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Passage of murine scrapie prion protein across the mouse vascular blood–brain barrier

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Abstract

Prions are the infectious agents associated with transmissible spongiform encephalopathies and are composed mainly of a misfolded form of the endogenous prion protein. Prion protein must enter the brain to produce disease. Previous work has emphasized various mechanisms which partially bypass the blood–brain barrier (BBB). Here, we used the brain perfusion method to directly assess the ability of mouse scrapie protein (PrP^{SC}) to cross the mouse BBB independent of the influences of neural pathways or circulating immune cells. We found that PrP^{SC} oligomers rapidly crossed the BBB without disrupting it with a unidirectional influx rate of about 4.4 μl/g-min. HPLC and capillary depletion confirmed that PrP^{SC} crossed the entire width of the capillary wall to enter brain parenchyma. PrP^{SC} also entered the cerebrospinal fluid (CSF) compartment. These results show that a prion protein can cross the intact BBB to enter both the parenchymal and CSF compartments of the brain.
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Prion diseases represent a diverse group of species specific, communicable disorders of the central nervous system (CNS) transmitted by infectious proteins. In mouse scrapie, the transmissible agent is a glycoprotein containing sialic acid and other oligosaccharides with about a 30,000 MW protein core, designated as PrP^{SC}, which binds to the endogenous protein PrPc. After binding, the tertiary structure of PrP^{C} is converted to that of PrP^{SC}. PrP^{C} and PrP^{SC} contain the same amino acid structure and conversion does not involve covalent modification. After an extended period of accumulation within the CNS, PrP^{SC} eventually produces clinical disease that is progressive and fatal.

To produce CNS disease, PrP^{SC} must enter the brain, which requires it to negotiate the blood–brain barrier (BBB). How PrP^{SC} negotiates the BBB is unclear. The BBB consists largely of endothelial cells which comprise the capillary bed of the brain and the ependymal cells which define the choroid plexus and are modified by intercellular tight junctions, a lack of intracellular pores, and a low rate of vesicular transcytosis. These modifications allow the BBB to severely restrict the entry of proteins into the CNS.

Two mechanisms for the transport of prion into the CNS have been proposed: retrograde spread through thoracic spine peripheral nerves and involvement of immune cells. Kimberlin and Walker [1] noted that the thoracic spinal cord is the first region of the CNS to become infected in mouse scrapie. Because they did not envision a mechanism by which hematogenous spread could account for selective uptake, they postulated that PrP^{SC} might enter the brain by selective uptake by sympathetic neurons which would provide a pathway into the spinal cord. They postulated that the thoracic cord was particularly vulnerable because it received a higher neural input from the spleen, an area of early PrP^{SC} replication. But later studies have shown that various species of scrapie tend to also target the midbrain and pons medulla as well as the spinal cord [2]. These sites of brain infection do not fit the splenic model because they are not the major sites of projection for the thoracic splenic nerves.

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Direct immune invasion is supported by the observation that the lymphoreticular system has been shown to be important in the infectivity of prions [3]. In particular, B lymphocytes which express native PrPc are required for peripherally inoculated PrP^{SC} to infect the CNS. As a result, SCID mice, which lack lymphocytes, do not develop scrapie after inoculation with PrP^{SC} [3]. These observations have led to the suggestion that the B lymphocyte is needed to deliver prion to the CNS [4–6]. However, the results are also readily explained by the lymphoreticular system being a critical site of replication, much as it is for HIV-1 [7,8]. Without lymphocytes, PrP^{SC} may not be able to reach titers capable of producing disease.

Both of these models are based on the anatomical distribution of prion lesions in the CNS. Because prion diseases progress slowly, a long time has elapsed since the initial inoculation and the appearance of the CNS patterns. These patterns could be the result of areas where prions can more easily reproduce because of high levels of PrPc or favorable conditions for conversion. Indeed, microglial activation and behavioral changes are evident in the preclinical stage, long before classic neuroanatomical lesions can be observed [9,10]. As such, it is difficult to deduce the initial pathway of entry into the CNS based on histologic patterns which occur months after inoculation. These methods cannot be used, for example, to determine whether PrP^{SC} can interact with the BBB directly, as such events would occur within minutes for circulating glycoproteins [11–14].

A mechanism which has not been well explored is the possibility that prion could directly cross the BBB. Here we used radioactively labeled, highly purified murine PrP^{SC} to study uptake by the mouse BBB. We used the brain perfusion method which has numerous advantages for these studies, because it eliminates circulating factors so that PrP^{SC} uptake by BBB can be studied without the influences of circulating immune cells or circulating proteins. Since PrP^{SC} is only in contact with the vasculature supplying the brain, influences of thoracic or splenic uptake are negated. By delivering PrP^{SC} directly to the brain vasculature, the direct interactions of PrP^{SC} with the BBB can be investigated.

Experimental procedures

Isolation of prion protein. PrP^{SC} was purified from mice infected with the 139A scrapie strain as previously described [15]. Briefly, brain tissue was solubilized in sarkosyl and subjected to a series of differential centrifugations employing a Beckman TL100 ultracentrifuge with the final step consisting of a sucrose gradient. After this procedure, PrP^{SC} represented 50–60% of the total protein as evaluated by SDS–PAGE and silver staining. The material was then treated with proteinase K (50 μg/ml) for 2 h followed by a sucrose gradient. Final PrP concentration was estimated by immunoblot analysis and by micro Bradford protein assay, using mouse recombinant PrP and monoclonal antibody to prion protein (6H4) purchased from Prionics (Zurich, Switzerland). Final protein was infective, estimated to be >90% PrP^{SC}, and had a concentration estimated by immunoblot analysis and by micro Bradford protein assay, using as standard recombinant PrP purchased from Prionics (Zurich, Switzerland), to be about 1.5 mg/ml [16].

Labeling of prion protein and albumin. PrP^{SC} was radioactively labeled with ^{131}I with the use of iodobeads (Pierce Biotechnology, Rockford, IL). Briefly, iodobeads were added to 0.1 ml of chloride free phosphate buffer (pH 7.5) containing 0.1% of SB3-14 (Sigma Chemical, St. Louis, MO) and 2 mCi ^{131}I. After 2 min, 50 μl of PrP^{SC} was added and incubated 3 min. Radioactively labeled PrP^{SC} (I-PrP^{SC}) was separated from free ^{131}I by eluting it on a column of G-10 Sephadex in 0.1 ml fractions of buffer. An equal volume of Ringer's lactated solution containing 1% bovine serum albumin was added to the purified fraction and this was stored at 4 °C until use. Radioactivity in the purified peak was more than 90% acid precipitable.

Albumin was labeled with ^{99m}Tc (T-Alb). A mixture of 240 mg/ml stannous tartrate and 1 mg/ml albumin was adjusted to pH 3.0 with HCl. One millicurie of $^{99m}TcNaOH_4$ was added to this mixture and allowed to incubate for 20 min. The T-Alb was purified on a column of G-10 Sephadex.

Brain perfusion. Mice were anesthetized with ethyl carbamate, the thorax opened, and the heart exposed. Both jugular veins were severed and the descending thoracic aorta was clamped. A 26 gauge butterfly needle was inserted into the left ventricle of the heart and Zlokovic's buffer (7.19 g/L NaCl, 0.3 g/L KCl, 0.28 g/L $CaCl_2$, 2.1 g/L $NaHCO_3$, 0.16 g/L KH_2PO_4, 0.17 g/L anhydrous $MgCl_2$, 0.99 g/L D-glucose, and 10 g/L bovine serum albumin added the day of perfusion) containing 10^5 cpm/ml I-PrP^{SC} and 10^5 cpm/ml T-Alb was infused at a rate of 2 ml/min for 1–5 min [17]. This rate of perfusion quickly fills the brain's vascular space without disrupting the BBB [18]. An injection check of 10 μl of the buffer solution was taken before and after perfusion, so the exact concentration of radioactivity could be calculated. After perfusion, the needle was removed and the mouse was decapitated. Brain/perfusion ratios were calculated by dividing the cpm/brain by the weight in g of the brain and by the cpm in a μl of perfusion fluid to yield units of μl/g. Influx rate (K_i) was determined from the slope of the relation between brain/perfusion ratios and time in min.

In other mice, the vascular space of the brain was washed free of it contents (vascular washout). To do this, 20 ml of Ringer's lactated solution was infused through the left ventricle of the heart in 60 s after brain perfusion had been completed but before decapitation.

Capillary depletion. After 5 min of brain perfusion with I-PrP^{SC} and T-Alb followed by vascular washout, the mouse was decapitated and the brain harvested. The cerebral cortex was isolated, weighed, and placed in ice-cold physiologic buffer (10 mM Hepes, 141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, and 10 mM D-glucose adjusted to pH 7.4). The cortex was then homogenized using a glass tissue grinder (10 strokes) in 0.8 ml physiologic buffer. Dextran solution, 1.6 ml of a 26% solution in physiologic buffer, was added to the homogenate, mixed vigorously, and homogenized (3 strokes). The homogenate was centrifuged at 5400*g* for 15 min at 4 °C in a swing bucket rotor. The pellet which contains the brain vasculature and the supernatant which contains the brain parenchyma were carefully separated and the radioactivity of each component was determined using a gamma counter. The parenchyma/serum and capillary/serum ratios (μl/g) were calculated by the equation:

$$\text{Ratio} = (\text{cpm Fr})/(w)(\text{cpm}/\mu\text{l serum}),$$

where cpm Fr is the cpm in either the parenchyma or supernatant fraction, w is the weight of the cortex, and cpm/μl serum is the level cpm in a μl of serum. The values for the parenchymal space of the brain for PrP^{SC} were corrected for any vascular residual by subtracting the values for T-Alb.

Entry into CSF. Cerebrospinal fluid was obtained from the posterior fossa 30 min after the iv injection of 10^6 cpm/mouse of PrP^{SC}. The CSF/serum ratio was calculated as μl of serum/ml of CSF so as to be analogous to the brain/serum ratio of μl of serum/g of brain (the brain/serum ratio is calculated as [cpm/g of brain]/[cpm/μl of serum] = μl/g).

HPLC identification of radioactivity entering brain. Brains were removed from mice that had been perfused with I-PrP^{SC} for 10 min and then had received vascular washout. Brains were homogenized in 5 ml of 0.25 M phosphate buffer, pH 7.0. The homogenate was centrifuged at 5400*g* for 20 min at 4 °C and the resulting supernatant lyophilized. A portion of the supernatant was mixed with 30% trifluoroacetic acid to determine whether radioactivity could be precipitated with acid. The remainder of the supernatant was reconstituted in 0.2 ml of distilled water before injecting onto a Bio-Sep size exclusion column. To assess the effects of processing, I-PrP^{SC} was added ex vivo to the brain of a non-perfused mouse and processed as above.

Statistics. Statistical analysis was performed with the use of the Prism 3.0 program (GraphPad Software, San Diego, CA). Regression lines were calculated by the least squares method and are reported with their correlation coefficient (r), n, and p value. Means are reported with their standard error terms and n.

Results

The rates of uptake into brain for I-PrP and T-Alb were compared by simultaneously perfusing them through brain. As the upper panel of Fig. 1 shows, the brain/perfusion ratio for Tc-Alb did not change with time, demonstrating that perfusion did not disrupt the blood–brain barrier. There was a trend for a correlation with time for radioactive PrP^{SC} ($p = 0.08$) with a K_i of 4.40 ± 2.18 μl/g-min. The lower panel of Fig. 1 shows the brain/serum ratios for PrP^{SC} corrected for vascular space by subtraction of the Tc-Alb ratios. The resulting relation between brain/serum ratios for PrP^{SC} and time was highly significant ($r = 0.792$, $n = 10$, $p < 0.01$) with a K_i of 5.56 ± 1.51 μl/g-min.

To further determine the ability of prion to cross the BBB, we repeated the study with a larger n and performing vascular washout at the end of perfusion. Brain/perfusion ratios for I-PrP^{SC} increased over time ($r = 0.602$, $n = 17$, $p < 0.05$) with a $K_i = 3.30 \pm 1.130$ μl/g-min, whereas essentially all of the Tc-Alb was washed out (Fig. 2, upper panel). With correction of vascular

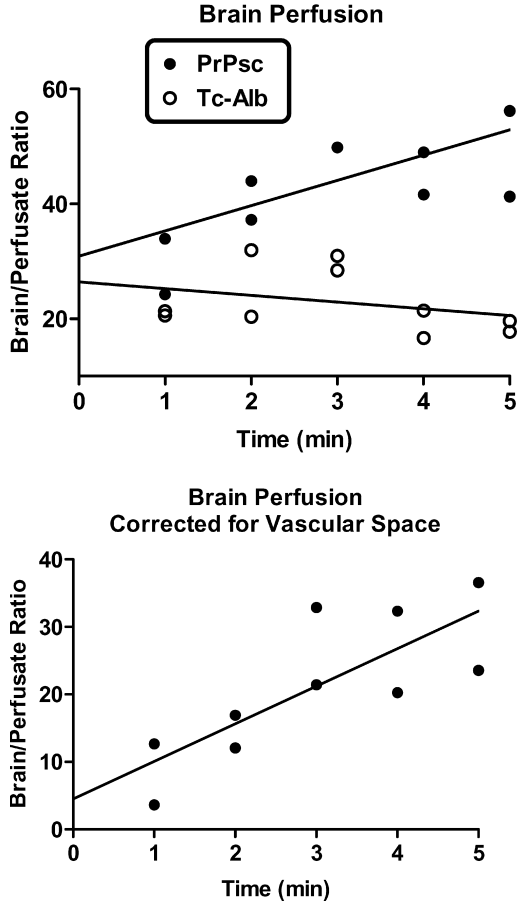

Fig. 1. Brain perfusion. Mice were perfused simultaneously with I-PrP^{SC} and Tc-Alb. Upper panel shows that I-PrP^{SC} crossed the BBB at a rate of 4.40 ± 2.18 μl/g-min. Tc-Alb did not penetrate the BBB, demonstrating an intact BBB. Lower panel shows I-PrP^{SC} brain/serum ratios corrected for vascular space as measured by Tc-Alb. This confirmed blood to brain entry of I-PrP^{SC} independently of albumin space or BBB disruption.

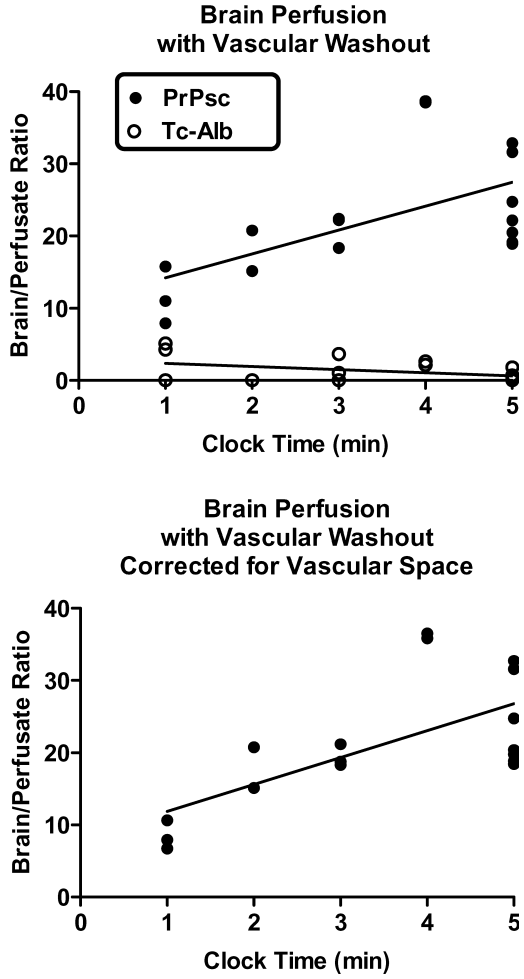

Fig. 2. Brain perfusion: vascular washout. Mice were perfused simultaneously with I-PrP^{SC} and Tc-Alb. Upper panel shows penetration of I-PrP^{SC} across the BBB at a rate of $K_i = 3.30 \pm 1.130$ μl/g-min. Almost all of the Tc-Alb was removed with vascular washout, demonstrating an intact BBB. Lower panel shows I-PrP^{SC} brain/serum ratios corrected for residual Tc-Alb.

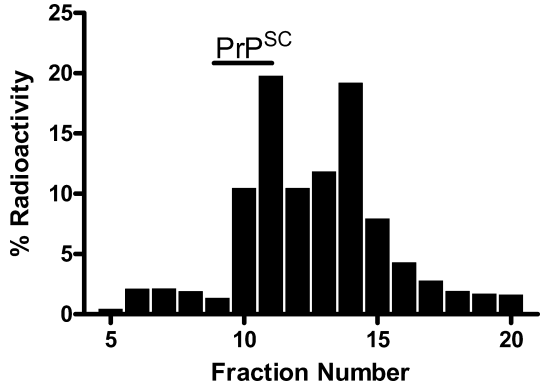

Fig. 3. HPLC on size exclusion column. About 40% of radioactivity extracted from brain eluted in the position of I-PrP^{SC}.

space, the uptake of PrP^{SC} by brain was more statistically robust ($r = 0.666$, $n = 17$, $p < 0.005$) and the K_i was 3.74 ± 1.08 μl/g-min (Fig. 2, lower panel).

Capillary depletion was performed after a 5 min brain perfusion with washout ($n = 5$). For PrP^{SC}, the capillary/perfusion ratio was 15.8 ± 2.4 μl/g and the parenchyma/perfusion ratio (after correction for Tc-Alb) was 8.56 ± 0.90 μl/g. No Tc-Alb was detected in the capillary fraction and the parenchyma/perfusion ratio was 0.21 ± 0.08 μl/g.

The CSF/serum ratio was 8.4 ± 1.8 ($n = 4$) μl/ml. In a second experiment, the CSF/serum ratio was 5.5 ± 1.2 μl/ml ($n = 3$).

Acid precipitation of the radioactivity in the brain supernatant from mice after brain perfusion of I-PrP^{SC} showed that $89.5 \pm 1.0\%$ ($n = 4$) of radioactivity was precipitable. Size exclusion HPLC showed that about 80% of the radioactivity eluted as a broad peak with a molecular weight between 30 and 110 kDa, for both G-10 purified I-PrP^{SC} and for the I-PrP^{SC} added to the brain processing control. Radioactivity extracted from the brain after 10 min of perfusion showed that 40% eluted at the position of PrP^{SC} and 34% eluted as a lower molecular weight fragment of about 3500 Da (Fig. 3).

Discussion

Here, we used the brain perfusion method to determine whether murine PrP^{SC} can directly cross the BBB. The brain perfusion method is an established method widely used to determine the ability of a substance to cross the BBB [19–21]. It has several advantages over other methods which make it particularly useful for the current study of prions. Replacing blood in the brain's vascular space with buffer eliminates the possibility that the prion might be interacting with cells, binding proteins, or other circulating substances which might affect passage. It also eliminates blood-borne enzymes, preventing degradation of the test material. Finally, it limits presentation of substances to the perfused region. In this case, spleen and the thoracic spinal cord are not included in the perfusion and so they are not possible routes of entry.

We were able to use established techniques to study the permeability of the BBB to PrP^{SC} because of the availability of highly purified mouse scrapie. This material is infectious [16] and was easily labeled with radioactive iodine.

We found a rapid uptake of PrP^{SC} from the vascular space into both brain and CSF. Uptake rates were at least 10 times faster than that of other toxic glycoproteins, including wheatgerm agglutinin and gp120, the viral coat of HIV [11,22], of cytokines which cross the blood–brain barrier by saturable transport systems [23], and even of morphine [24]. The rate of 3–5 μl/g-min is similar to that of small, CNS-active peptides transported across the BBB by saturable transport systems [25–27]. Therefore, the uptake rate that we measured for PrP^{SC} is well within the range for substances known to have effects on the CNS. In comparison, albumin was not taken up by brain, showing that neither perfusion nor PrP^{SC} disrupted the BBB during the course of these experiments.

A substance taken up by the BBB is not necessarily transported into the brain. Glycoproteins in particular are often recycled back into the circulation or sequestered by the BBB itself [12,13,28–32]. To determine the distribution of the PrP^{SC} taken up by the BBB, we performed capillary depletion, a method which separates capillaries from the brain parenchyma [33]. We used the brain washout version of capillary depletion, which eliminates any PrP^{SC} which would be reversibly bound to the luminal surface of the BBB. After a 5 min perfusion, capillary depletion showed that PrP^{SC} was able to cross the full width of the capillary wall to enter the brain parenchymal and interstitial fluid space. About 2/3 of the PrP^{SC} was retained by the brain endothelial cells. It may be that this material would eventually complete its passage across the BBB or it may be that this PrP^{SC} is permanently sequestered by the BBB. This raises the possibility that the brain vasculature may act as a reservoir for prion or even be a site for its replication, as has been shown for some viruses [34].

We showed with HPLC that much of the radioactivity entering the brain parenchymal space represented the intact PrP^{SC}. Acid precipitation agreed with this assessment. In comparison to the HPLC pattern for the perfused PrP^{SC}, the HPLC pattern for PrP^{SC} recovered from brain tended to be lower molecular weight. This suggests that the BBB may be preferentially permeable to the smaller forms of PrP^{SC}.

We also found that PrP^{SC} entered the CSF. Many substances which enter the brain do so primarily at the vascular BBB or the choroid plexus, whereas others enter at both locations. The pattern of distribution

within the CNS is different for these two routes of entry. Substances entering at the vascular BBB will be distributed throughout the parenchymal space of the brain, whereas substances entering at the choroid plexus diffuse throughout the cranial CSF but do not penetrate deeply beyond the CSF contacting brain surface [35–38]. Entry of PrP^{SC} at both the vascular BBB and the choroid plexus would give it a wide, immediate distribution throughout the brain.

Taken together, these results suggest a new mechanism for prion invasion into the CNS: direct hematogenous invasion of the prion protein across the intact BBB. These results do not negate the role of the lymphoreticular system in prion reproduction or in prion sequestration, nor do they address the question of prion transport within immune cells. They also do not negate the possibility that visceral autonomic nerves are a pathway for prion invasion into the thoracic spinal cord. However, they do demonstrate that neither the lymphoreticular system nor the splenic outflow is needed or required for prion invasion of the brain.

In conclusion, we show that PrP^{SC} can directly and rapidly cross the BBB to enter brain and CSF. This passage is independent of immune cells or peripheral afferent nerves. This suggests that one mechanism by which prions can enter the CNS is by direct hematogenous spread.

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