Localized changes to glycogen synthase kinase-3 and collapsin response mediator protein-2 in the Huntington’s disease affected brain

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All cases of Huntington’s disease (HD) are caused by mutant huntingtin protein (mhtt), yet the molecular mechanisms that link mhtt to disease symptoms are not fully elucidated. Given glycogen synthase kinase-3 (GSK3) is implicated in several neurodegenerative diseases as a molecular mediator of neuronal decline and widely touted as a therapeutic target, we investigated GSK3 in cells expressing mhtt, brains of R6/1 HD mice and post-mortem human brain samples. Consistency in data across the two models and the human brain samples indicate decreased GSK3 signalling contributes to neuronal dysfunction in HD. Inhibitory phosphorylation of GSK3 (pGSK3) was elevated in mhtt cells and this appeared related to an overall energy metabolism deficit as the mhtt cells had less ATP and inhibiting ATP production in control cells expressing non-pathogenic htt with paraquat also increased pGSK3. pGSK3 was increased and ATP levels decreased in the frontal cortex and striatum of R6/1 mice and levels of cortical pGSK3 inversely correlated with cognitive function of the mice. Consistent with decreased GSK3 activity in the R6/1 mouse brain, β-catenin levels were increased and phosphorylation of collapsin response mediator protein-2 (CRMP2) decreased in the frontal cortex where inhibitory phosphorylation of GSK3 was the greatest. pGSK3 was predominantly undetectable in HD and healthy control human brain samples, but levels of total GSK3 were decreased in the HD-affected frontal cortex and this correlated with decreased pCRMP2. Thus, disruptions to cortical GSK3 signalling, possibly due to localized energy metabolism deficits, appear to contribute to the cognitive symptoms of HD.

INTRODUCTION

Huntington’s disease (HD) is a fatal neurodegenerative disorder in which all cases are caused by an expanded polyglutamine-encoding CAG repeat sequence in the huntingtin gene (1). A repeat sequence encoding more than 36 consecutive glutamine residues in the huntingtin protein is invariably pathogenic and following a prodromal period with a duration inversely proportional to the number of CAG repeats (2) the symptoms of HD manifest as loss of motor control, cognitive deficits and mood imbalances. These symptoms progressively worsen until death occurs 15–20 years after symptom onset. Despite the unambiguous genetic basis for the inheritance and development of HD, the molecular mechanisms triggered by the mutant protein that contribute to neuronal dysfunction and ultimately give rise to the symptoms of the disease are yet to be fully elucidated. As a consequence, there is no cure for HD and effective disease-modifying therapies still do not yet exist.

Glycogen synthase kinase-3 (GSK3) is a highly conserved enzyme first isolated from muscle tissue and named for its ability to phosphorylate glycogen synthase (3). Two mammalian forms, GSK3α and GSK3β, are encoded by separate genes which despite differences in substrate preference in vivo (4) are very similar in structure and regulation (5). Unlike many kinases, GSK3 is constitutively active and its activity primarily regulated by inhibitory phosphorylation (at serine-21 in GSK3α and serine-9 in GSK3β) which renders the kinase inactive (6).

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Despite its name, the number of substrates for GSK3 extends well beyond glycogen synthase. GSK3 is a highly promiscuous kinase with substrates involved in many aspects of cell biology, including energy metabolism, transcription factor activation, cell development, division and proliferation, inflammation and microtubule stability (7). Furthermore, the number of recognized and putative roles for GSK3 specifically within the central nervous system continues to expand (8). Given its broad range of substrates, it is unsurprising that disrupted regulation and function of GSK3 has been associated with many diverse diseases, including diabetes (9), cancer (10), Alzheimer’s disease (11,12) and mental illnesses such as bipolar disorder (13). The pathogenic role for GSK3 in these diseases is relatively well studied and in all cases GSK3 has been touted as a viable therapeutic target.

Altered energy metabolism has been proposed as an important factor that contributes to neuronal decline in HD (14), but despite the apparent obligate requirement for appropriate regulation of GSK3 in neuronal function and energy metabolism, relatively little is known about the role of GSK3 in HD. Of those studies that have examined GSK3 in HD, the outcomes appear to be conflicting; one study has shown that inhibitory phosphorylation of GSK3 is elevated in response to expression of mutant huntingtin (15), whereas others have demonstrated decreased inhibitory phosphorylation and shown efficacy of a GSK3 inhibitor in mouse models of HD (16). To date, GSK3 has not been examined in the human HD-affected brain, and there is a paucity of data to show whether GSK3 substrates associated with neuronal function are altered in vivo. Nonetheless, some reports have proposed GSK3 inhibitors as a therapeutic strategy for HD (16,17). Given the large number of substrates for GSK3 and the central role for this kinase in seemingly all aspects of cell biology, the development of GSK3 inhibitors to treat human disease needs to proceed with caution (7). The present study was undertaken to progress our understanding of GSK3 in HD and therefore help guide therapy development.

RESULTS

Increased GSK3 phosphorylation and decreased ATP in a cell culture model of HD

Cells derived from the striata of mice expressing non-pathogenic huntingtin encoding 7 glutamine repeats (STHdh$^{Q7/7}$) or mutant huntingtin encoding 111 glutamine repeats (STHdh$^{Q111/111}$) (18) were analysed for levels of GSK3 and ATP. Supporting and extending results from a previous study (15), the presence of mutant huntingtin in these cells was associated with an increase in GSK3β phosphorylation at the inhibitory serine-9 residue (Fig. 1A). Phosphorylation of GSK3α (serine-21) was also elevated and a statistically significant correlation existed between the phosphorylation state of both GSK3 isoforms (Fig. 1B).

The inhibitory activity of mutant huntingtin towards mitochondrial function has been reported (19–23), and although a relatively crude measure of mitochondrial function we found the STHdh$^{Q111/111}$ cells contained less ATP (Fig. 1C). These cells were also more sensitive to the mitochondrial toxin paraquat when compared with the STHdh$^{Q7/7}$ cells (Fig. 1D and E). To assess whether the observed changes to pGSK3 in the STHdh$^{Q111/111}$ cells may be secondary to the effects of mutant huntingtin on mitochondrial function, we examined the apparent link between ATP generation and pGSK3 independently of mutant huntingtin by treating STHdh$^{Q7/7}$ cells with paraquat. Consistent with the effects of mutant huntingtin (Fig. 1A and C), exposure to paraquat increased GSK3 phosphorylation (Fig. 1F) and decreased ATP levels (Fig. 1G) in the STHdh$^{Q7/7}$ cells.

Increased GSK3 phosphorylation and decreased ATP in the R6/1 mouse model of HD

Brain tissue from R6/1 mice (24) showing impairment in the Morris water maze and decreased brain mass relative to their non-transgenic littermates (Fig. 2) was assessed for levels of GSK3 and ATP. Consistent with the effects of mutant huntingtin on GSK3 phosphorylation in vitro (Fig. 1), levels of phosphorylated GSK3 were also elevated in the brains of R6/1 mice (Fig. 3A and B). Despite ubiquitous expression of the polyglutamine repeat-expanded huntingtin in the brains of these mice (24), increased GSK3β phosphorylation was restricted to the cerebral cortex and striatum, sparing the hippocampus and cerebellum (Fig. 3A–D). The changes to pGSK3β in these brain regions correlated with changes in pGSK3α (Fig. 3E and F). Supporting the apparent relationship between GSK3β phosphorylation and ATP levels identified in vitro (Fig. 1), ATP levels were also decreased in the brains of R6/1 mice, but only in those brain regions where levels of phosphorylated GSK3β were increased (Fig. 3G–J). Although in situ microwave fixation of rodent brains provides higher levels of brain ATP (25), this method of preparing brain samples was not used in our study and the effects of post-mortem interval on ATP levels in the mouse brain samples therefore a possibility. However, data in Figure 3 are consistent with data from a recent study which did employ microwave fixation and also showed ATP levels are decreased in the brains of HD model mice (26). Thus, while our method of collecting brain samples may have contributed to lower ATP levels in all samples, the ability to ascertain the in vivo effects of mutant huntingtin on ATP levels appears unaffected.

Altered GSK3 phosphorylation in the cerebral cortex correlates with cognitive ability of R6/1 mice

To assess whether changes to GSK3β phosphorylation in the brains of R6/1 mice were associated with the ability of individual mice to perform in the Morris water maze, a series of correlation analyses were performed. Levels of phosphorylated GSK3β in the cortex showed a significant inverse correlation with performance of individual mice in the recall phase of the Morris water maze (Fig. 4A). Changes to ATP in the same brain region did not correlate with cognitive ability ($R^2 = 0.24$, $P = 0.24$), indicating that although impaired energy metabolism contributes to changes in GSK3β phosphorylation, the extent of impaired energy metabolism is not proportional to the cognitive phenotype of the mice. Despite increased GSK3β phosphorylation in striatum samples from the mice (Fig. 3B), these changes did not correlate with cognitive ability (Fig. 4B), nor did levels of phosphorylated GSK3β in the hippocampus or cerebellum (Fig. 4C and D).
Biochemical consequences of increased GSK3 phosphorylation in the brains of R6/1 mice

Increased phosphorylation of GSK3β at serine-9 inhibits its kinase activity (6). To investigate potential biochemical consequences of inhibitory phosphorylation of GSK3β in the cerebral cortex and striatum of R6/1 mice, levels of downstream targets predicted to change in response to GSK3β inhibition were investigated. Consistent with inhibitory phosphorylation of GSK3β, levels of phosphorylated collapsin response mediator

Figure 1. Phosphorylated GSK3 and ATP in cells grown in culture. (A) Levels of phosphorylated GSK3 (pGSK3) were assessed by western blot and using an antibody that recognizes GSK3 phosphorylated at serine-9 on GSK3β or serine-21 on GSK3α. pGSK3α is the fainter band immediately above pGSK3β in all representative western blot images. Values from densitometry analyses show pGSK3β levels in STHdhQ111/111 cells (111/111) relative to STHdhQ7/7 cells (7/7). (B) Correlation between levels of pGSK3β and pGSK3α in STHdhQ111/111 cells and STHdhQ7/7 cells. Statistical significance of correlation determined using the Pearson correlation test. Dashed lines in (B) represent 95% confidence intervals. (C) ATP levels in STHdhQ111/111 cells and STHdhQ7/7 cells expressed per mg protein. (D) Effects of paraquat on MTT reduction by STHdhQ111/111 cells and STHdhQ7/7 cells. Values are expressed relative to cells treated with 0 μM paraquat. (D) Effects of paraquat on levels of lactate dehydrogenase (LDH) released from cells into the culture medium. Values are expressed relative to cells treated with 1% (v/v) triton X-100. (F) Levels of pGSK3β assessed as per (A), and (G) levels of ATP in STHdhQ111/111 cells treated with 0 mM (control) or 1 mM paraquat. All densitometry values are normalized to levels of the GAPDH loading control. Asterisks indicate statistically significant differences between 7/7 cells and 111/111 cells (A, C, D, E) or control and paraquat treated cells (F and G) (P < 0.05, unpaired t test, Mann–Whitney test or two-way ANOVA with Bonferroni post tests). All values represent the mean ± SEM from 6–8 (A, C, D, E) or 4 (F and G) replicates.
protein-2 (CRMP2) were decreased in the cerebral cortex of R6/1 mice and levels of β-catenin were increased (Fig. 5A and C). Levels of phosphorylated CRMP2 were not changed in the striatum (Fig. 5B), whereas β-catenin levels were increased (Fig. 5D).

**Decreased GSK3 in the HD-affected post-mortem human brain**

Phosphorylated GSK3 was predominantly not detectable in post-mortem human brain samples (Fig. 6A and F) despite using the same western blot procedure as for all cell and mouse brain analyses. Non-phosphorylated GSK3 by contrast was readily detectable. These analyses revealed a 50% decrease in GSK3β in the frontal cortex of the human HD-affected brain (Fig. 6A) and this correlated with changes to GSK3α (Fig. 6C). Consistent with data for phosphorylated GSK3 in the brains of R6/1 mice (Fig. 3A–D), changes to GSK3 in the human HD brain were not evident in all brain regions as GSK3β levels were not altered in the cerebellum relative to healthy controls (Fig. 6F).

Despite an inability to detect phosphorylated GSK3β in the post-mortem human brain samples, the overall decrease in GSK3 in the HD brain suggests an overall decrease in GSK3 kinase activity. Supporting this, levels of phosphorylated CRMP2 were also decreased in the human frontal cortex (Fig. 6B), the levels of which correlated directly with GSK3β (Fig. 6D). The same correlation did not exist in the cerebellum (Fig. 6H). As per all western blot results presented for cell and mouse brain samples, these human brain results are expressed relative to GAPDH which was not changed in either of the brain regions analysed. The correlation between GSK3β and phosphorylated CRMP2 in the frontal cortex therefore does not represent non-specific protein loss in HD. Levels of β-catenin were not altered (Fig. 6E and I).

**DISCUSSION**

Two transgenic models of HD as well as post-mortem human HD brain samples were analysed for the present study and consistency in the results across both models and the human brain samples suggests that GSK3 may be a significant molecular
Figure 3. Phosphorylated GSK3 and ATP in brain regions of R6/1 mice. Levels of phosphorylated GSK3 (pGSK3) were assessed by western blot and using an antibody that recognizes GSK3 phosphorylated at serine-9 on GSK3β or serine-21 on GSK3α. pGSK3α is the fainter band immediately above pGSK3β in all representative western blot images. Values from densitometry analyses show pGSK3β levels in the cortex (A), striatum (B), hippocampus (C) and cerebellum (D) of R6/1 mice relative to their non-transgenic (non-Tg) littermates. All densitometry values are normalized to levels of the GAPDH loading control and are expressed relative to non-Tg mice. Correlation analyses show changes to pGSK3α relative to pGSK3β in the cortex (E) and striatum (F). Levels of ATP in cortex (G), striatum (H), hippocampus (I) and cerebellum (J) of R6/1 mice and their non-Tg littermates expressed per mg protein. Asterisks indicate statistically significant differences between R6/1 and non-Tg mice ($P < 0.05$, unpaired $t$ test or Mann–Whitney test, $n = 7–8$). All values represent the mean ± SEM. $P$-value shown in (E) and (F) represents statistical significance of correlation determined using the Pearson correlation test and dashed lines represent 95% confidence intervals.
mediator of neuronal dysfunction in HD. Decreased energy metabolism due to the presence of mutant huntingtin appears to initiate the molecular events that lead to disrupted GSK3, and altered CRMP2 phosphorylation appears to be one of the GSK3 substrates that may have direct effects on neuronal function. These events were evident in the frontal cortex of R6/1 mice and the human HD-affected brain, and indicated disrupted cortical GSK3 signalling contributes to the cognitive symptoms of HD.

The association between HD and disrupted energy metabolism in the brain has been recognized for many years. Assessing uptake of the glucose analogue 18F-FDG via positron emission tomography (PET) provides a relatively broad indication of energy metabolism in the brain and most 18F-FDG-PET studies with HD subjects have demonstrated that glucose utilization is altered in HD (reviewed in 27). Some of these studies pre-date discovery of the huntingtin mutation (28,29), and more recent studies indicate that altered glucose metabolism in pre-manifest HD may be a better predictor of the age of symptom onset than the number of CAG repeats (30,31). An early 18F-FDG-PET study was one of the first to indicate CNS anomalies in HD extend beyond sub-cortical regions of the brain, and it was also one of the first to indicate energy metabolism deficiencies in the cerebral cortex contribute to the cognitive symptoms of HD (32).

At the biochemical level, a number of key regulators of energy metabolism are altered in cases of HD and models of HD, including peroxisome proliferator-activated receptor-γ coactivator-1α, AMP-activated protein kinase and creatine kinase B (reviewed in 14). The stimuli that induce these biochemical changes and the tissues within which they occur vary across different study groups and the animal models examined, but a consistent feature which appears to unify all biochemical markers of impaired energy metabolism to the global changes in glucose utilisation as detected via PET imaging is mitochondrial dysfunction. Mitochondria in HD have an abnormal morphology, their cellular distribution is altered, their axonal transport disrupted and their ability to generate ATP via oxidative phosphorylation impaired (33,34). These mitochondrial deficiencies appear to be the result of the direct effects of mutant huntingtin on mitochondria (20,35–37) as well as indirect effects on mitochondrial biogenesis (38) and fission (22). Underscoring the contribution of mitochondrial deficiencies to the symptoms of HD, therapeutic up-regulation of mitochondrial biogenesis is effective in attenuating the phenotype of HD model mice (39) and has therefore been proposed as a therapeutic option for HD.

Relatively generic consequences of mitochondrial dysfunction such as decreased ATP generation, elevated ROS production and initiation of pro-apoptotic cascades have all been described as contributors to the neuronal decline in HD and subsequent manifestation of characteristic symptoms. There is little doubt that these aspects of mitochondrial dysfunction will contribute to overt aspects of neuronal dysfunction in HD. However, the molecular pathways triggered by mutant huntingtin-induced mitochondrial dysfunction are likely to be many and varied, with seemingly minor biochemical events also contributing to the symptoms of the disease. Data presented in this study indicate that decreased activity of GSK3 may be one of these biochemical events. Elevated inhibitory phosphorylation of GSK3 was evident in STHdhQ111/111 cells (Fig. 1A and B) and appeared to be driven by impaired mitochondrial energy metabolism; it was associated with decreased ATP levels in the STHdhQ7/7 cells (Fig. 1C and D) and recapitulated in STThdhQ7/7 cells by exposing them to the mitochondrial inhibitor paraquat (Fig. 1F). Further to this, GSK3 phosphorylation was selectively elevated in regions of the R6/1 mouse brain where ATP levels were decreased (Fig. 3). Although ATP levels are a relatively crude indicator of mitochondrial function, they are nonetheless consistent with previous studies which have demonstrated the inhibitory effects of mutant huntingtin towards mitochondria using more direct measures of mitochondrial function (19–23).

Two independent studies have shown that treating cells with chemical inhibitors of GSK3 provides protection against mutant huntingtin expression (40,41). The latter of these also analysed lipid rafts derived from brains and neuronal cells from mutant huntingtin knock-in HD model mice and identified accumulation of mutant huntingtin and GSK3 in the lipid rafts (41). This accumulation was evident prior to overt symptoms indicating that the association of both proteins within lipid rafts could contribute to disease pathogenesis. Further implicating a
role for GSK3 in HD, neuronal expression of expanded RNA repeat sequences in *Drosophila*, such as CAG found in HD, has been shown to alter transcripts that encode elements of GSK3 signalling pathways (42). Collectively, these studies support our observations that GSK3 homeostasis is disrupted due to expression of mutant huntingtin. However, the conclusion that GSK3 inhibitors may be a viable therapeutic option for HD (40,41) opposes outcomes from our own study (Figs 1, 3 and 4) which indicate that inhibitory phosphorylation of GSK3 is already elevated in HD. Our observations are consistent with a previous study that demonstrated increased Akt (protein kinase B) signalling in STHdh<sup>Q111/Q111</sup> cells and the brains of mutant huntingtin mice and consequent inhibitory phosphorylation of GSK3 in the STHdh<sup>Q111/Q111</sup> cells (15).

Differences in the experimental models used across the limited number of studies that have investigated GSK3 in HD are considerable. The *in vitro* studies have used different cell lines transfected with different *huntingtin*-expression constructs or have used primary cell lines derived from HD model mice, and the *in vivo* studies have used different HD model mice and/or used

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**Figure 5.** Phosphorylated CRMP2 and β-catenin in brains of R6/1 mice. Levels of phosphorylated CRMP2 (pCRMP2) in the cortex (A), striatum (B), hippocampus (C), and cerebellum (D) of the brains of R6/1 mice and their non-transgenic (non-Tg) littermates were assessed by western blot and using an antibody that recognizes CRMP2 phosphorylated at threonine-514. Levels of β-catenin in the cortex (E), striatum (F), hippocampus (G), and cerebellum (H) of the brains of R6/1 mice and their non-transgenic (non-Tg) littermates were assessed by western blot. All densitometry values are normalized to levels of the GAPDH loading control and expressed relative to non-Tg mice. Asterisks indicate statistically significant differences between R6/1 and non-Tg mice (P < 0.05, unpaired t test or Mann–Whitney test, n = 6–8). All values represent the mean ± SEM.
models created in a different species (Drosophila). At this stage, it is challenging to reconcile apparent conflicting results regarding whether GSK3 activity is elevated or decreased in HD and therefore whether GSK3 inhibition represents a viable therapeutic strategy. To partly address these challenges, we therefore analysed human brain tissue. Phosphorylated GSK3 was not
readily detected in human brain tissue, but total levels of GSK3 were decreased in the HD-affected frontal cortex (Fig. 6A). These data represent the first direct evidence for altered GSK3 homeostasis in the human HD-affected brain. Consistency across the cell culture, R6/1 mouse brain and human brain data indicate that the presence of mutant huntingtin contributes to decreased GSK3 activity in HD, and that despite ubiquitous expression of mutant huntingtin within the brain the frontal cortex is the region in which GSK3 homeostasis is primarily affected.

The relevance of decreased GSK3 activity to the manifestation of HD symptoms is indicated by data from R6/1 mice which revealed a correlation between levels of phosphorylated GSK3β in the frontal cortex and performance of the mice in the recall phase of the Morris water maze (Fig. 4). Although predominantly regarded as a test for hippocampal-dependent spatial learning and memory, the contribution of non-hippocampal brain regions to performance in this task has been articulated (43). Thus, the data for ATP and GSK3β phosphorylation in the frontal cortex of R6/1 mice indicate that disrupted GSK3 signalling, possibly initiated by impaired mitochondrial energy metabolism, contributes to cognitive decline in HD. This is supported by 18F-FDG-PET imaging data that show that the rate of glucose consumption in the cerebral cortex of HD patients correlates with the clinical rating of severity of dementia (32), but significance of the seemingly central role for GSK3 still requires further validation. Not least of which, the GSK3 substrates that directly affect neuronal and synaptic function need to be examined in greater detail. Our examination of brain material from the R6/1 mice revealed decreased CRMP2 phosphorylation and increased levels of β-catenin in the frontal cortex (Fig. 5). Both of these results are consistent with the observed elevation in inhibitory phosphorylation of GSK3 and both have the potential to contribute to neuronal dysfunction. The β-catenin data support a previous study which demonstrated β-catenin levels (or analogues thereof) were increased in response to mutant huntingtin expression in cells grown in culture, in flies and in the brains of mice (44). This increase was attributed to pathological polyglutamine expansion within the huntingtin protein destabilizing the β-catenin destruction complex, thereby causing accumulation of β-catenin (44). Although the regulation of β-catenin levels via this pathway involves GSK3 kinase activity (45), GSK3 was not examined in the study (44). Regardless, altered β-catenin levels can directly impact on neuronal function and memory formation (46). Data generated from the present study (Fig. 5E–H) and previously (44) therefore support a potential contribution of elevated β-catenin levels to the phenotypes of animal models of HD. This is supported by data that show suppression of β-catenin levels by treating with indomethacin was protective in mutant huntingtin expressing cells and attenuated the phenotype of HD model flies (44). The involvement of β-catenin in the human HD-affected brain, however, is less clear. Data generated by analysing human brain samples indicated that β-catenin levels are not altered in the HD-affected frontal cortex (Fig. 6D) or cerebellum (Fig. 6H). In contrast, and despite a limited number of samples analysed (n = 2 control and n = 2 HD cases), an analysis of striatal samples has indicated that β-catenin levels are elevated in the human HD-affected striatum (44). Further analyses are clearly required to better understand β-catenin in human HD.

As per elevated β-catenin levels, decreased phosphorylation of CRMP2 in the frontal cortex of R6/1 mice (Fig. 5A) and human HD subjects (Fig. 6B) is also consistent with decreased GSK3 kinase activity (47). These data represent the first evidence for altered CRMP2 in HD, but it is not yet clear whether decreased CRMP2 phosphorylation contributes to neuronal dysfunction and the symptoms of HD or whether it represents a relatively inconsequential result of altered GSK3 activity. Supporting the latter, levels of pCRMP2 in the brain, unlike pGSK3, did not correlate with performance of R6/1 mice in the Morris water maze (data not shown). However, CRMP2 is one of five known homologous proteins (CRMP1-5) that regulate a range of cellular features central to highly specialized cells such as the neuron, including establishment of neuronal polarity, axon guidance, dendritic organisation, neurotransmitter release and regulation of synaptic plasticity (reviewed in 48,49). Relative to other family members, CRMP2 is highly expressed and phosphorylated in the adult nervous system, particularly in regions that necessitate a high degree of plasticity (50), suggesting sustained phosphorylation of the protein is indicative of status quo under healthy conditions. Thus, CRMP2 appears to be an important contributor to normal neuronal function and disruption to its phosphorylation state likely to impede normal neuronal function.

Most information on the function of CRMP2 in the CNS has come from studies pertaining to its activity during neurogenesis or in the developing brain where many of its effects are generally recognized as mediated by non-phosphorylated CRMP2. For example, non-phosphorylated CRMP2 promotes axon outgrowth in neurons grown in culture and an increase in CRMP2 phosphorylation is inhibitory towards this function (47). Thus, phosphorylated CRMP2 is often regarded as deleterious to neuronal function. The effects of CRMP2 phosphorylation on its functionality within a mature neuron or synapse, however, are less well known, and the consequences of CRMP2 hyper-phosphorylation compared with hypo-phosphorylation in this context yet to be fully appreciated. CRMP2 is hyper-phosphorylated in the Alzheimer’s disease-affected brain (51) and in neurodegenerative conditions associated with HIV infection (52), thereby associating hyper-phosphorylation with neuronal failure in the adult brain. However, presynaptic interaction between CRMP2 and N-type Ca2+ channels has been demonstrated, and CRMP2 has been described as a ‘neuromodulator’ due to its effects on Ca2+ flux after membrane depolarisation, synaptic vesicle loading and release of the neurotransmitter glutamate (53). It was proposed that post-translational modification of CRMP2 by kinases such as GSK3 and the GSK3 priming kinase Cdk5 could decrease binding between CRMP2 and the N-type Ca2+ channels, thereby suppressing neurotransmission (53). This proposal was supported when the phosphorylation of CRMP2 by Cdk5 was shown to decrease the association between CRMP2 and N-type Ca2+ channels (54). GSK3-mediated phosphorylation is yet to be directly examined in this mechanism of neurotransmission, but data presented in the present study are consistent with decreased phosphorylation of CRMP2 in the frontal cortex being a potential pathway through which altered GSK signalling contributes to the symptoms of HD.

Collectively, the data presented in this study indicate impaired energy metabolism due to expression of mutant huntingtin
suppresses GSK3 activity, be it through elevated inhibitory phosphorylation or down-regulation of overall GSK3 levels. Data from the R6/1 mouse model of HD indicate that altered GSK3 in the brain is a correlate of the cognitive symptoms of HD and the GSK3 substrates β-catenin and CRMP2 may be effector molecules in this degenerative pathway. However, the precise contribution of β-catenin or CRMP2 to neuronal decline in HD and the potential contribution of additional GSK3 substrates still require further examination. In light of these data, we concur with previous assertions (7) that due to the broad range of known and putative substrates for GSK3 and the seemingly central role for GSK3 in neuronal function, the development of GSK3 inhibitors for HD and other neurodegenerative conditions needs to proceed with caution.

MATERIALS AND METHODS

Chemicals and reagents

All cell culture reagents were from Life Technologies unless specified otherwise. All other chemicals and reagents were from Sigma-Aldrich unless specified otherwise.

Cell culture

Striatum-derived cells expressing non-pathogenic huntingtin encoding 7 glutamine repeats (STHdhQ7/7) or mutant huntingtin encoding 111 glutamine repeats (STHdhQ111/111) (18) were grown in high glucose DMEM medium supplemented with 50 U ml⁻¹ penicillin, 50 μg ml⁻¹ streptomycin, 500 μg ml⁻¹ geneticin, 2 mM L-glutamine and 10% (v/v) fetal bovine serum. Cultures were incubated in six-well plates at 33°C with 5% CO₂. Confluent cells were removed from the plates using a plastic cell scraper then pelleted by centrifugation (16 000 g, 3 min).

Cells at ~80% confluency were exposed to paclitaxel by supplementing the existing cell culture medium with paclitaxel up to a final concentration of 2 μM then incubating the cells for a further 24 h. Cells were collected as above or analysed for metabolic reductase activity or cell death using the 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) release assays, respectively. For the MTT assay, MTT was added to the existing culture medium (final concentration of 120 μM) for ~15 min after which the media were removed from the cells and reduced MTT solubilized with 200 μl DMSO then measured by absorbance at 585 nm. The LDH assay used the LDH Cytotoxicity Detection Kit (Roche) as per manufacturer’s instructions.

Transgenic mice

Procedures involving mice were approved by an Animal Experimentation Ethics Committee. R6/1 HD mice (24) purchased from The Jackson Laboratory (Bar Harbor, USA) were bred with non-transgenic CBAXC57/B6 mice. Mice were housed in standard boxes with ad libitum access to food and water and a 12/12 h light/dark cycle. The boxes contained saw dust and shredded paper with no other environmental enrichment. The mice were genotyped using the commercial Extract-N-Amp™ Plant PCR Kit (Sigma). Primers and cycling steps were as per The Jackson Laboratory website and the PCR product run on a 1.7% agarose gel (100 volts, 30 min). All mice used in this study were control mice from a separate study in which we examined efficacy of a potential HD therapeutic. They were treated daily (by gavage) with standard suspension vehicle (55) at 100 μl per 25 g body weight from the age of 7 weeks.

R6/1 mice and non-transgenic littermates were assessed at 12 weeks of age using the Morris water maze (pool diameter 1.2 m, pool depth 600 mm). Each mouse was allowed to swim for a maximum of 90 s and the time taken to reach a platform hidden 10 mm from the surface of the water measured. Mice were pre-tested the day before data collection by swimming in the pool for 1 min from the northwest quadrant, removed and dried and then placed into the pool again to swim for 1 min from the southwest quadrant. Mice were then tested for seven consecutive days. On the last day of testing, the hidden platform was removed and the amount of time the mice spent in the correct quadrant recorded (recall phase). At 17 weeks of age, all of the mice were killed by cervical dislocation. Brains were removed then dissected to separate frontal cortex, striatum, hippocampus and cerebellum. These brain regions were then frozen on dry ice and stored at −80°C.

Human brain tissues

All procedures involving human brain tissues were approved by a Human Ethics Committee. Post-mortem, fresh-frozen brain samples (frontal cortex Brodmann area 9 and cerebellum) from HD cases and age- and sex-matched healthy controls were obtained from the Victorian Brain Bank Network and stored at −80°C until analysed. Details for all HD cases and controls are in Table 1.

Preparation of brain samples and cell pellets for analysis

Brain samples and cell pellets were homogenized in Phospho-Safe Extraction Reagent (Calbiochem) supplemented with deoxyribonuclease I (DNase I, Sigma) at a ratio of ~4 vol g⁻¹ sample. Samples were homogenized with a plastic pestle, then soluble extracts obtained by centrifuging (18 000g, 3 min, 4°C) then collecting the supernatant. Protein content of the soluble extracts was determined (Pierce BCA Protein Assay, Thermo Scientific) and then the samples stored at −80°C.

Western blot

Samples were diluted in 4× sample buffer (250 mM Tris, 20% (v/v) glycerol, 8% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 0.1% (w/v) bromophenol blue), heated for 5 min at 95°C and then loaded onto 4–12% NuPAGE Novex Bis-Tris Midi gels in MES SDS running buffer. The amount of protein loaded per lane was as follows: 30 μg for pCRMP2, 20 μg for pGSK3α/β and β-catenin and10 μg for GSK3β. After electrophoresis (175 volts, 50 min), proteins were transferred onto PVDF membranes. Membranes were blocked with 4% (w/v) skin milk powder in phosphate buffered saline containing 0.05% (v/v) Tween-20 (PBST) for 1 h at room temperature and then incubated overnight at 4°C with antibodies to pGSK3α/β (Cell Signalling #9331), unphosphorylated GSK3β (Cell Signalling #9315), pCRMP2 (Abcam, #62478), β-catenin (BD Biosciences, #610153) or GAPDH (Cell Signalling #2118) in 4%...
outliers from data sets with Gaussian distribution were identified using the Extreme Studentised Deviate test. All data are expressed as mean values ± SEM. P-values < 0.05 are considered statistically significant and all significant results are indicated in the figures.

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Conflict of Interest statement. None declared.

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Table 1. Case information for individual HD and control cases from which post-mortem brain tissues were obtained

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Sex</th>
<th>Age</th>
<th>PMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD M</td>
<td>F</td>
<td>66.7</td>
<td>18.5</td>
</tr>
<tr>
<td>HD M</td>
<td>F</td>
<td>65.9</td>
<td>58.0</td>
</tr>
<tr>
<td>HD M</td>
<td>F</td>
<td>57.2</td>
<td>22.0</td>
</tr>
<tr>
<td>HD M</td>
<td>F</td>
<td>72.2</td>
<td>22.0</td>
</tr>
<tr>
<td>HD F</td>
<td>M</td>
<td>61.1</td>
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<td>M</td>
<td>66.6</td>
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<tr>
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<td>26.5</td>
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<tr>
<td>HD M</td>
<td>M</td>
<td>68.7</td>
<td>72.0</td>
</tr>
</tbody>
</table>

Post mortem interval (PMI) is in hours and age in years. Mean age and PMI for HD and control cases is shown (± SEM). M:F = ratio of males to females.
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