Research Proposal

Bridging the gap:
From aggregate-prone proteins and cellular dysfunction to neuronal cell death

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Date:
26 June 2015

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Abstract

A proper treatment for Alzheimer's disease (AD) is the largest unmet medical need in neurology. Despite the extend of Alzheimer research, the actual mechanisms that lead to neuronal death in AD are not yet identified. Up to now, research has mainly focussed on the characteristic appearance of β-amyloid plaques and neurofibrillary tangles. However, there is still a lack of unequivocal evidence that these misfolded and aggregate-prone proteins contribute to toxicity, cellular dysfunction and ultimate neuronal death. Emerging data point to the importance of other pathways (e.g. autophagy) and organelles (e.g. mitochondria) that may contribute to neuronal cell death in late-onset neurodegenerative diseases such as AD. Understanding the mechanisms underlying cell death in AD may contribute to the development of new therapeutics to ameliorate disease progression, since the clinical manifestation of AD correlates with the degree of neuronal loss in the brain. Moreover, by unravelling the pathways that lead to neuronal death, we may get an idea why specific cell populations are more sensitive to the effects of the disease than others. This may eventually be of value for other neurodegenerative diseases, including Parkinson's disease and Huntington's disease. We will use cutting edge genetic techniques to generate 'humanized' fish (*N.furzeri)* and worms (*C.elegans)* that carry a knock-in of human genes associated with AD (PSEN1/2 and APP), either in their normal form or with disease related mutations. Moreover, we will develop reporter fish and worm lines that express fluorescently tagged markers for cell death. After cellular and behavioural characterisation of these highly novel mutants and genetic crosses of the reporter lines with the disease-mimicking lines, unbiased forward genetic screens will be performed to find those genes that contribute to the increased neuronal cell death in AD.

Key words: Nothobranchius furzeri, Caenorhabditis elegans, neurodegeneration, ageing, Alzheimer's disease

a. Research Topic

I. Cell death as characteristic of neurodegenerative diseases

The hallmarks of many late-onset neurodegenerative diseases are the accumulation and aggregation of disease-specific, aggregate-prone proteins and neuronal cell death (fig. 1). Examples of neurodegenerative diseases with these characteristics, commonly referred to as proteinopathies, include but are not limited to Alzheimer's disease (AD), Huntington's disease (HD) and Parkinson's disease (PD) (Ross et al., 2004; Forman et al., 2004). The loss of brain cells, i.e. neurodegeneration, leads in many cases to severe cognitive impairments and ultimately death (Niikura et al., 2006). *Despite ongoing research, very little is understood about the processes and mechanisms that underlie the death of brain cells in neurodegenerative diseases.* Moreover, the problem with current models for neurodegenerative diseases is that they do show some pathological features but usually do not show cell death as observed in human (Citron, 2010).

A proper treatment for AD is the largest unmet medical need in neurology. Although current drugs alleviate some of the symptoms, they have no profound effect on the disease progression itself (Morris et al., 2014). Up to now, research has mainly focussed on the two striking hallmarks of the disease, namely: the mostly extracellular deposits of the β-amyloid peptide, called plaques, and the neurofibrillary tangles of the microtubule binding protein tau (Ittner et al., 2011; Morris et al., 2014). Especially the β-amyloid deposits have been of major interest as familiar forms of AD are caused by mutations in the amyloid precursor protein (APP), presenilin 1 and 2 (PSEN1 and PSEN2) (Selkoe, 2001). All genes are involved in the processing of APP and thus the generation of the β-amyloid peptide. Indeed, the amyloid hypothesis is one of the most accepted ones so far. β-amyloid, in various forms, is thought to trigger a cascade that hampers neuronal function, eventually leading to secondary pathology including the formation of neurofibrillary tangles, synapse loss and cell death (Morris et al., 2014).

Even though the amyloid hypothesis has been widely considered, there is still a lack of unequivocal evidence proving that either β-amyloid oligomers or deposits are toxic *in vivo* and thus actually cause cell death (Lee et al., 2007; Morris et al, 2014). Strikingly, many anti-amyloid treatments, extensively tested in model organisms, have broadly failed to meet their clinical endpoints and sometimes even exacerbated symptoms (Palop et al., 2006; Morris et al., 2014). Emerging data show that aberrations in several cell processes precede the actual formation of extracellular plaques and may contribute to the neuronal cell death observed in AD. Especially mitochondrial dysfunction and aberrations in the autophagy pathway, that acts as the intracellular recycling system of the cell, may directly contribute to neuronal cell death (Martin, 2012; Nixon et al., 2012). Mitochondria are the energy factories of the cell but also play a critical role in the initiation of programmed cell death (Martin, 2012). At the same time a specialized form of autophagy, called mitophagy, is responsible for removing damaged mitochondria and thereby protecting the cell against mitochondria-induced cell death (Arena et al., 2013). Both the mitochondria and the autophagy pathway are described to be affected in AD (Hirari et al., 2001; Nixon et al., 2005; Boland et al., 2008). Nonetheless, there may be multiple unexplored genes and pathways that contribute to cell death in neurodegenerative diseases.

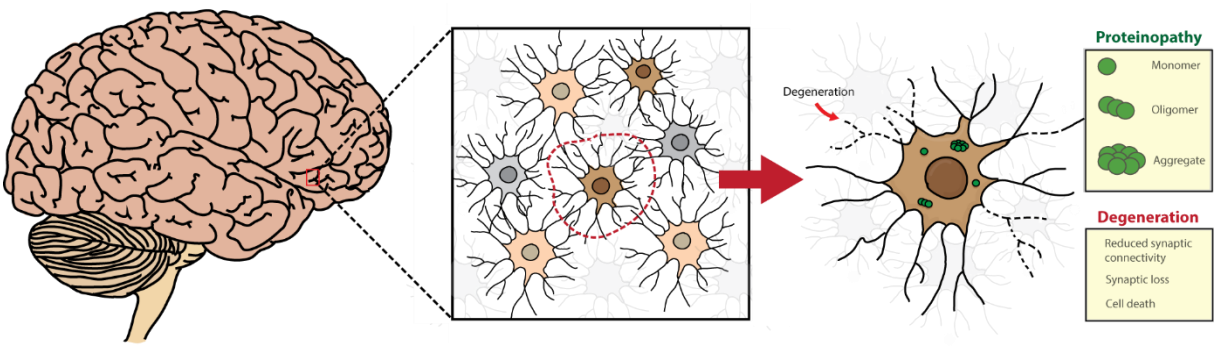

Figure 1. Protein aggregates are a characteristic shared by late-onset neurodegenerative diseases
Proteinopathies are characterised by the presence of misfolded, aggregation-prone proteins in different forms: monomers, oligomers and aggregates. The current view suggest that the accumulation of aggregation-prone proteins mediate toxicity via a gain-of-function mechanism, either directly or indirectly contributing to reduced synaptic connectivity, synaptic loss and eventually neuronal cell death.

II. Nothobranchius furzeri as novel model organism to study AD.

In this research project we will start making use of a novel vertebrate model, the African killifish *Nothobranchius furzeri*. Preliminary findings in *N. furzeri* shows that the fish undergoes an ageing process comparable to that observed in human (Cellerino et al., 2015). An age-dependent deterioration in several cellular and molecular functions has been observed, including disrupted circadian rhythmicity, cognitive impairments, neuronal cell death, mitochondrial impairments and telomere shortening (as described in: Cellerino et al., 2015). Especially the loss of neuronal cells during ageing seems to be a promising finding when considering to use the killifish for studying neuronal cell death in neurodegenerative diseases. Furthermore, *N. furzeri* has an average lifespan around ~3 months, making it the vertebrate species with the shortest lifespan recorded in captivity and a very useful organism for ageing studies. In addition, next to the killifish we will make use of *Caenorhabditis elegans*, a transparent nematode with extreme power when considering genetic manipulations.

We will make for the first time ever 'humanized' fish and worms by a knock-in of the human Alzheimer genes (APP, PSEN1 and PSEN2) with either a disease-related mutation or in its normal form. We will use these highly novel transgenic organisms to screen for disease phenotypes at both the behavioural and cellular level in order to confirm similarities with the human disease symptoms (e.g. impaired cognition). In turn, we will use these new models to perform an unbiased forward genetic screen to look for potential genetic hits that may contribute to neuronal cell death in AD. Finally, we will track these genes down in terms of underlying mechanisms.

In summary, this project proposes to use the powerful genetics of C. elegans and N. furzeri to explore novel genetic targets that may contribute to cell death in AD. By performing unbiased forward genetic screens, we may touch upon novel pathways that either protect or makes neurons more vulnerable to programmed cell death. It is of note that even when N. furzeri does not show AD-related phenotypes, the fish can still be used to examine those genes contributing to age-related neurodegeneration, as this age-related cell death has already been observed in the killifish

Aims:

1. Make an Alzheimer disease model for *N. furzeri* and *C. elegans*. CRISPR knock in of human APP and PSEN1/2 with and without common disease mutations.
2. Screen the highly novel model organisms for diseases phenotypes: both at the behavioural and cellular level
3. Identify an expression marker for neuronal cell death in neurodegeneration with RNA sequencing during normal aging (for *N.* furzeri) and make fluorescently-tagged lines (reporter lines) with this marker.
4. Full genome library genetic screens of our humanized worms crossed to the reporter lines to find genes functionally involved in neuronal death. RNA sequencing in *N. furzeri.*
5. Explore the contribution (mechanisms) of these 'hits' in the progression and pathology of AD: especially its role in neuronal death

Topic Significance:

Despite the extend of Alzheimer research, the actual mechanisms that lead to neuronal death in AD are not yet identified. This observation is underlined by a recent request for applications by a global funding collaboration of the Alzheimer's association, Alzheimer's Research UK and the Weston Brain institute. First of all, since the clinical manifestation of AD is considered to correlate with the degree of neuronal loss in the brain (Niikura et al., 2006), understanding the mechanisms underlying this type of cell death may contribute to the development of new therapeutics to ameliorate disease progression. Secondly, by developing a novel fish model for AD that potentially shows neuronal cell death as observed in human, we will get a step closer to mimic AD which may eventually contribute to a better understanding of the disease. Finally, neuronal cell death and the accumulation of mutant proteins are shared characteristics of proteinopathies (Ross et al., 2004; Forman et al., 2004). This potential unifying theory of pathogenesis and disease progression in late-onset neurodegenerative diseases provides an important avenue for developing pharmacological interventions. Therefore, finding mechanisms leading to

neuronal cell death in AD, may also provide useful knowledge for other proteinopathies. In fact, by unravelling the pathways that lead to neuronal death, we may get an idea why specific cell populations are more sensitive to the effects of the disease than others.

b. Approach

Aim 1: Making Alzheimer disease models

We will make use of the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system to generate a highly targeted knock-in of the human mutant or 'normal' APP, PSEN1 or PSEN2 genes in both N. *furzeri* and *C.elegans* (Friedland et al., 2013; Cellerino et al., 2015).

Aim 2: Screening for disease phenotypes

We will screen for common phenotypes in Alzheimer's disease at the behavioural and cellular levels. For behavioural phenotyping there is an existing set-up in Cologne in the group of Dario Valenzano, with whom we will collaborate. We will look for cognitive impairments with e.g. a modified shuttle-box experiment, in which the fish associates a light stimulus with a mechanical disturbance and learns to avoid the negative stimulus by crossing a hurdle upon light onset (Valenzano et al., 2006). We will look for, amongst others, cellular markers as lipofuscin (senescence marker), hyperphosphorylated tau and accumulation of amyloid beta with western blotting and immunohistochemistry. For *C. elegans*, we will screen phenotypes that are associated with the neurons that normally express *apl-1* ans *sel-1*, the worm orthologs of human APP and PSEN In addition, we will analyse behaviour in an unbiased manner, using automated worm trackers.

Aim 3: Identifying an expression marker for neuronal death

We will make use of time course RNA sequencing to find those genes that coincides with increased neuronal death and will validate these markers with in situ hybridization. Upon finding suitable markers, we will make stable fish and worm lines that express the markers tagged with a fluorophore. Multiple markers for programmed cell death are already described in *C.elegans* and those include: *ced-3, ced-4* and *egl-1* (Yuan et al., 1990). We will cross our 'humanized' worms and fish with the reporter lines to see whether (and at which age) mutations in the APP, PSEN1 or PSEN2 genes cause increased neuronal death (If not: we will explore those mechanisms leading to age-related cell death in killifish, as previously described).

Aim 4: Full genome library genetic screens

We will use full genome library RNAi screens in *C.elegans* (disease models crossed to reporter lines) to find those genes functionally involved in neuronal cell death. In addition, we will use RNA sequencing to compare the transcriptomes between the killifish that either have a 'normal' or mutant knock-in of one of the Alzheimer genes. Potential hits (i.e. upregulated or downregulated genes) will be clustered and used for further analysis. Importantly, to narrow down the hits we will do these screens at multiple time points: before neuronal death is observed with the reporter lines (aim 3) and during the phase of neuronal death.

Aim 5: Explore the contribution of the hits to neuronal cell death

After an intensive literature study, we will establish whether the function of the found hits relates to known protein quality control systems and stress responses (genetic epistasis, stress protein induction, proteasomal degradation, autophagy) or whether they act via novel pathways. Finally, we will investigate how the genetic hits contribute to cell death, i.e. which underlying pathways are involved, and whether rescuing expression of these hits affect – ameliorates or exacerbates - cognitive impairments.

C. Innovative potential:

This work is innovative in multiple ways. First of all, we will for the first time make 'humanized' animal models for AD. Recent developments in CRISPR technology will allow us for to make single copy replacements of the endogenous *C.elegans* and *N.furzeri* orthologues of APP and PSEN1/2, which is expected to closely resemble the human disease conditions. Secondly, the use of *N.furzeri* for genetic research of neurodegeneration is new and has a high potential for success, given that neurodegeneration occurs as part of the normal and rapid aging of these fish. The potential to genetically modify *N.furzeri* in combination with their short lifespan, will allow us to

identify genes that modify the earliest events that trigger degeneration of neurons. This has not been possible before with other models. Finally, the study will yield new models, reporter strains, and analysis tools that open up new avenues for research on mechanisms, identification of therapeutic targets and screens for therapeutic compounds that interfere with the early toxic events in AD.

D. Work plan

	Year 1				Year 2				Year 3			
Quarters	1	2	3	4	1	2	3	4	1	2	3	4
Literature search	x							x	x			
Aim 1: *C.elegans* AD model		x	x									
Aim 1: *N.furzeri* AD model		x	x	x	x							
Aim 2: Screening for disease phenotypes				x	x	x	x					
Aim 3: Neuronal death markers / RNA profiling		x	x	x	x							
Aim 4: Forward genetic screens					x	x	x	x	x			
Aim 5: Explore the contribution to neuronal cell death									x	x	x	x

A DEC application will be required for work with *N.furzeri.* Dario Valenzano, which whom we will collaborate, is experienced in this and will provide help before the start of the project.

Publication topic suggestions:

I. Behavioural and cellular characterisation of 'humanized' *C.elegans* and *N.furzeri* as model for Alzheimer's disease
II. Markers for neuronal cell death in *C.elegans* and *N.furzeri*
III. Forward genetic screen: genes involved in neuronal cell death in *N.furzeri*
IV. Forward genetic screen (II): genes in AD that affect neuronal cell death in *N.furzeri* and *C.elegans.* Including mechanism underlying the effects.

Collaborators:

1) Prof. dr. D.R. Valenzano (dario.valenzano@age.mpg.de)
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Depending on the outcomes of the screens:

2) Prof. dr. D.C. Rubinsztein (dcr100@cam.ac.uk)
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Word count: 1984

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