Published in final edited form as:

Nat Neurosci. 2008 January ; 11(1): 109–117. doi:10.1038/nn2028.

A versatile prion replication assay in organotypic brain slices

Jeppe Falsig¹, Christian Julius¹, Ilan Margalith¹, Petra Schwarz¹, Frank L Heppner^{1,2}, and Adriano Aguzzi¹

¹Institute of Neuropathology, University of Zürich, Schmelzbergstrasse 12, CH-8091 Zürich, Switzerland.

Abstract

Methods enabling prion replication *ex vivo* are important for advancing prion studies. However, few such technologies exist, and many prion strains are not amenable to them. Here we describe a prion organotypic slice culture assay (POSCA) that allows prion amplification and titration *ex vivo* under conditions that closely resemble intracerebral infection. Thirty-five days after contact with prions, mouse cerebellar slices had amplified the abnormal isoform of prion protein, PrP^{Sc}, >10⁵-fold. This is quantitatively similar to amplification *in vivo*, but fivefold faster. PrP^{Sc} accumulated predominantly in the molecular layer, as in infected mice. The POSCA detected replication of prion strains from disparate sources, including bovines and ovines, with variable detection efficiency. Pharmacogenetic ablation of microglia from POSCA slices led to a 15-fold increase in prion titers and PrP^{Sc} concentrations over those in microglia-containing slices, as well as an increase in susceptibility to infection. This suggests that the extensive microglial activation accompanying prion diseases represents an efficacious defensive reaction.

The defining trait of transmissible spongiform encephalopathies (TSEs) is transmissibility between individuals or across species¹. The infectious agent causing TSEs is the prion, which may be identical to PrP^{Sc}, a partially protease-resistant isoform of a membrane glycoprotein termed PrP^C. Mice devoid of the *Prnp* gene, which encodes PrP^C, resist prion infections².

To determine prion titers, endpoint dilutions of samples are inoculated into large numbers of indicator animals. The incubation time of prion infections is negatively correlated to the size of the inoculum, allowing a simplified incubation time bioassay that sacrifices precision, yet requires fewer animals³. Because small amounts of infectivity correlate with incubation times, the observation periods span the life of indicator animals. This is impractical and expensive. Minimizing the numbers of animals used for titration would also reduce the suffering wrought by prion infections.

The scrapie cell assay in endpoint format (SCEPA) eliminates some of the above concerns and allows dramatically faster assays⁴. Here, cells are exposed to endpoint titrations of prions and subsequently passaged several times. Prion titers are determined by counting PrP^{Sc}-positive

© 2008 Nature Publishing Group

Correspondence should be addressed to A.A. (adriano.aguzzi@usz.ch).

²Present address: Institute of Neuropathology, Charité - Universitätsmedizin Berlin, Augustenburger Platz 1, D-13353 Berlin, Germany.

AUTHOR CONTRIBUTIONS

P.S. performed histoblots and transmissions of RML into *tga20* and CD1 mice and C.J. performed SCEPAs. I.M. performed all experimental work in Figure 3 and in Supplementary Figure 7b. Development of POSCA, slice culture preparation, infections, western blots and all other experimental work were done by J.F. *CD11b-HSVTK* mice were generated by F.L.H. This study was conducted under the supervision of A.A. All authors contributed intellectually with practical or theoretical input.

Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions>

tissue-culture wells. The SCEPA is equivalent to animal bioassays in that it detects actual transfer of prion infectivity from samples to susceptible cells. However, the reactions of the CNS to prion infection include highly diverse cell types such as neurons, astrocytes, oligodendrocytes and microglia acting in concert. This diversity cannot be reproduced in the SCEPA, which is based on monoclonal, homogeneous cells. Also, the range of cell lines that have been identified as susceptible to prion infection *in vitro* is limited⁵. At present this restricts the applicability of the SCEPA to a subset of mouse-adapted prion strains. These two limitations may be interdependent, as the machinery necessary for overcoming strain and species barriers may require cells distinct from those that replicate prions. Hence any infectivity titrations of prion variants that do not efficiently infect cultured cells still require animal bioassays.

Here we show that chronically cultured organotypic cerebellar slices rapidly amplify PrP^{Sc} after exposure to prions, thereby circumventing some of the above limitations. This protocol allows dissection of CNS pathologies in a complex cellular environment morphologically similar to the intact brain. The sensitivity of the prion organotypic slice culture assay (POSCA) was marginally lower than that of the SCEPA. Although the POSCA makes use of animals as the original tissue donors, many genetically identical slices can be produced from each individual mouse. Hence genetic background variation—which may account for the variability of mouse bioassays—can be controlled by comparing samples prepared from the same individual mouse.

Activation of microglia in prion infections is very extensive and precedes neurodegeneration^{6,7}. Microglia are abundantly associated with prion plaques, and PrP^{Sc} can sometimes be found within microglia^{6,8}. However, it is unclear whether microglial activation represents a precipitating or a defensive reaction. Using the POSCA we found that microglial ablation from slices from *CD11b-HSVTK* transgenic mice markedly increased prion titers and PrP^{Sc} deposition. Thus microglia may be important in containing prion loads during the course of prion infections.

RESULTS

Longevity of mouse cerebellar slices in long-term cultures

As prion infections progress extremely slowly *in vivo*, we reasoned that slice infection would require organotypic cultures of great longevity. We tested various parameters, including the method used for preparing slices, age of the mice, culture medium composition, and volume and frequency of media changes (data not shown). We achieved the best results when preparing slices by vibratome sectioning and culturing them on Millicell-CM polytetrafluoroethylene membranes, exchanging the full volume of medium thrice each week.

We assessed slice viability after 5 weeks of culture by morphometric quantitation of cells permeable to propidium iodide (PI) and by DEVDase activity assays, which measure the prevalence of dead cells and the substrate turnover rate of executioner caspases, respectively. Control slices were treated with staurosporine, which induces widespread apoptosis (Supplementary Fig. 1a–c online). Untreated slices showed $0.1\% \pm 0.02\%$ (mean $\pm$ s.d.) of PI⁺ cells (or $<0.1\% \pm 0.02\%$ of the total tissue surface area) and $2.1\% \pm 0.6\%$ of DEVDase levels seen in staurosporine-treated slices. Hence, slices were viable for up to 5 weeks. Morphological and immunohistochemical analyses with various markers of CNS constituents (Supplementary Fig. 1d–h) supported this conclusion.

The POSCA assay

Most TSE assays rely on the differential susceptibility of PrP^C and PrP^{Sc} to digestion with proteinase K (PK)⁹. The relative resistance of PrP^{Sc} to PK depends on the prion strain and the composition of non-PrP contaminants within the samples. We therefore determined the minimal conditions under which all PrP^C present in 5-week-old slices is fully degraded by PK (Supplementary Fig. 2a online). We found that digestion of 20 µg protein lysate with 25 µg ml⁻¹ PK (in a reaction volume of 20 µl, corresponding to 1 µg ml⁻¹ total protein) ensured complete, reproducible removal of PrP^C. We used these conditions in all subsequent experiments.

In a first attempt, we prepared slices from prion-inoculated pups, but after 5 weeks *ex vivo*, we found no PrP^{Sc} in the slices (Supplementary Fig. 2b). Next, we prepared slices from 10-d-old PrP^C-overexpressing *tga20*^{TK} (offspring of *tga20*^{+/+} males crossed to *CD11b-HSVTK* females¹⁰) pups or from *Prnp*^{0/0} (mice devoid of PrP^C) pups¹¹ and incubated groups of ten slices with 1 ml of buffer containing 20 mg 'RML' (Rocky Mountain Laboratory strain) inoculum, consisting of crude brain homogenate from pooled, terminally ill wild-type mice on a CD1 background infected with sixth-passage RML prions. To minimize any toxicity from cellular debris, slices were exposed to RML for 1 h at 4 °C in the presence of kynurenic acid.

After 5 weeks, we analyzed slices by western blotting (Fig. 1a). In all experiments, we used monoclonal antibody POM1 (ref. ¹²) to detect PrP. To facilitate comparisons, we loaded 20 µg protein from the brain of a C57BL/6 mouse terminally ill with scrapie next to the marker on each blot. In lysates of RML-infected *tga20*^{TK} cultures, we observed a characteristic triplet band pattern representing di-, mono- and unglycosylated forms of PrP^{Sc} after treatment with 25 or 50 µg ml⁻¹ PK (Fig. 1a). RML-exposed *Prnp*^{0/0} slices and slices exposed to uninfected brain homogenate never showed any PK-resistant material (Fig. 1a).

Operating performance of the POSCA

The PrP^{Sc} signal observed in POSCA slices might partly represent residual inoculum adhering to slices. We therefore performed titration experiments using varying amounts of inoculum for the infection of slices. PrP^{Sc} was unambiguously detected in *tga20*^{TK} slices exposed to as little as 1 µg RML homogenate (Fig. 1b). The amount of PrP^{Sc} recovered after 5 weeks was ≥ 40-fold higher than the total inoculum used to infect the cultures (Supplementary Fig. 3 online). Hence all PrP signals resulted from bona fide slice infection and *de novo* PrP^{Sc} amplification within slices. In RML-treated *Prnp*^{0/0} cultures (*n* = 5) or in cultures treated with uninfected brain homogenates (*n* = 5), we never observed PK-resistant material, even in slices exposed to 10 mg of RML (Fig. 1b), indicating that PrP^{Sc} was amplified at least 10⁴-fold.

Slices infected with small amounts of RML showed less PrP^{Sc} than those infected with larger amounts (Fig. 1b). This correlation was not explainable by variations in PrP^C expression (Supplementary Fig. 4a online), and it suggests that PrP^{Sc} was accumulating exponentially at the time of analysis. We recorded PrP^{Sc} amplification within slices over time (Fig. 1c). One hour after inoculation we detected no residual inoculum (Fig. 1c), and 14 d after inoculation (dpi) we observed no indication of prion replication. After 21 d we observed a definite PrP^{Sc} signal (Fig. 1c). PrP^C expression was similar in all cultures (Supplementary Fig. 4b).

We then examined the impact of host PrP^C expression levels on prion replication. We infected cultures from *tga20*^{TK} mice (heterozygous *Prnp*^{+/-} mice carrying one copy of the *tga20* transgene), *Prnp*^{+/-} mice, or *tga20*⁺ mice (mice carrying one copy of the *tga20* transgene), and compared PrP^{Sc} amounts. We observed a positive correlation between host expression of PrP^C (*tga20*^{TK} > *tga20*⁺ > *Prnp*^{+/-}; Supplementary Fig. 4c) and the amount of PrP^{Sc} observed

in the cultures 5 weeks after inoculation ($tga20^{TK} > tga20^{+} > Prnp^{+/o}$; Fig. 1d), as has been seen after intracerebral inoculation of mice¹³.

Infectivity amplification and relative sensitivity of POSCA

We then tested whether deposition of PrP^{Sc} accompanies prion replication, defined as increase in prion infectivity. Samples from the experiment in Figure 1c were used to infect the PK1 subclone⁴ of N2a neuroblastoma cells. $Tga20^{TK}$ culture homogenates showed no infectivity when assayed <28 d after exposure to RML. Slices assayed at 28 and 35 d contained >6.8 log TCI₅₀ (tissue culture infectivity) units of prion infectivity per gram total protein (1 TCI₅₀ unit being the dose required to infect 50% of PK1 culture wells) as determined by SCEPA⁴ (Fig. 1e). We detected no infectivity in RML-exposed $Prnp^{o/o}$ slice-culture lysates at any time. We compared the amount of infectivity in POSCA slices to a calibration curve of RML (Fig. 1e). RML contained >6.8 log TCI₅₀ units g⁻¹ protein (1 g RML homogenate contained 138 mg protein), similar to the amount of infectivity measured in RML-infected slices. Therefore, both PrP^{Sc} and prions were amplified in slices to amounts similar to those detected in terminally ill mouse brains.

To determine the dose required to infect 50% of the slice cultures (slice culture infectivity; SCI₅₀), we performed an endpoint dilution experiment. We found that all slices infected with 1 µg RML, yet none of the cultures infected with 0.1 µg RML, accumulated PrP^{Sc} (Table 1, Supplementary Fig. 5a online and data not shown). The SCI₅₀ was calculated according to standard methods¹⁴. Accordingly, RML homogenate contained 6.7 ± 0.1 log SCI₅₀ g⁻¹. To compare the sensitivity of the POSCA with that of other bioassays, we measured the titers of serially diluted RML homogenate by intracerebral inoculation into $tga20$ and CD1 mice as well as by SCEPA (Table 1). The $tga20$ bioassay measured 9.9 ± 0.2 log ID₅₀ units (1 ID₅₀ unit being the amount of infectivity that will cause infection in 50% of experimentally infected animals) and CD1 mice 9.3 ± 0.9 log ID₅₀ units per gram of brain homogenate. In comparison, the SCEPA showed a sensitivity of 7.7 ± 0.5 log ID₅₀ g⁻¹ (Table 1).

Infected slices appear healthy

We assessed the morphological integrity of slices by immunohistochemistry 1, 7, 21 and 35 dpi. Neurons (calbindin), astrocytes (glial fibrillary acidic protein (GFAP)) and myelin (myelin basic protein (MBP)) appeared intact. A progressive broadening of cell layers became evident at 7–35 d of culture (Supplementary Fig. 1d–f). Microglia and macrophages increased over time, confirming that slices underwent progressive microgliosis (Supplementary Fig. 1g,h). No difference was detected between $tga20^{TK}$ cultures treated with uninfected brain homogenates or RML-treated $Prnp^{o/o}$ and $tga20^{TK}$ cultures at any time point (Supplementary Fig. 1d–h).

Unexpectedly, prion infection did not affect viability 5 weeks after prion infection (one-way analysis of variance (ANOVA) with Bonferroni multiple comparison test, $P > 0.05$, $n = 12$; Fig. 2a,b). We evaluated the localization of PrP^{Sc} replication and deposition using the histoblot technique¹⁵. We observed a strong PrP^{Sc} signal in the molecular and Purkinje cell layers, suggesting substantial PrP^{Sc} deposition in granule cell axons, Purkinje neurons and possibly astrocytes (Fig. 2c). An intermediate signal was observed in the granule cell layer, and a weak signal was present in the white matter.

The POSCA is applicable to a broad range of prion strains

We exposed $tga20^{+/+}$ slices to 100 µg of brain homogenate from mouse-adapted prion strains originally derived from scrapie-infected sheep, cows infected with bovine spongiform encephalopathy (BSE), or deer infected with chronic wasting disease (CWD) and analyzed PrP^{Sc} 5 weeks after inoculation (see Supplementary Table 1 online). Mouse-adapted scrapie

strains 79A and 139A, which replicate in PK1 cells (data not shown), led to an accumulation of PrP^{Sc} comparable to or higher than that in RML-exposed slices (Fig. 3a). The mouse-adapted BSE strain 301C and, to a lesser extent, three scrapie isolates passaged once (5114/2) or twice in *tga20* (5193/1) or wild-type mice (5192/2) also replicated in slices (Supplementary Fig. 5b and data not shown). PK1 cells are known to be resistant to infection with the mouse scrapie strain ME7 (ref. 4). In slices exposed to 100 µg ME7, we found only a faint PrP^{Sc} signal (Fig. 3b), but 1 mg of ME7 inoculum clearly induced PrP^{Sc} in all cultures (Fig. 3b).

Not all scrapie strains tested positive in the POSCA, and there was a correlation between short incubation times *in vivo* after intracerebral inoculation and the ability to replicate in the POSCA (Supplementary Fig. 5 and Supplementary Table 1). We also tested mouse-adapted CWD strains from a Colorado mule-deer herd¹⁶. Mouse-adapted CWD inoculum did not seem to replicate in *tga20* slices, irrespective of *in vivo* incubation times (data not shown and Supplementary Table 1).

Conditional microglial depletion in organotypic slices

CD11b-HSVTK mice allow conditional ablation of microglia *in vivo* and *in vitro* with the cytotoxic prodrug ganciclovir (GCV)¹⁰. We cultured organotypic cerebellar slices from 10-d-old *CD11b-HSVTK* mice with GCV (5 µg ml⁻¹). After 14 d, GCV led to an almost complete loss of cells positive for IB₄ and CD68 (two macrophage-specific markers) in *CD11b-HSVTK*⁺ (*tk*⁺) slices (Fig. 4a,b). The few remaining IB₄⁺ cells appeared fragmented or associated with IB₄⁺ blood vessels (Fig. 4a). Time-lapse microscopy showed that microglial cells underwent apoptotic cell death (Supplementary Video 1 online). Neighboring microglia took up the dying cells but eventually also underwent apoptosis and were removed by other microglia.

We quantified microglial depletion from images acquired by confocal laser scanning microscopy at a depth of 35–40 µm (that is, underneath the glial scar). *Tk*⁺ slices showed reductions in microglia of 97%, 98% and 99% after 14, 28, and 42 d of GCV treatment, respectively (ANOVA, *P* < 0.001, *n* = 4; Fig. 4c). Few if any microglia were found outside the tissue in treated *tk*⁺ slices (Fig. 4d). Quantification of mRNA by quantitative RT-PCR showed a 98% reduction in *CD11b* transcript expression in *tk*⁺ slices treated with GCV for 14 d (*P* < 0.001, *n* = 3), whereas GFAP, MBP and neurofilament heavy-chain transcripts (markers for astrocytes, oligodendrocytes and neurons, respectively) were unaffected (ANOVA, *P* > 0.05) (Fig. 4e). Note that GCV treatment had no effect on *tk*⁻ slices (Fig. 4e). GFAP protein expression increased over time, and isoforms of lower molecular masses could be observed after 7–21 d in culture (Fig. 4f). Depletion of microglia did not affect GFAP expression, suggesting that the astrogliosis induced by preparing and culturing the slices was independent of microglia activation. The concentration of PrP^C was unaltered in microglia-depleted slices (Fig. 4g).

Lack of ‘bystander killing’ after microglia depletion

Phosphorylated GCV released from dying microglia may cause bystander toxicity. Thus we assessed the viability of *CD11b-HSVTK*⁺ slices treated with GCV for 14 d with respect to PI incorporation and DEVDase activity. Microglia-depleted slice cultures showed a small but significant increase in PI⁺ cells from approximately 1% up to 2% of the total surface area (ANOVA, *P* < 0.001, *n* = 12) (Fig. 4h). We observed no difference in caspase activity (*n* = 3 pools of four slices) (Fig. 4h).

To evaluate whether the increase in cell death was due to impaired corpse removal, we reconstituted microglia-depleted slices with peritoneal wild-type macrophages resistant to GCV. Macrophages were placed either directly onto the slices or separated by Millicell-CM

membranes. Direct reconstitution of slices with wild-type macrophages placed onto slices partially reduced the GCV-induced increase in PI⁺ cells, suggesting a lack of removal of dead cells within microglia-depleted tissue (Supplementary Fig. 6 online). We speculated that the increased PI⁺ cells might represent dying or dead *tk*⁺ microglia. To test this, we performed dual labeling of unfixed, GCV-treated *CD11b-HSVTK* slices with PI and IB₄. In GCV-treated slices, most PI⁺ cells also stained with IB₄, indicating that the increase in PI⁺ cells was indeed due to dead microglia (Fig. 4i,j).

Phosphorylated GCV released from dying cells can also cause cell cycle arrest¹⁷. We therefore assayed bromodeoxyuridine (BrdU) incorporation into the DNA of dividing cells. GCV treatment of *tk*⁺ slices for 14 d abolished BrdU⁺CD68⁺ cells, as expected, whereas it did not affect the number of BrdU⁺CD68⁻ cells (non-microglial BrdU⁺ cells characterized by a large nucleus) (Fig. 4k,l). Also, GCV treatment of *tk*⁻ slices had no significant effect on BrdU incorporation. Therefore, depletion of CD68⁺ cells was selective and transgene-dependent, and did not elicit any significant bystander effects.

Microglial depletion enhances prion replication

To address the contribution of microglia to prion replication, we prepared slices from *tga20*^{TK} *CD11b-HSVTK*⁻ (*tga20*^{TK-}) and *tga20*^{TK} *CD11b-HSVTK*⁺ pups (*tga20*^{TK+}). *Tga20*^{TK+} and *tga20*^{TK-} slices infected with RML (100 µg RML, or 2.7 log SCI₅₀) were split into two pools, one of which was treated with GCV. After 30 d, similar amounts of PrP^{Sc} were observed in untreated *tga20*^{TK+} and in both untreated and GCV-treated *tga20*^{TK-} slices. Notably, microglial depletion by GCV treatment of *tga20*^{TK+} slices resulted in a fivefold increase in PK-resistant PrP^{Sc} (Fig. 5a). As this increase was independent of PrP^C expression (Supplementary Fig. 7a online), microglia either directly affected the conversion of PrP^C into PrP^{Sc} or, more likely, affected the half-life of PrP^{Sc} by phagocytosing and degrading prions. To test whether the observed increase in PrP^{Sc} was associated only with microglial expression of HSVTK, we infected slices prepared from 10-d-old *GFAP-HSVTK* mice allowing for conditional ablation of astrocytes¹⁸. In contrast to GCV treatment of *CD11b-HSVTK*⁺ cultures, GCV treatment of *GFAP-HSVTK*⁺ cultures had no major impact on PrP^{Sc} or PrP^C (Fig. 5b and Supplementary Fig. 7b). Hence, increased PrP^{Sc} levels were selectively associated with microglial depletion.

To further study whether the observed effect was due to GCV-mediated removal of microglia, we tested whether the enhanced accumulation of PrP^{Sc} could be counteracted by reconstituting the macrophage population. After RML inoculation, we separated *tga20*^{TK+} and *Prnp*^{0/0}/*TK*⁺ slices into four pools each and treated three pools per condition with GCV to deplete microglia. We added 250,000 *tga20*^{+/+} or *Prnp*^{0/0} macrophages to two GCV-treated cultures of each genotype immediately after RML infection and harvested and analyzed the tissues 30 d later. After microglial depletion, we observed a clear increase in PrP^{Sc}, as expected (Fig. 5c). This increase in PrP^{Sc} could be reduced upon reconstitution of slices with macrophages, irrespective of whether the macrophages expressed PrP^C (Fig. 5c). PrP^C amounts were equal in all samples (Fig. 5d). We verified microglial depletion and reconstitution by analyzing the expression levels of the macrophage marker Iba1 (Fig. 5e). We observed no differences in GFAP expression (Fig. 5f) or caspase activity (Fig. 5g) in either of the prion-infected tissues.

Next, we used SCEPA to analyze *tga20*^{TK+} samples, untreated or treated with GCV for 30 d, from three independent biological experiments performed on different days. Microglia-depleted cultures showed a significant 14.5 ± 13.2 fold increase over undepleted cultures in their prion titers (one-tailed paired Student's *t*-test, $P = 0.0474$, $n = 3$; Fig. 5h), which corresponded very well to the difference observed by western blot analysis of microglia-depleted samples (9 ± 11.8 fold over that in undepleted slices, $n = 6$). Despite a high interexperimental variance, all six independent replicas showed a clear increase in PrP^{Sc}.

accumulation after microglial depletion (2–33 fold). No residual inoculum was observed in *Prnp^{0/0}-tk⁺* culture, irrespective of microglia status (Fig. 5h). These results confirmed that microglia negatively regulated both PrP^{Sc} and prion formation.

Microglia-depleted tissue accumulated prions to a higher degree, begetting the question of whether GCV-treated *tk⁺* slices would be more sensitive to a prion infection. We therefore performed an endpoint titration experiment with RML on *tga20^{TK+}* slices. *Tga20^{TK+}* slices were infected with various concentrations of RML and separated into two pools, one of which was treated with GCV. After 5 weeks *in vitro*, we observed PK-resistant material in microglia-depleted tissue at a tenfold higher RML dilution. Hence, depletion of microglia rendered slices more sensitive to prion infections by 1 log (Fig. 5i and Supplementary Fig. 7c).

DISCUSSION

Analytical sensitivity of POSCA, SCEPA and other methods

As no certainty yet exists about the physical nature of the agent, transmission of serially diluted samples to susceptible hosts remains the gold standard for measuring prions. The POSCA strives to combine the versatility of the mouse bioassay, which can identify many strains of prions and take advantage of a large transgenic mouse repository, with the speed and precision of the SCEPA⁴. We evaluated the analytical sensitivity of the POSCA against the SCEPA⁴ and the mouse endpoint bioassay¹⁹. The sensitivity of SCEPA was <10-fold higher than that of POSCA, the limits of detection being 7.7 log ID₅₀ per gram of brain for SCEPA, 9.9 log ID₅₀ g⁻¹ for the *tga20* bioassay and >6.7 log ID₅₀ g⁻¹ for POSCA. Depletion of microglia led to a tenfold increase in analytical sensitivity, thereby approaching that of the SCEPA. As organotypic mouse slice cultures can be viable in culture for up to 26 weeks²⁰, sensitivity might be enhanced by harvesting slices at later time points or by optimizing the infection and washing steps, particularly because residual inoculum does not seem to interfere with POSCA readings.

Only few cell lines, highly selective in their permissivity to various prion strains, are efficiently and chronically infectible with prions, and the crucial determinants of infectibility are unknown. In contrast, the transgenic expression of xenogeneic *Prnp* minigenes from various species has substantially broadened the range of strains measurable by mouse bioassay. The POSCA can fully exploit this broad range of hosts, as organotypic brain slice cultures can be prepared from mice of any desired genotype. This yields a fast, flexible and robust experimental system to study prion infection and the molecular basis of strain adaptation²¹ in a highly controlled environment. Toward this goal, we have successfully infected POSCA slices with POSCA-derived inoculum (data not shown). We found that not only the chronically mouse-adapted RML prions but also a variety of sheep scrapie and bovine BSE strains replicate in slices derived from *tga20^{+/+}* pups. The collection of POSCA-compatible strains includes ME7 prions, which do not replicate in PK1 cells⁴.

Compared to its *in vivo* counterparts, the POSCA has a very short incubation time. Intracerebral inoculation of *tga20* mice with RML prions induces disease only after 60–150 d (ref. 22), yet we detected prion replication after 20 d in *tga20^{TK}* slices. Analogously, we observed prion replication in *Prnp^{+/-}* slices after 35 d, whereas *Prnp^{+/-}* mice show incubation times of 225–300 d after intracerebral inoculation². The rapid replication in slices indicates that there may be unidentified protective processes that operate *in vivo* but may be disabled in slices.

Unexpectedly, we did not observe any pathological changes in POSCA slices as a consequence of prion infection. Although neuronal death and astrogliosis are among the defining morphological characteristics of prion-infected brains, we saw no significant changes in slice viability and in GFAP expression. It is unlikely that any developing pathology was masked by continuous removal of dead cells by microglia, because there was no difference between

microglia-depleted and undepleted prion-infected slices. It is more likely that gliosis induced by tissue preparation may mask any further prion-induced pathology. Inasmuch as the relevant neuroectodermal cell types are preserved, we do not view these changes as limiting for the deployment of the POSCA.

Microglia in prion disease

Microglia activation is an early hallmark of prion diseases. Many studies point to potentially deleterious effects of microglia. First, microglia can release inflammatory and neurotoxic factors associated with neurodegenerative diseases^{23–25}. Second, microglial cells are often spatially associated with prion plaques, and infectivity was recovered from microglia purified from terminally ill mice^{6,8,26}, leading to the conjecture that microglia act as a carrier of prions within the brain. This scheme predicts that microglia might be detrimental at several levels, but cannot be easily reconciled with the observation that peripheral macrophages can reduce prion infectivity *in vitro* and splenic PrP^{Sc} accumulation *in vivo*^{27–29}. In addition, *Prnp*^{+/-} microglia do not support prion replication when grafted into *Prnp*^{0/0} brains³⁰.

Selective manipulation of microglial cells *in vivo* is hampered by their similarities to macrophages. In contrast, the POSCA is well suited to studying the impact of microglial manipulations on prion replication. We found that microglia could be completely ablated by treating *CD11b-HSVTK*⁺ cerebellar slice cultures¹⁰ with GCV without affecting the viability of other cell populations. Microglial removal drastically unleashed prion replication in organotypic slices, suggesting a role for microglia in containment of prion infections. Microglia may exert this effect by phagocytosing and degrading prions *in situ*^{27–30}, possibly explaining the presence of prions in microglia acutely isolated from mice sick with scrapie²⁶, despite the inability of microglia to replicate prions³⁰.

The above phenomena may have practical ramifications. One intracerebral ID₅₀ unit of prions has been reported to be equivalent to 10⁴–10⁵ PrP molecules³¹. This is in contrast to many viral diseases, in which 1–10 particles are equivalent to one ID₅₀, and has been taken to question the identity of the prion with PrP^{Sc} (ref. ³²). The priolytic activity of microglia uncovered here may contribute to increasing the minimal amount of PrP^{Sc} needed to infect individual organisms. By extension, one might speculate that medical conditions and iatrogenic interventions affecting microglia may heighten the susceptibility to prion infections.

METHODS

Culture preparation and prion inoculation

All mouse experiments conformed to Swiss law. The pups used for most experiments were F₁ offspring of *tga20*^{+/+} *Prnp*^{0/0} males on a 129Sv/BL6 background crossed to heterozygous *CD11b-HSVTK* females (*tg620*) on a C57BL/6 background¹⁰. We refer to the *Prnp*^{+/-} *tga20*⁺ *HSVTK* transgene-negative offspring as *tga20*^{TK-} and the transgene-positive littermates as *tga20*^{TK+}. *Prnp*^{0/0}, *Prnp*^{+/-} and *Prnp*^{0/0} *tga20*⁺ mice were all on a 129Sv/BL6 background. *CD11b-HSVTK* (*tg620*) and *GFAP-HSVTK* mice (line 7.1) were all on a C57BL/6 background and were genotyped as previously described^{10,33}. Rocky Mountain Laboratory strain passage 6 (RML) was amplified in CD1 mice, ME7 in 129/Sv mice, 5193/1 in *tga20* mice and 22F, 79A, 87A, 87V, 139V, 301V, 5192/2 and 301C (mouse-adapted BSE) were all amplified in C57BL/6 mice by intracerebral inoculation of 30 µl 10% wt/vol brain homogenate.

We prepared organotypic cerebellar slice cultures according to a modified version of a published protocol³⁴. We obtained cerebella from 9- to 12-d-old pups. Brain tissue was embedded in 5 ml liquid (2% wt/vol) ultralow-melting-point agarose dissolved in Gey's balanced salt solution (GBSS) (NaCl 8 g l⁻¹, KCl 0.37 g l⁻¹, Na₂HPO₄ 0.12 g l⁻¹,

CaCl₂·2H₂O 0.22 g l⁻¹, KH₂PO₄ 0.09 g l⁻¹, MgSO₄·7H₂O 0.07 g l⁻¹, MgCl₂·6H₂O 0.210 g l⁻¹, NaHCO₃ 0.227 g l⁻¹) supplemented with the glutamate receptor antagonist kynurenic acid (1 mM) (GBSSK). We transferred liquid agarose (kept at 37 °C using a water bath) to a small container, submerged the isolated brain tissue and cooled on ice until the agarose solidified (10 min). The agarose block was mounted onto a specimen disc, and 350-μm-thick slices were cut on a vibratome (VT1000, Leica) while submerged in a cooled reservoir (4 °C) containing GBSSK. We recovered slices from the agarose and transferred them in a small volume of liquid to a 24-well plate using the broad end of a Pasteur pipette. Cultures were treated with various concentrations of prion-infected or uninfected brain homogenates (homogenized in PBS) diluted in 1 ml GBSSK. Tissues were incubated with brain homogenates as free-floating sections for 1 h at 4 °C and washed twice by transfer (in ~0.5 ml) to a reservoir containing 6 ml fresh GBSSK. Five to ten slices were placed on a 6-well Millicell-CM Biopore PTFE membrane insert (Millipore). Residual GBSSK was removed and the inserts were transferred to a cell culture plate and cultured in slice culture medium (50% vol/vol MEM, 25% vol/vol basal medium Eagle and 25% vol/vol horse serum supplemented with 0.65% glucose (wt/vol), penicillin/streptomycin and Glutamax (Invitrogen). Cultures were kept in a standard cell incubator (37 °C, 5% CO₂ and 95% humidity) and all of the culture medium was exchanged three times a week.

For reconstitution experiments, we obtained peritoneal macrophages by lavage. We killed mice and removed the abdominal skin without disturbing the peritoneal cavity, then injected 5 ml ice-cold PBS into the peritoneal cavity using a 25-gauge needle. After massaging the abdomen, we recovered macrophages and determined cells densities using 0.4% trypan blue. Slices were reconstituted at the time of preparation at a density of 250,000 macrophages per insert (ten slices per 6-well insert) and GCV treatment was initiated immediately. GCV was used at a concentration of 5 μg ml⁻¹, a concentration previously shown to be optimal for microglia depletion *in vitro*¹⁰.

Western blot analyses

We washed cultures twice in PBS, removed residual PBS and scraped the tissue off the membrane using 10 μl lysis buffer per slice (0.5% wt/vol sodium deoxycholate and 0.5% vol/vol Nonidet P-40 in PBS). Tissue was lysed by three freeze-thaw cycles and triturated using a 200 μl pipette until homogeneous. For vol/vol SCEPA analysis, we harvested slices by scraping tissue off the membrane in PBS, triturated using a 29-gauge insulin syringe and sonicated for 30 s. Each sample was a pool of 5–10 slices grown on the same insert, yielding approximately 200–350 μg protein in total. We determined protein concentration using the bicinchoninic acid assay (Pierce) and digested the tissue with PK (Roche) in lysis buffer for 30 min at 37 °C. PK-resistant material was detected as a standard by digesting 20 μg protein lysate in a reaction volume of 20 μl (1 mg ml⁻¹ total protein) with 25 μg ml⁻¹ PK. This condition allowed specific detection of PrP^{Sc}. PK digestion was stopped by adding loading buffer (NuPAGE, Invitrogen) and boiling the samples at 95 °C for 5 min. Proteins were separated on a 12% Bis-Tris polyacrylamide gel (NuPAGE, Invitrogen) and blotted onto a nitrocellulose membrane. Membranes were blocked with 5% wt/vol Topblock (Fluka) in Tris-buffered saline supplemented with Tween (150 mM NaCl, 10 mM Tris HCl, 0.05% Tween 20 (vol/vol)) and incubated with primary antibodies in 1% Topblock. Primary antibodies used were POM1 mouse IgG₁ antibody to PrP^C (anti-PrP^C) (200 ng ml⁻¹), ascites fluid of mouse anti-glial fibrillary acidic protein (GFAP) IgG₁ (clone GA5) (1:3,000, Sigma-Aldrich) and goat anti-human Iba1 (0.5 μg ml⁻¹, Wako). Secondary antibodies used were horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG₁ (1:10,000, Zymed) and HRP-conjugated swine anti-rabbit IgG (260 ng ml⁻¹, DAKO). The blots were developed using SuperSignal West Pico chemiluminescent substrate (Pierce) and detected using the VersaDoc system (model 3000, Bio-Rad). The molecular weight marker was spiked with recombinant PrP^C, yielding a PrP

signal at 23 kDa with a cleavage product at 15 kDa. We loaded 10 μ l marker in the left lane on all blots.

Cell death measurements

We measured PI incorporation by incubating the slices with propidium iodide (10 μ g ml⁻¹, Molecular Probes) for 15 min. Images were acquired using a fluorescent microscope (Axiovert 200M equipped with AxioCam HRm, Zeiss) and analyzed using image analysis software analySIS vs5.0 (Olympus Soft Imaging Solutions GmbH). All images were acquired using the same exposure times and analyzed using the same software settings within a region of interest (the total area of tissue present in the image). After recording fluorescence images, we washed the tissue in PBS and lysed it in PBS containing 0.5% vol/vol Nonidet P-40, 0.5% wt/vol sodium deoxycholate and 5 mM 1,4-dithiothreitol. We used a caspase-3 fluorometric correlate assay (Assay Designs) to determine caspase-3 DEVDase activity: protein lysates were incubated with the fluorogenic caspase-3 substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl-coumarin, and enzymatic cleavage by caspase-3 was quantified by kinetic measurements (one measurement every 2 min for 2 h) using a fluorometer. The specific caspase activity was calculated from a standard curve generated using recombinant caspase-3 with a known enzymatic activity and normalized to the protein content. One unit of caspase-3 activity is defined as the amount of enzyme needed to convert 1 pmol of substrate per minute at 30 °C.

Immunocytochemistry

We performed histoblot analysis according to a standard protocol using 50 μ g ml⁻¹ PK (30 min, 37 °C)¹⁵. Proteins were transferred from slice cultures to nitrocellulose membranes by pressing the tissue onto membranes soaked in lysis buffer. After protein transfer, membranes were digested with PK and PrP^{Sc} was detected with POM1. For immunocytochemistry, the tissue was fixed in 2% wt/vol PFA overnight at 4 °C. Membrane inserts were washed and incubated for 1 h in blocking buffer (0.1% vol/vol Triton X-100 and 2% vol/vol goat serum dissolved in PBS) and incubated for 2 d with primary antibody diluted in blocking buffer. Primary antibodies and concentrations used were ascites fluid of anti-chicken calbindin IgG₁ antibody (1:2,000, Swant), rabbit anti-mouse GFAP polyclonal antibody (1:1,000, DAKO), rat anti-mouse CD68 IgG_{2a} (1 μ g ml⁻¹, Serotec) and ascites fluid of rat anti-bovine MBP IgG_{2a} (1:250, Serotec). The primary antibodies were detected using Alexa-conjugated secondary antibodies (4 μ g ml⁻¹, Molecular Probes) and counterstained with 4,6-diamidino-2-phenylindole (DAPI) (1 μ g ml⁻¹). Microglia were detected in fixed and in live tissue by staining with IB₄ (*Griffonia simplicifolia*) (2–4 μ g ml⁻¹, Molecular Probes). We removed the culture membranes from the inserts and mounted them directly onto a microscope slide using fluorescence mounting medium (DAKO). Images were acquired in a focal plane 35–40 μ m below the tissue surface using a laser-scanning confocal microscope (SP 2, Leica), by fluorescence microscopy (Axiovert 200M, Zeiss) or, for live imaging studies, by using wide-field microscope (DM IRBE, Leica) equipped with a temperature controller and a CO₂ box (37 °C, 5% CO₂). We assessed incorporation of 5-BrdU into the tissue after 24 h incubation with 25 μ g ml⁻¹ BrdU. DNA was denatured by treatment with 1M HCl for 1 h at 37 °C, followed by two 30-min neutralization steps with 0.1 M borate, pH 8.5, and two washes in PBS. We then stained the tissue with 15 μ g ml⁻¹ mouse IgG₁ anti-BrdU (clone BU-33, Sigma) and rat anti-mouse CD68 according to normal procedures. We counted cells manually, assisted by image analysis software analySIS vs5.0.

Quantitative PCR

Organotypic slice cultures were prepared and incubated as previously stated. We washed cultures once with PBS and extracted total RNA using TRIzol reagent (Invitrogen Life

Technologies) according to the manufacturer's protocol. Before cDNA synthesis, residual genomic DNA was removed using the DNA-free kit (Ambion); cDNA was synthesized from 1 µg total RNA with the QuantiTect reverse transcription kit (Qiagen) using random hexamers according to the manufacturer's protocol. We tested for successful cDNA synthesis (+ reverse transcriptase) and contamination of total RNA with genomic DNA (– reverse transcriptase) by PCR with primers specific for β-actin (*Actb*). Quantitative real-time PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems) on an ABI PRISM 7700 Sequence Detector (PerkinElmer). Regulation was calculated relative to untreated wild-type slices after normalization to the *Actb* signal. The following primer pairs were used: *Actb* sense (NM_007393), 5'-GACGGCCAGGTCATCACTAT-3'; antisense, 5'-ACATCTGCTGGAAGGTGGAC-3'. *Itgam* sense (NM_008401), 5'-GACTCAGTGAGCCCCATCAT-3'; antisense, 5'-AGATCGTCTTGGCAGATGCT-3'. *Gfap* sense (P03995), 5'-AAGGGACCATTCCCTGTC-3'; antisense, 5'-AGGTTAGCGGAGGTGGAA-3'. *Mbp* (NM_010777) and *Nefh* (NM_010904) we detected using the commercially available QuantiTect primer assay (Qiagen).

Scrapie cell assay in endpoint format (SCEPA)

Prion-susceptible neuroblastoma cells (subclone N2aPK1) were exposed to 300-µl brain homogenates in 96-well plates for 3 d. Cells were subsequently split three times 1:3 every 2 d, and three times 1:10 every 3 d. After they reached confluence, we filtered 25,000 cells from each well onto the membrane of an ELISPOT plate, treated them with PK (0.5 µg ml⁻¹ for 90 min. at 37 °C), denatured, and detected individual infected (PrP^{Sc}-positive) cells by immunocytochemistry using alkaline phosphatase-conjugated POM1 mouse anti-PrP and an alkaline phosphatase-conjugated substrate kit (Bio-Rad). We performed serial tenfold dilutions in cell culture medium containing healthy mouse brain homogenate. Scrapie-susceptible PK1 cells were then exposed to dilutions of experimental samples ranging from 10⁻³ to 10⁻⁶, or to a 10⁻³ dilution of healthy mouse brain homogenate or RML. Samples were quantified by total luminescence per well or, in endpoint format, by counting positive wells according to established methods⁴.

Statistical analysis

We used one-way ANOVA with Bonferroni post-test for selected pairs of columns for statistical analysis of experiments involving the comparison of three or more samples, and paired Student's *t*-test for comparing two samples. Results are displayed as the average of replicas ± s.d. For assay comparisons in Table 1, all values are given as the determined titer ± the standard error calculated within each single experiment (that is, reflecting technical rather than biological variations).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank L. Rietschin and B. Gähwiler for help with slice cultures, D. Marino and A. Marcel for technical help, and C.J. Sigurdson and M. Glatzel for prion strains. A.A. is supported by grants from the Sixth European Union Framework program (TSEUR, LSHB-CT-2005-018805), the Swiss National Foundation, the National Competence Center on Neural Plasticity and Repair, the Stambach Foundation, and the UK Department for Environment, Food and Rural Affairs. J.F. is supported by a grant from Zentrum für Neurowissenschaften Zurich, the Desiree and Niels Yde foundation, the Swiss Center of Transgenic Expertise, the Henny Sophie Clausen og møbelarkitekt Axel Clausens Foundation and the Ivan Nielsens Foundation, and F.L.H. was supported by US National Institutes of Health grant R01 NS046006 from the National Institute of Neurological Disorders and Stroke.

References

1. Aguzzi A, Polymenidou M. Mammalian prion biology. One century of evolving concepts. *Cell* 2004;116:313–327. [PubMed: 14744440]
2. Büeler H, et al. Mice devoid of PrP are resistant to scrapie. *Cell* 1993;73:1339–1347. [PubMed: 8100741]
3. Prusiner SB, et al. Measurement of the scrapie agent using an incubation time interval assay. *Ann. Neurol* 1982;11:353–358. [PubMed: 6808890]
4. Kohn PC, Stoltze L, Flechsig E, Enari M, Weissmann C. A quantitative, highly sensitive cell-based infectivity assay for mouse scrapie prions. *Proc. Natl. Acad. Sci. USA* 2003;100:11666–11671. [PubMed: 14504404]
5. Solassol J, Crozet C, Lehmann S. Prion propagation in cultured cells. *Br. Med. Bull* 2003;66:87–97. [PubMed: 14522851]
6. Manuelidis L, Fritch W, Xi YG. Evolution of a strain of CJD that induces BSE-like plaques. *Science* 1997;277:94–98. [PubMed: 9204907]
7. Williams A, Lucassen PJ, Ritchie D, Bruce M. PrP deposition, microglial activation, and neuronal apoptosis in murine scrapie. *Exp. Neurol* 1997;144:433–438. [PubMed: 9168844]
8. Andreoletti O, et al. Phenotyping of protein-prion (PrP^{Sc})-accumulating cells in lymphoid and neural tissues of naturally scrapie-affected sheep by double-labeling immunohistochemistry. *J. Histochem. Cytochem* 2002;50:1357–1370. [PubMed: 12364569]
9. Bolton DC, McKinley MP, Prusiner SB. Identification of a protein that purifies with the scrapie prion. *Science* 1982;218:1309–1311. [PubMed: 6815801]
10. Heppner FL, et al. Experimental autoimmune encephalomyelitis repressed by microglial paralysis. *Nat. Med* 2005;11:146–152. [PubMed: 15665833]
11. Büeler H, et al. Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. *Nature* 1992;356:577–582. [PubMed: 1373228]
12. Polymenidou M, et al. Coexistence of multiple PrP^{Sc} types in individuals with Creutzfeldt-Jakob disease. *Lancet Neurol* 2005;4:805–814. [PubMed: 16297838]
13. Büeler H, et al. High prion and PrP^{Sc} levels but delayed onset of disease in scrapie-inoculated mice heterozygous for a disrupted PrP gene. *Mol. Med* 1994;1:19–30. [PubMed: 8790598]
14. Kaerber G. Beitrag zur kollektiven behandlung pharmakologischer reihenversuche. *Arch. Exp. Pathol. Pharmacol* 1931;162:480–483.
15. Taraboulos A, et al. Regional mapping of prion proteins in brain. *Proc. Natl. Acad. Sci. USA* 1992;89:7620–7624. [PubMed: 1354357]
16. Sigurdson CJ, et al. Strain fidelity of chronic wasting disease upon murine adaptation. *J. Virol* 2006;80:12303–12311. [PubMed: 17020952]
17. Rubsam LZ, Davidson BL, Shewach DS. Superior cytotoxicity with ganciclovir compared with acyclovir and 1- β -D-arabinofuranosylthymine in herpes simplex virus-thymidine kinase-expressing cells: a novel paradigm for cell killing. *Cancer Res* 1998;58:3873–3882. [PubMed: 9731497]
18. Bush TG, et al. Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice. *Neuron* 1999;23:297–308. [PubMed: 10399936]
19. Fischer M, et al. Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. *EMBO J* 1996;15:1255–1264. [PubMed: 8635458]
20. Gogolla N, Galimberti I, DePaola V, Caroni P. Preparation of organotypic hippocampal slice cultures for long-term live imaging. *Nat Protoc* 2006;1:1165–1171. [PubMed: 17406399]
21. Weissmann C. The state of the prion. *Nat. Rev. Microbiol* 2004;2:861–871. [PubMed: 15494743]
22. Brandner S, et al. Normal host prion protein necessary for scrapie-induced neurotoxicity. *Nature* 1996;379:339–343. [PubMed: 8552188]
23. Dandoy-Dron F, et al. Gene expression in scrapie. Cloning of a new scrapie-responsive gene and the identification of increased levels of seven other mRNA transcripts. *J. Biol. Chem* 1998;273:7691–7697. [PubMed: 9516475]

24. Baker CA, Manuelidis L. Unique inflammatory RNA profiles of microglia in Creutzfeldt-Jakob disease. *Proc. Natl. Acad. Sci. USA* 2003;100:675–679. [PubMed: 12525699]
25. Baker CA, Lu ZY, Manuelidis L. Early induction of interferon-responsive mRNAs in Creutzfeldt-Jakob disease. *J. Neurovirol* 2004;10:29–40. [PubMed: 14982726]
26. Baker CA, Martin D, Manuelidis L. Microglia from Creutzfeldt-Jakob disease-infected brains are infectious and show specific mRNA activation profiles. *J. Virol* 2002;76:10905–10913. [PubMed: 12368333]
27. Carp RI, Callahan SM. Effect of mouse peritoneal macrophages on scrapie infectivity during extended in vitro incubation. *Intervirology* 1982;17:201–207. [PubMed: 6813286]
28. Michel B, Tamalet J, Bongrand P, Gambarelli D, Gastaut JL. Role of phagocytes in experimental scrapie in hamsters [in French with English abstract]. *Rev. Neurol. (Paris)* 1987;143:526–531. [PubMed: 3116644]
29. Beringue V, et al. Role of spleen macrophages in the clearance of scrapie agent early in pathogenesis. *J. Pathol* 2000;190:495–502. [PubMed: 10700001]
30. Priller J, et al. Early and rapid engraftment of bone marrow-derived microglia in scrapie. *J. Neurosci* 2006;26:11753–11762. [PubMed: 17093096]
31. McKinley MP, Bolton DC, Prusiner SB. A protease-resistant protein is a structural component of the scrapie prion. *Cell* 1983;35:57–62. [PubMed: 6414721]
32. Manuelidis L, Sklaviadis T, Manuelidis EE. Evidence suggesting that PrP is not the infectious agent in Creutzfeldt-Jakob disease. *EMBO J* 1987;6:341–347. [PubMed: 3556164]
33. Bush TG, et al. Fulminant jejuno-ileitis following ablation of enteric glia in adult transgenic mice. *Cell* 1998;93:189–201. [PubMed: 9568712]
34. Stoppini L, Buchs PA, Muller D. A simple method for organotypic cultures of nervous tissue. *J. Neurosci. Methods* 1991;37:173–182. [PubMed: 1715499]

Figure 1.

Prions in slice cultures. (a) Immunoblot from *tga20^{TK}* and *Prnp^{0/0}* slices exposed to RML-infected (R) or uninfected (Ø) brain homogenate, cultured for 35 d, optionally digested with PK, and probed with antibody POM1 to PrP. Here and in all following figures, sample labels indicate the PrP^C expression dictated by the slice genotype: o/o, *Prnp^{0/0}*; +/-, hemizygous *Prnp^{+/o}*; ++, wild type; ++, hemizygous *tga20* and *Prnp^{0/0}*; +++, hemizygous for the *tga20* allele and *Prnp^{+/o}*; +++++, homozygous *tga20* and *Prnp^{0/0}*; Sc, positive-control homogenate from brain of a mouse with scrapie; inoc., inoculum. Left lane on all blots, molecular weight marker spiked with recombinant PrP^C, yielding a PrP signal at 23 kDa with a cleavage product at 15 kDa. Arrows, PrP^{Sc} glycoforms. (b) Sensitivity of the POSCA. Immunoblot from *tga20^{TK}* and *Prnp^{0/0}* slices inoculated with decadal dilutions (10⁴–10⁰ µg; lane label, log(µg)) of prion-infected (RML) or uninfected (Ø) brain homogenate and cultured for 35 d before harvesting. (c) Time course of PrP^{Sc} accumulation in infected slices. Immunoblot from *tga20^{TK}* (+) and *Prnp^{0/0}* (–) slices inoculated with 100 µg prion-infected (RML) or uninfected (Ø) brain homogenate. (d) Immunoblot from *Tga20^{TK}*, *tga20⁺*, *Prnp^{+/o}* or *Prnp^{0/0}* slices inoculated with 1 mg brain homogenate and harvested 35 dpi. (e) Time-dependent buildup of prion titers in slices. RML-infected or uninfected brain homogenate and slice homogenates from the experiment reported in panel c were serially tenfold diluted in uninfected brain homogenate, and infectivity was determined by transfer to PK1 cells. Means of three independent biological replicas ± s.d. RLU, relative light units.

Figure 2.

Localization and impact of prion replication. **(a)** PI incorporation at 35 dpi in *Tga20^{TK}* and *Prnp^{o/o}* cultures inoculated with 100 µg RML or with uninfected homogenate (Ø). Average of 12 slices ± s.d. **(b)** Representative examples of images used for PI incorporation. Owing to its high cellularity, the granule cell layer of staurosporine-treated slices is prominently stained by PI. **(c)** Histoblot analysis of PrP^{Sc} deposition in POSCA slices. *Tga20^{TK}* and *Prnp^{o/o}* cultures were inoculated with 100 µg RML or uninfected homogenate (Ø). PrP^{Sc} was detected by immunoblotting with POM1 antibody. PrP^{Sc} was found to predominantly accumulate in the molecular layer.

Figure 3.

A broad range of strains is detectable by POSCA. **(a,b)** Immunoblots from cultures from 10-d-old *tga20^{+/+}* (+++++) and *Prnp^{o/o}* (o/o) pups, inoculated with various prion strains (100 µg) and harvested after 35 d. Ø, noninfectious brain homogenate. **(a)** Transmission of mouse-adapted scrapie strains RML, 139A, 79A and Ø. **(b)** Western blotting performed under less stringent conditions on 30 µg protein digested with 25 µg ml⁻¹ PK (+) or 10 µg undigested protein (-) and detected with POM1. Cultures were from 10-d-old *tga20^{+/+}* and *Prnp^{o/o}* pups, and were inoculated with 0.1 and 1 mg of RML, ME7 or Ø and cultured for 35 d.

Figure 4.

Microglial depletion in organotypic slice cultures. (**a,b**) Slices from 10-d-old *CD11b-HSVTK*⁺ mice cultured for 1, 14, 28 or 42 d (DIV) in the optional presence of GCV and stained with IB₄ (**a**) or anti-CD68 (**b**). (**c,d**) Quantification of cells resident within (**c**) or outside (**d**) of slices. (**e**) Real-time RT-PCR of *CD11b-HSVTK*⁺ (*tk*⁺) and *CD11b-HSVTK*⁻ (*tk*⁻) slices cultured for 14 d in presence or absence of GCV (*n* = 3). mRNAs encoding neurofilament (*Nefh*), CD11b (*Itgam*), GFAP (*Gfap*) and MBP (*Mbp*) are shown. (**f,g**) Western blotting from cultures prepared from 10-d-old *tk*⁺ and *tk*⁻ mice cultured with or without GCV, performed on 10 μ g protein and detected with mouse IgG₁ anti-GFAP (clone GA5) (**f**) or mouse IgG₁ anti-PrP^C (POM1) (**g**). (**h**) PI incorporation in cultures prepared as in **e** and treated with GCV for

5 weeks ($n = 12$). Positive control: tk^{-} tissue treated with 5 μ M staurosporine for 24 h. Caspase-3 enzymatic activity was measured and normalized to protein contents ($n = 3$ pools of four slices). **(i,j)** Tk^{+} slices untreated **(i)** or treated **(j)** with GCV for 10 d and then incubated while still alive with IB₄ (green) and PI (red). Open arrows, IB₄⁻PI⁺ cells; closed arrows, IB₄⁺PI⁺ cells. Scale bar in **k** applies to **i,j** as well. **(k,l)** BrdU incorporation in samples prepared as in **e** after 13 dpi. **(k)** Example of stained cells. Green, IgG₁ anti-BrdU; red, IgG_{2a} anti-CD68. Closed arrows, CD68⁺BrdU⁺ cells; open arrows, BrdU⁺CD68⁻ cells. **(l)** Counts of CD68⁺BrdU⁺ and CD68⁻BrdU⁺ cells ($n = 6$, each the average of five individual $\times 40$ images). Averages of n replicas $\pm$ s.d.

Figure 5.

Impact of microglial depletion on prion replication. Cultures were prepared from 10-d-old mice either *CD11b-HSVTK*⁺ (*tga20*^{TK+} or *Prnp*^{o/o/TK+}) or *CD11b-HSVTK*⁻ (*tga20*^{TK-}). **(a)** Western blot from infected *tga20*^{TK-} and *tga20*^{TK+} slices treated with GCV. Samples were PK-digested and PrP detected with POM1. **(b)** Western blot from cultures from 10-d-old *GFAP-HSVTK*⁻ (*Gfap*^{TK-}), *GFAP-HSVTK*⁺ (*Gfap*^{TK+}) or *Prnp*^{o/o} mice treated as in **a**. **(c-h)** Macrophage reconstitution of microglia-depleted tissue. Antigen detected is indicated in lower left corner of immunoblots. **(c)** Experiment performed as in **a**, showing marked increase in PrP^{Sc} accumulation upon GCV treatment of RML-infected *tga20*^{TK+} slices, which was counteracted by *Prnp*^{o/o} or *tga20*^{+/+} macrophages (genotype at bottom of lanes). **(d-f)** Undigested sample detected with POM1 **(d)**, rabbit anti-Iba1 **(e)**; *unidentified cross-reacting protein) or mouse anti-GFAP **(f)**. **(g)** Caspase-3 enzymatic activity, normalized to protein content. Averages of four biological replicas $\pm$ s.d. (NS, $P > 0.05$). **(h)** SCEPA of homogenates of *tga20*^{TK+} or *Prnp*^{o/o/TK+} slices treated as in **a**. Three independent biological replicas of *tga20*^{TK+} and single replicas of *Prnp*^{o/o/TK+} slices or RML were analyzed in tenfold dilution steps using 6–12 replica PK1-containing wells per dilution. Data are indicated as the number of infectious tissue culture units per gram of slice culture protein and are the averages of biological replicas $\pm$ s.d. Slices from the same animal (pairs of -GCV and +GCV samples) are represented by the same color. **(i)** Immunoblot from cultures from 10-d-old *tga20*^{TK+} or *Prnp*^{o/o/TK+} mice uninfected (Ø) or infected with RML in tenfold dilution steps from 1.7 to -1.3 log SCI₅₀; log(μ g) inoculum is

indicated above each lane. Each sample was divided into two pools (–GCV and +GCV) and harvested 35 dpi.

Table 1
Comparison of the sensitivity of four different prion infectivity assays

Dilution	Tga20 inoculation		CD1 inoculation		SCEPA (positive/total)	POSCA (positive/total)
	Sick/total	Incubation time (d) ± s.d.	Sick/total	Incubation time (d) ± s.d.		
10 ⁻¹	4/4	54 ± 1	7/7	119 ± 1	Not done	5/5
10 ⁻²	4/4	59 ± 2	7/7	124 ± 1	6/6	5/5
10 ⁻³	3/3	69 ± 1	7/7	126 ± 1	6/6	15/15
10 ⁻⁴	4/4	77 ± 2	6/6	132 ± 4	12/12	5/5
10 ⁻⁵	6/6	86 ± 2	5/5	135 ± 3	12/12	9/9
10 ⁻⁶	6/6	93 ± 2	5/5	159 ± 4	12/24	0/9 (2/2 ^a)
10 ⁻⁷	6/6	119 ± 4	2/5	167 ± 16	5/24	0/3
10 ⁻⁸	1/6	121, >236	1/5	202, >239	0/24	
10 ⁻⁹	0/5	>236	0/5	>239		
log ID ₅₀ g ⁻¹ ± s.e.m.	9.9 ± 0.2		9.3 ± 0.9		7.7 ± 0.5	>6.7 ± 0.1

The assay sensitivity of the *tga20* and CD1 bioassay, SCEPA and POSCA, compared by performing endpoint titrations of the same batch of RML brain homogenate in the various assays. The infectivity titers per gram of brain were determined according to ref. 14 and expressed as TCID₅₀ units for the SCEPA, SCI₅₀ for the POSCA, and LD₅₀ for *tga20* and CD1 mice upon intracerebral inoculation. Each data point represents the number of infected animals or cultures out by the total number of animals or cultures exposed to the inoculum. The sensitivity of POSCA lags behind that of SCEPA by 1 log, but is considerably enhanced by microglial depletion.

^aMicroglia-depleted slices.