

# PrP<sup>C</sup> expression in the peripheral nervous system is a determinant of prion neuroinvasion

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Transmissible spongiform encephalopathies are often propagated by extracerebral inoculation. The mechanism of spread from peripheral portals of entry to the central nervous system (neuroinvasion) is complex: while lymphatic organs typically show early accumulation of prions, and B-cells and follicular dendritic cells are required for efficient neuroinvasion, actual entry into the central nervous system occurs probably via peripheral nerves and may utilize a PrP<sup>C</sup>-dependent mechanism. This study shows that transgenic mice overexpressing PrP<sup>C</sup> undergo rapid and efficient neuroinvasion upon intranerval and footpad inoculation of prions. These mice exhibited deposition of the pathological isoform of the prion protein (PrP<sup>Sc</sup>) and infectivity in specific portions of the central and peripheral sensory pathways, but almost no splenic PrP<sup>Sc</sup> accumulation. In contrast, wild-type mice always accumulated splenic PrP<sup>Sc</sup>, and had widespread deposition of PrP<sup>Sc</sup> throughout the central nervous system even when prions were injected directly into the sciatic nerve. These results indicate that a lympho-neural sequence of spread occurs in wild-type mice even upon intranerval inoculation, while overexpression of PrP<sup>C</sup> leads to substantial predilection of intranerval over lymphoreticular spread. The rate of transport of infectivity in peripheral nerves was ca. 0.7 mm per day, and prion infectivity titres of sciatic nerves were much higher in *tga20* than in wild-type mice, suggesting that overexpression of PrP<sup>C</sup> modulates the capacity for intranerval transport.

## Introduction

Prion diseases are transmissible fatal neurodegenerative diseases. Although the infectious agent is most efficiently propagated through intracerebral inoculation, peripheral infection is the natural route of transmission in most prion diseases. Oral administration is most probably involved in kuru, BSE and the new variant of Creutzfeldt–Jakob disease, while parenteral administration of growth hormone and gonadotropins has resulted in iatrogenic Creutzfeldt–Jakob disease. In all these instances transport of the infectious agent from the site of entry into the body to the central nervous system (CNS) is of crucial importance (Hill *et al.*, 1997; Scott *et al.*, 1999). A large body of evidence indicates that both the lymphoreticular system (LRS) and the peripheral nervous system (PNS) are involved in scrapie neuroinvasion (Baldauf *et al.*, 1997; Kimberlin & Walker, 1988; Klein *et al.*, 1997; Lasmezas *et al.*, 1996). Transport along the PNS was suggested by experiments showing that intranerval (i.n.) injection of

infectivity could bypass the need for extraneural replication of the agent (Kimberlin *et al.*, 1983*b*). Also, the first pathological lesions and replication of infectivity after peripheral administration of the scrapie agent occur in spinal cord and medulla oblongata: these sites are consistent with entry via peripheral nerves like the vagal or splanchnic nerves (Beekes *et al.*, 1998; Cole & Kimberlin, 1985).

The LRS clearly plays an important role in the transport of the scrapie agent. In several animal models, including hamsters and mice, lymphoid organs such as the spleen are early sites of accumulation and replication of the agent following intraperitoneal (i.p.) inoculation (Eklund *et al.*, 1967; Kimberlin & Walker, 1986, 1989). Although B-lymphocytes are required for efficient neuroinvasion of the agent, they do not need to express PrP<sup>C</sup>. It appears that their role in neuroinvasion consists – at least in part – of lymphotoxin  $\beta$ -mediated induction of follicular dendritic cell maturation (Montrasio *et al.*, 2000; Klein *et al.*, 1998). In order to replicate prions within lymphatic tissues follicular dendritic cells may need to express PrP<sup>C</sup> (Brown *et al.*, 1999).

Although various components of the immune system play a pivotal role in scrapie neuroinvasion, there is substantial

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evidence that the PNS may be important for neuroinvasion of prions as well (Lasmez *et al.*, 1996). Adoptive bone marrow transfer of PrP<sup>C</sup>-expressing cells into PrP<sup>C</sup> knockout mice restored accumulation and replication of prions in the lymphatic tissue, yet not transport of the agent to the brain (Blättler *et al.*, 1997). These results indicated that a non-haematopoietic PrP<sup>C</sup>-expressing tissue is required for efficient neuroinvasion. Further experiments using *Prnp* knockout mice expressing transgenic PrP<sup>C</sup> under a neuron-specific promoter provided evidence that this tissue may be the PNS (Race *et al.*, 2000).

Here we show that transgenic mice overexpressing PrP<sup>C</sup> under the control of its own regulatory sequences (Fischer *et al.*, 1996) support rapid neuroinvasion upon i.n. and footpad (f.p.) inoculation of the infectious agent. The route of neuroinvasion was consistent with direct intranervial spread in all transgenic mice, and in only one subset of i.n. inoculated wild-type mice. The use of two different routes of inoculation in transgenic and wild-type mice enabled us to calculate the actual rate of spread of the infectious agent in the PNS of *tga20* mice as a function of PrP<sup>C</sup> expression.

## Methods

■ **Scrapie inoculation.** Mice were inoculated with a 1% homogenate of heat- and sarcosyl-treated brain prepared from mice infected with the Rocky Mountain Laboratory (RML) scrapie strain. Twenty  $\mu$ l was used for f.p. injections while 1  $\mu$ l was administered i.n. For f.p. and i.p. injections mice were anaesthetized with Rompun (Bayer). For i.n. injection, mice were anaesthetized with Xylazin/Ketamin and the right sciatic nerve was surgically exposed by dislodging the M. gluteus superficialis and the M. biceps femoris. The nerve was gently placed onto a metal plate (20  $\times$  5  $\times$  0.5 mm) and 1  $\mu$ l of RML inoculum was injected with a 34-gauge Hamilton syringe over a period of 5 min (Bassant *et al.*, 1986). The nerve was anatomically repositioned and the skin was closed with USP 4.0 nylon sutures. The i.p. injected mice received 100  $\mu$ l of a 0.1% (high dose) or 100  $\mu$ l of a 0.01% (low dose) homogenate of heat- and sarcosyl-treated RML inoculum. Mice of the respective control groups received the same amounts of mock inoculum, consisting of similarly prepared brain homogenate from uninfected mice. Mice were monitored every third day, and scrapie was diagnosed according to standard clinical signs.

■ **Western blot analysis.** Homogenates (10%, w/v) of sciatic nerve, spinal cord or brain were prepared as described (Büeler *et al.*, 1993) and, where indicated, digested with 20  $\mu$ g/ml proteinase K for 30 min at 37 °C. Unless otherwise stated in the figure legends, 50  $\mu$ g of total protein was then electrophoresed through 12%–SDS polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were probed with monoclonal antibody 6H4 to mouse PrP<sup>C</sup> (Korth *et al.*, 1997), and developed by enhanced chemiluminescence (Amersham). Quantification of protein was accomplished by scanning membranes with a Kodak image station 440. The signal intensities produced by serial dilutions of *tga20* sciatic nerve homogenates were measured and compared to the signal intensity of a specified amount of wild-type sciatic nerve homogenate using the 1D image analysis software (Kodak). All three bands corresponding to the different glycosylation states of PrP<sup>C</sup> (un-, mono- and diglycosylated) were included in the measurement.

■ **Histoblots.** The histoblot technique was performed according to protocols of Taraboulos *et al.* (1992). Frozen sections of 8  $\mu$ m thickness were mounted on uncoated glass slides and immediately pressed on a nitrocellulose membrane wetted in lysis buffer. Membranes were air-dried for at least 24 h. For detection, they were rehydrated in TBST, and limited proteolysis was performed using proteinase K concentrations of 50 and 100  $\mu$ g/ml at 37 °C for 4 h. Blots were then denatured in 3 M guanidinium thiocyanate for 10 min and blocked for 1 h in 5% non-fat milk serum. Incubation with primary antibody 6H4 (Korth *et al.*, 1997) was carried out at a dilution of 1:2000 in 1% non-fat milk serum at room temperature for 1 h. Detection was accomplished with an alkaline phosphatase-conjugated goat anti-mouse antibody at a concentration of 1:2000. Visualization was achieved with nitro blue tetrazolium and bromo-chloro-indolyl phosphate according to the protocols of the supplier.

■ **Histological studies.** Brain, spinal cord, sciatic nerves and muscles from selected mice were fixed with 4% buffered formalin, inactivated by 1 h with 98% formic acid and embedded in paraffin. Sections were cut to a thickness of 5  $\mu$ m. For cryosectioning tissues were snap-frozen in cryoprotectant compound and cut with a cryostat to a thickness of 6  $\mu$ m. Sections were subjected to routine stainings with haematoxylin–eosin and to immunostaining for glial fibrillary acidic protein (GFAP) according to standard procedures. Gliosis (a non-specific but early indicator of brain damage) was visualized by the presence of large immunostained reactive astrocytes. The clinical diagnosis of scrapie was confirmed by histological analysis of brains or spinal cords.

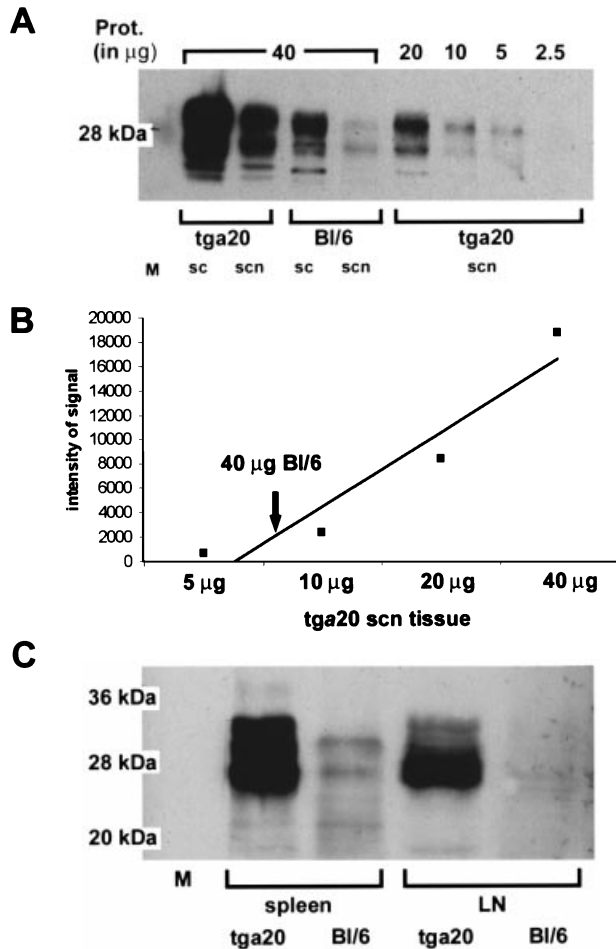
■ **Semi-thin sections.** After fixation with 0.5% glutaraldehyde, samples were fixed with osmic tetroxide and embedded in epoxy resin. Sections 3  $\mu$ m thick were stained with toluidine blue.

■ **Infectivity bioassays.** Spinal cord and sciatic nerve homogenates (10% in 0.32 M sucrose) were prepared from infected animals by homogenizing the tissues using a pellet mixer and by sonicating the samples for 5 min with a sonifier (Branson 450) at a constant output power of 400 W. Thirty  $\mu$ l (diluted 1:10 in PBS and 1% BSA) was administered intracerebrally to groups of four (in one sample three) *tga20* mice for each sample. The incubation time until development of terminal scrapie sickness was determined and infectivity titres were calculated (Prusiner *et al.*, 1982) using the relationship  $y = 11.45 - 0.088x$ , where  $y$  is LD<sub>50</sub> and  $x$  is incubation time (days) to terminal disease (Fischer *et al.*, 1996).

## Results

### *tga20* mice overexpress PrP<sup>C</sup> in the PNS

Previous studies have shown that *tga20* mice overexpress PrP<sup>C</sup> in the CNS about tenfold (Fischer *et al.*, 1996). We investigated the level of PrP<sup>C</sup> expression in the PNS in this mouse line. Western blot analysis of sciatic nerves of *tga20* mice compared to C57Bl/6 mice show that these mice also overexpress PrP<sup>C</sup> in the PNS (Fig. 1A). Quantification of the amount of PrP<sup>C</sup> expressed on the sciatic nerve was performed by measuring the signal intensity of serial dilutions of *tga20* and wild-type sciatic nerve extracts using a Kodak image station and appropriate software. The expression of PrP<sup>C</sup> in the PNS of *tga20* mice was determined to be five to seven times higher than in wild-type mice (Fig. 1B). In addition to the different expression level there is a marked difference between the various PrP<sup>C</sup> glycotypes as assessed by the electrophoretic pattern of PrP<sup>C</sup> between wild-type and *tga20* nerves. In *tga20*

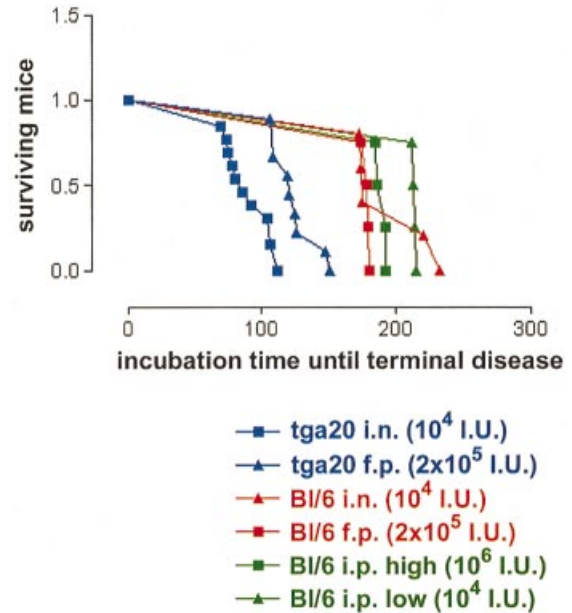


**Fig. 1.** (A) Western blot analysis of PrP<sup>C</sup> in the spinal cords (sc) and sciatic nerves (scn) of C57Bl/6 (Bl/6) and *tga20* mice. The amount of total protein loaded is indicated for each lane. (B) PrP<sup>C</sup> content in sciatic nerves was quantified by direct recording of Western blot chemiluminescence using the Kodak 1D image analysis system. A standard curve was produced by plotting the signal intensity produced by 5–40 μg of *tga20* sciatic nerve homogenate. The arrow indicates that the PrP<sup>C</sup> content of 40 μg wild-type sciatic nerve corresponds to 8 μg *tga20* sciatic nerve. (C) Western blot analysis of PrP<sup>C</sup> of spleens and lymph nodes (LN) of C57Bl/6 (Bl/6) and *tga20* mice. The amount of total protein loaded in each lane was 120 μg.

mice the strongest band is the high molecular mass band (diglycosylated PrP<sup>C</sup>), whereas in wild-type nerves a lower molecular mass band (monoglycosylated PrP<sup>C</sup>) gives the strongest signal (Fig. 1A).

### Overexpression of PrP<sup>C</sup> in the PNS leads to efficient neuroinvasion following intranerval and footpad inoculation

Studies aimed to investigate the transport of prions in the PNS often rely on inoculation routes that target specifically the PNS. In this study we used two different routes of administration of the infectious agent: the i.n. and f.p. routes. To study the effect of overexpression of PrP<sup>C</sup> on the PNS we inoculated



**Fig. 2.** Incubation time (days) from inoculation until terminal scrapie in i.n. and f.p. injected *tga20* mice compared to i.n., f.p. and i.p. injected C57Bl/6 (Bl/6) mice. *tga20* mice developed clinical scrapie at early time-points with a difference of about 30 days between the i.n. and f.p. injected group. Wild-type mice did not show such a clear difference between i.n. and f.p. routes of inoculation.

*tga20* mice and wild-type mice using these inoculation routes. All of the *tga20* mice inoculated with RML scrapie prions ( $n = 14$  for i.n.;  $n = 9$  for f.p.) developed scrapie (Fig. 2). The incubation time until terminal scrapie was  $89 \pm 16$  days for i.n. injected mice. F.p. injected *tga20* mice developed scrapie 33 days later ( $123 \pm 17$  days). Wild-type mice exposed to prions according to the same protocol ( $n = 5$  for i.n.;  $n = 4$  for f.p.) developed terminal disease  $195 \pm 29$  days after i.n. inoculation and  $178 \pm 3$  days after f.p. injection (Fig. 2).

Among wild-type mice inoculated i.n., segregation into two subgroups was observed: a 'fast-onset' group came down with scrapie very rapidly, while the remaining group of mice developed the disease later, with an incubation time similar to that of i.p. injected mice. A similar phenomenon was observed earlier (Kimberlin *et al.*, 1983b).

Control groups consisting of wild-type mice inoculated i.p. with different amounts of RML inoculum (high and low dose) developed terminal scrapie at  $190 \pm 5$  days (high dose of inoculum,  $n = 4$ ) and at  $214 \pm 2$  days (low dose of inoculum,  $n = 4$ ).

None of the mock injected *tga20* or wild-type mice developed scrapie ( $n = 4$  for *tga20* i.n.;  $n = 3$  for *tga20* f.p.; and  $n = 2$  for C57Bl/6 i.n. or f.p.). These mice were sacrificed at day 205 (*tga20*) or 333 (C57Bl/6) after injection. Finally, we did not observe any clinical or histopathological signs of disease in *Prnp*<sup>0/0</sup> mice following i.n. or f.p. injection of RML inoculum ( $n = 2$  for i.n.,  $n = 2$  for f.p.): these mice were sacrificed 333 days after inoculation.

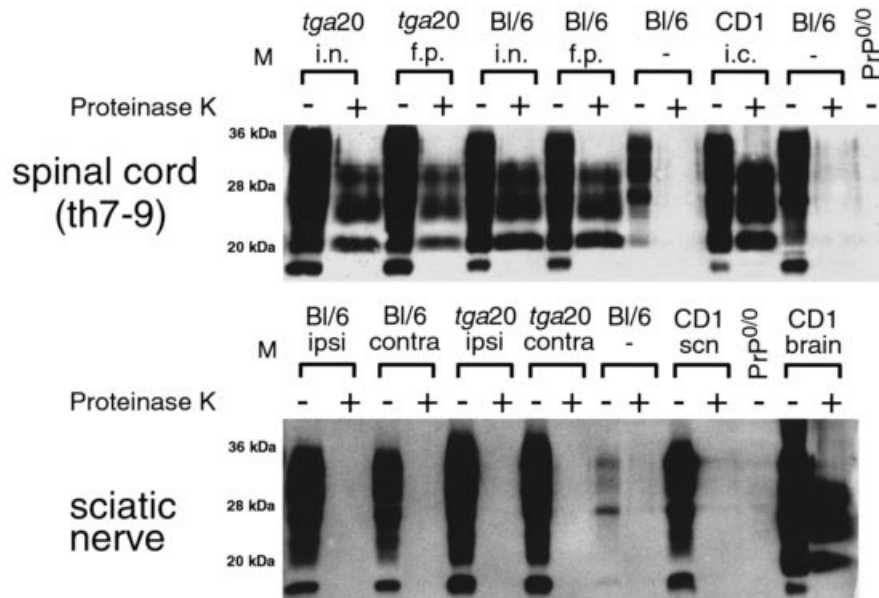


Fig. 3. Western blot detection of PrP<sup>C</sup> and PrP<sup>Sc</sup>. Upper blot, extracts of spinal cords; lower blot, extracts of sciatic nerves. Both the side of i.n. prion injection (ipsi) and the contralateral (contra) side were examined. Genotypes of mice, route of inoculation and proteinase K digest are indicated above each lane. As a control, we analysed terminally sick intracerebrally inoculated CD1 mice (CD1), mock injected wild-type mice (Bl/6) and PrP knockout mice (PrP<sup>0/0</sup>). The amount of total protein loaded in each lane was 50 µg except for the Bl/6 sciatic nerve, where it was 25 µg. While PrP<sup>Sc</sup> was readily detectable in the spinal cords of all examined mice, no proteinase K-resistant PrP could be detected in any of the investigated sciatic nerve samples.

**Table 1.** Incubation times and titres of infectivity of selected samples transmitted to indicator mice

In all except one case (Bl/6 sciatic nerve), groups of four mice were challenged. Incubation time until terminal disease is indicated. Sciatic nerves from *tga20* mice gave significantly higher titres ( $5.6$  and  $5.4$   $\log\text{LD}_{50} \times \text{g}^{-1}$ ) than sciatic nerves from wild-type mice ( $4.2$  and  $3.8$   $\log\text{LD}_{50} \times \text{g}^{-1}$ ) when injected into indicator mice.

Host	Transmitted sample	Incubation time of indicator mice (days)		Infectivity ( $\log\text{LD}_{50} \times \text{g}^{-1}$ )
		Individual mice	Mean $\pm$ SD	
Bl/6	Spinal cord	55, 57, 57, 57	$57 \pm 1$	6.43
Bl/6	Spinal cord	58, 62, 62, 64	$62 \pm 2.5$	6.08
Bl/6	Sciatic nerve	76, 79, 87	$81 \pm 5.7$	4.23
Bl/6	Sciatic nerve	81, 83, 86, 91	$85 \pm 4.3$	3.79
<i>tga20</i>	Sciatic nerve	68, 70, 70, 70	$70 \pm 1$	5.55
<i>tga20</i>	Sciatic nerve	67, 67, 67, 70	$68 \pm 1.5$	5.37

### Predominantly intranervial spread of prions in *tga20* mice

In order to elucidate the predominant route of transport to the CNS in *tga20* mice and in wild-type mice, we analysed the content and localization of PrP<sup>Sc</sup> and of scrapie infectivity in sciatic nerves, spinal cords and brains of inoculated mice. In the sciatic nerves of both *tga20* and wild-type mice we were not able to detect any proteinase K-resistant PrP by Western blot analysis (Fig. 3).

This was further investigated by inoculating *tga20* indicator

mice with homogenates of selected sciatic nerves: all transmitted sciatic nerves showed infectivity. When titres were calculated by the incubation time method, sciatic nerves of *tga20* mice were shown to contain significantly more infectivity than those of wild-type mice. The calculated titres were  $5.4$  and  $5.6$   $\log\text{LD}_{50} \times \text{g}^{-1}$  for *tga20* sciatic nerves, as opposed to  $3.8$  and  $4.2$   $\log\text{LD}_{50} \times \text{g}^{-1}$  for wild-type nerves (Table 1).

The sciatic nerves of seven wild-type and seven *tga20* mice were then examined histologically. In semi-thin sections we could see signs of axonal degeneration in the sciatic nerves of both i.n. and f.p. injected *tga20* mice. In contrast, no signs of

axonal degeneration were seen in the sciatic nerves of i.n. and f.p. injected wild-type mice or of mock injected *tga20* mice (Fig. 4). Because degeneration was observed in *tga20* mice but not in wild-type mice, it is likely to represent a consequence of retrograde prion spread within the PNS of *tga20* mice rather than Wallerian degeneration secondary to CNS affection.

#### ***tga20* mice injected i.n. and in the f.p. show selective accumulation of PrP<sup>Sc</sup> in specific areas of the CNS**

To further investigate the spread of infectivity from the PNS to the CNS we performed Western blot analyses of selected thoracic spinal cords of i.n. and f.p. injected *tga20* and wild-type mice. In all of the examined mice ( $n = 12$ ) we detected proteinase K-resistant PrP<sup>Sc</sup> in the spinal cord (Fig. 3). The large amount of PrP<sup>Sc</sup> detectable in the spinal cords of *tga20* stands in contrast to the very low amount of PrP<sup>Sc</sup> that was detected in the brains of these mice (Fischer *et al.*, 1996), whereas in wild-type mice the amount of PrP<sup>Sc</sup> in spinal cords and in brains was similar (Fig. 5).

The results above suggested selective targeting of specific CNS areas with PrP<sup>Sc</sup> in *tga20* mice following i.n. and f.p. injection of prions. This was studied in more detail by histoblot analyses of brains. Indeed, the topography of PrP<sup>Sc</sup> accumulation was strikingly different in *tga20* and wild-type mice. Wild-type mice exhibited abundant accumulation of PrP<sup>Sc</sup> in cortex and hippocampus, while in *tga20* transgenic mice these regions did not contain detectable PrP<sup>Sc</sup> (Fig. 5 A, B, C). The sites of accumulations in *tga20* mice were superimposable on the main targets of the projections of the sensory pathway, whereas wild-type mice accumulated PrP<sup>Sc</sup> much more diffusely in the telencephalon.

Selected spinal cords of i.n. injected wild-type mice were assayed for the presence of infectivity by bioassay with *tga20* indicator mice. Prion titres were calculated to be 6.1 and 6.4 logLD<sub>50</sub> × g<sup>-1</sup> (Table 1).

#### **No accumulation of PrP<sup>Sc</sup> in spleens of *tga20* mice following i.n. and f.p. injection**

In wild-type mice accumulation of PrP<sup>Sc</sup> in the LRS occurs very early following i.p. injection (Eklund *et al.*, 1967; Kimberlin & Walker, 1989). To examine the role of the spleen in neuroinvasion following i.n. and f.p. injection of *tga20* and wild-type mice, we performed Western blots of selected spleens from i.n. and f.p. injected mice. In all of the tested wild-type mice we could detect sizeable PrP<sup>Sc</sup> accumulation in spleens, whereas PrP<sup>Sc</sup> could not be detected in spleens of *tga20* mice, or was present in very low amounts (Fig. 6). This unexpected finding may, in principle, point to lower expression levels of PrP<sup>C</sup> in lymphoreticular organs of *tga20* transgenic mice: we therefore determined the expression levels of PrP<sup>C</sup> in spleens and in inguinal lymph nodes of *tga20* mice. However, similarly to what was observed in other tissues, PrP<sup>C</sup> was massively overexpressed in these tissues (Fig. 1). We conclude

that i.n. and f.p. injection of peripheral nerves overexpressing PrP<sup>C</sup> facilitates intranerval spread so extensively that lympho-invasion of prions becomes marginal or absent.

#### **Determination of the rate of spread in the PNS of *tga20* mice**

The results documented above clearly indicate that, in *tga20* mice, prions bypass the lymphoid tissue and spread intranervally. By comparing the incubation times of the f.p. and the i.n. injected mice we attempted to gain some insight into the rate of spread in the PNS in these mice. The average distance between the f.p. and the i.n. sites of inoculation was determined to be 2.1 cm. The difference in the incubation times of these two groups of animals is approximately 30 days. Therefore, the actual rate of spread in the PNS of the *tga20* mice is around 0.7 mm per day.

#### **Discussion**

A wealth of studies points to the importance of the PNS for neuroinvasion (Beekes *et al.*, 1998; Kimberlin *et al.*, 1983a; Kimberlin & Walker, 1988). We have previously shown that expression of PrP<sup>C</sup> in a sessile compartment that cannot be reconstituted by adoptive bone marrow transfer is necessary for neuroinvasion of prions (Blättler *et al.*, 1997). Several lines of evidence suggest that at least a part of this compartment is the PNS (Race *et al.*, 2000). To clarify the role of neural PrP<sup>C</sup> expression in scrapie neuroinvasion via the PNS, we took advantage of transgenic mice that overexpress PrP<sup>C</sup> (Fischer *et al.*, 1996). These mice were inoculated both i.n. and via the f.p. route. All of the inoculated *tga20* mice came down with clinical scrapie, with a difference in incubation times between the i.n. and f.p. routes of about 30 days. In contrast, the incubation times of i.n. and f.p. injected wild-type mice were not significantly different from those of mice inoculated i.p. A detailed breakdown of the incubation times of i.n. inoculated wild-type mice (Fig. 2) suggested that two groups can be differentiated: 'fast transporting' and 'slow transporting' mice. Kimberlin *et al.* (1983b) had observed this phenomenon in a previous study and remarked that the fast transporting group may possibly accomplish neuroinvasion directly via the injected nerve, whereas the slower transporting group may use other routes of neuroinvasion. Accordingly, the efficiency could be increased by damaging the injected nerve. In contrast to wild-type mice, in i.n. injected *tga20* mice there was no segregation in two groups.

The pattern of PrP<sup>Sc</sup> distribution in histoblots of *tga20* mice showed selective accumulation of PrP<sup>Sc</sup> in areas representing projections of the sensory pathway. It is conceivable that the selective accumulation of PrP<sup>Sc</sup> in *tga20* mice is due to an altered proteinase K sensitivity of cerebral versus spinal PrP<sup>Sc</sup>. To control for this possibility we performed Western blot analysis of various brain regions. Proteinase K-resistant PrP<sup>Sc</sup> could be demonstrated in cortical areas and in the brain stem of wild-type mice, whereas *tga20* mice were practically devoid of

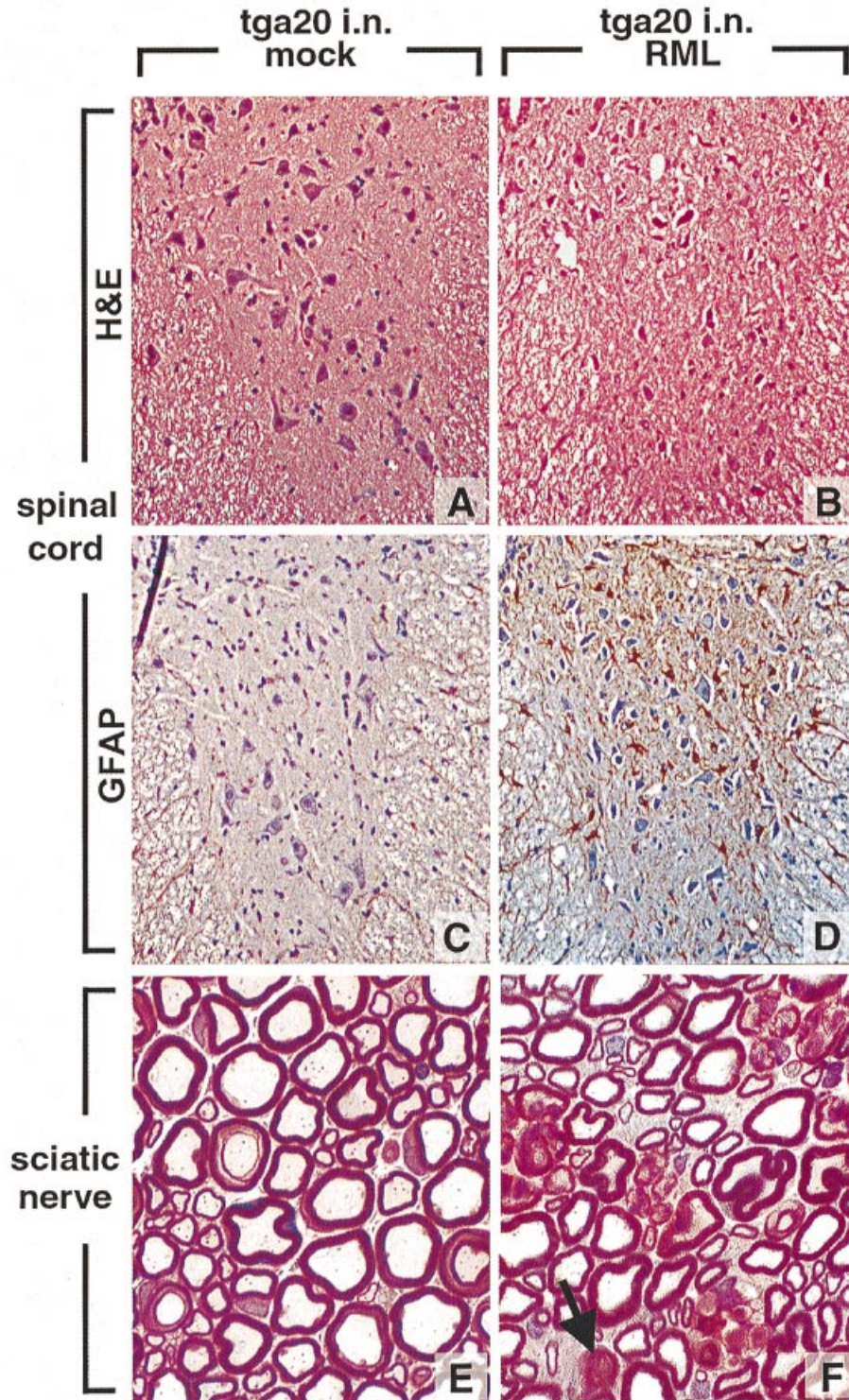
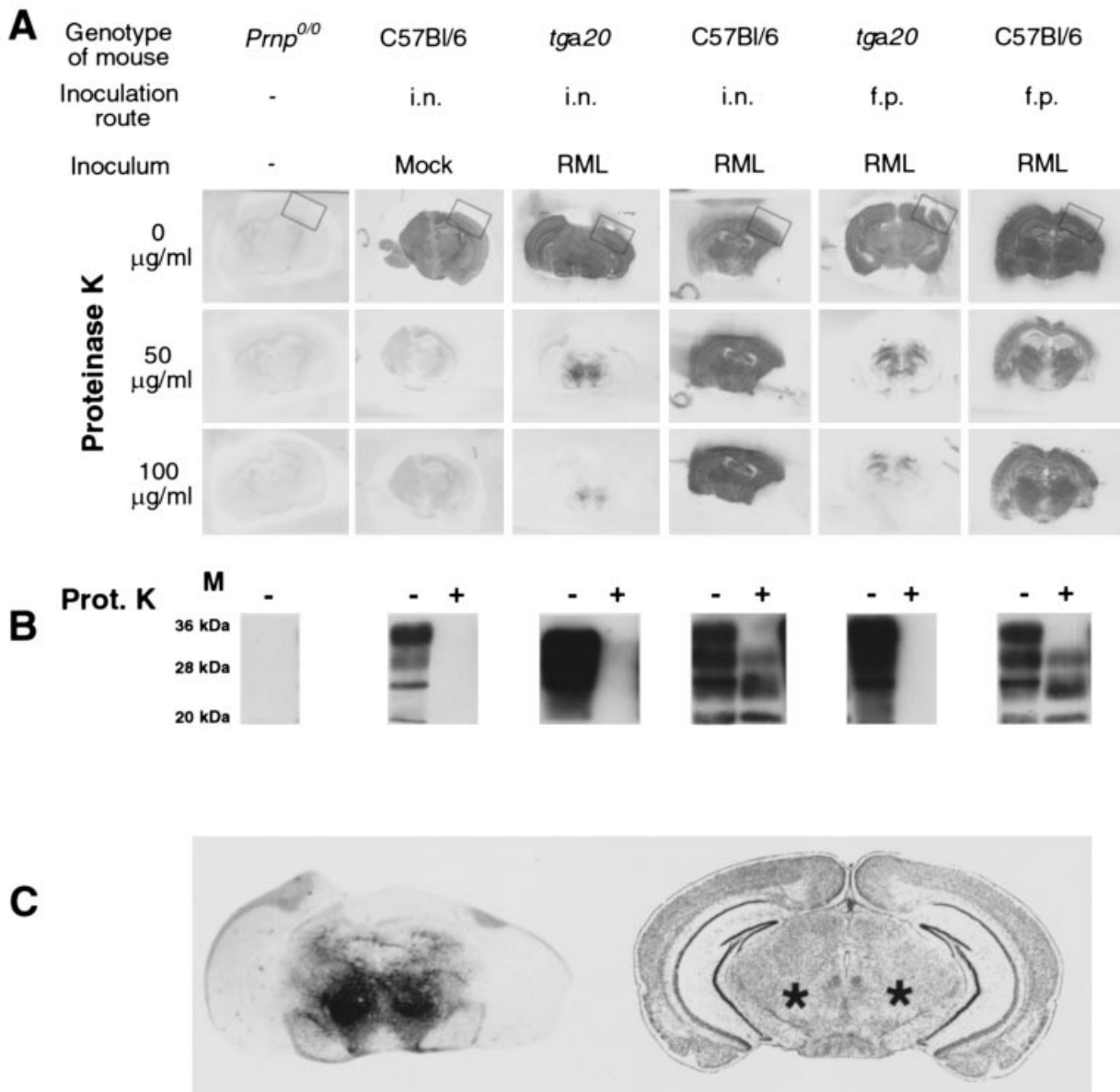


Fig. 4. Histology of thoracic spinal cords (A–D) and sciatic nerves (E, F) from a terminally sick *tga20* mouse compared to a mock injected *tga20* mouse. In both mice the injection was i.n. Strong gliosis (D) and spongiosis (B) are visible in the spinal cord of the terminally sick mouse. (A)–(B) Haematoxylin–eosin stain; (C)–(D) GFAP immunohistochemistry. Original magnifications,  $\times 100$ . (E)–(F) Semi-thin sections of injected sciatic nerves from a mock injected *tga20* mouse (E) and terminally sick *tga20* mouse (F). Axonal degeneration is obvious in (F): single axons undergoing Wallerian degeneration are indicated by an arrow. Original magnification,  $\times 260$ .

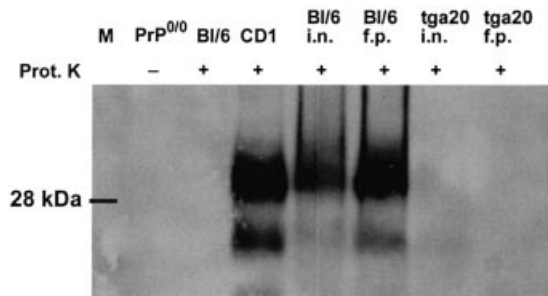


**Fig. 5.** (A) Accumulation of PrP<sup>Sc</sup> in brains. Histoblots showing immunoreactive PrP<sup>C</sup> in brain sections natively (upper row) and after digestion with increasing levels of proteinase K (second and third rows). *Prnp<sup>0/0</sup>* mice (first column) show no immunoreactivity, while mock inoculated wild-type mice show proteinase K-sensitive PrP<sup>C</sup> (second column), but no proteinase K-resistant PrP<sup>Sc</sup>. Terminally sick i.n. or f.p. injected wild-type mice contained large amounts of both PrP<sup>C</sup> and PrP<sup>Sc</sup> (fourth and sixth columns). Terminally sick i.n. and f.p. injected *tga20* mice accumulated PrP<sup>Sc</sup> selectively in specific brain areas belonging to the sensory nervous system (thalamus) (third and fifth columns). The boxes indicate areas where material for Western blotting was obtained. (B) Western blot analysis of cortical areas of terminally sick i.n. or f.p. injected wild-type and transgenic mice. The treatment and genotype of the mice correspond to (A). No PrP<sup>Sc</sup> was detected in the cortex of transgenic mice, while wild-type mice showed abundant cortical PrP<sup>Sc</sup>. (C) Left side, histoblot [close-up from (A), second row third column] showing accumulation of PrP<sup>Sc</sup> in the thalamus of an i.n. inoculated *tga20* mouse. Right side, coronal section through a mouse brain (modified after Sidman *et al.*, 1971). Asterisks indicate the posterior nucleus of the thalamus.

proteinase K-resistant PrP<sup>Sc</sup> in cortical areas. The fact that we found proteinase K-resistant PrP<sup>Sc</sup> in the brain stem of *tga20* mice (data not shown) demonstrates that this is not due to an altered proteinase K sensitivity of PrP<sup>Sc</sup>.

The selective accumulation of PrP<sup>Sc</sup> speaks in favour of neuroinvasion via the PNS and strengthens the hypothesis that

*tga20* mice transport the scrapie agent mainly in the PNS. In wild-type mice we could not see such a targeted distribution of PrP<sup>Sc</sup>. This prompted us to assess the involvement of the LRS in i.n. and f.p. injected *tga20* mice. As expected, wild-type mice showed typical accumulation of PrP<sup>Sc</sup> in the spleen, indicating colonization of the immune system (Lasmezas *et al.*, 1996;



**Fig. 6.** Western blot analysis of spleens. Immunodetection with antibody 1B3 (Farquhar *et al.*, 1996) revealed the presence of proteinase K-resistant PrP<sup>Sc</sup> in spleens of terminally sick wild-type ( $n = 2$ ) and *tga20* ( $n = 2$ ) mice after i.n. and f.p. injection of scrapie prions. No or very little proteinase K-resistant PrP<sup>Sc</sup> could be detected in the i.p. or f.p. injected *tga20* mice. Indicated above each lane is the genotype of the mouse, the route of inoculation of infectivity, and proteinase K digest. Control experiments were performed with terminally sick intracerebrally inoculated CD1 mice (CD1); mock injected wild-type mice (BI/6) and PrP knockout mice (PrP<sup>0/0</sup>). The amount of total protein loaded in each lane was 80  $\mu$ g.

Mabbott *et al.*, 1998). In contrast, *tga20* mice injected i.n. and in the f.p. did not show significant PrP<sup>Sc</sup> accumulation in spleens. These results indicate that wild-type mice respect a lympho-neural sequence of pathogenesis even after direct administration of prions into nerves, while *tga20* mice transport prions predominantly in the PNS. Finally, a subset of i.n. injected wild-type mice may use direct PNS neuroinvasion, and develop disease significantly earlier.

The fact that we did not find any PrP<sup>Sc</sup> by Western blot analysis of the sciatic nerves of wild-type and transgenic mice may surprise, especially in view of the infectivity readily detectable by bioassay of the same samples. However, considerable amounts of infectivity that are not associated with detectable PrP<sup>Sc</sup> deposits have been observed before (Manson *et al.*, 1999), and are probably due to the limited sensitivity of the Western blot technique.

Using the difference in incubation times of the f.p. and the i.n. inoculated *tga20* mice we attempted to estimate the velocity of transport of infectivity in the PNS. The distance between the footpad and the mid sciatic nerve, where the i.n. injection is performed, is 2.1 cm on average, and the difference in incubation times of the i.n. and f.p. injected mice is about 30 days. Because *tga20* mice transport primarily in the PNS after inoculation at either of these two sites, we calculated the speed of transport in the PNS by dividing the distance between the different sites of inoculation by the difference in incubation time. The calculated rate of spread of infectivity is 0.7 mm per day. This velocity is similar to that reported for wild-type mice where the rate of spread was calculated to be around 1 to 2 mm per day (Kimberlin *et al.*, 1983*b*). Neither of these values correspond to fast axonal transport or to slow axonal transport (McEwen & Grafstein, 1968), whereas PrP<sup>C</sup> was reported to be transported with fast axonal transport with a velocity of about 1 cm/h (Borchelt *et al.*, 1994). The possibility that the transport of PrP<sup>Sc</sup> in the PNS may not occur through axonal transport

mechanisms was raised recently (Groschup *et al.*, 1999; Hainfellner & Budka, 1999) and is compatible with our data.

The bioassay data gathered in this study provide intriguing insights into the kinetics of intranerval spread. Prion infectivity titres of *tga20* sciatic nerves were up to 1.8 log higher than those observed in wild-type mice, yet the velocity of transport was similar in wild-type and in transgenic mice. Therefore, PrP<sup>C</sup> availability in the nerve modulates the capacity of intranerval spread, but does not affect its velocity. Perhaps the significantly higher titres in the sciatic nerves of *tga20* mice are indicative of a mode of transport in which PrP<sup>C</sup> localized on the PNS is converted into PrP<sup>Sc</sup> in a 'domino' fashion centripetally towards the CNS. A similar phenomenon may occur in the CNS (Brandner *et al.*, 1996). Another possibility to explain the difference between wild-type and *tga20* mice is the difference in the glyco-type ratio of PrP<sup>C</sup> expressed by the electrophoretic pattern of PrP<sup>C</sup> between wild-type and *tga20* nerves. In sciatic nerves of *tga20* mice the diglycosylated form of PrP<sup>C</sup> seems to be abundant, whereas in wild-type mice monoglycosylated PrP<sup>C</sup> is predominant. It is conceivable that different glycosylation states of PrP<sup>C</sup> may influence the transport of PrP<sup>Sc</sup>.

Besides confirming a central role of PrP<sup>C</sup> in the PNS in prion neuroinvasion, the present study provides surprising evidence that mice which overexpress PrP<sup>C</sup> can effect strictly intranerval neuroinvasion and bypass LRS pathogenesis. One may wonder whether similar phenomena may underlie neuroinvasion of BSE prions in cows, which also appear to bypass the LRS.

Because overexpression of PrP<sup>C</sup> leads to increased intranerval prion titres, PrP<sup>C</sup> may well be rate-limiting for prion spread. In order to test this hypothesis (which bears some relevance to the prospect of post-exposure prophylaxis for prion diseases) we are currently attempting to express PrP<sup>C</sup> conditionally in the PNS using virus vector-mediated gene transfer (Glatzel *et al.*, 2000).

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