermine critical neuronal stress responses that enable survival of a cell facing extreme and relentless metabolic pressure. The work described herein starts to unpick the complex molecular circuits that integrate cooling-induced preconditioning with proteostasis, and re-invests the concept of tau as a guardian of neural integrity. Prospects for realising 'tau-driven' rather than 'tau-targeted' therapies are thus a step closer. To date, hypothermic preconditioning has protected hCNs against oxidative, excitotoxic and ER stress, all of which feature in traumatic as well as degenerative processes. This 'cross-tolerance' effect (Rutkowski et al. 2006; Stetler et al., 2014) highlights the potential of molecular neurocryobiology to yield innovative therapeutic targets for devastating disorders. Overall, this thesis delivers mechanistic insight into hypothermic neuroprotection, in a system highly relevant to its clinical application.

# References


Aarts E, Verhage M, Veenvliet JV, Dolan CV, van der Sluis S (2014) A solution to dependency: using multilevel analysis to accommodate nested data. Nat Neurosci 17:491-6.

Abdel Malek MA, Jagannathan S, Malek E, Sayed DM, Elgammal SA, Abd El-Azeem HG, Thabet NM, Driscoll JJ (2014) Molecular chaperone GRP78 enhances aggresome delivery to autophagosomes to promote drug resistance in multiple myeloma. Oncotarget 6:3098-110.

Abisambra JF, Jinwal UK, Blair LJ, O'Leary JC 3rd, Li Q, Brady S, Wang L, Guidi CE, Zhang B, Nordhues BA, Cockman M, Suntharalingham A, Li P, Jin Y, Atkins CA, Dickey CA (2013) Tau accumulation activates the unfolded protein response by impairing endoplasmic reticulum-associated degradation. J Neurosci 33:9498-507.

Adachi M, Sohma O, Tsuneishi S, Takada S, Nakamura H (2001) Combination effect of systemic hypothermia and caspase inhibitor administration against hypoxic-ischaemic brain damage in neonatal rats. Pediatr Res 50:590-5.

Adams SJ, Crook RJ, Deture M, Randle SJ, Innes AE, yu XZ, Lin WL, Dugger BN, McBride M, Hutton M, Dickson DW, McGowan E (2009) Overexpression of wild-type murine tau results in progressive tauopathy and neurodegeneration. Am J Pathol 175:1598-609.

Adembri C, Venturi L, Tani A, Chiarugi A, Gramigni E, Cozzi A, Pancani T, De Gaudio RA, Pellegrini-Giampietro DE (2006) Neuroprotective effects of propofol in models of cerebral ischemia: inhibition of mitochondrial swelling as a possible mechanism. Anesthesiology 104:80-9.

Ahmadian-Attari MM, Dargahi L, Mosaddegh M, Kamalinejad M, Khallaghi B, Noorbala F, Ahmadiani A (2015) Impairment of rat spatial leanring and memory in a new model of cold water-induced chronic hypothermia: implication for Alzheimer's disease. Neurotox Res [Epub ahead of print].

Akaji K, Suga A, Fujino T, Mayanagi K, Inamasu J, Horiguchi T, Sato S, Kawase T (2003) Effect of intra-ischaemic hypothermia on the expression of c-Fos and c-Jun, and DNA binding activity of AP-1 after focal cerebral ischaemia in rat brain. Brain Res 975:149-57.

Al-Fageeh MB, Marchant RJ, Carden MJ, Smales CM (2006) The cold-shock response in cultured mammalian cells: harnessing the response for the improvement of recombinant protein production. Biotechnol Bioeng 93:829-35.

Al-Fageeh MB, Smales CM (2006) Control and regulation of the cellular responses to cold shock: the responses in yeast and mammalian systems. Biochem J 397:247-259.

Ali YO, Ruan K, Zhai RG (2012) NMNAT suppresses tau-induced neurodegeneration by promoting clearance of hyperphosphorylated tau oligomers in a Drosophila model of tauopathy. Hum Mol Genet 21:237-50.

Allen B, Ingram E, Takao M, Smith MJ, Jakes R, Virdee K, Yoshida H, Holzer M, Craxton M, Emson PC, Atzori C, Migheli A, Crowther RA, Ghetti B, Spillantini MG, Goedert M (2002) Abundant tau filaments and nonapoptotic neurodegeneration in transgenic mice expressing human P301S tau protein. J Neurosci 22:9340-51.

Alonso AC, Grundke-Iqbal I, Iqbal K (1996) Alzheimer's disease hyperphosphorylated tau sequesters normal tau into tangles of filaments and disassembles microtubules. Nat Med 2:783-787.

Alonso Adel C, Li B, Grundke-Iqbal K (2006) Polymerization of hyperphosphorylated tau into filaments eliminates its inhibitory activity. Proc Natl Acad Sci USA 103:8864-9.

Alonso AD, Zaidi T, Novak M, Barra HS, Grundke-Iqbal I, Iqbal K (2001)
Interaction of tau isoforms with Alzheimer's disease abnormally hyperphosphorylated tau and in vitrophosphorylation into the disease-like protein. J Biol Chem 276:37967-73.

Alzheimer A (1907) Ubereine eigenartige Erkrankung der Hirnrinde. Allgemeine Zeitschrift fur Psychiatrie und Psychisch-Gerichtliche Medizin 64:146-148.

Amos LA (2014) Why do brains need tau (MAPT)? FEBS J 281:iv-v.

Andorfer C, Acker CM, Kress Y, Hof PR, Duff K, Davies P (2005) Cell-cycle reentry and cell death in transgenic mice expressing nonmutant human tau isoforms. J Neurosci 25:5446-54.

Andorfer C, Kress Y, Espinoza M, de Silva R, Tucker KL, Barde YA, Duff K, Davies P (2003) Hyperphosphorylation and aggregation of tau in mice expressing normal human tau isoforms. J Neurochem 86:582-90.

Andreadis A (2006) Misregulation of tau alternative splicing in neurodegeneration and dementia. Prog Mol Subcell Biol 44:89-107.

Andreadis A (2005)
Tau gene alternative splicing: expression patterns, regulation and modulation of function in normal br ain andneurodegenerative diseases. Biochim Biophys Acta 1739:91-103.

Andreadis A (2012) Tau splicing and the intricacies of dementia. J Cell Physiol 227:1220-5.

Andreadis A, Broderick JA, Kosik KS (1995) Relative exon affinities and suboptimal splice site signals lead to non-equivalence of two cassette exons. Nucleic Acids Res 23: 3585-93.

Andreadis A, Brown WM, Kosik KS (1992) Structure and novel exons of the human tau gene. Biochemistry 31:10626-33.

Angel P, Karin M (1991) The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim Biophys Acta 1072:129-57.

Antonic A, Dottori M, Leung J, Sidon K, Batchelor PE, Wilson W, Macleod MR, Howells DW (2014) Hypothermia protects human neurons. Int J Stroke 9:544-52.

Aoki M, Tamatani M, Taniguchi M, Yamaguchi A, Bando Y, Kasai K, Miyoshi Y, Nakamura Y, Vitek MP, Tohyama M, Tanaka H, Sugimoto H (2001)
Hypothermic treatment restores glucose regulated protein 78 (GRP78) expression in ischemic brain. Brain Res Mol Brain Res 95:117-28.

Archer SN, Laing EE, Möller-Levet CS, van der Veen DR, Bucca G, Lazar AS, Santhi N, Slak A, Kabiljo R, von Schantz M, Smith CP, Dijk DJ (2014)
Mistimed sleep disrupts circadian regulation of the human transcriptome. Proc Natl Acad Sci U S A 111:E682-91.

Arendt T (2004) Neurodegeneration and plasticity. Int J Dev Neurosci 22:507-14.

Arendt T, Brückner MK (2007) Linking cell-cycle dysfunction in Alzheimer's disease to a failure of synaptic plasticity. Biochim Biophys Acta 1772:413-21

Arendt T, Brückner MK, Bigl V, Marcova L (1995a) Dendritic reorganisation in the basal forebrain under degenerative conditions and its defects in Alzheimer's disease. II. Ageing, Korsakoff's disease, Parkinson's disease, and Alzheimer's disease. J Comp Neurol 351:189-222.

Arendt T, Brückner MK, Bigl V, Marcova L (1995b) Dendritic reorganisation in the basal forebrain under degenerative conditions and its defects in Alzheimer's disease. III. The basal forebrain compared with other subcortical areas. J Comp Neurol 351:223-46.

Arendt T, Brückner MK, Gertz HJ, Marcova L (1998) Cortical distribution of neurofibrillary tangles in Alzheimer's disease matches the pattern of neurons that retain their capacity of plastic remodelling in the adult brain. Neuroscience 83:991-1002.

Arendt T, Bullmann T (2013) Neuronal plasticity in hibernation and the proposed role of the microtubule-associated protein tau as a "masterswitch" regulating synaptic gain in neuronal networks. Am J Physiol Regul Integr Comp Physiol 305:R478-89.

Arendt T, Marcova L, Bigl V, Brückner MK (1995c) Dendritic reorganisation in the basal forebrain under degenerative conditions and its defects in Alzheimer'sdisease. I. Dendritic organisation of the normal human basal forebrain. J Comp Neurol 351:169-88.

Arendt T, Stieler J, Strijkstra AM, Hut RA, Rüdiger J, Van der Zee EA, Harkany T, Holzer M, Härtig W (2003) Reversible paired helical filament-like phosphorylation of tau is an adaptive process associated with neuronal plasticity in hibernating animals. J Neurosci 23:6972-81.

Arrasate M, Mitra S, Schweitzer ES, Segal MR, Finkbeiner S (2004) Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. Nature 431:805-10.

Arriagada PV, Growdon JH, Hedley-Whyte ET, Hyman BT (1992) Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. Neurology 42:631-639.

Arrich J, Holzer M, Havel C, Mullner M, Herkner H (2012) Hypothermia for neuroprotection in adults after cardiopulomonary resuscitation. Cochrane Database Syst Rev 9:CD004128.

Artero-Castro A, Callejas FB, Castellvi J, Kondoh H, Carnero A, Fernández-Marcos PJ, Serrano M, Ramón y Cajal S, Lleonart ME (2009) Cold-inducible RNA-binding protein bypasses replicative senescence in primary cells through extracellular signal-regulated kinase 1 and 2 activation. Mol Cell Biol 29:1855-68.

Atlas R, Behar L, Sapoznik S, Ginzburg I (2007) Dynamic association with polysomes during P19 neuronal differentiation and an untranslated-region-dependenttranslation regulation of the tau mRNA by the tau mRNA-associated proteins IMP1, HuD, and G3BP1. J Neurosci Res 85:173-83.

Avila J, Díaz-Nido J (2004) Tangling with hypothermia. Nat Med 10:460-1.

Avila J, León-Espinosa G, García E, García-Escudero V, Hernández F, Defelipe J (2012) Tau Phosphorylation by GSK3 in Different Conditions. Int J Alzheimers Dis 2012:578373.

Avila J, Lucas JJ, Perez M, Hernandez F (2004) Role of tau protein in both physiological and pathological conditions. Physiol Rev 84:361-84.

Azzimondi G, Bassein L, Nonino F, Fiorani L, Vignatelli L, Re G, D'Alessandro R (1995) Fever in acute stroke worsens prognosis. A prospective study. Stroke 26:2040-3.

Bading H, Ginty DD, Greenberg ME (1993) Regulation of gene expression in hippocampal neurons by distinct calcium signalling pathways. Science 260:181-6.

Baker CJ, Fiore AJ, Frazzini VI, Choudhri TF, Zubay GP, Solomon RA (1995) Intraischemic hypothermia decreases the release of glutamate in the cores of permanent focal cerebral infarcts. Neurosurgery 36:994-1001.

Baker FC, Waner JI, Vieira EF, Taylor SR, Driver HS, Mitchell D (2001)
Sleep and 24 hour body temperatures:
a comparison in young men, naturally cycling women and women taking hormonal contraceptives. J Physiol 530:565-74.

Balch WE, Morimoto RI, Dillin A, Kelly JW (2008) Adapting proteostasis for disease intervention. Science 319:916-9.

Baldi A, Battista T, De Luca A, Santini D, Rossiello L, Baldi F, Natali PG, Lombardi D, Picardo M, Felsani A, Paggi MG (2003) Identification of genes down-regulated during melanoma progression: a cDNA array study. Exp Dermatol 12:213-8.

Ballesteros MA, Marin MJ, Martin MS, Rubio-Lopez MI, Lopez-Hoyos M, Minambres E (2013) Effect of neuroprotective therapies (hypothermia and cyclosporine a) on dopamine-induced apoptosis in human neuronal SH-SY5Y cells. Brain Inj 27:354-60.

Bancher C, Brunner C, Lassmann H, Budka H, Jellinger K, Wiche G, Seitelberger F, Grundke-Iqbal I, Iqbal K, Wisniewski HM (1989)
Accumulation of abnormally phosphorylated tau precedes the formation of neurofibrillary tangles in Alzheimer's disease. Brain Res 477:90-9.

Bando Y, Katayama T, Kasai K, Taniguchi M, Tamatani M, Tohyama M (2003) GRP94 (94 kDa glucose-regulated protein) suppresses ischemic neuronal cell death against ischemia/reperfusion injury. Eur J Neurosci 18:829-40.

Baranov D, Bickler PE, Crosby GJ, Culley DJ, Eckenhoff MF, Eckenhoff RG, Hogan KJ, Jevtovic-Todorovic V, Palotás A, Perouansky M, Planel E, Silverstein JH, Wei H, Whittington RA, Xie Z, Zuo Z (2009) First International Workshop on Anesthetics and Alzheimer's Disease. Consensus statement: First International Workshop on Anesthetics and Alzheimer's disease. Anesth Analg 108:1627-30.

Barclay JW, Robertson RM (2003) Role for calcium in heat shock-
mediated synaptic thermoprotection in Drosophila larvae. J Neurobiol 56:360-71.

Barone FC, Feuerstein GZ, White RF (1997)
Brain cooling during transient focal ischemia provides complete neuroprotection. Neurosci Biobehav Rev 21:31-44.

Batulan Z, Shinder GA, Minotti S, He BP, Doroudchi MM, Nalbantoglu J, Strong MJ, Durham HD (2003) High threshold for induction of the stress response in motor neurons is associated with failure to activate HSF1. J Neurosci 23:5789-5798.

Baxter SL, Allard DE, Crowl C, Sherwood NT (2014)
Cold temperature improves mobility and survival in Drosophila models of autosomal-dominant hereditary spasticparaplegia (AD-HSP). Dis Model Mech 7:1005-12.

Baxter P (2011) Neuronal activity-dependent protection against apoptotic and oxidative insults. Thesis, The Univeristy of Edinburgh, p32, p102.

Bechtold DA, Rush SJ, Brown IR (2000) Localization of the heat-shock protein Hsp70 to the synapse following hyperthermic stress in the brain. J Neurochem 74:641-6.

Beer C, Buhr P, Hahn H, Laubner D, Wirth M (2003)
Gene expression analysis of murine cells producing amphotropic mouse leukaemia virus at a cultivationtemperature of 32 and 37 degrees C. J Gen Virol 84:1677-86.

Begum G, Kintner D, Liu Y, Cramer SW, Sun D (2012) DHA inhibits ER $Ca^{2+}$ release and ER stress in astrocytes following in vitro ischemia. J Neurochem 120:622-630.

Begum G, Yan HQ, Li L, Singh A, Dixon CE, Sun D (2014)
Docosahexaenoic acid reduces ER stress and abnormal protein accumulation and improves neuronal f unction following traumatic brain injury. J Neurosci 34:3743-55.

Belinksy GS, Antic SD (2013) Mild hypothermia inhibits differentiation of human embryonic and induced pluripotent stem cells. Biotechniques 55:79-82.

Bell KF, Al-Mubarak B, Fowler JH, Baxter PS, Gupta K, Tsujita T, Chowdhry S, Patani R, Chandran S, Horsburgh K, Hayes JD, Hardingham GE (2011) Mild oxidative stress activates Nrf2 in astrocytes, which contributes to neuroprotective ischemic preconditioning. Proc Natl Acad Sci U S A 108(1):E1-2; author reply E3-4.

Bell-Pedersen D, Cassone VM, Earnest DJ, Golden SS, Hardin PE, Thomas TL, Zoran MJ (2005) Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nat Rev Genet 6:544-56.

Ben-Zvi A, Miller EA, Morimoto RI (2009) Collapse of proteostasis represents an early molecular event in Caenorhabditis elegans aging. Proc Natl Acad Sci U S A 106:14914-9.

Bernard SA, Gray TW, Buist MD, Jones BM, Silvester W, Gutteridge G, Smith K (2002)
Treatment of comatose survivors of out-of-hospital cardiac arrest with induced hypothermia. N Engl J Med 346:557-63.

Bernert G, Fountoulakis M, Lubec G (2002) Manifold decreased protein levels of matrin 3, reduced motor protein HMP and hlark in foetal Down's syndrome brain. Proteomics 2:1752-1757.

Berns M, Seeberg L, Schmidt M, Kerner T (2009) High-dose propofol triggers short-
term neuroprotection and long-term neurodegeneration in primary neuronalcultures from rat embryos. J Int Med Res 37:680-8.

Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D (2000) Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nat Cell Biol 2:326-32.

Bhat R, Xue Y, Berg S, Hellberg S, Ormo M, Nilsson Y, Radesater AC, Jerning E, Markgren PO, Borgegard T, Nylof M, Gimenez-Cassina A, Hernandez F, Lucas JJ, Diaz-Nido J, Avila J (2003) Structural insights and biological effects of glycogen synthase kinase 3-specific inhibitor AR-A014418. J Biol Chem 278: 45937-45.

Bickler PE, Warren DE, Clark JP, Gabatto P, Gregersen M, Brosnan H (2012)
Anesthetic protection of neurons injured by hypothermia and rewarming: roles of intracellular Ca2+ andexcitotoxicity. Anesthesiology 117:280-92.

Bienik KF, Murray ME, Rutherford NJ, Castanedes-Casey M, DeJesus-Hernandez M, Liesinger AM, Baker MC, Boylan KB, Rademkers R, Dickson DW (2013) Tau pathology in frontotemporal lobar degeneration with C9ORF72 hexanucleotide repeat expansion. Acta Neuropathol 125: 289-302.

Biernat J, Wu YZ, Timm T, Zheng-Fischhöfer Q, Mandelkow E, Meijer L, Mandelkow EM (2002)
Protein kinase MARK/PAR-
1 is required for neurite outgrowth and establishment of neuronal polarity. Mol Biol Cell 13:4013-28.

Binder LI, Frankfurter A, Rebhun KI (1985) The distribution of tau in the mammalian central nervous system. J Cell Biol 101:1371-78.

Bilican B, Livesey MR, Haghi G, Qiu J, Burr K, Siller R, Hardingham GE, Wyllie DJ, Chandran S (2014) Physiological normoxia and absence of EGF is required for the long-term propagation of anterior neural precursors from human pluripotent cells. PLoS One 9:e85932.

Bilican B, Serio A, Barmada SJ, Nishimura AL, Sullivan GJ, Carrasco M, Phatnani HP, Puddifoot CA, Story D, Fletcher J, Park IH, Friedman BA, Daley GQ,Wyllie DJ, Hardingham GE, Wilmut I, Finkbeiner S, Maniatis T, Shaw CE, Chandran S (2012) Mutant induced pluripotent stem cell lines recapitulate aspects of TDP-43 proteinopathies and reveal cell-specific vulnerability. Proc Natl Acad Sci U S A 109:5803-8.

Bingol B, Schuman EM (2006) Activity-dependent dynamics and sequestration of proteasomes in dendritic spines. Nature 441:1144-8.

Bini R, Olivero G, Trombetta A, Castagna E, Cotogni P (2008) Effects of dimethyl sulfoxide, pyrrolidine dithiocarbamate, and methylprednisolone on nuclear factor-kappaB andheat shock protein 70 in a rat model of hemorrhagic shock. J Trauma 64:1048-54.

Blackstone E, Morrison M, Roth MB (2005) H2S induces a suspended animation-like state in mice. Science 308:518.

Blard O, Frébourg T, Campion D, Lecourtois M (2006) Inhibition of proteasome and Shaggy/Glycogen synthase kinase-3beta kinase prevents clearance ofphosphorylated tau in Drosophila. J Neurosci Res 84:1107-15.

Bliss TV, Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361:31-9.

Bodner RA, Housman DE, Kazantsev AG (2006) New directions for neurodegenerative disease therapy: using chemical compounds to boost the formation of mutant protein inclusions. Cell Cycle 5:1477-80.

Boerema AS, Keijser JN, Bouma HR, van der Zee EA, Strijkstra AM (2012) Chapter 17: 'The Brain at Low Temperature: Tau Hyperphosphorylation Dynamics in Hibernation Torpor' T. Ruf et al. (eds.), Living in a Seasonal World, DOI: 10.1007/978-3-642-28678-0_17, _ Springer-Verlag Berlin Heidelberg 2012 191-199.

Bolkan BJ, Kretzschmar D (2014) Loss of Tau results in defects in photoreceptor development and progressive neuronal degeneration in Drosophila. Dev Neurobiol 74:1210-25.

Boluda S, Iba M, Zhang B, Raible KM, Lee VM, Trojanowski JQ (2015) Differential induction and spread of tau pathology in young PS19 tau transgenic mice following intracerebral injections of pathological tau from Alzheimer's disease or corticobasal degeneration brains. Acta Neuropathol 129:221-37.

Bonda DJ, Castellani RJ, Zhu X, Nunomura A, Lee HG, Perry G, Smith MA (2011) A novel perspective on tau in Alzheimer's disease. Curr Alzheimer Res 8:639-42.

Bossenmeyer-Pourié C, Koziel V, Daval JL (2000) Effects of hypothermia on hypoxia-induced apoptosis in cultured neurons from developing rat forebrain: comparison with preconditioning. Pediatr Res 47:385-91.

Borg J (1980) Axonal refractory period of single short toe extensor motor units in man. J Neurol Neurosurg Psychiatry 43:917-24.

Bottley A, Phillips NM, Webb TE, Willis AE, Spriggs KA (2010) eIF4A inhibition allows translational regulation of mRNAs encoding proteins involved in Alzheimer's disease. PLoS One 5pii: e13030.

Bouchecareilh M, Higa A, Fribourg S, Moenner M, Chevet E (2011) Peptides derived from the bifunctional kinase/RNase enzyme IRE1α modulate IRE1α activity and protect cells from endoplasmic reticulum stress. FASEB J 25:3115-29.

Bown CD, Wang JF, Young LT (2000)
Increased expression of endoplasmic reticulum stress proteins following chronic valproate treatment o f rat C6 glioma cells. Neuropharmacology 39:2162-9.

Boyce M, Bryant KF, Jousse C, Long K, Harding HP, Scheuner D, Kaufman RJ, Ma D, Coen DM, Ron D, Yuan J (2005) A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress. Science 307:935-9.

Braak H, Braak E (1991) Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol 82:239-59.

Braak H, Braak E (1994) A sequence of cytoskeleton changes related to the formation of neurofibrillary tangles and neuropil threads. Acta Neuropathol 87: 554-567.

Braak H, Braak E (1998) Evolution of neuronal changes in the course of Alzheimer's disease. J Neural Transm Suppl 53:127-140.

Braak H, Del Tredici K (2011) Alzheimer's pathogenesis: is there neuron-to-neuron propagation? Acta Neuropathol 121:589-95.

Braak H, Thal DR, Ghebremedhin E, Del Tredici K (2011) Stages of the pathologic process in Alzheimer disease: age categories from 1 to 100 years. J Neuropathol Exp Neurol 70:960-9.

Bramblett GT, Goedert M, Jakes R, Merrick SE, Trojanowski JQ, Lee VM (1993)
Abnormal tau phosphorylation at Ser396
in Alzheimer's disease recapitulates development and contributes toreduced microtubule binding. Neuron 10:1089-99.

Bramlett HM, Dietrich WD (2004) Pathophysiology of cerebral ischemia and brain trauma: similarities and differences. J Cereb Blood Flow Metab 24:133-50.

Brandon Westover M, Ching S, Kumaraswamy VM, Akeju O, Pierce E, Cash SS, Kilbride R, Brown EN, Purdon PL (2015) The human brust suppression electroencephalogram of deep hypothermia. Clin Neurophysiol S1388-2457:00014. [Epub ahead of print].

Brandt R (1996) The tau proteins in neuronal growth and development. Front Biosci 1:d118-30.

Brandt R, Léger J, Lee G (1995) Interaction of tau with the neural plasma membrane mediated by tau's amino-terminal projection domain. J Cell Biol 131:1327-40.

Brennand K, Savas JN, Kim Y, Tran N, Simone A, Hashimoto-Torii K, Beaumont KG, Kim HJ, Topol A, Ladran I, Abdelrahim M, Matikainen-Ankney B, Chao SH, Mrksich M, Rakic P, Fang G, Zhang B, Yates JR 3rd, Gage FH (2014)
Phenotypic differences in hiPSC NPCs derived from patients with schizophrenia. Mol Psychiatry doi: 10.1038/mp.2014.22. [Epub ahead of print]

Bretteville A, Marcouiller F, Julien C, El Khoury NB, Petry FR, Poitras I, Mouginot D, Lévesque G, Hébert SS, Planel E (2012) Hypothermia-induced hyperphosphorylation: a new model to study tau kinase inhibitors. Sci Rep 2:480.

Bretteville A, Planel E (2008) Tau aggregates: toxic, inert, or protective species? J Alzheimers Dis 14:431-6.

Brewer LD, Thibault O, Staton J, Thibault V, Rogers JT, Garcia-Ramos G, Kraner S, Landfield PW, Porter NM (2007)
Increased vulnerability of hippocampal neurons with age in culture: temporal association with increas

es inNMDA receptor current, NR2A subunit expression and recruitment of L-type calcium channels. Brain Res 1151:20-31.

Bright J, Hussain S, Dang V, Wright S, Cooper B, Byun T, Ramos C, Singh A, Parry G, Stagliano N, Griswold-Prenner I (2014) Human secreted tau increases amyloid-beta production. Neurobiol Aging pii: S0197-4580(14)00602-2.

Briner A, Nikonenko I, De Roo M, Dayer A, Muller D, Vutskits L (2011) Developmental Stage-dependent persistent impact of propofol anesthesia on dendritic spines in the rat medial prefrontal cortex. Anesthesiology 115:282-93.

Brion JP, Smith C, Couck AM, Gallo JM, Anderton BH (1993) Developmental changes in tau phosphorylation: foetal tau is transiently phosphorylated in a manner similar to paired helical filament-tau characteristic of Alzheimer's disease. J Neurochem 61:2071-80.

Brochu C, Cabrita MA, Melanson BD, Hamill JD, Lau R, Pratt MA, McKay BC (2013) NF-κB-dependent role for cold-inducible RNA binding protein in regulating interleukin 1β. PLoS One 8:e57426.

Brown V, Jin P, Ceman S, Darnell JC, O'Donnell WT, Tenenbaum SA, Jin X, Feng Y, Wilkinson KD, Keene JD, Darnell RB, Warren ST (2001) Microarray identification of FMRP-associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome. Cell 107:477-487.

Bruening W, Roy J, Giasson B, Figlewicz DA, Mushynski WE, Durham HD (1999) Up-regulation of protein chaperones preserves viability of cells expressing toxic Cu/Zn-superoxide dismutase mutants associated with amyotrophic lateral sclerosis. J Neurochem 72:693-699.

Brun A, Englund E (1981)
Regional pattern of degeneration in Alzheimer's disease: neuronal loss and histopathological grading. Histopathology 5:549-64.

Brush MH, Shenolikar S (2008) Control of cellular GADD34 levels by the 26S proteasome. Mol Cell Biol 28:6989-7000.

Buée L, Troquier L, Burnouf S, Belarbi K, Van der Jeugd A, Ahmed T, Fernandez-Gomez F, Caillierez R, Grosjean ME, Begard S, Barbot B, Demeyer D, Obriot H, Brion I, Buée-Scherrer V, Maurage CA, Balschun D, D'hooge R, Hamdane M, Blum D, Sergeant N (2010)
From tau phosphorylation to tau aggregation: what about neuronal death? Biochem Soc Trans 38:967-72.

Bullmann T, de Silva R, Holzer M, Mori H, Arendt T (2007)
Expression of embryonic tau protein isoforms persist during adult neurogenesis in the hippocampus. Hippocampus 17:98-102.

Burd CG, Dreyfuss G (1994) Conserved structures and diversity of functions of RNA-binding proteins. Science 265:615-21.

Burdon RH (1987) Temperature and animal cell protein synthesis. Symp Soc Exp Biol 41:113-133.

Burke D, Mogyoros I, Vagg R, Kiernan MC (1999)
Temperature dependence of excitability indices of human cutaneous afferents. Muscle Nerve 22:51-60.

Burattini S, Battistelli M, Falcieri E (2010) Morpho-functional features of in-vitro cell death induced by physical agents. Curr Pharm Des 16:1376-86.

Busciglio J, Lorenzo A, Yeh J, Yankner BA (1995) β-amyloid fibrils induce tau phosphorylation and loss of microtubule binding. Neuron 14:879-888.

Bush KT, Goldberg AL, Nigam SK (1997) Proteasome inhibition leads to a heat-shock response, induction of endoplasmic reticulum chaperones, and thermotolerance. J Biol Chem 272:9086-92.

Busija DW, Leffler CW (1987)
Hypothermia reduces cerebral metabolic rate and cerebral blood flow in newborn pigs. Am J Physiol 25:H869-73.

Busto R, Dietrich WD, Globus MY, Valdés I, Scheinberg P, Ginsberg MD (1987)
Small differences in intraischemic brain temperature critically determine the extent of ischemic neuro nal injury. J Cereb Blood Flow Metab 7:729-38.

Busto R, Globus MY, Dietrich WD, Martinez E, Valdés I, Ginsberg MD (1989)
Effect of mild hypothermia on ischemia-
induced release of neurotransmitters and free fatty acids in rat brain. Stroke 20:904-10.

Buttgereit F, Brand MD (1995) A hierarchy of ATP-consuming processes in mammalian cells. Biochem J 312:163-7.

Caffrey TM, Wade-Martins R (2007) Functional MAPT haplotypes: bridging the gap beween genotype andneuropathology. Neurobiol Dis 27: 1-10.

Calkins MJ, Vargas MR, Johnson DA, Johnson JA (2010) Astrocyte-
specific overexpression of Nrf2 protects striatal neurons from mitochondrial complex II inhibition. Toxicol Sci 115:557-68.

Campos F, Pérez-Mato M, Agulla J, Blanco M, Barral D, Almeida A, Brea D, Waeber C, Castillo J, Ramos-Cabrer P (2012) Glutamate excitoxicity is the key molecular mechanism which is influenced by body temperature during theacute phase of brain stroke. PLoS One 7:e44191.

Cano M, Wang L, Wan J, Barnett BP, Ebrahimi K, Qian J, Handa JT (2014) Oxidative stress induces mitochondrial dysfunction and a protective unfolded protein response in RPE cells. Free Radic Biol Med 69:1-14.

Cantero JL, Hita-Yañez E, Moreno-Lopez B, Portillo F, Rubio A, Avila J (2010) Tau protein role in sleep-wake cycle. J Alzheimers Dis 21:411-21.

Capani F, Saraceno GE, Botti V, Aon-Bertolino L, de Oliveira DM, Barreto G, Galeano P, Giraldez-Alvarez LD, Coirini H (2009)
Protein ubiquitination in postsynaptic densities after hypoxia in rat neostriatum is blocked by hypother mia. Exp Neurol 219:404-13.

Caradec J, Sirab N, Keumeugni C, Moutereau S, Chimingqi M, Matar C, Revaud D, Bah M, Manivet P, Conti M et al. (2010) 'Desperate house genes': the dramatic example of hypoxia. Br J Cancer 102: 1037-1043.

Cash AD, Aliev G, Siedlak SL, Nunomura A, Fujioka H, Zhu X, Raina AK, Vinters HV, Tabaton M, Johnson AB, Paula-Barbosa M, Avíla J, Jones PK, Castellani RJ, Smith MA, Perry G (2003) Microtubule reduction in Alzheimer's disease and aging is independent of tau filament formation. Am J Pathol 162:1623-7.

Castellani RJ, Nunomura A, Lee HG, Perry G, Smith MA (2008)
Phosphorylated tau: toxic, protective, or none of the above. J Alzheimers Dis 14:377-83.

Caviness VS Jr, Takahashi T, Nowakowski RS (1995) Numbers, time and neocortical neurogenesis: a general developmental and evolutionary model. Trends Neurosci 18:379-83.

Cerezo D, Cánovas M, García-Peñarrubia P, Martín-Orozco E (2015)
Collateral sensitivity to cold stress and differential BCL-2 family expression in new daunomycin-resistantlymphoblastoid cell lines. Exp Cell Res 331:11-20.

Chakrabarti A, Chen AW, Varner JD (2011) A review of the mammalian unfolded protein response. Biotechnol Bioeng 108:2777-93.

Chalmers KA, Love S (2007) Neurofibrillary tangles may interfere with Smad 2/3 signaling in neurons. J Neuropathol Exp Neurol 66:158-167.

Chan SF, Sucher NJ (2001) An NMDA receptor signaling complex with protein phosphatase 2A. J Neurosci 21:7985-92.

Chang NT, Huang LE, Liu AY (1993) Okadaic acid markedly potentiates the heat-induced hsp70 promoter activity. J Biol Chem 268:1436-9.

Chappell SA, Owens GC, Mauro VP (2001) A 5' leader of Rbm3, a cold-stress induced mRNA, mediates internal initiation of translation with increased efficiency under conditions of mild hypothermia. J Biol Chem 276:36917-22.

Chandran S, Svendsen C, Compston A, Scolding N (1998) Regional potential for oligodendrocyte generation in the rodent embryonic spinal cord following exposure to EGF and FGF-2. Glia 24:382-9.

Chau KW, Chan WY, Shaw PC, Chan HY (2006)
Biochemical investigation of Tau protein phosphorylation status and
its solubility properties in Drosophila. Biochem Biophys Res Commun 346:150-9.

Chen J, Herrup K (2012) Glutamine acts as a neuroprotectant against DNA damage, beta-amyloid and $H_2O_2$-induced stress. PLoS One 7:e33177.

Chen J, Kanai Y, Cowan NJ, Hirokawa N (1992) Projection domains of MAP2 and tau determine spacings between microtubules in dendrites and axons. Nature 360:674-7.

Chen B, Retzlaff M, Roos T, Frydman J (2011) Cellular strategies of protein quality control. Cold Spring Harb Perspect Biol 3:a004374.

Chen Y, Run X, Liang Z, Zhao Y, Dai CL, Iqbal K, Liu F, Gong CX (2014)
Intranasal insulin prevents anesthesia-induced hyperphosphorylation of tau in 3xTg-AD mice. Front Aging Neurosci 6:100.

Chen PC, Vargas MR, Pani AK, Smeyne RJ, Johnson DA, Kan YW, Johnson JA (2009) Nrf2-mediated neuroprotection in the MPTP mouse model of Parkinson's disease: Critical role for the astrocyte. Proc Natl Acad Sci U S A 106:2933-8.

Chen CM, Wu CT, Chiang CK, Liao BW, Liu SH (2012a)
C/EBP homologous protein (CHOP) deficiency aggravates hippocampal cell apoptosis and impairs m emory performance. PLoS One 7:e40801.

Chen J, Yuan L, Sun M, Zhang L, Zhang S. Screening of hibernation-related genes in
the brain of Rhinolophus ferrum equinum during hibernation (2008) Comp Biochem Physiol B Biochem Mol Biol 149:388-93.

Chen Q, Zhou Z, Zhang L, Wang Y, Zhang YW, Zhong M, Xu SC, Chen CH, Li L, Yu ZP (2012b) Tau protein is involved in morphological plasticity in hippocampal neurons in response to BDNF. Neurochem Int 60:233-42.

Cheng BC, Huang HS, Chao CM, Hsu CC, Chen CY, Chang CP (2013) Hypothermia may attenuate ischemia/reperfusion-induced cardiomyocyte death by reducing autophagy. Int J Cardiol 168:2064-9.

Chesta ME, Carbajal A, Arce CA, Bisig CG (2014) Serum-induced neurite retraction in CAD cells-involvement of an ATP-actin retractile system and the lack of microtubule-associated proteins. FEBS J 281:4767-78.

Childs C, Hiltunen Y, Vidyasagar R, Kauppinen RA (2007)
Determination of regional brain temperature using proton magnetic resonance spectroscopy to assess b rain-body temperature differences in healthy human subjects. Magn Reson Med 57:59-66.

Childs C, Vail A, Protheroe R, King AT, Dark PM (2005)
Differences between brain and rectal temperatures during routine critical care of patients with severe t raumatic brain injury. Anaesthesia 60:759-65.

Chip S, Zelmer A, Ogunshola OO, Felderhoff-Mueser U, Nitsch C, Bührer C, Wellmann S (2011) The RNA binding protein RBM3 is involved in hypothermia induced neuroprotection. Neurobiol Dis 43:388-396.

Chiu R, Boyle WJ, Meek J, Smeal T, Hunter T, Karin M (1988) The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. Cell 54:541-52.

Choi HA, Badjatia N, Mayer SA (2012) Hypothermia for acute brain injury – mechanisms and practical aspects. Nat Rev Neurol 8:214-222.

Choi SH, Kim YH, Hebisch M, Sliwinski C, Lee S, D'Avanzo C, Chen H, Hooli B, Asselin C, Muffat J, Klee JB, Zhang C, Wainger BJ, Peitz M,Kovacs DM, Woolf CJ, Wagner SL, Tanzi RE, Kim DY (2014) A three-dimensional human neural cell culture model of Alzheimer's disease. Nature 515:274-8.

Choi DW, Maulucci-Gedde M, Kriegstein AR (1987) Glutamate neurotoxicity in cortical cell culture. J Neurosci 7:357-68.

Choi DW, Rothman SM (1990) The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. Annu Rev Neurosci 13:171-82.

Clavaguera F, Akatsu H, Fraser G, Crowther RA, Frank S, Hench J, Probst A, Winkler DT, Reichwald J, Staufenbiel M, Ghetti B, Goedert M, Tolnay M (2013)
Brain homogenates from human tauopathies induce tau inclusions in mouse brain. Proc Natl Acad Sci U S A 110:9535-40.

Clavaguera F, Bolmont T, Crowther RA, Abramowski D, Frank S, Probst A, Fraser G, Stalder AK, Beibel M, Staufenbiel M, Jucker M, Goedert M, Tolnay M (2009)
Transmission and spreading of tauopathy in transgenic mouse brain. Nat Cell Biol 11:909-13.

Cleveland DW, Hwo SY, Kirschner MW (1977a) Purification of tau, a microtubule-associated protein that induces assembly of microtubules from purified tubulin. J Mol Biol 116:207-25.

Cleveland DW, Hwo SY, Kirschner MW (1977b) Physical and chemical properties of purified tau factor and the role of tau in microtubule assembly. J Mol Biol 116:227-47.

Cleveland DW, Rothstein JD (2001) From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS. Nat Rev Neurosci 2:806-819.

Clifton GL, Miller ER, Choi SC, Levin HS, McCauley S, Smith KR Jr, Muizelaar JP, Wagner FC Jr, Marion DW, Luerssen TG, Chesnut RM, Schwartz M (2001) Lack of effect of induction of hypothermia after acute brain injury. N Engl J Med 344:556-63.

Colbourne F, Sutherland GR, Auer RN (1999) Electron microscopic evidence against apoptosis as the mechanism of neuronal death in global ischemia. J Neurosci 19:4200-10.

Colby DW, Prusiner SB (2011) Prions. Cold Spring Harb Perspect Biol 3:a006833.

Coleshaw SR, Van Someren RN, Wolff AH, Davis HM, Keatinge WR (1983) Impaired memory registration and speed of reasoning caused by low body temperature. J Appl Physiol Respir Environ Exerc Physiol 55:27-31.

Colla E, Jensen PH, Pletnikova O, Troncoso JC, Glabe C, Lee MK (2012) Accumulation of toxic α-synuclein oligomer within endoplasmic reticulum occurs in α-synucleinopathy in vivo. J Neurosci 32:3301-5.

Compta Y, Martí MJ, Rey MJ, Ezquerra M (2009) Parkinsonism, dysautonomia, REM behaviour disorder and visual hallucinations mimicking synucleinopathy in a patient with progressive supranuclear palsy. J Neurol Neurosurg Psychiatry 80:578-9.

Conde C, Cáceres A (2009) Microtubule assembly, organization and dynamics in axons and dendrites. Nat Rev Neurosci 10:319-32.

Coomber B, Edwards D, Jones SJ, Shackleton TM, Goldschmidt J, Wallace MN, Palmer AR (2011) Cortical inactivation by cooling in small animals. Front Syst Neurosci 5:53.

Corbett D, Thornhill J (2000) Temperature modulation (hypothermic and hyperthermic conditions) and its influence on histological andbehavioral outcomes following cerebral ischemia. Brain Pathol 10:145-52.

Cork LC, Powers RE, Selkoe DJ, Davies P, Geyer JJ, Price DL (1988) Neurofibrillary tangles and senile plaques in aged bears. J Neuropathol Exp Neurol 47:629-41.

Corwin WL, Baust JM, Baust JG, Van Buskirk RG (2014) Characterization and modulation of human mesenchymal stem cell stress pathway response following hypothermic storage. Cryobiology 68:215-26.

Costa M, Squassina A, Piras IS, Pisanu C, Congiu D, Niola P, Angius A, Chillotti C, Ardau R, Severino G, Stochino E, Deidda A, Persico AM, Alda M, Del Zompo M (2015) Preliminary Transcriptome Analysis in Lymphoblasts from Cluster Headache and Bipolar Disorder Patients Implicates Dysregulation of Circadian and Serotonergic Genes. J Mol Neurosci [Epub ahead of print].

Costa-Mattioli M, Gobert D, Stern E, Gamache K, Colina R, Cuello C, Sossin W, Kaufman R, Pelletier J, Rosenblum K, Krnjević K, Lacaille JC, Nader K,Sonenberg N (2007) eIF2alpha phosphorylation bidirectionally regulates the switch from short- to long-term synaptic plasticity and memory. Cell 129:195-206.

Cowan CM, Mudher A (2013) Are tau aggregates toxic or protective in tauopathies? Front Neurol 4:114.

Couchie D, Mavilia C, Georgieff IS, Liem RK, Shelanski ML, Nunez J (1992) Primary structure of high molecular weight tau present in the peripheral nervous system. Proc Natl Acad Sci U S A 89:4378-81.

Crespo-Biel N, Theunis C, Borghgraef P, Lechat B, Devijver H, Maurin H, Van Leuven F (2014) Phosphorylation of protein Tau by GSK3β prolongs survival of bigenic Tau.P301L×GSK3β mice by delaying brainstem tauopathy. Neurobiol Dis 67:119-32.

Crimins JL, Pooler A, Polydoro M, Luebke JI, Spires-Jones TL (2013) The intersection of amyloid β and tau in glutamatergic synaptic dysfunction and collapse in Alzheimer's disease. Ageing Res Rev 12:757-63.

Crimins JL, Rocher AB, Luebke JI (2012) Electrophysiological changes precede morphological changes to frontal cortical pyramidal neurons in the rTg4510 mouse model of progressive tauopathy. Acta Neuropathol 124:777-95.

Crimins JL, Rocher AB, Peters A, Shultz P, Lewis J, Luebke JI (2011) Homeostatic responses by surviving cortical pyramidal cells in neurodegenerative tauopathy. Acta Neuropathol 122:551-564.

Cripps D, Thomas SN, Jeng Y, Yang F, Davies P, Yang AJ (2006) Alzheimer disease-specific conformation of hyperphosphorylated paired helical filament-Tau is polyubiquitinatedthrough Lys-48, Lys-11, and Lys-6 ubiquitin conjugation. J Biol Chem 281:10825-38.

Cullinan SB, Zhang D, Hannink M, Arvisais E, Kaufman RJ, Diehl JA (2003) Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. Mol Cell Biol 23:7198-209.

Czeisler CA, Weitzman Ed, Moore-Ede MC, Zimmerman JC, Knauer RS (1980) Human sleep: its duration and organization depend on its circadian phase. Science 210:1264-7.

Daan S, Barnes BM, Strijkstra AM (1991) Warming up for sleep? Ground squirrels sleep during arousals from hibernation. Neurosci Lett 128:265-8.

Dalle-Donne I, Rossi R, Milzani A, Di Simplicio P, Colombo R (2001) The actin cytoskeleton response to oxidants: from small heat shock protein phosphorylation to changes in the redox state of actin itself. Free Radic Biol Med 31:1624-32.

Danbolt NC. Glutamate uptake. Prog Neurobiol 65:1-105.

Danno S, Nishiyama H, Higashitsuji H, Yokoi H, Xue JH, Itoh K, Matsuda T, Fujita J (1997) Increased transcript level of RBM3, a member of the glycine-rich RNA-binding protein family, in human cells in response to cold stress. Biochem Biophys Res Commun 236:804-807.

D'Antonio M, Musner N, Scapin C, Ungaro D, Del Carro U, Ron D, Feltri ML, Wrabetz L (2013) Resetting translational homeostasis restores myelination in Charcot-Marie-Tooth disease type 1B mice. J Exp Med. 210:821-38.

Darusman HS, Gjedde A, Sajuthi D, Schapiro SJ, Kalliokoski O, Kristianingrum YP, Handaryani E, Hau J (2014) Amyloid Beta1-42 and the Phoshorylated Tau Threonine 231 in Brains of Aged Cynomolgus Monkeys (Macacafascicularis). Front Aging Neurosci 6:313.

Das I, Krzyzosiak A, Schneider K, Wrabetz L, D'Antonio M, Barry N, Sigurdardottir A, Bertolotti A (2015) Preventing proteostasis diseases by selective inhibition of a phosphatase regulatory subunit. Science 348:239-42.

Dausmann KH, Glos J, Ganzhorn JU, Heldmaier G (2004) Physiology: hibernation in a tropical primate. Nature 429:825-6.

Dave KR, Christian SL, Perez-Pinzon MA, Drew KL (2012) Neuroprotection: lessons from hibernators. Comp Biochem Physiol B Biochem Mol Biol 162:1-9.

David DC, Layfield R, Serpell L, Narain Y, Goedert M, Spillantini MG (2002) Proteasomal degradation of tau protein. J Neurochem 83:176-85.

Davis FA, Schauf CL, Reed BJ, Kesler RL (1976) Experimental studies of the effects of extrinsic factors on conduction in normal and demyelinated nerve. 1. Temperature. J Neurol Neurosurg Psychiatry 39:442-8.

Dawson HN, Ferreira A, Eyster MV, Ghoshal N, Binder LI, Vitek MP (2001)
Inhibition of neuronal maturation in primary hippocampal neurons from tau deficient mice. J Cell Sci 114:1179-87.

De Boni U, McLachlan DR (1985) Controlled induction of paired helical filaments of the Alzheimer type in cultured human neurons, by glutamate and aspartate. J Neurol Sci 68:105-18.

de Calignon A, Polydoro M, Suárez-Calvet M, William C, Adamowicz DH, Kopeikina KJ, Pitstick R, Sahara N, Ashe KH, Carlson GA, Spires-Jones TL, Hyman BT (2012) Propagation of tau pathology in a model of early Alzheimer's disease. Neuron 73:685-97.

De Cock VC, Lannuzel A, Verhaeghe S, Roze E, Ruberg M, Derenne JP, Willer JC, Vidailhet M, Arnulf I (2007) REM sleep behavior disorder in patients with guadeloupean parkinsonism, a tauopathy. Sleep 30:1026-32.

Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, Cuny GD, Mitchison TJ, Moskowitz MA, Yuan J (2005)
Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol 1:112-9.

DeKosky ST, Abrahamson EE, Taffe KM, Dixon CE, Kochanek PM, Ikonomovic MD (2004)
Effects of post-injury hypothermia and nerve growth factor infusion on antioxidant enzyme activity in the rat:implications for clinical therapies. J Neurochem 90:998-1004.

De Leeuw F, Zhang T, Wauquier C, Huez G, Kruys V, Gueydan C (2007) The cold-inducible RNA-binding protein migrates from the nucleus to cytoplasmic stress granules by amethylation-dependent mechanism and acts as a translational repressor. Exp Cell Res 313:4130-44.

Denys EH (1991) AAEM minimonograph #14: The influence of temperature in clinical neurophysiology. Muscle Nerve 14:795-811.

Derkinderen P, Scales TM, Hanger DP, Leung KY, Byers HL, Ward MA, Lenz C, Price C, Bird IN, Perera T, Kellie S, Williamson R, Noble W, Van Etten RA,Leroy K, Brion JP, Reynolds CH, Anderton BH (2005)
Tyrosine 394 is phosphorylated in Alzheimer's paired helical filament tau and in foetal tau with c-Abl as thecandidate tyrosine kinase. J Neurosci 25:6584-93.

Derry JM, Kerns JA, Francke U (1995) RBM3, a novel human gene in Xp11.23 with a putative RNA binding domain. Hum Mol Genet 4:2307-2311.

Deshpande A, Win KM, Busciglio J (2008) Tau isoform expression and regulation in human cortical neurons. FASEB J 22:2357-2367.

Devi L, Ohno M (2014) PERK mediates eIF2α phosphorylation responsible for BACE1 elevation, CREB dysfunction and neurodegeneration in a mouse model of Alzheimer's disease. Neurobiol Aging 35:2272-81.

De Vito D, Piraino S, Schmich J, Bouillon, Boero F (2005) Evidence of reverse development in Leptomedusae (Cnidaria, Hydrozoa): the case of Laodicea undulata (Forbes and Goodsir 1851) Marine Biol DOI 10.1007/ S00227-005-0182-3.

Devlin AC, Burr K, Borooah S, Foster JD, Cleary EM, Geti I, Vallier L, Shaw CE, Chandran S, Miles GB (2015) Human iPSC-
derived motoneurons harbouring TARDBP or C9ORF72 ALS mutations are dysfunctional despite maintaining viability. Nat Commun 6:5999. doi: 10.1038/ncomms6999.

Dickey CA, Kamal A, Lundgren K, Klosak N, Bailey RM, Dunmore J, Ash P, Shoraka S, Zlatkovic J, Eckman CB, Patterson C, Dickson DW, Nahman NS Jr,Hutton M, Burrows F, Petrucelli L (2007)

The high-affinity HSP90-CHIP complex recognizes and selectively degrades phosphorylated tau client proteins. J Clin Invest 117:648-58.

Dietrich WD, Atkins CM, Bramlett HM (2009) Protection in animal models of brain and spinal cord injury with mild to moderate hypothermia. J Neurotrauma 26:301-12.

Dietrich WD, Busto R, Bethea JR (1999) Postischemic hypothermia and IL-10 treatment provide long-lasting neuroprotection of CA1 hippocampusfollowing transient global ischemia in rats. Exp Neurol 158:444-50.

Dietrich WD, Lin B, Globus MY, Green EJ, Ginsberg MD, Busto R (1995) Effect of delayed MK-801 (dizocilpine) treatment with or without immediate postischemic hypothermia on chronicneuronal survival after global forebrain ischemia in rats. J Cereb Blood Flow Metab 15:960-8.

Di Fonzo A, Ronchi D, Gallia F, Cribiù FM, Trezzi I, Vetro A, Della Mina E, Limongelli I, Bellazzi R, Ricca I, Micieli G, Fassone E, Rizzuti M, Bordoni A, Fortunato F, Salani S, Mora G, Corti S, Ceroni M, Bosari S, Zuffardi O, Bresolin N, Nobile-Orazio E, Comi GP (2014) Lower motor neuron disease with respiratory failure caused by a novel MAPT mutation. Neurology 82:1990-8.

Dirnagl U, Simon RP, Hallenbeck JM (2003) Ischemic tolerance and endogenous neuroprotection. Trends Neurosci 26:248-54.

Dixit R, Ross JL, Goldman YE, Holzbaur EL (2008) Differential regulation of dynein and kinesin motor proteins by tau. Science 319:1086-9.

Dong H, Moody-Corbett F, Colbourne F, Pittman Q, Corbett D (2001) Electrophysiological properties of CA1 neurons protected by postischemic hypothermia in gerbils. Stroke 32:788-95.

Donnelly CJ, Zhang PW, Pham JT, Haeusler AR, Mistry NA, Vidensky S, Daley EL, Poth EM, Hoover B, Fines DM, Maragakis N, Tienari PJ, Petrucelli L,Traynor BJ, Wang J, Rigo F, Bennett CF, Blackshaw S, Sattler R, Rothstein JD (2013) RNA toxicity from the ALS/FTD C9ORF72 expansion is mitigated by antisense intervention. Neuron 80:415-28.

Dou F, Netzer WJ, Tanemura K, Li F, Hartl FU, Takashima A, Gouras GK, Greengard P, Xu H (2003) Chaperones increase association of tau protein with microtubules. Proc Natl Acad Sci U S A. 100:721-6.

Douglas P, Moorhead GB, Ye R, Lees-Miller SP (2001) Protein phosphatases regulate DNA-dependent protein kinase activity. J Biol Chem 276:18992-8.

Dresios J, Aschrafi A, Owens GC, Vanderklish PW, Edelman GM, Mauro VP (2005) Cold stress-induced protein Rbm3 binds 60S ribosomal subunits, alters microRNA levels, and enhances global protein synthesis. Proc Natl Acad Sci USA 102:1865-1870.

Drew KL, Rice ME, Kuhn TB, Smith MA (2001) Neuroprotective adaptations in hibernation: therapeutic implications for ischemia-reperfusion, traumatic braininjury and neurodegenerative diseases. Free Radic Biol Med 31:563-73.

Drexler HC (2009) Synergistic apoptosis induction in leukemic cells by the phosphatase inhibitor salubrinal and proteasome inhibitors. PLoS One 4:e4161.

Drubin D, Kobayashi S, Kellogg D, Kirschner M (1988) Regulation of microtubule protein levels during cellular morphogenesis in nerve growth factor-treated PC12cells. J Cell Biol 106:1583-91.

Duff K, Knight H, Refolo LM, Sanders S, Yu X, Picciano M, Malester B, Hutton M, Adamson J, Goedert M, Burki K, Davies P (2000) Characterization of pathology in transgenic mice over-expressing human genomic and cDNA tau transgenes. Neurobiol Dis 7:87-98.

Dujardin S, Lécolle K, Caillierez R, Bégard S, Zommer N, Lachaud C, Carrier S, Dufour N, Aurégan G, Winderickx J, Hantraye P, Déglon N, Colin M[1], Buée L (2014) Neuron-to-neuron wild-type Tau protein transfer through a trans-synaptic mechanism: relevance to sporadictauopathies. Acta Neuropathol Commun 2:14.

Durandy Y (2008) Pediatric myocardial protection. Curr Opin Cardiol 23:85-90.

Ebneth A, Godemann R, Stamer K, Illenberger S, Trinczek B, Mandelkow E (1998) Overexpression of tau protein inhibits kinesin-dependent trafficking of vesicles, mitochondria, and endoplasmic reticulum: implications for Alzheimer's disease. J Cell Biol 143:777-94.

Eckermann K, Mocanu MM, Khlistunova I, Biernat J, Nissen A, Hofmann A, Schönig K, Bujard H, Haemisch A, Mandelkow E, Zhou L, Rune G, Mandelkow EM (2007) The beta-propensity of Tau determines aggregation and synaptic loss in inducible mouse models of tauopathy. J Biol Chem 282:31755-65.

El Khoury NB, Gratuze M, Papon MA, Bretteville A, Planel E (2014) Insulin dysfunction and Tau pathology. Front Cell Neurosci 8:22.

Endres K, Reinhardt S (2013) ER-stress in Alzheimer's disease: turning the scale? Am J Neurodegener Dis 2:247-65.

Engel T, Sanz-Rodgriguez A, Jimenez-Mateos EM, Concannon CG, Jimenez-Pacheco A, Moran C, Mesuret G, Petit E, Delanty N, Farrell MA, O'Brien DF, Prehn JH, Lucas JJ, Henshall DC (2013) CHOP regulates the p53-MDM2 axis and is required for neuronal survival after seizures. Brain 136:577-92.

Engin F, Hotamisligil GS (2010) Restoring endoplasmic reticulum function by chemical chaperones: an emerging therapeutic approach formetabolic diseases. Diabetes Obes Metab 2:108-15.

Esclaire F, Terro F, Yardin C, Hugon J (1998) Neuronal apoptosis is associated with a decrease in tau mRNA expression. Neuroreport 9:1173-7.

Espuny-Camacho I, Michelsen KA, Gall D, Linaro D, Hasche A, Bonnefont J, Bali C, Orduz D, Bilheu A, Herpoel A, Lambert N, Gaspard N, Péron S, Schiffmann SN, Giugliano M, Gaillard A, Vanderhaeghen P (2013) Pyramidal neurons derived from human pluripotent stem cells integrate efficiently into mouse brain circuits in vivo. Neuron 77:440-456.

Fan MM, Raymond LA (2007) N-methyl-D-aspartate (NMDA) receptor function and excitotoxicity in Huntington's disease. Prog Neurobiol 81:272-93.

Fanara P, Husted KH, Selle K, Wong PY, Banerjee J, Brandt R, Hellerstein MK (2010) Changes in microtubule turnover accompany synaptic plasticity and memory formation in response to contextual fear conditioning in mice. Neuroscience 168:167-78.

Faridar A, Bershad EM, Emiru T, Iaizzo PA, Suarez JI, Divani AA (2011) Therapeutic hypothermia in stroke and traumatic brain injury. Front Neurol 2:80.

Fay T (1943) Observations on generalized refrigeration in cases of severe cerebral trauma. Assoc Res Nerv Ment Dis Proc 24:611–619.

Fein JA, Sokolow S, Miller CA, Vinters HV, Yang F, Cole GM, Gylys KH (2008) Co-localization of amyloid beta and tau pathology in Alzheimer's disease synaptosomes. Am J Pathol 172:1683-92.

Feiner JR, Bickler PE, Estrada S, Donohoe PH, Fahlman CS, Schuyler JA (2005) Mild hypothermia, but not propofol, is neuroprotective in organotypic hippocampal cultures. Anesth Analg 100:215-25.

Feng CS, Qiu JP, Ma HC, Yue Y (2007) Effect of propofol on synaptic long-term potentiation in hippocampal slices of rats. Zhonghua Yi Xue Za Zhi 87:763-7.

Ferreiro E, Pereira CM (2012) Endoplasmic reticulum stress: a new playER in tauopathies. J Pathol 226:687-92.

Ferry AL, Vanderklish PW, Dupont-Versteegden EE (2011)
Enhanced survival of skeletal muscle myoblasts in response to overexpression of cold shock protein R BM3. Am J Physiol Cell Physiol 301:C392-402.

Feuillette S, Miguel L, Frébourg T, Campion D, Lecourtois M (2010)
Drosophila models of human tauopathies indicate that Tau protein toxicity in vivo is mediated by soluble cytosolic phosphorylated forms of the protein. J Neurochem 113:895-903.

Flach K, Hilbrich I, Schiffmann A, Gärtner U, Krüger M, Leonhardt M, Waschipky H, Wick L, Arendt T, Holzer M (2012) Tau oligomers impair artificial membrane integrity and cellular viability. J Biol Chem 287:43223-33.

Flechsig PE (1920) Anatomie des menschlichen Gehirns und Rueckenmarks auf myelogenetischer Grundlage. Thieme, Leipzig.

Fogolín MB, Wagner R, Etcheverrigaray M, Kratje R (2004)
Impact of temperature reduction and expression of yeast pyruvate carboxylase on hGM-CSF-producing CHOcells. J Biotechnol 109:179-91.

Fong H, Wang C, Knoferle J, Walker D, Balestra ME, Tong LM, Leung L, Ring KL, Seeley WW, Karydas A, Kshirsagar MA, Boxer AL, Kosik KS, Miller BL, Huang Y (2013) Genetic correction of tauopathy phenotypes in neurons derived from human induced pluripotent stem cells. Stem Cell Reports 1:226-234.

Fonnum F (1984) Glutamate: a neurotransmitter in mammalian brain. J Neurochem 42:1-11.

Forman MS, Zhukareva V, Bergeron C, Chin SS, Grossman M, Clark C, Lee VM, Tojanowksi JQ (2002) Signature tau neuropathology in gray and white matter of corticobasal degeneration. Am J Pathol 160: 2045-53.

Fotuhi M, Hachinski V, Whitehouse PJ (2009) Changing perspectives regarding late-life dementia. Nat Rev Neurol 5:649-58.

Fouillet A, Levet C, Virgone A, Robin M, Dourlen P, Rieusset J, Belaidi E, Ovize M, Touret M, Nataf S, Mollereau B (2012) ER stress inhibits neuronal death by promoting autophagy. Autophagy 8:915-26.

Fox LM, William CM, Adamowicz DH, Pitstick R, Carlson GA, Spires-Jones TL, Hyman BT (2011) Soluble tau species, not neurofibrillary aggregates, disrupt neural system integration in a tau transgenic model. J Neuropathol Exp Neurol 70:588-595.

Frandemiche ML, De Seranno S, Rush T, Borel E, Elie A, Arnal I, Lanté F, Buisson A (2014) Activity-
dependent tau protein translocation to excitatory synapse is disrupted by exposure to amyloid-betaoligomers. J Neurosci 34:6084-97.

Frankenhauser B, Moore LE (1963) The effect of temperature on the sodium and potassium permability changes in myelinated nerve fibres of Xenopus laevis. J Physiol 169:431-7.

Friedhoff P, von Bergen M, Mandelkow EM, Mandelkow E (2000) Structure of tau protein and assembly into paired helical filaments. Biochim Biophys Acta 1502:122-32.

Friman G, Schiller HH, Schwartz M (1977) Disturbed neuromuscular transmission in viral infections. Scand J Infect Dis 9:99-103.

Frost B, Hemberg M, Lewis J, Feany MB (2014) Tau promotes neurodegeneration through global chromatin relaxation. Nat Neurosci 17:357-66.

Frost B, Jacks RL, Diamond MI (2009) Propagation of tau misfolding from the outside to the inside of a cell. J Biol Chem 284:12845-52.

Fu ZQ, Yang Y, Song J, Jiang Q, Lin ZC, Wang Q, Zhu LQ, Wang JZ, Tian Q (2010) LiCl attenuates thapsigargin-induced tau hyperphosphorylation by inhibiting GSK-3β in vivo and in vitro. J Alzheimers Dis 21:1107-17.

Fuentealba LC, Eivers E, Geissert D, Taelman V, De Robertis EM (2008) Asymmetric mitosis: Unequal segregation of proteins destined for degradation. Proc Natl Acad Sci U S A 105:7732-7.

Fujita J (1999) Cold shock response in mammalian cells. J Mol Microbiol Biotechnol 1:243-255.

Gagnon JF, Postuma RB, Mazza S, Doyon J, Montplaisir J (2006) Rapid-eye-movement sleep behaviour disorder and neurodegenerative diseases. Lancet Neurol 5:424-32.

Gao XY, Huang JO, Hu YF, Gu Y, Zhu SZ, Huang KB, Chen JY, Pan SY (2014) Combination of mild hypothermia with neuroprotectants has greater neuroprotective effects during ox ygen-glucose deprivation and reoxygenation-mediated neuronal injury. Sci Rep 4:7091.

Garcia ML, Cleveland DW (2001) Going new places using an old MAP: tau, microtubules and human neurodegenerative disease. Curr Opin Cell Biol 13:41-8.

Gärtner U, Janke C, Holzer M, Vanmechelen E, Arendt T (1998) Postmortem changes in the phosphorylation state of tau-protein in the rat brain. Neurobiol Aging 19:535-43.

Gasparini L, Terni B, Spillantini MG (2007) Frontotemporal dementia with tau pathology. Neurodegener Dis 4:236-53.

Ge PF, Luo TF, Zhang JZ, Chen DW, Luan YX, Fu SL (2008) Ischemic preconditioning induces chaperone hsp70 expression and inhibits protein aggregation in the CA1neurons of rats. Neurosci Bull 24:288-96.

Gemignani A, Pietrini P, Murrell JR, Glazier BS, Zolo P, Guazzelli M, Ghetti B (2005) Slow wave and REM sleep mechanisms are differently altered in hereditary pick disease associated with the TAU G389R mutation. Arch Ital Biol 143:65-79.

Gillingwater TH, Wishart TM (2013) Mechanisms underlying synaptic vulnerability and degeneration in neurodegenerative disease. Neuropathol Appl Neurobiol 39:320-34.

Ginsberg MD, Belayev L (2005) Chapter 4: Biological and Molecular Mechanisms of Hypothermic Neuroprotection in: 'Therapeutic Hypothermia' edited by Mayer SA and Sessler DI, Marcel Dekker, New York, p85-140.

Ginsberg MD, Busto R (1998) Combating hyperthermia in acute stroke: a significant clinical concern. Stroke 29:529-34.

Gisselsson LL, Matus A, Wieloch T (2005) Actin redistribution underlies the sparing effect of mild hypothermia on dendritic spine morphology after in vitro ischemia. J Cereb Blood Flow Metab 25:1346-55.

Globus MY, Alonso O, Dietrich WD, Busto R, Ginsberg MD (1995a)
Glutamate release and free radical production following brain injury: effects of posttraumatic hypother mia. J Neurochem 65:1704-11.

Globus MY, Busto R, Lin B, Schnippering H, Ginsberg MD (1995b)
Detection of free radical activity during transient global ischemia and recirculation: effects of intraisch emic braintemperature modulation. J Neurochem 65:1250-6.

Gluckman PD, Wyatt JS, Azzopardi D, Ballard R, Edwards AD, Ferriero DM, Polin RA, Robertson CM, Thoresen M, Whitelaw A, Gunn AJ (2005)
Selective head cooling with mild systemic hypothermia after neonatal encephalopathy: multicentre ran domisedtrial. Lancet 365:663-70.

Goedert M, Clavaguera F, Tolnay M (2010) The propagation of prion-like protein inclusions in neurodegenerative diseases. Trends Neurosci 33:317-25.

Goedert M, Falcon B, Clavaguera F, Tolnay M (2014) Prion-like mechanisms in the pathogenesis of tauopathies and synucleinopathies. Curr Neurol Neurosci Rep 14:495.

Goedert M, Jakes R (1990) Expression of separate isoforms of human tau protein: correlation with the tau pattern in brain and effects on tubulin polymerization. EMBO J 9:4225-30.

Goedert M, Jakes R, Crowther RA, Six J, Lübke U, Vandermeeren M, Cras P, Trojanowski JQ, Lee VM (1993) The abnormal phosphorylation of tau protein at $Ser^{202}$ in Alzheimer's disease recapitulates phosphorylation during development. Proc Natl Acad Sci USA 90:5066-5070.

Goedert M, Spillantini MG (2011) Pathogenesis of the tauopathies. J Mol Neurosci 45:425-31.

Goedert M, Spillantini MG, Jakes R, Rutherford D, Crowther RA (1989a) Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. Neuron 3:519-26.

Goedert M, Spillantini MG, Potier MC, Ulrich J, Crowther RA (1989b) Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein tau containing four tandem repeats: differential expression of tau protein mRNA's in human brain. EMBO J 8:393-399.

Goedert M, Wischik CM, Crowther RA, Walker JE, Klug A (1988) Cloning and sequencing of the cDNA encoding a core protein of the paired helical filament of Alzheimer disease: identification as the microtubule-associated protein tau. Proc Natl Acad Sci U S A 85:4051-5.

Goldbaum O, Oppermann M, Handschuh M, Dabir D, Zhang B, Forman MS, Trojanowski JQ, Lee VM, Richter-Landsberg C (2003)
Proteasome inhibition stabilizes tau inclusions in oligodendroglial cells that occur after treatment with okadaicacid. J Neurosci 23:8872-80.

Gómez-Isla T, Hollister R, West H, Mui S, Growdon JH, Petersen RC, Parisi JE, Hyman BT (1997) Neuronal loss correlates with but exceeds neurofibrillary tangles in Alzheimer's disease. Ann Neurol 41:17-24.

Gómez-Ramos A, Díaz-Nido J, Smith MA, Perry G, Avila J (2003) Effect of the lipid peroxidation product acrolein on tau phosphorylation in neural cells. J Neurosci Res 71:863-870.

Gong WJ, Golic KG (2006) Loss of Hsp70 in Drosophila is pleiotropic, with effects on thermotolerance, recovery from heat shock and neurodegeneration. Genetics 172:275-86.

Gong CX, Lidsky T, Wegiel J, Zuck L, Grundke-Iqbal I, Iqbal K (2000) Phosphorylation of microtubule-associated protein tau is regulated by protein phosphatase 2A in mammalian brain. Implications for neurofibrillary degeneration in Alzheimer's disease. J Biol Chem 275:5535-44.

Gong CX, Singh TJ, Grundke-Iqbal I, Iqbal K (1993) Phosphoprotein phosphatase activities in Alzheimer disease brain. J Neurochem 61:921-7.

Goode BL, Feinstein SC (1994) Identification of a novel microtubule binding and assembly domain in the developmentally regulated inter-repeatregion of tau. J Cell Biol 124:769-82.

Grasshoff C, Gillessen T (2002) The effect of propofol on increased superoxide concentration in cultured rat cerebrocortical neurons after stimulation of N-methyl-d-aspartate receptors. Anesth Analg 95:920-2

Green EJ, Pazos AJ, Dietrich WD, McCabe PM, Schneiderman N, Lin B, Busto R, Globus MY, Ginsberg MD (1995) Combined postischemic hypothermia and delayed MK-801 treatment attenuates neurobehavioral deficitsassociated with transient global ischemia in rats. Brain Res 702:145-52.

Greenwood SM, Connolly CN (2007) Dendritic and mitochondrial changes during glutamate excitotoxicity. Neuropharmacology 53:891-8.

Grulova I, Slovinska L, Nagyova M, Cizek M, Cizkova D (2013) The effect of hypothermia on sensory-motor function and tissue sparing after spinal cord injury. Spine J 13:1881-91.

Grundke-Iqbal I, Iqbal K, Tung YC, Quinlan M, Wisniewski HM, Binder LI (1986) Abnormal phosphorylation of the microtubule-associated protein tau in Alzheimer cytoskeletal pathology. Proc Natl Acad Sci USA 83:4913-17.

Grupp K, Wilking J, Prien K, Hube-Magg C, Sirma H, Simon R, Steurer S, Budäus L, Haese A, Izbicki J, Sauter G, Minner S, Schlomm T, Tsourlakis MC (2014) High RNA-binding motif protein 3 expression is an independent prognostic marker in operated prostate cancer and tightly linked to ERG activation and PTEN deletions. Eur J Cancer 50:852-61.

Gu J, Zheng JQ (2009) Microtubules in Dendritic Spine Development and Plasticity. Open Neurosci J 3:128-133.

Gualerzi CO, Giuliodori AM, Pon CL (2003) Transcriptional and post-transcriptional control of cold-shock genes. J Mol Biol 331:527-539.

Guo JL, Lee VM (2011) Seeding of normal Tau by pathological Tau conformers drives pathogenesis of Alzheimer-like tangles. J Biol Chem 286:15317-31.

Guo JL, Lee VM (2013) Neurofibrillary tangle-like tau pathology induced by synthetic tau fibrils in primary neurons over-expressingmutant tau. FEBS Lett 587:717-23.

Gupta K, Hardingham GE, Chandran S (2013) NMDA receptor-dependent glutamate excitotoxicity in human embryonic stem cell-derived neurons. Neurosci Lett 543:95-100.

Gustke N, Trinczek B, Biernat J, Mandelkow EM, Mandelkow E (1994) Domains of tau protein and interactions with microtubules. Biochemistry 33: 9511-22.

Guthrie TC, Nelson DA (1995) Influence of temperature changes on multiple sclerosis: critical review of mechanisms and research potential. J Neurol Sci 129:1-8.

Guven H, Amanvermez R, Malazgirt Z, Kaya E, Doganay Z, Celik C, Ozkan K (2002) Moderate hypothermia prevents brain stem oxidative stress injury after hemorrhagic shock. J Trauma 53:66-72.

Hajat C, Hajat S, Sharma P (2000) Effects of poststroke pyrexia on stroke outcome: a meta-analysis of studies in patients. Stroke 31:410-4.

Hall GF, Patuto BA (2012) Is tau ready for admission to the prion club? Prion 6:223-33.

Halliday M, Mallucci GR (2014) Modulating the unfolded protein response to prevent neurodegeneration and enhance memory. Neuropathol Appl Neurobiol doi: 10.1111/nan.12211. [Epub ahead of print].

Halterman MW, Gill M, DeJesus C, Ogihara M, Schor NF, Federoff HJ (2010) The endoplasmic reticulum stress response factor CHOP-10 protects against hypoxia-induced neuronal death. J Biol Chem 285:21329-40.

Han D, Lerner AG, Vande Walle L, Upton JP, Xu W, Hagen A, Backes BJ, Oakes SA, Papa FR (2009) IRE1alpha kinase activation modes control alternate endoribonuclease outputs to determine divergent cell fates. Cell 138:562-75.

Han HS, Park J, Kim JH, Suk K (2012) Molecular and cellular pathways as a target of therapeutic hypothermia: pharmacological aspect. Curr Neuropharmacol 10:80-7.

Han HS, Qiao Y, Karabiyikoglu M, Giffard RG, Yenari MA (2002) Influence of mild hypothermia on inducible nitric oxide synthase expression and reactive nitrogen pro duction inexperimental stroke and inflammation. J Neurosci 22:3921-8.

Hanger DP, Anderton BH, Noble W (2009) Tau phosphorylation: the therapeutic challenge for neurodegenerative disease. Trends Mol Med 15:112-9.

Harada A, Oguchi K, Okabe S, Kuno J, Terada S, Ohshima T, Sato-Yoshitake R, Takei Y, Noda T, Hirokawa N (1994) Altered microtubule organization in small-calibre axons of mice lacking tau protein. Nature 369:488-91.

Harding HP, Zhang Y, Ron D (1999) Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature 397:271-4.

Hardingham GE (2009) Coupling of the NMDA receptor to neurprotective and neurodestructive events. Biochem Soc Trans 37:1147-60.

Hardingham GE, Bading H (2003) The Yin and Yang of NMDA receptor signalling. Trends Neurosci 26:81-9.

Hardingham GE, Bading H (2010) Synaptic versus extrasynaptic NMDA receptor signalling: implications for neurodegenerative disorders. Nat Rev Neurosci 11:682-96.

Hardingham GE, Chawla S, Cruzalegui FH, Bading H (1999) Control of recruitment and transcription-activating function of CBP determines gene regulation by NMDA receptors and L-type calcium channels. Neuron 22:789-798.

Hardingham GE, Chawla S, Johnson CM, Bading H (1997) Distinct functions of nuclear and cytoplasmic calcium in the control of gene expression. Nature 385:260-265.

Hardingham GE, Fukunaga Y, Bading H (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat Neurosci 5:405-414.

Hardingham NR, Larkman AU (1998) Rapid report: the reliability of excitatory synaptic transmission in slices of rat visual cortex in vitro is temperature dependent. J Physiol 507:249-56.

Harris BA (2010) Heat loss from the upper airways and through the skull: studies of direct brain cooling in humans. Thesis, The University of Edinburgh, p101, p1376.

Harris BA, Andrews PJ (2014) A lesson on induction of hypothermia and measurement of efficacy. Crit Care 18: 710.

Harris B, Andrews PJD, Murray GD, Forbes J, Moseley O (2012) Systematic review of head cooling in adults after traumatic brain injury and stroke. Health Technol Assess 16:1-175.

Harris KM, Kater SB (1994)
Dendritic spines: cellular specializations imparting both stability and flexibility to synaptic function. Annu Rev Neurosci 17:341-71.

Härtig W, Stieler J, Boerema AS, Wolf J, Schmidt U, Weissfuss J, Bullmann T, Strijkstra AM, Arendt T (2007) Hibernation model of tau phosphorylation in hamsters: selective vulnerability of cholinergic basal forebrain neurons - implications for Alzheimer's disease. Eur J Neurosci 25:69-80.

Hasbani MJ, Schlief ML, Fisher DA, Goldberg MP (2001)
Dendritic spines lost during glutamate receptor activation reemerge at original sites of synaptic contact. J Neurosci 21:2393-403.

Hasel P, Mckay S, Qiu J, Hardingham GE (2014) Selective dendritic susceptibility to bioenergetic, excitotoxic and redox perturbations in cortical neurons. Biochim Biophys Acta pii: S0167-4889(14)00449-2.

Hashimoto-Torii K, Torii M, Fujimoto M, Nakai A, El Fatimy R, Mezger V, Ju MJ, Ishii S, Chao SH, Brennand KJ, Gage FH, Rakic P (2014)
Roles of heat shock factor 1 in neuronal response to foetal environmental risks and its relevance to braindisorders. Neuron 82:560-72.

Heller, J (1961) *Catch-22*. New York: Simon and Schuster.

Henker RA, Brown SD, Marion DW (1998)
Comparison of brain temperature with bladder and rectal temperatures in adults with severe head injur y. Neurosurgery 42:1071-5.

Henkins KM, Sokolow S, Miller CA, Vinters HV, Poon WW, Cornwell LB, Saing T, Gylys KH (2012) Extensive p-tau pathology and SDS-stable p-tau oligomers in Alzheimer's cortical synapses. Brain Pathol 22:826-33.

Hetz C, Mollereau B (2014) Disturbance of endoplasmic reticulum proteostasis in neurodegenerative diseases. Nat Rev Neurosci 15:233-49.

Hibaoui Y, Grad I, Letourneau A, Sailani MR, Dahoun S, Santoni FA, Gimelli S, Guipponi M, Pelte MF, Béna F, Antonarakis SE, Feki A (2014)
Modelling and rescuing neurodevelopmental defect of Down syndrome using induced pluripotent ste m cells frommonozygotic twins discordant for trisomy 21. EMBO Mol Med 6:259-77.

Higuchi M, Trojanowksi JQ, Lee VM-Y (2002) Chapter 94 'Tau protein and tauopathy' in: 'Neurospsychopharamcology: The Firth Generation of Progress' Edited by Davis KL, Charney D, Coyle JT, Nemeroff C. American College of Neuropsychopharmacology p1339-1354.

Hill RS, Walsh CA (2005) Molecular insights into human brain evolution. Nature 437:64-7.

Hiller G, Weber K (1978) Radioimmunoassay for tubulin: a quantitative comparison of the tubulin content of different established tissue culture cells and tissues. Cell 14:795-804.

Himmler A, Drechsel D, Kirschner MW, Martin DW Jr (1989) Tau consists of a set of proteins with repeated C-terminal microtubule-binding domains and variable N-terminal domains. Mol Cell Biol 9:1381-8.

Hindle AG, Martin SL (2013) Cytoskeletal regulation dominates temperature-sensitive proteomic changes of hibernation in forebrain of 13-lined ground squirrels. PLoS One 8:e71627.

Hirokawa N, Shiomura Y, Okabe S (1988) Tau proteins: the molecular structure and mode of binding on microtubules. J Cell Biol 107:1449-59.

Hlatky R, Robertson CS (2005) 'Temperature Monitoring' in 'Therapeutic Hypothermia' edited by Mayer SA, Sessler DI, Marcel Dekker, New York p61-84.

Ho YS, Yang X, Lau JC, Hung CH, Wuwongse S, Zhang Q, Wang J, Baum L, So KF, Chang RC (2012) Endoplasmic reticulum stress induces tau pathology and forms a vicious cycle: implication in Alzheimer's disease pathogenesis. J Alzheimers Dis 28:839-54.

Hodgkin AL, Katz B (1949) The effect of temperature on the electrical activity of the aquid giant axon. J Physiol 109: 240-9.

Holmes BB, DeVos SL, Kfoury N, Li M, Jacks R, Yanamandra K, Ouidja MO, Brodsky FM, Marasa J, Bagchi DP, Kotzbauer PT, Miller TM, Papy-Garcia D,Diamond MI (2013) Heparan sulfate proteoglycans mediate internalization and propagation of specific proteopathic seeds. Proc Natl Acad Sci U S A 110:E3138-47.

Hodgkin AL, Huxley AF, Katz B (1952) Measurement of current-volatge relations in the membrane of the giant axon of Loligo. J Phyiol 116:424-48.

Hodgkin AL, Katz B (1949) The effect of temperature on the electrical activity of the giant axon of the squid. J Physiol 109:240-9.

Hofmann S, Cherkasova V, Bankhead P, Bukau B, Stoecklin G (2012) Translation suppression promotes stress granule formation and cell survival in response to cold shock. Mol Biol Cell 23:3786-800.

Höglinger GU, Carrard G, Michel PP, Medja F, Lombès A, Ruberg M, Friguet B, Hirsch EC (2003) Dysfunction of mitochondrial complex I and the proteasome: interactions between two biochemical deficits in a cellular model of Parkinson's disease. J Neurochem 86:1297-307.

Höglinger GU, Melhem NM, Dickson DW, Sleiman PM, Wang LS, Klei L, Rademakers R, de Silva R, Litvan I, Riley DE, van Swieten JC, Heutink P, Wszolek ZK, Uitti RJ, Vandrovcova J, Hurtig HI, Gross RG, Maetzler W, Goldwurm S, Tolosa E, Borroni B, Pastor P; PSP Genetics Study Group, Cantwell LB, Han MR,Dillman A, van der Brug MP, Gibbs JR, Cookson MR, Hernandez DG, Singleton AB, Farrer MJ, Yu CE, Golbe LI, Revesz T, Hardy J, Lees AJ, Devlin B, Hakonarson H, Müller U, Schellenberg GD (2011) Identification of common variants influencing risk of the tauopathy progressive supranuclear palsy. Nat Genet 43:699-705.

Holmes BB, Diamond MI (2014) Prion-like properties of Tau protein: the importance of extracellular Tau as a therapeutic target. J Biol Chem 289:19855-61.

Holth JK, Bomben VC, Reed JG, Inoue T, Younkin L, Younkin SG, Pautler RG, Botas J, Noebels JL (2013)
Tau loss attenuates neuronal network hyperexcitability in mouse and Drosophila genetic models of epi lepsy. J Neurosci 33:1651-9.

Hong M, Zhukareva V, Vogelsberg-Ragaglia V, Wszolek Z, Reed L, Miller BI, Geschwind DH, Bird TD, McKeel D, Goate A, Morris JC, Wilhelmsen KC,Schellenberg GD, Trojanowski JQ, Lee VM (1998) Mutation-specific functional impairments in distinct tau isoforms of hereditary FTDP-17. Science 282:1914-7.

Hoover BR, Reed MN, Su J, Penrod RD, Kotilinek LA, Grant MK, Pitstick R, Carlson GA, Lanier LM, Yuan LL, Ashe KH, Liao D (2010)
Tau mislocalization to dendritic spines mediates synaptic dysfunction independently of neurodegenera tion. Neuron 68:1067-81.

Hoozemans JJ, Scheper W (2012) Endoplasmic reticulum:
the unfolded protein response is tangled in neurodegeneration. Int J Biochem Cell Biol 44:1295-8.

Hoozemans JJ, van Haastert ES, Nijholt DA, Rozemuller AJ, Eikelenboom P, Scheper W (2009)
The unfolded protein response is activated in pretangle neurons in Alzheimer's disease hippocampus. Am J Pathol 174:1241-51.

Hosie KA, King AE, Blizzard CA, Vickers JC, Dickson TC (2012) Chronic excitotoxin-
induced axon degeneration in a compartmented neuronal culture model. ASN Neuro 4:e00076.

Howells J, Czesnik D, Trevillion L, Burke D (2013) Excitability and
the safety margin in human axons during hyperthermia. J Physiol 591:3063-80.

Hsu HK, Shao PL, Tsai KL, Shih HC, Lee TY, Hsu C (2005) Gene regulation by NMDA receptor activation in the SDNPOA neurons of male rats during sexual development. J Mol Endocrinol 34:433-445.

Hu X, Viesselmann C, Nam S, Merriam E, Dent EW (2008) Activity-dependent dynamic microtubule invasion of dendritic spines. J Neurosci 28:13094-105.

Huang R, Shuaib A, Hertz L (1993)
Glutamate uptake and glutamate content in primary cultures of mouse astrocytes during anoxia, substr ate deprivation and simulated ischemia under normothermic and hypothermic conditions. Brain Res 618:346-51.

Hudson AE, Hemmings HC Jr (2011) Are anaesthetics toxic to the brain? Br J Anaesth 107:30-7.

Huh PW, Belayev L, Zhao W, Koch S, Busto R, Ginsberg MD (2000)
Comparative neuroprotective efficacy of prolonged moderate intraischemic and postischemic hypothe rmia infocal cerebral ischemia. J Neurosurg 92:91-9.

Hunsberger HC, Rudy CC, Batten SR, Gerhardt GA, Reed MN (2015) P301L tau expression affects glutamate release and clearance in the hippocampal trisynaptic pathway. J Neurochem 132:169-82.

Hussain M, Berger M, Eckenhoff RG, Seitz DP (2014) General anesthetic and
the risk of dementia in elderly patients: current insights. Clin Interv Aging 9:1619-28.

Hutchison JS, Ward RE, Lacroix J, Hébert PC, Barnes MA, Bohn DJ, Dirks PB, Doucette S, Fergusson D, Gottesman R, Joffe AR, Kirpalani HM, Meyer PG,Morris KP, Moher D, Singh RN, Skippen PW (2008) Hypothermia Pediatric Head Injury Trial Investigators and the Canadian Critical Care Trials Group. Hypothermia therapy after traumatic brain injury in children. N Engl J Med 358:2447-56.

Hutton M, Lendon CL, Rizzu P, Baker M, Froelich S, Houlden H, Pickering-Brown S, Chakraverty S, Isaacs A, Grover A, Hackett J, Adamson J, Lincoln S,Dickson D, Davies P, Petersen RC, Stevens M, de Graaff E, Wauters E, van Baren J, Hillebrand M, Joosse M, Kwon JM, Nowotny P, Che LK, Norton J, Morris JC, Reed LA, Trojanowski J, Basun H, Lannfelt L, Neystat M, Fahn S, Dark F, Tannenberg T, Dodd PR, Hayward N, Kwok JB, Schofield PR, Andreadis A,Snowden J, Craufurd D, Neary D, Owen F, Oostra BA, Hardy J, Goate A, van Swieten J, Mann D, Lynch T, Heutink P (1998) Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17. Nature 393:702-05.

Hyman BT (2014) Tau propagation, different tau phenotypes, and prion-like properties of tau. Neuron 82:1189-90.

Hyman BT, Van Hoesen GW, Damasio AR, Barnes CL (1984) Alzheimer's disease: cell-specific pathology isolates the hippocampal formation. Science 225:1168-70.

Hypothermia after Cardiac Arrest Study Group (2002) Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest. N Engl J Med 346:549-56.

Ihara Y (1988) Massive somatodendritic sprouting of cortical neurons in Alzheimer's disease. Brain Res 459:138-44.

Ihara Y (2001) PHF and PHF-like fibrils--cause or consequence? Neurobiol Aging 22:123-6.

Iijima T, Mishima T, Akagawa K, Iwao Y (2006) Neuroprotective effect of propofol on necrosis and apoptosis following oxygen-glucose deprivation--relationship between mitochondrial membrane potential and mode of death. Brain Res 1099:25-32.

Ikeda Y, Ishiguro K, Fujita SC (2007) Ether stress-induced Alzheimer-like tau phosphorylation in the normal mouse brain. FEBS Lett 581:891-7.

Ikegaya Y, Kim JA, Baba M, Iwatsubo T, Nishiyama N, Matsuki N (2001)
Rapid and reversible changes in dendrite morphology and synaptic efficacy following NMDA recepto r activation:implication for a cellular defense against excitotoxicity. J Cell Sci 114:4083-93.

Ikonomovic MD, Uryu K, Abrahamson EE, Ciallella JR, Trojanowski JQ, Lee VM, Clark RS, Marion DW, Wisniewski SR, DeKosky ST (2004) Alzheimer's pathology in human temporal cortex surgically excised after severe brain injury. Exp Neurol 190:192-203.

Illenberger S, Zheng-Fischhöfer Q, Preuss U, Stamer K, Baumann K, Trinczek B, Biernat J, Godemann R, Mandelkow EM, Mandelkow E (1998) The endogenous and cell cycle-dependent phosphorylation of tau protein in living cells: implications forAlzheimer's disease. Mol Biol Cell 9:1495-512.

Ingram EM, Spillantini MG (2002) Tau gene mutations: dissecting the pathogenesis of FTDP-17. Trends Mol Med 8: 555-562.

Iovino M, Patani R, Watts C, Chandran S, Spillantini SG (2010) Human stem cell-derived neurons: a system to study tau function and dysfunction. PLoS One 5:e13947.

Iovino M, Pfisterer U, Holton JL, Lashley T, Swingler RJ, Calo L, Treacy R, Revesz T, Parmar M, Goedert M, Muqit MM, Spillantini MG (2013) The novel MAPT mutation K298E: mechanisms of mutant tau toxicity, brain pathology and tau expression in induced fibroblast-derived neurons. Acta Neuropathol 127:283-95.

Iqbal K, Grundke-Iqbal I, Zaidi T, Merz PA, Wen GY, Shaikh SS, Wisniewski HM, Alafuzoff I, Winblad B (1986) Defective brain microtubule assembly in Alzheimer's disease. Lancet 2:421-6.

Iqbal K, Liu F, Gong CX, Alonso Adel C, Grundke-Iqbal I (2009) Mechanisms of tau-induced neurodegeneration. Acta Neuropathol 118:53-69.

Ishihara T, Hong M, Zhang B, Nakagawa Y, Lee MK, Trojanowski JQ, Lee VM (1999) Age-dependent emergence and progression of a tauopathy in transgenic mice overexpressing the shortest human tau isoform. Neuron 24:751-762.

Isopi E, Granzotto A, Corona C, Bomba M, Ciavardelli D, Curcio M, Canzoniero LM, Navarra R, Lattanzio R, Piantelli M, Sensi SL (2014) Pyruvate prevents the development of age-dependent cognitive deficits in
a mouse model of Alzheimer's diseasewithout reducing amyloid and tau pathology. Neurobiol Dis S0969-9961(14)00358-1.

Israel MA, Yuan SH, Bardy C, Reyna SM, Mu Y, Herrera C, Hefferan MP, Van Gorp S, Nazor KL, Boscolo FS, Carson CT, Laurent LC, Marsala M, Gage FH,Remes AM, Koo EH, Goldstein LS (2012) Probing sporadic and familial Alzheimer's disease using induced pluripotent stem cells. Nature 482:216-220.

Ito D, Komatsu T, Gohara K (2013) Measurement of saturation processes in glutamatergic and GABAergic synapse densities during long-term development of cultured rat cortical networks. Brain Res 1534:22-32.

Ittner LM, Götz J (2011) Amyloid-β and tau--a toxic pas de deux in Alzheimer's disease. Nat Rev Neurosci 12:65-72.

Ittner LM, Ke YD, Delerue F, Bi M, Gladbach A, van Eersel J, Wölfing H, Chieng BC, Christie MJ, Napier IA, Eckert A, Staufenbiel M, Hardeman E, Götz J (2010) Dendritic function of tau mediates amyloid-beta toxicity in Alzheimer's disease mouse models. Cell 142:387-397.

Jäättelä M (1999) Heat shock proteins as cellular lifeguards. Ann Med 31:261-71.

Jackson GR, Wiedau-Pazos M, Sang TK, Wagle N, Brown CA, Massachi S, Geschwind DH (2002) Human wild-type tau interacts with wingless pathway components and produces neurofibrillary pathology inDrosophila. Neuron 34:509-19.

Jacobs SE, Berg M, Hunt R, Tarnow-Mordi WO, Inder TE, Davis PG (2013) Cooling for newborns with hypoxic ischaemic encephalopathy. Cochrane Database Syst Rev 1:CD003311.

James OT, Livesey MR, Qiu J, Dando O, Bilican B, Haghi G, Rajan R, Burr K, Hardingham GE, Chandran S, Kind PC, Wyllie DJ (2014)
Ionotropic GABA and glycine receptor subunit composition in human pluripotent stem cell-derived excitatorycortical neurones. J Physiol 592:4353-63.

Janke C, Beck M, Stahl T, Holzer M, Brauer K, Bigl V, Arendt T (1999) Phylogenetic diversity of the expression of the microtubule associated protein tau: implications for neurodegenerative disorders. Brain Res Mol Brain Res 68:119-28.

Janson J, Laedtke T, Parisi JE, O'Brien P, Petersen RC, Butler PC (2004) Increased risk of type 2 diabetes in Alzheimer disease. Diabetes 53:474-81.

Janssen R (1992) Thermal influences on nervous system function. Neurosci Biobehav Rev 16:399-413.

Jantzie LL, Talos DM, Jackson MC, Park HK, Graham DA, Lechpammer M, Folkerth RD, Volpe JJ, Jensen FE (2015) Developmental Expression of N-Methyl-d-
Aspartate (NMDA) Receptor Subunits in Human White and Gray Matter:Potential Mechanism of Incr eased Vulnerability in the Immature Brain. Cereb Cortex 25:482-95.

Jedema HP, Grace AA (2003)
Chronic exposure to cold stress alters electrophysiological properties of locus coeruleus neurons recor ded invitro. Neuropsychopharmacology 28:63-72.

Jellinger KA (2010) Basic mechanisms of neurodegeneration: a critical update. J Cell Mol Med 14:457-87.

Ji X, Luo Y, Ling F, Stetler RA, Lan J, Cao G, Chen J (2007)
Mild hypothermia diminishes oxidative DNA damage and pro-
death signaling events after cerebral ischemia: amechanism for neuroprotection. Front Biosci 12:1737-47.

Jiang HQ, Ren M, Jiang HZ, Wang J, Zhang J, Yin X, Wang SY, Qi Y, Wang XD, Feng HL (2014) Guanabenz delays the onset of disease symptoms, extends lifespan, improves motor performance anda ttenuates motor neuron loss in the SOD1 G93A mouse model of amyotrophic lateral sclerosis. Neuroscience 277:132-8.

Jin T, Gu Y, Zanusso G, Sy M, Kumar A, Cohen M, Gambetti P, Singh N (2000) The chaperone protein BiP binds to a mutant prion protein and mediates its degradation by the proteasome. J Biol Chem 275:38699-704.

Jögi A, Brennan DJ, Rydén L, Magnusson K, Fernö M, Stål O, Borgquist S, Uhlen M, Landberg G, Påhlman S, Pontén F, Jirström K (2009) Nuclear expression of the RNA-binding protein RBM3 is associated with an improved clinical outcome in breast cancer. Mod Pathol 22:1564-74.

Johnson GV, Seubert P, Cox TM, Motter R, Brown JP, Galasko D (1997) The
tau protein in human cerebrospinal
fluid in Alzheimer's disease consists of proteolytically derivedfragments. J Neurochem 68:430-3.

Johnson MA, Weick JP, Pearce RA, Zhang S-C (2007) Functional neural development from human embryonic stem cells: accelerated synaptic activity via astrocyte co-culture. J Neurosci 27:3069-3077.

Jones PG, Inouye M (1994) The cold-shock response - a hot topic. Mol Microbiol 11:811-8.

Julien C, Marcouiller F, Bretteville A, El Khoury NB, Baillargeon J, Hébert SS, Planel E (2012) Dimethyl sulfoxide induces both direct and indirect tau hyperphosphorylation. PLoS One 7:e40020.

Kadoshima T, Sakaguchi H, Nakano T, Soen M, Ando S, Eiraku M, Sasai Y (2013) Self-organization of axial polarity, inside-out layer pattern, and species-specific progenitor dynamics in human ES cell–derived neocortex. Proc Natl Acad Sci USA 110:20284-9.

Kalmbach AS, Waters J (2012) Brain surface temperature under a craniotomy. J Neurophysiol 108:3138-46.

Kamat PK, Rai S, Swarnkar S, Shukla R, Ali S, Najmi AK, Nath C (2013) Okadaic acid-
induced Tau phosphorylation in rat brain: role of NMDA receptor. Neuroscience 238:97-113.

Kamme F, Campbell K, Wieloch T (1995) Biphasic expression of
the fos and jun families of transcription factors following transient forebrain ischaemia in the rat. Effect of hypothermia. Eur J Neurosci 7:2007-16.

Kammersgaard LP, Jørgensen HS, Rungby JA, Reith J, Nakayama H, Weber UJ, Houth J, Olsen TS (2002) Admission body temperature predicts long-term mortality after acute stroke: the Copenhagen Stroke Study. Stroke 33:1759-62.

Kanamaru T, Kamimura N, Yokota T, Iuchi K, Nishimaki K, Takami S, Akashiba H, Shitaka Y, Katsura K, Kimura K, Ohta S (2015) Oxidative stress accelerates amyloid deposition and memory impairment in a double-transgenic mouse model of Alzheimer's disease. Neurosci Lett 587:126-31.

Kandel ER, Schwartz JH, Jessell TM (2000) Principles of Neural Science Fourth Edition McGraw Hill, p 128-9.

Kaneko T, Kibayashi K (2012) Mild hypothermia facilitates the expression of cold-inducible RNA-binding protein and heat shock protein 70.1 in mouse brain. Brain Res 1466:128-36

Kapfhammer JP, Schwab ME (1994) Inverse patterns of myelination and GAP-43 expression in the adult CNS: neurite growth inhibitors as regulators of neuronal plasticity? J Comp Neurol 340:194-206.

Kapoor S, Berishvili E, Bandi S, Gupta S (2014) Ischemic preconditioning affects long-term cell fate through DNA damage-related molecular signaling andaltered proliferation. Am J Pathol 184:2779-90.

Kar A, Havlioglu N, Tarn W-Y, Wu JY (2006) RBM4 interacts with an intronic element and stimulates tau exon 10 inclusion. J Biol Chem 281:24479-24488.

Karch CM, Jeng AT, Goate AM (2012)
Extracellular Tau levels are influenced by variability in Tau that is associated with tauopathies. J Biol Chem 287:42751-62.

Karibe H, Zarow GJ, Graham SH, Weinstein PR (1994)
Mild intraischemic hypothermia reduces postischemic hyperperfusion, delayed postischemic hypoperf usion, blood-brain barrier disruption, brain edema,
and neuronal damage volume after temporary focal cerebralischemia in rats. J Cereb Blood Flow Metab 14:620-7.

Katano H, Masago A, Yamada K (1995) Marked alteration of c-fos and c-jun but not hsp70 messenger RNA expression in rat brain after cold-induced trauma: An in situ hybridization study. Eur J Neurosci 7:2007-16.

Kato H, Liu Y, Araki T, Kogure K (1991) Temporal profile of
the effects of pretreatment with brief cerebral ischemia on
the neuronal damage followingsecondary ischemic insult in
the gerbil: cumulative damage and protective effects. Brain Res 553:238-42.

Katz B, Miledi R (1965) The effect of temperature on the synaptic delay at the neuromuscular junction. J Physiol 181:656-670.

Kaufman RJ, Scheuner D, Schröder M, Shen X, Lee K, Liu CY, Arnold SM (2002) The unfolded protein response in nutrient sensing and differentiation. Nat Rev Mol Cell Biol 3:411-21.

Ke YD, Delerue F, Gladbach A, Götz J, Ittner LM (2009) Experimental diabetes mellitus exacerbates tau pathology in a transgenic mouse model of Alzheimer's disease. PLoS One 4:e7917.

Ke YD, Suchowerska AK, van der Hoven J, De Silva DM, Wu CW, van Eersel J, Ittner A, Ittner LM (2012) Lessons from tau-deficient mice. Int J Alzheimers Dis 2012:873270.

Keck S, Nitsch R, Grune T, Ullrich O (2003) Proteasome inhibition by paired helical filament-tau in brains of patients with Alzheimer's disease. J Neurochem 85:115-22.

Kedersha N, Chen S, Gilks N, Li W, Miller IJ, Stahl J, Anderson P (2002) Evidence that ternary complex (eIF2-GTP-tRNA(i)(Met))-deficient preinitiation complexes are core constituents of mammalian stress granules. Mol Biol Cell 13:195-210.

Keller JN, Hanni KB, Markesbery WR (2000) Impaired proteasome function in Alzheimer's disease. J Neurochem 75:436-9.

Kew JNC, Trube G, Kemp JA (1996) A novel mechanism of activity-dependent NMDA receptor antagonism describes the effect of ifenprodil in rat cultured cortical neurones. J Physiol 497:761-722.

Khatri N, Man HY (2013) Synaptic activity and bioenergy homeostasis: implications in brain trauma and neurodegenerative diseases. Front Neurol 4:199.

Khurana V, Lu Y, Steinhilb ML, Oldham S, Shulman JM, Feany MB (2006) TOR-mediated cell-cycle activation causes neurodegeneration in a Drosophila tauopathy model. Curr Biol 16:230-41.

Kieran D, Kalmar B, Dick JR, Riddoch-Contreras J, Burnstock G, Greensmith L (2004) Treatment with arimoclomol, a coinducer of heat shock proteins, delays disease progression in ALS mice. Nat Med 10:402-5.

Kiernan MC, Cikurel K, Bostock H (2001) Effects of temperature on the excitability properties of human motor axons. Brain 124:816-25.

Kil HY, Zhang J, Piantadosi CA (1996) Brain temperature alters hydroxyl radical production during cerebral ischemia/reperfusion in rats. J Cereb Blood Flow Metab 16:100-6.

Kim W, Lee S, Hall GF (2010) Secretion of human tau fragments resembling CSF-tau in Alzheimer's disease is modulated by the presence of the exon 2 insert. FEBS Lett 584:3085-8.

Kim HJ, Raphael AR, LaDow ES, McGurk L, Weber RA, Trojanowski JQ, Lee VM, Finkbeiner S, Gitler AD, Bonini NM (2014) Therapeutic modulation of eIF2α phosphorylation rescues TDP-43 toxicity in amyotrophic lateral sclerosisdisease models. Nat Genet 46:152-60.

Kim AJ, Shi Y, Austin RC, Werstuck GH (2005) Valproate protects cells from ER stress-induced lipid accumulation and apoptosis by inhibiting glycogen synthase kinase-3. J Cell Sci 118:89-99.

Kim I, Xu W, Reed JC (2008) Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. Nat Rev Drug Discov 7:1013–1030.

King A, AL-Sarraj S, Troakes C, Smith BN, Maekawa S, Iovino M, Spillantini MG, Shaw CE (2013) Mixed tau, TDP-43 and p62 pathology in FTLD associated with C9ORF72 repeat expansion and p.Ala239Thr MAPT (tau) variant. Acta Neuropathol 125:303-10.

King L, Weber G (1986) Conformational drift and cryoinactivation of lactate dehydrogenase. Biochemistry 25:3637-40.

Kingston S, Mao L, Yang L, Arora A, Fibuch EE, Wang JQ (2006) Propofol inhibits phosphorylation of N-methyl-D-aspartate receptor NR1 subunits in neurons. Anesthesiology 104:763-9.

Kirov SA, Petrak LJ, Fiala JC, Harris KM (2004) Dendritic spines disappear with chilling but proliferate excessively upon rewarming of mature hippoca mpus. Neuroscience 127:69-80.

Kiskinis E, Sandoe J, Williams LA, Boulting GL, Moccia R, Wainger BJ, Han S, Peng T, Thams S, Mikkilineni S, Mellin C, Merkle FT, Davis-Dusenbery BN, Ziller M, Oakley D, Ichida J, Di Costanzo S, Atwater N, Maeder ML, Goodwin MJ, Nemesh J, Handsaker RE, Paull D, Noggle S, McCarroll SA, Joung JK, Woolf CJ, Brown RH, Eggan K (2014) Pathways disrupted in human ALS motor neurons identified through genetic correction of mutant SO D1. Cell Stem Cell 14:781-95.

Kita H, Carmichael J, Swartz J, Muro S, Wyttenbach A, Matsubara K, Rubinsztein DC, Kato K (2002) Modulation of polyglutamine-induced cell death by genes identified by expression profiling. Hum Mol Genet 11:2279-2287.

Kitagawa K, Matsumoto M, Kuwabara K, Tagaya M, Ohtsuki T, Hata R, Ueda H, Handa N, Kimura K, Kamada T (1991) 'Ischemic tolerance' phenomenon detected in various brain regions. Brain Res 561:203-11.

Kiyatkin EA (2010) Brain temperature homeostasis: physiological fluctuations and pathological shifts. Front Biosci (Landmark Ed) 15:73-92.

Klose MK, Armstrong G, Robertson RM (2004) A role for the cytoskeleton in heat-shock-mediated thermoprotection of locust neuromuscular junctions. J Neurobiol 60:453-62.

Klose MK, Atwood HL, Robertson RM (2008) Hyperthermic preconditioning of presynaptic calcium regulation in Drosophila. J Neurophysiol 99:2420-30.

Koga H, Kaushik S, Cuervo AM (2011) Protein homeostasis and aging:
The importance of exquisite quality control. Ageing Res Rev 10:205-15.

Koh JY, Choi DW (1987) Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. J Neurosci Methods 20: 83–90.

Köhler C, Dinekov M, Götz J (2014)
Granulovacuolar degeneration and unfolded protein response in mouse models of tauopathy and Aβamyloidosis. Neurobiol Dis 71:169-79.

Kojima S, Matsumoto K, Hirose M, Shimada M, Nagano M, Shigeyoshi Y, Hoshino S, Ui-Tei K, Saigo K, Green CB, Sakaki Y, Tei H (2007) LARK activates posttranscriptional expression of an essential mammalian clock protein, PERIOD1. Proc Natl Acad Sci USA 104:185964.

Kopeikina KJ, Hyman BT, Spires-Jones TL (2012) Soluble forms of tau are toxic in Alzheimer's disease. Transl Neurosci 3:223–233.

Kopeikina KJ, Polydoro M, Tai HC, Yaeger E, Carlson GA, Pitstick R, Hyman BT, Spires-Jones TL (2013) Synaptic alterations in the rTg4510 mouse model of tauopathy. J Comp Neurol 521:1334-53.

Köpke E, Tung YC, Shaikh S, Alonso AC, Iqbal K, Grundke-Iqbal I (1993) Microtubule-associated protein tau. Abnormal phosphorylation of a non-paired helical filament pool in Alzheimer disease. J Biol Chem 268:24374-84.

Kosik KS, Orecchio LD, Bakalis S, Neve RL (1989)
Developmentally regulated expression of specific tau sequences. Neuron 2:1389-97.

Kraft AD, Johnson DA, Johnson JA (2004) Nuclear factor E2-related factor 2-dependent antioxidant response element activation by tert-
butylhydroquinoneand sulforaphane occurring preferentially in astrocytes conditions neurons against oxidative insult. J Neurosci 24:1101-12.

Krishnamoorthy J, Rajesh K, Mirzajani F, Kesoglidou P, Papadakis AI, Koromilas AE (2014)
Evidence for eIF2α phosphorylation-independent effects of GSK2656157,
a novel catalytic inhibitor of PERK withclinical implications. Cell Cycle 13:801-6.

Kudo T, Kanemoto S, Hara H, Morimoto N, Morihara T, Kimura R, Tabira T, Imaizumi K, Takeda M (2008) A molecular chaperone inducer protects neurons from ER stress. Cell Death Diffe 15:364-75

Kumura E, Yoshimine T, Takaoka M, Hayakawa T, Shiga T, Kosaka H (1996) Hypothermia suppresses nitric oxide elevation during reperfusion after focal cerebral ischemia in rats. Neurosci Lett 220:45-8.

Kunz AR, Iliadis C (2007) Hominid evolution of the arteriovenous system through the cranial base and its relevance for craniosynostosis. Childs Nerv Syst 23:1367-77.

Kuo JR, Lo CJ, Wang CC, Lu CL, Lin SC, Chen CF (2011) Measuring brain temperature while maintaining brain normothermia in patients with severe traumatic braininjury. J Clin Neurosci. 18:1059-63.

Kräuchi K, Deboer T (2010) Chapter 28 'Body Temperatures, Sleep, and Hibernation', Part I Section 4: Physiology in Sleep in: 'Principles and Practice of Sleep Medicine', Fifth Edition, edited by Kryger MH, Roth T and Dement WC, Elsevier Health Sciences, p323-334.

Krstic D, Knuesel I (2013) Deciphering the mechanism underlying late-onset Alzheimer disease. Nat Rev Neurol 9:25-34.

Kuchibhotla KV, Wegmann S, Kopeikina KJ, Hawkes J, Rudinskiy N, Andermann ML, Spires-Jones TL, Bacskai BJ, Hyman BT (2014) Neurofibrillary tangle-bearing neurons are functionally integrated in cortical circuits in vivo. Proc Natl Acad Sci U S A 111:510-4.

Ksiezak-Reding H, Liu WK, Yen SH (1992) Phosphate analysis and dephosphorylation of modified tau associated with paired helical filaments. Brain Res 597:209-19.

Kuo JR, Lo CJ, Wang CC, Lu CL, Lin SC, Chen CF (2001) Measuring brain temperature while maintaining brain normothermia in patients with severe traumatic brain injury. J Clin Neurosci 18:1059-63.

Lai MC, Kuo H-W, Chang W-C, Tarn W-Y (2003) A novel splicing regulator shares a nuclear import pathway with SR proteins. EMBO J 22:1359-69.

Landolt HP, Moser S, Wieser HG, Borbély AA, Dijk DJ (1995) Intracranial temperature across 24-hour sleep-wake cycles in humans. Neuroreport 6:913-7.

Larner SF, Hayes RL, Wang KK (2006) Unfolded protein response after neurotrauma. J Neurotrauma 23:807-829.

Larson M, Sherman MA, Amar F, Nuvolone M, Schneider JA, Bennett DA, Aguzzi A, Lesné SE (2012) The complex PrP(c)-Fyn couples human oligomeric Aβ with pathological tau changes in Alzheimer's disease. J Neurosci 32:16857-71a.

Lasagna-Reeves CA, Castillo-Carranza DL, Sengupta U, Clos AL, Jackson GR, Kayed R (2011) Tau oligomers impair memory and induce synaptic and mitochondrial dysfunction in wild-type mice. Mol Neurodegener 6:39.

Lee VM, Balin BJ, Otvos L Jr, Trojanowski JQ (1991) A68: a major subunit of paired helical filaments and derivatized forms of normal Tau. Science 251:675-8.

Lee VM, Brunden KR, Hutton M, Trojanowski JQ (2011) Developing therapeutic approaches to tau, selected kinases, and related neuronal protein targets. Cold Spring Harb Perspect Med 1:a006437.

Lee YJ, Mou Y, Klimanis D, Bernstock JD, Hallenbeck JM (2014) Global SUMOylation is a molecular mechanism underlying hypothermia-induced ischemic tolerance. Front Cell Neurosci 8:416.

Lee HG, Perry G, Moreira PI, Garrett MR, Liu Q, Zhu X, Takeda A, Nunomura A, Smith MA (2005) Tau phosphorylation in Alzheimer's disease: pathogen or protector? Trends Mol Med 11:164-9.

Le Grevès P, Sharma HS, Westman J, Alm P, Nyberg F (1997)
Acute heat stress induces edema and nitric oxide synthase upregulation and down-regulates mRNA levels of theNMDAR1, NMDAR2A and NMDAR2B subunits in the rat hippocampus. Acta Neurochir Suppl 70:275-8.

Lei P, Ayton S, Finkelstein DI, Spoerri L, Ciccotosto GD, Wright DK, Wong BX, Adlard PA, Cherny RA, Lam LQ, Roberts BR, Volitakis I, Egan GF, McLean CA,Cappai R, Duce JA, Bush AI (2012) Tau deficiency induces parkinsonism with dementia by impairing APP-mediated iron export. Nat Med 18:291-5

León-Espinosa G, García E, García-Escudero V, Hernández F, Defelipe J, Avila J (2013) Changes in tau phosphorylation in hibernating rodents. J Neurosci Res 91:954-62.

Lesort M, Blanchard C, Yardin C, Esclaire F, Hugon J (1997)
Cultured neurons expressing phosphorylated tau are more resistant to apoptosis induced by NMDA or serum deprivation. Brain Res Mol Brain Res 45:127-32.

Lewerenz J, Maher P (2009)
Basal levels of eIF2alpha phosphorylation determine cellular antioxidant status by regulating ATF4 an d xCTexpression. J Biol Chem 284:1106-15.

Lewis J, McGowan E, Rockwood J, Melrose H, Nacharaju P, Van Slegtenhorst M, Gwinn-Hardy K, Paul Murphy M, Baker M, Yu X, Duff K, Hardy J, Corral A, Lin WL, Yen SH, Dickson DW, Davies P, Hutton M (2000) Neurofibrillary
tangles, amyotrophy and progressive motor disturbance in mice expressing mutant (P301L) tau protein. Nat Genet 25:402-5.

Li XM, Delaunay F, Dulong S, Claustrat B, Zampera S, Fujii Y, Teboul M, Beau J, Lévi F (2010) Cancer inhibition through circadian reprogramming of tumor transcriptome with meal timing. Cancer Res 70:3351-60.

Li PA, He QP, Miyashita H, Howllet W, Siesjö BK, Shuaib A (1999) Hypothermia ameliorates ischemic brain damage and suppresses the release of extracellular amino acids in both normo- and hyperglycemic subjects. Exp Neurol 158:242-53.

Li HL, Wang HH, Liu SJ, Deng YQ, Zhang YJ, Tian Q, Wang XC, Chen XQ, Yang Y, Zhang JY, Wang Q, Xu H, Liao FF, Wang JZ (2007)
Phosphorylation of tau antagonizes apoptosis by stabilizing beta-catenin,
a mechanism involved in Alzheimer'sneurodegeneration. Proc Natl Acad Sci U S A 104:3591-6.

Li G, Yin H, Kuret J (2004)
Casein kinase 1 delta phosphorylates tau and disrupts its binding to microtubules. J Biol Chem 279:15938-45.

Li S, Zhang Z, Xue J, Liu A, Zhang H (2012) Cold-inducible RNA binding protein inhibits $H_2O_2$-induced apoptosis in rat cortical neurons. Brain Res 1441:47-52.

Lin JC, Hsu M, Tarn W-Y (2007a) Cell stress modulates the function of splicing regulatory protein RBM4 in translational control. Proc Natl Acad Sci USA 104:2235-40.

Lin JH, Li H, Yasumura D, Cohen HR, Zhang C, Panning B, Shokat KM, Lavail MM, Walter P (2007b) IRE1 signaling affects cell fate during the unfolded protein response. Science 318:944-9.

Lindwall G, Cole RD (1984) Phosphorylation affects the ability of tau protein to promote microtubule assembly. J Biol Chem 259:5301-5.

Lipton SA, Nakanishi N (1999) Shakespeare in love--with NMDA receptors? Nat Med 5:270-1.

Lipton SA, Rosenberg PA (1994) Excitatory amino acids as a final common pathway for neurologic disorders. N Engl J Med 330:613-22.

Liu AY, Bian H, Huang LE, Lee YK (1994) Transient cold shock induces the heat shock response upon recovery at 37 degrees C in human cells. J Biol Chem 269:14768-75.

Liu L, Drouet V, Wu JW, Witter MP, Small SA, Clelland C, Duff K (2012a) Trans-synaptic spread of tau pathology in vivo. PLoS One 7:e31302.

Liu ZC, Fu ZQ, Song J, Zhang JY, Wei YP, Chu J, Han L, Qu N, Wang JZ, Tian Q (2012b) Bip enhanced the association of GSK-3β with tau during ER stress both in vivo and in vitro. J Alzheimers Dis 29:727-40.

Liu F, Grundke-Iqbal I, Iqbal K, Gong CX (2005) Contributions of protein phosphatases PP1, PP2A, PP2B and PP5 to the regulation of tau phosphorylation. Eur J Neurosci 22: 1942-50.

Liu Y, Hu W, Murakawa Y, Yin J, Wang G, Landthaler M, Yan J (2013) Cold-induced RNA-binding proteins regulate circadian gene expression by controlling alternative polyadenylation. Sci Rep 3:2054.

Liu XA, Song J, Jiang Q, Wang Q, Tian Q, Wang JZ (2012c) Expression of the hyperphosphorylated tau attenuates ER stress-induced apoptosis with upregulation of unfolded protein response. Apoptosis 17:1039-49.

Liu Y, Szaro BG (2011) hnRNP K post-transcriptionally co-regulates multiple cytoskeletal genes needed for axonogenesis. Development 138:3079-90.

Liu A, Zhang Z, Li A, Xue J (2010) Effects of hypothermia and cerebral ischemia on cold-inducible RNA-binding protein mRNA expression in ratbrain. Brain Res 1347:104-10.

Litersky JM, Johnson GV (1992) Phosphorylation by cAMP-dependent protein kinase inhibits the degradation of tau by calpain. J Biol Chem 267:1563-8.

Livesey MR, Bilican B, Qiu J, Rzechorzek NM, Haghi G, Burr K, Hardingham GE, Chandran S, Wyllie DJ (2014) Maturation of AMPAR composition and the $GABA_AR$ reversal potential in hPSC-derived cortical neurons. J Neurosci 34:4070-5.

Lleonart ME (2010) A new generation of proto-oncogenes: cold-inducible RNA binding proteins. Biochim Biophys Acta 1805:43-52.

Lopez M, Meier D, Müller A, Franken P, Fujita J, Fontana A (2014) Tumor necrosis factor and transforming growth factor β regulate clock genes by controlling the expression of the cold inducible RNA-binding protein (CIRBP). J Biol Chem 289:2736-44.

Lopresti P, Szuchet S, Papasozoomenos SC, Zinkowski RP, Binder LI (1995) Functional implications for the microtubule-associated protein tau: localization in oligodendrocytes. Proc Natl Acad Sci USA 92:10369-73.

Louis AA, Hotson JR (1986) Regional cooling of human nerve and slowed Na+ inactivation. Electroencephalogr Clin Neurophysiol 63:371-5.

Lowitzsch K, Hopf HC, Galland J (1977)
Changes of sensory conduction velocity and refractory periods with decreasing tissue temperature in man. J Neurol 216:181-8.

Lu R, Wang H, Liang Z, Ku L, O'donnell WT, Li W, Warren ST, Feng Y (2004) The fragile X protein controls microtubule-associated protein 1B translation and microtubule stability in brain neuron development. Proc Natl Acad Sci USA 101:15201-6.

Ludin HP, Beyeler F (1977) Temperature dependence of normal sensory nerve action potentials. J Neurol 216:173-80.

Luo Y, Ji X, Ling F, Li W, Zhang F, Cao G, Chen J (2007) Impaired DNA repair via the base-excision repair pathway after focal ischemic brain injury: a proteinphosphorylation-dependent mechanism reversed by hypothermic neuroprotection. Front Biosci 12:1852-62.

Lyeth BG, Jiang JY, Robinson SE, Guo H, Jenkins LW (1993)
Hypothermia blunts acetylcholine increase in CSF of traumatically brain injured rats. Mol Chem Neuropathol 18:247-56.

Ma Y, Hendershot LM (2003) Delineation of a negative feedback regulatory loop that controls protein translation during endoplasmic reticulum stress. J Biol Chem. 278:34864-73.

Ma QL, Zuo X, Yang F, Ubeda OJ, Gant DJ, Alaverdyan M, Kiosea NC, Nazari S, Chen PP, Nothias F, Chan P, Teng E, Frautschy SA, Cole GM (2014) Loss of MAP function leads to hippocampal synapse loss and deficits in the Morris Water Maze with aging. J Neurosci 34:7124-36.

Maekawa S, Aibiki M, Si QS, Nakamura Y, Shirakawa Y, Kataoka K (2002)
Differential effects of lowering culture temperature on mediator release from lipopolysaccharide-stimulatedneonatal rat microglia. Crit Care Med 30:2700-4.

Mah VH, Eskin TA, Kazee AM, Lapham L, Higgins GA (1992)
In situ hybridization of calcium/calmodulin dependent protein kinase II andtau mRNAs; species differ ences and relative preservation in Alzheimer'sdisease. Brain Res Mol Brain Res 12:85-94.

Maier CM, Chan PH (2002) Role of superoxide
dismutases in oxidative damage and neurodegenerative disorders. Neuroscientist 8:323-34.

Malchiodi-Albedi F, Petrucci TC, Picconi B, Iosi F, Falchi M (1997)
Protein phosphatase inhibitors induce modification of synapse structure and tau hyperphosphorylation incultured rat hippocampal neurons. J Neurosci Res 48:425-38.

Malhotra JD, Kaufman RJ (2011) ER stress and its functional link to mitochondria: role in cell survival and death. Cold Spring Harb Perspect Biol 3:a004424.

Mandelkow EM, Mandelkow E (2012) Biochemistry and cell biology of tau protein in neurofibrillary degeneration. Cold Spring Harb Perspect Med 2:a006247.

Marber MS, Mestril R, Chi SH, Sayen MR, Yellon DM, Dillmann WH (1995) Overexpression of the rat inducible 70-kD heat stress protein in a transgenic mouse increases the resistance of the heart to ischemic injury. J Clin Invest 95:1446-56.

Marion DW, Penrod LE, Kelsey SF, Obrist WD, Kochanek PM, Palmer AM, Wisniewski SR, DeKosky ST (1997) Treatment of traumatic brain injury with moderate hypothermia. N Engl J Med 336:540-6.

Markus MA, Morris BJ (2006) Lark is the splicing factor RBM4 and exhibits unique subnuclear localization properties. DNA Cell Biol 25:457-64.

Markus MA, Morris BJ (2009) RBM4: a multifunctional RNA-binding protein. Int J Biochem Cell Biol 41:740-3.

Martin S, Lamb HK, Brady C, Lefkove B, Bonner MY, Thompson P, Lovat PE, Arbiser JL, Hawkins AR, Redfern CP (2013) Inducing apoptosis of cancer cells using small-molecule plant compounds that bind to GRP78. Br J Cancer 109:433-43.

Martínez-Arribas F, Agudo D, Pollán M, Gómez-Esquer F, Díaz-Gil G, Lucas R, Schneider J (2006) Positive correlation between the expression of X chromosome RBM genes (RBMX, RBM3, RBM10) and the proapoptotic Bax gene in human breast cancer. J Cell Biochem 97:1275-1282.

Marzec M, Eletto D, Argon Y (2012) GRP94: An HSP90-like protein specialized for protein folding and quality control in the endoplasmic reticulum. Biochim Biophys Acta 1823:774-87.

Masino SA, Dunwiddie TV (1999) Temperature-dependent modulation of excitatory transmission in hippocampal slices is mediated by extracellularad enosine. J Neurosci 19:1932-9.

Matijasevic Z, Snyder JE, Ludlum DB (1998) Hypothermia causes a reversible, p53-mediated cell cycle arrest in cultured fibroblasts. Oncol Res 10:605-610.

Matsuda T, Fujita J (1997) Increased transcript level of RBM3, a member of the glycine-rich RNA-binding protein family, in human cells in response to cold stress. Biochem Biophys Res Commun 236:804-7.

Matsuda A, Ogawa M, Yanai H, Naka D, Goto A, Ao T, Tanno Y, Takeda K, Watanabe Y, Honda K, Taniguchi T (2011) Generation of mice deficient in RNA-binding motif protein 3 (RBM3) and characterization of its role in innateimmune responses and cell growth. Biochem Biophys Res Commun 411:7-13.

Matsuo ES, Shin RW, Billingsley ML, Van deVoorde A, O'Connor M, Trojanowski JQ, Lee VM (1994) Biopsy-derived adult human brain tau is phosphorylated at many of the same sites as Alzheimer's disease paired helical filament tau. Neuron 13:989-1002.

Mattson MP (1990) Antigenic changes similar to those seen in neurofibrillary tangles are elicited by glutamate and Ca2+ influx in cultured hippocampal neurons. Neuron 4:105-17.

Mattson MP, Dou P, Kater SB (1988) Outgrowth-regulating actions of glutamate in isolated hippocampal pyramidal neurons. J Neurosci 8:2087-100.

Mattson MP, Guo Q (1997) Cell and molecular neurobiology of presenilins: a role for the endoplasmic reticulum in the pathogenesis ofAlzheimer's disease? J Neurosci Res 50:505-13.

Mattson MP, Guthrie PB, Hayes BC, Kater SB (1989) Roles for mitotic history in the generation and degeneration of hippocampal neuroarchitecture. J Neurosci 9:1223-32.

Maurin H, Lechat B, Borghgraef P, Devijver H, Jaworski T, Van Leuven F (2014) Terminal hypothermic Tau.P301L mice have increased Tau phosphorylation independently of glycogen synthase kinase 3α/β. Eur J Neurosci 40:2442-53.

Mawal-Dewan M, Henley J, Ven de Voorde A, Trojanowski JQ, Lee VM (1994) The Phosphorylation state of tau in the developing rat brain is regulated by phosphoprotein phosphatases. J Biol Chem 269:30981-7.

Mayer PJ, Bradley MO, Nichols WW (1987) The effect of mild hypothermia (34 degrees C) and mild hyperthermia (39 degrees C) on DNA damage, repair andaging of human diploid fibroblasts. Mech Ageing Dev 39:203-22.

Mayer MP, Bukau B (2005) Hsp70 chaperones: cellular functions and molecular mechanism. Cell Mol Life Sci 62:670-84.

Mayer SA, Sessler DI (2005) 'Therapeutic Hypothermia', Marcel Dekker, New York.

McKee AC, Cantu RC, Nowinski CJ, Hedley-Whyte ET, Gavett BE, Budson AE, Santini VE, Lee HS, Kubilus CA, Stern RA (2009) Chronic traumatic encephalopathy in athletes: progressive tauopathy after repetitive head injury. J Neuropathol Exp Neurol 68:709-35.

McKee AC, Stern RA, Nowinski CJ, Stein TD, Alvarez VE, Daneshvar DH, Lee HS, Wojtowicz SM, Hall G, Baugh CM, Riley DO, Kubilus CA, Cormier KA,Jacobs MA, Martin BR, Abraham CR, Ikezu T, Reichard RR, Wolozin BL, Budson AE, Goldstein LE, Kowall NW, Cantu RC (2013) The spectrum of disease in chronic traumatic encephalopathy. Brain 136:43-64.

McMillan P, Korvatska E, Poorkaj P, Evstafjeva Z, Robinson L, Greenup L, Leverenz J, Schellenberg GD, D'Souza I (2008) Tau isoform regulation is region- and cell-specific in mouse brain. J Comp Neurol 511:788-803.

McNaughton BL, Shen J, Rao G, Foster TC, Barnes CA (1994)
Persistent increase of hippocampal presynaptic axon excitability after repetitive electrical stimulation: dependence on N-methyl-D-aspartate receptor activity, nitric-oxide synthase, and temperature. Proc Natl Acad Sci U S A 91:4830-4.

McShea A, Lee HG, Petersen RB, Casadesus G, Vincent I, Linford NJ, Funk JO, Shapiro RA, Smith MA (2007) Neuronal cell cycle re-entry mediates Alzheimer disease-type changes. Biochim Biophys Acta 1772:467-72

Meares GP, Mines MA, Beurel E, Eom TY, Song L, Zmijewska AA, Jope RS (2011)
Glycogen synthase kinase-3 regulates endoplasmic reticulum (ER) stress-
induced CHOP expression in neuronalcells. Exp Cell Res 317:1621-8.

Mellergård P (1992)
Changes in human intracerebral temperature in response to different methods of brain cooling. Neurosurgery 31:671-7.

Mellergård P, Nordström CH, Christensson M (1990) A method for monitoring intracerebral temperature in neurosurgical patients. Neurosurgery 27:654-7.

Mendes CS, Levet C, Chatelain G, Dourlen P, Fouillet A, Dichtel-Danjoy ML, Gambis A, Ryoo HD, Steller H, Mollereau B (2009) ER stress protects from retinal degeneration. EMBO J 28:1296-307.

Menuet C, Cazals Y, Gestreau C, Borghgraef P, Gielis L, Dutschmann M, Van Leuven F, Hilaire G (2011) Age-
related impairment of ultrasonic vocalization in Tau.P301L mice: possible implication for progressive language disorders. PLoS One 6:e25770.

Merino-Serrais P, Benavides-Piccione R, Blazquez-Llorca L, Kastanauskaite A, Rábano A, Avila J, DeFelipe J (2013) The influence of phospho-τ
on dendritic spines of cortical pyramidal neurons in patients with Alzheimer'sdisease.
Brain 136:1913-28.

Mertens J, Stüber K, Poppe D, Doerr J, Ladewig J, Brüstle O, Koch P (2013) Embryonic stem cell-based modelling of tau pathology in human neurons. Am J Pathol 182:1769-79.

Mewes A, Franke H, Singer D (2012)
Organotypic brain slice cultures of adult transgenic P301S mice--a model for tauopathy studies. PLoS One 7:e45017.

Miller JA, Ding SL, Sunkin SM, Smith KA, Ng L, Szafer A, Ebbert A, Riley ZL, Royall JJ, Aiona K, Arnold JM, Bennet C, Bertagnolli D, Brouner K,Butler S, Caldejon S, Carey A, Cuhaciyan C, Dalley RA, Dee N, Dolbeare TA, Facer BA, Feng D, Fliss TP, Gee G, Goldy J, Gourley L, Gregor BW, Gu G, Howard RE, Jochim JM, Kuan CL, Lau C, Lee CK, Lee F, Lemon TA, Lesnar P, McMurray B, Mastan N, Mosqueda N, Naluai-Cecchini T, Ngo NK, Nyhus J, Oldre A, Olson E, Parente J, Parker PD, Parry SE, Stevens A, Pletikos M, Reding M, Roll K, Sandman D, Sarreal M, Shapouri S, Shapovalova NV, Shen EH, Sjoquist N, Slaughterbeck CR, Smith M, Sodt AJ, Williams D, Zöllei L, Fischl B, Gerstein MB, Geschwind DH, Glass IA, Hawrylycz MJ, Hevner RF, Huang H, Jones AR, Knowles JA, Levitt P, Phillips JW, Sestan N, Wohnoutka P, Dang C, Bernard A, Hohmann JG, Lein ES (2014) Transcirptional landscape of the prenatal human brain. Nature 508: 199-206.

Miller EC, Teravskis PJ, Dummer BW, Zhao X, Huganir RL, Liao D (2014) Tau phosphorylation and tau mislocalization mediate soluble Aβ oligomer-induced AMPA glutamate receptor signaling deficits. Eur J Neurosci 39:1214-24.

Mills JC, Lee VM, Pittman RN (1998) Activation of a PP2A-like phosphatase and dephosphorylation of tau protein characterize onset of the execution phase of apoptosis. J Cell Sci 111:625-36.

Milnerwood AJ, Gladding CM, Pouladi MA, Kaufman AM, Hines RM, Boyd JD, Ko RW, Vasuta OC, Graham RK, Hayden MR, Murphy TH, Raymond LA (2010)
Early increase in extrasynaptic NMDA receptor signaling and expression contributes to phenotype ons et inHuntington's disease mice. Neuron 65:178-90.

Minamisawa H[1], Nordström CH, Smith ML, Siesjö BK (1990)
The influence of mild body and brain hypothermia on ischemic brain damage. J Cereb Blood Flow Metab 10:365-74.

Mintz CD, Smith SC, Barrett KM, Benson DL (2012) Anesthetics interfere with the polarization of developing cortical neurons. J Neurosurg Anesthesiol 24:368-75.

Modic M, Ule J, Sibley CR (2013) CLIPing the brain: studies of protein-RNA interactions important for neurodegenerative disorders. Mol Cell Neurosci 56:429-35.

Mocanu MM, Nissen A, Eckermann K, Khlistunova I, Biernat J, Drexler D, Petrova O, Schönig K, Bujard H, Mandelkow E, Zhou L, Rune G, Mandelkow EM (2008) The potential for beta-structure in the repeat domain of tau protein determines aggregation, synaptic decay,neuronal loss, and coassembly with endogenous Tau in inducible mouse models of tauopathy. J Neurosci 28:737-48.

Moldovan M, Krarup C (2004) Mechanisms of hyperpolarization in regenerated mature motor axons in cat. J Physiol 560:807-19.

Mollereau B (2013) Establishing links between endoplasmic reticulum-mediated hormesis and cancer. Mol Cell Biol 3:2372-4.

Mollereau B (2015) Cooling-Induced ER Stress is Good for Your Brain. EBioMedicine http://dx.doi.org/10.1016/j.ebiom.2015.05.008.

Mondragón-Rodríguez S, Trillaud-Doppia E, Dudilot A, Bourgeois C, Lauzon M, Leclerc N, Boehm J (2012) Interaction of endogenous tau with synaptic proteins is regulated by NMDA-receptor dependent tau phosphorylation. J Biol Chem 287:23040-53.

Money TG, Anstey ML, Robertson RM (2005) Heat stress-mediated plasticity in a locust looming-sensitive visual interneuron. J Neurophysiol 93:1908-19.

Moore S, Evans LDB, Andersson T, Portelius E, Smith J, Dias TB, Saurat N, McGlade A, Kirwan P, Blennow K, Hardy J, Zetterberg H, Livesey FJ (2015) APP metabolism regulates tau proteostasis in human cerebral cortex neurons. Cell Reports 11:1-8.

Morales R, Estrada LD, Diaz-Espinoza R, Morales-Scheihing D, Jara MC, Castilla J, Soto C (2010) Molecular cross talk between misfolded proteins in animal models of Alzheimer's and prion diseases. J Neurosci 30:4528-35.

Moreno JA, Halliday M, Molloy C, Radford H, Verity N, Axten JM, Ortori CA, Willis AE, Fischer PM, Barrett DA, Mallucci GR (2013) Oral treatment targeting the unfolded protein response prevents neurodegeneration and clinical disease in prion-infected mice. Sci Transl Med 5:206ra138.

Moreno JA, Radford H, Peretti D, Steinert JR, Verity N, Martin MG, Halliday M, Morgan J, Dinsdale D, Ortori CA, Barrett DA, Tsaytler P, Bertolotti A, Willis AE,Bushell M, Mallucci GR (2012) Sustained translational repression by eIF2α-P mediates prion neurodegeneration. Nature 485:507-11.

Morf J, Rey G, Schneider K, Stratmann M, Fujita J, Naef F, Schibler U (2012) Cold-inducible RNA-binding protein modulates circadian gene expression posttranscriptionally. Science 338:379-83.

Morimoto RI, Cuervo AM (2009) Protein homeostasis and aging: taking care of proteins from the cradle to the grave. J Gerontol A Biol Sci Med Sci 64:167-70.

Morris JA, Dorner AJ, Edwards CA, Hendershot LM, Kaufman RJ (1997) Immunoglobulin binding protein (BiP) function is required to protect cells from endoplasmic reticulum stress but is not required for the secretion of selective proteins. J Biol Chem 272:4327-34.

Morris M, Maeda S, Vossel K, Mucke L (2011) The many faces of tau. Neuron 70:410-26.

Morishima-Kawashima M, Hasegawa M, Takio K, Suzuki M, Yoshida H, Titani K, Ihara Y (1995) Proline-directed and non-proline-directed phosphorylation of PHF-tau. J Biol Chem 270: 823-9.

Morsch R, Simon W, Coleman PD (1999) Neurons may live for decades with neurofibrillary tangles. J Neuropathol Exp Neurol 58:188-97.

Moschner K, Sündermann F, Meyer H, da Graca AP, Appel N, Paululat A, Bakota L, Brandt R (2014) RNA protein granules modulate tau isoform expression and induce neuronal sprouting. J Biol Chem 289:16814-25.

Moser E, Mathiesen I, Andersen P (1993) Association between brain temperature and dentate field potentials in exploring and swimming rats. Science 259:1324-6.

Muchowski PJ, Wacker JL (2005) Modulation of neurodegeneration by molecular chaperones. Nat Rev Neurosci 6:11-22.

Mueller-Burke D, Koehler RC, Martin LJ (2008)
Rapid NMDA receptor phosphorylation and oxidative stress precede striatal neurodegeneration after h ypoxicischemia in newborn piglets and are attenuated with hypothermia. Int J Dev Neurosci 26:67-76.

Mukrasch MD, Bibow S, Korukottu J, Jeganathan S, Biernat J, Griesinger C, Mandelkow E, Zweckstetter M (2009) Structural polymorphism of 441-residue tau at single residue resolution. PLoS Biol 7:e34.

Mulkey RM, Herron CE, Malenka RC (1993) An essential role for protein phosphatases in hippocampal long-term depression. Science 261:1051-5.

Murphy TH, Baraban JM (1990) Glutamate toxicity in cortical neurons precedes development of glutamate receptor currents. Brain Res Dev Brain Res 57:146-50.

Myers AJ, Pittman AM, Zhao AS, Rohrer K, Kaleem M, Marlowe L, Lees A, Leung D, McKeith IG, Perry RH, Morris CM, Trojanowski JQ, Clark C, Karlawish J,Arnold S, Forman MS, Van Deerlin V, de Silva R, Hardy J (2007) The MAPT H1c risk haplotype is associated with increased expression of tau and especially of 4 repeat containing transcripts. Neurobiol Dis 25:561-70.

Nakanishi N, Ryan SD, Zhang X, Khan A, Holland T, Cho EG, Huang X, Liao FF, Xu H, Lipton SA, Tu S (2013) Synaptic protein α1-takusan mitigates amyloid-β-induced synaptic loss via interaction with tau and postsynaptic density-95 at postsynaptic sites. J Neurosci 33:14170-83.

Nakayama T, Goshima Y, Misu Y, Kato T (1999)
Role of cdk5 and tau phosphorylation in heterotrimeric G protein-mediated retinal growth cone collapse. J Neurobiol 41:326-39.

Neutelings T, Lambert CA, Nusgens BV, Colige AC (2013) Effects of mild cold shock ($25^0$C) followed by warming up at $37^0$C on the cellular stress response. PLoS One 8:e69687.

Neve RL, Harris P, Kosik KS, Kurnit DM, Donlon TA (1986) Identification of cDNA clones for the human microtubule-associated protein tau and chromosomal localization of the genes for tau and microtubule-associated protein 2. Brain Res 387:271-80.

Newell DW, Barth A, Papermaster V, Malouf AT (1995) Glutamate and non-glutamate receptor mediated toxicity caused
by oxygen and glucose deprivation inorganotypic hippocampal cultures. J Neurosci 15:7702-11.

Nielsen N, Wetterslev J, Cronberg T, Erlinge D, Gasche Y, Hassager C, Horn J, Hovdenes J, Kjaergaard J, Kuiper M, Pellis T, Stammet P, Wanscher M, Wise MP, Åneman A, Al-Subaie N, Boesgaard S, Bro-Jeppesen J, Brunetti I, Bugge JF, Hingston CD, Juffermans NP, Koopmans M, Køber L, Langørgen J, Lilja G,Møller JE, Rundgren M, Rylander C, Smid O, Werer C, Winkel P, Friberg H; TTM Trial Investigators (2013) Targeted Temperature Management at 33°C versus 36°C after Cardiac Arrest. N Engl J Med 369:2197-206.

Nijholt DA, van Haastert ES, Rozemuller AJ, Scheper W, Hoozemans JJ (2012) The unfolded protein response is associated with early tau pathology in the hippocampus of tauopathies. J Pathol 226:693-702.

Nikoletopoulou V, Papandreou M, Tavernarakis N (2014) Autophagy in
the physiology and pathology of the central nervous system. Cell Death Differ doi: 10.1038/cdd.2014.204 [Epub ahead of print].

Nikkel AL, Martino B, Markosyan S, Brederson JD, Medeiros R, Moeller A, Bitner RS (2012) The novel calpain inhibitor A-705253 prevents stress-induced tau hyperphosphorylation in vitro and in vivo. Neuropharmacology 63:606-12.

Nishio S, Yunoki M, Chen ZF, Anzivino MJ, Lee KS (2000) Ischemic tolerance in
the rat neocortex following hypothermic preconditioning. J Neurosurg 93:845-51.

Nishiyama H, Higashitsuji H, Yokoi H, Itoh K, Danno S, Matsuda T, Fujita J (1997a) Cloning and characterization of human CIRP (cold-inducible RNA-binding protein) cDNA and chromosomal assignment of the gene. Gene 208:115-120.

Nishiyama H, Itoh K, Kaneko Y, Kishishita M, Yoshida O, Fujita J (1997b) A glycine-rich RNA-binding protein mediating cold-inducible suppression of mammalian cell growth. J Cell Biol 137:899-908.

Nishiyama H, Xue JH, Sato T, Fukuyama H, Mizuno N, Houtani T, Sugimoto T, Fujita J (1998) Diurnal change of the cold-inducible RNA-binding protein (Cirp) expression in mouse brain. Biochem Biophys Res Commun 245:534-538.

Noël A, Poitras I, Julien J, Petry FR, Morin F, Charron J, Planel E (2015) ERK (MAPK) does not phosphorylate tau under physiological conditions in vivo or in vitro. Neurobiol Aging 36: 901-2.

Northington FJ, Ferriero DM, Flock DL, Martin LJ (2001a)
Delayed neurodegeneration in neonatal rat thalamus after hypoxia-ischemia is apoptosis. J Neurosci 21:1931-8.

Northington FJ, Ferriero DM, Graham EM, Traystman RJ, Martin LJ (2001b)
Early Neurodegeneration after Hypoxia-
Ischemia in Neonatal Rat Is Necrosis while Delayed Neuronal Death IsApoptosis. Neurobiol Dis 8:207-19.

Nunomura A, Perry G, Aliev G, Hirai K, Takeda A, Balraj EK, Jones PK, Ghanbari H, Wataya T, Shimohama S, Chiba S, Atwood CS, Petersen RB, Smith MA (2001) Oxidative damage is the earliest event in Alzheimer disease. J Neuropathol Exp Neurol 60:759–767.

O'Collins VE, Macleod MR, Donnan GA, Horky LL, van der Worp BH, Howells DW (2006)
1,026 experimental treatments in acute stroke. Ann Neurol 59:467-77.

Ohsaka Y, Ohgiya S, Hoshino T, Ishizaki K (2002) Phosphorylation of c-Jun N-
terminal kinase in human hepatoblastoma cells is transiently increased by coldexposure and further enhanced by subsequent warm incubation of the cells. Cell Physiol Biochem 12:111-8.

Okamoto S, Pouladi MA, Talantova M, Yao D, Xia P, Ehrnhoefer DE, Zaidi R, Clemente A, Kaul M, Graham RK, Zhang D, Vincent Chen HS, Tong G, Hayden MR, Lipton SA (2009) Balance between synaptic versus extrasynaptic NMDA receptor activity influences inclusions and neurotoxicity of mutant huntingtin. Nat Med 15:1407-13

Olney JW (1969) Brain lesions, obesity, and other disturbances in mice treated with monosodium glutamate. Science 164:719-21.

Orozco D, Tahirovic S, Rentzsch K, Schwenk BM, Haass C, Edbauer D (2012) Loss of fused in sarcoma (FUS) promotes pathological tau splicing. EMBO Rep 13:759-64.

Oscarsson A, Massoumi R, Sjölander A, Eintrei C (2001) Reorganization of actin in neurons after propofol exposure. Acta Anaesthesiol Scand 45:1215-20.

Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO, Görgün CZ, Hotamisligil GS (2006) Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. Science 313:1137-40.

Paintal AS (1965)
Effects of temperature on conduction in single vagal and saphenous myelinated nerve fibres of the cat. J Physiol 180:20-49.

Papadia S, Soriano FX, Léveillé F, Martel MA, Dakin KA, Hansen HH, Kaindl A, Sifringer M, Fowler J, Stefovska V, McKenzie G, Craigon M, Corriveau R,Ghazal P, Horsburgh K, Yankner BA, Wyllie DJ, Ikonomidou C, Hardingham GE (2008) Synaptic NMDA receptor activity boosts intrinsic antioxidant defences. Nat Neurosci 11:476-87.

Papon MA, Whittington RA, El-Khoury NB, Planel E (2011) Alzheimer's disease and anesthesia. Front Neurosci 4:272.

Park SY, Ferreira A (2005) The generation of a 17 kDa neurotoxic fragment:
an alternative mechanism by which tau mediates beta-amyloid-induced neurodegeneration. J Neurosci 25:5365-75.

Parsell DA, Lindquist S (1993) The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annu Rev Genet 27:437-96.

Paschen W (2003) Endoplasmic reticulum: a primary target in various acute disorders and degenerative diseases of the brain. Cell Calcium 34:365-83.

Pazos AJ, Green EJ, Busto R, McCabe PM, Baena RC, Ginsberg MD, Globus MY, Schneiderman N, Dietrich WD (1999) Effects of combined postischemic hypothermia and delayed N-tert-butyl-alpha-pheylnitrone (PBN) administrationon histopathologicaland behavioral deficits associated with transient global ischemia in rats. Brain Res 846:186-95.

Pearson RC, Esiri MM, Hiorns RW, Wilcock GK, Powell TP (1985) Anatomical correlates of the distribution of the pathological changes in the neocortex in Alzheimer disease. Proc Natl Acad Sci U S A 82:4531-4.

Pelsman A, Hoyo-Vadillo C, Gudasheva TA, Seredenin SB, Ostrovskaya RU, Busciglio J (2003) GVS-111 prevents oxidative damage and apoptosis in normal and Down's syndrome human cortical neurons . Int J Dev Neurosci 21:117-24.

Peretti D, Bastide A, Radford H, Verity N, Molloy C, Martin MG, Moreno JA, Steinert JR, Smith T, Dinsdale D, Willis AE, Mallucci GR (2015) RBM3 mediates structural plasticity and protective effects of cooling in neurodegeneration. Nature doi: 10.1038/nature14142 [Epub ahead of print].

Pérez-Alvarez A, Araque A (2013) Astrocyte-neuron interaction at tripartite synapses. Curr Drug Targets 14:1220-4.

Perman SM, Goyal M, Neumar RW, Topjian AA, Gaieski DF (2014) Clinical applications of targeted temperature management. Chest 145:386-93.

Petrucelli L, Dickson D, Kehoe K, Taylor J, Snyder H, Grover A, De Lucia M, McGowan E, Lewis J, Prihar G, Kim J, Dillmann WH, Browne SE, Hall A, Voellmy R, Tsuboi Y, Dawson TM, Wolozin B, Hardy J, Hutton M (2004) CHIP and Hsp70 regulate tau ubiquitination, degradation and aggregation. Hum Mol Genet 13:703-14.

Petry FR, Pelletier J, Bretteville A, Morin F, Calon F, Hébert SS, Whittington RA, Planel E (2014) Specificity of anti-tau antibodies when analyzing mice models of Alzheimer's disease: problems and solutions. PLoS One 9:e94251.

Phadtare S, Alsina J, Inouye M (1999) Cold-shock response and cold-shock proteins. Curr Opin Microbiol 2:175-80.

Phanithi PB, Yoshida Y, Santana A, Su M, Kawamura S, Yasui N (2000) Mild hypothermia mitigates post-ischemic neuronal death following focal cerebral ischemia in rat brain: immunohistochemical study of Fas, caspase-3 and TUNEL. Neuropathology 20:273-82.

Pilotte J, Cunningham BA, Edelman GM, Vanderklish PW (2009) Developmentally regulated expression of the cold-inducible RNA-binding motif protein 3 in euthermic rat brain. Brain Res 1258:12-24.

Piraino S, De Vito D, Schmich J, Bouillon J, Boero F (2004) Reverse development in Cnidaria Can. J. Zool. 82: 1748–1754

Planel E, Bretteville A, Liu L, Virag L, Du AL, Yu WH, Dickson DW, Whittington RA, Duff KE (2009) Acceleration and persistence of neurofibrillary pathology in a mouse model of tauopathy following anesthesia. FASEB J 23:2595-604.

Planel E, Krishnamurthy P, Miyasaka T, Liu L, Herman M, Kumar A, Bretteville A, Figueroa HY, Yu WH, Whittington RA, Davies P, Takashima A, Nixon RA,Duff KE (2008) Anesthesia-induced hyperphosphorylation detaches 3-repeat tau from microtubules without affecting their stability in vivo. J Neurosci 28:12798-807.

Planel E, Miyasaka T, Launey T, Chui DH, Tanemura K, Sato S, Murayama O, Ishiguro K, Tatebayashi Y, Takashima A (2004) Alterations in glucose metabolism induce hypothermia leading to tau hyperphosphorylation through differential inhibition of kinase and phosphatase activities: implications for Alzheimer's disease. J Neurosci 24:2401-2411.

Planel E, Richter KE, Nolan CE, Finley JE, Liu L, Wen Y, Krishnamurthy P, Herman M, Wang L, Schachter JB, Nelson RB, Lau LF, Duff KE (2007a) Anesthesia leads to tau hyperphosphorylation through inhibition of phosphatase activity by hypothermia. J Neurosci 27:3090-3097.

Planel E, Tatebayashi Y, Miyasaka T, Liu L, Wang L, Herman M, Yu WH, Luchsinger JA, Wadzinski B, Duff KE, Takashima A (2007b) Insulin dysfunction induces in vivo tau hyperphosphorylation through distinct mechanisms. J Neurosci 27:13635-48.

Planel E, Yasutake K, Fujita SC, Ishiguro K (2001) Inhibition of protein phosphatase 2A overrides tau protein kinase I/glycogen synthase kinase 3b and cyclin-dependent kinase 5 inhibition and results in tau hyperphosphorylation in the hippocampus of starved mouse. J Biol Chem 276:34298-306.

Poljak A, Hill M, Hall RJ, MacLullich AM, Raftery MJ, Tai J, Yan S, Caplan GA (2014) Quantitative proteomics of delirium cerebrospinal fluid. Transl Psychiatry 4:e477.

Pooler AM, Phillips EC, Lau DH, Noble W, Hanger DP (2013) Physiological release of endogenous tau is stimulated by neuronal activity. EMBO Rep 14:389-94.

Pooler AM, Usardi A, Evans CJ, Philpott KL, Noble W, Hanger DP (2012) Dynamic association of tau with neuronal membranes is regulated by phosphorylation. Neurobiol Aging 33:431.e27-38.

Poorkaj P, Bird TD, Wijsman E, Nemens E, Garruto RM, Anderson L, Andreadis A, Wiederholt WC, Raskind M, Schellenberg GD (1998) Tau is a candidate gene for chromosome 17 frontotemporal dementia. Ann Neurol 43:815-25.

Popov VI, Bocharova LS (1992) Hibernation-induced structural changes in synaptic contacts between mossy fibres and hippocampal pyramidalneur ons. Neuroscience 48:53-62.

Popov VI, Medvedev NI, Patrushev IV, Ignat'ev DA, Morenkov ED, Stewart MG (2007) Reversible reduction in dendritic spines in CA1 of rat and ground squirrel subjected to hypothermia-normothermia in vivo: A three-dimensional electron microscope study. Neuroscience 149:549-60.

Popovic M, Fabjan A, Mraz J, Magdic J, Glavac D, Zupan A, Novak MD, Bresjanac M (2014) Tau protein mutation P364S in two sisters: clinical course and neuropathology with emphasis on new, composite neuronal tau inclusions. Acta Neuropathol 128: 155-7.

Powers ET, Morimoto RI, Dillin A, Kelly JW, Balch WE (2009) Biological and chemical approaches to diseases of proteostasis deficiency. Annu Rev Biochem 78:959-91.

Pritchard SM, Dolan PJ, Vitkus A, Johnson GV (2011) The toxicity of tau in Alzheimer disease: turnover, targets and potential therapeutics. J Cell Mol Med 15:1621-35.

Prokop A (2013) The intricate relationship between microtubules and
their associated motor proteins during axon growth andmaintenance. Neural Dev 8:17.

Povellato G, Tuxworth RI, Hanger DP, Tear G (2014) Modification of the Drosophila model of in vivo Tau toxicity reveals protective phosphorylation by GSK3β. Biol Open 3:1-11.

Prell T, Lautenschläger J, Weidemann L, Ruhmer J, Witte OW, Grosskreutz J (2014)
Endoplasmic reticulum stress is accompanied by activation of NF-κB in amyotrophic lateral sclerosis. J Neuroimmunol 270:29-36.

Prusiner SB (1984) Some speculations about prions, amyloid, and Alzheimer's disease. N Engl J Med 310:661-3.

Pruunsild P, Sepp M, Orav E, Koppel I, Timmusk T (2011) Identification of cis-elements and transcription factors regulating neuronal activity-dependent transcription of human BDNF gene. J Neurosci 31:3295-3308.

Qian W, Liang H, Shi J, Jin N, Grundke-Iqbal I, Iqbal K, Gong CX, Liu F (2011) Regulation of the alternative splicing of tau exon 10 by SC35 and Dyrk1A. Nucleic Acids Res 39:6161-71.

Qiang X, Yang WL, Wu R, Zhou M, Jacob A, Dong W, Kuncewitch M, Ji Y, Yang H, Wang H, Fujita J, Nicastro J, Coppa GF, Tracey KJ, Wang P (2013) Cold-inducible RNA-binding protein (CIRP) triggers inflammatory responses in hemorrhagic shock and sepsis. Nat Med 19:1489-95.

Rahman A, Ting K, Cullen KM, Braidy N, Brew BJ, Guillemin GJ (2009)
The excitotoxin quinolinic acid induces tau phosphorylation in human neurons. PLoS One 4:e6344.

Ramaswami M, Taylor JP, Parker R (2013) Altered ribostasis: RNA-protein granules in degenerative disorders. Cell 154:727-36.

Rapoport SI (1999a)
In vivo PET imaging and postmortem studies suggest potentially reversible and irreversible stages of brainmetabolic failure in Alzheimer's disease. Eur Arch Psychiatry Clin Neurosci 249 Suppl 3:46-55.

Rapoport SI (1999b) How did the human brain evolve? A proposal based on new evidence from in vivo brain imaging during attentionand ideation. Brain Res Bull 50:149-65.

Rapoport M, Dawson HN, Binder LI, Vitek MP, Ferreira A (2002) Tau is essential to beta -amyloid-induced neurotoxicity. Proc Natl Acad Sci U S A 99:6364-9.

Rasminsky M (1973) The Effects of Temperature on Conduction in Demyelinated Single Nerve Fibers. Arch Neurol 28:287-292.

Reisberg B, Doody R, Stöffler A, Schmitt F, Ferris S, Möbius HJ (2003) Memantine Study Group. Memantine in moderate-to-severe Alzheimer's disease. N Engl J Med 348:1333-41.

Reith J, Jørgensen HS, Pedersen PM, Nakayama H, Raaschou HO, Jeppesen LL, Olsen TS (1996) Body temperature in acute stroke: relation to stroke severity, infarct size, mortality, and outcome. Lancet 347:422-5.

Rejdak R, Rejdak K, Sielucka-Dziuba M, Stelmasiak Z, Grieb P (2001) Brain tolerance and preconditioning. Pol J Pharamcol 53:73-9.

Resende R, Ferreiro E, Pereira C, Oliveira CR (2008) ER stress is involved in Abeta-induced GSK-3beta activation and tau phosphorylation. J Neurosci Res 86:2091-9.

Reyes O, Sosa I, Kuffler DP (2006) Neuroprotection of adult human neurons against ischemia by hypothermia and alkalinization. P R Health Sci J 25:43-50.

RichardsV, Pinto D, Coombs P (1963) Studies in suspended animation by hypothermia combined with hyperbaric oxygenation. Ann Surg 158:349-62.

Richter-Landsberg C, Goldbaum O (2003) Stress proteins in neural cells: functional roles in health and disease. Cell Mol Life Sci 60:337-49.

Ritchie JM, Straub RW (1956) The effect of cooling on the size of the action potential of mammalian non-medullated fibres. J Physiol 134:712-7.

Robertson RM, Money TG (2012) Temperature and neuronal circuit function: compensation, tuning and tolerance. Curr Opin Neurobiol 22:724-34.

Roberson ED, Scearce-Levie K, Palop JJ, Yan F, Cheng IH, Wu T, Gerstein H, Yu GQ, Mucke L (2007) Reducing endogenous tau ameliorates amyloid beta-induced deficits in an Alzheimer's disease mouse model. Science 316:750-4.

Rocher AB, Crimins JL, Amatrudo JM, Kinson MS, Todd-Brown MA, Lewis J, Luebke JI (2010) Structural and functional changes in tau mutant mice neurons are not linked to the presence of NFTs. Exp Neurol 223:385-393.

Rodgers CI, Armstrong GA, Shoemaker KL, LaBrie JD, Moyes CD, Robertson RM (2007) Stress preconditioning of spreading depression in the locust CNS. PLoS One 2:e1366.

Rodríguez EC, Robertson RM (2012) Protective effect of hypothermia on brain potassium homeostasis during repetitive anoxia in Drosophil a melanogaster. J Exp Biol 215:4157-65.

Rodriguez-Echandia EL, Piezzi RS (1968) Microtubules in the nerve fibers of the toad Bufo arenarum Hensel. Effect of low temperature on the sciatic nerve. J Cell Biol 39:491-7.

Rodríguez-Vázquez J, Camacho-Arroyo I, Velázquez-Moctezuma J (2012) Differential impact of REM sleep deprivation on cytoskeletal proteins of brain regions involved in sleep regulation. Neuropsychobiology 65:161-7.

Roelandse M, Matus A (2004) Hypothermia-associated loss of dendritic spines. J Neurosci 24:7843-7.

Rohani L, Johnson AA, Arnold A, Stolzing A (2014) The aging signature: a hallmark of induced pluripotent stem cells? Aging cell 13:2-7.

Roobol A, Carden MJ, Newsam RJ, Smales CM (2009) Biochemical insights into the mechanisms central to the response of mammalian cells to cold stress and subsequent rewarming. FEBS J 276:286-302.

Roth LE (1967) Electron microscopy of mitosis in amebae. 3. Cold and urea treatments: a basis for tests of direct effects of mitotic inhibitors on microtubule formation. J Cell Biol 34:47-59.

Rothman SM, Olney JW (1986) Glutamate and the pathophysiology of hypoxic--ischemic brain damage. Ann Neurol 19:105-11.

Ruediger J, Van der Zee EA, Strijkstra AM, Aschoff A, Daan S, Hut RA (2007) Dynamics in the ultrastructure of asymmetric axospinous synapses in the frontal cortex of hibernating Europeanground squirrels (Spermophilus citellus). Synapse 61:343-52.

Rujano MA, Bosveld F, Salomons FA, Dijk F, van Waarde MA, van der Want JJ, de Vos RA, Brunt ER, Sibon OC, Kampinga HH (2006)
Polarised asymmetric inheritance of accumulated protein damage in higher eukaryotes. PLoS Biol 4:e417.

Rumana CS, Gopinath SP, Uzura M, Valadka AB, Robertson CS (1998)
Brain temperature exceeds systemic temperature in head-injured patients. Crit Care Med 26:562-7.

Run X, Liang Z, Gong CX (2010) Anesthetics and tau protein: animal model studies. J Alzheimers Dis 22 Suppl 3:49-55.

Rutkove SB (2001) Effects of temperature on neuromuscular electrophysiology. Muscle Nerve 24:867-82.

Rutkowski DT, Arnold SM, Miller CN, Wu J, Li J, Gunnison KM, Mori K, Sadighi Akha AA, Raden D, Kaufman RJ (2006) Adaptation to ER stress is mediated by differential stabilities of pro-survival and pro-apoptotic mRNAs andproteins. PLoS Biol 4:e374.

Rzechorzek NM, Connick P, Patani R, Selvaraj BT, Chandran S (2015) Hypothermic preconditioning of human cortical neurons requires proteostatic priming. *EBioMedicine In press* DOI: http://dx.doi.org/10.1016/j.ebiom.2015.04.004.

Sachdev RN, Ebner FF, Wilson CJ (2004) Effect of subthreshold up and down states on the whisker-evoked response in somatosensory cortex. J Neurophysiol 92:3511-21.

Sahara N, Murayama M, Mizoroki T, Urushitani M, Imai Y, Takahashi R, Murata S, Tanaka K, Takashima A (2005) In vivo evidence of CHIP up-regulation attenuating tau aggregation. J Neurochem 94:1254-63.

Saidi LJ, Polydoro M, Kay KR, Sanchez L, Mandelkow EM, Hyman BT, Spires-Jones TL (2014) Carboxy Terminus Heat Shock Protein 70 Interacting Protein Reduces Tau-Associated Degenerative Changes. J Alzheimers Dis [Epub ahead of print].

Saito K, Fukuda N, Matsumoto T, Iribe Y, Tsunemi A, Kazama T, Yoshida-Noro C, Hayashi N (2010) Moderate low temperature preserves the stemness of neural stem cells and suppresses apoptosis of the cells via activation of the cold-inducible RNA binding protein. Brain Res 1358:20-29.

Saitoh M, Kunitomo J, Kimura E, Iwashita H, Uno Y, Onishi T, Uchiyama N, Kawamoto T, Tanaka T, Mol CD, Dougan DR, Textor GP, Snell GP, Takizawa M, Itoh F, Kori M (2009) 2-{3-[4-(Alkylsulfinyl)phenyl]-1-benzofuran-5-yl}-5-methyl-1,3,4-oxadiazole derivatives as novel inhibitors of glycogen synthase kinase-3beta with good brain permeability. J Med Chem 52(20):6270-86.

Sakagami Y, Kudo T, Tanimukai H, Kanayama D, Omi T, Horiguchi K, Okochi M, Imaizumi K, Takeda M (2013) Involvement of endoplasmic reticulum stress in tauopathy. Biochem Biophys Res Commun 430:500-4.

Sakamoto KI, Fujisawa H, Koizumi H, Tsuchida E, Ito H, Sadamitsu D, Maekawa T (1997) Effects of mild hypothermia on nitric oxide synthesis following contusion trauma in the rat. J Neurotrauma 14:349-53.

Sakurai T, Itoh K, Higashitsuji H, Nonoguchi K, Liu Y, Watanabe H, Nakano T, Fukumoto M, Chiba T, Fujita J (2006) Cirp protects against tumor necrosis factor-alpha-induced apoptosis via activation of extracellular signal-regulated kinase. Biochim Biophys Acta 1763:290-5.

Sanders DW, Kaufman SK, DeVos SL, Sharma AM, Mirbaha H, Li A, Barker SJ, Foley AC, Thorpe JR, Serpell LC, Miller TM, Grinberg LT, Seeley WW, Diamond MI.
Distinct tau prion strains propagate in cells and mice and define different tauopathies.
Neuron 82:1271-88.

Santacruz K, Lewis J, Spires T, Paulson J, Kotilinek L, Ingelsson M, Guimaraes A, DeTure M, Ramsden M, McGowan E, Forster C, Yue M, Orne J, Janus C,Mariash A, Kuskowski M, Hyman B, Hutton M, Ashe KH (2005) Tau suppression in
a neurodegenerative mouse model improves memory function.
Science 309:476-81.

Saraste J, Palade GE, Farquhar MG (1986) Temperature-sensitive steps in
the transport of secretory proteins through the Golgi complex in exocrinepancreatic cells. Proc Natl Acad Sci U S A 83:6425-9.

Sarkar M, Kuret J, Lee G (2008) Two motifs within the tau microtubule-binding domain mediate its association with the hsc70 molecular chaperone. J Neurosci Res 86:2763-73.

Sato-Numata K, Numata T, Okada Y (2014) Temperature sensitivity of acid-sensitive outwardly rectifying (ASOR) anion channels in cortical neurons is involved
in hypothermic neuroprotection against acidotoxic necrosis. Channels (Austin) 8:278-83.

Saxena S, Cabuy E, Caroni P (2009) A role for motoneuron subtype-
selective ER stress in disease manifestations of FALS mice. Nat Neurosci 12:627-36.

Schauf CL, Davis FA (1974) Impulse conduction in multiple sclerosis:
a theoretical basis for modification by temperature andpharmacological agents. J Neurol Neurosurg Psychiatry 37:152-61.

Schiff SJ, Somjen GG (1985) The effects of temperature on synaptic transmission in hippocampal tissue slices. Brain Res 345:279-84.

Schmich J, Kraus Y, De Vito D, Graziussi D, Boero F, Piraino S (2007)
Induction of reverse development in two marine Hydrozoans. Int J Dev Biol 51:45-56.

Schneider A, Biernat J, von Bergen M, Mandelkow E, Mandelkow EM (1999) Phosphorylation that detaches tau protein from microtubules (Ser262, Ser214) also protects it against aggregation into Alzheimer paired helical filaments. Biochemistry 38:3549-58.

Schubert M, Gautam D, Surjo D, Ueki K, Baudler S, Schubert D, Kondo T, Alber J, Galldiks N, Küstermann E, Arndt S, Jacobs AH, Krone W, Kahn CR, Brüning JC (2004) Role for neuronal insulin resistance in neurodegenerative diseases. Proc Natl Acad Sci U S A 101:3100-5.

Schwartz AL, Ciechanover A (1999) The ubiquitin-proteasome pathway and pathogenesis of human diseases. Annu Rev Med 50:57-74.

Schwartz C, Hampton M, Andrews MT (2013)
Seasonal and regional differences in gene expression in the brain of a hibernating mammal. PLoS One 8:e58427.

Schwarz JR (1986) The effect of temperature on Na currents in rat myelinated nerve fibres. Pflugers Arch 406:397-404.

Schwarz JR, Eikhof G (1987)
Na currents and action potentials in rat myelinated nerve fibres at 20 and 37 degrees C. Pflugers Arch 409:569-77.

Schweers O, Schönbrunn-Hanebeck E, Marx A, Mandelkow E (1994) Structural studies of tau protein and Alzheimer paired helical filaments show no evidence for beta-structure. J Biol Chem 269:24290-7.

Sea T, Ballinger ML, Bittner GD (1995) Cooling of peripheral myelinated axons retards Wallerian degeneration. Exp Neurol 133:85-95.

Sebastià J, Cristòfol R, Pertusa M, Vílchez D, Torán N, Barambio S, Rodríguez-Farré E, Sanfeliu C (2004) Down's syndrome astrocytes have greater antioxidant capacity than euploid astrocytes. Eur J Neurosci 20:2355-66.

Sergeant N, Bussière T, Vermersch P, Lejeune JP, Delacourte A (1995) Isoelectric point differentiates PHF-tau from biopsy-derived human brain tau proteins. Neuroreport 6:2217-20.

Sergeant N, Delacourte A, Buée L (2005) Tau protein as a differential biomarker of tauopathies. Biochim Biophys Acta 1739:179-97.

Serio A, Bilican B, Barmada SJ, Ando DM, Zhao C, Siller R, Burr K, Haghi G, Story D, Nishimura AL, Carrasco MA, Phatnani HP, Shum C, Wilmut I, Maniatis T,Shaw CE, Finkbeiner S, Chandran S (2013) Astrocyte pathology and the absence of non-cell autonomy in an induced pluripotent stem cell model of TDP-43 proteinopathy. Proc Natl Acad Sci USA 110:4697-702.

Seubert P, Mawal-Dewan M, Barbour R, Jakes R, Goedert M, Johnson GV, Litersky JM, Schenk D, Lieberburg I, Trojanowski JQ, Lee VM-Y (1995) Detection of phosphorylated Ser262 in foetal tau, adult tau, and paired helical filament tau. J Biol Chem 270:18917-22.

Shahani N, Subramaniam S, Wolf T, Tackenberg C, Brandt R (2006) Tau aggregation and progressive neuronal degeneration in the abence of changes in spine density and morphology after targeted expression of Alzheimer's disease-relevant tau constructs in organotypic hippocampal slices. J Neurosci 26: 6103-14.

Shankaran S, Laptook AR, McDonald SA, Higgins RD, Tyson JE, Ehrenkranz RA, Das A, Sant'Anna G, Goldberg RN, Bara R, Walsh MC; Eunice Kennedy Shriver National Institute of Child Health and Human Development Neonatal Research Network (2012) Temperature profile and outcomes of neonates undergoing whole body hypothermia for neonatal hypoxic-ischemic encephalopathy. Pediatr Crit Care Med 13:53-9.

Shattock MJ, Tipton MJ (2012) 'Autonomic conflict': a different way to die during cold water immersion? J Physiol 590:3219-30.

Shay J, Gonatas NK (1973) Electron microscopy of cat spinal cord subject to circulatory arrest and deep local hypothermia (15 C). Am J Pathol 72:369-96.

Shchipakina TG, Zharikova AD, Arkhipov VI (1995) Protein phosphorylation of synaptic membranes isolated from the brain of ground squirrels during hibernation. Neuroreport 7:278-80.

Sheng M, McFadden G, Greenberg ME (1990) Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. Neuron 4:571-582.

Sherratt RM, Bostock H, Sears TA (1980) Effects of 4-aminopyridine on normal and demyelinated mammalian nerve fibres. Nature 283:570-2.

Shi Y, Kirwan P, Smith J, MacLean G, Orkin SH, Livesey FJ (2012a) A human stem cell model of early Alzheimer's disease pathology in Down syndrome. Sci Transl Med 4:124ra29.

Shi Y, Kirwan P, Smith J, Robinson HPC, Livesey FJ (2012b) Human cerebral cortex development from pluripotent stem cells to functional excitatory synapses. Nat Neurosci 15:477-486.

Shi J, Zhang T, Zhou C, Chohan MO, Gu X, Wegiel J, Zhou J, Hwang YW, Iqbal K, Grundke-Iqbal I, Gong CX, Liu F (2008) Increased dosage of Dyrk1A alters alternative splicing factor (ASF)-regulated alternative splicing of tau in Down syndrome. J Biol Chem 283:28660-9.

Shiarli AM, Jennings R, Shi J, Bailey K, Davidson Y, Tian J, Bigio EH, Ghetti B, Murrell JR, Delisle MB, Mirra S, Crain B, Zolo P, Arima K, Iseki E, Murayama S,Kretzschmar H, Neumann M, Lippa C, Halliday G, Mackenzie J, Khan N, Ravid R, Dickson D, Wszolek Z, Iwatsubo T, Pickering-Brown S, Mann DM (2006)
Comparison of extent of tau pathology in patients with frontotemporal dementia with Parkinsonism lin ked tochromosome 17 (FTDP-
17), frontotemporal lobar degeneration with Pick bodies and early onset Alzheimer'sdisease. Neuropathol Appl Neurobiol 32:374-87.

Shimura H, Schwartz D, Gygi SP, Kosik KS (2004) CHIP-
Hsc70 complex ubiquitinates phosphorylated tau and enhances cell survival. J Biol Chem 279:4869-76.

Shintani Y, Terao Y (2012) Molecular Mechanisms Underlying the Neuroprotective Effect of Hypothermia in Cerebral Ischemia, Advances in the Treatment of Ischemic Stroke, Dr. Maurizio Balestrino(Ed.), ISBN: 978-953-51-0136-9, InTech, Available from: http://www.intechopen.com/books/advances-in-thetreatment-of-ischemic-stroke/molecular-mechanisms-involved-in-the-neuroprotective-effect-of-hypothermia-incerebral-ischemia.

Shintani Y, Terao Y, Ohta H (2010) Molecular mechanisms underlying hypothermia-induced neuroprotection. Stroke Res Treat 2011:809874.

Si QS, Nakamura Y, Kataoka K (1997) Hypothermic suppression of microglial activation in culture: inhibition of cell proliferation and production of nitric oxide and superoxide. Neuroscience 81:223-9.

Sidrauski C, Acosta-Alvear D, Khoutorsky A, Vedantham P, Hearn BR, Li H, Gamache K, Gallagher CM, Ang KK, Wilson C, Okreglak V, Ashkenazi A, Hann B,Nader K, Arkin MR, Renslo AR, Sonenberg N, Walter P (2013) Pharmacological brake-
release of mRNA translation enhances cognitive memory. Elife 2:e00498.

Silasi G, Colbourne F (2011) Unilateral brain hypothermia as a method to examine efficacy and mechanisms of neuroprotection against global ischemia. Ther Hypothermia Temp Manag 1:87-94.

Simón D, García-García E, Royo F, Falcón-Pérez JM, Avila J (2012) Proteostasis
of tau. Tau overexpression results in its secretion via membrane vesicles. FEBS Lett 586:47-54.

Singh P, Mann KA, Mangat HK, Kaur G (2003)
Prolonged glutamate excitotoxicity: effects on mitochondrial antioxidants and antioxidant enzymes. Mol Cell Biochem 243:139-45.

Singhvi A, Garriga G (2009) Asymmetric divisions, aggresomes and apoptosis. Trends Cell Biol 19:1-7.

Smart F, Aschrafi A, Atkins A, Owens GC, Pilotte J, Cunningham BA, Vanderklish PW (2007) Two isoforms of the cold-inducible mRNA-binding protein RBM3 localize to dendrites and promote translation. J Neurochem 101:1367-1379.

Smeal T, Angel P, Meek J, Karin M (1989)
Different requirements for formation of Jun: Jun and Jun: Fos complexes. Genes Dev 3:2091-100.

Smith MA, Casadesus G, Joseph JA, Perry G (2002) Amyloid-beta and tau serve antioxidant functions in the aging and Alzheimer brain. Free Radic Biol Med 33:1194-9.

Smith DH, Chen XH, Nonaka M, Trojanowski JQ, Lee VM, Saatman KE, Leoni MJ, Xu BN, Wolf JA, Meaney DF (1999) Accumulation of amyloid beta and tau and the formation of neurofilament inclusions following diffuse brain injury in the pig. J Neuropathol Exp Neurol 58:982-92.

Smoter M, Bodnar L, Duchnowksa R, Stec R, Grala B, Szczylik C (2011) The role of tau protein in resistance to paclitaxel. Cancer Chemother Pharmacol 68:553-7.

Soderling TR, Derkach VA (2000) Postsynaptic protein phosphorylation and LTP. Trends Neurosci 23:75-80.

Sokka AL, Putkonen N, Mudo G, Pryazhnikov E, Reijonen S, Khiroug L, Belluardo N, Lindholm D, Korhonen L (2007) Endoplasmic reticulum stress inhibition protects against excitotoxic neuronal injury in the rat brain. J Neurosci 27:901-8.

Sokolow S, Henkins KM, Bilousova T, Gonzalez B, Vinters HV, Miller CA, Cornwell L, Poon WW, Gylys KH (2014) Pre-synaptic C-terminal truncated tau is released from cortical synapses in Alzheimer's disease. J Neurochem doi: 10.1111/jnc.12991.

Sonna LA, Fujita J, Gaffin SL, Lilly CM (2002) Invited review: Effects of heat and cold stress on mammalian gene expression. J Appl Physiol 92:1725-1742.

Soriano FX, Baxter P, Murray LM, Sporn MB, Gillingwater TH, Hardingham GE (2009) Transcriptional regulation of the AP-1 and Nrf2 target gene sulfiredoxin. Mol Cells 27:279-82.

Soriano FX, Papadia S, Hofmann F, Hardingham NR, Bading H, Hardingham GE (2006) Preconditioning doses of NMDA promote neuroprotection by enhancing neuronal excitability. J Neurosci 26:4509-18.

Spillantini MG, Bird TD, Ghetti B (1998) Frontotemporal dementia and Parkinsonism linked to chromosome 17: a new group of tauopathies. Brain Pathol 8:387-402.

Spillantini MG, Goedert M (2013) Tau pathology and neurodegeneration. Lancet Neurol 12:609-22.

Spires-Jones TL, Stoothoff WH, de Calignon A, Jones PB, Hyman BT (2009) Tau pathophysiology in neurodegeneration: a tangled issue. Trends Neurosci 32:150-9.

Spittaels K, Van den Haute C, Van Dorpe J, Bruynseels K, Vandezande K, Laenen I, Geerts H, Mercken M, Sciot R, Van Lommel A, Loos R, Van Leuven F (1999) Prominent axonopathy in the brain and spinal cord of transgenic mice overexpressing four-repeat human tau protein. Am J Pathol 155:2153-65.

Spriggs KA, Bushell M, Willis AE (2010) Translational regulation of gene expression during conditions of cell stress. Mol Cell 40:228-37.

Stapulionis R[1], Kolli S, Deutscher MP. Efficient mammalian protein synthesis requires an intact F-actin system. J Biol Chem. 1997 Oct 3;272(40):24980-6.

Stacpoole SR, Bilican B, Webber DJ, Luzhynskaya A, He XL, Compston A, Karadottir R, Franklin RJ, Chandran S (2011) Derivation of neural precursor cells from human ES cells at 3% $O_2$ is efficient, enhances survival and presents no barrier to regional specification and functional differentiation. Cell Death Differ 18:1016-1023.

Stargardt A, Swaab DF, Bossers K (2015) The storm before the quiet: neuronal hyperactivity and Aβ in the presymptomatic stages of Alzheimer's disease. Neurobiol Aging 36:1-11.

Stefansson H, Helgason A, Thorleifsson G, Steinthorsdottir V, Masson G, Baranrd J, Baker A, Jonasdottr A, Ingason A, Gudnadottir VG, Desnica N, Hicks A, Gylfason A, Gudbjartsson DF, Jonsdottir GM, Sainz J, Agnarsson K, Birgisdottir B, Ghosh S, Olafsdottir A, Cazier JB, Kristjansson

K, Frigge ML, Thorgeirsson TE, Gulcher JR, Kong A, Stefansson K (2005) A common inversion under selection in Europeans. Nat Genet. 37: 129-137.

Steiner B, Mandelkow EM, Biernat J, Gustke N, Meyer HE, Schmidt B, Mieskes G, Söling HD, Drechsel D, Kirschner MW (1990) Phosphorylation of microtubule-associated protein tau: identification of the site for Ca2(+)-calmodulin dependent kinase and relationship with tau phosphorylation in Alzheimer tangles. EMBO J 9: 3539–3544.

Stenzel-Poore MP, Stevens SL, Xiong Z, Lessov NS, Harrington CA, Mori M, Meller R, Rosenzweig HL, Tobar E, Shaw TE, Chu X, Simon RP (2003)
Effect of ischaemic preconditioning on genomic response to cerebral ischaemia: similarity to neuropro tectivestrategies in hibernation and hypoxia-tolerant states. Lancet 362:1028-37.

Stetler RA, Leak RK, Gan Y, Li P, Zhang F, Hu X, Jing Z, Chen J, Zigmond MJ, Gao Y (2014) Preconditioning provides neuroprotection in models of CNS disease: paradigms and clinical significan ce. Prog Neurobiol 114:58-83.

Stieler JT, Bullmann T, Kohl F, Tøien Ø, Brückner MK, Härtig W, Barnes BM, Arendt T (2011) The physiological link between metabolic rate depression and tau phosphorylation in mammalian hibernation. PLoS One 6:e14530.

Stolzing A, Sethe S, Scutt AM (2006)
Stressed stem cells: Temperature response in aged mesenchymal stem cells. Stem Cells Dev 15:478-87.

Stoveken BJ (2013) Tau pathology as a cause and consequence of the UPR. J Neurosci 33:14285-7.

Strack S, Zaucha JA, Ebner FF, Colbran RJ, Wadzinski BE (1998)
Brain protein phosphatase 2A: developmental regulation and distinct cellular and subcellular localizati on by Bsubunits. J Comp Neurol 392:515-27.

Stutzbach LD, Xie SX, Naj AC, Albin R, Gilman S; PSP Genetics Study Group, Lee VM, Trojanowski JQ, Devlin B, Schellenberg GD (2013)
The unfolded protein response is activated in disease-affected brain regions in progressive supranuclear palsyand Alzheimer's disease. Acta Neuropathol Commun 1:31.

Su JH, Cummings BJ, Cotman CW (1993) Identification and distribution of axonal dystrophic neurites in Alzheimer's disease. Brain Res 625:228-37.

Su B, Wang X, Drew KL, Perry G, Smith MA, Zhu X (2008) Physiological regulation of tau phosphorylation during hibernation. J Neurochem 105:2098-108.

Sultan A, Nesslany F, Violet M, Bégard S, Loyens A, Talahari S, Mansuroglu Z, Marzin D, Sergeant N, Humez S, Colin M, Bonnefoy E, Buée L, Galas MC (2011) Nuclear tau, a key player in neuronal DNA protection. J Biol Chem 286:4566-75.

Sumitomo Y, Higashitsuji H, Higashitsuji H, Liu Y, Fujita T, Sakurai T, Candeias MM, Itoh K, Chiba T, Fujita J (2012) Identification of a novel enhancer that binds Sp1 and contributes to induction of cold-inducible RNA-binding protein (cirp) expression in mammalian cells. BMC Biotechnol 12:72.

Sumpter PQ, Mann DM, Davies CA, Yates PO, Snowden JS, Neary D (1986) An ultrastructural analysis of the effects of accumulation of neurofibrillary tangle in pyramidal neurons of the cerebral cortex in Alzheimer’s disease. Neuropathol Appl Neurobiol 12:305–319.

Sun XY, Wei YP, Xiong Y, Wang XC, Xie AJ, Wang XL, Yang Y, Wang Q, Lu YM, Liu R, Wang JZ (2012) Synaptic released zinc promotes tau hyperphosphorylation by inhibition of protein phosphatase 2A (PP2A). J Biol Chem 287:11174-82.

Sureban SM, Ramalingam S, Natarajan G, May R, Subramaniam D, Bishnupuri KS, Morrison AR, Dieckgraefe BK, Brackett DJ, Postier RG, Houchen CW, Anant S (2008) Translation regulatory factor RBM3 is a proto-oncogene that prevents mitotic catastrophe. Oncogene 27:4544-4556.

Sydow A, Van der Jeugd A, Zheng F, Ahmed T, Balschun D, Petrova O, Drexler D, Zhou L, Rune G, Mandelkow E, D'Hooge R, Alzheimer C, Mandelkow EM (2011) Tau-induced defects in synaptic plasticity, learning, and memory are reversible in transgenic mice afterswitching off the toxic Tau mutant. J Neurosci 31:2511-25.

Tabas I, Ron D (2011) Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. Nat Cell Biol 13:184-90.

Tai HC, Schuman EM (2008) Ubiquitin, the proteasome and protein degradation in neuronal function and dysfunction. Nat Rev Neurosci 9:826-38.

Tai HC, Serrano-Pozo A, Hashimoto T, Frosch MP, Spires-Jones TL, Hyman BT (2012) The synaptic accumulation of hyperphosphorylated tau oligomers in Alzheimer disease is associated withdysfunction of the ubiquitin-proteasome system. Am J Pathol 181:1426-35.

Tai HC, Wang BY, Serrano Pozo A, Frosch MP, Spires-Jones TL, Hyman BT (2014) Frequent and symmetric deposition of misfolded tau oligomers within presynaptic and postsynaptic te rminals inAlzheimer's disease. Acta Neuropathol Commun 2:146.

Takahashi RH, Capetillo-Zarate E, Lin MT, Milner TA, Gouras GK (2010) Co-occurrence of Alzheimer's disease ß-amyloid and τ pathologies at synapses. Neurobiol Aging 31:1145-52.

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663-76.

Takamatsu I, Sekiguchi M, Wada K, Sato T, Ozaki M (2005) Propofol-mediated impairment of CA1 long-term potentiation in mouse hippocampal slices. Neurosci Lett 389:129-32.

Tan W, Cao X, Wang J, Lv H, Wu B, Ma H (2010) Tau hyperphosphorylation is associated with memory impairment after exposure to 1.5% isoflurane without temperature maintenance in rats. Eur J Anaesthesiol 27:835-41.

Taylor DM, De Koninck PD, Minotti S, Durham HD (2007) Manipulation of protein kinases reveals different mechanisms for upregulation of heat shock proteins in motor neurons and non-neuronal cells. Mol Cell Neurosci 34:20-33.

Taylor RC, Dillin A (2011) Aging as an event of proteostasis collapse. Cold Spring Harb Perspect Biol 3(5).

Terao Y, Miyamoto S, Hirai K, Kamiguchi H, Ohta H, Shimojo M, Kiyota Y, Asahi S, Sakura Y, Shintani Y (2009) Hypothermia enhances heat-shock protein 70 production in ischemic brains. Neuroreport 20:745-9.

Texel SJ, Mattson MP (2011) Impaired adaptive cellular responses to oxidative stress and the pathogenesis of Alzheimer's disease. Antioxid Redox Signal. 14:1519-34.

Thompson SM, Masukawa LM, Prince DA (1985) Temperature dependence of intrinsic membrane properties and synaptic potentials in hippocampal CA1 neurons in vitro. J Neurosci 5:817-824.

Thoresen M, Satas S, Puka-Sundvall M, Whitelaw A, Hallström A, Løberg EM, Ungerstedt U, Steen PA, Hagberg H (1997) Post-hypoxic hypothermia reduces cerebrocortical release of NO and excitotoxins. Neuroreport 8:3359-62.

Thrippleton MJ, Parikh J, Harris BA, Hammer SJ, Semple SI, Andrews PJ, Wardlaw JM, Marshall I (2014) Reliability of MRSI brain temperature mapping at 1.5 and 3T. NMR Biomed 27:183-90.

Tilney LG, Porter KR (1967) Studies on the microtubules in heliozoa. II. The effect of low temperature on these structures in the formation and maintenance of the axopodia. J Cell Biol 34:327-43.

Tipton MJ (1989) The initial responses to cold-water immersion in man. Clin Sci (Lond) 77:581-8.

Tobaben S, Thakur P, Fernández-Chacón R, Südhof TC, Rettig J, Stahl B (2001)
A trimeric protein complex functions as a synaptic chaperone machine. Neuron 31:987-99.

Tøien Ø, Blake J, Edgar DM, Grahn DA, Heller HC, Barnes BM (2011)
Hibernation in black bears: independence of metabolic suppression from body temperature. Science 331:906-9.

Tong G, Endersfelder S, Rosenthal LM, Wollersheim S, Sauer IM, Bührer C, Berger F, Schmitt KR (2013) Effects of moderate and deep hypothermia on RNA-binding proteins RBM3 and CIRP expressions in murine hippocampal brain slices. Brain Res 1504:74-84.

Tovar KR, Westbrook GL (1999) The incorporation of NMDA receptors with
a distinct subunit composition at nascent hippocampal synapses invitro. J Neurosci 19:4180-8.

Trabzuni S, Wray S, Vandrovcova J, Ramasamy A, Walker R, Smith C, Luk C, Gibbs JR, Dilman A, Hernandez DG, Arepalli S, Singleton AB, Cookson MR, Pittman AM, de Silva R, Weale ME, Hardy J, Ryten M (2012) MAPT expression and splicing is differentially regulated by brain region: relation to genotype ad implication for tauopathies. Hum Mol Genet 21: 4094-103.

Trojanowski JQ, Lee VM-Y (1995) Phosphorylation of paired helical filament tau in Alzheimer's disease: focusing on phosphatases. FASEB J. 9:1570-1576.

Trojanowski JQ, Lee VM (2000) "Fatal attractions" of proteins. A comprehensive hypothetical mechanism underlying Alzheimer's disease and other neurodegenerative disorders. Ann N Y Acad Sci 924:62–67.

Turina D, Loitto VM, Björnström K, Sundqvist T, Eintrei C (2008)
Propofol causes neurite retraction in neurones. Br J Anaesth 101:374-9.

Tyedmers J, Mogk A, Bukau B (2010) Cellular strategies for controlling protein aggregation. Nat Rev Mol Cell Biol 11:777-88.

Tymianski M, Sattler R, Zabramski JM, Spetzler RF (1998)
Characterization of neuroprotection from excitotoxicity by moderate and profound hypothermia in cul turedcortical neurons unmasks a temperature-insensitive component of glutamate neurotoxicity. J Cereb Blood Flow Metab 18:848-67.

Ule J, Darnell RB (2006) RNA binding proteins and the regulation of neuronal synaptic plasticity. Curr Opin Neurobiol 16:102-110.

Unterberger U, Höftberger R, Gelpi E, Flicker H, Budka H, Voigtländer T (2006) Endoplasmic reticulum stress features are prominent in Alzheimer disease but not in prion diseases in vivo. J Neuropathol Exp Neurol 65:348-57.

Valdés P, Mercado G, Vidal RL, Molina C, Parsons G, Court FA, Martinez A, Galleguillos D, Armentano D, Schneider BL, Hetz C (2014) Control of dopaminergic neuron survival by the unfolded protein response transcription factor XBP1. Proc Natl Acad Sci U S A 111:6804-9.

Vallone D, Frigato E, Vernesi C, Foà A, Foulkes NS, Bertolucci C (2007) Hypothermia modulates circadian clock gene expression in lizard peripheral tissues. Am J Physiol Regul Integr Comp Physiol 292:R160-6.

van der Harg JM, Nölle A, Zwart R, Boerema AS, van Haastert ES, Strijkstra AM, Hoozemans JJ, Scheper W (2014) The unfolded protein response mediates reversible tau phosphorylation induced by metabolic stress. Cell Death Dis 5:e1393.

van der Worp HB, Sena ES, Donnan GA, Howells DW, Macleod MR (2007) Hypothermia in animal models of acute ischaemic stroke: a systematic review and meta-analysis. Brain 130:3063-74.

van Eersel J, Stevens CH, Przybyla M, Gladbach A, Stefanoska K, Chan CK, Ong WY, Hodges JR, Sutherland GT, Kril JJ, Abramowski D, Staufenbiel M,Halliday GM, Ittner LM (2015) Early-onset Axonal Pathology in a Novel P301S-Tau Transgenic Mouse Model of Frontotemporal Lobar Degeneration. Neuropathol Appl Neurobiol doi: 10.1111/nan.12233 [Epub ahead of print].

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3:RESEARCH0034.

Vargas MR, Johnson DA, Sirkis DW, Messing A, Johnson JA (2008) Nrf2 activation in astrocytes protects against neurodegeneration in mouse models of familial amyotrop hic lateralsclerosis. J Neurosci 28:13574-81.

Velly LJ, Guillet BA, Masmejean FM, Nieoullon AL, Bruder NJ, Gouin FM, Pisano PM (2003) Neuroprotective effects of propofol in a model of ischemic cortical cell cultures: role of glutamate and its transporters. Anesthesiology 99:368-75.

Vieira M, Fernandes J, Carreto L, Anuncibay-Soto B, Santos M, Han J, Fernández-López A, Duarte CB, Carvalho AL, Santos AE (2014) Ischemic insults induce necroptotic cell death in hippocampal neurons through the up-regulation of endogenousRIP3. Neurobiol Dis 68:26-36.

Vogelsberg-Ragaglia V, Schuck, T, Trojanowski JG, Lee VM (2001) PP2A mRNA expression is quantitatively decreased in Alzheimer's disease hippocampus. Exp Neurol 168:402-12.

Volgushev M, Vidyasagar TR, Chistiakova M, Eysel UT (2000) Synaptic transmission in the neocortex during reversible cooling. Neuroscience 98:9-22.

Wade-Martins R (2012) Genetics: The MAPT locus-a genetic paradigm in disease susceptibility. Nat Rev Neurol 8:477-8.

Walker GA, Thompson FJ, Brawley A, Scanlon T, Devaney E (2003) Heat shock factor functions at the convergence of the stress response and developmental pathways in Caenorhabditis elegans. FASEB J 17:1960-2.

Walsh AH, Cheng A, Honkanen RE (1997) Fostriecin, an antitumor antibiotic with inhibitory activity against serine/threonine protein phosphatases types 1 (PP1) and 2A (PP2A), is highly selective for PP2A. FEBS Lett 416:230-4.

Walter J, Davenne D, Shoham S, Dinarello CA, Krueger JM (1986) Brain temperature changes coupled to sleep states persist during interleukin 1-enhanced sleep. Am J Physiol 250:R96-103.

Walter P, Ron D (2011) The unfolded protein response: from stress pathway to homeostatic regulation. Science 334:1081-6.

Walther DM, Kasturi P, Zheng M, Pinkert S, Vecchi G, Ciryam P, Morimoto RI, Dobson CM, Vendruscolo M, Mann M, Hartl FU (2015) Widespread proteome remodeling and aggregation in aging C. elegans. Cell 161:919-32.

Wang G, Grone B, Colas D, Appelbaum L, Mourrain P (2011) Synaptic plasticity in sleep: learning, homeostasis and disease. Trends Neurosci 34:452-63.

Wang JZ, Grundke-Iqbal I, Iqbal K (2007) Kinases and phosphatases and tau sites involved in Alzheimer neurofibrillary degeneration. Eur J Neurosci 25:59-68.

Wang JZ, Liu F (2008) Microtubule-associated protein tau in development, degeneration and protection of neurons. Prog Neurobiol 85:148-75.

Wang Y, Martinez-Vicente M, Krüger U, Kaushik S, Wong E, Mandelkow EM, Cuervo AM, Mandelkow E (2009) Tau fragmentation, aggregation and clearance: the dual role of lysosomal processing. Hum Mol Genet 18:4153-70.

Wang LY, Orser BA, Brautigan DL, MacDonald JF (1994) Regulation of NMDA receptors in cultured hippocampal neurons by protein phosphatases 1 and 2A. Nature 369:230-2.

Wang X, Takata T, Bai X, Ou F, Yokono K, Sakurai T (2012) Pyruvate prevents the inhibition of the long-termpotentiation induced by amyloid-β through protein phosphatase 2A inactivation. J Alzheimers Dis 30:665–673.

Wang JZ, Wang ZF (2006) Role of melatonin in Alzheimer-like neurodegeneration. Acta Pharmacol Sin 27:41-9.

Wang H, Wang B, Normoyle KP, Jackson K, Spitler K, Sharrock MF, Miller CM, Best C, Llano D, Du R (2014) Brain temperature and its fundamental properties: a review for clinical neuroscientists. Front Neurosci 8:307.

Warmus BA, Sekar DR, McCutchen E, Schellenberg GD, Roberts RC, McMahon LL, Roberson ED (2014) Tau-mediated NMDA receptor impairment underlies dysfunction of a selectively vulnerable network in a mouse model of frontotemporal dementia. J Neurosci 34:16482-95.

Wassmann H, Greiner C, Hülsmann S, Moskopp D, Speckmann EJ, Meyer J, Van Aken H (1998) Hypothermia as cerebroprotective measure. Experimental hypoxic exposure of brain slices and clinical application in critically reduced cerebral perfusion pressure. Neurol Res 20 Suppl 1:S61-5.

Wei H, Xie Z (2009) Anesthesia, calcium homeostasis and Alzheimer's disease. Curr Alzheimer Res 6:30-5.

Weil ZM, Norman GJ, DeVries AC, Nelson RJ (2008) The injured nervous system: a Darwinian perspective. Prog Neurobiol 86:48-59.

Weingarten MD, Lockwood AH, Hwo SY, Kirschner MW (1975) A protein factor essential for microtubule assembly. Proc Natl Acad Sci U S A 72:1858-62.

Weisenberg RC (1972) Microtubule formation in vitro in solutions containing low calcium concentrations. Science 177:1104-5.

Wellmann S, Bührer C, Moderegger E, Zelmer A, Kirschner R, Koehne P, Fujita J, Seeger K (2004) Oxygen-regulated expression of the RNA-binding proteins RBM3 and CIRP by a HIF-1-independent mechanism. J Cell Sci 117:1785-1794.

Wellmann S, Truss M, Bruder E, Tornillo L, Zelmer A, Seeger K, Bührer C (2010) The RNA-binding protein RBM3 is required for cell proliferation and protects against serum-deprivation-induced cell death. Pediatr Res 67:35-41.

Wen Y, Yang S, Liu R, Brun-Zinkernagel AM, Koulen P, Simpkins JW (2004) Transient cerebral ischemia induces aberrant neuronal cell cycle re-entry and Alzheimer's disease-like tauopathy in female rats. J Biol Chem 279:22684-92.

Werner C, Engelhard K (2007) Pathophysiology of traumatic brain injury. Br J Anaesth 99:4-9.

Westerfield M, Joyner RW, Moore JW (1978) Temperature-sensitive conduction failure at axon branch points. J Neurophysiol 41:1-8.

White F, McCaig D, Brown SM, Graham DI, Harland J, Macrae IM (2004) Up-regulation of a growth arrest and DNA damage protein (GADD34) in the ischaemic human brain: implicationsfor protein synthesis regulation and DNA repair. Neuropathol Appl Neurobiol 30:683-91.

Whittington RA, Virág L, Marcouiller F, Papon MA, El Khoury NB, Julien C, Morin F, Emala CW, Planel E (2011) Propofol directly increases tau phosphorylation. PLoS One 6:e16648.

Wilhemsen KC, Lynch T, Pavlou E, Higgins M, Nygaard TG (1994) Localization of disinhibition-dementia-parkinsonism-amyotrophy complex to 17q21-22. Am J Hum Genet 55:1159-65.

Williams DR, Epperson LE, Li W, Hughes MA, Taylor R, Rogers J, Martin SL, Cossins AR, Gracey AY (2005) Seasonally hibernating phenotype assessed through transcript screening. Physiol Genomics 24:13-22.

Williams A, Jahreiss L, Sarkar S, Saiki S, Menzies FM, Ravikumar B, Rubinsztein DC (2006) Aggregate-prone proteins are cleared from the cytosol by autophagy: therapeutic implications. Curr Top Dev Biol 76:89-101.

Wilson NR, Kang J, Hueske EV, Leung T, Varoqui H, Murnick JG, Erickson JD, Liu G (2005) Presynaptic regulation of quantal size by the vesicular glutamate transporter VGLUT1. J Neurosci 25:6221-34.

Wisniewski KE, Wisniewski HM, Wen GY (1985) Occurrence of neuropathological changes and dementia of Alzheimer’s disease in Down’s syndrome. Ann Neurol 17:278-282.

Wong E, Cuervo AM (2010) Integration of clearance mechanisms: the proteasome and autophagy. Cold Spring Harb Perspect Biol 2:a006734.

Wood JG, Mirra SS, Pollock NJ, Binder LI (1986) Neurofibrillary tangles of Alzheimer disease share antigenic determinants with the axonal microtubule-associated protein tau (tau) Proc Natl Acad Sci U S A 83:4040-3.

Wulhfard S (2009) Transient Recombinant Protein Expression in Mammalian Cells: the Role of mRNA Level and Stability. Thesis, Ecole Polytechnique Federale de Lausanne.

Xiao C, Mileva-Seitz V, Seroude L, Robertson RM (2007) Targeting HSP70 to motoneurons protects locomotor activity from hyperthermia in Drosophila. Dev Neurobiol 67:438-55.

Xiong M, Chen LX, Ma SM, Yang Y, Zhou WH (2013) Short-term effects of hypothermia on axonal injury, preoligodendrocyte accumulation and oligodendrocytem yelination after hypoxia-ischemia in the hippocampus of immature rat brain. Dev Neurosci 35:17-27.

Xu SY, Hu YF, Li WP, Wu YM, Ji Z, Wang SN, Li K, Pan SY (2014) Intermittent hypothermia is neuroprotective in an in vitro model of ischemic stroke. Int J Biol Sci 10:873-81.

Xu D, Yang W, Zhao G (2012) Effect of propofol and inhalation anesthesia on postoperative cognitive dysfunction in the elderly: a meta-analysis. Nan Fang Yi Ke Da Xue Xue Bao 32:1623-7.

Yablonskiy DA, Ackerman JJ, Raichle ME (2000) Coupling between changes in human brain temperature and oxidative metabolism during prolonged visual stimulation. Proc Natl Acad Sci U S A 97:7603-8.

Yamada K, Cirrito JR, Stewart FR, Jiang H, Finn MB, Holmes BB, Binder LI, Mandelkow EM, Diamond MI, Lee VM, Holtzman DM (2011) In vivo microdialysis reveals age-dependent decrease of brain interstitial fluid tau levels in P301S human tautransgenic mice. J Neurosci 31:13110-7.

Yamada K, Holth JK, Liao F, Stewart FR, Mahan TE, Jiang H, Cirrito JR, Patel TK, Hochgräfe K, Mandelkow EM, Holtzman DM (2014) Neuronal activity regulates extracellular tau in vivo. J Exp Med 211:387-93.

Yamaguchi S, Midorikawa Y, Okuda Y, Kitajima T (1999) Propofol prevents delayed neuronal death following transient forebrain ischemia in gerbils. Can J Anaesth 46:593-8.

Yamamoto H, Saitoh Y, Yasugawa S, Miyamoto E (1990) Dephosphorylation of tau factor by protein phosphatase 2A in synaptosomal cytosol fractions, and inhibition by aluminium. J Neurochem 55:683-90.

Yamashita K, Eguchi Y, Kajiwara K, Ito H (1991) Mild hypothermia ameliorates ubiquitin synthesis and prevents delayed neuronal death in the gerbilhippocampus. Stroke 22:1574-81.

Yanagisawa M, Planel E, Ishiguro K, Fujita SC (1999) Starvation induces tau hyperphosphorylation in mouse brain: implications for Alzheimer's disease. FEBS Lett 461:329–333.

Yang C, Carrier F (2001) The UV-inducible RNA-binding protein A18 (A18 hnRNP) plays a protective role in the genotoxic stress response. J Biol Chem 276:47277-84.

Yang G, Paschos G, Curtis AM, Musiek ES, McLoughlin SC, FitzGerald GA (2013) Knitting up the raveled sleave of care. Sci Transl Med 5:212rv3.

Yang XF, Ouyang Y, Kennedy BR, Rothman SM (2005) Cooling blocks rat hippocampal neurotransmission by a presynaptic mechanism: observations using 2-photon microscopy. J Physiol 567:215-24.

Yang R, Weber DJ, Carrier F (2006) Post-transcriptional regulation of thioredoxin by the stress inducible heterogenous ribonucleoprotein A18. Nucleic Acids Res 34:1224-1236.

Yang R, Zhan M, Nalabothula NR, Yang Q, Indig FE, Carrier F (2010) Functional significance for a heterogenous ribonucleoprotein A18 signature RNA motif in the 3'-untranslated region of ataxia telangiectasia mutated and Rad3-related (ATR) transcript. J Biol Chem 285:8887-93.

Yardin C, Terro F, Esclaire F, Rigaud M, Hugon J (1998) Brefeldin A-induced apoptosis is expressed in rat neurons with dephosphorylated tau protein. Neurosci Lett 250:1-4.

Yasuda M, Yokoyama K, Nakayasu T, Nishimura Y, Matsui M, Yokoyama T, Miyoshi K, Tanaka C (2000) A Japanese patient with frontotemporal dementia and parkinsonism by a tau P301S mutation. Neurology 55:1224-7.

Yenari MA, Han HS (2012) Neuroprotective mechanisms of hypothermia in brain ischaemia. Nat Rev Neurosci 13:267-278.

Yenari MA, Iwayama S, Cheng D, Sun GH, Fujimura M, Morita-Fujimura Y, Chan PH, Steinberg GK (2002) Mild hypothermia attenuates cytochrome c release but does not alter Bcl-2 expression or caspase activation afterexperimental stroke. J Cereb Blood Flow Metab 22:29-38.

Yenari M, Kitagawa K, Lyden P, Perez-Pinzon M (2008) Metabolic downregulation: a key to successful neuroprotection? Stroke 39:2910-7.

Yenari MA, Liu J, Zheng Z, Vexler ZS, Lee JE, Giffard RG (2005) Antiapoptotic and anti-inflammatory mechanisms of heat-shock protein protection. Ann N Y Acad Sci 1053:74-83.

Yoshida H, Ihara Y (1993) Tau in paired helical filaments is functionally distinct from foetal tau: assembly incompetence of paired helical filament-tau. J Neurochem 61:1183-6.

Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 107:881-91.

Yoshiyama Y, Higuchi M, Zhang B, Huang SM, Iwata N, Saido TC, Maeda J, Suhara T, Trojanowski JQ, Lee VM (2007) Synapse loss and microglial activation precede tangles in a P301S tauopathy mouse model. Neuron 53:337-351.

Yu DX, Di Giorgio FP, Yao J, Marchetto MC, Brennand K, Wright R, Mei A, McHenry L, Lisuk D, Grasmick JM, Silberman P, Silberman G, Jappelli R, Gage FH (2014) Modeling hippocampal neurogenesis using human pluripotent stem cells. Stem Cell Reports 2:295-310.

Yu Z, Luo H, Fu W, Mattson MP (1999) The endoplasmic reticulum stress-responsive protein GRP78 protects neurons against excitotoxicity and apoptosis: suppression of oxidative stress and stabilization of calcium homeostasis. Exp Neurol 155:302-14.

Yu Y, Run X, Liang Z, Li Y, Liu F, Liu Y, Iqbal K, Grundke-Iqbal I, Gong CX (2009) Developmental regulation of tau phosphorylation, tau kinases, and tau phosphatases. J Neurochem 108:1480-1494.

Yuan HB, Huang Y, Zheng S, Zuo Z (2004) Hypothermic preconditioning increases survival of purkinje neurons in rat cerebellar slices after an in vitrosimulated ischemia. Anesthesiology 100:331-7.

Zempel H, Mandelkow E (2014) Lost after translation: missorting of Tau protein and consequences for Alzheimer disease. Trends Neurosci 37:721-32.

Zeng H, Guo M, Martins-Taylor K, Wang X, Zhang Z, Park JW, Zhan S, Kronenberg MS, Lichtler A, Liu HX, Chen FP, Yue L, Li XJ, Xu RH (2010) Specification of region-specific neurons including forebrain glutamatergic neurons from human induced pluripotent stem cells. PLoS One 5:e11853.

Zeng Y, Wodzenski D, Gao D, Shiraishi T, Terada N, Li Y, Vander Griend DJ, Luo J, Kong C, Getzenberg RH, Kulkarni P (2013) Stress-response protein RBM3 attenuates the stem-like properties of prostate cancer cells by interfering with CD44 variant splicing. Cancer Res 73:4123-33.

Zhang YQ, Broadie K (2005) Fathoming fragile X in fruit flies. Trends Genet 21:37-45.

Zhang HT, Zhang ZW, Xue JH, Kong HB, Liu AJ, Li SC, Liu YX, Xu DG (2013) Differential expression of the RNA-binding motif protein 3 in human astrocytoma. Chin Med J 126:1948-52.

Zhang YQ, Bailey AM, Matthies HJ, Renden RB, Smith MA, Speese SD, Rubin GM, Broadie K (2001) Drosophila fragile X-related gene regulates the MAP1B homolog Futsch to control synaptic structure and function. Cell 107:591-603.

Zhao X, Jin YW, Wang DY, Wang ZG (2009) Effect of propofol upon brain after whole-body hyperthermia in rats] Zhonghua Yi Xue Za Zhi 89:2356-9.

Zhao H, Steinberg GK, Sapolsky RM (2007) General versus specific actions of mild-moderate hypothermia in attenuating cerebral ischemic damage. J Cereb Blood Flow Metab 27:1879-94.

Zhang F, Phiel CJ, Spece L, Gurvich N, Klein PS (2003) Inhibitory phosphorylation of glycogen snthase kinase-3 (GSK-3) in response to lithium: evidence for autoregulation of GSK-3. J Biol Chem 278: 33067-33077.

Zheng Z, Kim JY, Ma H, Lee JE, Yenari MA (2008) Anti-inflammatory effects of the 70 kDa heat shock protein in experimental stroke. J Cereb Blood Flow Metab 28:53-63.

Zhong Q, Congdon EE, Nagaraja HN, Kuret J (2012) Tau isoform composition influences extent of filament formation. J Biol Chem 287: 20711-9.

Zhou M, Yang WL, Ji Y, Qiang X, Wang P (2014) Cold-inducible RNA-binding protein mediates neuroinflammation in cerebral ischemia. Biochim Biophys Acta 1840:2253-61.

Zhou F, Zhu X, Castellani RJ, Stimmelmayr R, Perry G, Smith MA, Drew KL (2001) Hibernation, a model of neuroprotection. Am J Pathol 158:2145-51.

Zhu Y, Fenik P, Zhan G, Sanfillipo-Cohn B, Naidoo N, Veasey SC (2008) Eif-2a protects brainstem motoneurons in a murine model of sleep apnea. J Neurosci 28:2168-78.

# Appendix (published works)

EBioMedicine xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Original Article

# Hypothermic Preconditioning of Human Cortical Neurons Requires Proteostatic Priming

Nina Marie Rzechorzek [a,b,*], Peter Connick [a,c], Rickie Patani [c,d], Bhuvaneish Thangaraj Selvaraj [a,b], Siddharthan Chandran [a,b,c,**]

[a] Centre for Clinical Brain Sciences, University of Edinburgh, Midlothian EH16 4SB, United Kingdom
[b] MRC Centre for Regenerative Medicine, University of Edinburgh, Midlothian EH16 4SB, United Kingdom
[c] The Anne Rowling Regenerative Neurology Clinic, University of Edinburgh, Midlothian EH16 4SB, United Kingdom
[d] Department of Molecular Neuroscience, UCL Institute of Neurology, Queen Square, London WC1N 3BG, United Kingdom

ARTICLE INFO

*Article history:*
Received 25 February 2015
Received in revised form 6 April 2015
Accepted 8 April 2015
Available online xxxx

*Keywords:*
Endoplasmic reticulum stress
Unfolded protein response
Hypothermia
Preconditioning
Proteostasis
Neuroprotection

ABSTRACT

Hypothermia is potently neuroprotective but poor mechanistic understanding has restricted its clinical use. Rodent studies indicate that hypothermia can elicit preconditioning, wherein a subtoxic cellular stress confers resistance to an otherwise lethal injury. The molecular basis of this preconditioning remains obscure. Here we explore molecular effects of cooling using functional cortical neurons differentiated from human pluripotent stem cells (hCNs). Mild-to-moderate hypothermia (28–32 °C) induces cold-shock protein expression and mild endoplasmic reticulum (ER) stress in hCNs, with full activation of the unfolded protein response (UPR). Chemical block of a principal UPR pathway mitigates the protective effect of cooling against oxidative stress, whilst pre-cooling neurons abrogates the toxic injury produced by the ER stressor tunicamycin. Cold-stress thus preconditions neurons by upregulating adaptive chaperone-driven pathways of the UPR in a manner that precipitates ER-hormesis. Our findings establish a novel arm of neurocryobiology that could reveal multiple therapeutic targets for acute and chronic neuronal injury.



## 1. Introduction

Therapeutic cooling offers robust protection against ischaemic brain damage, but its practical challenges and risks have limited its application to specific patient groups (Choi et al., 2012; Yenari and Han, 2012). Advancing our insight into cooling-induced neuroprotection at the cellular level could provide new molecular targets to bypass the need for cooling — whilst expanding its therapeutic potential. Preconditioning describes the tolerance achieved against an intensively toxic insult by subjecting cells or tissue to a sublethal stress (Stetler et al., 2014). Neuronal preconditioning can be effected by many and varied stimuli, including hypothermia (Dirnagl et al., 2003; Yuan et al., 2004; Stetler et al., 2014). In rodents, this cooling-induced tolerance requires de novo protein synthesis (Nishio et al., 2000) — a fundamental arm of the cold-shock response (Fujita, 1999), for which data in human neurons is lacking. Depending on the depth of cooling, this response leads to cell-cycle arrest with shut-down of transcription and translation (Yenari and Han, 2012). Simultaneously, a subset of highly conserved 'cold-inducible' RNA chaperones including RNA binding motif 3 (RBM3) and cold-inducible RNA binding protein (CIRBP) is rapidly upregulated (Lleonart, 2010). These 'cold-shock' proteins mediate important survival functions including facilitated translation of essential mRNAs and suppression of apoptosis (Lleonart, 2010; Saito et al., 2010).

Aside from induction of cold-shock proteins however, little is known of other fundamental cellular stress pathways in relation to cooling and their potential relevance to hypothermic preconditioning (Hofman et al., 2012; van der Harg et al., 2014). Hypothermia can induce protein unfolding and disrupt the cell secretory pathway (Saraste et al., 1986; Liu et al., 1994; Fujita, 1999), both of which would result in endoplasmic reticulum (ER) stress (Kim et al., 2008). However, mammalian cell lines have produced conflicting data regarding the ability of cooling to trigger ER stress and downstream events coordinated by the unfolded protein response (UPR) (Hofman et al., 2012; van der Harg et al., 2014). Although this may relate to the variable depths of hypothermia studied, it likely also reflects the resistance of immortal cell types to physiological stress (Abdel Malek et al., 2015; Cerezo et al., 2015). Furthermore, the ER-UPR cascade as a whole has never been explored at clinically-relevant hypothermic temperatures. Potentially, such a moderate level of cold-stress might bring about an adaptive proteostatic response in

* Correspondence to: N.M. Rzechorzek, Centre for Clinical Brain Sciences, University of Edinburgh, Midlothian EH16 4SB, United Kingdom.
** Correspondence to: S. Chandran, Centre for Clinical Brain Sciences, University of Edinburgh, Midlothian EH16 4SB, United Kingdom.
*E-mail addresses:* nina.rzechorzek@ed.ac.uk (N.M. Rzechorzek), siddharthan.chandran@ed.ac.uk (S. Chandran).

http://dx.doi.org/10.1016/j.ebiom.2015.04.004

post-mitotic neurons (Mendes et al., 2009; Fouillet et al., 2012). Here we test this hypothesis by characterizing the cold-shock response to protective hypothermia in functional cortical neurons differentiated from human pluripotent stem cells (hCNs) (Bilican et al., 2014), using this model to explore the molecular basis of hypothermic preconditioning.

## 2. Materials and Methods

### 2.1. Human Brain Tissue

Human post-mortem cortical brain tissue was obtained under full ethical and Institutional Review Board approval of the University of Edinburgh. Adult samples (healthy control, 17 y) were provided by the MRC Edinburgh Brain & Tissue Bank. Foetal samples were procured after elective surgical abortion (gestation 16 w), with full ethics permission of the NHS Lothian Research Ethics Committee (REC 08/S1101/1). Post-mortem samples were included as positive (foetal) and negative (adult) controls for RBM3 and CIRBP expression, which is developmentally-regulated in the human cortex (Miller et al., 2014 and The Allen Institute for Brain Science).

### 2.2. Cell Culture

All culture experiments were performed using hCNs derived from human pluripotent stem cell lines. Two human embryonic stem cell (hES) lines (H9, female, WiCell, Madison, WI and Shef 4, male, UK Stem Cell Bank, designated HES1 and HES2) were obtained under full ethical and Institutional Review Board approval of the University of Edinburgh. One human induced pluripotent stem cell (iPS) line (IPS1, healthy female control) was reprogrammed in-house after obtaining written informed consent and ethics permission (REC/10/S1103/10). hCN differentiation and immunocytochemistry protocols are described in Bilican et al. (2014). Primary antibodies (Abs) included: βIII-tubulin (mouse monoclonal, Sigma), RBM3 (rabbit monoclonal, Abcam) and CIRBP (rabbit polyclonal, Pierce). Cell counts were performed blind to the temperature variable.

### 2.3. Cooling and Multiplexed Injury Assays

Hypothermia was induced at 5 w when >90% of hCNs are functional (Bilican et al., 2014; Livesey et al., 2014). Identical plates were cultured at 28, 32 or 37 °C to simulate 'moderate hypothermia', 'mild hypothermia' or 'normothermia', respectively (Yenari and Han, 2012). Samples for transcript analysis were lifted at 3 and 24 h, after which additional samples were processed for immunocytochemistry and biochemistry. For oxidative injury experiments, neurons were switched to minimal medium (MiM (Gupta et al., 2013)) containing no antioxidants 12 h prior to temperature shift as above. After 24 h, $H_2O_2$ (diluted in MiM) was applied at 0, 50, 100 or 200 μM via 50% media exchange. Control wells received MiM with vehicle only. After a further 24 h at the respective temperatures, culture media was harvested for the cytotoxicity assay (CytoTox-One™, Promega), and cells lysed for the viability assay (CellTiter-Glo®, Promega). LDH release (cytotoxicity) was read fluorometrically (excitation 560 and emission 590 nm), whilst ATP production (viability) was measured via luminescence (Promega Glomax). Readings were taken in triplicate and averaged for each condition, after subtracting values for MiM only (fluorescence) and no cell control (luminescence). The derived 'injury ratio' (cytotoxicity in relative fluorescent units (RFU) divided by viability in relative luminescent units (RLU)) obtained for each well of cells adjusted for any potential inter-well variation in cell number. To evaluate baseline toxicity of tunicamycin (Tm) and protein kinase R (PKR)-like ER kinase (PERK) inhibitor, neurons were switched to MiM 12 h before a 24 h exposure to these compounds at 37 °C followed by multiplexed injury analysis as above.

### 2.4. Quantitative Real-Time PCR (qRT-PCR)

RNA extraction, cDNA synthesis and qRT-PCR were performed as described (Bilican et al., 2014) using primers listed in Supplementary Materials and Methods. Validation of reference target stability in hCNs under hypothermic conditions was determined with a combination of geNorm (qbase+, Biogazelle) and NormFinder (Excel) analysis (Vandesompele et al., 2002). qRT-PCR reactions were performed in triplicate and average target transcript expression was normalized to the geometric mean of eukaryotic translation initiation factor 4A2 (EIF4A2) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression for each sample.

### 2.5. Quantitative Western Analysis

Cell pellets were harvested in ice-cold Tris-buffered saline (TBS) containing protease inhibitors (cOmplete ULTRA, Roche), and where necessary, phosphatase inhibitors (PhosSTOP, Roche). Post-mortem samples were divided into 200–300 mg pieces at 4 °C. Protein was extracted on ice in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris pH 8, 150 mM NaCl, 1% Triton-X 100, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Na·deoxycholate (w/v), 0.1% sodium dodecyl sulphate (SDS)) containing protease inhibitors (as above, plus 100 μM phenylmethanesulfonylfluoride (PMSF), Fluka BioChemika). Tissue samples required homogenization (Precyllys®24). Lysates were ultracentrifuged (20 min, 50,000 ×*g*, 4 °C, Beckman). RIPA-insoluble pellets were further extracted for 20 min in 2% SDS RIPA followed by repeat ultracentrifugation to isolate nuclear proteins. Protein concentration was measured (BCA assay, Pierce) and samples boiled prior to SDS-polyacrylamide gel electrophoresis (PAGE) (4–20% gradient gels, Thermoscientific). Proteins were transferred onto Immobilon®-FL polyvinylidene fluoride (PVDF) membranes (Millipore) and blocked for 45 min at room temperature (Odyssey™ Blocking Buffer, LI-COR® Biosciences). Membranes were incubated overnight at 4 °C with primary Abs: activating transcription factor 6 (ATF6 at 1:100, mouse monoclonal, Abcam), binding immunoglobulin protein (BiP; also known as glucose-regulated protein, 78 kDa (GRP78) or heat shock 70 kDa protein 5 (HSPA5) at 1:1000, rabbit monoclonal, Abcam), CIRBP (at 1:500, rabbit polyclonal, Proteintech), eukaryotic initiation factor 2α (eIF2α at 1:1000, mouse monoclonal, Abcam), phospho-eIF2α (p-eIF2α at 1:100, rabbit monoclonal, Cell Signalling), PERK (at 1:100, rabbit monoclonal, Cell Signalling), RBM3 (at 1:100, rabbit monoclonal, Abcam), GAPDH (at 1:10,000, mouse monoclonal, Calbiochem) or heterogeneous nuclear ribonucleoprotein (hnRNP) A1 (at 1:1000, mouse monoclonal, Santa Cruz), then probed for 1 h at room temperature with Fluorescent conjugated secondary Abs (IRDye®680RD Goat (polyclonal) Anti-Rabbit IgG (H + L) and IRDye® 800CW Goat (polyclonal) Anti-Mouse IgG (H + L), LI-COR® Biosciences). Blots were exposed for 10 min (at 700 nm and/or 800 nm, LI-COR® Odyssey Fc Dual-Mode Imaging System), with band intensities quantified in Image Studio. Samples for each independently plated batch of cells were run in triplicate and average intensity readings were normalized to their respective loading control expression or total eIF2α expression (for p-eIF2α quantification).

### 2.6. XBP1 Splicing Assay

hCNs were treated for 24 h with hypothermia or Tm (0.3 μg/ml). qRT-PCR was performed as above. Conventional RT-PCR was performed using Quick-Load® Taq 2X Master Mix (New England Biolabs) and a BioRad C1000 Thermal Cycler (annealing temperature 60 °C). GAPDH and c-Myc (MYC) were included for reference and to confirm a stress response respectively. Products were resolved on 2.5% agarose gels.

Please cite this article as: Rzechorzek, N.M., et al., Hypothermic Preconditioning of Human Cortical Neurons Requires Proteostatic Priming, EBioMedicine (2015), http://dx.doi.org/10.1016/j.ebiom.2015.04.004

### 2.7. Media and Supplements

Components were purchased from Invitrogen unless otherwise stated. hCN differentiation medium is described elsewhere (Bilican et al., 2014; Livesey et al., 2014). MiM comprised 90% salt–glucose–glycine solution (Bading et al., 1993) with 10% Minimal Eagle's Medium (+ Earle's, − Glutamine) and 0.5% Penicillin–Streptomycin. Tm (0.3 μg/ml, Sigma) and PERK inhibitor (GSK2606414, 500 nM, Calbiochem) were applied from the start of the temperature shift (they were present at these concentrations throughout the 24 h preconditioning phase and were then diluted by 50% upon the addition of $H_2O_2$ for the 24 h injury phase).

### 2.8. Statistical Analysis

Pairwise correlations were performed by two-tailed Pearson correlation. 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, random effects for coefficient by concentration or time. *N* denotes the number of individual cell lines and *n* describes the total number of independently differentiated batches of hCNs used as the statistical *n* for each experiment (the number of independent observations). The number ($n_{line}$) of batches derived from each cell line is then stated in parenthesis to show the contribution of each biological entity to the pooled total. Unless otherwise stated, data are presented as standardized point estimates (SPE) + standardized estimated standard error (SESE) after normalizing to control values (normothermic or untreated cells). Asterisks denote significance of the test statistic: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0005$.

## 3. Results

### 3.1. Human Neurons Exhibit an Archetypal Cold-Shock Response

To confirm the utility of hCNs to study cryobiological phenomena – and noting that hallmark cold-shock protein induction has not previously been reported in human neurons – we first tested their capacity to elicit this response at mild-to-moderate hypothermic temperatures (Danno et al., 1997; Fujita, 1999; Chip et al., 2011; Yenari and Han, 2012; Tong et al., 2013; Peretti et al., 2015). Within 24 h of cooling, RBM3 and CIRBP transcripts were both increased in hCNs at 28 °C and 32 °C relative to 37 °C, with a concomitant increase in the proportion of RBM3- and CIRBP-positive cells (Fig. 1A and B). RBM3 displayed a more acute and robust response to hypothermia than CIRBP, and both proteins exhibited a predominantly nuclear expression pattern (Fig. 1C and D). Biochemical analysis confirmed upregulation of these chaperones in response to cooling (Fig. 1E and F), and correlation of RBM3 and CIRBP transcript levels at each temperature was supportive of their co-regulation under hypothermic conditions (Fig. 1G). In addition, hypothermic hCNs exhibited a time- and temperature-dependent induction of immediate early transcripts c-Fos (FOS) and c-Jun (JUN) (Yenari and Han, 2012) (Fig. S1 available online). Together, these findings are consistent with a physiological cold-shock response in hCNs.

### 3.2. Cooling Induces ER Stress in Human Neurons with Activation of All UPR Branches

Since cold-shock can activate PERK (Hofman et al., 2012) and JUN (Fig. S1), both of which are components of the UPR, we postulated that cooling could trigger ER stress — the principal driver of UPR activity (Walter and Ron, 2011). We tested this using Tm as a positive control (Lin et al., 2007). BiP and 94 kDa glucose-regulated protein (GRP94) are key chaperones that respond to ER stress and regulate protein folding (Walter and Ron, 2011). We observed a temperature-dependent induction of BiP transcript, at an order of magnitude less than that produced by Tm (Fig. 2A), whilst GRP94 transcript was elevated at 24 h only in response to Tm treatment (Fig. S2A). BiP protein also showed an increasing trend with cooling (Fig. 2B and D). ER stress initiates a tripartite signalling cascade via 3 ER membrane-associated signal transducers (Walter and Ron, 2011). Once activated by autophosphorylation, the first of these transducers (inositol requiring enzyme 1α (Ire1α)) directs non-conventional splicing of its downstream target, x-box binding protein-1 (XBP1) (Lin et al., 2007). Spliced XBP1 (XBP1s) regulates transcription of several UPR target genes including chaperones and ER-associated degradation (ERAD) components which serve to alleviate ER stress (Hetz and Mollereau, 2014). We found a significant upregulation of total XBP1 and Ire1α (ERN1) transcripts at 28 and 32 °C relative to 37 °C (Figs. 2C and S2B). We also observed an increase in XBP1s transcript after cooling, again to a lesser extent than that produced by Tm (Fig. 2E and F). A second UPR pathway involving cleavage of ATF6 was induced at 28 °C (Hetz and Mollereau, 2014) (Fig. 2D). ATF6 activates transcription of ERAD genes and XBP1 (Hetz and Mollereau, 2014). Within the third pathway, total PERK expression decreased after 24 h cooling and eIF2α was inactive at this time point according to biochemical analysis of its phosphorylated form (Fig. S2C) (Rutkowski et al., 2006). There was however a significant increase in their downstream targets, activating transcription factor 4 (ATF4), DNA damage-inducible transcript 3 (DDIT3, or CHOP) and growth arrest and DNA damage 34 (GADD34) at hypothermic temperatures (Figs. 2G, H, and S2E). In summary, these findings demonstrate a mild ER stress in cooled hCNs, sufficient to activate all branches of the UPR. To our knowledge, this is the first description of a full UPR cascade in cells under hypothermic conditions.

### 3.3. Hypothermic Preconditioning of Human Neurons Requires UPR-Driven ER-Hormesis

Mild ER stress with UPR activation inhibits apoptosis (Rutkowski et al., 2006) and pre-conditions neurons to resist more stressful insults — an effect termed ER-hormesis (Mendes et al., 2009; Fouillet et al., 2012). To determine whether ER preconditioning contributes to hypothermic protection of hCNs we chemically modified the ER-UPR cascade during the pre-cooling phase, prior to inducing a standard oxidative stress protocol. PERK inhibitor was used to block the third UPR pathway, whilst Tm was added to induce ER stress (Lin et al., 2007). First we determined dose response curves for each compound in normothermic hCNs to identify concentrations that were non-toxic at baseline (Fig. S3). Multiplexed injury analysis (Materials and Methods) was then applied to hCNs exposed to increasing concentrations of $H_2O_2$, after pre-incubation at 28 °C or 37 °C, with or without PERK inhibitor or Tm. As expected, moderate hypothermia was protective of hCNs (Fig. 3A and B). However, PERK inhibition increased hCN injury at each temperature, abrogating the protective effect of cooling at all but the highest concentration of $H_2O_2$ (Fig. 3A). Tm exacerbated oxidative stress-mediated injury at 37 °C, but this effect was attenuated by pre-conditioning at 28 °C (Fig. 3B), thus directly demonstrating cooling-mediated ER-hormesis. These results confirm that full hypothermic neuroprotection requires an intact UPR to prime the ER against intensively toxic insults. The influence of cooling on this proteostatic cascade in hCNs is summarized in Fig. 3C.

## 4. Discussion

In acute injury, mildly enhancing the UPR can rescue neurons from programmed cell death and instigate adaptive ER preconditioning (Hetz and Mollereau, 2014). In hCNs, PERK activity was essential for hypothermic preconditioning against an oxidative challenge. Mild XBP1 splicing after 24 h of cooling, together with a substantial increase in unspliced XBP1 mRNA (Figs. 2E, F, and S2B) indicate that Ire1α and ATF6 were active within the cooling period (Hetz and Mollereau, 2014). Moreover, the increase in BiP transcript after 24 h is consistent

Please cite this article as: Rzechorzek, N.M., et al., Hypothermic Preconditioning of Human Cortical Neurons Requires Proteostatic Priming, EBioMedicine (2015), http://dx.doi.org/10.1016/j.ebiom.2015.04.004

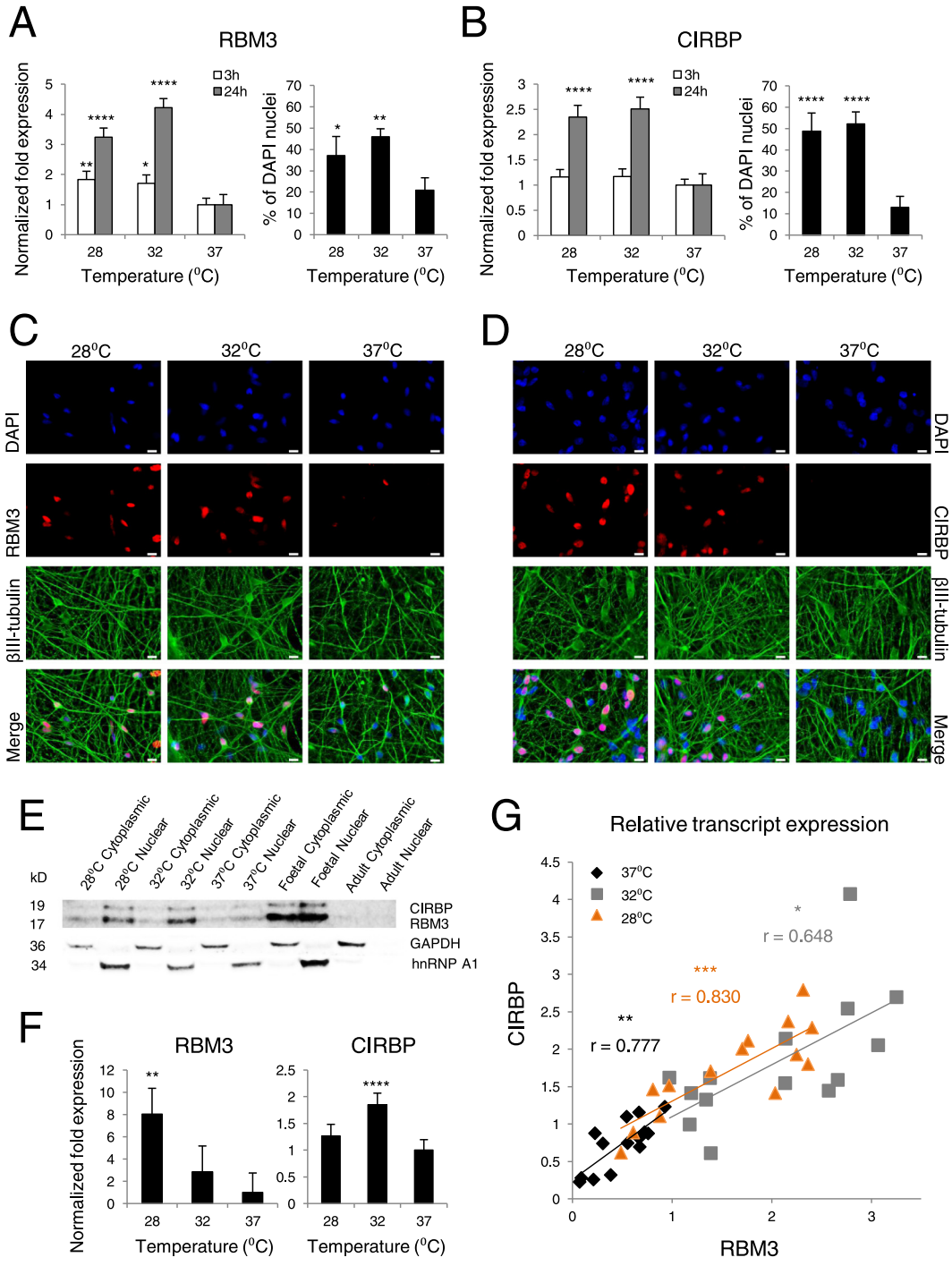


**Fig. 1.** Mild-to-moderate hypothermia elicits a cold-shock response in hCNs. (A) RBM3 transcripts (left, $N = 3$; $n = 14$; $n_{HESI} = 7$, $n_{HES2} = 4$, $n_{IPS1} = 3$) with significant increases after 3 h (32 °C $P = 0.011$, 28 °C $P = 0.003$) and 24 h ($P < 0.0005$). Cell counts for RBM3-positive nuclei (right, $N = 3$; $n = 6$; $n_{HES1} = 4$, $n_{HES2} = 1$, $n_{IPS1} = 1$, mean 37.2% (total 902 out of 2096 cells) at 28 °C, $P = 0.039$; mean 46.0% (total 1050 out of 2377 cells) at 32 °C, $P = 0.001$; mean 20.8% (total 531 out 2062 cells) at 37 °C). For each independent hCN batch and temperature condition, a minimum of 10 fields of view at 63× were counted (pooled from two replicate coverslips). Counts are presented as mean % + standard error of the mean (SEM). (B) CIRBP transcripts (left, $N = 3$; $n = 14$; $n_{HES1} = 7$, $n_{HES2} = 4$, $n_{IPS1} = 3$, $P < 0.0005$ at 24 h) and cell counts (right, $N = 3$; $n = 6$; $n_{HES1} = 4$, $n_{HES2} = 1$, $n_{IPS1} = 1$, mean 48.9% (total 719 out of 1416 cells) at 28 °C; mean 52.3% (total 985 out of 1882 cells) at 32 °C; mean 13.1% (total 252 out of 1850 cells) at 37 °C, $P < 0.0005$). (C and D) Fluorescent micrographs of hCNs co-stained for neuronal and cold-shock markers, scale bar = 10 μm. (E) Subcellular expression of RBM3 and CIRBP by immunoblot, alongside human foetal and adult cortex. GAPDH and hnRNP A1 are loading controls. The stability of hnRNP A1 expression under mild hypothermic conditions in human cells has reported elsewhere (Danno et al., 1997). (F) Quantitative Western analysis of RBM3 and CIRBP ($N = 3$; $n \geq 4$; $n_{HES1} \geq 2$; $n_{HES2} = 1$, $n_{IPS1} = 1$). RBM3 expression was greatest at 28 °C ($P = 0.002$); CIRBP expression peaked at 32 °C ($P < 0.0005$). (G) Correlation of RBM3 and CIRBP transcripts (37 °C $P = 0.001$, 32 °C $P = 0.012$, 28 °C $P < 0.0005$). See also Fig. S1.

with prior activation of ATF6 and splicing of XBP1 (Hetz and Mollereau, 2014). Enhanced injury with PERK inhibition at 37 °C may reflect a constitutive proteostatic function of the UPR in long-term culture — potentially through buffering oxidative processes (Cullinan et al., 2003). It might also explain why hypothermic induction of some PERK branch-specific components was not observed; eIF2α phosphorylation does occur under deep hypothermic conditions (10 °C) and contributes to the global suppression of protein translation in mammalian cell lines (Roobol et al., 2009; Hofman et al., 2012), but it may be undetectable biochemically in the context of mild ER stress (Rutkowski et al., 2006).

Please cite this article as: Rzechorzek, N.M., et al., Hypothermic Preconditioning of Human Cortical Neurons Requires Proteostatic Priming, EBioMedicine (2015), http://dx.doi.org/10.1016/j.ebiom.2015.04.004

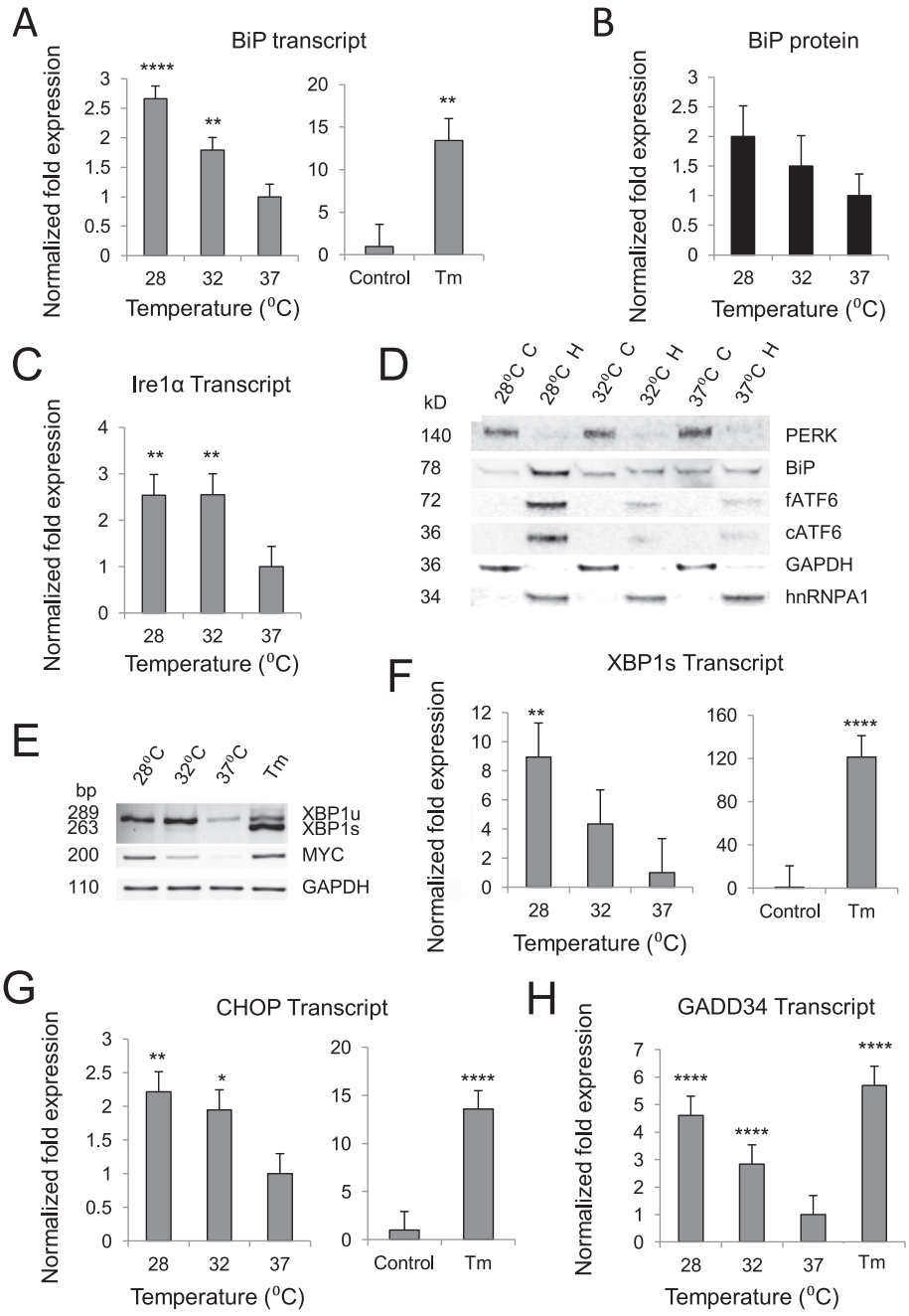


**Fig. 2.** Hypothermia induces mild ER stress in hCNs with full activation of the UPR. (A) BiP transcripts after cooling (left, $N = 3$; $n = 22$; $n_{HES1} = 11$, $n_{HES2} = 6$, $n_{IPS1} = 5$; 32 °C, $P = 0.006$; 28 °C, $P < 0.0005$) or Tm treatment (right, $N = 3$; $n = 8$; $n_{HES1} = 4$, $n_{HES2} = 2$, $n_{IPS1} = 2$, $P = 0.004$). (B) Total BiP protein expression ($N = 2$; $n = 3$; $n_{HES1} = 2$; $n_{HES2} = 1$; 28 °C, $P = 0.051$). (C) IRE1α transcripts ($P < 0.01$, $N = 3$; $n = 14$; $n_{HES1} = 7$, $n_{HES2} = 4$, $n_{IPS1} = 3$). (D) Immunoblots of fractionated lysates (C = cytoplasmic, H = high-detergent) from hCNs. Note increased BiP, full length (fATF6), and cleaved (cATF6) sitting in the high detergent fraction at 28 °C. This is consistent with nuclear translocation of cATF6 and upregulation of its target transcripts (BiP and unspliced XBP1 — as shown in Figs. 2A, E, and S2B). (E) Gel images of RT-PCR products. Faint bands at 263 bp confirm mild splicing of XBP1 in hypothermic hCNs relative to negative (37 °C) and positive (Tm-treated) controls. GAPDH = reference target. (F) qRT-PCR analysis of XBP1s transcript after cooling (left, $N = 3$; $n = 22$; $n_{HES1} = 11$, $n_{HES2} = 6$, $n_{IPS1} = 5$; 28 °C, $P = 0.003$) or Tm-treatment (right, $N = 3$; $n = 8$; $n_{HES1} = 4$, $n_{HES2} = 2$, $n_{IPS1} = 2$, $P < 0.0005$). (G) CHOP transcripts after cooling (left, $N = 3$; $n = 22$; $n_{HES1} = 11$, $n_{HES2} = 6$, $n_{IPS1} = 5$; 32 °C, $P = 0.011$; 28 °C, $P = 0.001$) or Tm treatment (right, $N = 3$; $n = 8$; $n_{HES1} = 4$, $n_{HES2} = 2$, $n_{IPS1} = 2$, $P < 0.0005$). (H) GADD34 transcripts ($N = 3$; $n = 7$; $n_{HES1} = 3$, $n_{HES2} = 2$, $n_{IPS1} = 2$, 32 °C, $P < 0.0005$; 28 °C, $P < 0.0005$; Tm, $P < 0.0005$). See also Fig. S2.

Equally, since PERK-mediated translational repression is subject to homeostatic autoregulation by phosphatases (Lin et al., 2007), a resolving influence of cooling on eIF2α activation is supported by hypothermic induction of GADD34 in hCNs (Figs. 2H and 3C) (Ma and Hendershot, 2003). In this respect, rather than signifying the duration limit of protective cooling, the CHOP induction observed would be a pre-requisite for GADD34-mediated negative feedback on eIF2α (Halterman et al., 2010). Accordingly, others have highlighted the protective role of CHOP in neuronal systems (Chen et al., 2012; Engel et al., 2013). The fact that 24 h cooling did not increase levels of the pro-apoptotic marker Bax (Fig. S2E) is in line with previous studies (Yenari et al., 2002) and further supports our conclusion that this duration and depth of hypothermia produced an adaptive UPR. Potentially, cold-shock proteins may complement this cascade by relieving translational repression of critical mRNAs (Peretti et al., 2015), and limiting CHOP-mediated apoptosis (Saito et al., 2010).

During an adaptive stress response UPR branches undergo complex homeostatic self-regulation (Fig. 3C). Thus the cross-sectional UPR

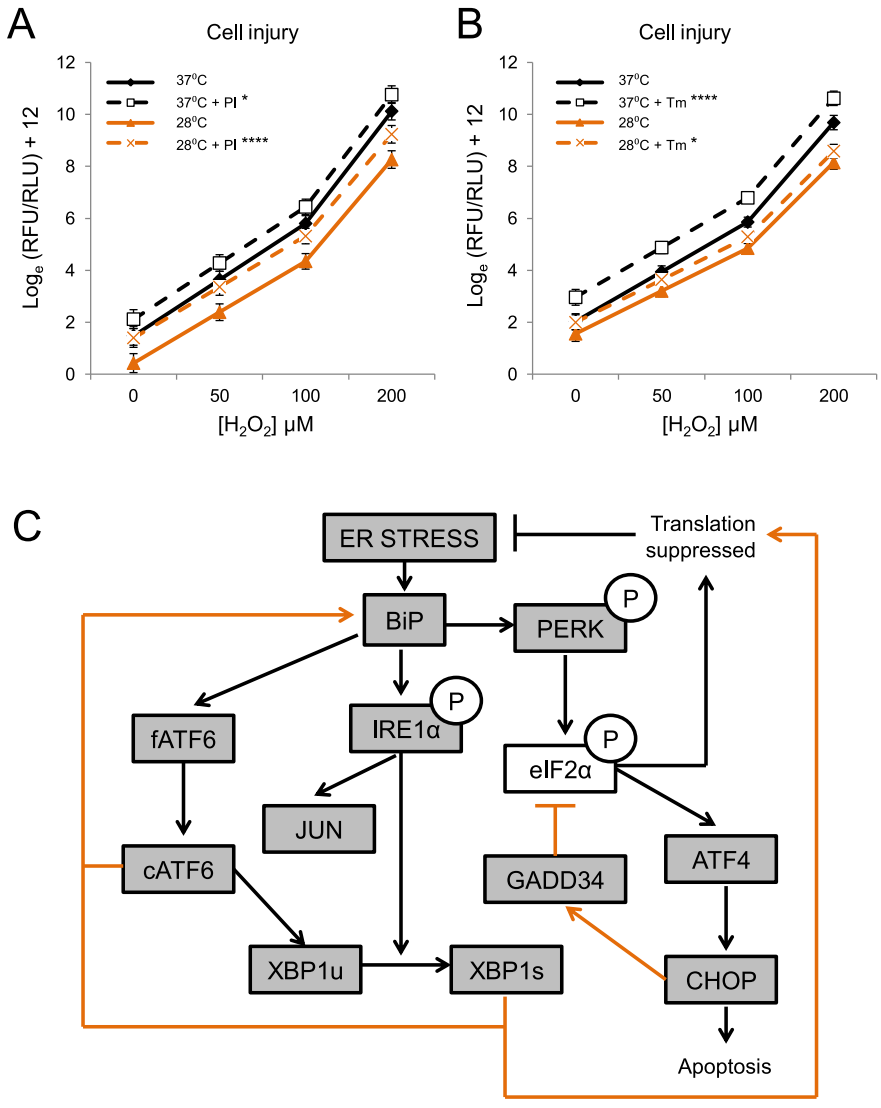


**Fig. 3.** Hypothermic UPR-mediated preconditioning of the ER protects hCNs against oxidative stress. (A) Oxidative stress-mediated injury is increased by PERK inhibitor (PI) ($N = 3$; $n = 3$; $n_{HES1} = 1$, $n_{HES2} = 1$, $n_{IPS1} = 1$; 28 °C, $P < 0.0005$; 37 °C, $P = 0.016$). Hypothermia remained protective only at 200 µM $H_2O_2$ ($P = 0.023$). (B) $H_2O_2$ injury is increased by Tm ($N = 3$; $n = 7$; $n_{HES1} = 5$, $n_{HES2} = 1$, $n_{IPS1} = 1$; 28 °C, $P < 0.05$; 37 °C, $P < 0.0005$). Hypothermia reduced the toxic effect of Tm ($P = 0.062$). Note that log scale was required to accommodate magnitude of injury changes across $H_2O_2$ concentrations in Fig. 3A and B. (C) Proposed mechanism of ER-hormesis in cooled hCNs. UPR pathways are depicted, together with known regulatory feedback pathways. Filled boxes denote components induced at transcript and/or protein level in hCNs with cooling. Phospho-IRE1α and phospho-PERK were not assessed. Orange arrows indicate hormetic elements that resolve the UPR and increase ER resilience to stress. See also Fig. S3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

profile captured in our hCN system cannot convey the dynamic nature of these pathways. However, our analysis at 24 h intimately links UPR activation to neuronal preconditioning, since this was the point at which $H_2O_2$ was applied, and it further indicates co-ordination of ER-hormesis with cold-shock protein induction. The lack of GRP94 induction after 24 h cooling may reflect the half-life of its transcript or the selective nature of this chaperone, whose client list is smaller than that of BiP — in particular, GRP94 is not induced at high temperatures (Marzec et al., 2012). Furthermore, prolonged ER stress leads to sequential activation then deactivation of Ire1α, ATF6 and PERK pathways respectively — which might explain the bias of UPR components towards the PERK arm at 24 h (Tabas and Ron, 2011). Nevertheless, our transcript analysis revealed distinct patterns of UPR responses resulting from two different stresses; whilst BiP, GRP94, XBP1s and CHOP dramatically increased with Tm, ATF4 and GADD34 induction were comparable between Tm and cooling. Therefore, in contrast to models described elsewhere (Rutkowski et al., 2006), the negative regulation of eIF2α appears to take precedence over unloading the ER in cooled human neurons. This relief of translational repression may confer tolerance to a prolonged hypothermic state (Peretti et al., 2015; Moreno et al., 2013).

In the clinic, 'preconditioning' is typically ascribed to a transient mild stress followed by a recovery interval (Nishio et al., 2000; Stetler et al., 2014). Here we have applied this term in its broadest sense — i.e., a subtoxic cellular stress that can lead to a protective state (Stetler et al., 2014) in order to account for the proteostatic priming observed during our pre-incubation phase of cooling. This definition circumvents the need for a re-warming phase which would confound analysis of oxidative injury by inducing relative hyperthermic and hypoxic stresses (Liu et al., 1994; Lleonart, 2010; Chip et al., 2011; Neutelings et al., 2013). Hypothermic preconditioning may reconcile conflicting data describing UPR modulation in neuronal health; (1) that ER stress can elicit UPR-mediated hormesis (Mendes et al., 2009; Fouillet et al., 2012), (2) that circumventing UPR-mediated translational repression promotes long-term survival (Moreno et al., 2013), and (3) that inhibiting eIF2α phosphatases resolves ER stress (Kiskinis et al., 2014). This highlights the importance of fine-tuning the entire network, rather than adjusting a single pathway or component — such a combinatorial approach has

been proposed for amyotrophic lateral sclerosis (Kiskinis et al., 2014). Whilst hypothermic preconditioning originates from the acute injury setting, impaired stress responses underlie several neurodegenerative disorders (Hetz and Mollereau, 2014) and preconditioning in general is a proposed target (Stetler et al., 2014). Cooling has recently demonstrated some benefit in an in vivo model of spastic paraplegia (Baxter et al., 2014) and Peretti et al. (2015) observed that neurodegenerative synaptic loss could be partially rescued through early cooling-induced enhancement of RBM3 expression. Conceivably, this temporal dependency might relate to hypothermia-mediated proteostatic priming, elicited prior to the build-up of a significant protein aggregate load. Whether the hypothermic preconditioning described here is linked to a cytoprotective mechanism that is synergistic with the preservation of synaptic plasticity is worthy of further investigation (Peretti et al., 2015). Ultimately, disease stage and neuronal subtype would determine whether enhanced or prophylactic preconditioning could be useful in the context of neurodegeneration (Saxena et al., 2009).

In response to cooling, hCNs displayed all the hallmarks of an adaptive, preconditioning UPR response: mild ER stress and activation of all 3 ER-stress transducers, a low level of CHOP induction that was insufficient to effect apoptosis, absence of detectable levels of phospho-eIF2α, and residual expression of key ER chaperones (Rutkowski et al., 2006; Tabas and Ron, 2011). The reversibility of these effects and the period over which they would remain protective is currently unknown and is part of ongoing work. Since our cooling paradigm can be used to titrate UPR activation, it represents a simple method to address subtle but important effects dictating adaptive versus maladaptive outcomes of this cascade in any cell type. We propose that ER-hormesis is an important outcome of the cold-shock response that protects human neurons from both ER and oxidative stress. This 'cross-tolerance' effect (Rutkowski et al., 2006; Stetler et al., 2014) places exponential value on the molecular neurobiology of cooling, which may deliver novel therapeutic targets for an unmet need.

## Author Contributions

N.M.R. conceived the research, conducted the literature search, designed and performed the experiments, compiled the figures and wrote the manuscript. P.C. analysed the data. N.M.R., P.C., R.P. and B.T.S. interpreted the results. S.C. supervised the project. All authors contributed to the discussion and the final draft of the paper.

## Role of the Funding Sources

Funding sources did not have any involvement in the study design; the collection, analysis and interpretation of data; writing of the report; or the decision to submit the article for publication. The authors declare no conflicts of interest.

## Acknowledgements

This research was funded by a Wellcome Trust Integrated Training Fellowship for Veterinarians (096409/Z/11/Z to N.M.R.), an Anne Rowling Senior Clinical Research Fellowship (to P.C.), an Anne Rowling Fellowship and Wellcome Trust Intermediate Clinical Fellowship (101149/Z/13/Z to R.P.) and the Euan MacDonald centre and the Fidelity Foundation (to S.C.). We thank the following at the University of Edinburgh: MRC Edinburgh Brain & Tissue Bank and Nusrat Khan, Peault lab, MRC Centre for Regenerative Medicine (human post-mortem brain tissue); David Hay lab, MRC centre for Regenerative Medicine (Glomax luminometer); Steve West, Wellcome Trust Centre for Cell Biology (MYC primers).

## Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2015.04.004.

## References

Abdel Malek, M.A., Jagannathan, S., Malek, E., Sayed, D.M., Elgammal, S.A., Abd El-Azeem, H.G., Thabet, N.M., Driscoll, J.J., 2015. Molecular chaperone GRP78 enhances aggresome delivery to autophagosomes to promote drug resistance in multiple myeloma. Oncotarget 6, 3098–3110.

Bading, H., Ginty, D.D., Greenberg, M.E., 1993. Regulation of gene expression in hippocampal neurons by distinct calcium signalling pathways. Science 260, 181–186.

Baxter, S.L., Allard, D.E., Crowl, C., Sherwood, N.T., 2014. Cold temperature improves mobility and survival in *Drosophila* models of autosomal-dominant hereditary spastic paraplegia (AD-HSP). Dis. Model. Mech. 7, 1005–1012.

Bilican, B., Livesey, M.R., Haghi, G., Qiu, J., Burr, K., Siller, R., Hardingham, G.E., Wyllie, D.J., Chandran, S., 2014. Physiological normoxia and absence of EGF is required for the long-term propagation of anterior neural precursors from human pluripotent cells. PLoS One 9, e85932.

Cerezo, D., Cánovas, M., García-Peñarrubia, P., Martín-Orozco, E., 2015. Collateral sensitivity to cold stress and differential BCL-2 family expression in new daunomycin-resistant lymphoblastoid cell lines. Exp. Cell Res. 331, 11–20.

Chen, C.M., Wu, C.T., Chiang, C.K., Liao, B.W., Liu, S.H., 2012. C/EBP homologous protein (CHOP) deficiency aggravates hippocampal cell apoptosis and impairs memory performance. PLoS One 7, e40801.

Chip, S., Zelmer, A., Ogunshola, O.O., Felderhoff-Mueser, U., Nitsch, C., Bührer, C., Wellmann, S., 2011. The RNA binding protein RBM3 is involved in hypothermia induced neuroprotection. Neurobiol. Dis. 43, 388–396.

Choi, H.A., Badjatia, N., Mayer, S.A., 2012. Hypothermia for acute brain injury — mechanisms and practical aspects. Nat. Rev. Neurol. 8, 214–222.

Cullinan, S.B., Zhang, D., Hannink, M., Arvisais, E., Kaufman, R.J., Diehl, J.A., 2003. Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. Mol. Cell. Biol. 23, 7198–7209.

Danno, S., Nishiyama, H., Higashitsuji, H., Yokoi, H., Xue, J.H., Itoh, K., Matsuda, T., Fujita, J., 1997. Increased transcript level of RBM3, a member of the glycine-rich RNA-binding protein family, in human cells in response to cold stress. Biochem. Biophys. Res. Commun. 236, 804–807.

Dirnagl, U., Simon, R.P., Hallenbeck, J.M., 2003. Ischemic tolerance and endogenous neuroprotection. Trends Neurosci. 26, 248–254.

Engel, T., Sanz-Rodgriguez, A., Jimenez-Mateos, E.M., Concannon, C.G., Jimenez-Pacheco, A., Moran, C., Mesuret, G., Petit, E., Delanty, N., Farrell, M.A., O'Brien, D.F., Prehn, J.H., Lucas, J.J., Henshall, D.C., 2013. CHOP regulates the p53-MDM2 axis and is required for neuronal survival after seizures. Brain 136, 577–592.

Fouillet, A., Levet, C., Virgone, A., Robin, M., Dourlen, P., Rieusset, J., Belaidi, E., Ovize, M., Touret, M., Nataf, S., et al., 2012. ER stress inhibits neuronal death by promoting autophagy. Autophagy 8, 915–926.

Fujita, J., 1999. Cold shock response in mammalian cells. J. Mol. Microbiol. Biotechnol. 1, 243–255.

Gupta, K., Hardingham, G.E., Chandran, S., 2013. NMDA receptor-dependent glutamate excitotoxicity in human embryonic stem cell-derived neurons. Neurosci. Lett. 543, 95–100.

Halterman, M.W., Gill, M., DeJesus, C., Ogihara, M., Schor, N.F., Federoff, H.J., 2010. The endoplasmic reticulum stress response factor CHOP-10 protects against hypoxia-induced neuronal death. J. Biol. Chem. 285, 21329–21340.

Hetz, C., Mollereau, B., 2014. Disturbance of endoplasmic reticulum proteostasis in neurodegenerative diseases. Nat. Rev. Neurosci. 15, 233–249.

Hofman, S., Cherkasova, V., Bankhead, P., Bukau, B., Stoecklin, G., 2012. Translation suppression promotes stress granule formation and cell survival in response to cold shock. Mol. Biol. Cell 23, 3786–3800.

Kim, I., Xu, W., Reed, J.C., 2008. Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. Nat. Rev. Drug Discov. 7, 1013–1030.

Kiskinis, E., Sandoe, J., Williams, L.A., Boulting, G.L., Moccia, R., Wainger, B.J., Han, S., Peng, T., Thams, S., Mikkilineni, S., et al., 2014. Pathways disrupted in human ALS motor neurons identified through genetic correction of mutant SOD1. Cell Stem Cell 14, 781–795.

Lin, J.H., Li, H., Yasumura, D., Cohen, H.R., Zhang, C., Panning, B., Shokat, K.M., Lavail, M.M., Walter, P., 2007. IRE1 signalling affects cell fate during the unfolded protein response. Science 318, 944–949.

Liu, A.Y., Bian, H., Huang, L.E., Lee, Y.K., 1994. Transient cold shock induces the heat shock response upon recovery at 37 degrees C in human cells. J. Biol. Chem. 269, 14768–14775.

Livesey, M.R., Bilican, B., Qiu, J., Rzechorzek, N.M., Haghi, G., Burr, K., Hardingham, G.E., Chandran, S., Wyllie, D.J., 2014. Maturation of AMPAR composition and the $GABA_AR$ reversal potential in hPSC-derived cortical neurons. J. Neurosci. 34, 4070–4075.

Lleonart, M.E., 2010. A new generation of proto-oncogenes: cold-inducible RNA binding proteins. Biochim. Biophys. Acta 1805, 43–52.

Ma, Y., Hendershot, L.M., 2003. Delineation of a negative feedback regulatory loop that controls protein translation during endoplasmic reticulum stress. J. Biol. Chem. 278, 34864–34873.

Marzec, M., Eletto, D., Argon, Y., 2012. GRP94: an HSP90-like protein specialized for protein folding and quality control in the endoplasmic reticulum. Biochim. Biophys. Acta 1823, 774–787.

Mendes, C.S., Levet, C., Chatelain, G., Dourlen, P., Fouillet, A., Dichtel-Danjoy, M.L., Gambis, A., Ryoo, H.D., Steller, H., Mollereau, B., 2009. ER stress protects from retinal degeneration. EMBO J. 28, 1296–1307.

Miller, J.A., Ding, S.-L., Sunkin, S.M., Smith, K.A., Ng, L., Szafer, A., Ebbert, A., Riley, Z.L., Royall, J.J., Aiona, K., et al., 2014. Transcriptional landscape of the prenatal human brain. Nature 508, 199–206 (http://dx.doi.org/10.1038/nature13185 and Website: ©2014 Allen Institute for Brain Science. BrainSpan Atlas of the Developing Human Brain. Available from: http://brainspan.org/).

Please cite this article as: Rzechorzek, N.M., et al., Hypothermic Preconditioning of Human Cortical Neurons Requires Proteostatic Priming, EBioMedicine (2015), http://dx.doi.org/10.1016/j.ebiom.2015.04.004

Moreno, J.A., Halliday, M., Molloy, C., Radford, H., Verity, N., Axten, J.M., Ortori, C.A., Willis, A.E., Fischer, P.M., Barrett, D.A., et al., 2013. Oral treatment targeting the unfolded protein response prevents neurodegeneration and clinical disease in prion-infected mice. Sci. Transl. Med. 5, 206ra138.

Neutelings, T., Lambert, C.A., Nusgens, B.V., Colige, A.C., 2013. Effects of mild cold shock (25 °C) followed by warming up at 37 °C on the cellular stress response. PLoS One 8, e69687.

Nishio, S., Yunoki, M., Chen, Z.-F., Anzivino, M.J., Lee, K.S., 2000. Ischaemic tolerance in the rat neocortex following hypothermic preconditioning. J. Neurosurg. 93, 845–851.

Peretti, D., Bastide, A., Radford, H., Verity, N., Molloy, C., Martin, M.G., Moreno, J.A., Steinert, J.R., Smith, T., Dinsdale, D., et al., 2015. RBM3 mediates structural plasticity and protective effects of cooling in neurodegeneration. Nature http://dx.doi.org/10.1038/nature14142.

Roobol, A., Carden, M.J., Newsam, R.J., Smales, C.M., 2009. Biochemical insights into the mechanisms central to the response of mammalian cells to cold stress and subsequent rewarming. FEBS J. 276, 286–302.

Rutkowski, D.T., Arnold, S.M., Miller, C.N., Wu, J., Li, J., Gunnison, K.M., Mori, K., Sadighi Akha, A.A., Raden, D., Kaufman, R.J., 2006. Adaptation to ER stress is mediated by different stabilities of pro-survival and pro-apoptotic mRNAs and proteins. PLoS Biol. 4, e374.

Saito, K., Fukuda, N., Matsumoto, T., Iribe, Y., Tsunemi, A., Kazama, T., Yoshida-Noro, C., Hayashi, N., 2010. Moderate low temperature preserves the stemness of neural stem cells and suppresses apoptosis of the cells via activation of the cold-inducible RNA binding protein. Brain Res. 1358, 20–29.

Saraste, J., Palade, G.E., Farquhar, M.G., 1986. Temperature-sensitive steps in the transport of secretory proteins through the Golgi complex in exocrine pancreatic cells. Proc. Natl. Acad. Sci. U. S. A. 83, 6425–6429.

Saxena, S., Cabuy, E., Caroni, P., 2009. A role for motoneuron subtype-selective ER stress in disease manifestations of FALS mice. Nat. Neurosci. 12, 627–636.

Stetler, R.A., Leak, R.K., Gan, Y., Li, P., Zhang, F., Hu, X., Jing, Z., Chen, J., Zigmond, M.J., Gao, Y., 2014. Preconditioning provides neuroprotection in models of CNS disease: paradigms and clinical significance. Prog. Neurobiol. 114, 58–83.

Tabas, I., Ron, D., 2011. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. Nat. Cell Biol. 13, 184–190.

Tong, G., Endersfelder, S., Rosenthal, L.M., Wollersheim, S., Sauer, I.M., Bührer, C., Berger, F., Schmitt, K.R., 2013. Effects of moderate and deep hypothermia on RNA-binding proteins RBM3 and CIRP expressions in murine hippocampal brain slices. Brain Res. 1504, 74–84.

van der Harg, J.M., Nölle, A., Zwart, R., Boerema, A.S., van Haastert, E.S., Strijkstra, A.M., Hoozemans, J.J., Scheper, W., 2014. The unfolded protein response mediates reversible tau phosphorylation induced by metabolic stress. Cell Death Dis. 5, e1393.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3 (RESEARCH0034).

Walter, P., Ron, D., 2011. The unfolded protein response: from stress pathway to homeostatic regulation. Science 334, 1081–1086.

Yenari, M.A., Han, H.S., 2012. Neuroprotective mechanisms of hypothermia in brain ischaemia. Nat. Rev. Neurosci. 13, 267–278.

Yenari, M.A., Iwayama, S., Cheng, D., Sun, G.H., Fujimura, M., Morita-Fujimura, Y., Chan, P.H., Steinberg, G.K., 2002. Mild hypothermia attenuates cytochrome c release but does not alter Bcl-2 expression or caspase activation after experimental stroke. J. Cereb. Blood Flow Metab. 22, 29–38.

Yuan, H.B., Huang, Y., Zheng, S., Zuo, Z., 2004. Hypothermic preconditioning increases survival of purkinje neurons in rat cerebellar slices after an in vitro simulated ischemia. Anesthesiol. 100, 331–337.

Please cite this article as: Rzechorzek, N.M., et al., Hypothermic Preconditioning of Human Cortical Neurons Requires Proteostatic Priming, EBioMedicine (2015), http://dx.doi.org/10.1016/j.ebiom.2015.04.004

Brief Communications

# Maturation of AMPAR Composition and the $GABA_AR$ Reversal Potential in hPSC-Derived Cortical Neurons

**Matthew R. Livesey,[1,2,3]* Bilada Bilican,[2,3,4]* Jing Qiu,[1] Nina M. Rzechorzek,[2,3,4] Ghazal Haghi,[1,2,3] Karen Burr,[2,3,4] Giles E. Hardingham,[1] Siddharthan Chandran,[2,3,4] and David J.A. Wyllie[1]**

[1]Centre for Integrative Physiology, University of Edinburgh, Edinburgh EH8 9XD, United Kingdom; and [2]Euan MacDonald Centre for MND Research, [3]Centre for Clinical Brain Sciences, and [4]MRC Centre for Regenerative Medicine, University of Edinburgh, Edinburgh EH16 4SB, United Kingdom

**Rodent-based studies have shown that neurons undergo major developmental changes to ion channel expression and ionic gradients that determine their excitation-inhibition balance. Neurons derived from human pluripotent stem cells theoretically offer the potential to study classical developmental processes in a human-relevant system, although this is currently not well explored. Here, we show that excitatory cortical-patterned neurons derived from multiple human pluripotent stem cell lines exhibit native-like maturation changes in AMPAR composition such that there is an increase in the expression of GluA2(R) subunits. Moreover, we observe a dynamic shift in intracellular $Cl^-$ levels, which determines the reversal potential of $GABA_AR$-mediated currents and is influenced by neurotrophic factors. The shift is concomitant with changes in KCC2 and NKCC1 expression. Because some human diseases are thought to involve perturbations to AMPAR GluA2 content and others in the chloride reversal potential, human stem-cell-derived neurons represent a valuable tool for studying these fundamental properties.**

*Key words:* **GABA; glutamate; neurotransmitter; patch clamp; qRT-PCR; stem cells**

## Introduction

The ability to generate *in vitro* cortical neuronal populations from human pluripotent stem cells (hPSCs) provides an experimental resource for the investigation of human cortical physiology and disease (Hansen et al., 2011). hPSCs have been used to derive human excitatory cortical neurons (hECNs) *in vitro* that appear to recapitulate aspects of *in vivo* cortical development (Johnson et al., 2007; Zeng et al., 2010; Shi et al., 2012; Espuny-Camacho et al., 2013). We have developed a protocol that enables the generation of a propagatable pool of anterior neural precursor cells (aNPCs) from human embryonic stem (ES) cell and induced pluripotent stem cell lines (Bilican et al., 2014). aNPCs can be efficiently differentiated as a monolayer of excitatory cortical neurons that are representative of neuronal populations present within native upper and lower cortical layers. Our recent study (Bilican et al., 2014) demonstrated the ability of hECNs to fire action potentials, express voltage-gated and ligand-gated ion channels, and exhibit putative synaptic activity; however, there is a need to identify specific physiologically postnatal/adult properties of such neurons to aid *in vitro* human studies of neurodevelopmental physiology and modeling of neurodevelopmental and adult diseases (Yang et al., 2011; Sandoe and Eggan, 2013).

Here, we demonstrate that hECNs differentiated from aNPCs exhibit native-like maturation shifts in AMPAR subunit composition and in the relative expression of the $Na^+$-$K^+$-$Cl^-$ cotransporter-1 (NKCC1) and $K^+$-$Cl^-$ cotransporter-2 (KCC2) subunits to reduce intracellular chloride concentration ($[Cl^-]_{INT}$), which determine the nature of GABA receptor type-A ($GABA_AR$) function (Blaesse et al., 2009). Beyond a platform for the study of maturation, these hECNs are of interest for studies of human diseases in which AMPAR and $[Cl^-]_{INT}$ regulation are disrupted (Blaesse et al., 2009; Wright and Vissel, 2012).

## Materials and Methods

*In vitro hECN preparation.* A detailed description of the derivation of hECNs, including immunohistochemistry protocols, can be found in Bilican et al. (2014) and is summarized in Figure 1*A*. The H9 (female), hereafter referred to as ES1 (WiCell) and SHEF4 (male), referred to as ES2 (UK Stem Cell Bank) human ES cell lines were obtained under full ethical/Institutional Review Board approval of the University of Edinburgh. For IPS1 (male M337V mutant line 1; Bilican et al., 2012) and IPS2 (female in-house control line) human IPS cell lines, written informed consent was obtained from each participant.

*hECN culture media supplements.* Forskolin (10 $\mu$M) was present during weeks 1–3 of differentiation. For AMPAR characterization, medium was supplemented with BDNF and GDNF (both 5 ng/ml) from week 3 onward. For experiments to determine $[Cl^-]_{INT}$, BDNF and GDNF were omitted unless otherwise stated. When cultures were maintained beyond 5 weeks, the culture medium was supplemented with IGF1 (10 ng/ml) from week 4 of differentiation.

Received Dec. 27, 2013; revised Feb. 3, 2014; accepted Feb. 11, 2014.

Author contributions: M.R.L., B.B., J.Q., N.M.R., G.E.H., S.C., and D.J.A.W. designed research; M.R.L., B.B., J.Q., N.M.R., G.H., and K.B. performed research; M.R.L., B.B., J.Q., N.M.R., G.E.H., S.C., and D.J.A.W. analyzed data; M.R.L., B.B., N.M.R., G.E.H., S.C., and D.J.A.W. wrote the paper.

This research was funded by The Wellcome Trust (Grant 092742/Z/10/Z to D.J.A.W., S.C. and G.E.H.), the Medical Research Council (Senior Nonclinical Research Fellowship to G.E.H.), a Wellcome Trust Integrated Training Fellowship for Veterinarians (096409/Z/11/Z to N.M.R.), and the Euan MacDonald Centre and the Fidelity Foundation (to S.C.).

*M.R.L. and B.B. contributed equally to this work.

This article is freely available online through the *J Neurosci* Author Open Choice option.

Correspondence should be addressed to either Siddharthan Chandran, Centre for Clinical Brain Sciences, University of Edinburgh, Edinburgh EH16 4SB, United Kingdom, E-mail: siddharthan.chandran@ed.ac.uk; or David J.A. Wyllie, Centre for Integrative Physiology, Hugh Robson Building, George Square, University of Edinburgh, Edinburgh EH8 9XD, United Kingdom, E-mail: dwyllie1@staffmail.ed.ac.uk.

DOI:10.1523/JNEUROSCI.5410-13.2014

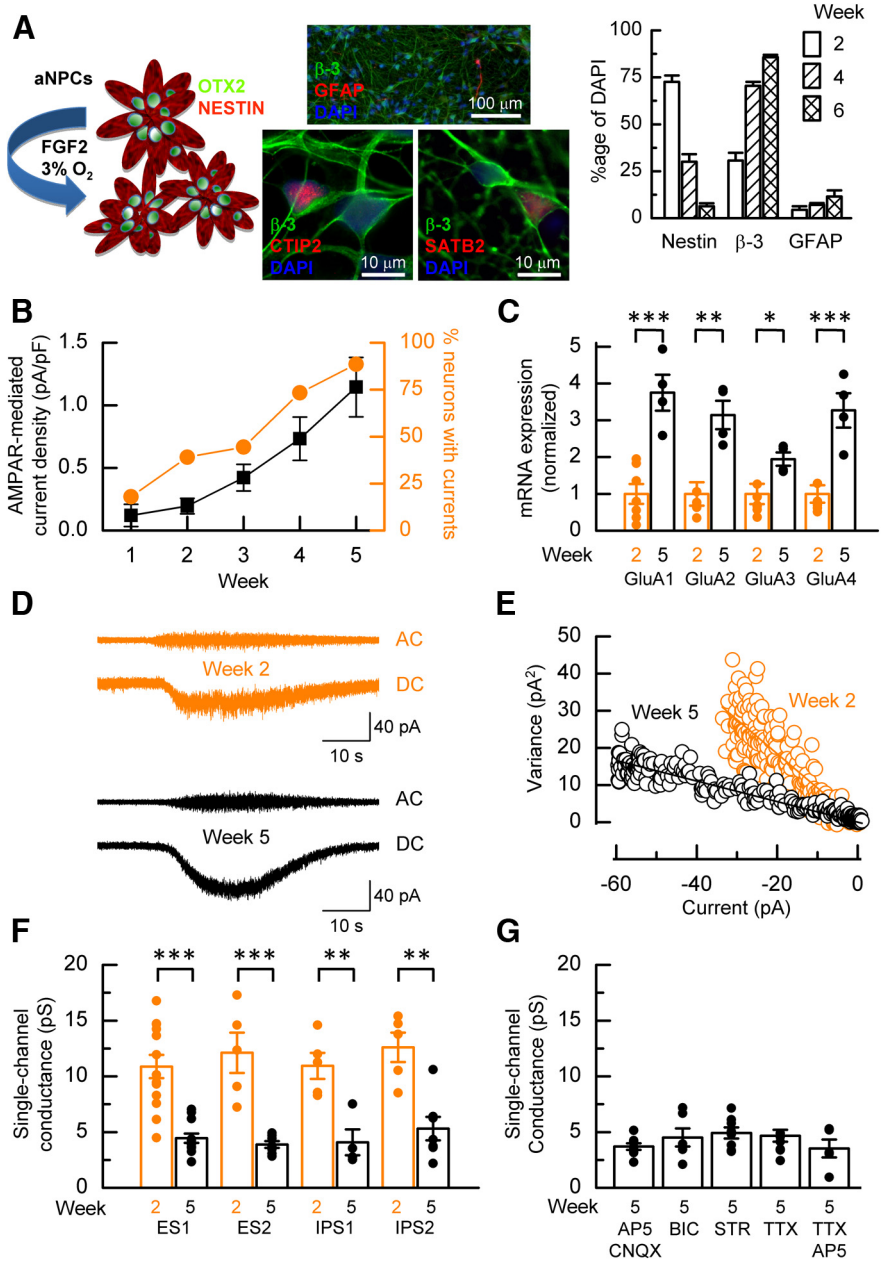


**Figure 1.** AMPAR maturation. ***A***, Schematic representation of hECNs derived from OTX2$^+$ and Nestin$^+$ aNPCs. Neuronal differentiation is initiated by the removal of FGF2 and cultures are maintained in a 3% $O_2$ atmosphere (Bilican et al., 2014). Immunocytochemistry for neuronal ($\beta$3-tubulin) astrocyte (glial fibrillary acidic protein; GFAP) and aNPC (Nestin) markers indicates increasing neuronal differentiation with time in culture and sparse astrocytes throughout. Bar graph displays cell marker counts as a percentage of DAPI-stained nuclei. hECNs are also immunopositive for deep and superficial cortical layer markers CTIP2 and SATB2, respectively. ***B***, Weekly percentage response (orange) to AMPA and the mean AMPA-mediated current density (black). $n = 22–35$, $N > 3$. ***C***, Mean normalized mRNA fold expression data for AMPAR subunits GluA1-GluA4 in week 2 and 5 cells as assessed by qRT-PCR. $n = 4–6$, $N = 3$, unpaired *t* test. Relative expression of each subunit of one of the week 2 samples is set to 1 after normalizing to $\beta$-actin. ***D***, Example nonstationary fluctuation analysis of AMPAR-mediated whole-cell currents from a week 2 (orange) and 5 (black) neurons. ***E***, Plot of the relationship between the variance of the AC-coupled current and DC-current amplitude for the respective recordings of week 2 (orange) and week 5 neurons (black) shown in ***D***. Linear regression analysis of the relationships for week 2 and 5 data gave respective unitary single-channel current amplitude estimates of −1 pA and −0.3 pA from which the unitary conductance was calculated. ***F***, A decrease in mean conductance is observed in other hPSC lines. $n = 4–13$, $N = 1–3$, unpaired *t* test. ***G***, The switch to lower conductance AMPARs was not prevented by antagonists. $n = 5–9$, $N = 1–2$, one-way ANOVA with *post hoc* Tukey's test.

*qRT-PCR.* Protocols were performed as described previously (Bilican et al., 2014). Forward and reverse sequences (5′→3′) of primers used were as follows: *GRIA1*: TGCTTTGTCGCAACTCACAGA, GGCATAGACTCCTTTGGAGAAC; *GRIA2*: CATTCAGATGAGACCCGACCT, GGTATGCAAACTTGTCCCATTGA; *GRIA3*: ACCATCAGCATAGGTGGACTT, GGTTGGTGTTGTATAACTGCACG; *GRIA4*: TTCCGAGCAGCGTGCAAATA, GCATTGGGGCTGGTGTTATGA; *SLC12A2*: TGGTGGTGCAATTGGTCTAA, TCCCACTCCATTCCAGCTAC; *SLC12A5*: GGAAGGAAATGAGACGGTGA, TCCCACTCCTCTCCACAATC.

*Electrophysiology.* Whole-cell patch-clamp recordings were made from hECNs using an Axon Multiclamp 700B amplifier (Molecular Devices). AMPA-evoked currents were made at a holding potential of −74 mV in the presence of picrotoxin (50 $\mu$M), strychnine (20 $\mu$M), and TTX (300 nM). GABA-evoked currents were made at a holding potential of −14 mV in the presence of CNQX (5 $\mu$M), D-APV (50 $\mu$M), strychnine, and TTX. Patch electrodes were filled with a solution containing the following (in mM): 155 K-gluconate, 2 $MgCl_2$, 10 Na-HEPES, 10 Na-PiCreatine, 2 $Mg_2$-ATP, and 0.3 $Na_3$-GTP, pH 7.3, 300 mOsm, resistances 4–7 MΩ. Coverslips containing hECNs were placed in the recording chamber and perfused using gravity feed with an extracellular solution composed of the following (in mM): 152 NaCl, 2.8 KCl, 10 HEPES, 2 $CaCl_2$, 10 glucose, pH 7.3, 320–330 mOsm. Recordings were made at 20–23°C. The liquid junction potential (LJP) was calculated to be +14 mV (JPCalc; Clampex). Perforated whole-cell recordings were performed using a gramicidin (50–100 $\mu$g/ml) supplemented patch-pipette solution containing the following (in mM): 145 KCl, 1 $MgCl_2$, 0.1 $CaCl_2$, 1 EGTA, 10 HEPES, pH 7.3, 320–330 mOsm; the LJP was calculated to be −4.3 mV. The progress of perforation in the voltage-clamp configuration was monitored by the development of capacitive transients. Abrupt rises in transients indicated membrane rupture to the whole-cell configuration and the experiment was terminated. Once perforation stability was established, series resistances were <40 MΩ.

*Data analysis.* Nonstationary fluctuation analysis of slowly rising whole-cell currents evoked by AMPA were used to estimate the AMPAR unitary single-channel current. The analysis of AC and DC currents was performed as described previously (Brown et al., 1998) with the exception that the AC-coupled signal was filtered with a 1–1200 Hz band-pass frequency. All other recordings were low-pass filtered online at 2 kHz and all recordings were digitized at 10 kHz and recorded to computer hard disk using the WinEDR V2.7.6 Electrophysiology Data Recorder (J. Dempster, University of Strathclyde, United Kingdom; www.strath.ac.uk/Departments/PhysPharm/). $[Cl^-]_{INT}$ was calculated using the Nernst equation in which extracellular $[Cl^-]$ was converted into activity using an activity coefficient.

Data are presented as mean ± SEM. The number of experimental replicates is denoted as *n* and *N* represents the number of *de novo* preparations of batches from which *n* is obtained.

## Results

### Developmental maturation of AMPARs in hECNs

Functional AMPAR expression was assessed, at weekly intervals, by the ability of AMPA (50 $\mu$M) to elicit whole-cell currents. With

time, hECNs displayed an increase in AMPAR-mediated current densities together with an increase in the proportion of hECNs that responded to application of AMPA. Five weeks after aNPC plate-down, ~90% of all cells gave currents (Fig. 1*B*) that could be blocked by CNQX. AMPARs are tetrameric assemblies of different combinations of GluA1, GluA2, GluA3, or GluA4 subunits. AMPAR composition in rodent and human excitatory cortical neurons is developmentally regulated; GluA1/GluA4-containing stoichiometry predominates in immature neurons, whereas mature neurons have an increased expression of GluA1 and GluA2 subunits (Monyer et al., 1991; Talos et al., 2006a, 2006b; Orlandi et al., 2011). We assessed relative AMPAR subunit mRNA expression levels at 2 and 5 weeks after aNPC plate-down by qRT-PCR (Fig. 1*C*) and found significant increases in the relative mRNA expression levels for each of the AMPAR subunits.

GluA2 subunits are RNA edited, resulting in a gene-encoded glutamine codon in the M2 reentrant loop region being replaced by an arginine codon in ~99% of mRNA transcripts (for review, see Wright and Vissel, 2012). Moreover, the biophysical properties of AMPARs are considerably influenced by the presence of edited GluA2 [GluA2(R)] subunits that give rise to AMPARs that possess low single-channel conductances, low $Ca^{2+}$ permeability, and insensitivity to polyamine-mediated channel block (Traynelis et al., 2010). To assess whether AMPARs expressed by hECNs show a developmental increase in the proportion of receptors containing GluA2(R) subunits, we performed nonstationary fluctuation analysis to estimate the mean unitary conductance ($\gamma$) of AMPARs at weeks 2 and 5 after aNPC plate-down. Current-variance plots constructed from AC- and DC-coupled components of whole-cell AMPAR-mediated responses were used to estimate unitary conductances (Fig. 1*D*,*E*). Figure 1*F* illustrates that, for the ES1 cell line, the mean unitary conductance decreases at week 2 from 10.9 ± 1.0 pS ($n = 13$, $N = 2$) to 4.5 ± 0.4 pS at week 5 ($n = 12$, $N = 3$, $p < 0.001$, unpaired *t* test). Significant decreases in conductance over this same period were also observed in hECNs derived from a separate ES cell line (denoted ES2) and from two independent IPS cell lines (Fig. 1*F*). The factors that control the developmental increase in the expression of GluA2(R)-containing AMPARs remain largely unknown (Traynelis et al., 2010) and we found no evidence that this regulation is mediated by excitatory or inhibitory neurotransmitter action or is regulated by an activity-dependent process, because pharmacological blockade from weeks 1–5 of AMPAR, NMDAR, $GABA_AR$, and glycine receptors by CNQX (15 $\mu$M), D-AP5 (25 $\mu$M), bicuculline (100 $\mu$M), and strychnine (400 $\mu$M), respectively, and voltage-dependent $Na^+$ channels by TTX (1 $\mu$M) did not prevent the decrease in conductance that occurs between weeks 2 and 5 after aNPC differentiation (Fig. 1*G*). Furthermore, media supplements (see Materials and Methods) are not implicated in the reduction of conductance, because hECNs maintained in the absence of supplements displayed equivalent estimates of conductance at week 2 (12.7 ± 1.5; $n = 11$, $N = 3$) and week 5 (5.0 ± 1.1; $n = 5$, $N = 1$).

An increase in the proportion of AMPARs containing GluA2(R) subunits was confirmed in hECNs by assessing the polyamine sensitivity of AMPAR-mediated current to 1-naphthyl acetyl spermine (NASPM), a selective antagonist of GluA2(R)-lacking AMPARs (Koike et al., 1997). At week 2, NASPM (3 $\mu$M) strongly inhibited (77 ± 5%) AMPA-evoked steady-state whole-cell currents (Fig. 2*A*). The extent of this block is consistent with the expression of AMPARs that lack GluA2(R) subunits. At week 5, NASPM gave only modest inhibition (Fig. 2*B*,*C*). Overall, these data, together

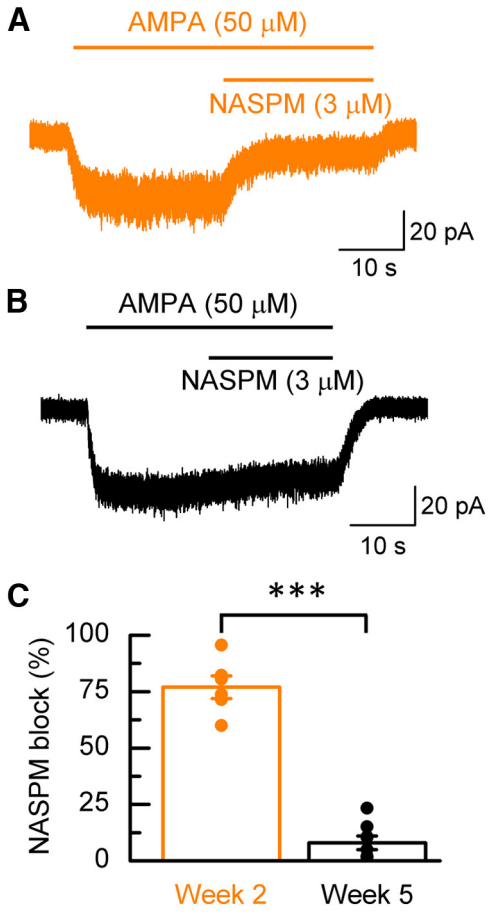


**Figure 2.** NASPM block. Example recordings from week 2 (***A***) and week 5 (***B***) neurons demonstrating NASPM inhibition of the steady-state current response evoked by AMPA. ***C***, Mean percentage block of AMPA currents by NASPM. $n = 6–8$, $N = 2$, unpaired *t* test.

with a development switch in unitary conductance of AMPARs, are entirely consistent with a switch from a GluA2(R)-lacking to a GluA2(R)-containing AMPAR population.

### hECNs show a developmental reduction in $[Cl^-]_{INT}$

Functional $GABA_AR$ expression was assessed at weekly intervals by the ability of GABA (100 $\mu$M) to elicit whole-cell currents. The magnitude of steady-state GABA-evoked currents increased with increasing periods of culture and, after 4 weeks of differentiation, all cells responded to GABA (Fig. 3*A*) with robust currents that were blocked by picrotoxin.

Fast depolarizing actions of GABA on neural precursor cells and immature cortical neurons are well documented and are a consequence of higher $[Cl^-]_{INT}$ (~25 mM) due to the higher relative expression level of NKCC1 over KCC2 (Ben-Ari et al., 2007). In both rodent and human cortex, the postnatal expression levels of KCC2 increase (Dzhala et al., 2005), leading to a net extrusion of $Cl^-$ from excitatory neurons and levels of $[Cl^-]_{INT}$ of 5–10 mM, which results in a net inward movement of $Cl^-$ and a hyperpolarizing response in neurons at or near their resting membrane potentials (Blaesse et al., 2009). Using gramicidin-perforated current-clamp recording to prevent disruption of $[Cl^-]_{INT}$ (Kyrozis and Reichling, 1995), we assessed the nature of the GABA response in hECNs when held at a potential of −60 mV. After 1 week of culture after aNPC plate-down, GABA elicited a depolarizing response (Fig. 3*B*), whereas at later periods, the response was hyperpolarizing (Fig. 3*C*). To determine the

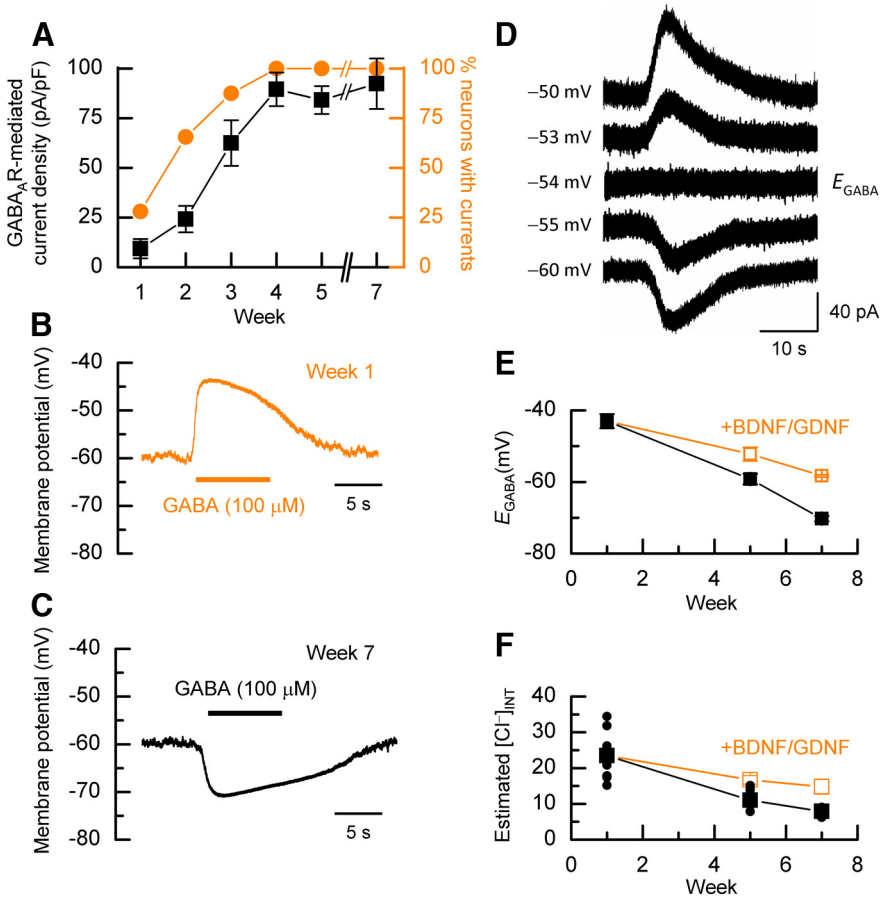


**Figure 3.** Determining $[Cl^-]_{INT}$. ***A***, Weekly percentage response (orange) to GABA and the mean GABA-mediated current density (black; $n = 20–34$, $N = 1–4$). ***B***, ***C***, Example perforated current-clamp recordings, at −60 mV from week 1 (***B***) and week 7 (***C***) neurons in which either a depolarizing or hyperpolarizing response to GABA is observed. ***D***, Representative voltage-clamp recordings used to determine $E_{GABA}$. ***E***, The relationship between mean $E_{GABA}$ in the absence ($n = 12–13$, $N = 2–3$) and presence of BDNF and GDNF ($n = 4–19$, $N = 1–3$). ***F***, Mean $[Cl^-]_{INT}$ development. Data at week 7 were obtained in the presence of IGF1. $n = 12–13$, $N = 2–3$.

reversal potential for GABA$_A$R-mediated responses ($E_{GABA}$), perforated voltage-clamp recordings were made from hECNs at various time points after aNPC differentiation. Figure 3*D* illustrates a series of GABA-activated current traces obtained from a week 5 hECNs and recorded at holding potentials that allowed $E_{GABA}$ to be determined. Figure 3*E* summarizes the extent to which $E_{GABA}$ changes with time in culture. At week 1, the mean $E_{GABA}$ is $-43.0 \pm 2.0$ mV ($n = 12$, $N = 3$) and becomes progressively more negative such that, at week 5, it is $-59.2 \pm 1.5$ mV ($n = 11$, $N = 2$, $p < 0.001$). To examine the possibility that $E_{GABA}$ could become more negative, we extended the culture period of hECNs to 7 weeks, which necessitated the inclusion of IGF1 in the culture media to improve cell viability (see Materials and Methods), and observed a further decrease in $E_{GABA}$ to $-70.3 \pm 0.7$ mV ($n = 13$, $N = 3$, $p < 0.001$). IGF1 was unlikely to have directly affected the change in $E_{GABA}$ because hECNs at week 5 cultured in the presence of IGF1 did not show any change in $E_{GABA}$ ($-62.9 \pm 2.3$ mV; $n = 10$, $N = 2$). In these experiments, BDNF and GDNF were omitted because these neurotrophic factors have been reported to cause alterations in $Cl^-$ transporter function and expression (Blaesse et al., 2009). Indeed, although $E_{GABA}$ becomes more negative in the presence of BDNF and GDNF, it remains ~10 mV more positive than is observed for hECNs cultured in the absence of neurotrophic factors (Fig. 3*E*).

Figure 3*F* illustrates the estimated $[Cl^-]_{INT}$ derived using the Nernst equation at each of the time points studied in Figure 3*E*. At week 1, the mean $[Cl^-]_{INT}$ is $23.5 \pm 1.9$ mM and this decreases by week 7 to $7.9 \pm 0.2$ mM. In the presence of BDNF and GDNF, $[Cl^-]_{INT}$ remains elevated at weeks 5 and 7. A reduction in $[Cl^-]_{INT}$ was also observed in hECNs derived from other hPSC lines (Fig. 4*A*). The underlying regulation of native neuronal NKCC1/KCC2/$[Cl^-]_{INT}$ development is controversial (Ben-Ari et al., 2012). We have demonstrated that the reduction in $[Cl^-]_{INT}$ in hECNs cannot be prevented by pharmacological blockade, from weeks 1 to 7, of ion channels and neurotransmitter systems, as described previously for AMPAR development (Fig. 4*B*). The reduction of $[Cl^-]_{INT}$ in hECNs is therefore activity-independent. Finally, using qRT-PCR analysis, we examined whether hECNs displayed shifts in NKCC1/KCC2 mRNA transcript levels. At week 1, NKCC1 mRNA levels were 4.8-fold higher than that of KCC2, but by week 7, KCC2 mRNA levels were 3.1-fold higher than NKCC1 (Fig. 4*C*). Such findings are consistent with the change in the expression levels of NKCC1 and KCC2 that are observed in rodent neurons when $[Cl^-]_{INT}$ decreases during development (Blaesse et al., 2009). In addition, hECNs cultured in the presence of BDNF and GDNF, which resulted in a smaller fall in $[Cl^-]_{INT}$, exhibited smaller relative shifts in the expression of KCC2 mRNA to that of NKCC1.

## Discussion

We have demonstrated the ability of hECNs to functionally express mature GluA2(R)-containing AMPARs and exhibit a reduction in $[Cl^-]_{INT}$ to adult-like levels. Importantly for the interpretation of our results, differentiating aNPCs are likely to be the predominate cellular phenotype at early culture time points (weeks 1 and 2) and, by later time points (≥week 4), the cultures are predominantly of hECN identity (Fig. 1*A*; Bilican et al., 2014).

We demonstrate that AMPAR single-channel conductance at weeks 2 and 5 significantly reduces from 10.9 to 4.5 pS in accordance with increased functional expression of the GluA2(R) subunit that confers a lower conductance to the AMPAR complex. Indeed, the conductance values at weeks 2 and 5 are directly comparable to conductance measurements of AMPAR-mediated events from rodent excitatory cortical neurons during a postnatal developmental period involving the upregulation of the GluA2(R) subunit (Brill and Huguenard, 2008). Furthermore, week 5 data correspond well to those of recombinantly expressed heteromeric GluA2(R)-containing AMPARs (Swanson et al., 1997). A GluA2(R)-lacking to GluA2(R)-containing switch in AMPAR subunits is confirmed by the observed high sensitivity of

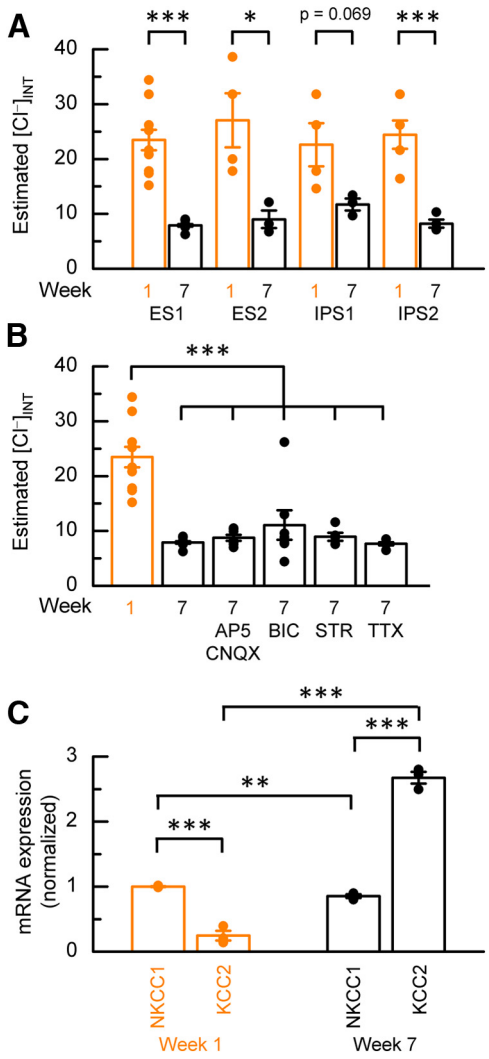


**Figure 4.** The maturation of $[Cl^-]_{INT}$. ***A***, Decrease in mean $[Cl^-]_{INT}$ are observed in other hPSC lines. $n = 3–13$, $N = 1–3$, unpaired *t* test. ***B***, The switch to low $[Cl^-]_{INT}$ is independent of neurotransmitter action and action potential driven activity. $n = 5–12$, $N = 2–3$, Kruskal–Wallis test with *post hoc* Dunn's test. ***C***, Mean normalized mRNA fold expression data for $Cl^-$ transporter NKCC1 and KCC2 subunits as assessed by qRT-PCR. $n = 3$, $N = 2$, paired *t* test. Expression is normalized to week 1 NKCC1 data after normalizing to GAPDH.

AMPAR-mediated currents at week 2, but not at week 5, to GluA2(R)-lacking the AMPAR blocker NASPM.

The principal AMPAR composition in native cortical preparations has been described to develop from an immature GluA1/GluA4-containing to an adult GluA1/GluA2-containing AMPAR complex (Monyer et al., 1991; Talos et al., 2006a, 2006b; Orlandi et al., 2011). Our data are consistent with an upregulation of the GluA2 subunit from weeks 2 to 5, however, the mRNA data for hECNs also demonstrate an equivalent upregulation of the GluA4 subunit. Nevertheless, these data do not indicate the level of functional protein. At week 2, the cultures contain a mixture of neurons and aNPCs, so it is interesting that native primary human embryonic cortical NPCs express unedited forms of the GluA2 subunit [GluA2(Q)] and, upon neuronal differentiation, express the edited GluA2(R) subunit over an equivalent culture period (Whitney et al., 2008).

The underlying mechanism of this AMPAR subunit shift has not yet been described in native excitatory cortical neurons. Here, we demonstrate that the reduction of AMPAR conductance is not prevented by antagonists of neurotransmitter ligand-gated ion channels and voltage-gated ion channels, indicating that the developmental upregulation of GluA2(R)-containing AMPARs is activity-independent. Indeed, it has been reported that NPC differentiation results in a large upregulation of the ADAR2 enzyme responsible for the editing of the GluA2 subunit Q/R site (Whitney et al., 2008).

We next investigated the regulation of $[Cl^-]_{INT}$. The understanding regarding the regulation, expression, and activity of $Cl^-$ transporters and $[Cl^-]_{INT}$ regulation is relevant to development and disease associated with $GABA_AR$ (and other $Cl^-$ channel) function (Blaesse et al., 2009). Perforated-patch clamp measurements of $E_{GABA}$ from weeks 1 to 7 show that $[Cl^-]_{INT}$ reduces from 24 to 8 mM. This is directly comparable to studies examining the maturation of $[Cl^-]_{INT}$ from NPCs to postnatal neurons in the rodent cortex (Owens et al., 1996; Yamada et al., 2004). In direct accordance with native mechanisms regulating $[Cl^-]_{INT}$, a reduction in $[Cl^-]_{INT}$ coincided with a switch in the relative mRNA expression levels of the $Cl^-$ transporters NKCC1 and KCC2 (Blaesse et al., 2009). This implies that the high $[Cl^-]_{INT}$ observed at week 1 is due to dominant NKCC1 activity and the low $[Cl^-]_{INT}$ observed at week 7 is a result of overriding KCC2-mediated $Cl^-$ extrusion.

To obtain a reduction in $[Cl^-]_{INT}$, hECNs had to be cultured for extended periods in the absence of BDNF and GDNF but with IGF supplementation. IGF1 was shown not to have an effect upon $E_{GABA}$ in week 5 hECNs, but has previously been associated with an upregulation of KCC2 expression in rodent hippocampal neurons (Kelsch et al., 2001). The inclusion of BDNF and GDNF, however, appears to slow the developmental $[Cl^-]_{INT}$ reduction. Neurotrophic supplements are commonly used in hPSC-derived neuronal cultures with little regard for their potential impact on neuronal $Cl^-$ transporter function (Blaesse et al., 2009) and receptor densensitization (Frank et al., 1996). We thus advocate caution when using these agents, particularly for functional studies.

The mechanistic control of neuronal of $[Cl^-]_{INT}$ development has received considerable attention and specific reports of mechanisms of NKCC1 and KCC2 regulation have been controversial (Ben-Ari et al., 2012). The inability to prevent the reduction of $[Cl^-]_{INT}$ by chronic blockade of ion channel activity are in agreement with an activity-independent mechanism in hECNs that has been observed in native neuronal preparations (Ben-Ari et al., 2007).

In summary, we show the ability of hECNs to functionally express mature GluA2(R)-containing AMPARs. This enables investigation into disease mechanisms that may affect the editing at the Q/R site. Furthermore, the ability of hECNs to exhibit a reduction in $[Cl^-]_{INT}$ to adult levels enables homeostatic and disease studies that potentially affect $Cl^-$ transporter function.

## References

Ben-Ari Y, Gaiarsa JL, Tyzio R, Khazipov R (2007) GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. Physiol Rev 87:1215–1284. CrossRef Medline

Ben-Ari Y, Woodin MA, Sernagor E, Cancedda L, Vinay L, Rivera C, Legendre P, Luhmann HJ, Bordey A, Wenner P, Fukuda A, van den Pol AN, Gaiarsa JL, Cherubini E (2012) Refuting the challenges of the developmental shift of polarity of GABA actions: GABA more exciting than ever! Front Cell Neurosci 6:35. CrossRef Medline

Bilican B, Serio A, Barmada SJ, Nishimura AL, Sullivan GJ, Carrasco M, Phatnani HP, Puddifoot CA, Story D, Fletcher J, Park IH, Friedman BA, Daley GQ, Wyllie DJ, Hardingham GE, Wilmut I, Finkbeiner S, Maniatis T, Shaw CE, Chandran S (2012) Mutant induced pluripotent stem cell lines recapitulate aspects of TDP-43 proteinopathies and reveal cell-

specific vulnerability. Proc Natl Acad Sci U S A 109:5803–5808. CrossRef Medline

Bilican B, Livesey MR, Haghi G, Qiu J, Burr K, Siller R, Hardingham GE, Wyllie DJ, Chandran S (2014) Physiological oxygen levels and EGF withdrawal are required for scalable generation of functional cortical neurones from human pluripotent stem cells. Plos One 9:e85932. CrossRef Medline

Blaesse P, Airaksinen MS, Rivera C, Kaila K (2009) Cation-chloride cotransporters and neuronal function. Neuron 61:820–838. CrossRef Medline

Brill J, Huguenard JR (2008) Sequential changes in AMPA receptor targeting in the developing neocortical excitatory circuit. J Neurosci 28:13918–13928. CrossRef Medline

Brown AM, Hope AG, Lambert JJ, Peters JA (1998) Ion permeation and conduction in a human recombinant 5-HT3 receptor subunit (h5-HT3A). J Physiol 507:653–665. CrossRef Medline

Dzhala VI, Talos DM, Sdrulla DA, Brumback AC, Mathews GC, Benke TA, Delpire E, Jensen FE, Staley KJ (2005) NKCC1 transporter facilitates seizures in the developing brain. Nat Med 11:1205–1213. CrossRef Medline

Espuny-Camacho I, Michelsen KA, Gall D, Linaro D, Hasche A, Bonnefont J, Bali C, Orduz D, Bilheu A, Herpoel A, Lambert N, Gaspard N, Péron S, Schiffmann SN, Giugliano M, Gaillard A, Vanderhaeghen P (2013) Pyramidal neurons derived from human pluripotent stem cells integrate efficiently into mouse brain circuits in vivo. Neuron 77:440–456. CrossRef Medline

Frank L, Ventimiglia R, Anderson K, Lindsay RM, Rudge JS (1996) BDNF down-regulates neurotrophin responsiveness, TrkB protein and TrkB mRNA levels in cultured rat hippocampal neurons. Eur J Neurosci 8:1220–1230. CrossRef Medline

Hansen DV, Rubenstein JL, Kriegstein AR (2011) Deriving excitatory neurons of the neocortex from pluripotent stem cells. Neuron 70:645–660. CrossRef Medline

Johnson MA, Weick JP, Pearce RA, Zhang SC (2007) Functional neural development from human embryonic stem cells: accelerated synaptic activity via astrocyte coculture. J Neurosci 27:3069–3077. CrossRef Medline

Kelsch W, Hormuzdi S, Straube E, Lewen A, Monyer H, Misgeld U (2001) Insulin-like growth factor 1 and a cytosolic tyrosine kinase activate chloride outward transport during maturation of hippocampal neurons. J Neurosci 21:8339–8347. Medline

Koike M, Iino M, Ozawa S (1997) Blocking effect of 1-naphthyl acetyl spermine on Ca(2+)-permeable AMPA receptors in cultured rat hippocampal neurons. Neurosci Res 29:27–36. CrossRef Medline

Kyrozis A, Reichling DB (1995) Perforated-patch recording with gramicidin avoids artifactual changes in intracellular chloride concentration. J Neurosci Methods 57:27–35. CrossRef Medline

Monyer H, Seeburg PH, Wisden W (1991) Glutamate-operated channels: developmentally early and mature forms arise by alternative splicing. Neuron 6:799–810. CrossRef Medline

Orlandi C, La Via L, Bonini D, Mora C, Russo I, Barbon A, Barlati S (2011) AMPA receptor regulation at the mRNA and protein level in rat primary cortical cultures. Plos One 6:e25350. CrossRef Medline

Owens DF, Boyce LH, Davis MB, Kriegstein AR (1996) Excitatory GABA responses in embryonic and neonatal cortical slices demonstrated by gramicidin perforated-patch recordings and calcium imaging. J Neurosci 16:6414–6423. Medline

Sandoe J, Eggan K (2013) Opportunities and challenges of pluripotent stem cell neurodegenerative disease models. Nat Neurosci 16:780–789. CrossRef Medline

Shi Y, Kirwan P, Smith J, Robinson HP, Livesey FJ (2012) Human cerebral cortex development from pluripotent stem cells to functional excitatory synapses. Nat Neurosci 15:477–486, S1. CrossRef Medline

Swanson GT, Kamboj SK, Cull-Candy SG (1997) Single-channel properties of recombinant AMPA receptors depend on RNA editing, splice variation, and subunit composition. J Neurosci 17:58–69. Medline

Talos DM, Fishman RE, Park H, Folkerth RD, Follett PL, Volpe JJ, Jensen FE (2006a) Developmental regulation of alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor subunit expression in forebrain and relationship to regional susceptibility to hypoxic/ischemic injury. I. Rodent cerebral white matter and cortex. J Comp Neurol 497:42–60. CrossRef Medline

Talos DM, Follett PL, Folkerth RD, Fishman RE, Trachtenberg FL, Volpe JJ, Jensen FE (2006b) Developmental regulation of alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor subunit expression in forebrain and relationship to regional susceptibility to hypoxic/ischemic injury. II. Human cerebral white matter and cortex. J Comp Neurol 497:61–77. CrossRef Medline

Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R (2010) Glutamate receptor ion channels: structure, regulation, and function. Pharmacol Rev 62:405–496. CrossRef Medline

Whitney NP, Peng H, Erdmann NB, Tian C, Monaghan DT, Zheng JC (2008) Calcium-permeable AMPA receptors containing Q/R-unedited GluR2 direct human neural progenitor cell differentiation to neurons. FASEB J 22:2888–2900. CrossRef Medline

Wright A, Vissel B (2012) The essential role of AMPA receptor GluR2 subunit RNA editing in the normal and diseased brain. Front Mol Neurosci 5:34. CrossRef Medline

Yamada J, Okabe A, Toyoda H, Kilb W, Luhmann HJ, Fukuda A (2004) $Cl^-$ uptake promoting depolarizing GABA actions in immature rat neocortical neurones is mediated by NKCC1. J Physiol 557:829–841. CrossRef Medline

Yang N, Ng YH, Pang ZP, Südhof TC, Wernig M (2011) Induced neuronal cells: how to make and define a neuron. Cell Stem Cell 9:517–525. CrossRef Medline

Zeng H, Guo M, Martins-Taylor K, Wang X, Zhang Z, Park JW, Zhan S, Kronenberg MS, Lichtler A, Liu HX, Chen FP, Yue L, Li XJ, Xu RH (2010) Specification of region-specific neurons including forebrain glutamatergic neurons from human induced pluripotent stem cells. Plos One 5:e11853. CrossRef Medline