In nature prion diseases are usually transmitted by extracerebral prion infection, but clinical disease results only after invasion of the central nervous system (CNS). Prion protein (PrP), a host-encoded glycosylphosphatidylinositol (GPI)-anchored membrane glycoprotein, is necessary for prion infection and disease. Here, we investigated the role of the anchoring of PrP on prion neuroinvasion by studying various inoculation routes in mice expressing either anchored or anchorless PrP. In control mice with anchored PrP, intracerebral or sciatic nerve inoculation resulted in rapid CNS neuroinvasion and clinical disease (154 to 156 days), and after tongue, ocular, intravenous, or intraperitoneal inoculation, CNS neuroinvasion was only slightly slower (193 to 231 days). In contrast, in anchorless PrP mice, these routes resulted in slow and infrequent CNS neuroinvasion. Only intracerebral inoculation caused brain PrPres, a protease-resistant isoform of PrP, and disease in both types of mice. Thus, anchored PrP was an essential component for the rapid neural spread and CNS neuroinvasion of prion infection.

Prion diseases, also known as transmissible spongiform encephalopathies (TSE diseases), are fatal neurodegenerative diseases of humans and animals. TSE diseases include scrapie in sheep, chronic wasting disease (CWD) in cervids, and bovine spongiform encephalopathy (BSE) in cattle as well as kuru, Gerstmann-Sträussler-Scheinker disease (GSS), and familial, sporadic, iatrogenic, and variant forms of Creutzfeldt-Jakob disease (CJD) in humans. Within a species, TSE diseases are easily transmissible by intracerebral inoculation of infected tissue homogenates, whereas transmission to a new species is usually inefficient (16). A hallmark of prion diseases is the accumulation in infected tissues of a partially protease-resistant isoform of the prion protein ([PrP] PrPres). The detection of PrPres by immunoblotting or immunohistochemistry is often used as an important diagnostic feature of prion disease. PrPres is generated by misfolding and aggregation of host-encoded protease-sensitive prion protein, PrPsen, which is attached to the outer leaflet of the plasma membrane via a glycosylphosphatidylinositol (GPI) moiety (59). PrPsen is required for susceptibility to prion infection and for pathogenesis and transmission of prion diseases (10, 14).

The fastest route of TSE infection is via direct entry to the central nervous system (CNS) by intracerebral (i.c.) or intraspinal inoculation (39, 42). However, natural and experimental prion infection models include a number of different routes of exposure outside the CNS where disease onset is usually slower. Most notably these include intraperitoneal (i.p.), intravenous (i.v), intraneural (i.n.), intratongue (i.t.), subcutaneous, intranasal, and oral routes of exposure (7, 9, 13, 26, 27, 40, 43, 51). Peripheral infection is often accelerated by local amplification of agent in follicular dendritic cells (FDC) of lymphoid organs, followed by spread via local nerves to the CNS (12, 30, 43, 47, 49, 53). Alternatively, neuroinvasion via peripheral nerves after i.p., i.v., i.n., i.t., or ocular inoculation may occur without agent amplification in lymphoid tissues (5, 40, 42, 43, 55). In most cases neurons are the final route of spread to the CNS, but these neurons must express PrP to be functional in this respect (8, 55). The mechanism by which scrapie infectivity is transported along peripheral nerves to the CNS is not well understood, and some studies suggest that conventional axonal transport is not the main mechanism (28, 40, 44, 45).

To study if PrPres spread to the CNS in peripheral nerves was dependent on membrane anchoring of PrP, we compared wild-type mice expressing anchored PrP with transgenic mice (tg44+/-) expressing only anchorless PrP, i.e., lacking the usual GPI membrane anchor (18). Intracerebral scrapie inoculation of tg44+/- mice leads to high levels of infectivity and amyloid PrPres in CNS and results in a fatal amyloid brain disease (17). In these mice the PrP amyloid-associated neuropathology and cerebral amyloid angiopathy were similar to observations in several human GSS patients expressing PrP molecules lacking the GPI anchor (25, 34, 56). Thus, anchorless PrP transgenic mice appeared to be an excellent model for these unusual forms of familial prion disease.

In the present experiments we employed five different routes of inoculation outside the CNS. For all extracerebral routes of inoculation, GPI anchoring of PrP was required for efficient spread of infectivity to the brain. Therefore, anchored PrP appeared to be an essential part of the mechanism of rapid neural spread of PrPres and prion infectivity to the CNS.
MATERIALS AND METHODS
Experimental mice and tissue collection for histochemical and biochemical analyses. All mice were housed at the Rocky Mountain Laboratories (RML) in an AAALAC-accredited facility, and research protocols and experimentation were approved by the NIH RML Animal Care and Use Committee (protocol number 2007-50). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Transgenic GPI anchorless PrP mice (tg44) expressed the transgene in homozygous form (+/+) and did not express normal GPI-anchored mouse PrP (17). Mice were bred and genotyped at RML. Weaning C57BL/10 mice were obtained from Harlan Laboratories, Indianapolis, IN. Animals were observed daily for onset and progression of scrapie and euthanized when late-stage scrapie signs were consistently present. One C57BL/10 mouse from each inoculation route was euthanized at 100 days postinfection (dpi) for histochemical and biochemical analyses. Tissues were also collected from C57BL/10 mice when they were in an advanced stage of scrapie. In a similar fashion, some tg44-/-mice from each group were euthanized at various days postinfection and at a terminal stage of disease or no later than 600 dpi for histochemical and biochemical analyses. A portion of each tissue was flash frozen in liquid nitrogen and kept at -80°C for future use in biochemical analyses. A second portion was immersed in 10% neutral buffered formalin (3.7% formaldehyde) for histochmistry studies.

At necropsy of mice, brain was collected first to avoid contamination with PrPres from non-brain tissues. Scrapie inoculation of mice. Young adult mice were inoculated with an RML-Chandler scrapie brain homogoneate from C57BL/10 mice. For intracerebral, intraperitoneal, intravenous, and intravenous injection followed with a mock intracerebral needle stab, 50 µl of a 1% brain suspension containing 1.0 × 10^9 50% infective doses (ID_{50}) was inoculated. One ID_{50} is the dose causing infection in 50% of C57BL/10 mice. Some routes of inoculation did not allow injection of large volumes, so we reduced the volume and increased the percentage of brain suspension to more closely match the ID_{50} inoculated. Mice inoculated by intracutaneous, pericutsaneous, intravital, and supracutaneous routes received 2 µl of a 10% brain suspension containing 4 × 10^9 ID_{50}, and mice inoculated in the tongue received 5 µl of a 10% suspension containing 1 × 10^9 ID_{50}.

Route-specific techniques are described below.

For intracerebral inoculation, mice were anesthetized with isoflurane and injected in the left parietal lobe with a 27-gauge, 0.5-in. needle. For intravenous injections mice were restrained in a chute and inoculated in the tail vein. Groups of mice receiving an intracerebral needle stab were anesthetized with isoflurane immediately following their i.v. inoculation, and a stab wound was made in the left parietal lobe using a sterile 27-gauge needle with no inoculum (23, 39). The needle was inserted through the skull and directed into the left parietal lobe; following a slight retraction of the needle, it was redirected into a second region of the parietal lobe.

For intravenous inoculation mice were anesthetized with an intraperitoneal injection of a combination of ketamine (80 mg/kg), xylazine (16 mg/kg), and acepromazine (2 mg/kg). The lateral surface of the left rear leg was clipped and prepared for surgery. Once mice had reached a medium-to-deep plane of anesthesia, a 1-cm incision was made just caudal to the midfemoral region. Blunt dissection was used to retract muscles and expose the sciatic nerve. After visualization of the nerve, a curved forceps was placed under the nerve and used to elevate the nerve for easier inoculation. A 30-gauge needle attached to a Hamilton syringe was inserted through the nerve sheath and threaded into the nerve. The needle was then moved back and forth 1 to 2 mm within the nerve sheath approximately 10 times (6), and 2 µl of 10% brain suspension was then injected within the nerve sheath. The skin incision was closed with 7-mm surgical skin staples.

For perineural inoculation, the same methods described for the intraneural inoculation were used to expose the nerve, but the nerve sheath was not penetrated by the needle, and the inoculum was placed outside the nerve rather than directly into the nerve sheath.

For tongue inoculation mice were anesthetized as described in the intraneural section. A 30-gauge needle was directed at an acute angle into the right side of the tongue to the level of the subepithelium where 5 µl of inoculum was injected.

For intraocular inoculation mice were anesthetized as described for the intraneural nerve inoculations. Inoculum was deposited either in the vitreous chamber (i.v.) or the subarachnoid space of the brain (i.c.) using a 32-gauge needle attached to a Hamilton syringe. Results were similar for both ocular routes.

Immunoblotting analysis of PrPres. To detect PrPres by immunoblotting, tissue homogenates (20%) were prepared in 10 mM Tris-HCl (pH 7.4) using a Mini-Beadbeater (Biospec products, Bartlesville, OK). All homogenates were sonicated for 1 min using a VibraCell cup-horn sonicator (Sonics, Newtown, NJ) and briefly vortexed. PrPres preparation was done as previously described (48). Briefly, an aliquot of a 20% tissue homogenate was adjusted to 100 mM Tris-HCl (pH 8.3), 1% Triton X-100, and 1% sodium deoxycholate and treated for 45 min at 37°C with proteinase K at a final concentration of 50 µg/ml. The reaction was stopped by the addition of Pefabloc SC (Roche) to a final concentration of 4 mM. Nerve samples were homogenized for 5 min. An equal volume of 2× Laemmli sample buffer (Bio-Rad, Hercules, CA) containing 10% β-mercaptoethanol was added; samples were boiled for 5 min and then frozen at -80°C until needed. Freshly boiled samples were electrophoresed on 16% SDS-PAGE gels (Invitrogen, CA). Immunoblots were probed with D13 anti-PrP antibody (InPro Bio-technology, S. San Francisco, CA) followed by a peroxidase-conjugated anti-human IgG secondary antibody (Sigma, St. Louis, MO). Bands were detected using enhanced chemiluminescence substrate (ECL) as directed by the manufacturer (GE Healthcare Life Sciences, Piscataway, NJ).

The amount of brain PrPres detected by immunoblotting was defined relative to the signal detected in i.c. inoculated tg44-/- mice at terminal stage, and scoring was as follows: 3, to 100% of terminal mouse; 2, to 24% of terminal mouse; 1, to 4% of terminal mouse; 0, no detectable signal (<1% of signal in terminal mouse).

Neuropathology and IHC. Tissues were removed and placed in 10% neutral buffered formalin (3.7% formaldehyde) for 3 to 5 days before dehydration and embedding in paraffin. Serial 5-µm sections were cut using a standard Leica microtome, placed on positively charged glass slides, and dried overnight at 56°C. Slides were then deparaffinized using standard procedures, rehydrated to aqueous conditions, and processed for standard hematoxylin and eosin (H&E) staining or immunohistochemistry (IHC) analysis. For immunohistochemical detection of PrPres using Fast Red chromogen (Ventana, Tucson, AZ), tissue sections were pretreated in citrate buffer, pH 6.0, and heated to 120°C at 20 °C/in² for 20 min in a Decloaking Chamber (Biocare, Walnut Creek, CA) for antigen retrieval, followed by staining in the Ventana automated NexES stainer. Staining for PrP was done using human anti-mouse PrP antibody D13 at a dilution of 1:500 (InPro Biotechnology) at 4°C for 16 h, followed by a biotinylated anti-human IgG at 1:500 (Jackson ImmunoResearch, West Grove, PA) and streptavidin-alkaline phosphatase with Fast Red chromogen. Immunohistochemical detection of PrPres using diaminobenzidine (DAB) chromogen (DAB Map kit; Ventana) was done on selected slides at later times in the course of the experiments as follows. Antigen retrieval and staining were performed using a Ventana automated Discovery XT stainer. PrPres antigens were exposed by incubation in 1% CC1 buffer (Ventana) containing Tris-borate-EDTA, pH 8.0, for 188 min at 95°C. Staining for PrP was done using human anti-mouse PrP antibody D13 at a dilution of 1:500 (InPro Biotechnology) at 37°C for 2 h, followed by a biotinylated anti-human IgG at 1:500 (Jackson ImmunoResearch) and avidin-horseradish peroxidase with DAB as chromogen. The staining for PrPres was observed using an Olympus BX51 microscope using Microsuite FIVE software.

Immunofluorescence microscopy. For immunofluorescence staining for PrPres and astroglia or PrPres and microglia, tissue sections were pretreated in citrate buffer, pH 6.0, and heated to 120°C at 20 °C/in² for 20 min for PrPres antigen retrieval. Staining was performed using D13 human anti-mouse PrP (1:500), rabbit anti-glia fibrillary acidic protein (GFAP) 1:1,000; Dako, Carpinteria, CA), and rabbit anti-Iba1 (1:1,000; Wako, Richmond, VA). D13 plus either anti-GFAP or anti-Iba1 antibodies was coincubated for 2 h at room temperature, followed by incubation with Alexa-Fluor 568-labeled anti-human IgG plus Alexa-Fluor 488-labeled anti-rabbit IgG (Invitrogen, Carlsbad, CA) for 30 min at room temperature in darkness. Both secondary antibodies were used at a 1:200 dilution. After samples were rinsed with double-distilled H₂O (ddH₂O), coverslips were applied to tissue sections with ProLong Gold antifade reagent with DAPI to stain nuclei and protect fluorescence from fading. The staining for PrPres, astroglia, and microglia was observed using an Olympus BX51 microscope using Microsuite FIVE software.

RESULTS
Slow neural spread after intraneural scrapie inoculation. In mice and hamsters, i.n. inoculation in the sciatic nerve is known to be an efficient extracerebral route of scrapie infection, which results in rapid neuroinvasion and clinical scrapie (2, 6, 40). In agreement with these results, we found similar rapid development of clinical brain disease after both i.c. and i.n. inoculation in C57BL/10 mice expressing anchored PrP;
TABLE 1. Influence of inoculation route on incidence and incubation period of scrapie disease in C57BL/10 mice

<table>
<thead>
<tr>
<th>Inoculation routea</th>
<th>Incubation period (dpi)b</th>
<th>Incidence of diseasec</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.c.</td>
<td>154 ± 6</td>
<td>5/5</td>
</tr>
<tr>
<td>i.n.</td>
<td>156 ± 2</td>
<td>9/9</td>
</tr>
<tr>
<td>p.n.</td>
<td>233 ± 7</td>
<td>4/4</td>
</tr>
<tr>
<td>t.t.</td>
<td>198 ± 12</td>
<td>7/7</td>
</tr>
<tr>
<td>i.o.</td>
<td>231 ± 32</td>
<td>6/6</td>
</tr>
<tr>
<td>i.p.</td>
<td>193 ± 5</td>
<td>3/3</td>
</tr>
<tr>
<td>i.v.</td>
<td>203 ± 6</td>
<td>6/6</td>
</tr>
<tr>
<td>i.v. + i.c. stab</td>
<td>190 ± 6</td>
<td>9/9</td>
</tr>
</tbody>
</table>

a The ID_{50} dose of RML scrapie inoculated via the different routes is described in Materials and Methods. i.c., intracerebral; i.n., intraneural; p.n., perineural; t.t., intratongue; i.o., intracranial; i.p., intraperitoneal; i.v., intravenous.

b Number of days postinoculation when mice were euthanized due to clinical scrapie. Values are means ± 1 standard deviation.

c Number of mice positive for clinical scrapie/total number of mice in group.

However, after perineural inoculation clinical disease was significantly delayed (Table 1). To test whether PrP anchoring had a role in neural spread and neuroinvasion, we compared i.c. and i.n. routes in homozygous anchorless PrP mice, line tg44+/+ (17). In these transgenic mice the two routes were quite different. After i.c. inoculation PrPres amyloid deposition was detected in the brain starting at 146 dpi (Fig. 1A), and clinical neurological disease was seen at 298 to 321 dpi (Fig. 1A). In contrast, after i.n. inoculation, mice had no PrPres amyloid in brain or clinical brain disease even after 600 dpi (Fig. 1A). However, i.n. inoculation resulted in some neural spread and accumulation as PrPres was detected by immunoblotting (Fig. 1B) and by IHC (Fig. 1C to F) in spinal cord and sciatic nerve and nerve roots on the ipsilateral (inoculated) side at late times postinfection. Furthermore, a higher frequency of PrPres detection was found in nerve and lumbar spinal cord than in thoracic and cervical spinal cord regions, which suggested slow progression of PrPres up the nerve and the spinal cord (Table 2). In addition, between 500 and 600 dpi, clinical signs of rear leg weakness and/or paralysis were noted on the ipsilateral side, indicating that significant damage might have been caused by the local PrPres deposition in these nerves or in lumbar spinal cord (Fig. 1C to F). However, the lack of PrPres in brain and the small amount in upper spinal cord following i.n. inoculation, in spite of extensive PrPres deposition in the sciatic nerve, indicated that neural spread of PrPres was slow in tg44+/+ mice. Therefore, GPI-anchored PrP was associated with rapid neural spread and brain infection following scrapie inoculation via sciatic nerve, whereas anchorless PrP was not.

PrPres deposition in nerves and muscles of the tongue. We next tested tongue inoculation as this extraneural route is known to result in CNS neuroinvasion via the hypoglossal nerve in animals expressing anchored PrP (4, 5, 7, 50). In C57BL/10 mice we also found 100% incidence of clinical scrapie after inoculation via the i.t. route (Table 1). In contrast, in tg44+/+ mice, i.t. inoculation resulted in deposition of PrPres in tongue muscle and nerves (Fig. 2A and B). However, no PrPres was found in brain (Fig. 2C), and no clinical signs were observed in any of the six mice analyzed from 354 to 600 dpi. The absence of CNS neuroinvasion, together with the presence of PrPres in nerve branches in the tongue, suggested that spread of infectivity by cranial nerves was very inefficient in tg44+/+ mice compared to spread in C57BL/10 mice. This conclusion was in agreement with results from sciatic nerve inoculation.

Infection of retina and brain after ocular scrapie inoculation. Ocular (i.o.) scrapie inoculation was also tested as this route is known to produce significant local infection as well as CNS neuroinvasion via the optic nerve and optic tract in mice expressing anchored PrP (22, 24, 42, 57). In C57BL/10 mice we found 100% incidence of clinical scrapie and abundant PrPres in brain at 231 ± 32 days after inoculation via the i.o. route (Table 1). In contrast, in tg44+/+ mice, after i.o. inoculation PrPres was not detected in brain until 357 dpi. PrPres amyloid plaques were found in the brains of 8 of 11 tg44+/+ mice between 357 and 604 dpi (Fig. 3A and B). Two of the eight positive mice also had clinical signs of CNS disease (Fig. 3A).

Interestingly, the majority of PrPres plaques in brain were not located in visual areas but were in cerebellum and cerebral cortex, often associated with the pia mater of the meninges and adjacent leptomeningeal blood vessels entering the brain (Fig. 3C and D).

After i.o. inoculation of tg44+/+ mice, large perivascular PrPres amyloid deposits were found in the inoculated eye by immunohistochemistry at 232 dpi (Fig. 4A and C). The most extensive PrPres accumulation was found surrounding blood vessels in the retinal ganglion layer (Fig. 4A, B, F, and G). At the site where the optic nerve enters the retina, PrPres deposition was readily detected in optic nerve at 232 dpi (Fig. 4C to E) and was extracellular but closely associated with GFAP-expressing astrocytes (Fig. 4E and F) and Iba1-positive activated microglia and/or macrophages with large plump cell bodies and thickened processes (Fig. 4G). PrPres was also found in close association with the pia mater in optic nerve (Fig. 4D and E). From this location PrPres might spread from the pia to the cerebrospinal fluid (CSF) in the adjacent subarachnoid space and subsequently via CSF to cortical and cerebellar meninges (Fig. 3C and D). The relatively high incidence of neuroinvasion in i.o. inoculated tg44+/+ mice indicated that spread of infectivity from retina to brain was more efficient than spread from sciatic nerve or tongue inoculation sites, but the progression was still very slow compared to that in mice with anchored PrP (Table 1).

Limited brain infection after intravenous or intraperitoneal scrapie inoculation. In a previous study, i.c. and i.p. inoculated heterozygous tg44+/− mice had readily detectable levels of infectivity in blood plasma (54). This finding together with the prominent perivascular distribution of PrPres in brain after i.c. inoculation (17, 18) prompted us to investigate the possibility of hematogenous neuroinvasion in tg44+/− mice. In C57BL/10 mice, i.v. and i.p. scrapie inoculation caused 100% incidence of brain disease at 203 ± 6 and 193 ± 5 dpi, respectively (Table 1), and accumulation of high levels of PrPres was detected in brain at similar times (Fig. 5B, lanes 2 and 3). However, perivascular PrPres accumulation was not a prominent feature in C57BL/10 mice. Therefore, there was no obvious morphological evidence for hematogenous neuroinvasion in C57BL/10 mice. Furthermore, previous experiments by several laboratories using mice expressing anchored PrP suggested that neuroinvasion after either i.v. or i.p. inoculation proceeds by amplification in lymphoid tissues, followed by spread to the CNS.
by peripheral nerves rather than by hematogenous neuroinvasion (8, 12, 29, 30, 43, 47, 49, 53).

In contrast to mice with anchored PrP, tg44+/+ mice inoculated by i.v. or i.p. routes had no brain PrPres until after 384 dpi (Fig. 5A). However, by 600 dpi, 3 of 12 i.p. inoculated mice and 3 of 11 i.v. inoculated mice had PrPres detectable by immunoblotting in brain (Fig. 5A). Two of these i.v. inoculated mice had clinical signs at late times (Fig. 5A). Representative immunoblots are shown in Fig. 5B. By immunohistochemistry, PrPres was found primarily adjacent to meninges of cerebel-
TABLE 2. PrPres deposition in brain, three levels of spinal cord, and ipsilateral sciatic nerve following sciatic nerve scrapie inoculation of tg44+/− mice

<table>
<thead>
<tr>
<th>Site</th>
<th>155–230 dpi</th>
<th>349–454 dpi</th>
<th>544–600 dpi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>0/2</td>
<td>0/2</td>
<td>0/7</td>
</tr>
<tr>
<td>Cervical cord</td>
<td>0/2</td>
<td>0/2</td>
<td>2/7</td>
</tr>
<tr>
<td>Thoracic cord</td>
<td>0/2</td>
<td>0/2</td>
<td>1/7</td>
</tr>
<tr>
<td>Lumbar cord</td>
<td>0/2</td>
<td>1/2</td>
<td>4/7</td>
</tr>
<tr>
<td>Sciatic nerveb</td>
<td>1/2</td>
<td>2/2</td>
<td>7/7</td>
</tr>
</tbody>
</table>

a Mice were inoculated in sciatic nerve with RML scrapie at 4 × 10^5 ID_{50}.
b At 15 dpi, sciatic nerve near the inoculation site was negative for PrPres by immunoblotting and IHC (data not shown), suggesting that PrPres detected at later times was newly generated.

DISCUSSION

Most cases of natural transmission of prion infection are believed to occur at peripheral sites, followed by spread to the CNS. In the present study we investigated the role of PrP membrane anchoring in scrapie neuroinvasion using peripheral sites of infection which mimic various types of natural or iatrogenic infection. Five different routes of scrapie inoculation outside the brain, i.e., i.t., i.o., i.p., and i.v., were used in C57BL/10 mice expressing anchored PrP and in tg44+/+ transgenic mice that express only PrP lacking the GPI anchor. In all

lum and cerebrum (Fig. 5C and D) and in rare cases was also seen in the choroid plexus (Fig. 5C). This was similar to the meningeal PrPres seen after i.o. inoculation (Fig. 3C and D). The low incidence and slow tempo of CNS infection after i.v. and i.p. scrapie inoculation in mice expressing anchorless PrP compared to mice expressing anchored PrP supported our previous conclusions that anchored PrP had an important influence on neuroinvasion after peripheral prion inoculation.

To investigate the role of the blood-brain barrier (BBB) in hematogenous spread of scrapie to the brain, we mechanically disturbed the BBB at the time of i.v. inoculation. Mice were inoculated by the i.v. route with scrapie, and within 2 min thereafter a needle stab to the brain was made with a clean needle (23, 39). In C57BL/10 mice the mean incubation period was 190 dpi after i.v. inoculation with the i.c. stab compared to 203 dpi without the i.c. stab, which was a small but statistically significant difference (Mann-Whitney; 95% confidence interval [CI], P = 0.0024). In tg44+/− mice, the combined i.v. and stab (i.v.+stab) method of infection compared to i.v. inoculation alone increased the incidence of neuroinvasion (Fig. 6A). Furthermore, PrPres deposition was clearly evident in the brain along the i.c. stab needle track at 225 dpi (Fig. 6B and C), suggesting that scrapie invasion of the brain occurred via leakage from blood into the stab wound. Thus, these experiments supported the conclusion that the BBB was an obstacle to early neuroinvasion by blood infectivity in both C57BL/10 mice and tg44+/− mice.

Spread of PrPres to extraneural tissues after peripheral infection routes in tg44+/− mice. In several previous studies after either i.c. or i.p. scrapie infection of heterozygous tg44+/− mice, extensive prion infectivity and/or PrPres deposition was detectable by immunoblotting or IHC at extraneural sites such as lymphoid tissues, brown and white fat, heart, tongue, skeletal muscle, and plasma (18, 54, 60). In the present studies after inoculation of tg44+/− mice by five peripheral routes described above, brown fat had prominent PrPres detectable starting at 146 to 230 dpi (Table 3). PrPres was also found in heart, spleen, white fat, and colon (lamina propria) at times prior to detection in brain. Thus, in the absence of PrP anchoring, spread of PrPres to extraneural sites was rapid, in marked contrast to the slow and inefficient spread to the CNS found in these same mice. Therefore, spread to these different locations appeared to be mediated by distinct mechanisms.
of these routes, neuroinvasion was severely affected by the lack of anchored PrP. Strikingly, in tg44+/+ mice, three routes (i.n., i.t., and i.o.) resulted in no or slow neuroinvasion of the brain (Fig. 1A, 2C, and 3A), whereas these same routes gave efficient neuroinvasion via neural spread in mice with anchored PrP (Table 1) (5, 22, 40). However, the poor neuroinvasion of brain after i.n., i.t., and i.o. inoculation in tg44+/H/H11001 mice was not due to a lack of PrPres generation in nerves as PrPres could be detected in sciatic nerve, branches of the hypoglossal nerve in tongue, and optic nerve at later times after inoculation. Thus, a lack of anchored PrP appeared to affect the speed and efficiency of neuroinvasion via nerves in tg44+/+ mice but did not block accumulation of PrPres in peripheral nerves.

Another possible interpretation of these findings is that presence of anchorless PrP, rather than absence of anchored PrP, might alter neuroinvasion. However, in our previous experiments using mice coexpressing anchored and anchorless PrP, i.p. scrapie inoculation resulted in CNS neuroinvasion in 100% of mice between 275 and 357 dpi (18; also and B. Race and B. Chesebro, unpublished data). Therefore, lack of neuroinvasion after peripheral scrapie inoculation in tg44+/+ mice appears to depend more on the absence of anchored PrP rather than on the presence of anchorless PrP.

Two other caveats might also influence the interpretation of our results. First, peripheral nerves in mice expressing no PrPsen (Prnp−/− mice) or mice expressing only anchorless PrPsen (heterozygous tg44+/− mice) were recently found to develop abnormal axonal morphology at around 60 weeks of age (11), which might influence spread of PrPres. However, this axonal problem was not detected in younger mice such as those used for our infections and thus would not be expected to influence the tempo of neuroinvasion in our experiments. Second, PrPsen expression levels in tg44+/+ mice are 8-fold lower than in C57BL/10 mice (17), and this lower expression level might also influence the rate of neuroinvasion by neural and other peripheral routes tested here. However, in other transgenic mice expressing anchored PrP at 10-fold lower levels than C57BL/10, we previously found that i.p. scrapie infection gave 90 to 100% incidence of neuroinvasion and clinical scrapie with incubation periods somewhat longer than the i.c.
FIG. 4. Immunohistochemical and immunofluorescence detection of PrPres deposition in eye and optic nerve at 232 dpi after ocular scrapie inoculation in several tg441/1 mice. (A) Extensive PrPres deposition (red, Fast Red staining) in retinal ganglion layer at 232 dpi. The eye was cut at an angle tangential to the ganglion layer, resulting in the appearance of a flap of ganglion layer extending into the vitreal space in the center of the section. Fewer PrPres deposits are present in the inner nuclear layer (INL) and only rarely were deposits detected in the outer nuclear layer (ONL) or other retinal cell layers outside INL. (B) At higher magnification of the boxed area in panel A, PrPres deposits are evident in the ganglion layer, and many PrPres plaques have a distinct perivascular appearance (arrows). (C) In a different mouse at 232 dpi the section shows widespread PrPres accumulation (brown, DAB staining) in the optic nerve entering the retina as well as in the ganglion layer of the retina and in the vitreous fluid. (D) At higher magnification of the boxed area in panel C, PrPres can be seen in optic nerve (ON), vitreous fluid (VF), ganglion layer (G), and inner nuclear layer (INL) and around two retinal blood vessels (arrows). PrPres plaques adjacent to the pia mater of optic nerve are indicated.
Therefore, lack of PrP anchoring, rather than low PrPsen levels, appears to account for the low incidence of neuroinvasion seen after i.p. infection in tg44+/−/− mice.

Slow neural spread of PrPres in tg44+/−/− mice was particularly apparent after i.n. inoculation, where the earliest detection of PrPres in lumbar spinal cord was at 349 dpi (Table 2). With an average distance of 20 mm from the inoculation site in the sciatic nerve to lumbar spinal cord, the rate of neural PrPres spread was calculated to be roughly 0.06 mm per day. Previous studies have reported rates of spread of scrapie infectivity and/or PrPres ranging from 0.5 to 3.3 mm per day in mice and hamsters with anchored PrP (6, 28, 40, 41, 46). Thus, the rate of neural PrPres spread in tg44+/−/− mice was approximately 10 to 50 times lower than in mice and hamsters with anchored PrP, demonstrating that neural PrPres spread was severely affected in the absence of the PrP GPI anchor. In both tg44+/−/− mice and mice with anchored PrP, neural spread of PrPres was calculated to be roughly 0.06 mm per day. Previous studies have reported rates of spread of scrapie infectivity and/or PrPres ranging from 0.5 to 3.3 mm per day in mice and hamsters with anchored PrP (6, 28, 40, 41, 46). Thus, the rate of neural PrPres spread in tg44+/−/− mice was approximately 10 to 50 times lower than in mice and hamsters with anchored PrP, demonstrating that neural PrPres spread was severely affected in the absence of the PrP GPI anchor. In both tg44+/−/− mice and mice with anchored PrP, neural spread of

by arrowheads. (E) Double immunofluorescence staining of the optic nerve of mouse shown in panels C and D at the level of entry into the retina. PrPres deposits (red) and an extensive network of GFAP-expressing astrocytes (green) can be seen in the optic nerve. This astrocyte network was also seen in optic nerve from uninfected tg44+/−/− mice (data not shown). Arrows indicate PrPres plaques in close proximity to the pia mater on the edge of the nerve. (F) Double immunofluorescence staining of retina at high magnification shows amyloid PrPres deposits (red), mostly perivascular and in close proximity to perivascular GFAP-expressing astrocytes (green). Close to the lower edge of the picture, the outer nuclear layer of the retina stains strongly with the nuclear stain DAPI (blue). (G) Iba1-positive microglia or macrophages (green) were detected in close proximity to PrPres deposits (red) in the retinal ganglion layer. Most of the Iba1-positive cells have large plump cell bodies (arrows) and thickened processes, suggesting an activated phenotype. Bars, 500 µm (A and C), 100 µm (D and E), and 50 µm (B, F, and G).
PrPres was much slower than conventional retrograde axonal transport (i.e., 85 mm to 430 mm per day [31]). The very slow neural spread in tg44+/+ mice might be due to limited and slow diffusion of both inoculated and newly formed PrPres in interstitial spaces of the nerve. In contrast, in the presence of anchored PrP, neural spread is likely to be a faster cell-associated process. Our data show that anchored PrP is a vital part of this process of neural transmission after infection at peripheral sites. The exact details of this process remain unclear (33), but they appear to differ from the mechanisms involved in neuroinvasion by Aβ-amyloid as this material showed no evidence for neuroinvasion after inoculation by i.v., i.o., oral, and intranasal routes (20). However, in a more recent report, neuroinvasion after i.p. inoculation of anchored Aβ-amyloid was observed (21).

Our results show that both i.v. and i.p. routes of scrapie inoculation in tg44+/+ mice resulted in slow and inefficient neuroinvasion compared to mice expressing anchored PrP (Fig. 5A). Previous studies have shown that PrP expression in nerves is necessary for neuroinvasion of the CNS after both of these routes of scrapie inoculation in mice (8, 28, 42, 55). After both i.v. and i.p. scrapie inoculation in mice with anchored PrP, CNS neuroinvasion is thought to occur via splanchnic nerves innervating lymphoid tissues, where scrapie infectivity is amplified (3, 19, 29). Thus, the poor neuroinvasion of the CNS in tg44+/+ mice after i.v. and i.p. inoculation might be due to poor spread of PrPres in splanchnic nerves, similar to sciatic and cranial nerves innervating the tongue. Similar results were seen in recent studies of G3 transgenic mice which produce only unglycosylated PrP (15). In G3 mice PrP is GPI anchored to membranes in the Golgi apparatus inside the cell rather than on the plasma membrane. G3 mice are susceptible to i.c. infection by 79A scrapie, but after i.p. inoculation they do not develop clinical disease or PrPres in brain. These results suggest that PrP must be anchored to the cell plasma membrane for effective CNS neuroinvasion.

As has been discussed in a preceding paper, lack of PrP anchoring in tg44+/+ mice leads to extensive formation of PrPres in an amyloid form (17). Possibly, large aggregates of amyloid PrPres might spread from peripheral inoculation sites to CNS less efficiently than the usual PrPres aggregates found in typical scrapie disease in mice, sheep, and other models. However, this conclusion seems unlikely for several reasons. First, tg44+/+ mice were inoculated with brain homogenates from C57BL/10 mice expressing anchored PrP, and the inoculum contained little, if any, amyloid PrPres. Second, in tg44+/+ mice only CNS neuroinvasion was slow, whereas PrPres and infectivity appeared to spread efficiently in extraneuronal tissues such as brown fat, heart, colon, and spleen (Table 3). Since plasma of infected tg44+/+ mice also contained significant amounts of infectivity (54), it is possible that extraneural spread in tg44+/+ mice is hematogenous or lymphatic.

Interestingly, in mice with anchored PrP, there is also evidence against the hematogenous route of neuroinvasion. After i.p. and i.v scrapie inoculation, CNS neuroinvasion was not observed in mice lacking PrP expression in nerves (8) or after i.p. inoculation of sympathectomized mice (29). In contrast, studies in sheep and goats (32, 58) found that initial sites of PrPres deposition in CNS were near the circumventricular organs (CVO) of the brain, which might indicate their role as a possible site of entry of prions from blood. However, the fenestrated capillaries of the CVO, which allow passage of small peptides (up to 50 amino acids), would not be likely to allow penetration by full-length PrPsen or larger multimers of PrPres and infectivity.

### FIG. 6. The effect of blood-brain barrier manipulation on PrPres deposition in brain of tg44+/+ mice after intravenous scrapie inoculation. (A) Relative amount of PrPres detected by immunoblotting in brains of tg44+/+ mice after intravenous scrapie inoculation followed by a mock intracerebral needle stab (i.v. + stab) was higher and detected earlier than after intravenous inoculation alone. Solid symbols represent mice with clinical neurological signs, and open symbols represent mice without clinical signs. (B) Immunohistochemical staining with monoclonal antibody D13 at the site of the mock needle stab (i.c. i.v. i.p. i.t. i.o. i.p. i.v. i.v. + stab inoculation) was higher and detected earlier than after intravenous scrapie inoculation alone. Solid symbols represent mice with clinical neurological signs, and open symbols represent mice without clinical signs. (C) H&E staining shows disruption of several neuronal cell layers along the needle track corresponding to locations of PrPres amyloid plaques seen in panel B. Bar, 200 μm.

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<th>Tissue</th>
<th>Earliest PrPres detection (dpi) by inoculation routea</th>
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<tr>
<td>Brain</td>
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<td>Brown fatb</td>
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a As detected by immunoblotting or IHC. Inoculation details are presented in the Materials and Methods section. See Table 1 for abbreviations of the inoculation routes.

b PrPres was usually seen first in brown fat and subsequently in white fat, heart, spleen, and colon.

c After i.t. inoculation, earliest PrPres was found in white fat.
PrPres (52). After i.v. and i.p. inoculation of tg44+/− mice, no PrPres deposition was found in CVO regions (data not shown). The PrPres found in brain of the few positive i.v. or i.p. inoculated mice was mostly in close proximity to the meninges of the cerebral cortex (Fig. 3D) and occasionally also in the choroïd plexus (Fig. 5C), suggesting that spread of infectivity from blood to CSF was a possible route of neuroinvasion at very late times after i.p. or i.v. inoculation.

Ocular inoculation of tg44+/− mice resulted in more frequent CNS invasion than with the other peripheral routes tested. Extensive PrPres was found in the retina and optic nerve of i.o. inoculated tg44+/− mice (Fig. 4). In mice and hamsters with anchored PrP, the optic tract is thought to be the main route for cerebral neuroinvasion of scrapie after i.o. inoculation (22, 24, 42, 57). However, in tg44+/− mice inoculated by the i.o. route, the majority of PrPres plaques detected at early stages of neuroinvasion were associated with meninges and submeningeal brain parenchyma and were not associated with the optic tract in the brain (Fig. 3C and D). This suggested that neural spread via the optic tract was not the main route of spread to the CNS in these mice. Alternatively, spread from retina to brain might be via CSF, which is in the subarachnoid space of the optic nerve and can flow between the optic nerve and the brain (37, 38). If PrPres could cross the pial membrane to the subarachnoid space (Fig. 4D and E, arrows), it could enter the CSF and spread to other pial surfaces in brain. This appeared to occur after i.o. inoculation of tg44+/− mice as most PrPres plaques in the brain were found in close proximity to the meninges in the cerebellum and cerebral cortex (Fig. 3D).

In summary, the present data appeared to support the conclusion that the presence of GPI-anchored PrP is an important factor in facilitating efficient CNS scrapie infection after inoculation by several different peripheral routes. Anchored PrP might be part of the transport process of PrPres by peripheral nerves to the CNS. However, the precise mechanism of this process remains to be elucidated. The current experiments also suggested that CNS neuroinvasion by the hematogenous route is unlikely to occur at high incidence in tg44+/− mice because after i.p. inoculation these mice have significant blood infectivity (54) but only rare late neuroinvasion. Thus, the BBB seems to be a barrier to prion infectivity in tg44+/− mice, and this is similar to previous conclusions from studies in nontransgenic mice with anchored PrP (8, 29).

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