Prion Infection in Cells is Abolished by a Mutated Manganese Transporter but shows no Relation to Zinc.

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Short Title: Metal Transport and Prions

Key words: prion, manganese, zinc, ZnT-1, SPCA

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ABBREVIATIONS

AMPA - α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BSE – bovine spongiform encephalopathy

CJD – Creutzfeldt-Jakob disease

vCJD – the variant of CJD

F14 – PrP knockout fusion cell line

F21 – wild-type fusion cell line

ICP-MS - inductively-coupled plasma mass spectrometry

MTF-1 – metal-regulatory transcription factor 1

N2a – neuro 2a neuroblastoma cell line

PK – proteinase K

PrP – prion protein

PrP^{Sc} – abnormal isoform of PrP

SPCA - secretory pathway calcium ATPase-1

SPCA-Q746A – mutant of SPCA

SMB – scrapie mouse brain cell line

SMB-PS – SMB cells cured by pentosan sulphate

ZnT-1 – zinc transporter 1

ABSTRACT

The cellular prion protein has been identified as a metalloprotein that binds copper. There have been some suggestions that prion protein also influences zinc and manganese homeostasis. In this study we used a series of cell lines to study the levels of zinc and manganese under different conditions. We overexpressed either the prion protein or known transporters for zinc and manganese to determine relations between the prion protein and both manganese and zinc homeostasis. Our observations supported neither a link between the prion protein and zinc metabolism nor any effect of altered zinc levels on prion protein expression or cellular infection with prions. In contrast we found that a gain of function mutant of a manganese transporter caused reduction of manganese levels in prion infected cells, loss of observable PrP^{Sc} in cells and resistance to prion infection. These studies strengthen the link between manganese and prion disease.

INTRODUCTION

The prion diseases include a range of human and animal diseases that are associated with the deposition of an abnormal isoform of the prion protein (PrP) in tissues (Prusiner, 1998), especially those of the central nervous system. These diseases include inherited, transmitted and sporadic forms of disease (Collinge, 2001). While low level sporadic diseases such as scrapie in sheep and Creutzfeldt-Jakob disease (CJD) in humans have been known for some time, the prion diseases really became prominent when the transmitted cattle disease bovine spongiform encephalopathy was believed to have been transmitted to humans as variant CJD (vCJD) (Will et al., 1996). The incidence of these two diseases has largely abated but concern over the mechanism of transmission and general aetiology of prion diseases remains to be clarified (Kretzschmar and Tatzelt, 2013). While formation of the abnormal isoform (PrP^{Sc}) of PrP has been accepted as the causative agent, there is currently no conclusive mechanism identified for its formation or how it initiates catastrophic neuronal loss in a very age specific manner.

The association between metals and prion diseases is now well established (Brown et al., 1997; Davies et al., 2009; Davies et al., 2011; Jackson et al., 2001; Jones et al., 2005; Kralovicova et al., 2009; Liu et al., 2011; Stevens et al., 2009; Stockel et al., 1998; Walter et al., 2009; Wells et al., 2006), but the exact role, if any, that the metals play in the disease process is not fully understood. Initially, PrP was identified as a copper binding protein *in vivo* (Brown et al., 1997). Significant effort was undertaken to determine the co-ordination sites and affinity of copper binding to the octameric repeat region of the protein but it took quite some years until reproducible values were obtained and PrP is now accepted to have nanomolar affinity for Cu(II) (Nadal et al.,

2009). Cyclic voltammetry has been used to verify that copper bound to PrP can cycle between Cu(II) and Cu(I) (Davies et al., 2009). Copper binding has been associated with a number of possible cellular functions for PrP which include copper uptake (Brown, 1999), cell survival (Brown et al., 1996) and antioxidant activity (Brown et al., 1999). Attempts to show that PrP can bind other metals have been less profitable. Both Zn(II) and Fe(II) show some very low affinity for PrP (Davies et al., 2009) and only Mn(II) shows affinity equivalent to other manganese binding proteins (Brazier et al., 2008).

The key event in conversion of cellular PrP (PrP^{c}) to PrP^{Sc} requires a conformational change in the protein. Metals have been investigated in relation to conversion of PrP to a protease resistant form. Although copper can cause increased protease resistance, it has not been linked to the formation of PrP^{Sc} (Quaglio et al., 2001; Roberts et al., 2013) but has been shown to accelerate its formation when PrP^{Sc} is already present (McKenzie et al., 1998). In contrast, manganese has been suggested to cause spontaneous PrP^{Sc} formation (Brown et al., 2000) and manganese chelation has been shown to decrease PrP^{Sc} present in infected rodent brains (Brazier et al., 2010). Manganese has also been shown to be associated with PrP^{Sc} plaques (Johnson et al., 2013) and general levels of manganese are increased in the brains and blood of cases of BSE, scrapie and CJD as well as experimentally infected rodents (Wong et al., 2001). Manganese is also found associated with PrP extracted from diseased brains (Thackray et al., 2002). Along with this change there was found a parallel reduction in the amount of copper bound to PrP. However, experiments with recombinant protein have shown that Cu(II) and Mn(II) can bind to PrP at the same time, occupying different sites on the protein (Brazier et al., 2008). Manganese has been suggested to

play a role environmentally, as its presence dramatically increases the survival of full length PrP in a clay matrix (Davies and Brown, 2009). Lastly, manganese has been shown to increase the infectivity of PrP^{Sc} to cultured cells (Davies and Brown, 2009).

A recent report has suggested that PrP expression by cells increases entry of zinc into cells (Watt et al., 2012). The reported mechanism of action is via AMPA receptors that allow entry of zinc. Interaction between the AMPA receptor and PrP has been suggested to increase the rate of zinc entry in uninfected cells but not prion-infected cells (Watt et al., 2012). There has been some interest in PrP function and zinc for some time (Brown and Harris, 2003; Jobling et al., 2001; Watt and Hooper, 2003). Zinc has also been suggested to alter PrP internalisation (Perera and Hooper, 2001). In contrast to copper and manganese there is little evidence for changes in zinc in disease. A single report suggested changes in zinc in experimental rodent brain (Wong et al., 2001) but none of the other studies looking at metal concentrations was able to confirm this (Thackray et al., 2002). However, any relation of zinc to prion disease is not known.

In this study we have looked at the effect of PrP expression on zinc concentrations and AMPA receptor expression in different cell types. We also looked at the effect of altering zinc concentration in cells experimentally. In parallel we also looked at a manganese transporter and its relation to prion infection. While we detected no relation between zinc levels and PrP expression or infection, overexpression of a mutated manganese transporter that causes efflux of manganese from cells, resulted in both a dramatic loss of PrP^{Sc} from cells and in increased resistance to prion infection.

METHODS

Reagents were purchased from Sigma-Aldrich unless stated otherwise.

Cell Culture

All cell lines were maintained at 37ºC and 5% CO_2 in a humidified incubator. Cell lines used for these studies were predominantly mouse N2a cells (Neuro-2A, ATCC Number: CCL-131). Other cell lines used were F14 and F21 cerebellar granule cells/neuroblastoma fusions cell lines (Holme et al., 2003). For infection studies we used scrapie mouse brain cells (SMB)(Birkett et al., 2001) and the pentosan sulphate cured control cells (SMB-PS). Cell lines were cultured in DMEM with 10% fetal bovine serum (Lonza) and 1% penicillin–streptomycin. Preparation of protein extracts from cells and proteinase K (PK) treatment of extracts was as previously described (Davies and Brown, 2009; Kralovicova et al., 2009). Infection of SMB-PS cells was carried out as previously described (Davies and Brown, 2009). Basically, SMB cells overexpressing PrP were grown and a protein extract prepared and treated with PK. After dialysis the PK resistant material was concentrated and added to 6 well cultures of SMB-PS cells at 500 µg/well. This was the maximum concentration that did not disrupt cell viability.

Stable cell lines were generated by transfection of N2A, F14, SMB or SMB-PS cells with the appropriate plasmid containing the ORF of the gene of interest. Transfection was carried out using Fugene (Promega) according to the manufacturers specifications. After transfection, stable cell lines were generated by growth of the cells in medium containing 400 µg/ml G418. This concentration was sufficient to completely eliminate non-transfected cells in parallel cultures (non-transfected) in seven days or less. Cell lines were maintained constantly in this concentration of

G418 to ensure only transfected cells survived. Stable cell lines were screened to ensure that high levels of the protein of interest was overexpressed and rescreened frequently to ensure high level of expression was maintained.

Western Blot

Cell extracts were prepared from cells by scraping cells from T75 flasks in PBS with 0.5% Tween 20. Extracts were then sonicated briefly and left for 30 min on ice. The debris was cleared by centrifugation. Cell extracts were electrophoresed on 10% polyacrylamide gels. Protein immobilised within the gel was transferred to a PVDF membrane (Millipore) by a semi-dry transfer method. The transfer was run for 120 minutes at 50 mA, 25 V using a Bio-Rad semi-dry blotter. The membrane was then blocked in Tris buffered saline containing 0.5% (v/v) Tween 20 and 5% (w/v) (TBS-T) milk powder for 1 hour with shaking. Membranes were probed with a primary antibody. This was either a monoclonal antibody against PrP (ICMS-18, D-Gen), a polyclonal ZnT-1 antibody (D-20, Santa Cruz), a polyclonal anti-SPCA antibody (Abcam), a polyclonal GluR1 antibody (Millipore), a monoclonal MTF-1 antibody (Abcam) or a monoclonal antitubulin antibody. Incubation with the primary antibody was in TBS-T containing 5 % milk powder at 4 ºC overnight. The membrane was then washed again in TBS-T 5 times before being incubated with a secondary HRP-conjugated antibody, in TBS-T containing 5 % milk powder for 45 minutes at room temperature. Following extensive washing in TBS-T, the membrane was developed for 2 minutes with ECL reagent and exposed to film. A Compact X-ray processor was used to develop the film. Membranes were then stripped with 100 mM glycine, 2%

SDS pH 3 stripping buffer for half an hour and reprobed with further antibodies as required.

Cellular metal contents

Cells were harvested from culture flasks by removal from the surface with trypsin. The cells were pelleted by centrifugation and then washed with metal-free PBS (DPBS, Lonza) to remove traces of medium and trypsin. Cell pellets were then weighed and digested with TMAH (tetramethylammonium hydroxide, 25%, TraceSelect, Fluka). Digestion was at room temperature for 7 days.

Digested samples were diluted 25-fold into a solution of 0.05% (*w/v*) EDTA (Fisher Scientific), 0.005% (*v/v*) Triton X-100 (Sigma) and centrifuged at 12,000 x *g* for 10 minutes to remove any particulate material prior to analysis by inductively-coupled plasma mass spectrometry (ICP-MS). Calibration of the ICP-MS instrument (Agilent 7500 series ICP mass spectrometer equipped with a cross flow nebulizer, quartz spray chamber, and an Octopole Reaction System (ORS®) cell, Agilent Technologies, USA) was performed in the range 0-500 ppb using external ICP-MS standards (Sigma), prepared in 0.4 % (*v/v*) TMAH, 0.05 % (*w/v*) EDTA and 0.005% (*v/v*) Triton X-100. 0.4 % TMAH was used as the rinsing solution between samples. Samples were measured in triplicate in helium gas-mode to remove matrix interferences.

RESULTS

PrP and Metal Transporters

The cellular concentrations of key trace elements such as manganese and zinc are maintained by a range of transporting and storage mechanisms. Altering the expression of key proteins in the cell can alter the level of trace elements. In the case of zinc the overexpression of zinc transporter-1 (ZnT-1) can increase zinc efflux from cells (Palmiter and Findley, 1995; Sankavaram and Freake, 2012). Similarly, a mutant of a Ca/Mn transporter protein expressed in the Golgi known as SPCA (secretory pathway calcium ATPase-1) has been shown to increase manganese efflux from the cell (Mukhopadhyay and Linstedt, 2011). The role of PrP in metal concentrations in cells has been a subject of study for some time, especially in regards to copper and manganese. However, much less is known about the relation of PrP to zinc homeostasis.

In this study we employed three different cell models. We used a mouse cerebellar granule cell/neuroblastoma fusion cell line pair. F14 was derived from knockout mice and lacks PrP expression, while the co-derived cell line, F21, has neuronal levels of PrP expression. We also used mouse N2a cells which could easily be transfected to overexpress mouse PrP or mutants. Lastly, we used the SMB cell line which is constitutively infected with scrapie and a cured cell line derived from this (SMB-PS) which does not have detectible infection.

We used western blotting to determine the difference in expression between these cell lines in terms of the level of expression of ZnT-1 and SPCA. In Figure 1A it can be seen that F21and F14 cell lines shown no difference in expression of these proteins. Neither N2a cells with and without PrP overexpression nor SMB and SMB-PS cell

show any difference in the expression of these transporters. This suggests that PrP has no effect on the expression of ZnT-1 or SPCA.

It has recently been suggested that the expression of PrP causes increased zinc flux into cells via AMPA receptors (Watt et al., 2012). We therefore took these same cell lines and examined them for difference in expression of the AMPA receptor subunit GluR1. As shown in Figure 1B, while the different cell lines have different levels of GluR1 expression, there is no effect of PrP expression on GluR1 expression. This implies that the level of PrP expression has no effect on AMPA receptor expression.

Effect of PrP on Cellular Zinc Concentrations

It has been suggested that altered expression of PrP results in a change in zinc flux into cells (Watt et al., 2012) . Also, it was suggested in the same work that the octameric repeat region of the N-terminus of PrP was required for this effect. While increased PrP expression had no effect on zinc retention, effects of PrP expression on basal zinc concentrations were not determined. For this reason we created stable N2a cell lines overexpressing either PrP or two mutants of PrP which were a deletion of the octameric repeat region or a deletion of the palindromic region of the hydrophobic domain. Control cells were transfected with the empty vector (pCDNA3.1). In addition we also transfected cells with pCDNA3.1 constructs containing the open reading frames for ZnT-1 or the metal-regulatory transcription factor-1 (MTF-1). Both proteins may alter zinc concentrations in cells (Choi and Bird, 2014; Sankavaram and Freake, 2012). The level of overexpression for these proteins is shown in Figure 2.

As we used different cell lines in this study, we first established whether there was any difference in the basal level of zinc concentration in these different cell lines. Therefore Figure 3A shows wet weight zinc concentrations for N2a, SMB, SMB-PS, F14 and F21 cells. The data showed that the approximate zinc concentration in all the cell types was in the order of 20-30 ppm (or 20-30 mg/kg). There was no significant difference between F14 and F21 cells in terms of zinc concentration (Student's t test, $p < 0.05$). These two cell lines differ greatly in the level of PrP they express which implies that PrP expression level has no effect on cellular zinc concentration under normal culture conditions. N2a cells have significantly higher levels of zinc than either F14 or F21 cells and these differences are likely to be due to factors other than PrP expression levels. SMB and SMB-PS cells shown no significant difference to each other ($p > 0.1$) in terms of zinc concentrations and this suggests that the presence of scrapie infection in the cells had no effect on cellular zinc concentration.

Having established the relatively similar level of zinc present in different cell types with different levels of AMPA receptor expression, we then looked at the effect of PrP overexpression (both wild-type and mutant) on cellular zinc levels in N2a cells. As shown in Figure 3B neither overexpression of wild-type nor mutant PrP caused any significant change to the levels of zinc present in the cells. In comparison, overexpression of MTF-1 caused a significant increase in zinc levels and overexpression of ZnT-1 caused a small but significant decrease in zinc levels. The increased zinc content of MTF-1 overexpressing cells may be due to increased expression of metallothioneins, which can be induced by MTF-1 binding to the MT promoters (Gunther et al., 2012; Laity and Andrews, 2007), whilst a decrease in zinc levels in the ZnT-1 overexpressing cells is likely due to enhanced efflux (Fukada and

Kambe, 2011). This demonstrates that protein overexpression could modulate zinc levels in the cells but that PrP overexpression did not.

Effect of Metal Transporters on PrP expression.

We have previously shown that PrP is interconnected with the regulation of expression of metal transport proteins and also antioxidants such as superoxide dismutase. Similarly, the expression of PrP can also be modulated by changing the expression of some metal transport proteins (Kralovicova et al., 2009). It has recently been shown that a mutant of SPCA (Q746A) can increase the rate of manganese transport out of the cell. We looked to see whether the overexpression of ZnT-1, SPCA and the SPCA mutant (Q746A) could alter the expression of PrP in N2a cells. As shown in Figure 4 neither overexpression of ZnT-1 nor SPCA caused a change in expression of PrP.

Manganese and Prion Infection

We have previously shown that prion infection increases the level of manganese in cells and also that increased manganese levels increase susceptibility to prion infection (Davies and Brown, 2009). We were therefore interested to see what effect increased manganese efflux from cells has on the level of prion infection in cells. We transfected SMB with SPCA, SPCA (Q746A) and the empty vector (pCDNA3.1) and analysed the manganese content of the cells. The results from the analysis are shown in Figure 5. As shown previously, scrapie infected SMB cells show significantly higher levels of manganese than the uninfected controls (SMB-PS). Overexpression

of SPCA had no significant effect on the levels of manganese in either cell line. In contrast, the SPCA mutant Q746A significantly reduced the level of manganese in SMB cells. However, Q746A had no significant effect on manganese concentrations in SMB-PS cells. These results suggested that the SPCA Q764A mutant caused a significant reduction of manganese in infected cells only.

We then looked to see if the overexpression of SPCA-Q746A had any effect on the production of protease resistant prion protein in SMB cells. Protein was extracted from SMB cells transfected to overexpress SPCA, SPCA-Q746A, ZnT-1 or transfected with the empty vector (pCDNA3.1) and treated with proteinase K (25 μg/ml, 30 min). Western blot and immunodetection for PrP was used to assess any differences in the level of proteinase K resistant protein in the extracts. As can be seen in Figure 6, the cell lines all showed similar levels of protease resistant protein except the Q746A cells. This cell line showed no apparent protease resistant protein. The results suggest that transfection with the SPCA-Q746A mutant resulted in a significant loss of detectible protease resistant protein from the cells.

Lastly, we carried out an infection study on SMB-PS cells either transfected with the SPCA-Q746A or the empty vector. The cells were exposed to extracts from SMB cells for four days and then cultured for a further week. Extracts were then prepared from the infected cells and the level of proteinase K resistant protein determined. As can be seen in Figure 7, while we successfully re-infected the SMB-PS cells, the SPCA-Q746A cells showed no protease resistant PrP. This implies that overexpression of SPCA-Q746A protected cells from prion infection in this assay.

DISCUSSION

The prion protein has been referred to as a metalloprotein because it binds copper with relatively high affinity (Brown, 2004; Brown et al., 1997; Davies et al., 2011). There is clear evidence that links PrP to metal metabolism. However, the extent and the importance of this remains unclear. While considerable research has focused on copper, the relation of PrP to other metals remains patchy. The three metals that have gained some interest in this regard are zinc, manganese and iron (Brown, 2011; Singh et al., 2014; Watt and Hooper, 2003). This current investigation was inspired in part by a recent paper suggesting that PrP mediated increased zinc flux through AMPA receptors (Watt et al., 2012). There has also been a suggestion that PrP is structurally similar to the ectodomain of some zinc transporters of the ZIP family (Ehsani et al., 2011). In addition we wished to continue our investigation of the relation of PrP and manganese.

There has been little research relating zinc and PrP. The possibilities in terms of any relationship between the two could either be a result of zinc interacting with PrP or PrP altering zinc metabolism indirectly. The possibility of a direct interaction between zinc and PrP depends upon the affinity of PrP for zinc being sufficiently high relative to the prevailing conditions. Affinity data for binding of zinc to PrP suggest that free zinc concentrations would have to be of the range of 25-300 μM (Davies et al., 2009; Walter et al., 2007). PrP is an extracellular protein and most synapses would not contain this level of zinc under normal conditions. The exception are zincergic neurons where free zinc levels after synaptic release have been estimated to be of the order of 1-100 μM (Ayton et al., 2013). In this case PrP could bind zinc during this

transient pulse. However, despite this possibility there is currently no evidence that PrP binds zinc *in vivo*.

Our results suggest that PrP expression does not alter total zinc contents of cells under any condition or cell type that we tested. We ensured that some of these cells expressed AMPA receptors as Watt et al. (2012) proposed that PrP modulates zinc entry through cells with AMPA receptors. There is sufficient evidence to link a proportion of zinc entry into the cell to AMPA receptor channel promiscuity in conditions such as epilepsy and ischemia where abnormally high extracellular levels of Zn^{2+} are encountered (Jia et al., 2002; Weiss and Sensi, 2000). However, under our study conditions, differences in AMPA receptor expression had no impact on total zinc contents as different cell types with differing levels of AMPA receptor expression showed almost no difference in cellular zinc concentrations.

The main cellular role of AMPA receptors is as ligand gated calcium channels. Therefore the main ion associated with the opening of AMPA receptor channels is calcium. There is a significant body of literature that suggests PrP modulates calcium entry/concentrations in cells, with PrP-deficient cells showing lower calcium entry (Ferreiro et al., 2008; Herms et al., 2000; Powell et al., 2008; Torres et al., 2010; Whatley et al., 1995). In this context one possible interpretation of the data showing that PrP modulates zinc entry (Watt et al., 2012) is that the effect of PrP on AMPA receptors is to increase ion permeability through the receptor. In this case what is observed is increased diffusion down the concentration gradient when excess zinc is applied to the outside of the cell. This explanation does not require any interaction of zinc with PrP.

Although PrP had no effect on total zinc levels, it could be possible for altered zinc concentrations to modulate PrP expression as the zinc sensitive transcription factor MTF-1 has been shown to alter the expression of PrP (Bellingham et al., 2009). MTF-1 modulates expression of many of the known transporters for zinc including ZnT-1 (Choi and Bird, 2014), but has also been associated with copper metabolism (Mattie and Freedman, 2004), which has been more frequently linked to PrP. We have shown in this study that altering the expression of ZnT-1 altered total cellular zinc quotas, but without any detectible change in PrP expression.

The final possibility is that zinc could be related to the level of prion infection in cells, either by zinc altering the level of protease resistant PrP, or by PrP^{Sc} altering zinc metabolism. Again, we have not been able to demonstrate any relation between zinc concentration and infection in the current study and altering the expression of ZnT-1 had no effect on the level of protease resistant protein in SMB cells. Our previous studies on tissue from CJD, experimental mouse scrapie, sheep scrapie and BSE showed no relation between zinc and disease (Hesketh et al., 2008; Hesketh et al., 2007; Thackray et al., 2002). We did observe an increase in blood zinc in the late stage of experimental scrapie (Thackray et al., 2002). One other study looked at zinc in experimental scrapie and found decreases in zinc in two different scrapie strains in the brains of infected rodents at the end stage of the disease (Wong et al., 2001). However, as our time course study on mouse scrapie did not show similar changes to the previous study, then these changes in zinc may have been a result of end stage pathology. Therefore, we feel that there is insufficient reason to link zinc to prion disease. In summary, our studies did reveal neither any relationship between PrP expression and total cellular zinc quotas nor between zinc levels and PrP^{Sc} infection.

In mammals, manganese is a less abundant metal than zinc and the regulation of its cellular concentration is less well studied. Transport of manganese into and out of cells is largely mediated through proteins that allow entry of multiple cation species. The principal transport protein for manganese into cells is the divalent metal transporter 1 (DMT-1), also known as natural resistance-associated macrophage protein 2 (NRAMP-2) (Erikson and Aschner, 2006). Another family member, NRAMP-1, is associated with manganese flux into the cytosol from the endoplasmic reticulum (Evans et al., 2001). Transport of manganese into cells has also been associated with the activity of the transferrin receptor through uptake of transferrin with manganese bound (Connor et al., 1990). Transport of manganese out of cells is believed to occur through the Golgi and the only known transporter of manganese here is SPCA (Van Baelen et al., 2004; Van Baelen et al., 2003). There are at least two family members to the SPCA family, but only SPCA-1 (referred to as SPCA in this work) is known to transport manganese. Manipulation of expression levels of SPCA has not been shown to impact cellular manganese levels. This is consistent with our observations. However, a mutation in this protein has been shown to greatly increase manganese efflux from cells. This mutation at residue Q746 was referred to as Q747A in the original publication but this was based on an error in the original published sequence (Mukhopadhyay and Linstedt, 2011). It was postulated that this mutation increases the pore cavity size within the transporter, allowing more rapid entry and exit of the cation and thus a greater rate of flux. There has been no structural study to confirm this suggestion.

Our data using the mutant Q746A of SPCA confirm that it can cause a reduced intracellular manganese concentration in cells, but only in the prion infected SMB

cells. The reason why non-infected cells did not show the same decrease in manganese content is unclear. However, clearly cellular manganese concentrations are dependent on a variety of mechanisms interacting (such as uptake and intracellular binding proteins) which might be quite different between the two cell lines. Additionally, manganese levels in cultured cells are quite low and there is likely to be a concentration-dependence to the increased manganese efflux that only SMB cells, with a higher cellular concentration to begin with, would be susceptible to. Also, we have previously shown that scrapie infected mice have elevated levels of DMT-1 which demonstrates the potential for altered manganese metabolism specific to infected cells (Kralovicova et al., 2009).

The most important finding in the current work is that cellular expression of mutant SPCA results in a significant loss of PK resistant PrP from SMB cells. Furthermore, SPCA-mutant overexpressing cells appeared to be resistant to prion infection at high titre. This would suggest that reducing the intracellular manganese concentration is effective in reducing cellular levels of PrP^{Sc}. In our previous work we have shown that increased expression of DMT-1 (Kralovicova et al., 2009), which is associated with uptake of manganese, increases cellular levels of PrP^{Sc}. Also, cells incubated in higher levels of manganese in the medium are more susceptible to prion infection at lower titres (Davies and Brown, 2009). Other work has shown that treating infected mice with CDTA, a manganese chelator that causes efflux of manganese from the brain, results in a marked decrease of detectible PrP^{Sc} in the brain (Brazier et al., 2010). Taken together these results strongly support the notion that the levels of PrP^{Sc} in cells, and their propensity to be infected with prion disease are influenced by cellular concentrations of manganese. Therefore, the possible mechanism of action of

SPCA-Q746A on PrP^{Sc} level is to reduce the amount of manganese that can interact with PrP. This would destabilise PrP^{Sc}, allowing it to be degraded by the proteasome and diminishing detectible levels, in a similar way to chelation as mentioned above.

Recombinant PrP has been shown to bind Mn(II) with an affinity similar to other known manganese binding proteins (Brazier et al., 2008). The metal can either bind at His95 in the N-terminal region in addition to Cu(II) binding in the octameric repeat region or can bind instead of Cu(II) at two sites (Brazier et al., 2008; Brown et al., 2000). The binding of manganese to PrP has a number of effects which include altering its conformation and increasing its protease resistance (Tsenkova et al., 2004; Zhu et al., 2008). The interaction of manganese with PrP also increases its toxicity to cells in culture and creates seeds with a high potential to initiate polymerisation of the protein into aggregates (Hesketh et al., 2012; Uppington and Brown, 2008). The presence of manganese in clay matrices enables PrP to survive in a soil-like environment for at least two years, much longer than metal free protein (Davies and Brown, 2009). This previous work has provided mechanistic insight into how manganese could be a risk factor for prion infection. The current finding supports this, but also shows that reversal of manganese levels in cells also reverses a sign of prion infection. It would be very important to determine if the expression of SPCA-Q746A in an animal model of prion disease would be protective or able to reverse the disease pathology.

It is important to note that manganese is essential for glycosylation of proteins (Powell and Brew, 1976). As PrP is a glycoprotein, this places PrP during it synthesis in a location with high manganese. Therefore, increased cellular concentrations of manganese are likely to increase the possibility of PrP-manganese interactions and

misfolding as we have previously demonstrated. SPCA as a key protein that supplies manganese to the compartment where glycosylation occurs is a likely candidate to alter the potential interaction of manganese and PrP. What is important to note is that the pumping of manganese by mutant SPCA causes increased secretion of manganese from the cell rather than an increased static level of Mn in the Golgi (Mukhopadhyay and Linstedt, 2011). Therefore, the mutant form of SPCA mechanistically is ideal for diminishing PrP-manganese interactions.

In summary, we have been unable to find any relation between cellular zinc concentrations and either PrP expression or infection. In contrast, prion infection increased cellular manganese levels and expression of a gain of function manganese transporter mutant decreases both levels of PrP^{Sc} and susceptibility to prion infection in cells.

ACKNOWLEDGEMENTS

The authors would like to thank Professor Arie Moran for the gift of the pCDNA3.1-ZnT-1 plasmid.

FIGURE LEGENDS

Figure 1 Effect of PrP on Protein Expression

Protein extracts were prepared from different cell lines and run on a PAGE gel and transferred to a membrane with western blotting. The level of expression of SPCA, ZnT-1 and the AMPA receptor subunit GluR1 were determined using specific antibodies and chemiluminescence. Tubulin was also detected to ensure equivalent loading. The cell lines used were F14, F21, N2a (transfected with the empty vector

pCDNA.3.1), N2a-PrP (transfected to overexpress mouse PrP), SMB-PS and SMB cells. The bands generated were densitometrically analysed and normalised to the N2a values (equated to 100%). There was no significant difference in the expression of any of the protein in the different cell lines for SPCA and ZnT-1. However, both F14 and F21 showed significantly higher levels of GluR1 (Student's t test, $p < 0.05$) and SMB-PS and SMB showed significantly less GluR1 (effectively undetectable). In no case did differences in PrP expression or infection cause a significant ($p > 0.05$) effect on the expression of the proteins analysed. Shown are the mean and s.e. for four experiments.

Figure 2 Validation of protein overexpression

This research required the overexpression of a number of proteins in different cell lines. The generation of N2a based cell lines overexpressing MTF-1 and ZnT-1 are presented here as representative of procedures to verify overexpression. N2a cell lines were transfected using pCDNA.3.1 based vectors carrying the open reading frame for either MTF-1 and ZnT-1. After at least two weeks of selection with G418 the protein extracts from the cells were tested for overexpression using western blot and detection with specific antibodies. As can be seen both MTF-1 and ZnT-1 were similarly overexpressed when compared to N2a cells transfected with the vector only (N2a).

Figure 3 Zinc levels in Cells

A Comparison of zinc concentrations in different cell types as determined by inductively-coupled plasma mass spectrometry. The mouse cell lines shown are a

neuroblastoma cell line (N2a, n=7), two fusion cell lines (F14 is PrP deficient, n=9: F21 has wild-type PrP expression, n=11), scrapie infected cells (SMB, n=5) and a cured version of SMB cells (SMB-PS, n=5). The only significant difference observed was that the F21 and F14 cells had significantly lower zinc levels than N2a cells ($p < 0.05$). Values are shown as parts per million (ppm) wet weight.

B Concentrations of zinc in N2a cells transfected to overexpress wild-type PrP (PrP-WT), a mutant of PrP lacking the octameric repeat region (PrP-ΔOcta), a mutant lacking the palindromic region (PrP-Δ112-119), MTF-1 or ZnT-1. Concentrations from wet weight analysis are shown as parts per million (ppm); n=4 for all measurements. An asterisk indicates significant difference ($p < 0.05$) to the control cells transfected with the empty vector (pCDNA).

Figure 4 PrP expression in cells overexpressing metal transporters.

Protein extracts were prepared from N2a cells transfected to overexpress either ZnT-1, SPCA or SPCA-Q746A (Q746A). After gel electrophoresis, and western blotting, the blot was analysed for the expression of PrP and tubulin. As PrP is a glycoprotein several bands can be observed ranging between 25 kD and 33 kD in size. Densitometrical analysis (graph) showed that the expression of PrP was not significantly affected by the overexpression of any of the proteins analysed. Shown are the mean and s.e. for four experiments.

Figure 5 Manganese levels in cells

Manganese concentrations were measured in SMB and SMB-PS that were transfected to overexpress either SPCA, SPCA-Q746A or transfected with the empty vector (pCDNA), (n=4 for each cell type). Values are shown in parts per billion (ppb) wet weight. The values of pCDNA for SMB and SMB-PS were significantly different ($p < 0.05$). The asterisk indicates that SPCA-Q746A was significantly different to pCDNA for SMB cells only.

Figure 6 SPCA overexpression and PK resistant PrP

SMB cells were transfected to overexpress either ZnT-1, SPCA or SPCA-Q746A. Extracts were prepared and treated with 50 μg/ml proteinase K (PK) for 1 hour and then inhibited with pefabloc. PK treated and untreated samples were then run on a PAGE gel and western blotted to a membrane. Detection with a specific antibody revealed PK resistant (PK +) bands. The level of PK resistant protein was compared to the total detected (PK -) for each cell line and is shown in the graph. There was no significant effect (Student's t test, $p > 0.05$) of ZnT-1 or SPCA on the amount of PK resistant PrP in the cells when compared to cells transfected with the vector only (pCDNA). However, PK resistant protein was effectively absent form SPCA-Q746A cells. Shown are the mean and s.e. for four experiments.

Figure 7 SPCA overexpression and prion infection.

SMB-PS cells were used to test whether SPCA-Q746A had an effect on prion infection. Both SMB-PS cells transfected with either pCDNA3.1 or SPCA-Q746A were exposed to SMB cell extract (500 ug) for 7 days. The cells were then passaged

four times before being assayed for PK resistant protein as described in Figure 6. While PK resistant protein could be observed in cells transfected with the empty vector (pCDNA) the cells transfected with SPCA-Q746A showed no PK resistant protein. Tubulin levels in the PK - lanes were assessed to verify equal loading (PK degrades tubulin).

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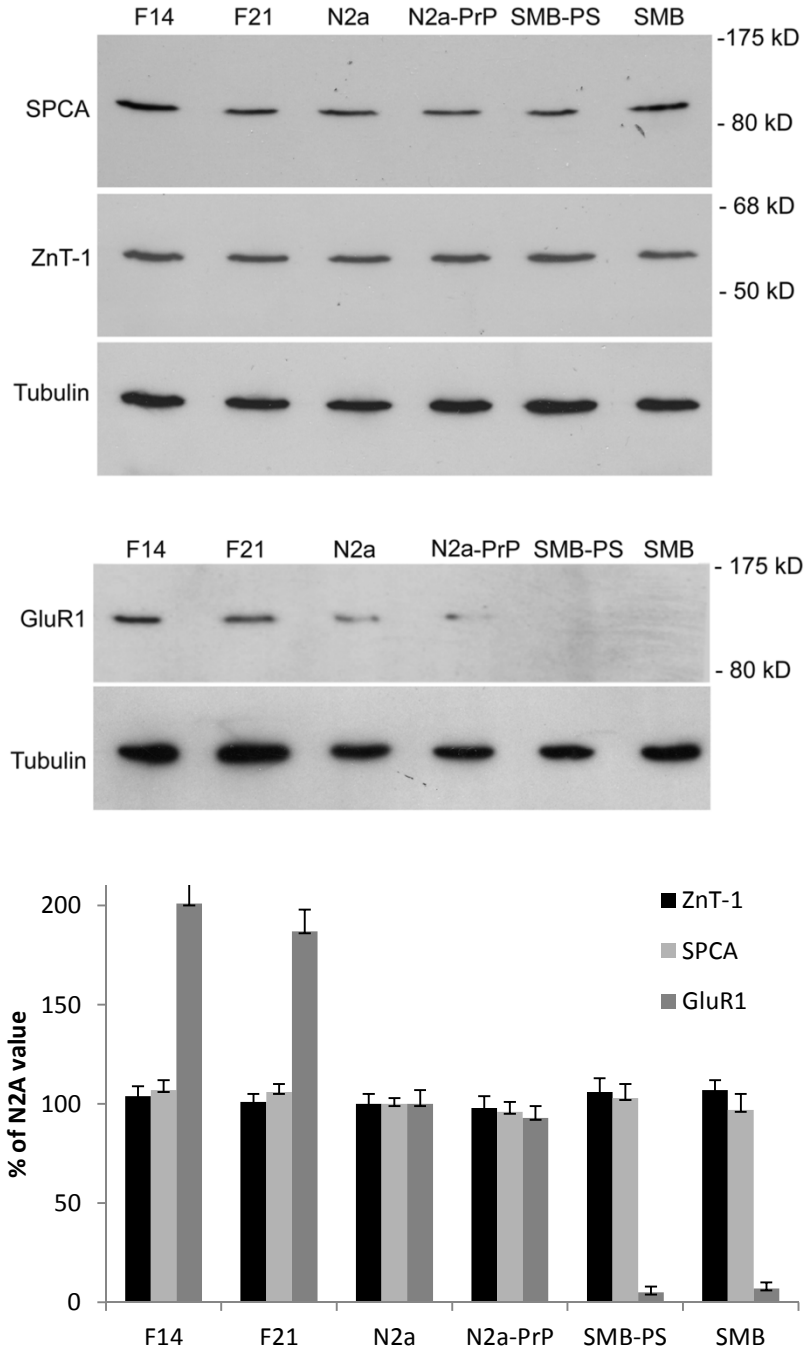
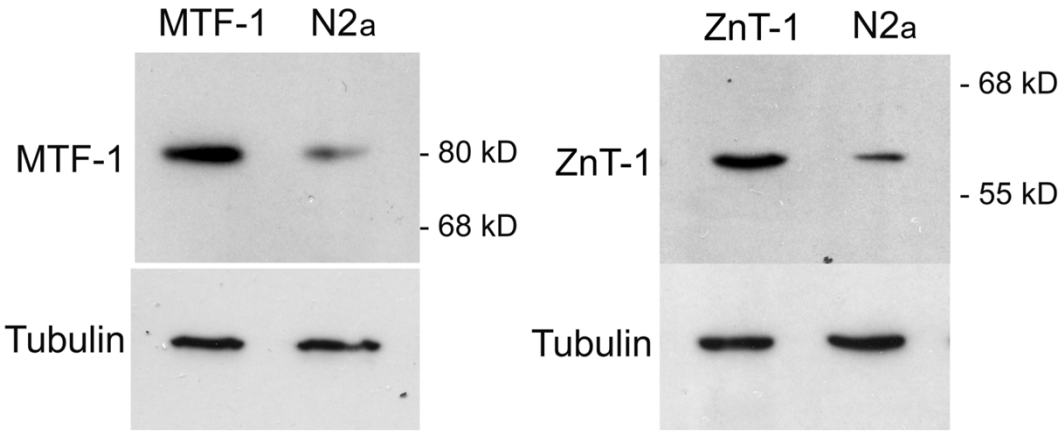

F14
F21
N2a
N2a-PrP
SMB-PS
SMB
-175 kD
SPCA
- 80 kD
- 68 kD
ZnT-1
- 50 kD
Tubulin
F14
F21
N2a
N2a-PrP
SMB-PS
SMB
- 175 kD
GluR1
- 80 kD
Tubulin
200
150
100
50
0
% of N2A value
ZnT-1
SPCA
GluR1
F14
F21
N2a
N2a-PrP
SMB-PS
SMB

Figure 1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N2a
MTF-1
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Tubulin
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ZnT-1
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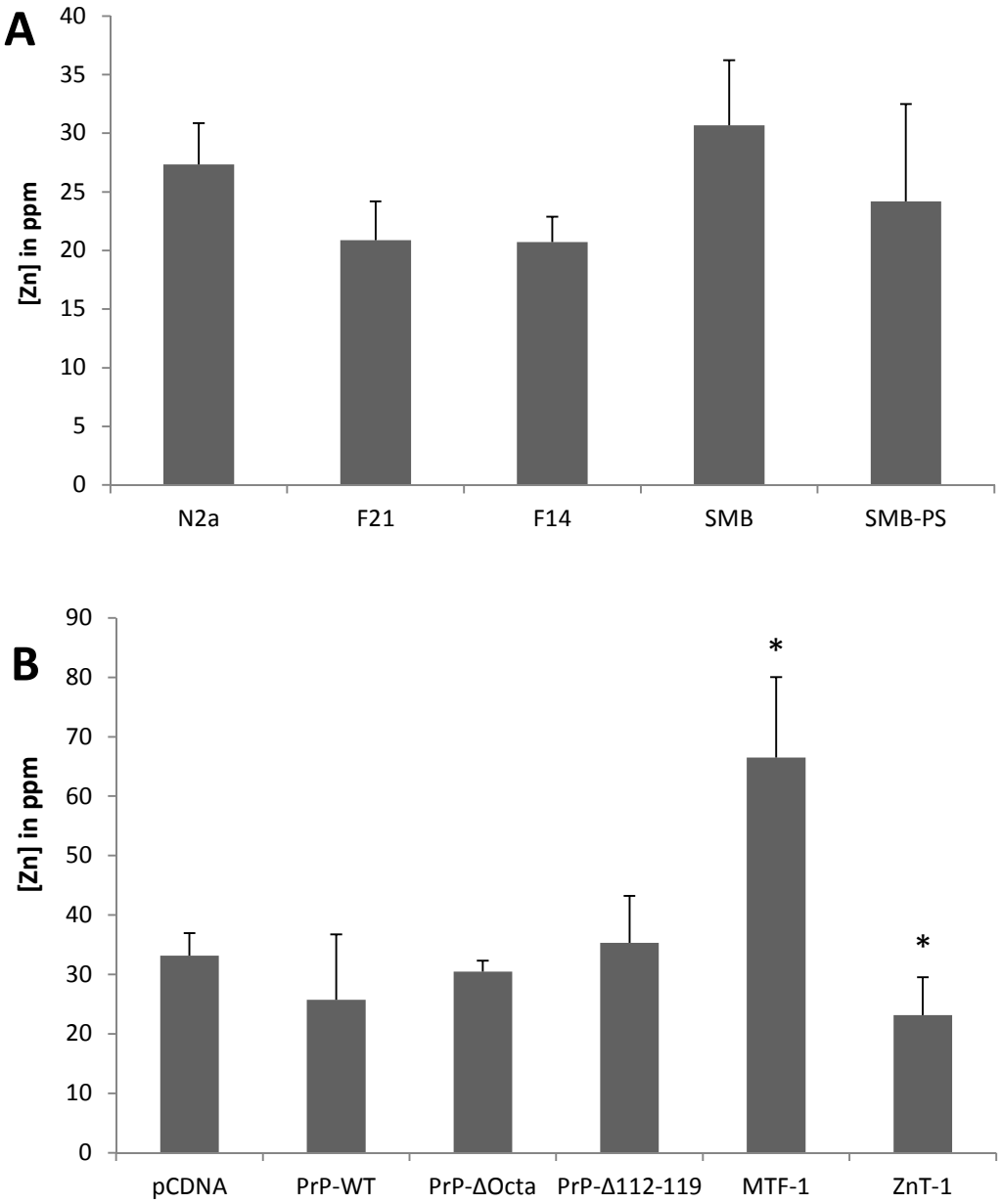
- 55 kD
Tubulin

Figure 2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30
25
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15
10
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N2a
F21
F14
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B
[Zn] in ppm
90
80
70
60
50
40
30
20
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PrP-WT
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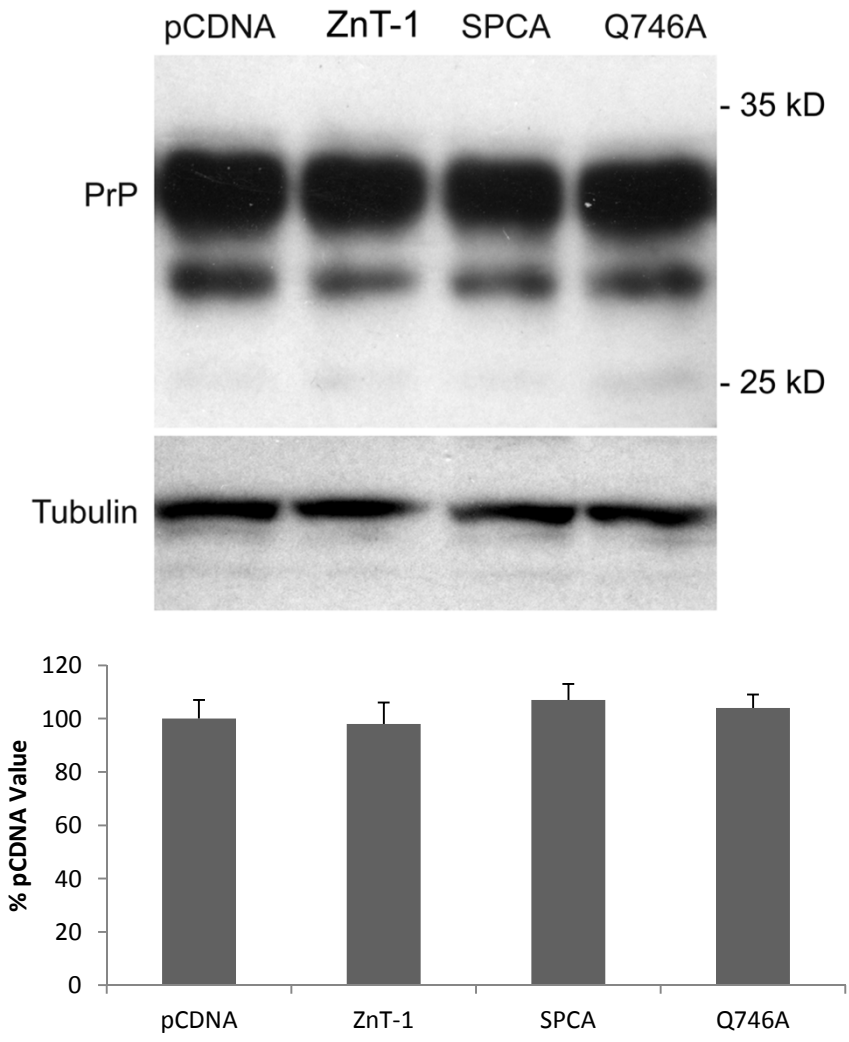
PrP-Δ112-119
MTF-1
ZnT-1

Figure 3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- 35 kD
PrP
- 25 kD
Tubulin
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SPCA
Q746A

Figure 4

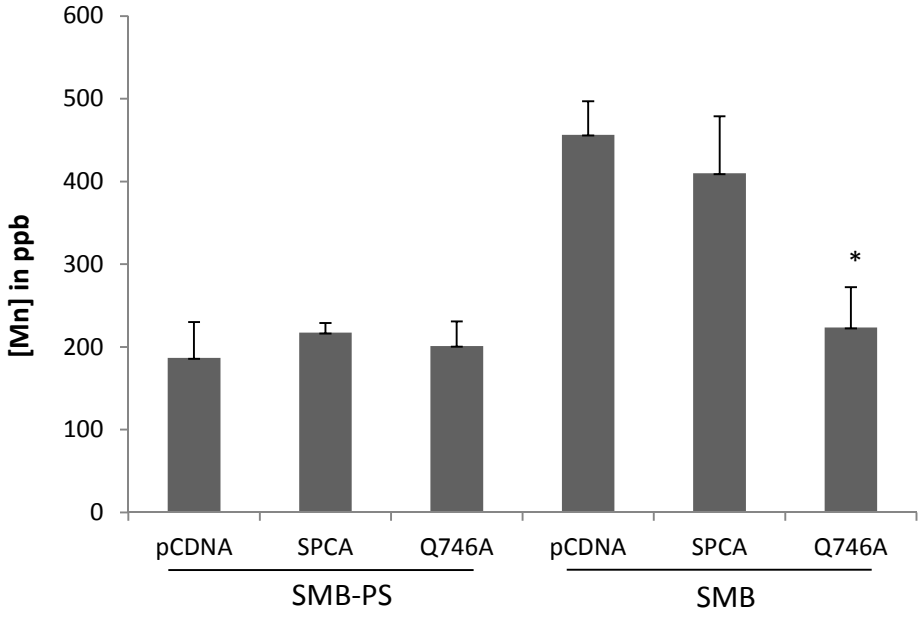
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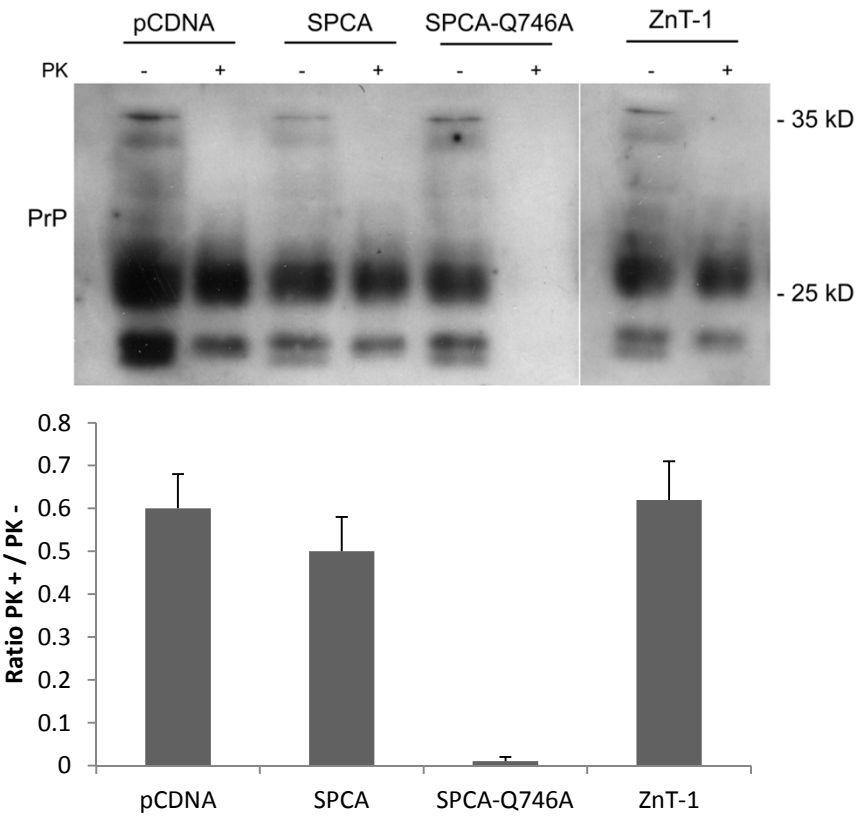
Figure 5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ZnT-1
PK
- + - + - + - +
PrP
- 35 kD
- 25 kD
Ratio PK + / PK -
0.8
0.7
0.6
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SPCA
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ZnT-1

Figure 6

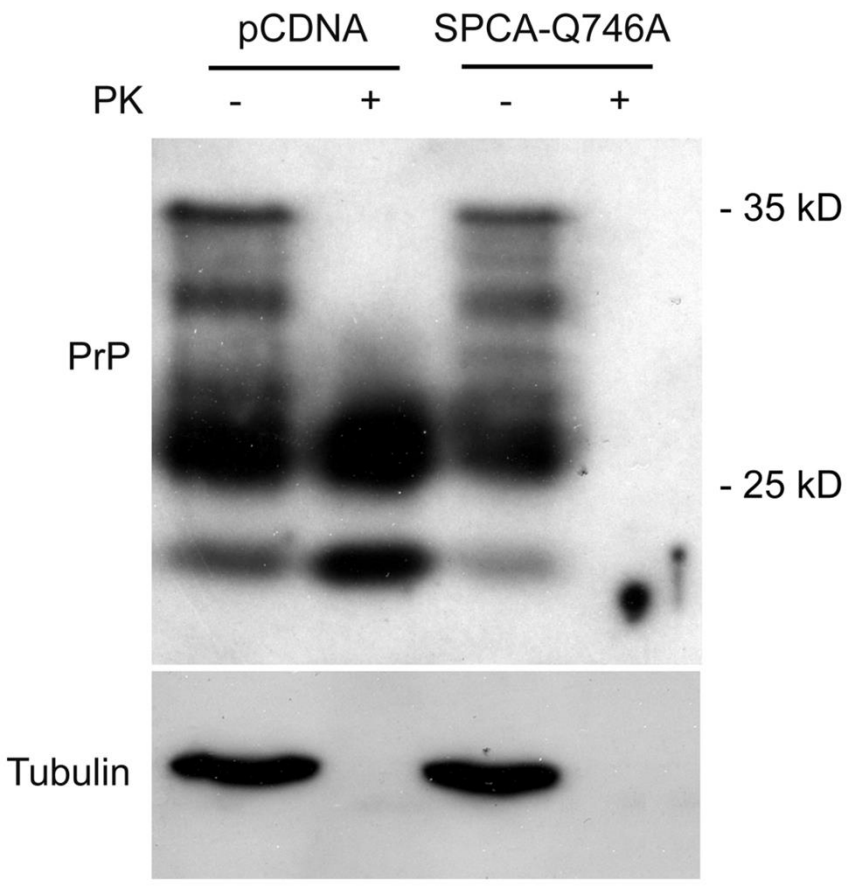

Figure 7