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HEPES inhibits the conversion of prion protein in cell culture

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HEPES is a well-known buffering reagent used in cell-culture medium. Interestingly, this compound is also responsible for significant modifications of biological parameters such as uptake of organic molecules, alteration of oxidative stress mechanisms or inhibition of ion channels. While using cell-culture medium supplemented with HEPES on prion-infected cells, it was noticed that there was a significant concentration-dependent inhibition of accumulation of the abnormal isoform of the prion protein (PrP^{Sc}). This effect was present only in live cells and was thought to be related to modification of the PrP environment or biology. These results could modify the interpretation of cell-culture assays of prion therapeutic agents, as well as of previous cell biology results obtained in the field using HEPES buffers. This inhibitory effect of HEPES could also be exploited to prevent contamination or propagation of prions in cell culture.

INTRODUCTION

Buffers and cell-culture media containing various concentrations of HEPES are widely used in biochemical and cell biology studies. This compound presents several advantages as a buffering agent when compared with bicarbonate or phosphate, as it is soluble, stable and undergoes minimum complexation with metal ions (Good & Izawa, 1972; Williamson & Cox, 1968). However, it has been demonstrated in different situations that HEPES could be responsible for significant modifications in biological parameters such as the uptake of organic molecules (Lelong & Rebel, 1998; Otero *et al.*, 1985), alteration of oxidative stress mechanisms (Habib & Tabata, 2004; Kirsch *et al.*, 1998) and inhibition of ion channels (Hanrahan & Tabcharani, 1990). Several molecular mechanisms have been proposed to explain these effects, including the production of oxidative radicals, modification of ATP metabolism and direct interactions with proteins (Habib & Tabata, 2004; Hanrahan & Tabcharani, 1990; Kihara *et al.*, 1983; Kirsch *et al.*, 1998; Lleu & Rebel, 1989; Luo *et al.*, 2010). HEPES-buffered medium is used in particular in the preparation of lentiviral vectors, which are now widely used tools for stable gene transfers (Salmon & Trono, 2006). HEPES is often added to the culture medium at a concentration of 25 mM to maintain human embryonic kidney 293 (HEK 293T) cells, as it ensures optimal virus production. Lentiviruses are very efficient delivery systems for post-mitotic cells and in particular for neurons (Salmon & Trono, 2006). They have therefore been used to develop gene therapy strategies in neurodegenerative disorders such as Alzheimer's disease and prion diseases (Marr *et al.*, 2004; Relaño-Ginés *et al.*, 2009). Whilst pursuing prion gene therapy approaches in our laboratory, we used HEK 293T supernatants containing relevant lentiviruses on prion-infected cells. In our control experiments, using irrelevant lentiviruses or medium alone, we observed a significant inhibitory effect on accumulation of the abnormal isoform of the prion protein (PrP^{Sc}) that was eventually linked to the presence of HEPES in the medium. This side effect of HEPES could modify the interpretation of cell-culture assays of prion therapeutic agents. It might also require us to revisit or question previous cell biology results obtained in the field using HEPES buffers.

RESULTS

HEPES inhibits PrP^{Sc} accumulation in cultured cells in a dose-dependent manner

As reported previously (Milhavet *et al.*, 2006), neural stem cell (NSC) cultures infected with prions of the 22L mouse-adapted scrapie strain accumulated high levels of PrP^{Sc} after 6, 8 or 10 days of culture (Fig. 1a). When the NSC medium was supplemented from the day of differentiation with 25 mM HEPES, a clear and rapid decrease in the amount of PrP^{Sc} was observed (Fig. 1a). When different

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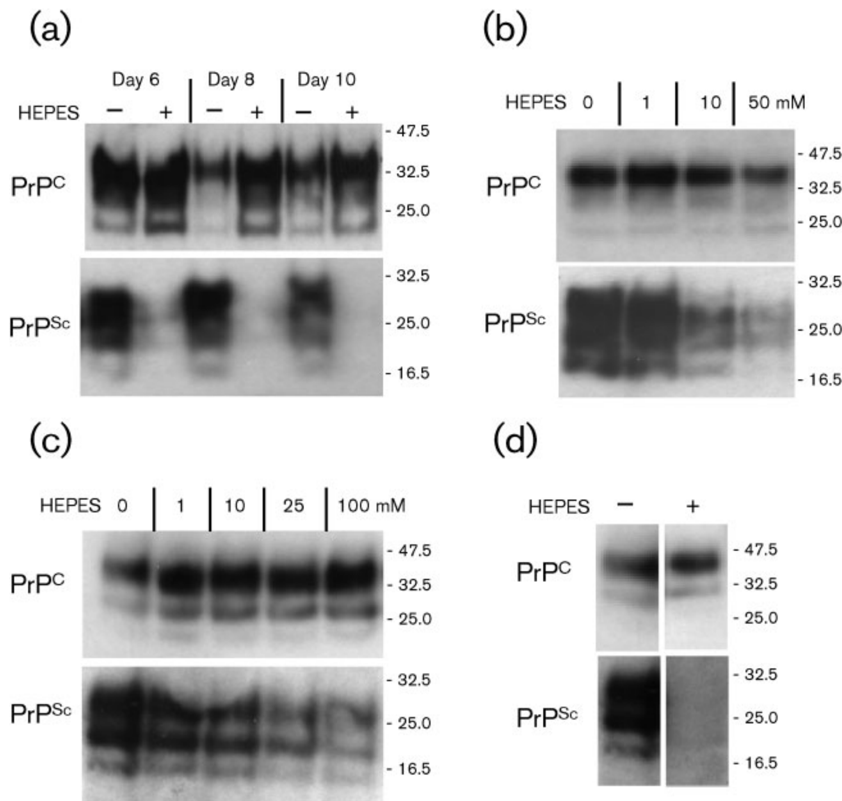

Fig. 1. HEPES inhibition of PrP^{Sc} accumulation in cultured cells. In each experiment, PrP^{C} expression and PrP^{Sc} production were analysed by Western blotting. PrP^{C} was detected in undigested cell lysates using SAF32 antibody, whilst PrP^{Sc} was revealed after digestion of cell lysates with proteinase K using SAFmix (see Methods). (a) Mouse cortical NSCs infected with 22L prions and treated with 25 mM HEPES were lysed at 6, 8 and 10 days after differentiation. (b) NSC cultures treated with increasing concentrations of HEPES and infected with 22L prions were lysed at 12 days after differentiation. (c) ScN2a cells were cultured for 5 days with increasing concentrations of HEPES and lysed prior to PrP^{C} and PrP^{Sc} detection. (d) ScN2a cells were cultured without (−) or with (+) 25 mM HEPES and lysed after four passages. Equal amounts of protein were digested (PrP^{Sc}) or loaded (PrP^{C}) in each lane in (a–d). Molecular mass markers (kDa) are indicated on the right.

concentrations of HEPES were used for 12 days, a significant effect was also observed starting from 10 mM (Fig. 1b). Repetition of similar experiments on a series of infected NSC cultures confirmed this inhibitory effect, albeit with small variations in its efficacy, as illustrated by the differences between Fig. 1(a) and (b). These differences were mostly related to variations in cell differentiation and PrP^{Sc} levels between cultures.

Importantly, in these experiments, the total amount of the normal prion protein (PrP^{C}) before protease digestion was also evaluated using the antibody SAF32 specific for the N terminus of PrP^{C}. Both the total amount of PrP^{C} and its Western blot pattern showed experimental variation that could not account for the effect of HEPES on PrP^{Sc} accumulation. Apparent differences in glycoform pattern in the presence and absence of HEPES, as visible on Fig. 1(a) for example, were probably related to differences in the overall Western blot signal.

To verify that the effect of HEPES was not restricted to NSCs, this compound was also added to scrapie-infected neuroblastoma N2a (ScN2a) cells, which constantly produce PrP^{Sc} following infection by the 22L mouse-adapted scrapie strain. After 5 days (Fig. 1c), a dose-dependent response was present, and after four passages in the presence of 25 mM HEPES at a split ratio of 1 : 10 (Fig. 1d), PrP^{Sc} was almost undetectable in the HEPES-treated cultures. PrP^{C} levels were very stable in these cells, and the PrP^{Sc} signal did not return after additional passages without HEPES (not shown).

Impact of HEPES on cell viability and differentiation

When N2a and NSC cells were cultured in the presence of HEPES up to a concentration of 50 mM, no obvious modifications in growth or cell culture were noticeable. This is shown for NSCs that were cultured and differentiated in the absence (Fig. 2a–c) or presence (Fig. 2d–f) of 50 mM HEPES. Labelling of the cultures with DAPI did not reveal a significant difference in the number of cells or the number of pyknotic nuclei between the culture conditions. The labelling of neuronal and glial cells using anti-β-tubulin type III antibody or anti-glial fibrillary acidic protein (GFAP) antibody as a marker was also similar in treated and non-treated cells.

HEPES does not modify PrP^{Sc} detection *in vitro*

To evaluate the possible direct impact of HEPES on PrP^{Sc} formation or on its detection independent of a cellular context, a cell lysate of prion-infected cells and a brain homogenate from prion-infected mice were incubated with HEPES for 24 h (Fig. 3). Unlike prion therapeutic or decontamination agents (Lehmann *et al.*, 2009; Solassol *et al.*, 2004), incubation with HEPES did not significantly modify the detection of PrP^{Sc}. The blots shown in Fig. 3 were representative of several independent experiments using different concentration of HEPES incubated at 4 °C or 37 °C.

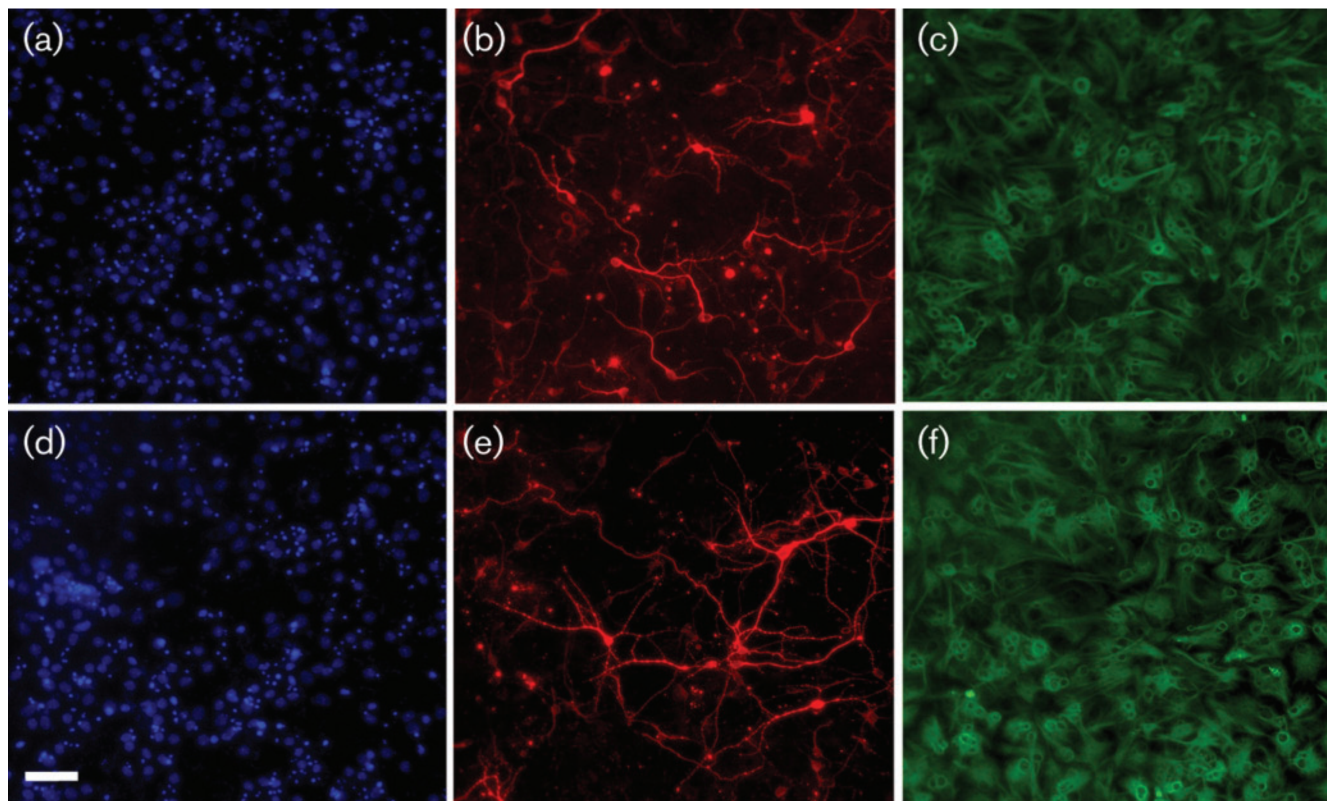

Fig. 2. Effect of HEPES on the viability and differentiation of NSCs. Immunostaining of mouse cortical NSCs after 12 days of differentiation in medium without (a–c) or with (d–f) 50 mM HEPES. Neurons were revealed with anti-β-tubulin type III antibody (b, e) and glial cells were detected using anti-GFAP antibody (c, f). Nuclei were counterstained with DAPI (blue) (a, d). Bar, 25 μm.

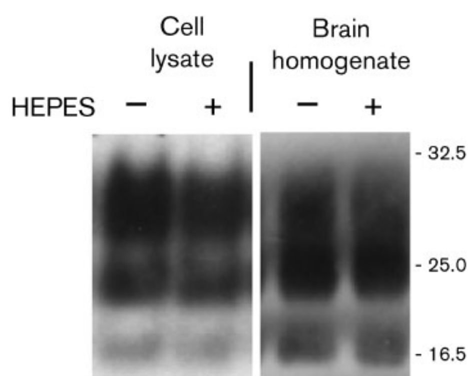

Fig. 3. Impact of HEPES on PrP^{Sc} detection *in vitro*. A lysate of prion-infected mouse cortical NSCs and a 22L mouse brain homogenate (10 % in PBS) were incubated for 24 h at 4 °C in the absence (−) or presence (+) of 100 mM HEPES. The samples were then digested with proteinase K before detection of PrP^{Sc} with SAFmix antibody. The result shown is representative of several experiments. Molecular mass markers (kDa) are indicated on the right.

PrP membrane localization is not significantly modified by HEPES

PrP molecules constitutively cycle between the plasma membrane and endocytic compartments, and it has been postulated that conversion of PrP^{C} to PrP^{Sc} occurs during this pathway (Borchelt *et al.*, 1992; Caughey & Raymond, 1991; Taraboulos *et al.*, 1992). To determine whether HEPES treatment influenced PrP localization, the cell-surface distribution of PrP was analysed by immunofluorescence. As reported previously (Shyng *et al.*, 1995), PrP was detected as a continuous, slightly punctated, cell-surface staining (Fig. 4). After 4 days of culture in the presence of 50 mM HEPES, PrP was still present on the surface of N2a cells and it was not possible to see significant modifications of its localization. When the labelling was performed after permeabilization, it was not possible to detect significant differences between the presence and absence of HEPES (not shown).

DISCUSSION

Cell lines that are chronically infected with prions, as well as cultures expressing variable amounts of wild-type,

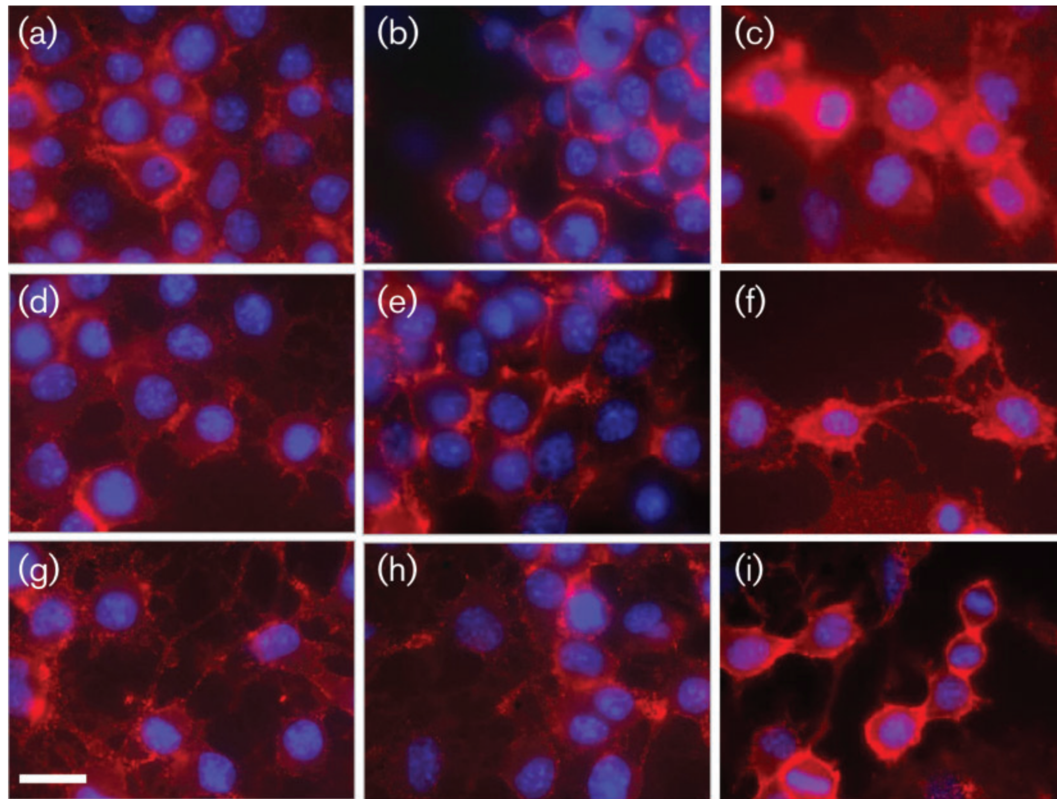

Fig. 4. Immunofluorescence distribution of PrP^{C} on the cell surface of ScN2a cells. ScN2a cells were incubated for 4 days in regular medium (a–c) or in the presence of 25 mM (d–f) or 50 mM (g–i) HEPES. After fixation, the cells were labelled overnight at 4 °C in PBS with SAF32 antibody to detect PrP^{C} on the cell surface (red). Nuclei were counterstained with DAPI (blue). Bar, 25 μm.

mutated or chimeric PrPs, have been widely used to investigate the biology of prions and to develop new therapeutic approaches for transmissible spongiform encephalopathies (Solassol *et al.*, 2003). The cell-culture media used in these experiments are diverse but in many instances include HEPES as the buffering agent. While testing gene therapy approaches using lentiviruses as a gene transfer tool, we discovered that HEPES, in the range of concentrations used in cell-culture media (10–50 mM), was able to interfere with PrP conversion. This effect was apparent after several days of culture in both NSC and prion-infected ScN2a cells (Fig. 1). We did not observe the reappearance of PrP^{Sc} after four passages of the N2a cells (10^{4} dilution of the cells) in medium containing 25 mM HEPES (not shown). This suggested, as observed with other compounds such as amphotericin B (Mangé *et al.*, 2000), that the cells were cured of prions. The parallel decrease in *in vivo* infectivity was not tested in this work; however, N2a cultures are well-established models where a close relationship exists between PrP^{Sc} accumulation and prion infectivity.

Several mechanisms of action could explain the disappearance of PrP^{Sc} in these cultures. Initially, we verified that HEPES treatment was not associated with a significant reduction in PrP^{C} levels (Fig. 1), which itself has a strong anti-prion effect (Daude *et al.*, 2003). We also verified that supplementation with HEPES, which is widely proposed in cell-culture protocols, did not modify the pH of the medium added to the cells and had no dramatic effect on cell survival, morphology or fate of the NSC (Fig. 2) or N2a (Fig. 4) cells. However, it is noteworthy that HEPES has been linked to the production of free radicals and oxidative damage (Habib & Tabata, 2004; Kirsch *et al.*, 1998), in particular when the culture medium is exposed to light (Zigler *et al.*, 1985), which was not tested specifically in this work but could indeed have influenced the effect. Hence, prion-infected cells that are more susceptible to oxidative stress (Milhavet *et al.*, 2000) could slowly disappear in long-term cultures in the presence of HEPES. The modification of redox status could also modify the biochemical environment of PrP, altering its conversion or modifying its biology (Schneider *et al.*, 2003). The possible '*in vitro*' effect of HEPES was also tested on both cell lysates and brain homogenates (Fig. 3). It is known that many therapeutic agents such as tetracycline (Forloni *et al.*, 2002) and polyamine dendrimers (Solassol *et al.*, 2004; Supattapone *et al.*, 1999), when mixed with prions, cause a

dose-dependent decrease in the protease resistance of PrP^{Sc}. HEPES, in contrast, had no significant '*in vitro*' effect, suggesting that its mechanism of action is linked to cellular events occurring during prion replication. Many anti-prion drugs proceed through modification of the endocytic trafficking of PrP^{C} (Shyng *et al.*, 1993; Taraboulos *et al.*, 1992), the integrity of which is essential to the conversion process. In our situation, HEPES at concentration of 50 mM did not significantly modify the Western blot pattern of PrP^{C} or its distribution on the cell membrane. Further investigations will be needed to pinpoint the action of HEPES in more detail. However, this is not simple, as the molecular determinants important for prion conversion in cell culture remain cryptic, as illustrated by our limited understanding of cell-culture susceptibility to prions (Chasseigneaux *et al.*, 2008). Of note, HEPES has recently been involved in modulation of the uptake and transport of permeability (P)-glycoprotein substrates (Luo *et al.*, 2010), whilst interaction between P-glycoprotein and PrP seems to be a determinant for the multidrug-resistant phenotype of cancer cells (Li *et al.*, 2009). It remains to be determined whether HEPES involvement in both phenomena is coincidental or not.

HEPES cannot be envisioned as a potential *in vivo* therapeutic agent, as it acts at a relatively high concentration, but its impact on prion replication needs to be taken into account. For example, many studies have relied on the use of the reduced-serum medium Opti-MEM (see, for example, Liu *et al.*, 2006; Nishida *et al.*, 2000; Raymond *et al.*, 2006; Taraboulos *et al.*, 1992), which is supplemented with HEPES at a concentration not disclosed by the manufacturing company. The difficulty in maintaining prion propagation in N2a cells has been a problem for years in the field. This could be related in part to culture conditions (Ghaemmaghami *et al.*, 2007), and several studies clearly mention that different batches of Opti-MEM are known to affect the status of prion infection (Kocisko & Caughey, 2006; Liu *et al.*, 2006; Raymond *et al.*, 2006). In addition to the use of Opti-MEM, it is of interest to note that several publications, looking for example at the half life of PrP^{C} and at the timing of PrP^{Sc} production (Borchelt *et al.*, 1992; Caughey & Raymond, 1991), have used chase media supplemented with HEPES at concentrations that could interfere with the conversion process. Additional studies will be necessary to evaluate the impact of HEPES in this context.

Finally, the inhibitory effect of HEPES supplementation in culture media could be exploited to prevent the possible propagation or contamination by prions of cultured cells, especially when used in graft and cell-therapy strategies.

METHODS

Cell cultures. NSC were obtained from 13.5-day-old CD1 embryos (Charles Rivers Laboratories) and cultured as described previously by Milhavet *et al.* (2006) with some minor modifications. Briefly, during proliferation, cells were cultured in Dulbecco's modified Eagle's medium with Ham's F-12 nutrient mixture (DMEM/F-12; Invitrogen) with modified N2 supplement and 25 ng basic fibroblast growth factor (bFGF; Invitrogen) ml^{-1} to support proliferation. bFGF was added daily and the medium changed every other day. At the time of differentiation, the medium was changed to a 50 : 50 mixture of DMEM/F-12 with modified N2 supplement and Neurobasal with B27 supplement. (Invitrogen).

The prion-infected N2a cells (ScN2a) used in this study correspond to N2a#58-22L cells described previously (Nishida *et al.*, 2000) and constantly produce PrP^{Sc} following infection by the 22L mouse-adapted scrapie strain. The infected status of the cells was assessed regularly by the detection of PrP^{Sc} (see below). They were cultured in DMEM without HEPES (containing 4.5 g glucose l^{-1} and L-glutamine, with no HEPES or sodium pyruvate; Invitrogen) with 10 % FCS and 0.1 mg Primocin ml^{-1} (InvivoGen).

For HEPES experiments, cells were either cultured in pre-prepared DMEM containing HEPES (containing 4.5 g glucose l^{-1}, L-glutamine and 25 mM HEPES but no sodium pyruvate; Invitrogen) or HEPES was added at the indicated concentration to DMEM without HEPES.

Prion infection of NSCs and detection of PrP^{Sc}. Prion infection of NSCs with the 22L homogenate was carried out as described previously (Milhavet *et al.*, 2006). For PrP^{Sc} detection, cells were lysed in a Triton/DOC lysis buffer [1 × buffer is 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate and 50 mM Tris/HCl (pH 7.5)] with 10 % sarkosyl, 50 U Benzonase (Sigma-Aldrich) ml^{-1} and 1 mM $MgCl_2$, before determining the protein concentration. In some experiments, HEPES was added to the lysate at 100 mM and the lysate was incubated at 4 °C for 24 h (see Fig. 3a). 22L mouse brain homogenate (10 % in PBS) was used as positive control and was pre-incubated with HEPES where indicated (Fig. 3).

The protein concentration of the different samples was quantified using a BCA protein assay kit (Pierce Thermo Fisher Scientific). The samples were then adjusted to the same concentration and 100 μg protein was digested with proteinase K (Roche Diagnostics) for 30 min at 37 °C. The reaction was stopped with 100 μM Pefabloc (Roche Diagnostics) for 5 min at 4 °C. Samples were incubated under agitation with phosphotungstic acid solution for 30 min at 37 °C before centrifugation at 20 000 ***g*** for 45 min. Samples were then Western blotted and detected using SAFmix (a mixture of mouse mAbs SAF60, SAF69 and SAF70; Nishida *et al.*, 2000) to detect PrP^{Sc} or SAF32 antibody to detect PrP^{C} (all kindly provided by J. Grassi, Centre d'Etudes Nucléaires de Saclay, France). Anti-actin antibody was purchased from Sigma-Aldrich, and HRP-conjugated secondary antibodies were from Jackson ImmunoResearch.

Immunofluorescence microscopy. ScN2a cells were fixed for 10 min with 4 % paraformaldehyde and 0.15 % picric acid, followed by standard immunohistochemical protocols. Briefly, PBS with 10 % FCS was used to block non-specific epitopes for 30 min at room temperature. This solution was supplemented with 0.1 % Triton X-100 when permeabilization was required. Cells were then incubated with the appropriate primary antibody for 1 h [mouse anti-β-tubulin type III mAb, clone TUJ1 (Covance Research Products) or rabbit anti-GFAP antibody (Dakocytomation) diluted 1 : 500] or overnight at 4 °C (SAF32 antibody diluted 1 : 500) and washed with PBS before incubation with the appropriate secondary antibody for 1 h [Alexa Fluor 555-conjugated anti-mouse or Alexa Fluor 488-conjugated anti-rabbit antibody, diluted 1 : 10 000 (Invitrogen Life Technologies)]. After washing with PBS, the nuclei were counterstained with DAPI. Preparations were observed with an Axiovert Zeiss microscope. The cells were mounted in FluorSave reagent (Calbiochem) and images were collected and processed using a Leica microscope.

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