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Review

Ageing and protein aggregation-mediated disorders: from invertebrates to mammals

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Late onset is a common hallmark character of numerous disorders including human neurodegenerative maladies such as Huntington's, Parkinson's and Alzheimer's diseases. Why these diseases manifest in aged individuals and why distinct disorders share strikingly similar emergence patterns were until recently unsolved enigmas. During the past decade, invertebrate-based studies indicated that the insulin/IGF signalling pathway (IIS) mechanistically links neurodegenerative-associated toxic protein aggregation and ageing; yet, until recently it was unclear whether this link is conserved from invertebrates to mammals. Recent studies performed in Alzheimer's mouse models indicated that ageing alteration by IIS reduction slows the progression of Alzheimer's-like disease, protects the brain and mitigates the behavioural, pathological and biochemical impairments associated with the disease. Here, we review these novel studies and discuss the potential of ageing alteration as a therapeutic approach for the treatment of late onset neurodegeneration.

Keywords: ageing; proteotoxicity; neurodegeneration; insulin/IGF-1 signalling

1. NEURODEGENERATIVE DISORDERS

Late age onset is the most striking common feature of human neurodegenerative disorders, such as Alzheimer's (AD), Parkinson's (PD), Huntington's (HD) and non-infectious prion diseases. In the cases of AD and PD, the minority of cases manifest as mutation-linked, familial cases during the patient's fifth decade, while most cases appear sporadically not earlier than the seventh decade of life. HD is a familial, monogenic disease that similarly to other familial neurodegenerative disorders onsets during the patient's fifth decade of life [1]. This common occurrence pattern defines ageing as the major risk factor for the development of these maladies [2] and raises the principal question of whether the ageing process plays an active mechanistic role in enabling neurodegeneration to onset late in life. During the past decade, studies based on invertebrate models revealed mechanistic links between ageing and the onset of toxic protein aggregation [3–5].

Aggregation, accumulation and deposition of aberrantly folded proteins are mechanistic unifying features of neurodegenerative disorders [6]. In AD, a dual digestion of the amyloid precursor protein (APP) by the proteases β and γ secretases, releases a subset of highly aggregative peptides, collectively termed amyloid β (Aβ) including Aβ_{1-40} and the highly aggregative Aβ_{1-42}. Aβ aggregation results in neural loss, cognitive impairments and eventually to death [7]. Similarly, the aggregation of α-synuclein underlies the development of PD [8]; the aggregation of mutated huntingtin (Htt), bearing an abnormally long polyglutamine stretch, causes HD [1]; and aggregation of the prion protein (PrP) leads to prion disorders [9]. Clinical observations [10] as well as experimental discoveries [11,12] indicated that not high-molecular weight (high-MW) Aβ fibrils but small oligomers are the major toxic species that are best correlated with neurotoxicity and with AD. Similarly, small PrP aggregates are the most toxic PrP species [13]. By the development of an automated microscope and the expression of fluorescently tagged aggregative mutated Htt, the Finkbeiner laboratory has found that the formation of inclusion bodies containing aggregated Htt promotes neuronal survival [14].

2. THE INSULIN/IGF-1 SIGNALLING PATHWAY REGULATES AGEING IN WORMS AND MAMMALS

At least three independent mechanisms regulate ageing and lifespan; dietary restriction (DR) [15], the mitochondrial electron transport chain [16–18] and the insulin/IGF signalling pathway (IIS) [19]. The IIS is the best studied and exhibits the most prominent effects on the lifespans of worms and flies. In the nematode *Caenorhabditis elegans*, the sole insulin/IGF receptor, DAF-2, initiates a signalling cascade that mediates the phosphorylation of its

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downstream FOXO transcription factor DAF-16. Phosphorylated DAF-16 is prevented from entering the nucleus and from regulating its target genes [20]. Thus, *daf-2* knockdown hyperactivates DAF-16 creating youthful, long-lived worms [21]. Several mouse models indicated that this ageing regulating pathway is conserved in mice. First, a mouse strain harbouring only one copy of the IGF-1 receptor (IGF-1R), the closest *daf-2* orthologue in mammals, exhibited long life and stress resistance [22].The longevity phenotype was more prominent in females but was also observed to a lesser extent in males. Similarly, the abolishment of the insulin receptor in the mouse adipose tissues (FIRKO mice) lead to longevity [23]. Although it is under debate whether the insulin receptor substrate (IRS) 2 is involved in the regulation of lifespan in the mouse [24,25], it was recently shown that mice lacking IRS 1 are long-lived [26].

Recently, several reports indicated correlation between lower IIS activity and extreme human longevity. Mutations in the IGF-1 receptor leading to lower activity of the IGF-1 signalling pathway were found to be more abundant among Jewish Ashkenazi centenarians than in control groups [27]. Similarly, mutations within FOXO3a (the closest *daf-16* mammalian orthologue) were found to be correlated with human longevity of two distinct centenarian groups, Japanese-Hawaiian [28] and German [29]. IRS2 variants were also reported to correlate with longevity in an Italian population [30]. Together these studies strongly suggest that the roles of the IIS as lifespan determinant are conserved in humans.

3. INSULIN/IGF SIGNALLING PATHWAY REDUCTION PROTECTS WORMS FOR TOXIC PROTEIN AGGREGATION

Slowing ageing by IIS reduction in worms expressing aggregative, disease-linked proteins, indicated that in these models, the IIS directly exposes the aged organism to the toxicity associated with protein aggregation (reviewed in Balch *et al.* [31]). Morley *et al.* [5] showed that IIS reduction by *age-1* RNAi or mutations mitigates the toxic effects of fluorescently tagged polyQ aggregates in the body wall muscles of worms as measured by impaired motility. The protective effect towards polyQ aggregation was found to be dependent on the heat shock factor 1 (HSF-1), a key player in the longevity effect of reduced IIS [4,32]. We reported previously that IIS reduction protects worms from the toxic effects of Aβ aggregation in DAF-16- and HSF-1-dependent manners. These transcription factors were found to mediate opposing activities: HSF-1 promotes disaggregation while DAF-16 facilitates protective active aggregation [3]. This protective effect was apparent in the worm model even when the alteration of IIS was applied late in life [33]. Together these studies point to IIS reduction as a promising novel approach for the development of counter-neurodegenerative therapy. However, rodent-based studies revealed that IGF-1 infusion protects from Aβ-mediated toxicity [34] and that blocking the IGF-1R exacerbates AD-like symptoms in model mice and results in neurodegeneration [35]. These studies, which apparently contradict the findings obtained from the worm systems, raised the prospect that IIS induction might protect from proteotoxicity [36]. Thus, it was critically required to evaluate whether IIS reduction delays the onset of the behavioural, pathological and biochemical impairments apparent in mammalian neurodegeneration models or whether IIS reduction accelerates neurodegeneration in mice.

4. THE COUNTER-PROTEOTOXIC EFFECT OF INSULIN/IGF SIGNALLING PATHWAY REDUCTION IS CONSERVED FROM WORMS TO MAMMALS

Three independent research groups have recently addressed these questions by crossing AD model mouse strains with animals that exhibit reduced IIS. Killick *et al.* [37] used transgenic mice that harbour mutated human APP gene carrying the Swedish mutation (K670N, M671L) under the PrP promoter (*Tg2576* mice [38]). These animals were created on the C57Bl/6 background and typically develop observable Aβ plaques in the brain at seven to eight month of age. *Tg2576* mice were crossed with C57Bl/6 mice that lack the insulin receptor substrate 2 (*Irs2*−/−) to obtain AD animals that lack the IRS2 gene (*Tg2576*/*Irs2*−/−). Comparison of 12 month old *Tg2576*/*Irs2*−/− and their age-matched *Tg2576* counterparts revealed that IIS reduction by *Irs2* deletion resulted in significant reduction in Aβ plaque burden in the brain. Furthermore, the plaques seen in brains of *Tg2576*/*Irs2*−/− animals were smaller than those of *Tg2576* controls. Interestingly, no differences in soluble and metabolized Aβ fractions were observed among the different mouse genotypes but *Tg2576*/*Irs2*−/− had significantly less aggregative Aβ, suggesting that the deletion of *Irs2* enhances Aβ clearance capabilities. Surprisingly, elevated phosphorylation rates of the microtubule associated protein TAU, a hallmark of AD [39], were detected in brains of *Tg2576*/*Irs2*−/− animals. Apparently, the elevated TAU phosphorylation resulted predominantly from the abolishment of IRS-2 and not the aggregation of Aβ. Next the researchers employed the fear conditioning test to measure whether *Irs2* knockout mice were rescued from learning and memory deficits associated with *Tg2576* animals [40] and found that *Tg2576*/*Irs2*−/− mice exhibit significantly improved performance when compared with the *Tg2576* controls. This study indicated that IIS reduction by the abolishment of IRS-2 confers protection from neurotoxicity associated with the aggregation of Aβ in the brain. Since IRS-2 null mice are short-lived [25,26], this study indicates that longevity and the counter-proteotoxic functions of IIS reduction can be uncoupled.

In a separate study, Freude *et al.* [41] adopted a similar approach and crossed *Tg2576* mice (on the C57Bl/6 background) with either: *Irs2*−/− mice (these animals had the same genetic design as those studied by Killick *et al.* [37], yet the *Irs2*−/− mice used in the two studies were created by different laboratories), mice which lack the IGF-1 receptor in neurons (*nIGF-1R*−/−), or mice lacking the

insulin receptor in their neurons (*nIR*−/−). The rationale behind these crosses was based on two observations: (i) the levels of IRS-2, IGF-1 receptor and of the insulin receptor have been reported to be abnormally low in brains of AD patients [42] and (ii) reduced levels of these molecules have been linked to longevity [22,23,43]. The abolishment of IRS-2 rescued *Tg2576*/*Irs2*−/− females, but not males, from premature mortality typical of *Tg2576* animals (it has been proposed that *Tg2576*/*Irs2*−/− males exhibited short lifespan owing to hyperglycemia) [41]. Although APP levels were comparable in 12 week old *Tg2576* and aged matched *Tg2576*/*Irs2*−/− counterparts, Western blot and ELISA analyses revealed significantly less $A\beta_{1-40}$ in the latter mouse group. By contrast, no differences in $A\beta_{1-40}$ and $A\beta_{1-42}$ levels could be detected in older animals (48 weeks of age). To explore the mechanism underlying the reduced Aβ levels observed in young *Tg2576*/*Irs2*−/− mice, the researchers measured the amounts of BACE-1 (the β secretase) and its activity levels in 12 week old *Tg2576* and *Tg2576*/*Irs2*−/− mice and found no significant differences. Yet the amounts of the βC terminal fragment (βCTF) were lower in *Tg2576*/*Irs2*−/− when compared with *Tg2576* animals suggesting an enhanced Aβ clearance capability in the absence of IRS-2 [41]. This result is correlated with the finding that the insulin degrading enzyme (IDE), a protease known to digest Aβ [44] is more abundant in the membrane fractions of *Tg2576*/*Irs2*−/− compared with their *Tg2576* counterparts [37].

To specifically investigate the roles of IGF-1 signalling in the brain, the researchers used the *Tg2576*/*nIGF-1R*−/− mouse strains. These animals exhibited reduced IGF-1R levels in the hippocampus but not in other brain structures or in peripheral organs. Lifespan experiments indicated that both *Tg2576*/*nIGF-1R*−/− males and females were protected from the premature death typical of *Tg2576* mice. Unlike old *Tg2576*/*Irs2*−/− (60 weeks of age) mice, *Tg2576*/*nIGF-1R*−/− animals had lower amounts of $A\beta_{1-40}$ and $A\beta_{1-42}$ when compared with their age matched *Tg2576* counterparts, showing that the abolishment of IGF-1R in the hippocampus has a more prominent counter-proteotoxic effect than organismal IRS-2 elimination. AD mice harbouring only one *Igf-1R* copy in neurons (*Tg2576*/*nIGF-1R*+/−) and those lacking the insulin receptor in their neurons (*Tg2576*/*nIR*+/−) showed no effect on survival when compared with *Tg2576* animals [41].

Both studies described above clearly indicated that IIS reduction by either the abolition of IRS-2 or by neuron-specific elimination of the IGF-1 receptor protected mice from toxicity associated with the aggregation of the human Aβ peptide. Yet the key questions of what is the protective mechanism and whether there are effects on a variety of AD-associated brain pathologies remained unanswered. To address whether the counter-proteotoxic effects of IIS reduction which we discovered in the worm system [3] are conserved in mice and in order to explore the underlying protective mechanism, we crossed AD-model mice with a well-established long-lived mouse strain that harbours only one copy of the gene encoding the *Igf-1* receptor to create long-lived AD animals. We selected an AD model strain that expresses two mutated AD-linked genes, humanized APP_{swe} (mouse APP that was mutated to harbour the human Aβ sequence) and human presenilin1 (PS1) lacking exon 9 (the regulatory exon of PS1). Both transgenes were driven by the mouse PrP promoter (APP_{swe}/P1ΔE9 mice) [45]. These mice develop relatively slow age-dependent neurodegenerative symptoms similarly to AD patients, including behavioural impairments [46], neuro-inflammation and Aβ plaque formation [45]. To achieve reduced IGF-1 signalling, we selected the long-lived, stress resistant *Igf1R*+/− mouse strain [22]. First, we equalized the genetic background of our mice by backcrossing both mouse strains with 129Xi females; next we crossed the strains to obtain APP_{swe}/*P1ΔE9* with reduced IGF-1 signalling (APP_{swe}/*P1ΔE9*/*Igf1R*+/−). The behavioural performances of 12–13 month old APP_{swe}/*P1ΔE9*/*Igf1R*+/− were significantly better than those of their age-matched APP_{swe}/*P1ΔE9* counterparts in a battery of behavioural assays, indicating that IGF-1 signalling reduction largely protected the mice from AD-associated memory and orientation impairments. Consistently, the rates of neuro-inflammation, neuronal and synaptic losses were all mitigated by IGF-1 signalling reduction in brains of aged mice (12–13 month of age) [47]. The total Aβ amounts as well as the levels of APP processing enzymes were indistinguishable in APP_{swe}/*P1ΔE9* and APP_{swe}/*P1ΔE9*/*Igf1R*+/− mice.

We found that Aβ plaques were smaller in size and of higher density in AD-model mice with reduced IGF-1 signalling when compared with their littermates which had natural IGF-1 signalling. To further establish this observation we employed post-embedding electron microscopy analysis and developed an automated algorithm designed to identify Aβ plaques by locating gold particles conjugated to Aβ antibodies, defining an area of interest around them and measuring their densities. This algorithm based assay, with *in vitro* and biochemical assays, confirmed that reduction of IGF-1 signalling led to the compaction of Aβ plaques in brains of APP_{swe}/*P1ΔE9*/*Igf1R*+/− mice. This compaction probably protects the brain by sequestering highly toxic Aβ oligomers [47]. This protective mechanism is common to Aβ worms whose IIS was reduced [3] and to the APP_{swe}/*P1ΔE9*/*Igf1R*+/− mice [47].

5. THE THERAPEUTIC POTENTIAL OF INSULIN/IGF SIGNALLING PATHWAY REDUCTION FOR NEURODEGENERATION

The three recent studies reviewed here adopted similar approaches, found similar findings (summarized in table 1) and reached consistent conclusions that IIS reduction protects AD-model mice from Aβ aggregation-associated proteotoxicity. These recent developments in the field and the discoveries that (i) Aβ quantities in brains of protected animals with reduced IIS were similar to those found in brains of their unprotected counterparts and (ii) IIS reduction led to the formation of Aβ plaques of higher densities, strongly support the theme that Aβ

Table 1. Comparison of AD-like associated parameters in the different mouse models.

	Tg2576/Irs2−/−[a] [37]	*Tg2576/Irs2−/−*[a] [41]	*Tg2576/nIGF-1R−/−*[a] [41]	APP_{swe}/*P1ΔE9/Igf1R* +/−[a] [3,33,47]
Aβ-associated premature death	ND	rescued (females)	rescued	ND
APP levels	UC	UC	ND	UC
soluble Aβ levels	UC	UC (48 month)	reduced (HC)	UC
number of Aβ plaques	reduced	ND	ND	UC
area covered by Aβ plaques	reduced	ND	ND	reduced
Aβ plaques density	ND	ND	ND	increased
βCTF levels[b]	UC	reduced	reduced (in HC)	UC
memory capabilities	increased	ND	ND	increased
orientation	ND	ND	ND	increased
neuro-inflammation	ND	ND	ND	reduced
neuronal density	ND	ND	ND	increased
BACE 1 level (β sec)[b]	ND	UC	ND	UC
ADAM17 level (α sec)	ND	ND	ND	UC
GSK-3 phosphorylation[b]	UC	UC	UC	ND

[a]For each mouse strain 'Increased' or 'reduced' indicates change of levels observed in AD mice with impaired IIS compared to littermates with natural IIS. ND, not detected; UC, unchanged; HC, Hippocampus.
[b]CTF, C terminal fragment; BACE, beta amyloid cleaving enzyme; GSK3, glycogen synthase kinase 3.

oligomers are the major toxic species that are correlated with AD. Accordingly, in mammals, hyper-aggregation probably serves as a protective mechanism that is negatively regulated by the ageing process. Thus, ageing exposes the organism to the toxicity of oligomers and enables neurodegenerative disorders to manifest late in life.

Together, the protection towards AD-like disorders in mice and the findings that the regulation of ageing by the IIS is conserved in humans point to the promising therapeutic potential of IIS reduction for the treatment of a variety of neurodegenerative disorders. IGF signalling inhibitors might be able to serve as novel, powerful drugs aimed at preventing the onset of neurodegenerative maladies and slowing their progression once emerged.

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