

RML prions act through Mahogunin and Attractin-independent pathways

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Abbreviations: CNS, central nervous system; HIV, human immunodeficiency virus; LYST, lysosomal trafficking regulator; PrP, prion protein; PrP^C, cellular (normal) prion protein; PrP^{Sc}, scrapie form of prion protein; *Mgrn1*, Mahogunin ring finger-1 gene; MGRN1, mahogunin ring finger-1 protein; *Atrn*, Attractin gene; ATRN, attractin protein; ESCRT-I, endosomal sorting complex required for transport-I; TSG101, tumor susceptibility gene 101 protein; MVBs, multivesicular bodies; siRNA, small interfering ribonucleic acid; RML, Rocky Mountain Laboratory; ALIX, ALG-2-interacting protein X (officially known as Programmed cell death 6-interacting protein, PDCD6IP); Fig4, FIG4 homolog gene; Vac14, Vac14 homolog gene; PI(3,5)P₂, phosphatidylinositol-3,5-bisphosphate; Ndufs4, NADH dehydrogenase (ubiquinone) Fe-S protein 4 gene; AIDS, acquired immunodeficiency syndrome; *Kcnj10*, potassium inwardly-rectifying channel, subfamily J, member 10 gene; *Gjb1*, gap junction protein, beta 1 gene; *Gjc2*, gap junction protein, gamma 2 gene

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While the conversion of the normal form of prion protein to a conformationally distinct pathogenic form is recognized to be the primary cause of prion disease, it is not clear how this leads to spongiform change, neuronal dysfunction and death. Mahogunin ring finger-1 (*Mgrn1*) and Attractin (*Atrn*) null mutant mice accumulate vacuoles throughout the brain that appear very similar to those associated with prion disease, but they do not accumulate the protease-resistant scrapie form of the prion protein or become sick. A study demonstrating an interaction between cytosolically-exposed prion protein and MGRN1 suggested that disruption of MGRN1 function may contribute to prion disease pathogenesis, but we recently showed that neither loss of MGRN1 nor MGRN1 overexpression influences the onset or progression of prion disease following intracerebral inoculation with Rocky Mountain Laboratory prions. Here, we show that loss of ATRN also has no effect on prion disease onset or progression and discuss possible mechanisms that could cause vacuolation of the central nervous system in *Mgrn1* and *Atrn* null mutant mice and whether the same pathways might contribute to this intriguing phenotype in prion disease.

Introduction

One of the most striking and intriguing neuropathological hallmarks of prion disease is the widespread appearance of vacuoles in the central nervous system (CNS). Other features, such as neuronal loss and

gliosis, are associated with many neurodegenerative disorders, but spongiform change is found in fewer, seemingly unrelated disorders, including prion disease, viral infection (i.e., HIV encephalopathy) and metabolic diseases.¹⁻⁴ In transmissible spongiform encephalopathies, vacuoles are most commonly observed within the neuropil and contain membrane fragments and amorphous material. Both the mechanism by which they arise and their role in prion disease remain unclear. The lysosomal pathway is thought to be involved since spongiform degeneration is reduced in the brains of aged prion-infected mink homozygous for the Aleutian allele, which is a loss-of-function mutation in the lysosomal trafficking regulator (LYST) gene.^{5,6} Interestingly, disease incubation time, clinical signs and gliosis are not altered, suggesting that the effect of the *LYST* mutation on the lysosomal pathway is specific to the formation of vacuoles and that this phenotype can be separated from other aspects of prion disease.

Vacuolation and gliosis of the CNS without accumulation of the protease-resistant scrapie form of the prion protein (PrP^{Sc}) occurs in several mouse mutants, including Mahogunin ring finger-1 (*Mgrn1*) and Attractin (*Atrn*) null animals.⁷ These mice develop widespread spongiform encephalopathy with a similar anatomical distribution and histological appearance to that associated with prion diseases (Fig. 1) but do not become clinically ill. If there is a shared mechanism underlying this phenotype in prion diseases and *Mgrn1* and *Atrn* mutant mice, then understanding the normal function of the MGRN1 and ATRN proteins in the

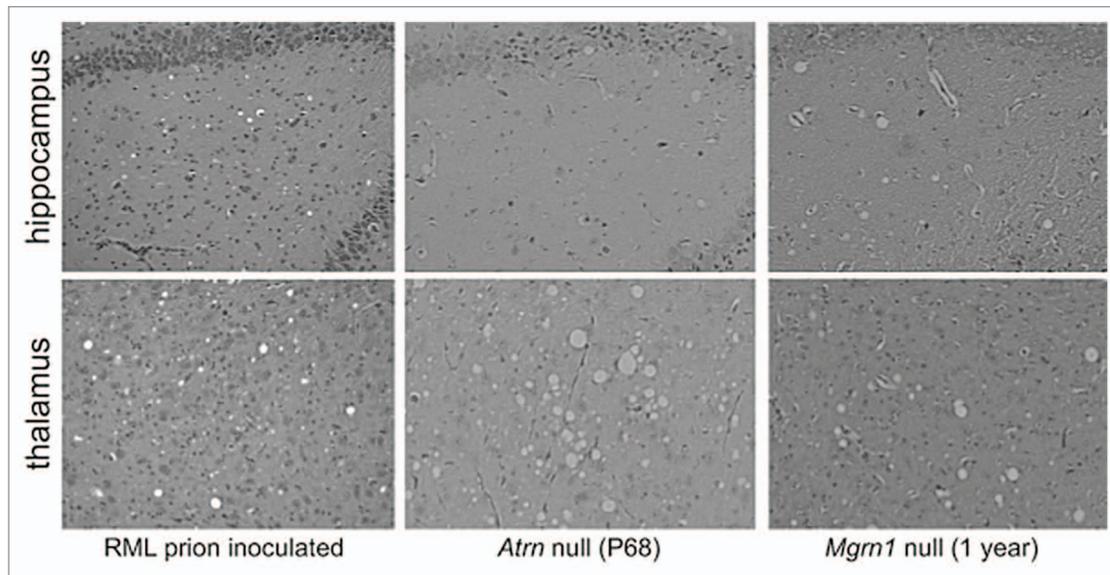


Figure 1. Histologically similar spongiform degeneration of the CNS with a similar anatomical distribution is observed in RML prion-inoculated mice and mice lacking ATRN or MGRN1. Top row, CA3 region of hippocampus; bottom row, thalamus.

brain and the pathways disrupted by their absence will provide insight into the cause of vacuolation in prion disease.

RML Prions Act Through MGRN1 and ATRN-Independent Pathways

ATRN is a widely expressed type I transmembrane protein, the CNS function of which remains unknown.⁸ MGRN1 is a RING-domain family E3 ubiquitin ligase, the only target of which identified to date is the endosomal sorting complex required for transport-I (ESCRT-I) protein, tumor susceptibility gene 101 protein (TSG101).^{9,10} TSG101 is required for the sorting of ubiquitinated proteins into multivesicular bodies (MVBs) and MVB formation.¹¹ Its depletion from mammalian cells results in the accumulation of vacuolar, multicisternal endosomes,¹² which is likely to have an indirect effect on membrane homeostasis. Loss of MGRN1 causes a partial loss of TSG101 function that leads to abnormalities in early and late endosomes and prolonged epidermal growth factor receptor signaling. In cultured cells, cytosolically-exposed PrP was shown to interact with and functionally sequester MGRN1, resulting in endosomal defects similar to those observed in cells in which *Mgrn1* expression was knocked down by siRNA.¹³ These defects could be rescued by *Mgrn1* overexpression.

Cytosolically-exposed PrP has been detected in both transmissible and inherited forms of prion disease,^{14,15} suggesting that CNS vacuolation associated with these diseases might be caused by disrupted MGRN1 function. If this were the case, a complete absence of MGRN1 might be expected to accelerate the onset of pathogenesis, while overexpression of *Mgrn1* could be protective.

We tested whether MGRN1 plays a role in the pathogenesis of transmissible prion disease by inoculating *Mgrn1* null mutant (*Mgrn1^{md-nc/md-nc}*) mice and transgenic mice that overexpress *Mgrn1* with Rocky Mountain Laboratory (RML) prions. MGRN1 levels had no effect on PrP^{Sc} accumulation or the onset, progression, symptoms or histopathology of disease.¹⁶ This suggests that cytosolically-exposed PrP either is not produced or does not play a significant role in the pathogenesis of prion disease caused by RML-prions. The fact that disease progression was not altered in mice lacking MGRN1 also suggests that partial disruption of TSG101-dependent endosomal trafficking does not significantly impact the conversion of PrP^C to PrP^{Sc}. This is consistent with a report that siRNA knockdown of ALIX (an ESCRT-associated protein) does not affect the production of PrP^{Sc} or the intracellular localization of PrP^C

or PrP^{Sc} in RML prion-infected GT1 or CAD cells.¹⁷

Mice homozygous for null alleles of *Atrn* also develop CNS vacuolation but at a much earlier age than *Mgrn1* null mutants.¹⁸ If ATRN and MGRN1 act in the same pathway, this would suggest that loss of ATRN causes a more severe disruption of that pathway and that prion disease might therefore be more likely to progress more rapidly in *Atrn* null mutant mice than in wild-type or *Mgrn1* null mutant mice. To test this, female *Atrn* null (*Atrn^{mg-31/mg-31}*) and wild-type control mice were inoculated with RML prions at 37–52 d of age. No significant genotype-dependent differences were observed in incubation or survival time (Fig. 2), indicating that RML-prions do not act through an ATRN-dependent pathway to cause disease. Although the role of ATRN in the CNS is not known, it appears to be required for normal membrane homeostasis since *Atrn* null mutant mice show age-dependent loss of detergent-resistant glycolipid-enriched membrane domains (sometimes referred to as membrane rafts).¹⁹ When brain protein lysates from adult *Atrn* null mutant mice are centrifuged through a discontinuous sucrose gradient, proteins normally found in detergent-resistant membrane fractions (i.e., flotillin-1) relocate to high-density membrane fractions (Fig. 3). The

distribution of membrane raft proteins was normal in brain lysates from both young (post-natal day 5) *Atrn* null mutant mice and adult animals homozygous for a hypomorphic allele (*Atrn^{mg-L}*), neither of which have histologically-detectable vacuoles in their brain (Fig. 3; refs. 18 and 19). Localization of PrP^C to lipid rafts has been implicated in its conversion to PrP^{Sc},²⁰ but the existence of these membrane domains remains controversial.²¹ The fact that neither the onset nor progression of disease is delayed in *Atrn* null mutant mice inoculated with RML-prions at an age by which they already show spongiform change and have begun to lose membrane raft domains suggests that the conversion of PrP^C to PrP^{Sc} can occur even as the ability of cells in the CNS to maintain membrane rafts is diminishing.

Are All Vacuoles Created Equal?

MGRN1 and ATRN are unlikely to act downstream of PrP^{Sc} to mediate prion disease pathogenesis since neither loss of MGRN1 or ATRN nor overexpression of MGRN1 altered the course of disease in RML-prion inoculated animals. The fact that *Mgrn1* and *Atrn* null mice develop spongiform change without overt neurological symptoms or illness suggests vacuoles may be a secondary phenotype, but understanding how they form could reveal primary mechanisms and perturbed pathways in prion disease, and it is possible that loss of MGRN1 or ATRN and the presence of PrP^{Sc} (and/or loss of PrP^C) act on the same downstream pathway(s) to cause spongiform change. Loss of MGRN1 has been associated with disrupted TSG101-dependent endo-lysosomal trafficking but

it is not known whether ATRN plays a role in this pathway, while age-dependent loss of lipid raft domains associated with loss of ATRN is not observed in the brains of *Mgrn1* null mutant mice (Fig. 3). A phenotype that is shared by *Atrn* and *Mgrn1* null mutant mice is elevated oxidative stress and mitochondrial dysfunction, apparent in the CNS by 1-mo of age.²² Interestingly, mitochondrial diseases and mutations that disrupt mitochondrial function can cause spongiform encephalopathy,²³⁻²⁹ and mitochondrial dysfunction has been observed in the brains of scrapie-infected mice^{30,31} and hamsters,³² as well as in mice lacking PrP^C.³³ These observations suggest that reduced energy production due to mitochondrial dysfunction may contribute to CNS vacuolation

in a variety of disorders, although in the case of prion disease this would most likely be a secondary effect of either loss of PrP^C or accumulation of PrP^{Sc} in neuronal membranes. An alternative mechanism could involve disrupted membrane functions due to the accumulation of PrP^{Sc}, in prion diseases, or an effect of loss of ATRN or MGRN1 on membrane homeostasis. Consistent with this hypothesis, widespread CNS vacuolation is observed in mice homozygous for loss-of-function mutations in *FIG4* homolog gene (*Fig4*) or *Vac14*,^{34,35} the products of which form a complex that regulates phosphatidylinositol-3,5-bisphosphate (PI[3,5]P₂) levels and select membrane trafficking pathways.

There is evidence for both neuronal and oligodendroglial-dependent forms of

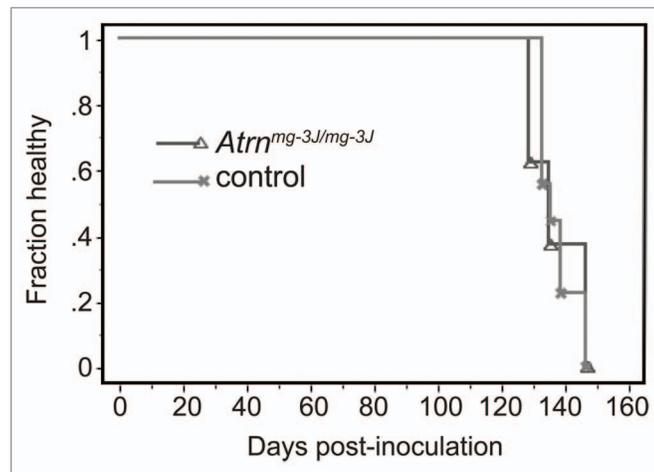


Figure 2. Kaplan-Meier plot showing health status following RML prion inoculation of *Atrn* null (C3HeB/FeJ-*Atrn^{mg-3J/mg-3J}*) and control (C3HeB/FeJ) mice. A total of 8 (5 female, 3 male) mutant and 10 (6 female, 4 male) control mice were inoculated intracerebrally with RML-prions at 37–52 d of age and monitored daily for general health status and thrice weekly for neurological symptoms. There was no significant difference in the time to onset of clinical symptoms of *Atrn*-deficient mice relative to controls.

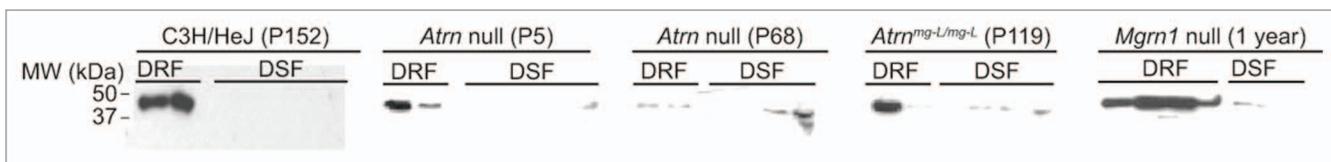


Figure 3. *Atrn* but not *Mgrn1* null mutant mice show age-dependent loss of membrane raft domains. Brain proteins were extracted in MES buffered saline containing 0.1% Triton-X100 and protease inhibitors from animals of the indicated genotypes and ages. Lysates were centrifuged through a discontinuous sucrose gradient as described in ref. 40 and fractions analyzed for the presence of the raft marker flotillin-1 by western blotting. Detergent-resistant membrane rafts float to the interface between 5% and 35% sucrose (detergent-resistant fraction, DRF). In samples from the brains of wild-type C3H/HeJ mice, young (postnatal day 5, P5) *Atrn* null mutants, adult mice homozygous for the hypomorphic *Atrn^{mg-L}* allele and aged (1 year old) *Mgrn1* null mutant mice, the vast majority of flotillin-1 localized to the DRF. In samples from the brains of adult *Atrn* null mutant mice (postnatal day 68 (P68) shown), there is progressive redistribution of flotillin-1 from the DRF to the detergent-soluble fraction (DSF), consistent with loss of membrane rafts.

spongiform degeneration, suggesting that either interactions between these two cell types is important or different mechanisms are responsible for vacuolation involving distinct CNS cell types in different diseases. Neuronal depletion of endogenous PrP^C in mice with established CNS prion disease (following inoculation with RML prions) prevented illness and reversed early spongiform change.³⁶ Similarly, neuron-specific expression of *Fig4* or *Ndufs4* (which encodes NADH dehydrogenase [ubiquinone] Fe-S protein 4) was sufficient to rescue the vacuolation observed in the respective null mutant mice.^{28,37} On the other hand, transgenic expression of HIV type I proteins in mice under control of the oligodendrocyte-specific myelin basic protein promoter caused vacuolar lesions in the spinal cord, similar to those found in AIDS patients,³⁸ and mice homozygous for mutations in oligodendroglial-specific genes *Kcnj10*, which encodes a potassium channel, and *Gjb1* and *Gjc2* (*connexin32*; *connexin47* double mutants) develop vacuoles predominantly in white matter tracts.³⁹ In the latter study, there was a positive correlation between the size and number of vacuoles and axonal activity, suggesting disrupted oligodendroglial-neuron interactions may play a role in vacuole formation. This could also explain why most forms of spongiform degeneration are associated with myelin defects. Further studies are needed to determine whether vacuoles themselves have a direct detrimental effect on CNS function and whether they form through perturbation of the same pathway in different diseases.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

Acknowledgments

RML-prion inoculated mouse brain histology images shown in **Figure 1** were provided by Dr Stephen DeArmond. RML-prion inoculation of *Atrn* null mutant mice was funded by National Institutes of Health program project grant NS041997 and performed in collaboration with Dr Gregory S Barsh; these data are shown with his permission.

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