Precision in the design of an experimental study reflects the significance of proteinase-activated receptor 2 expression in scrapie-inoculated mice

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Abstract

Proteinase-activated receptor 2 (PAR2) is suspected to modulate the pathogenesis of various neurodegenerative conditions. We previously described delayed onset of clinical symptoms and prolonged survival of PAR2-deficient mice after intracerebral inoculation with prions. Here we report the results from a refined blinded study that aimed to investigate the effects of PAR2 deletion on scrapie pathogenesis after peripheral infection. This study failed to confirm that PAR2 deficiency impacts on the length of the incubation period, with PAR2−/− and PAR2+/+ littermates developing scrapie at the same time. To clarify the discrepancy between the two observations, we repeated the intracerebral inoculation study while utilizing our refined protocol, which aimed to limit possible sources of experimental bias. The study again failed to confirm the significant effect of PAR2 expression on the course of prion infection. Our report emphasizes and discusses the importance of unbiased experimental design and the selection of proper genetic controls when using genetically altered animal models for prion pathogenesis studies.

Prion diseases and many other neurodegenerative disorders are characterized by the accumulation of misfolded forms of endogenous proteins in the brains of affected individuals. While the nature of these proteins is quite different, the structural features of their disease-associated, aggregation-prone forms are strikingly similar [1]. Apart from this, the mechanism by which misfolding of the proteins or their aggregation and accumulation triggers the neurodegenerative process remains obscure. The process is commonly accompanied by the activation of astrocytes and microglia, oxidative and endoplasmic reticulum stress, disturbed calcium homeostasis and mitochondrial dysfunction [2]. Recent studies suggested that the progress of neurodegeneration could be modulated by proteinase-activated receptors (PARs), a family of transmembrane G-protein-coupled receptors facilitating cell responses to extracellular proteases. PARs are activated by cleavage of their N-terminal region, which results in the exposure of a new N-terminus that serves as an intramolecular ligand for the receptor [3]. Four members of this family have been identified; PAR1, PAR3 and PAR4 are thrombin-interacting receptors, whereas PAR2 is a thrombin-insensitive receptor for trypsin and trypsin-like proteases [4]. Serine proteases and PARs are endogenously expressed in the brain and take part in the development and maintenance of the central nervous system [5]. The importance of PARs in inflammation and haemostasis is well recognized and it is anticipated that they are involved in other pathophysiological processes [6]. The trypsin receptor PAR2 (also called coagulation factor II receptor-like 1, F2RL1) was suggested to have a cell-type-specific role in Alzheimer’s disease. The activation of neuronal PAR2 seemed to have a neuroprotective effect against Aβ 1-42 and glutamate toxicity, whereas PAR2-mediated glial activation contributed to neuronal death [7, 8]. Likewise, increased PAR2 immunoreactivity was observed in glial cells in multiple sclerosis [9] and the inhibition of PAR2 signalling decreased the level of α-synuclein production in the rodent model of Parkinson’s disease [10].

We previously showed that PAR2-deficient mice displayed delayed onset of clinical symptoms and prolonged survival after intracerebral inoculation with RML prions [11]. The study was carried out using homozygous PAR2 knock-out
mice and wild-type control mice. PAR2 knock-out strain (B6.Cg-F2rl1tm1Mslb/J) was generated on a mixed FVB/N, 129SvJ and C57Bl/6 background [12] and back-crossed to C57Bl/6 mice, making the mice 98.4% congenic with C57Bl/6 controls. To elaborate on our original finding we initiated a subsequent study that aimed to examine whether PAR2 contributes to scrapie pathology after peripheral infection. To eliminate the possibility of experimental bias we utilized the following considerations in the experimental design: (1) to further limit genetic variability, we used sibling mice as controls; (2) to limit the possible effect of the environment, infected knock-out and control mice were housed together; (3) we monitored the weight of each mouse as an objective measure of disease progression; (4) we eliminated the observer-expectancy effect by performing a blinded experiment.

PAR2 knock-out mice (B6.Cg-F2rl1tm1Mslb/J) and wild-type C57Bl/6 mice were purchased from Jackson Laboratories and housed in a specific-pathogen-free (SPF) facility. Knock-out and wild-type individuals were mated and their heterozygous offspring (B6.Cg-F2rl1tm1Mslb/J × C57Bl/6)F1 were further bred together. Female littermates with different PAR2 genotypes from the resulting F2 generation were used in the study, which was approved by the Charles University Committee on the Ethics of Animal Experiments (protocol 390/11). The mice of unknown genotype were marked by ear tags (6–9 weeks old, n=48) and inoculated subcutaneously with 500 µl of 0.2% RML brain homogenate diluted in PBS. The RML strain of scrapie was provided by Adriano Aguzzi (Institute of Neuropathology, University of Zurich, Switzerland) and further propagated in CD1 mice. A group of mice (n=12) inoculated with normal CD1 brain homogenate served as a negative control. All inoculated mice were housed in non-SPF ventilated cabinets and periodically observed for clinical signs of scrapie, while their weight was also monitored. Symptoms of prion disease comprising decreased mobility and unkempt appearance were recorded 26 weeks post-inoculation (Table S1 and Fig. S1, available in the online Supplementary Material).

To compare the neuropathological features of RML-inoculated mice, the brain was removed at autopsy, and one hemisphere was frozen and stored in a −80°C freezer, while the other was fixed by 3.8% formaldehyde in PBS. The diagnosis of prion disease for every individual animal was confirmed by demonstrating the presence of pathological prion protein (PrP^{TSE}) in the brain homogenate after proteinase K (PK) treatment using Western blot as described previously [13]. A mix of the mouse monoclonal antibodies AH6 (TSE Resource Centre, Roslin Institute, UK) and 6D11 (Bio-Legend) were used for prion protein (PrP) detection. All mice inoculated with RML were found positive for PrP^{TSE}. The overall PrP quantity and electrophoretic mobility was similar among all groups of RML-infected mice (Fig. 1c). The effect of prion infection on brain expression of PAR1 and PAR2 mRNA was analysed using quantitative reverse transcriptase PCR (RT-PCR) in six PAR2^{+/−} mice as described in the supplementary data. Notably, the infection led to a 0.5-fold decrease of the PAR2 mRNA level (P=0.009, Mann–Whitney rank sum test; SigmaStat 3.5), while the decrease of PAR1 expression was not significant (Fig. S2).

The level of brain prion infectivity was evaluated using a standard scrapie cell assay (SSCA). Six individual brain homogenates from infected mice of the PAR2^{+/+} or PAR2^{−/−} genotype were pooled to obtain 10% mixed brain homogenate and the SSCA was performed as described previously [14]. Briefly, prion-susceptible CAD5 cells (donated by Charles Weissmann, Scripps Research Institute, USA) were incubated with the serially diluted brain homogenate mixture for 4 days and propagated for two additional 1:8 splits. After reaching confluence, the cells were counted using a haemocytometer and 1000 or 10,000 cells per well were filtered on the membrane of MultiScreen 96-well plates (Millipore). The plates were dried and after PK treatment and denaturation with 3 M guanidine isothiocyanate PrP^{TSE}-positive cells were identified by immunostaining using 6D11 primary antibody, AP-conjugated secondary donkey-anti-mouse antibody (Jackson ImmunoResearch) and an AP conjugate substrate kit (Bio-Rad). Stained cells were counted by an ELISPOT reader using NIS-Elements software (Nikon Instruments). We found no difference in the amount of RML infectivity present in PAR2^{+/−} and PAR2^{−/−} brain homogenates (Fig. 1d).

The presence of neuropathological hallmarks of prion disease was examined on 5 µm-thick sections of fixed brains embedded into paraffin. Haematoxylin–eosin (HE) staining was used to evaluate spongiform changes and labelling with antibody against glial fibrillary acidic protein (GFAP; Dako) was employed to determine the level of astrogliosis. Blinded scoring of vacuolization and astrocytic gliosis on a scale of 0–3 was carried out by the consensus of two experienced pathologists on frontal cortex, striatum, hippocampus, mesencephalon and cerebellar cortex regions; six individual animals were scored for each genotype. Spongiform degeneration was present at comparable levels in the brains of RML-inoculated PAR2^{+/+}, PAR2^{−/−} and PAR2^{+/−} mice (Fig. 2a, c–e), comprising various sized vacuoles located in different brain areas. All the groups of infected animals also exhibited similar levels of astrogliosis (Fig. 2b, f–h).

Taken together, our data did not confirm the effect of PAR2 deletion on the survival of prion-infected mice that was suggested by our previous study [11]. To clarify whether the
discrepancy was caused by different modes of inoculation (intracerebral versus peripheral) we repeated the intracerebral infectious study utilizing objective study conditions.

Female mice (6–9 weeks old) bred from heterozygous PAR2+/– parents were utilized to diminish genetic diversity. The mice were genotyped before inoculation and only PAR2+/+ (n=12) and PAR2–/– (n=14) animals were included in the study. However, the experiment was blinded to the main investigator and sibling mice of different genotypes were housed together to avoid the negative effect of subjective factors on experimental results. The mice were inoculated intracerebrally (i.c.) with 25 µl of 1 % RML brain homogenate diluted in PBS. Six PAR2+/+ and six PAR2–/– mice injected with normal CD1 brain homogenate served as negative controls. The mice were monitored as described in the peripheral study.

The first clinical signs of scrapie were observed 20–22 weeks after infection and animals were sacrificed in the terminal stage of the disease. Contrary to our expectations, PAR2–/– mice displayed only short, statistically insignificant prolongation of survival (median 198.5 days) compared to the group of PAR2+/+ mice (median 193.5 days) (Fig. 3a, b). The body-weight monitoring of infected animals also did not demonstrate any significant difference between PAR2+/+ and PAR2–/– mice (Table S2 and Fig. S3). The Western blot analysis confirmed the presence of PrP TSE with similar biochemical features in the brain homogenates of all i.c. infected mice (Fig. 3c) and the level of scrapie infectivity in the mixed brain homogenates measured using SSCA was equal for both PAR2+/+ and PAR2–/– mice (Fig. 3d). Interestingly, the level of PAR1 and PAR2 mRNA was significantly lower in the brains of infected PAR2+/+ mice (n=6) (Fig. 3e, f). Scrapie-associated neuropathological features were evaluated after HE and GFAP staining of brain tissue by semi-quantitative scoring. Spongiform degeneration with similar scoring patterns was observed in both PAR2+/+ and PAR2–/– RML-infected mice (n=6) (Fig. S4a); vacuoles with clusters of diverse size were identified through different brain regions (Fig. S4c, d). A slight decrease in the level of cerebellum astrogliosis in terminally sick PAR2–/– mice compared to PAR2+/+ mice was detected (P=0.015, Mann–Whitney rank sum test; SigmaStat 3.5; Fig. S4b). However, in other brain regions the differences in astrogliosis scores were not significant (Fig. S4e, f).

Altogether, neither of the above studies, which were designed to limit possible biases, reproduced the results of our previous study, in which the infection of PAR2–/– mice led to an extended incubation period following prion infection. Contrary to our expectation, the studies utilizing
improved experimental design suggested that the deletion of PAR2 does not significantly affect the pathogenesis of RML scrapie in mouse after either intracerebral or peripheral inoculation. Several factors deserve consideration to explain the observed discrepancy.

The different result of our initial infective study may stem from unpredicted non-genetic diversity of mouse cohorts. While the PAR2+/− mice were bred in-house under SPF conditions, the control C57Bl/6J mice were purchased directly from the producer. So the mice likely differ in their residential microbial flora [15]. While we doubt that such a difference can significantly affect length of survival after intracerebral inoculation of prions, the relationship between microbiome composition and neurodegeneration is gaining momentum also in the prion field [16]. Our present studies were well controlled for this factor, as they were carried out on sibling mice sharing the same environment. Another source of variation may paradoxically stem from housing cohorts of mice in separate cages. Under such conditions it is possible that some uninvited seasonal infection might affect just one cohort of animals, sparing the other. Whether such an event could influence the length of survival of prion-infected mice is unclear at the present time [17]. Another possible explanation might lie in the fact that the PAR2+/− and control wild-type mice in the original study were not fully congenic (98.4 %), with the study being influenced by genetic background differences. The mice utilized in our current studies were 99.2 % congenic. In addition, we followed the recommendation of utilizing wild-type littermates coming from the same breeding background as the controls when a fully congenic strain (>99.9 %) is not available [18].

![Diagram](image_url)

**Fig. 2.** Disease-associated brain pathology of PAR2+/+, PAR2+/− and PAR2+/− mice subcutaneously inoculated with RML scrapie strain. (a, b) Semi-quantitative scoring of spongiosis (a) and astrogliosis (b) for different brain regions of terminally sick PAR2+/+ mice (●, solid line, mean±SD, n=6), PAR2+/− mice (■, dashed line, mean±SD, n=6) and PAR2+/− mice (▲, grey line, mean±SD, n=6). FX, frontal cortex; ST, striatum; HC, hippocampus; MN, mesencephalon; CX, cerebellar cortex. (c-e) Representative images of vacuolization observed in the brain of PAR2+/+ (c), PAR2+/− (d) and PAR2+/− (e) mice (basal ganglia, HE, original magnification ×400). (f-h) Representative images of GFAP-labelled brain tissue of PAR2+/+ (f), PAR2+/− (g) and PAR2+/− (h) mice showing reactive astrogliosis (basal ganglia, immunohistochemical staining with anti-GFAP polyclonal antibody, original magnification ×400).
However, even the utilization of a fully congenic strain does not solve the problem of flanking gene regions, which are inherited by knock-out models created from a mixed genetic background. The observed phenotype may in fact be caused by the allele of a flanking gene in close proximity to the mutated locus, which is not derived from the inbred strain, but from the original donor strain [19]. Until now, this emerging phenomenon has been widely overlooked. Recently, concerns have also arisen about Prnp knock-out mice and phenotypes credited to the PrP deficiency. The enhanced phagocytic activity of Prnp−/− macrophages was shown to be controlled via the polymorphic gene encoding signal regulatory protein alpha, SIRPa [20]. Likewise, the recently created co-isogenic Prnp knock-out mice failed to reproduce many previously reported Prnp−/− phenotypes [21].

There are several polymorphic genes in close proximity to the F2rl1 gene encoding PAR2 in mice and some of them have been shown to be connected with brain-attacking
conditions, such as ARSB in Alzheimer’s disease [22], LHFPL2 in Parkinson’s disease [23] or CMYA5 in schizophrenia [24]. These facts suggest that the PAR2−/− (B6.Cg-F2r1tm1Mslb/C0) mouse utilized in our study might be affected by the flanking-gene effect. The development of fully co-isogenic strains of knock-out mice using gene editing techniques such as TALENs or CRISPR/Cas9 could help to overcome these problems in the future [25].

In conclusion, our blinded infection experiments using intracerebral and peripheral inoculation with RML prions did not confirm delayed onset of clinical symptoms and prolonged survival in mice lacking PAR2 expression. The disease manifested similarly in animals with diverse PAR2 genotypes. The objective monitoring of the disease progress by periodical measurement of the total body weight of individual animals revealed no difference between the groups. Similarly, the assessment of brain pathology did not show any substantial variation, with the exception of the isolated reduction of the astrogliosis level detected in the cerebellum of i.c. inoculated PAR2−/− mice.

Interestingly, the levels of PAR1 and PAR2 mRNAs were significantly lower in the brains of i.c. RML-infected mice than in those of healthy controls. It is tempting to speculate that this change may reflect neuronal loss in the diseased mice, but it is not clear if such a difference is also present at the protein level. A recent human study demonstrated similar levels of PAR2 in the cerebrospinal fluid of Creutzfeldt–Jakob disease patients and patients with other neurodegenerative disorders [26].

Our results suggest that PAR2 deletion probably was not solely responsible for the outcome of our original study [11] and that its effect on the course of prion infection is minor. These findings alerted us again to the crucial importance of experimental design when dealing with transgenic and knock-out model animals. The reproducibility of animal experiments represents a serious problem for current biomedical research [27]. Because of their exceptional length, prion infection studies are more vulnerable to bias than other studies. Utilizing sibling mice as controls and housing infected mice with different genotypes together should limit the possible effect of non-genetic factors on the experiments. The observer-expectancy effect could be mitigated by running the experiments blinded and by utilizing an objective method to assess disease progress. In the case where prion-strain-specific clinical signs include wasting, simply monitoring the body weight of individual infected mice may serve as an example of such a method. Finally, the use of fully co-isogenic strains of knock-out mice should alleviate the possible effects of flanking polymorphic genes.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
All animals were bred in SPF conditions at the animal facility of the First Faculty of Medicine, Charles University, Prague, Czech Republic. After inoculation, animals were housed in non-SPF ventilated cabinets placed within the BSL-2 laboratory at the Institute of Immunology and Microbiology, First Faculty of Medicine, Charles University, Prague, Czech Republic. Animal procedures were carried out in agreement with the good animal practice recommended by the Federation of Laboratory Animal Science Associations. Experiments involving animals were approved by the Charles University Committee on the Ethics of Animal Experiments under protocol number 390/11 and were performed in accordance with the Czech animal protection law (act no. 246/1992 Coll., as amended).

References


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