Prion protein and susceptibility to kainate-induced seizures

Genetic pitfalls in the use of PrP knockout mice

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Introduction

Expression of prion protein (PrP) is well-known to be essential for susceptibility to prion diseases, also known as transmissible spongiform encephalopathies (TSE). However, despite significant research efforts the normal physiological roles of the prion protein (PrP) remain poorly understood. Strategies to assign a function to PrP have typically relied on deletion or inactivation of Prnp gene expression in laboratory mice. While deletion of PrP results in no obvious physiological, developmental or anatomical insufficiencies in mice, investigations using PrP knockout mice have suggested a perplexing array of roles for PrP. Among these effects is a possible influence of PrP expression on susceptibility to seizure induction. Research in this area has produced conflicting results on the role of PrP. Two independent groups found Prnp−/− mice to be more susceptible than Prnp+/+ controls to seizures induced by kainic acid (KA), pentylenetetrazol (PTZ) or pilocarpine. In contrast, in our recent study C57BL/10SnJ Prnp−/− mice were less susceptible than Prnp+/+ controls to kainate-induced seizures. In ex vivo studies by another group using hippocampal slices treated with PTZ, bicuculline and zero-magnesium conditions, Prnp−/− slices were less susceptible to induction of seizure activity compared with Prnp+/+ controls. These confounding results have clouded our ability to draw accurate conclusions about the role of Prnp in seizure susceptibility. Moreover, these differences suggest the possibility that factors other than the deletion of Prnp may be influencing experimental outcomes. Possibly, differences in seizure induction protocols and/or variations in mouse background genes in the Prnp−/− and Prnp+/+ mice are responsible. Here, using our recently published results as a model, we elaborate on...
genetic pitfalls related to the use of Prnp<sup>−/−</sup> mice and the potential solutions to these problems.

**Lower Seizure Susceptibility of Prnp<sup>−/−</sup> Mice on C57/BL/10SnJ Background**

In our recent investigation of kainate-induced seizure susceptibility in mice on the C57BL/10SnJ background, seizure severity was significantly less in male Prnp<sup>−/−</sup> mice than in Prnp<sup>+/+</sup> mice (Fig. 1).<sup>8</sup> The greater seizure sensitivity seen in Prnp<sup>−/−</sup> mice compared with Prnp<sup>+/+</sup> mice was opposite to the findings of others,<sup>5,7</sup> and therefore we considered explanations other than Prnp deletion for these results.

In our own mouse system C57BL/10SnJ Prnp<sup>−/−</sup> mice were originally derived from the 129/Ola Prnp<sup>−/−</sup> mice constructed in Edinburgh.<sup>10</sup> Because these 129/Ola Prnp<sup>−/−</sup> mice were poor breeders, they were subsequently bred onto the C57BL/10SnJ background by serial backcrossing to C57BL/10SnJ mice for nine generations. As in all situations where genes are transferred between mouse strains by serial backcrossing, 129/Ola genes flanking the Prnp locus persisted in our C57BL/10SnJ Prnp<sup>−/−</sup> mice despite 9 backcrosses. To identify more accurately the extent of this 129-derived flanking region, we compared the DNA of Prnp<sup>+/+</sup> and Prnp<sup>−/−</sup> mice on the C57BL/10SnJ background using PCR analysis to detect previously mapped strain-specific single nucleotide polymorphisms (SNP). A 47.4 Mb region of 129/Ola genes flanking the Prnp<sup>−/−</sup> locus on chromosome 2 was the only 129-derived genetic region detected in the C57BL/10SnJ Prnp<sup>−/−</sup> mice (Fig. 2). Thus, our Prnp<sup>−/−</sup> mice differed from Prnp<sup>+/+</sup> controls not only in the deletion of Prnp, but also in the presence of over 400 protein coding genes in the adjacent flanking regions derived from the 129/Ola strain rather than the C57BL/10SnJ strain. In this region, by comparison of DNA sequences, strains 129/Ola and C57BL/10SnJ differ at >5000 SNPs. Therefore, the seizure phenotypes observed in Prnp<sup>−/−</sup> mice might be a result of either the deletion of Prnp or the presence of flanking genes derived from strain 129 where the Prnp gene was first propagated in embryonic stem cells.<sup>10</sup>

**Transgenic Rescue Experiment to Study Possible Influence of Flanking Genes**

To test the specificity of a deleted Prnp gene in knockout mice, one approach is genetic complementation or rescue, where a PrP expressing transgene located at a chromosomal site unrelated to the Prnp<sup>−/−</sup> gene is bred back into the C57BL/10SnJ background by serial backcrossing to C57BL/10SnJ mice for eight generations. To identify more accurately the extent of this 129-derived flanking region, we compared the DNA of Prnp<sup>+/+</sup> and Prnp<sup>−/−</sup> mice on the C57BL/10SnJ background using PCR analysis to detect previously mapped strain-specific single nucleotide polymorphisms (SNP). A 47.4 Mb region of 129/Ola genes flanking the Prnp<sup>−/−</sup> locus on chromosome 2 was the only 129-derived genetic region detected in the C57BL/10SnJ Prnp<sup>−/−</sup> mice (Fig. 2). Thus, our Prnp<sup>−/−</sup> mice differed from Prnp<sup>+/+</sup> controls not only in the deletion of Prnp, but also in the presence of over 400 protein coding genes in the adjacent flanking regions derived from the 129/Ola strain rather than the C57BL/10SnJ strain. In this region, by comparison of DNA sequences, strains 129/Ola and C57BL/10SnJ differ at >5000 SNPs. Therefore, the seizure phenotypes observed in Prnp<sup>−/−</sup> mice might be a result of either the deletion of Prnp or the presence of flanking genes derived from strain 129 where the Prnp gene was first propagated in embryonic stem cells.<sup>10</sup>

Interestingly, when tested for KA-induced seizure sensitivity tga20-PrP<sup>+/−</sup> and tga20-PrP<sup>−/−</sup> mice were not significantly different (Fig. 3), and their seizure phenotype matched that of the C57BL/10SnJ Prnp<sup>−/−</sup> mice. Thus, expression of the tga20-PrP transgene did not restore the phenotype seen in Prnp<sup>−/−</sup> mice. This result was not due to insufficient PrP expression as the transgene heterozygote expresses 3-fold more PrP than wildtype mice.<sup>11</sup> These findings indicated that the 129/Ola flanking genes rather than Prnp deletion were responsible for the difference in seizure susceptibility seen between Prnp<sup>−/−</sup> and Prnp<sup>+/−</sup> mice on the C57BL/10SnJ background.

**129/Ola Coisogenic Mice**

Ideally the simplest method for analyzing the effect a mouse with a targeted gene deletion is to maintain the deleted gene on the same mouse genetic background where it was created, and then compare co-isogenic mice with and without the targeted deletion to determine the effect of the deletion. In our KA seizure susceptibility experiments, co-isogenic Prnp<sup>−/−</sup> and Prnp<sup>+/−</sup> mice on the 129/Ola background did not differ significantly in seizure latency or severity.<sup>8</sup> Therefore, overall these data supported our previous findings that Prnp itself did not alter susceptibility to KA-induced seizures.

Because our experiments used mice on both 129/Ola and C57BL/10SnJ backgrounds, we cannot be certain that our
conclusions explain the results of previous workers who compared Prnp+/− and Prnp−/− mice which were on a mixed 129-C57BL/6J background.6,7 However, these latter studies also involved mice with 129-derived genes flanking the Prnp−/− locus, and thus it remains to be tested whether flanking genes might also influence these data.

**Pittfalls in the Use of Various Types of Prnp Knockout Mice**

In addition to the requirement for PrP in susceptibility to prion diseases, PrP has also been described to influence a large number of biological processes involving not only many aspects of brain function and biochemistry, but also immune system function, phagocytosis, hematopoietic stem cells, and even dentin formation in teeth (for review see ref. 2). This list continues to grow at the present time, but there has been little consensus from these studies regarding the overall main function(s) of PrP in cells. Most studies identifying new PrP functions have utilized comparisons between PrP-expressing and PrP-non-expressing mice or cells. Although the results have been distinct in regard to the requirement for PrP expression for prion disease susceptibility, the results for other processes requiring PrP expression have been less clear. This is likely due to the weaknesses of the phenotypic differences observed and also to the problem of the influence of non-Prnp background genes in many systems. For these reasons, we consider here some aspects of possible pitfalls in the use of Prnp KO mice and discuss possible approaches to circumventing these problems.

Currently available lines of Prnp KO mice can be considered in three different groups (Table 1) with increasing potential problems due to non-Prnp background gene effects: (1) co-isogenic mice on the same genetic background as the original ES cells, (2) congenic mice where the original construct in ES cells was moved to another genetic background by repeated backcrossing, and (3) mice on a mixed genetic background where the original construct was moved to a mixed background composed of segregating genes from 2 or more inbred mouse strains.

To date co-isogenic mice, where Prnp−/− and Prnp +/+ are on the same background as the embryo stem (ES) cells used to introduce the Prnp− mutation, have been derived using ES cells from strain 129 mice which were capable of fusing with early mouse embryos to form viable mosaic mice (Table 1).10 Since these mice differ only at the site of the targeted insertion of the Prnp−/− gene, functional differences found in studies using the mice or their cells can be presumed to be due to differences in the presence of Prnp. One caveat is the possibility that these paired lines can undergo genetic drift over time leading to differences in other genetic loci, but the influence of such unwanted mutations so far has not been a detectable problem. Other approaches such as genetic complementation (see below) could be used to rule out such a possibility.

Prnp−/− mice have also been generated on genetic backgrounds of mouse strains other than 129 (Table 1). In these cases, strain 129 ES cells were also used to insert the deleted Prnp gene. Mice containing the KO gene were then repeatedly backcrossed with a different mouse strain to generate mice expressing the Prnp− allele, and these mice were intercrossed to generate Prnp−/− mice lacking PrP expression. This is an effective strategy to allow the study of Prnp deletion on genetic backgrounds other than strain 129. If the backcross is done using a single inbred mouse line to create a congenic mouse strain, there is...
prion diseases, this approach has shown regarding the rescuing of susceptibility to the Prnp−/− genotype might instead be able to the Prnp−/− phenotype. 

10 to 500 flanking genes derived from two different strains, i.e., 129 and B6. 129 strain 129, and effects apparently attributable to the Prnp−/− locus. Such flanking gene regions cannot be removed by repeated backcrossing because the incidence of recombination becomes vanishingly low as the flanking gene region gets smaller. Thus there always remain at least 10 to 500 flanking genes derived from strain 129, and effects apparently attributable to the Prnp−/− genotype might instead be due to 129-derived flanking genes. Fortunately the effect of a flanking gene vs. that of the deleted gene can usually be distinguished by genetic complementation or rescue of the Prnp−/− phenotype by inserting a PrP expressing transgene at a different chromosomal site in the Prnp KO mice.

Genetic complementation using PrP transgenes has also utilized cell-type-specific promoters to not only show complementation but also to determine the cell type where PrP complementation is required to reverse the Prnp−/− phenotype. Regarding the rescuing of susceptibility to prion diseases, this approach has shown the effectiveness of PrP expression in both neurons and astrocytes, and the ineffectiveness of PrP expression in hepatocytes, T lymphocytes, oligodendroglia and Schwann cells.

Another elegant approach to genetic complementation involves the use of the Cre-lox system where two transgenes, a PrP transgene flanked by loxP sites plus a prototype expressing Cre recombinase under control of a cell-specific promoter, are bred into the Prnp−/− mice. Such mice later undergo cell-specific deletion of expression of the PrP transgene. If transgene-mediated deletion of PrP abolishes the rescue of the original Prnp−/− phenotype, this is strong evidence for the role of PrP expression in the phenotype tested.

Again it is critical that the background genes in these crosses be identical to each other to rule out mixed backgrounds as a possible explanation of the results.

The Zurich I Prnp KO line, which was the first Prnp−/− line produced, has the most complicated genetic background due to its breeding history (Table 1). Because of its early availability, this line has been used for the largest number of studies of the PrP knockout phenotype. With this line, the initial breeding generated a genetically mixed background derived from both strain 129 and strain B6 mice.

Table 1. Comparison of various lines of Prnp−/− mouse lines

| Type | Prnp−/− gene | ES cells | Flanking genes | Background | Prnp−/− control | Cerebellar degeneration |
|------|--------------|---------|--------------|------------|----------------|------------------------|
| Co-isogenic | Edin | 129/Ola | No | 129/Ola | 129/Ola | No |
| Congenic | Zu I | 129/Sv | Yes | FVB, BALB/c, 129 flanking genes | FVB, BALB/c, 129 flanking genes | No |
| Congenic | Ngsk | 129/Sv | Yes | 129 flanking genes | 129 flanking genes | Yes |
| Congenic | Rikn | 129/Ola | Yes | 129 flanking genes | 129 flanking genes | Yes |
| Congenic | Zu II | 129/Ola | Yes | 129 flanking genes | 129 flanking genes | Yes |
| Congenic | Rcm0 | 129/Ola | Yes | unknown | ? | Yes |
| Congenic | GFP | 129/Ola | Yes | 129 flanking genes | 129 flanking genes | NT |
| Mixed | Zu I | 129/Sv | Yes | Mixed B6–129 | None | No |
| Mixed | Zu I/tga20 | 129/Sv | Yes | Mixed B6–129 | None | No |

*Category of line. Co-isogenic, mice identical at all genes except gene targeted by knockout recombinant; Congenic, knockout gene backcrossed onto background genotype of an inbred strain different from that of the original ES cells; Mixed, background genotype from two different strains, i.e., 129 and B6. Abbreviated name for Prnp knockout construct described in reference cited in background column. 129 flanking gene issue as described. When genotype of ES cells differs from that of mice used to make a congenic line, there are 129-derived genes flanking the Prnp−/− locus which must be considered in the analysis. Use of a genetic complementation or rescue experiment using a PrP transgene is helpful to resolve this problem. Some strains of Prnp−/− mice develop spontaneous cerebellar degeneration as indicated in this Table. This process is blocked by expression of a normal PrP transgene, suggesting that it is caused by the Prnp knockout itself, but in fact it is actually caused by the knockout construct altering splice sites downstream from Prnp leading to an increased expression of the downstream Prn-d (doppel) gene. There are no publications stating whether or not these mice develop spontaneous cerebellar degeneration. During selection and maintenance of homozygous Prnp−/− genotype, if strict inbreeding is done, homozygosity for either the 129 or B6 allele of each gene is eventually achieved, but the genotype of each gene is randomly distributed between 129 and B6. If lines become separated as for Zu I and Zu I/tga20, mixture of 129 and B6 genes will not be the same in the 2 lines. At this point, they cannot be accurately compared with each other without intercrossing and analyzing littermates differing in transgene expression (see Fig. 2). tga20 transgene, which expresses mouse PrP, was inserted and maintained in Zu I Prnp−/− mice on a mixed 129-B6 background. However, the backgrounds of these mice and of the original Zu I Prnp−/− mice are likely to have diverged during the years since these 2 lines were created because 129 or B6 alleles at various loci are randomly selected during the breeding process.
similar enough in all the mice to identify effects of Prnp deletion. Genetic complementation experiments can also be done in these mice. However, all transgenic and knockout mice on the mixed 129-B6 background are unlikely to have the same mixture of these background genes. This is due in part, to the varying times of establishment of these mice, and continuing segregation ongoing in these lines. Thus the use of these lines requires the rigorous use of littermate mice to control for possible differences in the random mixture of B6 and 129 background genes. For a more detailed discussion of issues concerning genetic background and flanking gene problems in mouse mutant studies see ref 31.

Disclosures

Potential Conflicts of Interest

No potential conflicts of interest were identified.

Acknowledgments

The authors thank Jeff Severson, Donna Norton, and Nicolette Arndt for animal husbandry, Katie Phillips, Jay Carroll, and Kimberly Meade-White for technical assistance with seizure experiments, and Karin Peterson, Jay Carroll, and Alison Kraus for critique of the manuscript. This work was supported by the Division of Intramural Research of the National Institute of Allergy and Infectious Diseases.

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Conclusions

Because of the problems which occur with the use of mice with a mixed genetic background, future researchers wishing to analyze the effects of Prnp deletion should consider using either the co-isogenic Prnp−/− mice from Edinburgh or one of the congenic lines with either the Edinburgh or the Zurich I Prnp−/− which have been generated (Table I). The use of PrP transgene rescue experiments should always accompany the use of the congenic lines which all have 129-derived genes flanking the Prnp−/− locus. The use of the Prnp−/− lines which develop spontaneous cerebellar disease, i.e., Ngsk, Rkkn, Rcm0, and Zt II, is not recommended for most situations due to their dysregulation of the adjacent Prnd (doppel) gene (Table I).

The above discussion of the different types of Prnp−/− mice raises concerns about the previous conclusions concerning PrP functions found using many of the systems. Where co-isogenic mice were used, the results obtained should be valid from the genetic viewpoint. Similarly when appropriate genetic complementation experiments were done using mice with similar backgrounds, the effect described can likely be attributed to Prnp rather than flanking genes. However, there are many published studies which do not meet these criteria for genetic accuracy, and readers and reviewers should be cautious in accepting the conclusions of such studies without further experimentation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Jeff Severson, Donna Norton, and Nicolette Arndt for animal husbandry, Katie Phillips, Jay Carroll, and Kimberly Meade-White for technical assistance with seizure experiments, and Karin Peterson, Jay Carroll, and Alison Kraus for critique of the manuscript. This work was supported by the Division of Intramural Research of the National Institute of Allergy and Infectious Diseases.
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