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Prion Protein-Hemin Interaction Upregulates Hemoglobin Synthesis: Implications for Cerebral Hemorrhage and Sporadic Creutzfeldt-Jakob Disease

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Abstract

Hemin is known to induce endocytosis of prion-protein (PrP^{C}) from the neuronal plasma membrane, potentially limiting propagation of the disease causing PrP-scrapie (PrP^{Sc}) isoform. Hemin is therefore an attractive disease-modifying option for sporadic Creutzfeldt-Jakob disease (sCJD), a human prion disorder with no effective treatment. The hemin-PrP^{C} interaction is also of interest in cerebral-hemorrhage (CH), a condition where potentially toxic hemin molecules come in contact with neuronal PrP^{C}. Interestingly, PrP^{C} is upregulated in penumbric neurons surrounding CH and is known to confer neuroprotection in a dose-dependent manner. The underlying mechanism, however, is not clear. Here, we report that hemin binds PrP^{C} on diverse cell lines, resulting in its aggregation or degradation in a cell-type specific manner. Surprisingly, the hemin-PrP^{C} interaction upregulates Hb synthesis in hematopoietic cells, a response reversed by deleting the hemin-binding octa-peptide repeat region of PrP^{C}. A similar response is noted in brain organotypic cultures where exposure to hemin induces significantly more α-globin in wild-type ($PrP^{+/+}$) relative to PrP-knock-out ($PrP^{-/-}$) samples. Furthermore, red blood cells and brain tissue from $PrP^{-/-}$ mice show significantly less α-globin relative to $PrP^{+/+}$ controls, indicating a positive effect of PrP^{C} on Hb synthesis under physiological conditions as well. Surprisingly, levels of α-globin are significantly higher in sCJD brain tissue relative to controls, suggesting compensatory upregulation of Hb synthesis by surviving neurons or misregulation in diseased brains. These observations reveal a unique function of PrP^{C} that is likely to impact the therapeutic management of CH and sCJD.

Keywords

α-Globin; hemin; neuronal hemoglobin; prion protein; sCJD

INTRODUCTION

Prion protein (PrP^{C}) is a ubiquitously expressed plasma membrane glycoprotein most abundant on neuronal cells. Although associated with diverse physiological functions, PrP^{C} is mostly known as the substrate for PrP-scrapie (PrP^{Sc}), a β-sheet rich isoform implicated in

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the pathogenesis of neurodegenerative conditions known as prion disorders. Sporadic Creutzfeldt-Jakob disease (sCJD) is the most common human prion disorder for which there is no effective treatment [1]. Search for possible therapeutic drugs led to the identification of certain porphyrins that delayed disease progression in experimental models [2]. Among these, hemin emerged as an attractive option because it induced rapid endocytosis of PrP^{C}, thereby limiting further replication of PrP^{Sc} [3]. The interaction of hemin with PrP^{C} was therefore thoroughly examined using recombinant PrP^{C} and purified hemin *in vitro*, and in mouse neuroblastoma cells in culture where extracellular hemin induced rapid internalization of plasma membrane PrP^{C} followed by aggregation in an intracellular compartment. Deletion of the N-terminal octa-peptide repeats of PrP^{C} abolished this interaction, associating this region with hemin binding activity [3].

Besides sCJD, the interaction of hemin with PrP^{C} is of clinical significance in cerebral hemorrhage (CH) where heme released from lysed red blood cells (RBCs) is rapidly oxidized to hemin and comes in contact with neuronal PrP^{C} [4, 5]. It is believed that hemin can reach concentrations of up to 10 mM in affected brain regions [6], and is highly toxic due to its redox-active nature and the ability to intercalate within lipid membranes [6–8]. Mechanisms are therefore in place to clear free heme from the extra-cellular milieu by high affinity heme binding proteins such as hemopexin and haptoglobin [9]. Heme is also internalized by neurons, astrocytes, and macrophages by heme carrier protein 1 (HCP-1) and rapidly metabolized by the heme-oxygenase family of enzymes [10–14]. Despite these protective mechanisms, free hemin is a significant cause of neurotoxicity in CH. It is notable that PrP^{C} is upregulated in regions affected by CH and is believed to provide neuroprotection in a dose-dependent manner [15–17]. Thus, experimentally induced cerebral hemorrhage causes significantly more neuronal death in mice lacking PrP ($PrP^{-/-}$) [18] compared to wild-type controls ($PrP^{+/+}$) [16, 17, 19–21], and relatively less neuronal injury in mice overexpressing PrP^{C} [16, 22]. The underlying mechanism, however, has remained unclear.

Here, we evaluated the downstream effects of hemin-PrP^{C} interaction using a combination of cell lines, transgenic mouse models, and brain tissue from sCJD and age-matched non-dementia controls. The main emphasis was on hemin-induced synthesis of Hb, a phenomenon studied extensively in the erythroleukemia cell line K562 [23–28] and reported recently in neuronal and glial cells [29–33]. Since neurons express modulators of hemoglobin synthesis such as the erythropoietin receptor and the hypoxia-inducible factor HIF1 [34–36], modulation of neuronal Hb by PrP^{C} via hemin or otherwise is likely to improve neuronal viability in pathological conditions such as CH and sCJD.

In the experimental paradigms described below, expression of α-globin was considered representative of Hb concentration since this globin chain is common to both fetal Hb expressed by K562 cells and adult Hb synthesized by mouse and human RBCs and brain tissue. Under physiological conditions the expression of α-globin and β-globin chains is closely balanced since free α-globin has a tendency to aggregate and cause toxicity [37, 38].

We report that cell surface PrP^{C} binds and internalizes extracellular hemin in hematopoietic and neuronal cell lines. This interaction is reversed by deleting the octa-peptide repeats of

PrP^C [3], associating this region with hemin-binding activity. More importantly, PrP^C upregulates hemin-mediated synthesis of Hb in K562 cells and mouse brain organotypic cultures, indicating a positive role in hematopoietic and neuronal/glial cell Hb synthesis. Moreover, levels of α-globin and β-globin are reduced in RBCs and brain tissue of $PrP^{-/-}$ mice relative to $PrP^{+/+}$ controls, suggesting PrP^C-mediated modulation of Hb synthesis under physiological conditions as well. Unexpectedly, levels of α-globin are significantly increased in sCJD brain samples relative to non-dementia controls, indicating a compensatory response by surviving neurons or altered synthesis of Hb in diseased brains. These observations are discussed in the context of CH and sCJD where neuronal and glial cell Hb synthesis are likely to provide neuroprotection.

MATERIALS AND METHODS

Animals and ethics statement

Colonies of FVB/NJ wild type ($PrP^{+/+}$) and transgenic mice lacking PrP ($PrP^{-/-}$) [18] were housed in the animal facility at Case Western Reserve University (CWRU) under a 12-h day-night cycle and received *ad libitum* access to food and water. Animal protocols and procedures were approved by the Institutional Animal Care Committee and conformed to the recommendations of the American Veterinary Medical Association Panel on Euthanasia, United States Department of Agriculture, Public Health Service regulations and guidelines, and the Department of Health and Human Services, National Institutes of Health. The approved protocol number is 2015–0027. Standard Operating Procedures and reference materials were provided by the IACUC Office for animal use. The animal health program was directed by the Case Animal Resource Center Director, W. John Dur-fee, DVM, Diplomate ACLAM, and provided by two full-time veterinarians. Animals in each room were observed daily for signs of illness by the animal technician responsible for providing husbandry. Medical records and documentation of experimental use were maintained individually or by cage group. Veterinary technicians under the direction of the attending veterinarian provided routine veterinary medical care to all animals. Animal care and use was additionally monitored for training and compliance issues by the Training and Compliance Manager. AAALAC Accreditation, July 18, 2012 (current accreditation letter pending). USDA Registration is valid until August 23, 2017. The Case PHS Assurance number is A-3145-01, valid until 04/30/19.

Brain samples from autopsy-confirmed cases of sCJD (age 61, 70, 61, 65, 65, 73, 59, and 65 years) were obtained from the National Prion Disease Pathology Surveillance center (NPDPSC) at CWRU. Non-dementia human brain samples (age 74, 82, 77, 73, 73, 72, 77, and 79 years) were from Harvard Brain Tissue Resource Center. Pre-mortem cerebrospinal fluid (CSF) samples from autopsy-confirmed cases of sCJD (age 48, 49, and 50 years) and non-dementia controls (age 48, 48, and 49 years) were from NPDPSC.

Antibodies and reagents

Antibodies 3F4 and 8H4 specific for human and both human and mouse PrP^C, respectively, were obtained from Signet Laboratories (Dedham, MA), and Abcam (cat #ab61409, Boston, MA). Antibodies specific to ferritin and β-actin were obtained from Sigma (cat# F5012,

Sigma-Aldrich, St. Louis, MO) and Millipore (cat# MAB1501, Bedford, MA) respectively. Antibody to α-globin was from Abcam (cat# ab102758, Boston, MA), and for β-globin, neuroglobin, and glycophorin-A from Santa Cruz Biotechnology Inc. (cat# sc21757, sc30144, and sc19453, Dallas, Texas). Antibody specific for Tf was from GeneTex (cat# GTX21223, Irvine, CA) and for TfR from Millipore (cat# CBL47, Bedford, MA). Hemin (cat# 51280), protoporphyrin IX (cat# P8293) and FAC (cat# F5879) were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). Cell culture supplies were from GIBCO (Life Technologies). All other chemicals were purchased from Sigma Aldrich (St. Louis, MO).

Cell lines and culture conditions

The hematopoietic cell line K562 and neuroblastoma cell lines SH-SY5Y and M17 were obtained from ATCC (Manassas, VA). K562 cells were cultured in RPMI medium supplemented with 10% FBS and antibiotics. SHSY5Y and M17 cells were cultured in OPTI-MEM supplemented with 10% FBS and antibiotics. Stable cell lines expressing PrP^{C}, $PrP^{\Delta 51\text{-}89}$ with and without the green fluorescent protein inserted between amino acids 38 and 39 of the prion protein were generated as described in previous reports [39–41]. Stock solutions of hemin and protoporphyrin IX were prepared in 50 mM NaOH (in 100 mM Tris HCl, pH-7.4) and used at a concentration of 10–30 μM and 3 μM respectively [42–44]. FAC was used at a concentration of 30 μM. Benzidine staining for the detection of Hb was performed as described [45].

SDS page and western blotting

Cells exposed to different conditions were lysed and processed for western blotting as described in previous reports [40, 46]. Antibody concentrations used were 1:5000 for 3F4, 1:1000 for α-globin, 1:750 for β-globin, 1:2500 for ferritin, 1:7000 for transferrin, 1:2000 for TfR, 1:300 for glycophorin-A, and 1:10000 for β-actin. Protein band density was quantified with UN-SCAN-IT gel 6.1 gel analysis software (Silk Scientific, Inc. Orem, UT).

Mouse brain organotypic slice culture

Brains harvested from 8 day old $PrP^{+/+}$ and $PrP^{-/-}$ pups were sliced to a thickness of 300 μM and cultured on transwell membrane inserts (0.4 μ pore size) for 1 week in high glucose DMEM supplemented with 10% FBS and antibiotics before the addition of 20 μM hemin to experimental samples. Subsequently, brain slices were washed with PBS, incubated in RBC lysis buffer [47, 48], and processed for SDS-PAGE and western blotting. To detect α-globin expression in mice brain, 14d old mice pups ($PrP^{+/+}$ and $PrP^{-/-}$) were sacrificed as per approved protocol. Brain tissue was triturated, incubated in RBCs lysis buffer, washed with chilled PBS (2X), and processed for SDS-PAGE and western blotting.

Statistical analysis

Data were analyzed using GraphPad Prism5 software (GraphPad Software, Inc., La Jolla, CA) and presented as Mean ± SEM. Level of significance was calculated by unpaired *t*-test between the control and treatment group.

RESULTS

Hemin induces endocytosis of PrP^C in neuroblastoma and hematopoietic cells

To evaluate whether hemin induces endocytosis of PrP^C independent of its cellular micro-environment, neuroblastoma cell lines SH-SY5Y and the erythroleukemia cell line K562 expressing green-fluorescent-protein tagged PrP^C ($PrP^{C\text{-}GFP}$) or a deletion construct lacking the hemin-binding octa-peptide repeat domain ($PrP^{\Delta 51\text{-}89\text{-}GFP}$) were exposed to 10–30 μM of hemin, and images of live cells were captured at different time-points. A representative result from 30 minutes of exposure is shown (Fig. 1). Hemin induced rapid endocytosis and intracellular accumulation of $PrP^{C\text{-}GFP}$ expressed on SH-SY5Y cells, but had no effect on the distribution of $PrP^{\Delta 51\text{-}89\text{-}GFP}$ (Fig. 1, panels 1–4). Similar observations were noted in M17 cells expressing $PrP^{C\text{-}GFP}$ and $PrP^{\Delta 51\text{-}89\text{-}GFP}$ (data not shown). In K562 cells, on the other hand, hemin reduced the expression of $PrP^{C\text{-}GFP}$ from the plasma membrane without significant accumulation in intracellular vesicles. The expression pattern of $PrP^{\Delta 51\text{-}89\text{-}GFP}$ was unaltered by hemin as in neuroblastoma cell lines (Fig. 1, panels 5–8). Interestingly, exposure to hemin resulted in clustering of $PrP^{C\text{-}GFP}$ and $PrP^{\Delta 51\text{-}89\text{-}GFP}$-expressing K562 cells (Fig. 1, panels 6 & 8).

Exposure to hemin has been reported to cause aggregation of PrP^C in N2a cells [3]. To evaluate whether this phenomenon is common to all cell types, K562, SH-SY5Y, and M17 cells expressing untagged PrP^C, $PrP^{\Delta 51\text{-}89}$, or vector were cultured in complete medium in the presence of hemin for 24 h, and lysates of untreated and hemin-exposed cells were evaluated by western blotting (Fig. 2). Probing for PrP^C revealed the typical un-glycosylated, mono-glycosylated, and di-glycosylated forms migrating at 27 kDa and 30–37 kDa in control and hemin-treated cells in all three cell lines. The corresponding glycoforms of $PrP^{\Delta 51\text{-}89}$ migrated faster on SDS-PAGE as expected (Fig. 2, 1–18). However, hemin-exposed SH-SY5Y lysates revealed an additional, slower migrating form, indicating aggregation of PrP^C by hemin (Fig. 2, lane 11). In K562 and M17 cells hemin caused downregulation of PrP^C, suggesting intracellular degradation (Fig. 2, lane 2 versus 5 & 14 versus 17). $PrP^{\Delta 51\text{-}89}$ did not show a detectable change in migration pattern or expression by exposure to hemin in any cell line (Fig. 2, lanes 6, 12, 18).

Thus, hemin interacts with PrP^C independent of the cell line and associated plasma membrane microenvironment. This interaction results in endocytosis of the PrP^C-hemin complex. In SH-SY5Y cells, PrP^C undergoes aggregation, while in other cell lines it is partially degraded. Hemin has no effect on $PrP^{\Delta 51\text{-}89}$ in any cell line, implicating this region in hemin binding [3]. Alternately, PrP^C could undergo co-internalization with the low-density lipoprotein receptor related protein-1 (LRP1) that interacts with its octa-peptide repeat region and serves as a receptor for hemin-hemopexin complexes [49–52]. This possibility was checked by exposing SH-SY5Y cells expressing $PrP^{C\text{-}GFP}$ to heme-hemopexin or hemin, and images of live cells were captured at different time points as in Fig. 1. Rapid endocytosis of $PrP^{C\text{-}GFP}$ by hemin but not by heme-hemopexin indicated LRP1-independent endocytosis of PrP^C by hemin (data not shown).

PrP^C upregulates Hb synthesis in hematopoietic cells in vitro and in vivo

Hemin has been reported to upregulate Hb synthesis in K562 cells [26], and recently, in neuronal cells [29, 53]. To understand the role of PrP^C in this process, K562 cells expressing PrP^C, $PrP^{\Delta 51\text{-}89}$, vector, and non-transfected controls were cultured in complete medium supplemented with hemin for 24–48 h, and washed cell pellets were examined visually. Surprisingly, PrP^C-expressing cells showed significantly more hemoglobinization relative to cells expressing $PrP^{\Delta 51\text{-}89}$ and controls (Fig. 3A). Reaction with benzidine, a stain used for identifying hemoglobinized cells, revealed significantly more positive cells in PrP^C-expressing relative to vector controls (Fig. 3B). Culture of SH-SY5Y and M17 cells in different concentrations of hemin for 24–48 h did not induce Hb synthesis (data not shown).

To evaluate whether PrP^C influences Hb synthesis *in vivo*, lysates of washed RBCs from age and sex-matched $PrP^{+/+}$ and $PrP^{-/-}$ mice were fractionated on duplicate gels, and transferred proteins were probed for α-globin and β-globin (Fig. 4). Majority of α-globin and β-globin migrated as a monomer, and the levels were significantly lower in $PrP^{-/-}$ relative to $PrP^{+/+}$ controls (Fig. 4A, B). Dimeric forms of α-globin and β-globin were visible after a longer exposure (Fig. 4A, B, right panels). Quantification of data from different mice confirmed reduced Hb expression in $PrP^{-/-}$ samples (Fig. 4C, D).

PrP^C upregulates protoporphyrin IX-mediated synthesis of α-globin in K562 cells

PrP^C is known to increase uptake of iron, an essential component of Hb, through its ferrireductase activity [54]. Since hemin is comprised of ferric iron enclosed in protoporphyrin IX ring, PrP^C could facilitate transport of iron released from hemin across the endosomal membrane, promote protoporphyrin IX-mediated upregulation of α-globin and β-globin synthesis [55, 56], or increase de novo incorporation of hemin in newly synthesized Hb [57]. To distinguish between these possibilities, K562 cells expressing vector, PrP^C, or $PrP^{\Delta 51\text{-}89}$ were exposed to hemin or ferrous ammonium citrate (FAC) (Fig. 5), or protoporphyrin IX (Fig. 6), and expression of α-globin was evaluated by western blotting. Exposure to hemin induced a significant increase in α-globin levels relative to untreated controls (Fig. 5A, lanes 1–3 versus 4–6). However, the increase in α-globin was significantly more in PrP^C-expressing cells relative to $PrP^{\Delta 51\text{-}89}$ and vector-expressing cells (Fig. 5A, lane 5 versus 4 & 6; Fig. 5B). Re-probing for ferritin showed significant upregulation by hemin in all cell lines, indicating release of hemin-associated iron and storage in cytosolic ferritin. Consequently, expression of transferrin (Tf) and transferrin receptor (TfR) was downregulated in these samples (Fig. 5A, lanes 1–3 versus 4–6).

To evaluate whether upregulation of α-globin is due to increase in intracellular iron, K562 cells expressing PrP^C, $PrP^{\Delta 51\text{-}89}$, or vector were cultured in the presence of FAC or hemin, and lysates were processed as above. Probing for α-globin revealed upregulation by hemin in all three cell lines, and significantly more in PrP^C-expressing relative to other cell lines as in Fig. 5A (Fig. 5C, lanes 1–3 versus 4–6; Fig. 5D). In contrast, exposure to FAC did not induce α-globin in any of the three cell lines (Fig. 5C, lanes 7–9), though the increase in intracellular iron was significant in all three cell lines as indicated by increase in ferritin levels (Fig. 5C, lanes 1–3 versus 7–9).

The influence of porphyrin ring of hemin (without iron) on α-globin synthesis was assessed by exposing the same cell lines to protoporphyrin IX (PP IX) followed by western blotting (Fig. 6). Lysates of cells exposed to PP IX showed increased synthesis of α-globin relative to untreated controls (Fig. 6A, lanes 1–3 versus 4–6). However, the increase in α-globin was significantly more in PrP^{C}-expressing relative to vector and $PrP^{\Delta 51\text{-}89}$-expressing cells (Fig. 6A, lane 5 versus 4 & 6; Fig. 6B). Re-probing for ferritin, Tf, and TfR revealed downregulation of ferritin and upregulation of TfR in PP IX-treated cells, indicating depletion of cellular iron stores by this treatment due to stimulation of Hb synthesis (Fig. 6A, lanes 1–3 versus 4–6) [55, 58].

Together, the above observations suggest that PrP^{C} facilitates protoporphyrin IX-mediated upregulation of globin genes, thereby increasing Hb synthesis. Hemin and FAC-mediated increase in intracellular iron is likely to support heme synthesis necessary for assembling tetrameric Hb, but does not influence globin chain synthesis *per se*, and is therefore insufficient by itself in upregulating Hb.

PrP^{C} upregulates hemin-mediated synthesis of α-globin in mouse brain organotypic cultures

Since hemin upregulates Hb synthesis in neuronal and glial cells [53], it is likely that PrP^{C} modulates this process as well. To explore this possibility, freshly harvested brains from 8 day post-natal $PrP^{+/+}$ and $PrP^{-/-}$ pups were sliced and cultured in complete medium on filter supports in the absence or presence of hemin. After overnight treatment, sections were harvested, treated with RBC lysis buffer, and processed for western blotting. Lysates from human brain and mouse RBCs were fractionated in parallel as controls. Probing for α-globin revealed significant upregulation by hemin in $PrP^{+/+}$ relative to $PrP^{-/-}$ samples (Fig. 7 lanes 2 versus 4). Surprisingly, monomeric α-globin from mouse and human brain lysates migrated slower than mouse blood α-globin (Fig. 7, lanes 1–5 versus 6). Human brain sample revealed additional α-globin forms that were absent in mouse brain samples, and migrated faster than dimeric α-globin from mouse blood (Fig. 7, lane 5 versus 6,*). Reaction for PrP^{C} was as expected.

Thus, PrP^{C}-upregulates hemin-mediated synthesis of α-globin in brain sections as well. Although the identity of specific cell types that respond to hemin is not clear, these data confirm that α-globin is quantifiable in the brain, and surprisingly, more prominent in human relative to mouse samples. The distinct migration pattern of human brain α-globin on SDS-PAGE suggests the presence of post-translational modifications specific to human brain cells. In addition, the different migration pattern of α-globin from brain and RBCs rules out contamination of brain samples with blood (also see Figs. 8 & 9).

Expression of α-globin is reduced in $PrP^{-/-}$ mouse brains

To evaluate whether PrP^{C} plays a role in Hb synthesis in the brain under physiological conditions, α-globin expression in brain samples harvested from $PrP^{+/+}$ and $PrP^{-/-}$ was evaluated. Lysates from human brain and RBCs from $PrP^{+/+}$ mice were fractioned in parallel as controls. Reaction for α-globin revealed a prominent band in both $PrP^{+/+}$ and $PrP^{-/-}$ samples and human brain that migrated slower than α-globin from mouse RBCs as in Fig. 7

above (Fig. 8A, lanes 1–7 versus 9). Expression of α-globin was variable between mice, but was lower in $PrP^{-/-}$ relative to $PrP^{+/+}$ controls (Fig. 8A, lanes 1–3 versus 4–6; Fig. 8B).

The α-globin antibody used in this analysis did not cross-react with neuroglobin, another oxygen carrying globin expressed in the brain, and at much higher levels in retinal pigment epithelial (RPE) cells (Fig. 8A, lane 8) [59, 60]. A separate analysis of neuroglobin expression in mouse and human brain samples and RPE cells did not show a detectable difference (Fig. 8C, lanes 1–9).

Since contamination from lysed RBCs is a concern in brain tissue samples, lysates from mouse RBCs and mouse brain (used in panel A) containing varying amounts of total protein were probed for α-globin followed by glycophorin-A, a major sialoglycoprotein of red blood cell membranes. Reactivity for α-globin was detected in RBCs and brain sample as in panel A, and was 50-fold lower in mouse brain samples relative to RBCs (Fig. 8D, lanes 1–4, upper panels). However, expression of glycophorin-A was limited to RBCs, ruling out contamination of brain samples with RBCs (Fig. 8D, lanes 1–3, lower panel).

These results indicate that PrP^{C} modulates neuronal and glial cell Hb synthesis under physiological conditions, a function that gains increasing importance in pathological conditions where neurons are exposed to hemin.

α-Globin levels are increased in sCJD brains

Since PrP^{C} forms non-functional PrP^{Sc} aggregates in sCJD brains, loss or altered function of PrP^{C} is likely to alter α-globin synthesis in diseased brains, compromising the metabolic potential of affected neurons. To explore this possibility, lysates from frontal cortex of sCJD and non-dementia controls were analyzed for α-globin expression as above. Samples from human and mouse RBCs were fractionated in parallel as controls (Fig. 9). Surprisingly, α-globin expression was significantly increased in sCJD samples relative to controls (Fig. 9A, lanes 1–4 versus 5–8; Fig. 9B). Probing for the glial marker GFAP revealed a small increase in sCJD samples as expected. However, the difference in neuronal marker NeuN was minimal, indicating equivalent representation of neurons in control and sCJD samples (Fig. 9A, lanes 1–4 versus 5–8). Ferritin was upregulated in sCJD samples as reported earlier (Fig. 9A, lanes 5–8), and expression of PrP was as expected (Fig. 9A, lanes 1–8). As noted above, α-globin from brain samples migrated slower than its counterpart from RBCs (Fig. 9, lanes 1–8 versus 9 & 10). Additional forms of α-globin were detected in control and sCJD samples, probably representing modified isoforms whose identify requires further exploration (lanes 1–8,*).

Since contamination of brain tissue with RBCs is a major concern, representative samples from human brain and RBCs were fractionated in duplicate, and re-analyzed by western blotting as above (Fig. 9C). Probing for α-globin and β-globin revealed slower migrating bands in the brain sample relative to corresponding bands from RBCs (Fig. 9C, lane 1 versus 2 & 3; upper and middle panels). However, reaction with glycophorin-A was limited to RBC samples (Fig. 9C, lane 1 versus 2 & 3, bottom panel). These observations rule out any contamination of brain samples with RBCs. No reactivity for α-globin or β-globin was detected in the CSF of control and sCJD samples (Fig. 9D, lanes 1–6), though the expected

bands were detected in brain and RBC samples (Fig. 9D, lanes 7 & 8). Levels of CSF Tf were lower in sCJD samples relative to controls as reported earlier (Fig. 9D, lanes 1–3 versus 4–6) [61].

DISCUSSION

This report confirms and extends previous observations on the interaction of hemin with PrP^C [3]. We demonstrate that PrP^C binds and internalizes extra-cellular hemin regardless of the plasma membrane micro-environment. This interaction causes aggregation or degradation of PrP^C in a cell-type specific manner, while internalized hemin is degraded by all cell lines and the released iron stored in cytosolic ferritin. More importantly, PrP^C upregulates hemin-induced synthesis of fetal Hb in K562 cells and adult Hb in mouse brain organotypic cultures, and modulates the synthesis of adult Hb in hematopoietic, neuronal, and glial cells under physiological conditions. Surprisingly, levels of α-globin are increased in sCJD brains, suggesting a compensatory response by surviving neurons or proliferating glial cells, or dys-regulation of Hb synthesis due to sCJD-associated pathology. These observations are discussed in the context of CH and sCJD, pathological conditions where interaction of hemin with PrP^C and altered functional activity of PrP^{Sc} respectively are likely to play a significant role.

The neuroprotective role of PrP^C in CH is well-documented, but poorly understood [15–17, 62]. Our observations suggest that PrP^C protects neurons by promoting the endocytosis and degradation of extracellular hemin, helping in its clearance from the neuronal micro-environment. Exposure to hemin concentrations comparable to brain regions affected by CH [6] led to rapid internalization of PrP^C and hemin in both hematopoietic and neuronal cell lines. Internalized PrP^C was partially degraded in most cell lines and organotypic brain cultures. In SH-SY5Y cells, however, PrP^C formed detergent-insoluble aggregates that accumulated in intracellular vesicles as reported for N2a cells [3]. The differential fate of PrP^C in specific cell lines probably reflects the efficiency with which the hemin-PrP^C complex is degraded, influenced in part by factors that modify the secondary structure of PrP^C and resistance to proteolytic enzymes [3]. However, absence of such aggregates in hemin-exposed K562 cells and organotypic brain cultures indicates that hemin-induced aggregation of PrP^C is not a common phenomenon, and unlikely to occur *in vivo*.

The positive effect of PrP^C on hemin-induced synthesis of Hb is surprising and of clinical significance because of its potential to improve neuronal viability. PrP^C upregulated hemin-mediated synthesis of Hb 2-fold in K562 cells, and ~3-fold in mouse organotypic brain slice cultures. PrP^C also amplified the effect of protoporphyrin IX that lacks iron [56, 63] on α-globin synthesis, while extracellular iron had no effect. Since deletion of the octa-peptide repeat region abolished this effect, it is likely that PrP^C increases the uptake and possibly transport of hemin and protoporphyrin IX through heme transporters across the endosomal membrane [64], facilitating their influence on α-globin gene expression [23, 24, 65] or direct incorporation in Hb [66]. These observations explain expression of PrP^C on proerythroblasts in bone marrow niches where surrounding macrophages are likely to export hemin recycled from senescent RBCs for incorporation in newly synthesized Hb [66–69]. Decreased levels of α-globin and β-globin in $PrP^{-/-}$ RBCs support an active role of PrP^C in

this pathway. A hemin-independent role of PrP^C in hematopoiesis has also been described, though the biochemical pathways involved in this process are not entirely clear [67, 70, 71].

Upregulation of hemin-mediated synthesis of α-globin by PrP^C in organotypic brain cultures was surprising, and indicated common pathway(s) of Hb synthesis in hematopoietic precursors and brain cells. These observations are of significance in CH where endocytosis of hemin by PrP^C followed by upregulation of Hb is likely to improve neuronal viability by the dual mechanism of clearing extracellular hemin and increasing their respiratory potential. Upregulation of Hb in neurons and astrocytes has been reported in primary cells exposed to hemin [29] and in areas surrounding CH [33, 53], supporting our observations. Although it is difficult to conclude whether neurons, glia, or both cell types respond to hemin, significant upregulation of Hb in $PrP^{+/+}$ relative to $PrP^{-/-}$ samples suggests participation of neurons that express higher levels of PrP^C. A small but significant reduction of α-globin in cortical brain tissue from $PrP^{-/-}$ brains suggests a positive role of PrP^C in Hb synthesis under physiological conditions as well, though the underlying mechanism is not clear. Considering the high metabolic rate of neurons at steady state and their susceptibility to oxygen deprivation, a positive role of PrP^C in neuronal Hb synthesis has clinical implications.

A significant increase in α-globin in the brain tissue of autopsy-confirmed cases of sCJD was surprising especially since a significant amount of PrP^C is nonfunctional in diseased brains due to conversion to the PrP^{Sc} isoform. Lack of α-globin reactivity in CSF samples despite significant upregulation in the brain tissue indicates upregulation of Hb, not α-globin *per se*. The increase in α-globin did not correlate with the glial marker GFAP or the neuronal marker NeuN, making it difficult to attribute this change to gliosis or neuronal loss, two main attributes of sCJD. It is likely that surviving neurons upregulate Hb synthesis in response to sCJD-associated stress, increasing the levels of α-globin in diseased tissue. Upregulation of ferritin in sCJD brains has been reported [72], but increased availability of iron in the absence of globin chain upregulation is unlikely to upregulate Hb synthesis. Interestingly, increased levels of α-Hb stabilizing protein (AHSP) have been reported in the serum of sCJD cases [73], suggesting upregulation of α-globin in the peripheral blood of sCJD cases. However, absence of α-globin in the CSF of both control and sCJD cases argues against this conclusion which was refuted in a later report [74], leaving the matter unsettled. Altered levels of neuronal Hb have been reported in multiple sclerosis [75], Alzheimer's disease, Parkinson's disease, and dementia with Lewy bodies [76, 77], suggesting a complex interplay of disease pathology and neuronal Hb synthesis.

In conclusion, this report highlights the dual role of PrP^C in clearing hemin from the neuronal micro-environment and upregulating neuronal Hb, thereby promoting neuronal survival in CH. A positive role of PrP^C in Hb synthesis under physiological conditions reveals a novel function of this protein in hematopoietic and neuronal Hb synthesis that requires further exploration. Upregulation of Hb in sCJD brains may reflect a compensatory mechanism by surviving neurons, an observation with significant therapeutic implications. Further investigations are necessary to understand the mechanism underlying disease-specific changes in neuronal Hb in sCJD and other neurodegenerative conditions.

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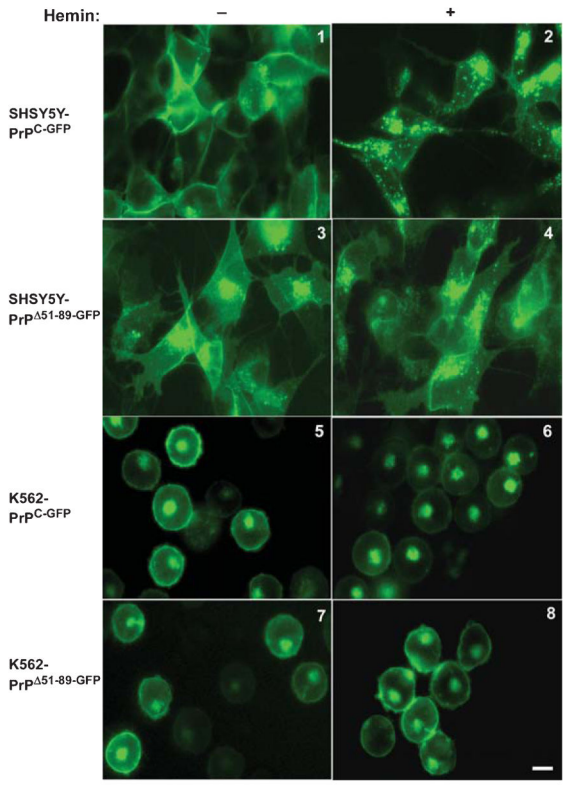

Fig. 1.

Hemin induces endocytosis of PrP^{C}. SH-SY5Y and K562 cells expressing $PrP^{C\text{-}GFP}$ or $PrP^{\Delta 51\text{-}89\text{-}GFP}$ were exposed to vehicle (left panels) or 30 μM hemin (right panels), and images of live cells were captured after 30 minutes. Hemin induces endocytosis of $PrP^{C\text{-}GFP}$ in both SH-SY5Y and K562 cells (panel 1 & 5 versus 2 & 6). In SH-SY5Y cells $PrP^{C\text{-}GFP}$ accumulates in intracellular endocytic vesicles (panel 2), while in K562 cells expression on the plasma membrane decreases without accumulation in endosomes. A small amount of $PrP^{C\text{-}GFP}$ and $PrP^{\Delta 51\text{-}89\text{-}GFP}$ are detected in the perinuclear region in the Golgi compartment (panel 6). Hemin has no effect on the distribution of $PrP^{\Delta 51\text{-}89\text{-}GFP}$ in either cell line (panels 3 & 7 versus 4 & 8). Bar: 10 μm.

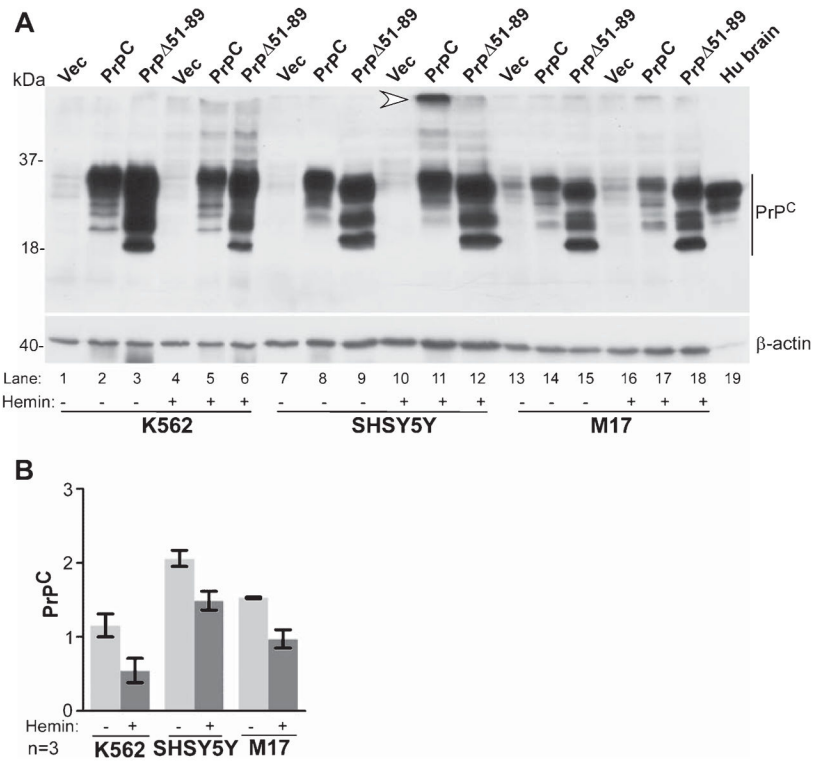

Fig. 2.

Hemin-induced aggregation of PrP^C is cell-type specific. A) Probing of lysates from K562, SH-SY5Y, and M17 cells for PrP^C reveals the expected glycoforms of full-length and octa-peptide-repeat deleted forms of $PrP^{\Delta 51\text{-}89}$ in all three cell lines (lanes 1–18). Exposure to hemin causes aggregation of PrP^C in SH-SY5Y cells (lane 11), while in other cell lines there is a small but noticeable decrease in PrP^C expression (lanes 2 versus 5 & 14 versus 17). Hemin has no effect on the expression of $PrP^{\Delta 51\text{-}89}$ in any cell line (lanes 3, 6, 9, 12, 15, 18). Human brain sample fractionated in parallel shows the expected reaction for PrP^C (lane 19). Re-probing for β-actin provides a loading control. B) Quantification by densitometry shows reduced expression of PrP^C in all cell lines exposed to hemin, including SH-SY5Y cells where a fraction of PrP^C is aggregated. Data represent Mean ± SEM of three independent experiments.

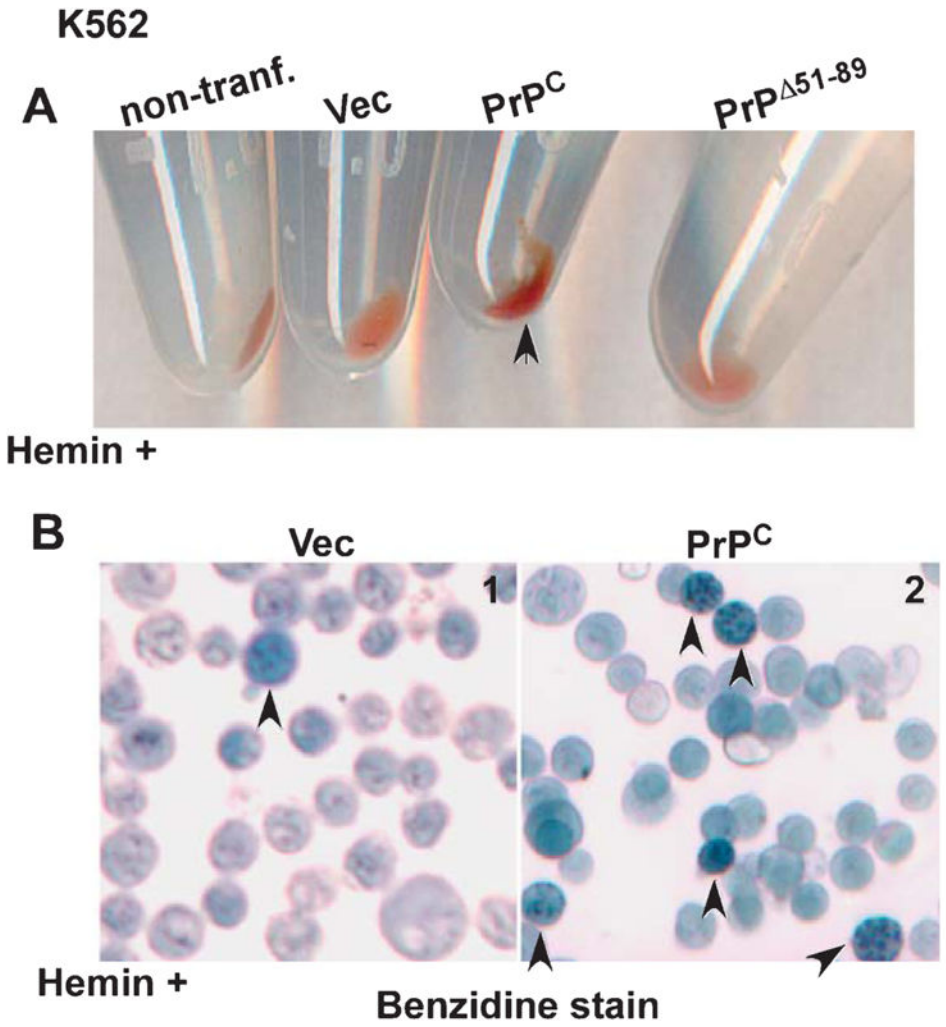

Fig. 3.

PrP^{C} upregulates hemin-mediated synthesis of Hb in K562 cells. A) Red color of Hb is more evident in cell pellets of PrP^{C}-expressing K562 cells (arrowhead) relative to non-transfected, vector, and $PrP^{\Delta 51\text{-}89}$-expressing cells. B) Staining with benzidine confirms significantly more Hb in PrP^{C}-expressing cells relative to vector controls (panel 1 versus 2, arrowhead).

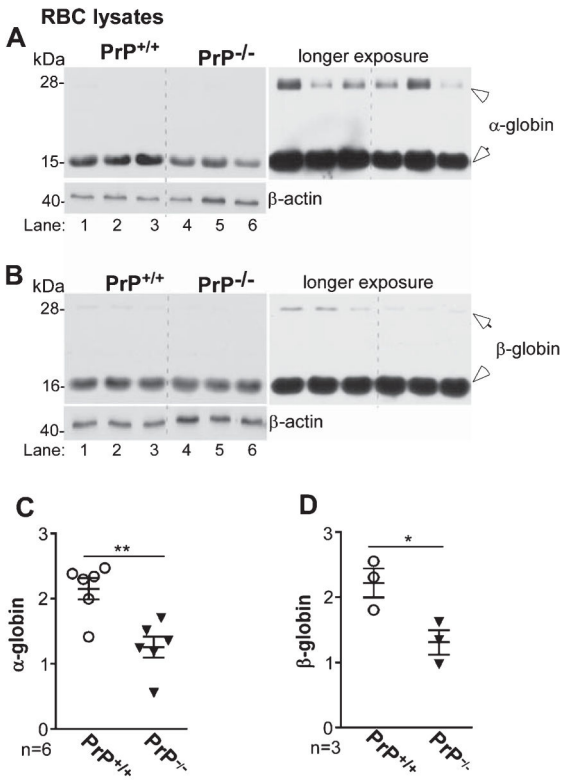

Fig. 4.

α- and α-globin expression is reduced in RBCs from $PrP^{-/-}$ mice. A, B) Lysates of washed RBCs from $PrP^{+/+}$ and $PrP^{-/-}$ mice reveal reduced expression of α-globin (A) and β-globin (B) in $PrP^{-/-}$ relative to $PrP^{+/+}$ samples (lanes 1–3 versus 4–6). Dimeric forms of α-globin and β-globin are evident after a longer exposure (right panels). C, D) Densitometry confirms significant reduction of α-globin and β-globin in $PrP^{-/-}$ samples relative to $PrP^{+/+}$ controls. Reaction for β-actin provides a loading control. Data are Mean ± SEM of three independent experiments on multiple mice. $^{*}p < 0.05$, $^{**}p < 0.01$.

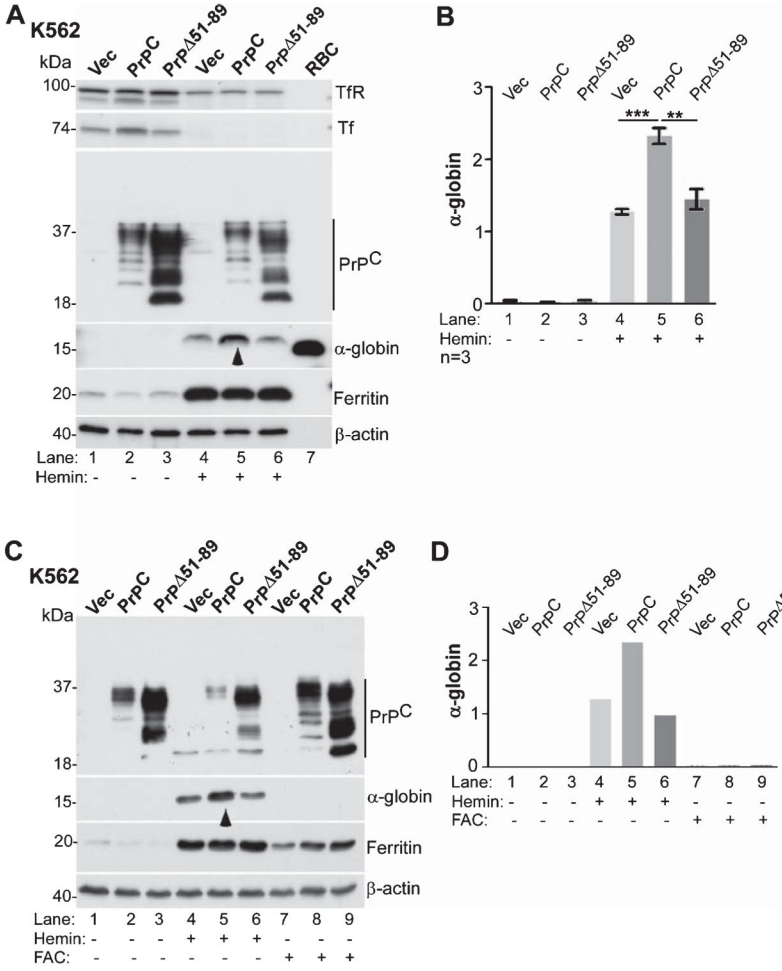

Fig. 5.

PrP^{C}-mediated upregulation of α-globin is not due to increase in intracellular iron. A) K562 cells expressing vector, PrP^{C}, or $PrP^{\Delta 51\text{-}89}$ were cultured in the absence or presence of hemin and subjected to western blotting. Probing for α-globin reveals significant upregulation by hemin in all cell lines (lanes 1–3 versus 4–6), but most prominent in PrP^{C}-expressing cells (lane 5 versus 4 & 6). Lysates from mouse RBCs fractionated in parallel show a strong reaction for α-globin as expected (lane 7). Ferritin is upregulated in hemin-treated samples due to the release of associated iron (lanes 1–3 versus 4–6). B) Quantification of α-globin by densitometry confirms significant upregulation by PrP^{C} in the presence of hemin relative to vector and $PrP^{\Delta 51\text{-}89}$-expressing cells. Data are Mean ± SEM of three independent evaluations. $^{**}p < 0.01$, $^{***}p < 0.001$. C) The above experiment was repeated with in the presence of FAC (lanes 7–9). Reaction for α-globin shows upregulation by hemin as in panel A, but not by FAC (lanes 4–6 versus 7–9). Ferritin is upregulated by hemin and FAC in all cell lines as expected (lanes 4–9). Reaction for β-actin provides a loading control. D) Quantification of the data in panel C by densitometry confirms upregulation of α-globin by hemin, not by FAC, and a positive effect of PrP^{C} on hemin-mediated increase in α-globin.

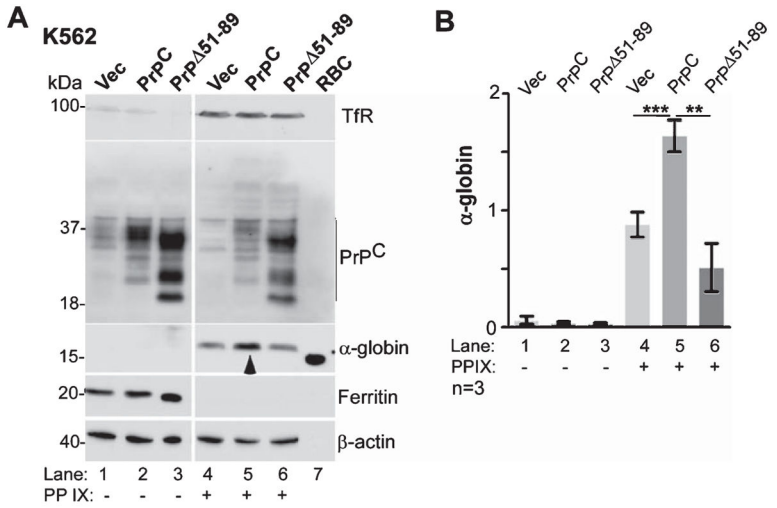

Fig. 6.

PrP^{C} promotes protoporphyrin IX-mediated synthesis of α-globin. A) K562 cells expressing vector, PrP^{C}, or $PrP^{\Delta 51\text{-}89}$ were cultured in the absence or presence of protoporphyrin IX and subjected to western blotting. Probing for α-globin reveals upregulation by protoporphyrin IX in all cell lines (lanes 1–3 versus 4–6), and significantly more in PrP^{C}-expressing cells (lane 5 versus 4 & 6). Ferritin is downregulated, and TfR upregulated by exposure to protoporphyrin IX, indicating depletion of cellular iron stores as expected (lanes 1–3 versus 4–6). Lysates from mouse RBCs provide a positive control for α-globin (lane 7). Reaction for β-actin provides a loading control. B) Quantification by densitometry confirms significantly more upregulation of α-globin by protoporphyrin IX in PrP^{C} relative to vector and $PrP^{\Delta 51\text{-}89}$-expressing cells. Data are Mean ± SEM of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

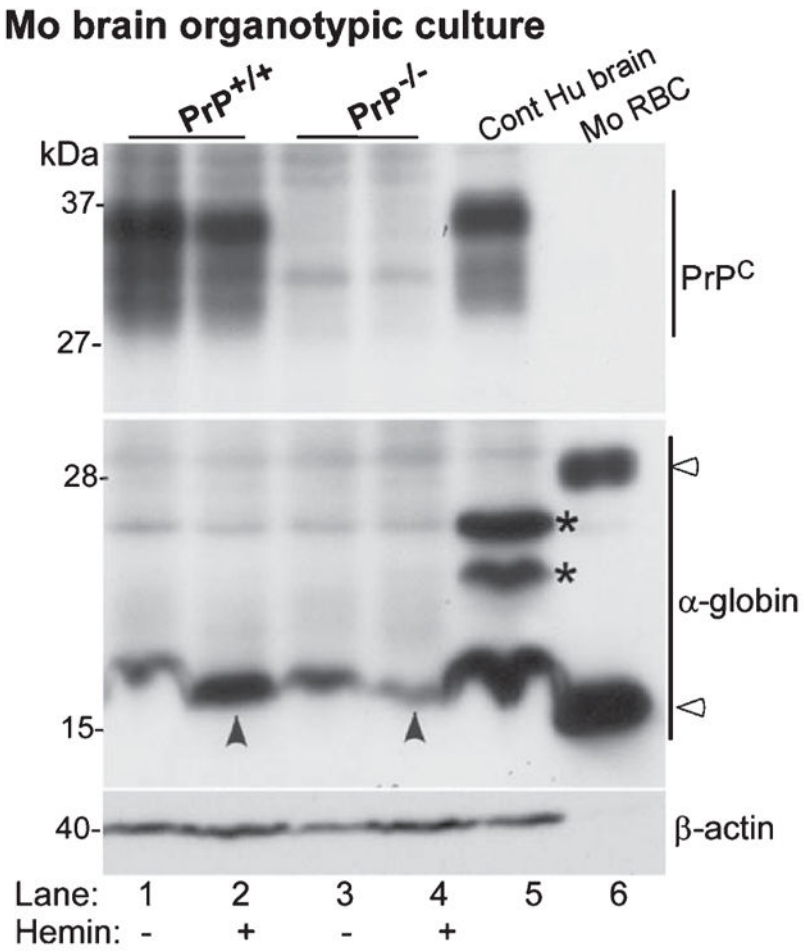

Fig. 7.

Hemin induces α-globin expression in mouse brain organotypic cultures. Lysates of organotypic brain cultures from $PrP^{+/+}$ and $PrP^{-/-}$ mice cultured in the absence or presence of hemin were processed for western blotting. Probing for α-globin reveals significantly more α-globin in hemin treated $PrP^{+/+}$ relative to $PrP^{-/-}$ samples (lanes 1 & 2 versus 3 & 4). Human brain sample fractionated in parallel shows significantly more α-globin relative to mouse samples (lanes 1–4 versus 5), and slower migrating forms that are absent in mouse brain and RBC lysates (lane 5, *). Expression of PrP^{C} is as expected (lanes 1–5). Reaction for β-actin provides a loading control.

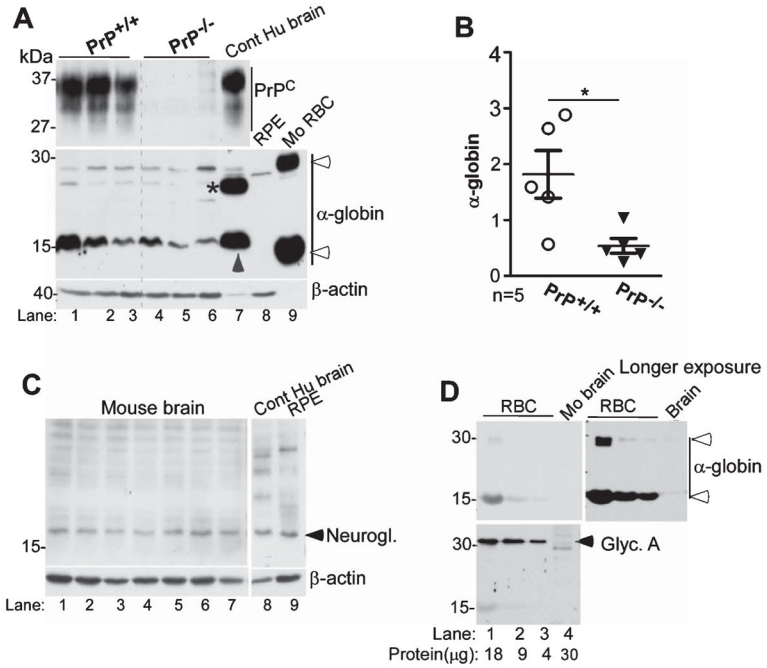

Fig. 8.

α-globin expression is reduced in $PrP^{-/-}$ mouse brain. A) Processing of brain homogenates by western blotting reveals reduced expression of α-globin in $PrP^{-/-}$ samples relative to $PrP^{+/+}$ controls (lanes 1–3 versus 4–6). Human brain sample fractionated in parallel shows monomeric α-globin that co-migrates with mouse brain samples (lane 7 versus 1–6), and slower than monomeric α-globin from mouse RBCs that show monomeric and dimeric α-globin as in Figs. 4 and 7 above (lane 7 versus 9). RPE cells that express significant amounts of neuroglobin do not show detectable reaction for α-globin (lane 8). Reaction for β-actin provides a loading control. Reaction for PrP^{C} is as expected (lanes 1–7). B) Quantification by densitometry shows significantly less α-globin in $PrP^{-/-}$ relative to $PrP^{+/+}$ samples. Data are Mean ± SEM of the indicated n ($n = 5$; $^{*}p < 0.05$). C) Reaction for neuroglobin shows no difference between mouse and human brain samples and human RPE cells (lanes 1–9). Reaction for β-actin provides a loading control. D) Processing of varying amounts of total protein from mouse RBC and brain samples by Western blotting reveals a strong reaction for α-globin in RBC samples, and significantly less in brain samples despite several-fold higher total protein (lanes 1–3 versus 4). Reaction for glycophorin-A is limited to RBC samples (lanes 1–3 versus 4, lower panel).

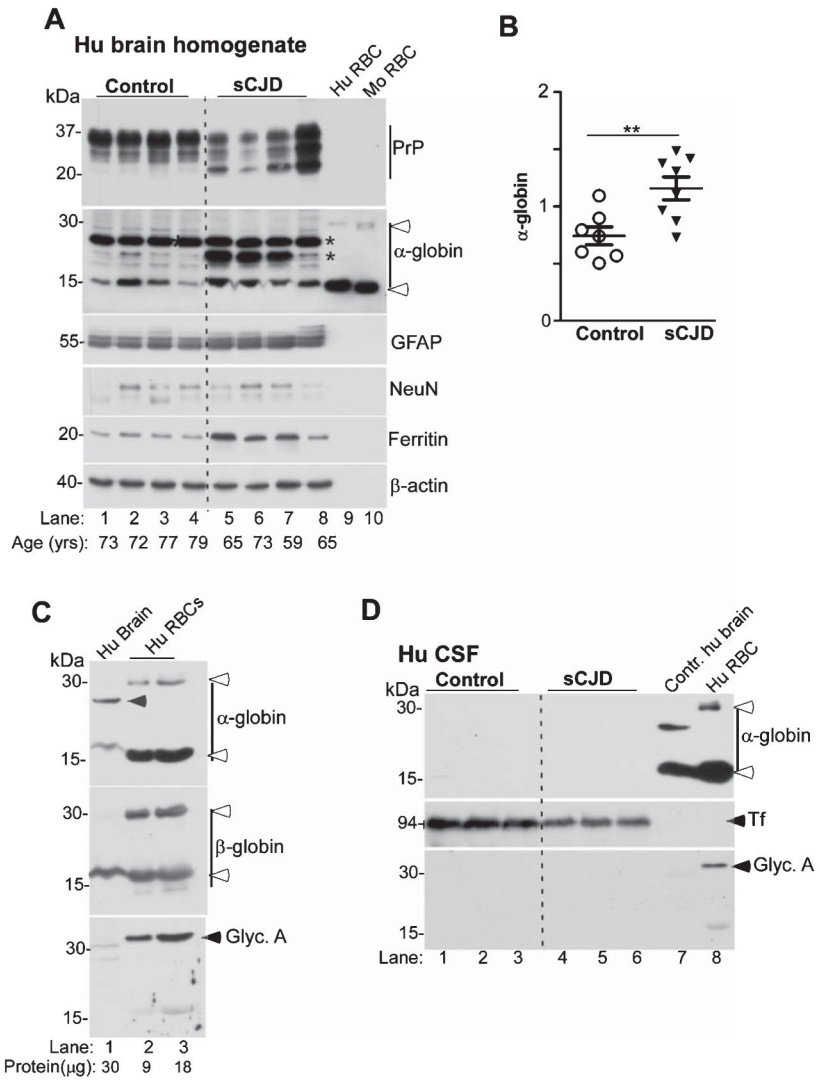

Fig. 9.

α-globin is increased in sCJD brains. A) Probing of lysates from the frontal cortex of sCJD and non-dementia controls shows significantly higher levels for α-globin in sCJD samples relative to controls (lanes 1–4 versus 5–8). Reaction for GFAP and ferritin is higher in sCJD, while NeuN is similar in control and sCJD samples (lanes 1–4 versus 5–8). Human and mouse RBCs fractionated in parallel confirm the presence of additional α-globin forms in human brain (lanes 1–8, * versus 9 & 10, open arrow-heads), and slower migration of monomeric α-globin from brain relative to RBC samples (lanes 1–8 versus 9 & 10). Reaction for β-actin provides a loading control. B) Quantification of α-globin by densitometry shows significantly higher levels in sCJD versus controls. C) Varying amounts of total protein from control human brain and human RBCs fractionated in parallel shows a strong reaction for α-globin and β-globin in brain and RBC samples (lanes 1–3, top and middle panels). A faster migrating form of brain α-globin that does not co-migrate with dimeric β-globin from RBCs is detected as in Figs. 7 and 8 above (lane 1, top panel, arrow-head). Reaction for glycophorin-A is limited to RBC samples (lanes 2 & 3, bottom panel),

ruling out contamination of brain samples with RBCs. D) Western blotting of equal volume of CSF from sCJD and control samples shows no reactivity for α-globin, though Tf is detected readily, and is reduced in sCJD samples (lanes 1–3 versus 4–6) [61]. Human brain and RBC samples react readily for α-globin as expected (lanes 7 & 8). Reaction for glycophorin-A is limited to the RBC sample (lane 8).