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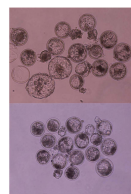
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EXTRA VIEW

Disease-associated protein seeding suggests a dissociation between misfolded protein accumulation and neurodegeneration in prion disease

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ABSTRACT. Chronic neurodegenerative diseases, such as prion diseases or Alzheimer's disease, are associated with progressive accumulation of host proteins which misfold and aggregate. Neurodegeneration is restricted to specific neuronal populations which show clear accumulation of misfolded proteins, whilst neighbouring neurons remain unaffected. Such data raise interesting questions about the vulnerability of specific neuronal populations to neurodegeneration and much research has concentrated only on the mechanisms of neurodegeneration in afflicted neuronal populations. An alternative, undervalued and almost completely unstudied question however is how and why neuronal populations are resilient to neurodegeneration. One potential answer is unaffected regions do not accumulate misfolded proteins, thus mechanisms of neurodegeneration do not become activated. In this perspectives, we discuss novel data from our laboratories which demonstrate that misfolded proteins do accumulate in regions of the brain which do not show evidence of neurodegeneration and further evidence that microglial responses may define the severity of neurodegeneration.

KEYWORDS. microglia, neurodegeneration, neurodegenerative disease, prion, protein misfolding

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Nascent proteins need to be specifically folded to gain functional activity and incorrect protein folding can be detrimental potentially resulting in cell death. Mechanisms maintaining regular protein folding and either refold or mark misfolded proteins for degradation are of critical importance for maintaining a healthy organism. This phenomenon is termed proteostasis, which has been extensively reviewed elsewhere.¹⁻³ A number of diseases are associated with the accumulation of misfolded or improperly degraded proteins, indicating that when the mechanisms of regulating proteostasis fail this can lead to severe manifestations of cellular and multi-cellular degradation and death. Over 50 human diseases have been identified to be related to protein misfolding,⁴ which manifest in many different tissues such as the heart, kidney, pancreas, liver and the brain.³⁻⁶

One of the most prevalent and increasingly common categories of protein misfolding disorders are age-related neurodegenerative diseases such as Alzheimer's disease.⁷ It is understood that the accumulation and aggregation of aberrantly folded host proteins in the central nervous system (CNS) is responsible for initiating or inhibiting a cascade of pathways which eventually lead to neuronal degeneration and death. However, the mechanism(s) of neurodegeneration and the exact role that the disease-associated misfolded proteins play remain elusive. The accumulation of misfolded proteins in chronic neurodegenerative diseases suggest a failure of proteostatic mechanisms. Indeed a number of studies have demonstrated deficits of proteostasis associated with neurodegenerative diseases, such as impairments in protein degradation pathways,⁸⁻¹¹ dysregulation or reduction of molecular chaperones¹² and improper or impaired stress response pathway activation.^{13,14}

It has been well documented that misfolded protein aggregates are targeted to specific neuronal populations of the CNS during chronic neurodegenerative diseases and that the distribution of these aggregates appears to spread between neuroanatomical connected regions with neighbouring neuronal populations remaining unaffected.¹⁵⁻¹⁹ These studies have utilised relatively insensitive pathology

techniques to detect the misfolded protein aggregates because no higher sensitive methods have existed for the detection of the misfolded protein isoforms within *in vivo* tissue. What is detected using these methods is almost certainly the end result of a progressive cascade of aberrant protein folding and proteostasis impairment. An exception to this rule is found in prion diseases, where several research groups have independently developed ultrasensitive methods for the detection of misfolded prion protein (PrP).²⁰⁻²² These methods work by using misfolded PrP to act as a "seed" to convert normally folded PrP to a disease-associated isoform. Due to the dynamics of these assays, extremely small quantities of misfolded protein can be used to seed the conversion process – thus in any sample, one can detect quantities of misfolded protein that cannot be detected by other methods. As a result, detection using these methods can identify isoforms of disease-associated protein which accumulate prior to forms characterised by traditional detection methods.²³ Recent advances have also utilised this same method to detect α -synuclein seeds, albeit to lower levels of sensitivity at present.²⁴

A recent study in our laboratory used these sensitive methods to probe the distribution of misfolded PrP, which act as seeds, over a time-course in the brains of mice experimentally infected with a prion disease.²³ In this mouse model of disease, neurons in specific brain regions progressively degenerate and eventually die within a predictable time-course. Importantly, there are brain regions which appear to remain completely unaffected by disease as assessed using a range of pathological markers of disease. Our study compared regions of neurodegeneration to those regions which appear resilient to neurodegeneration. Our hypothesis was that the early progenitors of misfolded PrP, detected using the highly sensitive techniques described above, would be processed and degraded by cells differently between brain regions and thus enable an in depth examination of the molecular events leading to impaired proteostasis and consequent misfolded protein aggregation. However, we observed an accumulation of misfolded PrP in all brain regions

tested using our sensitive techniques, regardless of whether a brain region was resilient or susceptible to neurodegeneration.²³ This data demonstrated that the molecular events of protein misfolding were widespread across the brain.

Further examination during the time-course revealed the initial time point of detection of misfolded PrP was the same in all brain regions tested, demonstrating that the protein misfolding events spread rapidly across the brain, rather than slowly progressing between neuronal populations as previously understood. Importantly, the accumulation across all brain regions tested showed no relationship to the appearance and progression of neurodegeneration. Another consideration was whether the misfolded PrP detected was present at different quantities, and if so, whether the regions exhibiting higher quantities would be correlated with neurodegeneration. We found that the levels of misfolded PrP were approximately equal across all brain regions tested. Taken together, this data showed that neither the time at which the initial seeds for protein misfolding accumulate, nor the quantity that misfolded PrP rises to, could define whether a brain region was susceptible or resilient to neurodegeneration. This data therefore suggests that the accumulation of misfolded PrP, alone, is not sufficient for neurodegeneration and thus raises important questions about the role of misfolded proteins within the mechanism of neurodegeneration.

One potential explanation is conformation and types of aggregate that form between brain regions may differ with some being proportionally more toxic compared to others. A second explanation would be the physiological and functional differences between cells in different brain regions, which contrast in their response to accumulation of misfolded protein aggregates. Taken individually both arguments are convincing, yet fail to fully explain the complexities of disease progression. For example, distinct conformations or aggregates have been extensively characterised in the literature (reviewed²⁵) with a number of different aggregates being associated with neurodegeneration, however it remains unclear how and why certain aggregates are able to form *in vivo*. At a most basic level, protein folding is controlled

by a preference to fold to the most thermodynamically stable state immediately, during and after translation and post-translational modifications. In all cases of chronic neurodegenerative disease associated with protein misfolding, the disease associated protein species is a host encoded protein which in the majority of individuals remain stable throughout life. Thus, for a host encoded protein to result in a 'misfolded' isoform which then aggregates would require a significant change in the thermodynamic state of the local microenvironment that the protein exists within. Therefore we argue the more relevant question is what are the major physiological differences between brain regions which might confer resilience or susceptibility to neurodegeneration?

In our study, for example, we used gross anatomical dissection of four different brain regions each containing an array of neuronal types unique to specific microdomains of the brain, each utilising different neurotransmitters, stress responses, metal ions and have widely varying metabolic activities, amongst many other differences. Each of these differences could, and likely does, contribute to determining selective vulnerability of specific neuronal populations but is a highly complex phenomenon and has been extensively reviewed elsewhere.²⁶ Recent studies, however, have demonstrated and characterised unique features amongst other cell types of various brain regions, such as microglia²⁷ and astrocytes.²⁸ For example, microglia have been demonstrated to exhibit significant differences in gene expression across brain regions of a healthy mouse brain which also alternately change expression during ageing. The major role for microglia in the healthy brain is to constantly evaluate their local microenvironment for insult and injury but also to provide trophic support to neurons. Due to their responsive nature, it is feasible the distinctive microglial profiles are the result of the underlying physiological differences between brain regions founded by the distinct resident neuronal populations, although this has not yet been studied. Thus, it is plausible that the varying glial cell responses could be major contributors to

region-specific vulnerabilities during chronic neurodegeneration.

In instances of disease, microglia respond by expressing a range of genes associated with a well characterised innate immune response.²⁹ Alternatively, astrocytes play an important, yet still poorly defined role in prion-associated immune responses. Astrocytes respond to disease by substantially upregulating their intermediate filamentary protein, GFAP, a marker for astrogliosis. It is unclear what impact this cellular activation has in prion disease mechanisms but there is growing evidence to suggest astrocytes play an important immune role during disease by their communication with microglia. For example, early studies suggest that a co-culture of astrocytes and neurons incubated with prions followed by experimental inoculation into mice had chemotactic properties that inoculation of prions alone, without co-culture, did not exhibit.³⁰ This suggests that astrocyte and/or neuronal signalling are responsible for microglial recruitment and activation to sites of prion infection. Other studies have indicated that the pro-inflammatory associated cytokine, IL-1 β , appears to play an important role in astrogliosis as mice deficient in IL-1 β exhibited attenuated astrogliosis.³¹ Another study showed by microinjection of IL-1 β or TNF α into mice experimentally infected with prion disease that IL-1 β synthesis is more robust but this occurred exclusively in microglia.³² This would indicate a communication between the microglia activated by such cytokines and astrocytes when considering this in the context of reduced astrogliosis in mice deficient in IL-1 β .³¹ Furthermore, the microinjection of IL-1 β or TNF α also demonstrated that astrocytes activate NF κ B which relates to the expression of chemokines in response to cytokine challenge.³² Taken together, the role of the astrocyte in mediating immune reactions appears intrinsically linked with microglial activation, whereby microglial activation may play a role in activating astrogliosis which in turn produces a robust chemokine signal to attract additional populations of microglia to the

areas of prion-associated pathology. One may then envisage this cellular activity as a chronic feedback loop which further propagates the immune response to prion disease.

In our study we examined the cellular responses at the transcriptional level within brain regions which show neurodegeneration and compared these to brain regions which appeared resilient to neurodegeneration, yet both accumulate misfolded proteins.²³ We identified major gene expression changes in all brain regions tested, including those that show no apparent neurodegeneration in the presence of misfolded protein aggregates. Upon further investigation of the most significantly altered profiles identified a set of genes which substantially correlated with previously published datasets of microglial transcriptomic experiments and gene ontology descriptions corresponding to those expected of a cell which controls the innate immune responses within the brain. Therefore we established that the major gene expression changes in all brain regions occur in microglia. Upon further analysis it was apparent that the microglial response was different between brain regions, with at least two distinct profiles of the microglia. Activation of the innate immune response was restricted to regions of neurodegeneration while activation of pathways which can regulate homeostasis was apparent in all brain regions tested. Therefore, we determined that microglia are the primary responsive cell in the brain during neurodegenerative disease but more importantly the specific type of response that microglia elicited corresponded to whether a brain region would succumb to neurodegeneration or remain apparently resilient.

Previous studies have shown changes in disease severity when manipulating the microglial response, for example by either knockout of specific pro- or anti-inflammatory genes,³³ by targeting specific pathways to limit microglial proliferation³⁴ or by microglial ablation.³⁵ When considering this data in the context of a diverse and complex microglial response, it can be argued that the expression profile of microglia is likely having a significant impact on the degree of severity of neurodegeneration between brain regions. In addition, current perspectives of microglia in the normal brain have produced

compelling evidence to indicate that microglia play an important role in regulating neuronal homeostasis, synaptic plasticity and providing trophic support.³⁶ In this context this data therefore would suggest that the upregulation of many genes associated with homeostasis by microglia in brain regions showing protein misfolding but no neurodegeneration, but importantly in the absence of an innate immune response, may be a neuroprotective mechanism. Furthermore, recent studies have demonstrated that specific activated microglial states can induce distinct astrocyte expression profiles and functions.²⁸ Importantly, the differences in induced astrocyte profile appears to have an important role in defining toxicity²⁸ and therefore further understanding the interaction between the different microglial activation states and their interaction with astrocytes could be valuable to aiding our understanding of why some brain regions appear resilient or susceptible to neurodegeneration in the presence of prion seeding activity.

One of the major challenges for drug development in chronic neurodegenerative diseases is firstly what pathological feature should be targeted in order to ameliorate pathogenesis, secondly how to specifically deliver therapy to areas of the central nervous system that are undergoing neurodegeneration and thirdly how to intervene at an early enough stage to deliver an effective therapy. Arguably, the most challenging answer to these is the first point, what pathology to therapeutically target. Neurodegenerative diseases are extremely complex and exhibit a great deal of pathologies, ranging from the protein misfolding, lack of proteostasis, mitochondrial deficits, ER stress, microglial responses, amongst many others. It is therefore unlikely that targeting a single aspect of the pathology will result in therapy that can either prevent clinical onset or offer substantial benefits to patients. Instead, a stronger approach would be to target a combination of pathologies. Thus we need to identify targets that would have the best chance of modifying disease. By their nature, microglia are responsive cells and our data combined with other studies indicates that the microglial response can be

actively manipulated during disease. As a result microglia could represent the most promising disease modifying target as they will be the most impressionable to change. Furthermore, if with future research efforts we can understand the complex microglial response and understand the driving processes behind the activation of the homeostasis response exhibited in brain regions that appear resilient in our study, it is feasible that microglia could not only be prevented from being a contributor to the severity of neurodegeneration, but instead be re-programmed to activate an alternate and potentially neuroprotective response. This may represent the first tangible step in a therapy which can then add new targets acting on other aspects of the neuropathology.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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