

Context dependent neuroprotective properties of prion protein (PrP)

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Although it has been known for more than twenty years that an aberrant conformation of the prion protein (PrP) is the causative agent in prion diseases, the role of PrP in normal biology is undetermined. Numerous studies have suggested a protective function for PrP, including protection from ischemic and excitotoxic lesions and several apoptotic insults. On the other hand, many observations have suggested the contrary, linking changes in PrP localization or domain structure— independent of infectious prion conformation—to severe neuronal damage. Surprisingly, a recent report suggests that PrP is a receptor for toxic oligomeric species of α - β , a pathogenic fragment of the amyloid precursor protein, and likely contributes to disease pathogenesis of Alzheimer's disease. We sought to access the role of PrP in diverse neurological disorders. First, we confirmed that PrP confers protection against ischemic damage using an acute stroke model, a well characterized association. After ischemic insult, PrP knockouts had dramatically increased infarct volumes and decreased behavioral performance compared to controls. To examine the potential of PrP's neuroprotective or neurotoxic properties in the context of other pathologies, we deleted PrP from several transgenic models of neurodegenerative disease. Deletion of PrP did not substantially alter the disease phenotypes of mouse models of Parkinson's disease or tauopathy. Deletion of PrP in one of two Huntington's disease models tested, R6/2, modestly slowed motor deterioration as measured on an accelerating rotarod but otherwise did not alter other major features of the disease. Finally, transgenic overexpression of PrP did not exacerbate the Huntington's motor phenotype. These results suggest that PrP has a context-dependent neuroprotective function and does not broadly contribute to the disease models tested herein.

Introduction

Neurodegenerative disorders are among the most common affliction of the aged population; millions suffer worldwide from Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis, Huntington's disease (HD), and several other more rare conditions, such as prion disease.^{1,2} Neurodegenerative diseases of aging typically manifest as clinically separable disorders in which certain regions of the brain deteriorate. One commonality for most common neurodegenerative diseases is the presence of specific misfolded protein(s).³ PD is characterized by the aggregation of α -synuclein in Lewy bodies within neuronal soma, and neuron loss in the *Substantia nigra* of the midbrain and other areas of the brain.⁴ An entire class of diseases, the tauopathies (TP), demonstrate an accumulation of the microtubule associated protein, tau.⁵ HD is a dominantly heritable, neurodegenerative disorder caused by a polyglutamine repeat expansion in the first exon of the huntingtin protein, resulting in neuronal

loss in the striatum and cerebral cortex.⁶ Developing therapies for these diseases is hampered by our poor understanding of the precise mechanism(s) of *how* misfolding of these proteins causes dysfunction and death and *which* misfolded species to target.

Prion diseases are an intensively studied class of neurodegenerative diseases, affecting a range of mammalian hosts, transmissible between species.⁷ Prion diseases also occur in sporadic and inherited forms. The prion protein (PrP) is an N-linked glycoprotein tethered to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor.⁸ The prion hypothesis posits that infectious prion disease arises when the normal host isoform of PrP, termed PrP^C, is converted into a misfolded conformer, PrP^{Sc} which is capable of further templating this process.⁸ Consistent with this notion, the higher the levels of PrP, the more rapidly prion disease progresses in transgenic mice overexpressing PrP.⁹ Advances into the understanding of infectious prion disease have shown that PrP expression in neurons is a requirement for neurotoxicity;¹⁰ moreover, PrP must retain its GPI anchor¹¹ to damage neural

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Table 1. Mouse strains used in this study

Mouse model	Common name	Genetic alteration	Original reference
Parkinson's Disease	D line	human α -synuclein overexpression	Masliah E, et al. 2000
Tauopathy	JNPL3	human P301L mutant Tau overexpression	Lewis J, et al. 2000
Huntington's Disease	R6/2, Bates	fragment of human Htt gene with n = 115–150 glutamine expansion	Mangiarini L, et al. 1996
Huntington's Disease	NI71-82Q, Ross-Borchelt	Fragment of human Htt with a n = 82 glutamine expansion	Schilling G, et al. 1999
PrP knockout	PrP KO, Edinburgh	Neomycin insertion into <i>Prnp</i> exon	Manson J, et al. 1994
PrP overexpression	Tga20	Mouse PrP overexpression	Fischer M, et al. 1996

tissue during prion disease. We have recently implicated heat shock factor 1 as a mediator of protection against prion disease *in vivo*¹² and other research suggests that inflammation is a major contributor to prion pathogenesis.¹³ Beyond these observations, the molecular mechanism(s) of prion-induced neuronal toxicity remain unresolved.^{14–17}

A multitude of biological functions have been attributed to PrP^C, with several lines of evidence suggesting a neuroprotective function.^{15,18–21} Studies by several groups have demonstrated that PrP knockout mice (KO) are more susceptible to ischemic damage^{22–24} and excitotoxic lesion by dampening activity of an NMDA receptor subtype.^{14,25} In addition, PrP can protect against toxicity associated with ectopic expression of its closest paralog, Doppel, as well as truncated PrP mutants.^{26–29} In cultured cells, PrP^C expression protects against apoptosis induced by overexpression of pro-apoptotic Bax³⁰ and against oxidative stress.^{31,32} Thus, there is ample evidence that PrP^C helps maintain cellular integrity during stressful conditions. However, this must be reconciled with the fact that PrP is a disease-causing agent when it misfolds during prion disease. Also, there is conflicting evidence suggesting that PrP^C is not neuroprotective and that it sensitizes cells to toxicity and death. Agents that cause ER stress show enhanced toxicity in cells expressing PrP.³² Also, when proteasome activity is compromised neurons expressing higher levels of PrP are more susceptible to apoptosis.³³ Mislocalizing PrP^C to the cytosol causes neurotoxicity *in vitro* and *in vivo*,^{33,34} possibly by inhibiting the activity of the proteasome.³⁴ To further complicate matters, several transgenic lines of mice overexpressing wild-type (WT) PrP develop neurologic disease,^{35,36} suggesting that too much PrP^C can wreak havoc on tissues.

A growing number of studies suggest indirect and direct influences of PrP on other neurodegenerative diseases. Several studies have implicated PrP in the pathogenesis of AD. One report reveals that PrP is a negative regulator of β -secretase activity, which leads to the production of a toxic fragment of the amyloid precursor protein, $a\text{-}\beta$.³⁷ The straightforward interpretation of this data is that PrP would be a protective factor against AD. However, Schwarze-Eicker and colleagues report that transgenic overexpression of PrP enhances amyloid plaque formation in a mouse model of AD.³⁸ Another striking finding is that PrP behaves as a cellular receptor for $a\text{-}\beta$ oligomeric species and thus appears to have a role in promoting neuronal dysfunction during AD.³⁹ Further experiments will be required to settle this question. Crosstalk between PrP and other neurodegenerative disease causing proteins is not limited to AD, as another report has suggested that PrP protects against polyglutamine aggregate toxicity.⁴⁰ Tau,

another pathological hallmark of AD, was recently implicated as an interaction partner of PrP in *in vitro* assays,⁴¹ raising a possible connection between TPs and PrP. Indeed, the deposition of neurofibrillary tangles of hyperphosphorylated tau protein, a pathological hallmark of AD and TP, has been noted in many cases of prion disease linked to mutations in PrP or sporadic Creutzfeldt Jacob disease (CJD).^{42,43} There is additional clinical overlap among neurodegenerative diseases that may involve PrP. Some cases of diagnosed HD are actually caused by a mutation in the gene encoding PrP (*PRNP*) rather than any alteration to the huntingtin gene.^{44,45} *PRNP* mutations can also be associated with some PD-like symptoms and neuropathology⁴⁶ and some demonstrate similarities to the tauopathies.⁴⁷ Collinge and colleagues described a kindred segregating pre-senile dementia, AD, HD, PD, Pick's disease, as well as prion disease (Gerstmann-Straussler-Scheinker syndrome and CJD). Remarkably, it was found that this family had a mutation within the *PRNP* coding sequence,⁴⁸ suggesting that mutant PrP could give rise to many different neurodegenerative disease symptomologies.

We conducted experiments to address two questions: (1) does the neuroprotective function of PrP extend to a broad spectrum of neurodegenerative pathologies? (2) Or alternatively, does PrP play a contributing role to neurodegenerative disease pathology? To address these questions we crossed transgenic models of PD, TP and HD (listed in Table 1) into a PrP KO background and followed the disease progression relative to that of control mice. We began by confirming the neuroprotective role of PrP in an acute ischemia model, where PrP deletion causes significantly higher levels of damage as measured by lesion volume and behavior. However, PrP deletion did not have a substantial impact on any of the neurodegenerative disease models tested, suggesting that—at least in so far as they are modeled in the mouse—PrP is not a major contributing factor to the disease processes we examined.

Results

PrP knockouts are more susceptible to ischemic injury. Reports by several groups have shown that PrP protects against ischemic damage.^{22–24} We performed middle cerebral artery occlusions (MCAO) on C57Bl/6J PrP KO and congenic control mice for 30 minutes followed by a 22 hour reperfusion. Cerebral blood flow was measured in PrP WT and KO mice undergoing MCAO to confirm that both groups had nearly equivalent blockages of blood flow in the brain (Fig. 1A). Similar to other reports, we observed that PrP KO mice were dramatically sensitized to ischemic insult. We performed behavioral testing on PrP KO mice and controls

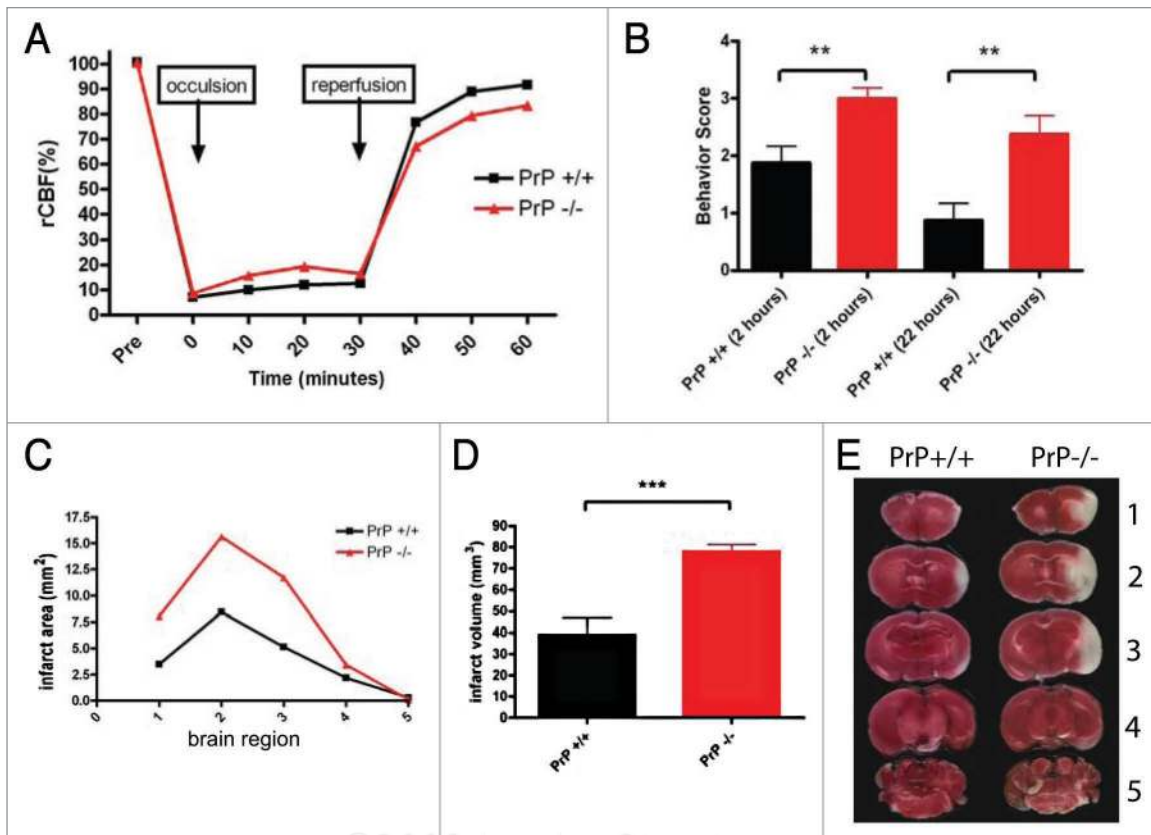


Figure 1. PrP knockout mice are more susceptible to ischemia induced by transient middle cerebral artery occlusion-reperfusion. (A) Cerebral blood flow was measured in PrP^{+/+} and PrP^{-/-} mice (n = 8 PrP WT and n = 7 PrP KO) undergoing MCAO to confirm that both groups had similar blockages of blood flow in the brain. (B) Behavioral testing of PrP KO and controls. Higher clinical scores indicate more severe injury both 2 and 24 hours after injury (p < 0.01, unpaired, two-tailed Student's t test). (C) Infarct area measured across five brain regions (1 anterior to 5 posterior shown in E). (D) Infarct volume in PrP^{+/+} and PrP^{-/-} brains (p < 0.001, two-tailed Student's t test). (E) Representative sections from brain regions 1–5 are shown for PrP^{+/+} and PrP^{-/-} brain sections from MCAO sections 1–5 are shown.

and noted that PrP KO mice present higher clinical scores, indicative of more severe injury (the details of clinical assessment can be found in the Methods) both 2 and 24 hours after injury (Fig. 1B). We next measured the infarct volume, the region of brain tissue that does not stain with vital dye, which was increased approximately 2-fold in PrP KO mice (Fig. 1C and D). An image of a control and PrP KO brain from MCAO treated mice is shown in Figure 1E.

Deletion of PrP in an α -synuclein transgenic model of parkinson's disease. Although no comprehensive model of PD existed at the onset of our studies, we chose to utilize a mouse model of PD which overexpresses human α -synuclein driven by a platelet-derived growth factor- β promoter⁵¹ (Table 1). This mouse was reported to mimic human pathologies seen in PD, such as loss of dopaminergic neurons, accumulation of α -synuclein aggregates, and motor dysfunction. Details of breeding and genetic backgrounds for all mouse lines are reported in the Methods section. Mice that were PD Tg⁺ and either wild-type (+/+), heterozygous (+/-) or null (-/-) for PrP were tested twice per month for motor performance by accelerating rotarod (Fig. 2A).⁵⁶ We observed only a small decline in motor performance in PrP^{+/+} α -synuclein Tg mice, suggesting that on the mixed genetic background of

our study there was not a significant progressive rotarod deficit in α -synuclein Tg⁺ mice or that the repeated testing had a masking effect on this phenotype (Fig. 2A). For example, the latency to fall off the accelerating rotarod was approximately 150 seconds at 5–8 months and even by 18 months the latency to fall had only decreased to approximately 100 seconds. We noted a trend that PrP^{-/-} and PrP^{+/+} mice had worse motor performance from 12–18 months, but it reached statistical significance only at 14 months. Given the high variability within these groups and the fact that at 19–21 months all the groups behaved similarly, we concluded that if PrP deletion enhanced the disease phenotype, the effect was modest. Alternative tests with higher sensitivity have revealed phenotypes in other α -synuclein Tg mice.⁵⁷

We also assessed survival in the α -synuclein Tg⁺ mice with different PrP gene dosage. There were no significant differences between α -synuclein Tg⁺ PrP^{+/+}, +/- or -/- in terms of survival (Fig. 2B). Most of these mice lived an apparently normal lifespan, although we did not include sufficient α -synuclein Tg^{-/-} controls to prove this point. Finally, we examined α -synuclein staining in several α -synuclein Tg⁺ PrP^{+/+} and PrP^{-/-} brains taken at 12–16 months of age. We did not detect any differences between groups in terms of α -synuclein deposition (Fig. 2C–E).

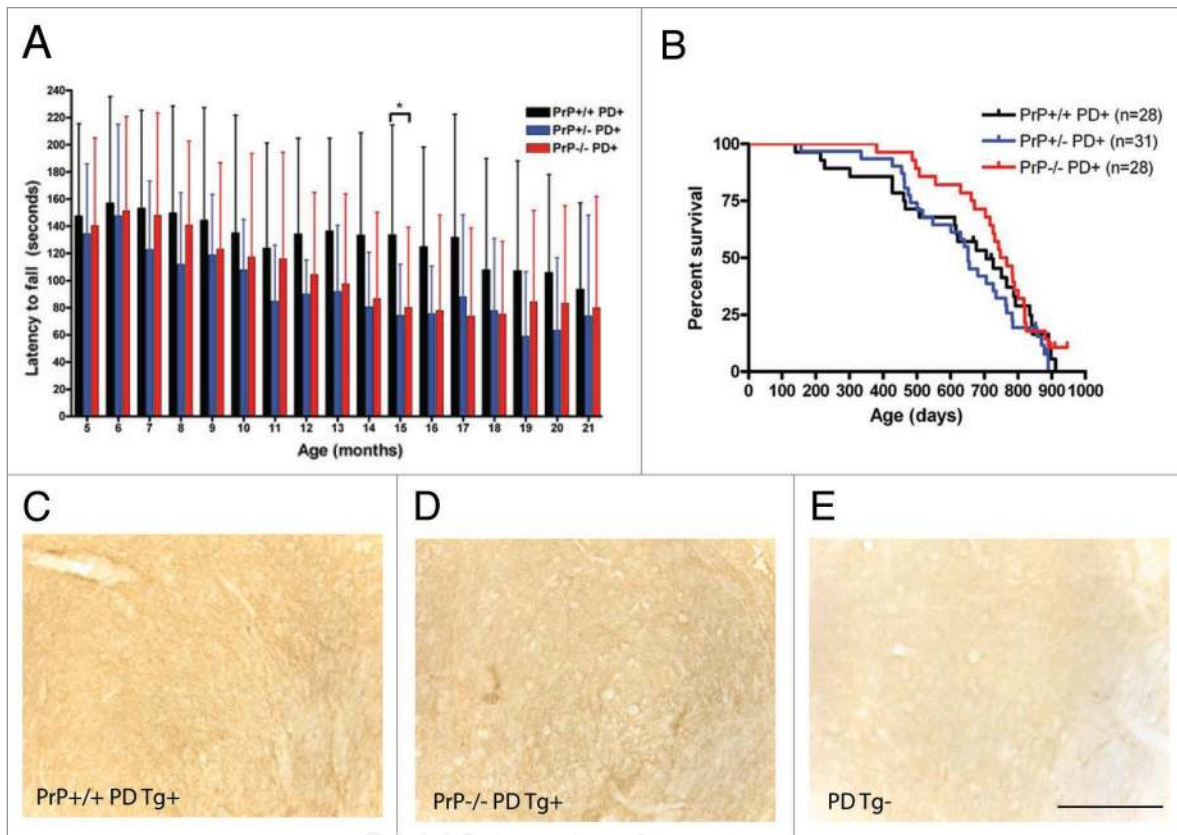


Figure 2. PrP deletion in α -synuclein Tg mice. (A) Motor performance was assessed on an accelerating rotarod on a monthly basis ($n = 13$ – 19 PrP^{+/+}, $n = 9$ – 17 PrP^{+/-} and $n = 16$ – 21 PrP^{-/-} PD Tg⁺ mice per time point; data shown as mean plus standard deviation). At 15 months of age PrP^{+/+} PD⁺ mice outperformed PrP^{-/-} PD⁺ mice (* $p < 0.05$, unpaired, two-tailed Student's t test). (B) Survival analysis did not show a significant differences between PrP^{+/+} ($n = 28$), PrP^{+/-} ($n = 31$) and PrP^{-/-} ($n = 28$) α -synuclein overexpression Tg mice (log rank test). (C and D) α -synuclein staining of the ventral midbrain (Substantia nigra). (E) a negative control from an α -synuclein Tg mouse is shown. Scale bar corresponds to 20 μ m.

Deletion of PrP in a mutant tau transgenic model of tauopathy. We utilized a transgenic model of TP that overexpresses mutant human Tau (P301L), which is associated with frontotemporal dementia associated with Parkinson's disease in humans, driven by a modified murine PrP promoter.⁵⁰ As previously described the P301L Tau Tg⁺ mice develop a severe motor phenotype, where their hind limbs eventually become completely paralyzed and can be utilized as a general model of TP.⁵⁰ Once signs of hind limb dysfunction were observed the mice typically had to be euthanized within 4 weeks. The motor performance of the populations of Tau Tg⁺ PrP^{+/+}, +/- and -/- mice showed a progressive decline in rotarod performance, but the kinetics of this decrease were indistinguishable between all groups of mice with different PrP levels (Fig. 3A). The decline in mean rotarod performance does not properly reflect the extreme paralysis of the Tau mutant mice because mice that developed paralysis were euthanized and removed from our rotarod study.

We determined the survival of the P301L Tau Tg mice with different PrP gene dosages. There was a clear difference in survival between Tau Tg⁺ and Tau Tg^{-/-} mice (both groups with WT levels of PrP) (data not shown). However, there were no statistically significant differences in the survival of any of the Tau Tg⁺ mice with 2, 1, or no copies of PrP (Fig. 3B). However, we

also assessed tau pathology in several mice from this study. As expected, we observed a similar distribution of tau aggregation in brains from Tau Tg⁺ PrP^{+/+} and Tau Tg⁺ PrP^{-/-} (Fig. 3C and D).

Deletion of PrP in polyglutamine expanded N-terminal huntingtin models of huntington's disease. We also tested whether PrP deletion would alter the phenotype of a mouse model of HD. This commonly used HD model, termed "R6/2", expresses the first exon of human huntingtin with an uninterrupted stretch of approximately 115–150 glutamines.⁵² These mice show severe motor impairments, dramatic weight loss, and many other phenotypes.^{52,55,58} Motor impairment was assessed by testing these mice twice per week on an accelerating rotarod. Average values of these bi-weekly trials suggested that the motor impairment was slightly less severe in HD Tg⁺ PrP^{-/-} mice (Fig. 4A). This subtle improvement persisted from 6 weeks until 12 weeks, but by 13 weeks and beyond there were no significant differences in rotarod performance between groups. We assessed survival in the HD Tg⁺ mice with differing PrP gene dosages. There were no significant differences in the survival of HD Tg⁺ PrP^{+/+}, +/- or -/- mice (Fig. 4B). We performed additional behavioral testing of HD mutant mice, including a tail suspension and escaping test; in this test a mouse is held suspended by its tail and healthy mice will typically "escape" onto the fingers of the experimenter and almost

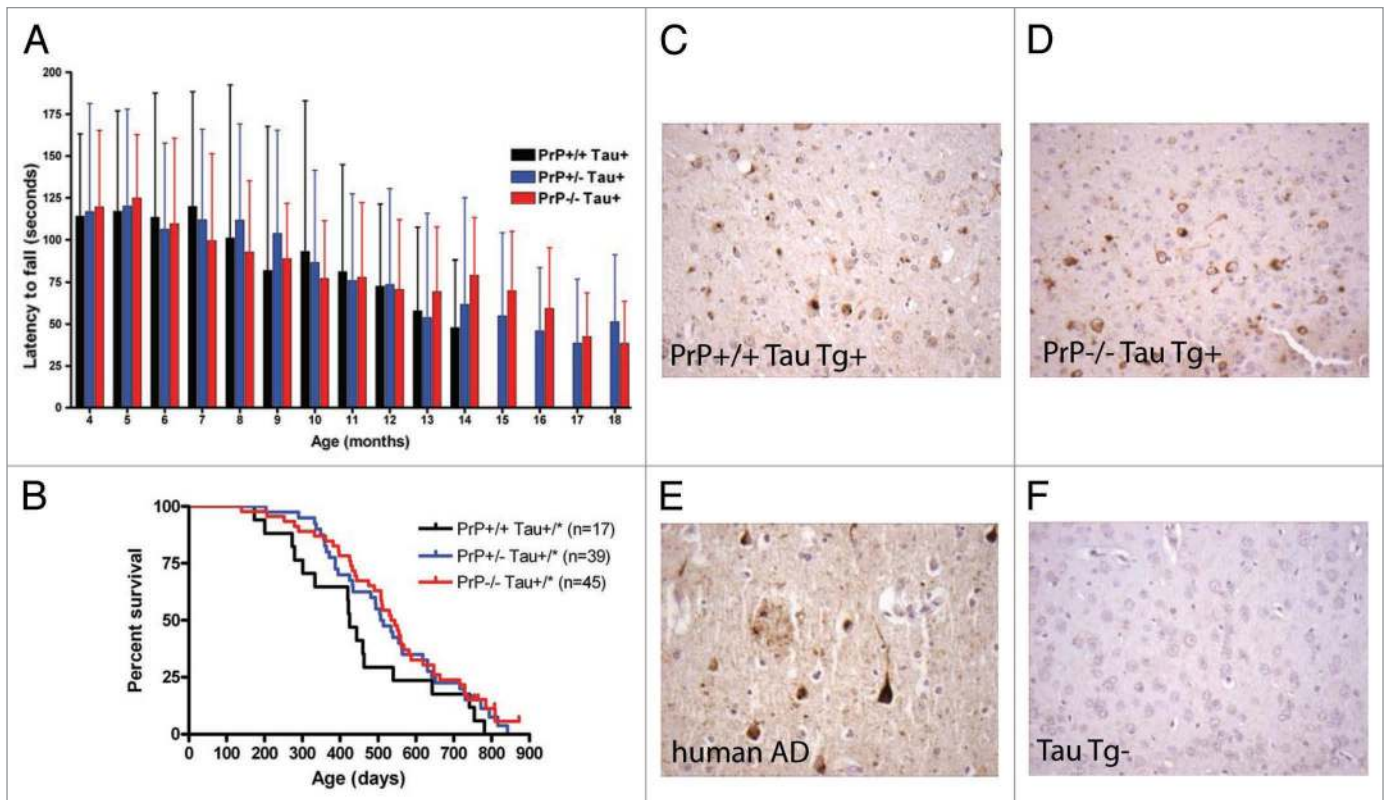


Figure 3. PrP deletion in P301L Tau Tg mice. (A) Motor performance was assessed on an accelerating rotarod; there were no significant differences (unpaired, two-tailed Student's t test) among the experimental groups (n = 9–14 PrP^{+/+} Tau Tg⁺, n = 7–19 PrP^{+/-} Tau Tg⁺, n = 15–26 PrP^{-/-} Tau Tg⁺). (B) Survival analysis did not show a significant differences between PrP^{+/+} (n = 17), PrP^{+/-} (n = 39) and PrP^{-/-} (n = 45) P301L Tau Tg mice (log rank test). (C and D) Representative image of neurofibrillary tangles detected by Tau immunohistochemistry. (E) A positive control of a human Alzheimer's and (F) a negative control from a Tau Tg⁻ mice is shown.

never clasp their fore and hind limbs. HD Tg⁺ mice progressively develop the clasp phenotype and lose their ability to escape, independent of PrP deletion (Fig. 4C and D). We measured body weights and observed a similar decline in body weight in HD Tg⁺ mice that was also independent of PrP levels (data not shown). Preliminary analysis of home cage behaviors of HD Tg⁺ PrP^{+/+} and HD Tg⁺ PrP^{-/-} using video based behavior recognition technology⁵⁵ also did not suggest any phenotypic differences between our experimental groups (data not shown). We also examined the brains of HD Tg⁺ PrP^{+/+} and HD Tg⁺ PrP^{-/-} mice for huntingtin aggregation. Dramatic nuclear aggregates of huntingtin were observed by immunohistochemistry with no notable differences between HD Tg⁺ PrP^{+/+} and HD Tg⁺ PrP^{-/-} (Fig. 4E–H). Thus, the HD Tg phenotype is mostly unaffected by PrP deletion with the exception of a modest improvement of rotarod performance in an early phase of disease. To further investigate the possible contribution of PrP to HD we also utilized a less severe HD model, termed “N171-82Q” (Table 1).⁵⁹ When deleted for PrP, the survival of this HD model was not altered (Fig. 4I), nor was motor performance, which was tested weekly from 6 to 19 weeks of age (data not shown).

Overexpression of PrP in polyglutamine expanded N-terminal huntingtin model of huntington's disease. Since we observed a subtle amelioration of the decline in rotarod performance in HD

Tg⁺ PrP^{-/-} mice, we predicted that overexpression of PrP might enhance the well-described HD Tg phenotype. To test this prediction, we crossed the well characterized PrP overexpression “Tga20” transgenic mouse⁹ on a C57Bl/6 genetic background⁵³ to the R6/2 HD Tg⁺ mice. With one copy of the Tga20 *Prnp* transgene (herein referred to as “PrP OE”) in a WT PrP background these mice express approximately 4–5 fold more PrP than WT mice.⁵³ Motor performance was assessed by rotarod and was indistinguishable between HD⁺ PrP OE⁺ mice and HD⁺ PrP OE⁻ mice (Fig. 5A). We did not observe any effect on the survival of the HD Tg⁺ mice whether PrP was expressed at a WT level or overexpressed (Fig. 5B). The clasp and escaping phenotype was also similar between both groups of mice (Fig. 5C and D). Home cage behavior analysis, measuring hanging, rearing, jumping, eating, drinking, and distance traveled did not reveal any substantial differences between HD⁺ PrP OE⁺ and HD⁺ PrP OE⁻ (data not shown, except for distance traveled, which is shown in Fig. 5E). From these experiments we concluded that PrP overexpression does not affect the R6/2 HD phenotype.

Discussion

In summary, we performed a series of long-term experiments designed to test whether PrP promotes or protects against disease

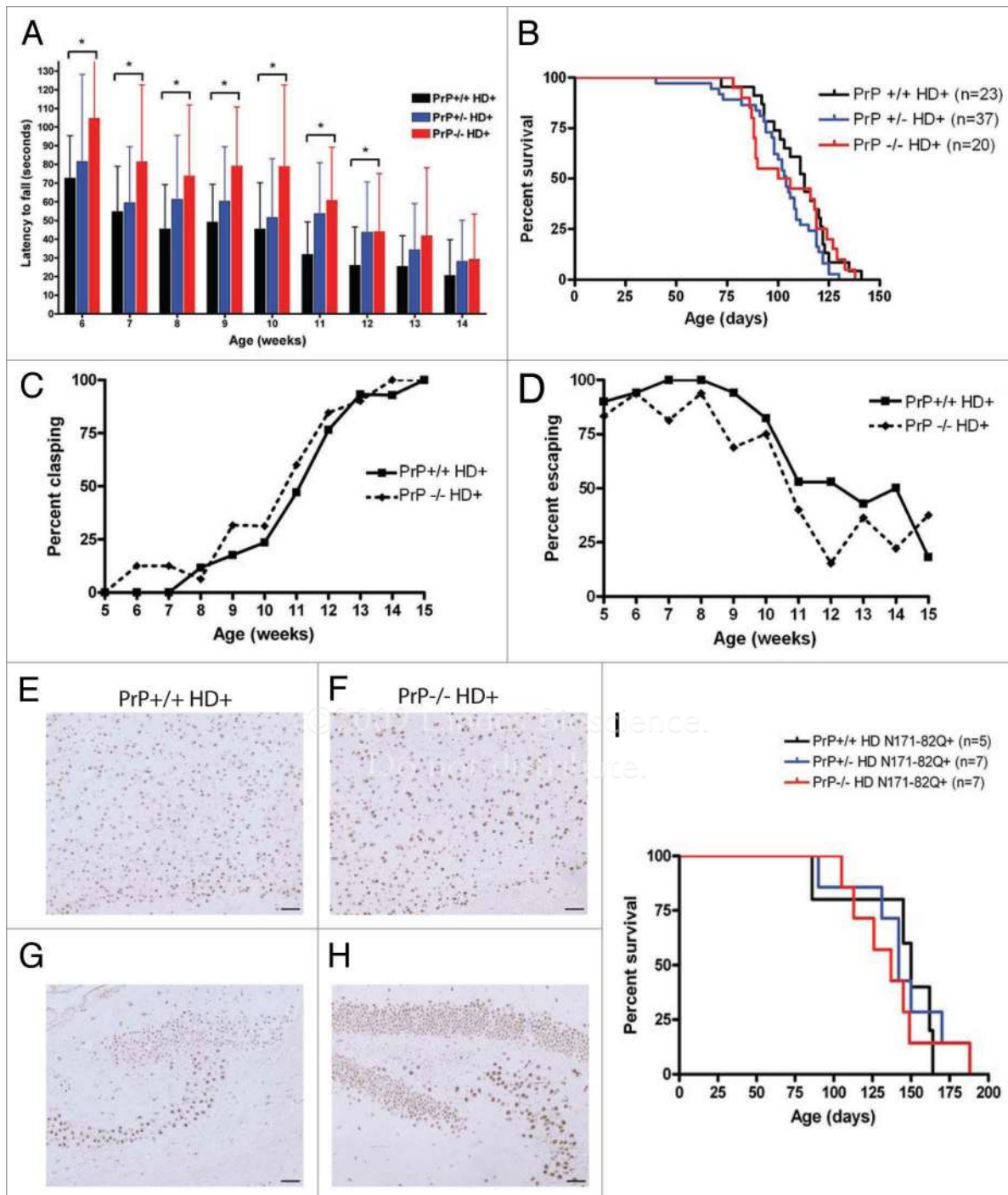


Figure 4. PrP deletion in Huntington's disease models. (A) Rotarod performance of PrP^{-/-} HD Tg⁺ mice was improved over that of PrP^{+/+} HD Tg⁺ until the later points of disease (12–14 weeks, *p < 0.05, unpaired, two-tailed Student's t test—individual comparisons of PrP^{+/+} to PrP^{-/-} at each time point). (B) Survival of R6/2 HD mice was not altered by deletion of PrP (n = 23 PrP^{+/+}, n = 37 PrP^{+/-}, and n = 20 PrP^{-/-} R6/2 HD Tg⁺). (C) Clamping and (D) escaping were not altered by PrP deletion in HD Tg⁺ mice (HD Tg⁺ mice all escape showed 100% escape at all time points and never clasp at any time point, data not shown). (E and F) Accumulation of nuclear aggregates of huntingtin in the cortex of PrP^{+/+} HD⁺ (E) and PrP^{-/-} HD⁺ (F) and hippocampus of PrP^{+/+} HD⁺ (G) and PrP^{-/-} HD⁺ (H). (I) Survival plot of N171-82Q HD mice with PrP^{+/+} (n = 5), PrP^{+/-} (n = 7) and PrP^{-/-} (n = 7); there were no significant differences in survival (log rank test).

in a panel of mouse models of neurodegenerative diseases. This study represents one of the most extensive cases where a single genetic mutation has been tested in combination with so many mammalian disease models. We observed that despite providing

strong protection against ischemia, PrP was not a major modulator of the respective disease phenotypes of models of PD, TP and HD except with the R6/2 HD Tg PrP^{-/-} mice there was a modest delay in the impairment of motor performance relative to R6/2

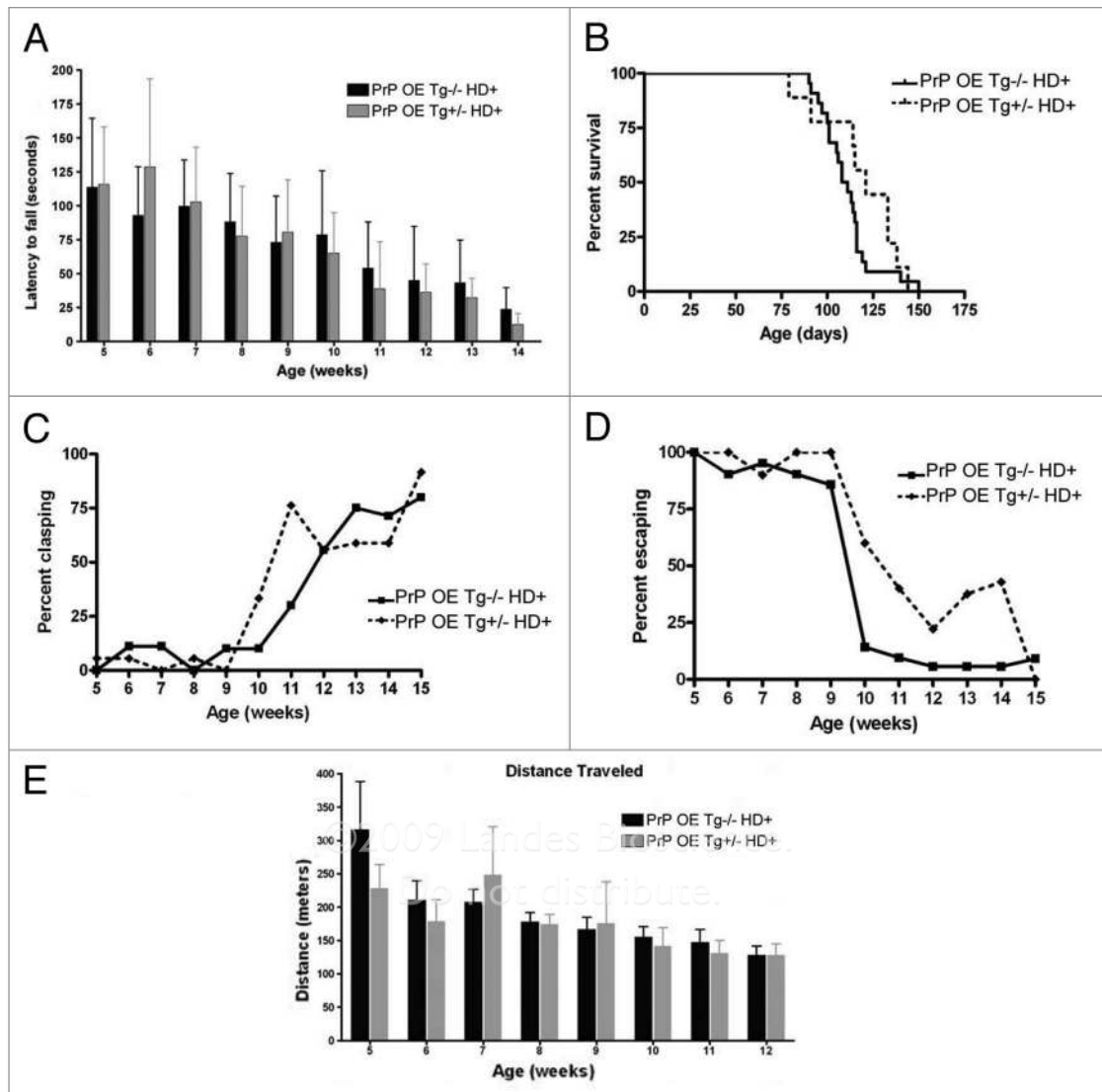


Figure 5. Overexpression of PrP in the R6/2 Huntington's disease Tg. (A) There were no significant differences in rotarod performance of HD Tg⁺ PrP OE⁻ and HD Tg⁺ PrP OE⁺ (n = 22 HD Tg⁺ PrP OE⁻; n = 9 HD Tg⁺ PrP OE⁺; unpaired, two-tailed Student's t test). (B) Survival of R6/2 HD mice was not altered by overexpression of PrP (same mice as used in survival study; log rank test). (C) Clamping and (D) escaping responses during tail suspension tests of HD Tg⁺ PrP OE⁻ and HD Tg⁺ PrP OE⁺ (HD Tg⁺ mice all showed 100% escaping responses at all time points tested and never clasped, data not shown). (E) Lateral distance traveled in the home cage over a 24 hour video recording (n = 5–9 mice per group).

HD Tg PrP^{+/+} mice. However, the other observed phenotypes of R6/2 mice, such as survival, were unaffected and transgenic overexpression of PrP did not affect the R6/2 phenotype. In addition, we crossed the PrP KO to a different transgenic model of HD⁵⁹ and did not observe any effect on the motor performance or survival dependent on PrP genotype. In the α -synuclein mouse model of PD there was a trend toward PrP^{+/+} mice performing better than PrP^{+/-} and PrP^{-/-} mice on the rotarod, but these animals failed to repeat the strong motor defects originally described in these transgenic mice.⁵¹ The lifespan of the α -synuclein transgenic mice (independent of PrP genotype) are quite long, on par with that of normal mice. Repeating this experiment with the next generation of α -synuclein transgenic mice^{57,60} may better address the potential role of PrP in contributing or protecting against α -synuclein toxicity.

Our behavioral and neuropathological assays could only detect a major modification in any of the disease phenotypes tested, with the exception of the HD model. A closer examination of behavior and/or pathology may have revealed a subtle role for PrP in promoting or protecting against neurodegenerative diseases. While intercrossing of strains on pure genetic backgrounds may have facilitated detection of small differences between PrP-deleted neurodegenerative disease models and controls, such backcrosses have their own limitations. Indeed, the R6/2 HD model is not viable on a pure background.⁵² Moreover, backcrossing to a pure background takes years and for reasons that are not clear, backcrossing neurodegenerative disease models onto pure backgrounds often does not increase the succinctness or robustness of their disease phenotypes (as with the P301L Tau transgenic [data not shown]). Thus, in our study we controlled for genetic background as well

as was feasible with our breeding strategy by obtaining littermate controls from intercrossing *Prnp*^{+/-} parents (one of which also carried the neurodegenerative disease transgene).

Our study touches upon a broader question of whether there is cross-talk among aggregation-prone proteins in neurodegenerative diseases, a notion well supported by the human disease literature (discussed in the Introduction). An elegant study in *C. elegans* demonstrated that under conditions of perturbed protein folding homeostasis, such as when a huntingtin fragment containing a polyglutamine expansion is expressed, a normally innocuous mutation became deleterious.⁶¹ Thus, misfolded proteins can act synergistically to harm cells. Whether these findings apply to human neurodegenerative diseases of aging is a pressing question and several findings suggest this question is worth further exploration, despite the failure of our study to reveal any contribution of wild-type PrP to other neurodegenerative diseases.

Based on our mostly negative findings we cannot rule out the importance of PrP as a contributing or protective factor to human neurodegenerative diseases. By using the best transgenic models of neurodegenerative disease available to us at the initiation of our study in 2002 our observations suggest that PrP does not have a major role in these models. Given the large number of mouse models of neurodegeneration, particularly AD, TP and PD that have been developed over the past seven years and the recent reports connecting PrP to β -amyloid generation and uptake, this question may be worth revisiting. A crucial experiment to test the relationship between PrP and AD will be to cross the PrP KO to a model mouse model of AD and assess memory loss and pathology. Although the results presented within this report are mainly negative, we report them because they represent a long and costly set of experiments that addresses a critical question to the field of neurodegenerative disease.

Materials and Methods

Mouse strains, genotyping and breeding strategy. All experiments using mice were approved by the institutional animal care and use committee. PrP KO mice⁴⁹ were obtained from Rick Race (Rocky Mountain Laboratories NIAID/NIH) on a mixed genetic background comprised of 129/Ola, C57Bl/10 and C57Bl/6 (2 backcrosses to C57Bl/6 were performed in our facility). The “JNPL3” tau mutant (P301L)⁵⁰ on a mixed genetic background comprised of C57Bl/6, DBA/2 and Swiss Webster was obtained from Taconic Farms. The PD mouse⁵¹ on a mixed genetic background composed of C57Bl/6 and DBA/2 was kindly provided by Brad Hyman (Harvard Medical School). The “R6/2” HD mouse⁵² on a mixed genetic background comprised of CBA and C57Bl/6 was obtained from Jackson Laboratories as ovarian transferred females. Transgenic mice overexpressing normal mouse *Prnp* (“Tga20”) was obtained from the European Mutant Mouse resource center and subsequently the PrP deletion alleles were bred out while the mice were backcrossed for at least six generations of C57Bl/6.⁵³ A summary of all mice used in this paper is presented in Table 1.

DNA was extracted from tail clippings digested with proteinase K by isopropanol extraction. Amplification of transgenes

was done according to published protocols for: PrP deletion or overexpression Tg,⁵³ human α -synuclein,⁵¹ huntingtin fragment,⁵² and the following primers were used to amplify P301L Tau 5'TGA ACC AGG ATG GCT GAG C3' and 5'TTG TCA TCG CTT CCA GTC C3'.

The general breeding strategy used to generate experimental mice was to cross PrP KO mice to Tg mice expressing either human α -synuclein, mutant Tau or polyglutamine expanded HD. The F1 progeny that were heterozygous for the PrP KO deletion allele and transgenic for the disease transgene were intercrossed, except for HD mice since Tg⁺ females do not breed. Thus, we crossed F₁ males that were HD Tg⁺ PrP^{+/-} to F₁ female HD Tg^{-/-} PrP^{+/-} to obtain our experimental F2 animals. Expected Mendelian ratios of progeny were obtained from F1 intercrosses of PD and HD mice, but there was a conspicuous lack of PrP^{+/-} mice in the TP crosses. We confirmed that this effect was independent of the mutant Tau transgene and was likely a polygenic interaction between PrP deletion and a combination of alleles in the mutant tau mouse genetic background, as PrP^{+/-} mice were recovered in expected Mendelian ratios in crosses to all the inbred lines (C57Bl/6, DBA/2 and Swiss Webster) that comprise the mixed background of the mutant tau line (data not shown).

Ischemia. For ischemia studies male mice aged 2–3 months and weighing between 25 to 30 grams were used. The PrP KO mice and controls were derived from the same breeding colony, which was at 10–11 generations of backcross of the PrP knockout allele onto a pure C57Bl/6J background. Transient middle cerebral artery occlusion was performed according to established protocols.⁵⁴ Briefly, animals were anesthetized with 2% isoflurane and maintained on 1.5% isoflurane in 70% N₂O and 30% O₂ by a face mask. Cerebral infarcts were produced by 30 minutes of MCA occlusion followed by reperfusion and monitored as previously described.⁵⁴ Cerebral infarct sizes were determined by image analysis (M4; Imaging Research) of vital dye (2,3,5-triphenyltetrazolium chloride) stained brain sections. Infarct volumes were calculated by integrating the infarct area in each brain section of the brain (shown in Fig. 1E). For neurological evaluation deficits were measured on a well established five-point neurological scale: 0, no neurologic deficit; 1, failure to extend the left forepaw fully; 2, circling to the left; 3, falling or leaning over to the left; 4, no spontaneous walking and a depressed level of consciousness; or 5, dead.⁵⁴ All animals tested had a score of 0 before undergoing fMCAO.

Behavioral analysis. For motor performance, all mice were tested on an Ugo Basile accelerating rotarod, which was modified by placing a rubber bike inner tube on the rod to deter gripping. Mice were placed on the rotarod, and then the rotarod was accelerated 5 seconds after the mice were placed on the rod. The amount of time the mice remained on the rotarod was recorded. HD mice were weighed, rotarod tested and clasp/escape tested twice weekly, beginning at 6 weeks of age (+/-1 week). For reporting of rotarod data an average value from two trials was taken for HD mice. HD mice were also tested for the ‘claspings’ phenotype⁵² by suspending them from their tails with close proximity to the experimenter’s hand to allow for “escaping” which was also recorded. For claspings values a mouse was

scored as positive if it clasped once or both times in twice-weekly trials. PrP OE Tg HD mice were tested similarly and in addition they were video recorded once per week for home cage behavior analysis as described previously.⁵⁵ PD mice were tested biweekly, beginning at 6 months old (+/- 2 months)—data is shown as a monthly average for PD mice. Mutant Tau Tg mice were tested weekly, beginning at 10 weeks old (+/- 2 weeks) then tested biweekly once the mice reached 6 months old until they were removed from the study due to paralysis. Rotarod data for TP mice is reported as group averages which were derived from averaging across each trial per month per mouse. For survival analysis, mice were sacrificed when they were moribund as determined in consultation with our veterinary staff. TP mice developed a severe hindlimb paresis and were sacrificed when they could not ambulate. HD mice were sacrificed when their body condition reached 1–2; many mice were found dead or died when being handled from severe seizures. PD mice were sacrificed when they displayed low body condition.

Immunohistochemistry. For α -synuclein staining, coronal brain sections (40 μ m) were cut on a Leica CM 1800 cryostat. Sections were washed in phosphate buffered saline (PBS, pH 7.4) and then incubated in 0.5% H₂O₂ in PBS for 15 min to block endogenous peroxidase. After washing in PBS, sections are incubated in mouse IgG blocking reagent (M.O.M. kit, Vector Laboratories, Burlingame, CA, USA) for 1 hour. Sections are then incubated in the primary antibody, mouse anti-alpha synuclein (1:250, BD Biosciences, San Jose, CA, USA) at 4°C overnight. Control sections are incubated with mouse IgG₁ (1 μ g/ml, Sigma). Sections were washed in PBS and then incubated in the secondary antibody, biotinylated goat anti-mouse IgG (1:250, Millipore, Billerica, MA) for 2 hours. The avidin-biotin complex method was used to detect the secondary antibody (ABC elite kit, Vector laboratories, Burlingame, CA) and the reaction

product was visualized by 3,3'-diaminobenzidine tetrachloride (DAB, Sigma, St. Louis, MO). Sections were mounted on gelatin coated slides and air dried overnight. Sections were then dehydrated and cleared with xylene, mounted with Eukit mounting medium (Calibrated Instruments, Hawthorne, NY), and examined under bright-field illumination with a Zeiss Axioskop microscope (Thornwood, NY). Digital images were captured by a Spot digital camera (Sterling Heights, MI). Neurofibrillary tangles of Tau were detected by immunostaining with a rabbit polyclonal anti-human Tau clone A 0024 (Dako) used at a 1:3,000 dilution. Paraffin sections were dewaxed and then formic acid (90%) treated for five minutes prior to standard immunohistochemical staining as described above. Huntingtin immunostaining was performed similarly, using 10% normal horse serum in PBS for 1 hour to block non-specific antibody binding and using goat anti-Huntingtin 1:100 (Santa Cruz Biotechnology, CA) at room temperature overnight. Control sections were incubated with goat IgG at 2 μ g/ml. After rinses in PBS, the sections were incubated in biotinylated horse anti-goat antibody (1:200) (Vector ABC Elite, Burlingame, CA) for 2 hr at room temperature. After several rinses in PBS, the sections were incubated for 2 hr in avidin-biotin complex (Vector ABC Elite) in PBS.

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