Targeted knock-down of cellular prion protein expression in myelinating Schwann cells does not alter mouse prion pathogenesis

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In naturally acquired transmissible spongiform encephalopathies, the pathogenic agents or prions spread from the sites of initial peripheral uptake or replication to the brain where they cause progressive and fatal neurodegeneration. Routing via the peripheral nervous system is considered to be one of the main pathways to the central nervous system. Replication of prions in Schwann cells is viewed as a potentially important mechanism for efficient prion spread along nerves. Here we used a Cre-loxP mouse transgenetic approach to disrupt host-encoded prion protein (PrP C) specifically in myelinating Schwann cells. Despite the use of infection routes targeting highly myelinated nerves, there was no alteration in mouse prion pathogenesis, suggesting that conversion-dependent, centripetal spread of prions does not crucially rely on PrP C expressed by myelinating Schwann cells.

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorders affecting farmed animals (e.g. sheep scrapie, bovine spongiform encephalopathy) and humans (e.g. Creutzfeldt–Jakob disease). TSEs are caused by prions, proteinaceous infectious agents mainly composed of PrP Sc, a misfolded, aggregated form of the ubiquitously expressed, host-encoded prion protein (PrP C). Upon infection, exogenous PrP Sc assemblies are thought to self-perpetuate by transferring their abnormal conformation onto host PrP C, leading to PrP Sc accumulation in both the peripheral (PNS) and central (CNS) nervous systems and a variety of extraneural tissues (reviewed by Aguzzi, 2003; Béringue et al., 2008a; Collinge, 2001).

The PNS is primarily involved in prion transport to the CNS (reviewed by Heikenwalder et al., 2007) and membrane-anchored PrP C expression within the PNS is required (Blättler et al., 1997; Klingeborn et al., 2011; Race et al., 2000). However, how prions spread along peripheral nerves remains unclear. The presence of PrP Sc deposits in Schwann cells ensheathing axons from scrapie-infected sheep and mice peripheral nerves and the sustained prion replication observable in immortalized cultured Schwann-like cells experimentally infected with scrapie prions (Archer et al., 2004; Follet et al., 2002) suggested a possible role for this cell type. Transgenic mice with reduced PrP C expression in Schwann cells did not show any deceleration in the transfer of orally or intraperitoneally injected prions to the CNS (Bradford et al., 2009). These routes of prion exposure may not be optimal if the assumption that the myelin protein zero (P0) promoter used in this study is active in both myelinating (MSCs) and non-myelinating Schwann cells (NMSCs) does not hold true. Although this promoter was reported to drive site-specific DNA recombinase Cre (Cre) expression in MSCs and putatively in NMSCs (Feltl et al., 2002; Yu et al., 2009), PrP C knock-down efficiency was only controlled in MSCs. However, the oral and intraperitoneal routes of infection employed rely on nerves particularly rich in NMSCs (Beekes et al., 1998; Kimberlin & Walker, 1988; McBride et al., 2001). The PrP C glycoform ratio in the transgenic line generated was also altered. This can powerfully affect prion neuroinvasion (Cancellotti et al., 2010). Lastly, the approach used by Bradford et al. did not prevent haematogenous prion spread to the CNS in a possible PNS-independent pathway (Aucouturier et al., 2001). Thus, we considered it relevant to reinvestigate this key issue with another conditional PrP C knock-down MSC-specific Cre-loxP-based transgenetic approach and
complementary peripheral routes of infection aimed at: (i) favouring prion propagation along MSC-enriched nerves; and (ii) better limiting prion escape and replication in the lymphoid tissue before peripheral nerves are reached.

Two transgenic mouse lines were crossed: tg37 and Krox20^Cre-. Prion susceptible tg37 mice express mouse PrP from ‘floxed’ PrP sequences on a FVB/N PrP-null background (Mallucci et al., 2002). This line has been extensively studied, both alone and after crossing with mice expressing Cre under the control of the NFH (neurofilament heavy gene) promoter (Mallucci et al., 2003, 2007). Krox20^{Cre-/+} mice express Cre in MSCs under the control of the endogenous (knock-in) Krox20 gene promoter.

**Fig. 1.** Targeted inactivation of PrP in myelinating Schwann cells. (a) Western blot analysis of alpha-tubulin (upper) and PrP (lower) in sciatic nerve homogenates prepared from adult WT FVB/N (WT), knockout (Prnp^+/+), tg37^+/+ and tg37^+/+Krox20^Cre-/+ mice. Note that full-length (Fl) and truncated (C) forms of α-, mono- and un-glycosylated (respectively, d, m and u) PrP^C are detected with the anti-PrP antibody used, hence the complex, multi-band pattern visualized here. (b) Relative PrP expression levels in sciatic nerves of WT FVB/N (WT), tg37^+/+ and tg37^+/+Krox20^Cre-/+ adult mice. PrP expression levels were quantified and normalized to x-tubulin expression levels from samples such as seen in (a). n=4 animals for FVB/N genotype and n=6 animals analysed for tg37^+/+ and tg37^+/+Krox20^Cre-/+ genotypes, ***, P<0.0001 (Student’s test). (c, d) Immunofluorescent labelling of transverse sections of sciatic nerves from WT FVB/N (c) and Prnp^+/+ (d) adult mice co-stained for PrP with the anti-PrP antibody Sha31 (Alexa 555, red), GFAP 200 (Sigma) (Alexa 488, green) and nuclear marker DAPI (blue). As expected, PrP^C labelling is only present in sciatic nerves from FVB/N mice (c). Strong PrP^C labelling is observed in Remak bundles (asterisk). The sectioned myelinated fibres exhibit PrP^C labelling in non-compact myelin, e.g. abaxonal regions (arrowhead). Bar, 5 µm. (e–g) Transverse sections of sciatic nerves from tg37^+/+ (left) and tg37^+/+Krox20^Cre-/+ (right) adult mice observed by light (e) or fluorescence microscopy (f, g). Sections were stained for PrP with the anti-PrP antibody Sha31 (f) or co-stained with the Epitomics anti-PrP antibody (g, top) and with DAPI (g, bottom). Note the strong PrP^C labelling in unmyelinated fibres for both genotypes (asterisks in f). In contrast, PrP^C labelling in myelinated fibres is weaker and varies between the two different genotypes. In tg37^+/+ genotype (left), a large part of the sectioned myelinated fibres exhibit PrP^C labelling in non-compact myelin, e.g. abaxonal regions (arrowheads, g). Peri-axonal regions (arrows, g) present PrP^C labelling, but at this resolution level, it is not possible to determine if it is expressed by Schwann cells, axons (a in g, top) or, both as is probable. Intensity of the PrP^C signal is highly variable among sectioned myelinated fibres. Note that perinuclear regions (Schwann cell nuclei are denoted nS in g) exhibit a strong PrP^C signal. In tg37^+/+Krox20^Cre-/+ genotype (right), the majority of the sectioned myelinated fibres do not exhibit PrP^C labelling at the abaxonal region even when the nucleus is visible (nS in g). They only exhibit PrP^C labelling at the periaxonal region, which is probably due to the axon (a in g, top)-restricted expression of PrP^P (arrow in g, top), (e, f); Bars, 10 µm (e, f); 5 µm (g). (h–i) Immunofluorescent labelling of longitudinal (h) and transverse (i) sections of sciatic nerves from tg37^+/+ adult mice stained for PrP with the anti-PrP antibody Sha31 (Alexa 555, red), GFAP (Alexa 488, green) and DAPI (blue). Note the co-staining of PrP and GFAP in cells showing high levels of PrP signal, on both longitudinal and transverse sections (arrows and asterisks, respectively). (h): Bars, 10 µm (h); 5 µm (i).
(Voiculescu et al., 2000) and therefore should undergo Cre-mediated recombination specifically in MSCs and a subpopulation of sensory neurons (Maro et al., 2004). Krox20Cre/+ mice were back-crossed with FVB/N-PrP-null mice for at least six successive generations before crossing with tg37 mice, to breed out the WT Prnp gene, avoiding any possible contribution of endogenously expressed mouse PrPSc to prion propagation, and to reduce background strain variation. Animal genotyping was performed after each cross (Mallucci et al., 2002; Maro et al., 2004). To quantify the reduction of PrPSc expression levels in the sciatic nerves, they were homogenized and analysed by Western blot analysis and densitometry. In order to make sure the transgenic approach used did not alter the ratio between the full-length and truncated forms of PrPSc, which is higher in sciatic nerves than in brain (Bremer et al., 2010) and could powerfully affect conversion into PrPSc (Westergard et al., 2011), the Sha31anti-PrP antibody recognizing full-length and truncated forms of PrPSc was used (Féraudet et al., 2005). tg37ctrl mice exhibited a ~40% reduction in the overall levels of PrPSc in sciatic nerves compared with FVB/N mice (Fig. 1a, b), although levels in the brain were ~1.7-fold higher (Fig. S1, available in JGV Online). Analysis of PrPSc expression levels in the sciatic nerves from tg37+/− (Krox20Cre/−) and tg37+/− mice (n=6 animals for each genotype) revealed a statistically significant >50% reduction for tg37+/− (Krox20Cre/−) animals compared with controls, consistent with an MSC-specific knock-down of PrPSc expression (Fig. 1a, b). Notably, the glycoform pattern of full-length and truncated PrPSc was essentially conserved between double-transgenic mice and FVB/N and tg37+−/− mice (Fig. 1a and Fig. S1), at variance with previous transgenic lines in which PrPSc expression in myelinating cells was altered (Bradford et al., 2009; Prinz et al., 2004). Conceivably, the use of a different genetic background (FVB/N vs 129/Ola mice) might explain these differences. This suggests that all PrPSc isoforms were equally affected by the transgenic knock-down. To validate the MSC-specificity of the approach, PrPSc disruption was also tested in the sciatic nerve by fluorescence microscopy (Zeiss). Choice between MSC and NMSC phenotypes is a function of axon diameter: NMSCs ensheathe multiple small axons, forming Remak bundles while MSCs associate in a 1:1 relationship with the larger axons they myelinate. PrPSc was detected in sciatic nerves from WT FVB/N (Fig. 1c), but not from Prnp knockout mice (Fig. 1d). The labelling was particularly intense in Remak bundles. PrPSc expression pattern was identical in sciatic nerves of WT (FVB/N) and tg37+/− littermate control (compare Fig. 1c and e–g, left). This was observed using Sha31 and the rabbit monoclonal anti-PrP antibody (Epitomics-2063-1). A very intense PrP signal was observed in Remak bundles identified by co-labelling of glial fibrillary acidic protein (GFAP), an NMSC marker, with anti-GFAP antibody (Dako-Z0334) (Fig. 1h, i). A weaker signal was obtained in myelinated fibres (Fig. 1c, f, left). A large part of the sectioned myelinated fibres exhibited PrPSc labelling, mainly in non-compact myelin – where cytoplasm is still present – and along axons (Fig. 1c, g, left), consistent with previous reports (Bremer et al., 2010; Follet et al., 2002; Ford et al., 2002). In tg37+/− (Krox20Cre/−) mice, the majority of the sectioned myelinated fibres showed no PrPSc labelling except along axons (Fig. 1e–g, right), most probably due to the axon-restricted

### Table 1. Survival time of mice expressing normal or reduced levels of PrPSc in myelinating Schwann cells after central or peripheral infection with ME7 prions

<table>
<thead>
<tr>
<th>Inoculation route</th>
<th>Volume inoculated* (µl)</th>
<th>Mean ± SEM survival time in days (n/n0)†</th>
<th>Mann–Whitney (p)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FVB/N tg37+/−§</td>
<td>tg37+/− Krox20Cre/−</td>
</tr>
<tr>
<td>Intracerebral</td>
<td>20 (1 %)</td>
<td>148 ± 2 (6/6)</td>
<td>111 ± 2 (4/4)</td>
</tr>
<tr>
<td>Intrapерitoneal</td>
<td>100 (0.2 %)</td>
<td>ND</td>
<td>179 ± 6 (12/22)</td>
</tr>
<tr>
<td>Footpad</td>
<td>5 (1 %)</td>
<td>278 ± 4 (7/7)</td>
<td>276 ± 45# (5/5)</td>
</tr>
<tr>
<td>Intralabral</td>
<td>1 (1 %)</td>
<td>ND</td>
<td>161 ± 7 (19/19)</td>
</tr>
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*Volume of ME7-infected brain homogenate inoculated to mice.  
†/n0, Diseased, proteinase K-resistant positive/inoculated animals.  
§The differences in survival times between tg37+/− and tg37+/− Krox20Cre/− mice were not statistically significant.  
¶The tg37 mice were genotyped using DNA probes previously described (Mallucci et al., 2002).  
||The Krox20Cre/− mice were genotyped by PCR and Southern blotting analysis using the primers Krox1219 (GTTCATCGTGT-CAAAGGGGCTTCCC), Krox1742 (CACTACACCGAACTCCTGGGTC) and Cre148 (ATGCTCAGAAACGCTGCGATCC) (Voiculescu et al., 2000).  
¶Mice that did not succumb to scrapie were followed until end of lifetime and euthanized (range of survival time: 630–973 days). Their brains were negative for PrPres content.  
#After footpad injection, mice succumbed in two groups (hence the small standard deviations), the second group developing disease 3 months later. This segregation was observed in both transgenic and control animals (Fig. S2).
expression of PrP<sup>C</sup>. Unmyelinated fibres were strongly PrP<sup>C</sup>-positive (Fig. 1f, right), consistent with the myelinating cell-specificity of the Krox20 promoter (Topilko et al., 1994). Altogether, these results show that our Cre-loxp model allowed reduction of PrP<sup>C</sup> levels specifically in MSCs without altering the overall ratio of PrP<sup>C</sup>-isoforms.

To examine whether PrP<sup>C</sup> knock-down in MSCs impacts prion spreading in the organism, tg<sup>37</sup><sup>+-</sup> and tg<sup>37</sup><sup>-/-</sup>-<br/>Krox20<sup>2cre-/+</sup> mice were inoculated with mouse ME7 prions by different routes of infection. FVB/N control mice were also inoculated to ascertain the efficiency of some routes of infection. These transmission studies were carried out in strict accordance with French national, ethical and welfare guidelines. Mice showing clinical signs of scrapie or at the endpoint of the experiment were euthanized and their brains and spleens removed to check for the presence of PrP<sub>Sc</sub>. Direct targeting of the CNS by intracerebral inoculation resulted in no statistical differences between <br/>tg<sup>37</sup><sup>+-</sup> and tg<sup>37</sup><sup>-/-</sup>-Krox20<sup>2cre-/+</sup> mice (Table 1), as expected. Intralabial and footpad inoculations were performed to restrict ME7 prions propagation to heavily myelinated somatic nerves such as the facial motor (Halata & Munger, 1983; Labetoulle et al., 2003) and sciatic nerves (Kratzel et al., 2007a). These two routes of infection did not affect the attack rate (100%) or survival times of MSC-specific PrP<sup>C</sup> knock-down mice as compared to control littermate animals (Table 1). Footpad injections resulted in two groups of diseased mice, with long and short incubation periods. The proportion of affected mice and the survival times in both groups were congruent among <br/>tg<sup>37</sup><sup>-/-</sup> and tg<sup>37</sup><sup>-/-</sup>-Krox20<sup>2cre-/+</sup> mice (Fig. S2). Whether this segregation is related to distinct neural prion spread (Glätzl & Aguzzi, 2000; Kimberlin et al., 1983) remains to be determined. Of note, the absence of a ‘fast-onset’ subgroup in FVB/N mice following footpad injection (Table 1; Fig. S2) would suggest that extraneural replication might somehow interfere with the most direct neuroinvasive pathway. After intraperitoneal inoculation, disease transmission was incomplete. However, the proportion of clinically affected mice was similar between <br/>tg<sup>37</sup><sup>+-</sup> and <br/>tg<sup>37</sup><sup>-/-</sup>-Krox20<sup>2cre-/+</sup> mice (Table 1). Incomplete disease transmission was observed upon intraperitoneal infection of prions in numerous immunodeficient mouse models, transgenic or WT (Beringue et al., 1999; Lasmézas et al., 1996; Mabbott et al., 2003; Prinz et al., 2002). To assess whether ME7 prions replication was altered in the lymphoid tissue, we checked for the presence of PrP<sub>Sc</sub> in the spleens of infected animals. The protein was not detectable in any of the <br/>tg<sup>37</sup><sup>-/-</sup> and <br/>tg<sup>37</sup><sup>-/-</sup>-Krox20<sup>2cre-/+</sup> mouse spleens analysed (Fig. 2a).

As ME7 prions are lymphoinvasive, we examined the reasons for impaired replication and found very little if any PrP<sup>C</sup> expression in the spleen tissue of the unaffected or infected animals by immunoblot (data not shown). Incidentally, this impaired replication in the spleen is likely to have minimized haematogenous, PNS-independent prion spread to the CNS (Aucouturier et al., 2001), at variance with Bradford et al. (2009). Taken together, these results argue against a major role of MSC-PrP<sup>C</sup> in prion spreading through the PNS.

**Fig. 2.** Abnormal PrP deposition in terminally infected mice expressing normal or reduced levels of PrP in myelinating Schwann cells. (a) Immunoblot analysis of proteinase K-resistant PrP<sub>Sc</sub> accumulation in tissue extracts (Br, brain; Sp, spleen) of FVB/N (WT),tg<sup>37</sup><sup>-/-</sup><br/>and <br/>tg<sup>37</sup><sup>-/-</sup>-Krox20<sup>2cre-/+</sup> mice inoculated by intracerebral (ic), footpad (fp), intralabial (il), intraperitoneal (ip) route. (b) Regional distribution of proteinase K-resistant PrP<sub>Sc</sub> in the brain of <br/>tg<sup>37</sup><sup>-/-</sup> and <br/>tg<sup>37</sup><sup>-/-</sup>-Krox20<sup>2cre-/+</sup> mice inoculated by the intralabial route of infection. Histoblot of four representative antero-posterior mouse brain sections are shown.

Brains of terminally ill mice were analysed for proteinase K-resistant PrP<sub>Sc</sub> content by immunoblot and histoblot techniques, using the Sha31 and 12F10 mouse anti-PrP
antibodies, respectively (Beringue et al., 2008b; Langevin et al., 2011). For Western blot analysis, PrPSc from homogenized brain tissues was extracted according to the Bio-Rad test protocol, by using 200 µg ml⁻¹ of protease K (TeSeE Western blot; Bio-Rad). For histoblot analysis, 10-µm-thick frozen sections were transferred to nitrocellulose membranes and treated with protease K (25 µg ml⁻¹). PrPSc knock-down in MSCs did not alter PrPSc levels and deposition patterns in the brain according to comparison of control and transgenic mice after infection by the same route (Fig. 2a, b and data not shown). This strengthens the view that ME7 disease pathogenesis was largely unaffected by PrPSc knock-down in MSC.

Here, we have shown that a dramatic reduction of all PrPSc isoforms in MSCs did not alter the capacity of ME7 prions to propagate centripetally from the peripheral nerve endings to the CNS and to exert their pathogenicity. Lack of effect has been shown using two additional peripheral routes of infection targeting highly myelinated peripheral nerves where we specifically demonstrated a strong reduction in PrPSc levels by our transgenic approach. The PNS exerts a major contribution to the transport of prions from the periphery to the CNS. It was proposed to rely on a continuous chain of cells expressing membrane-anchored PrPSc (Aguzzi, 2003; Heikenwalder et al., 2007; Klingeborn et al., 2011). Our study supports the view that PrPSc expression by MSCs is not mandatory in the process of prion neural spread. Of note, high levels of PrPSc expression were detected in NMSCs, but whether it plays a role in prion transport remains an open question. Because a major contribution of conventional retrograde axonal transport appears unlikely (Glatzel & Aguzzi, 2000; Groschup et al., 1999; Kratzel et al., 2007a, b; Künzi et al., 2002), further studies, including the possibility of cell-autonomous mechanisms – as recently suggested for prion transport to lymphoid tissues (Michel et al., 2012) – and/or at-distance PrPSc-independent prion transport (Magalhães et al., 2005) are required to elucidate how prions are conveyed within the PNS to the brain. This might have broad-range relevance as other abnormally folded protein aggregates may also be capable of propagating from the periphery to the brain (Hansen & Li, 2012).

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