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# Prion-like seeding and nucleation of intracellular amyloid-β

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ABSTRACT

Alzheimer's disease (AD) brain tissue can act as a seed to accelerate aggregation of amyloid-β (Aβ) into plaques in AD transgenic mice. Aβ seeds have been hypothesized to accelerate plaque formation in a prion-like manner of templated seeding and intercellular propagation. However, the structure(s) and location(s) of the Aβ seeds remain unknown. Moreover, in contrast to tau and α-synuclein, an *in vitro* system with prion-like Aβ has not been reported. Here we treat human APP expressing N2a cells with AD transgenic mouse brain extracts to induce inclusions of Aβ in a subset of cells. We isolate cells with induced Aβ inclusions and using immunocytochemistry, western blot and infrared spectroscopy show that these cells produce oligomeric Aβ over multiple replicative generations. Further, we demonstrate that cell lysates of clones with induced oligomeric Aβ can induce aggregation in previously untreated N2a APP cells. These data strengthen the case that Aβ acts as a prion-like protein, demonstrate that Aβ seeds can be intracellular oligomers and for the first time provide a cellular model of nucleated seeding of Aβ.

## 1. Introduction

Protein aggregation is a pathological hallmark of many neurodegenerative disorders such as amyloid-β (Aβ) plaques and tau tangles in Alzheimer's disease (AD) and α-synuclein containing Lewy bodies in Parkinson's disease (PD). However, how these proteins aggregate and spread throughout the brain remain poorly understood. A hypothesis that has been gaining traction the last decade is that these disease-linked proteins have prion-like properties. Prions are potentially infectious proteins that are capable of misfolding and aggregating (forming amyloid), inducing homologous proteins to misfold and, crucially, can spread and induce misfolding throughout the brain and even between organisms. There is evidence that α-synuclein pathology might spread from host to graft in PD patients who received embryonic stem cell grafts (Kordower et al., 2008; Li et al., 2008) and between cells in culture (Hansen et al., 2011). Moreover, treatment with fibrillar α-synuclein can seed intracellular inclusions in α-synuclein expressing cells (Luk et al., 2009) and intracerebral injection of pathological α-synuclein into α-synuclein expressing mice accelerated formation of Lewy bodies and neurites (Luk et al., 2012). Tau has also been shown to spread between cells in culture (Holmes et al., 2013). Intracellular tau inclusions can be formed after addition of fibrillar tau to tau-fragment expressing HEK-293 cells, and injecting these cells into the brains of transgenic tau mice induced tau pathology (Sanders et al., 2014). For Aβ, studies have shown that intracerebral injections of AD brain material in familial AD (FAD) transgenic mice accelerate amyloid pathology and that it is specifically Aβ that causes this as immuno-depletion of Aβ abolishes seeding activity (Kane et al., 2000; Meyer-Luehmann et al., 2006). Remarkably, as little as a femtogram of PBS soluble AD brain derived Aβ can seed pathology in a FAD mouse (Fritschi et al., 2014). In contrast, much larger amounts of synthetic Aβ either fail to seed plaques (Meyer-Luehmann et al., 2006) or require nitration or 72 h of agitation of the synthetic Aβ to augment pathology (Kummer et al., 2011; Stöhr et al., 2012). It appears that only particular form(s) of Aβ that is (are) present in AD but not normal brains are capable of seeding pathology, albeit in remarkably low quantities. On the basis of these findings it has been argued that Aβ is a prion-like protein (Frost and Diamond, 2010; Jucker and Walker, 2011; Morales et al., 2015). Understanding where "prion-like" Aβ can form and its structure would then be important for understanding the pathogenesis of AD.

While there is much *in vivo* work on prion-like Aβ, it has not been shown that one can induce inclusions of Aβ in cultured cells as has been shown for tau and α-synuclein. One reason is practical; Aβ is a low molecular weight metabolite cleaved from within the larger amyloid precursor protein (APP) and it is therefore less feasible to construct a cell line expressing physiologically generated, fluorescently labeled Aβ. This makes it difficult to study Aβ inclusions and transfer in cells. There

*Abbreviations:* FTIR, Fourier transform infrared spectroscopy; BN-PAGE, Blue native polyacrylamide gel electrophoresis; SDD-PAGE, Semi-denaturing detergent polyacrylamide gel electrophoresis

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is also a theoretical reason; as Aβ plaques are extracellular, intracellular Aβ has been viewed as less relevant. However, intraneuronal Aβ42 accumulation is seen before plaques in the brain areas first affected by AD and appears to be among the earliest changes in AD (Gouras et al., 2000) and is associated with synaptic pathology (Takahashi et al., 2002). Accumulation of intraneuronal Aβ coincides with cognitive symptoms and occurs before plaques in the 3xTg AD mouse (Billings et al., 2005). Recently it was also reported that FAD mutations in presenilin 2 specifically increase the intracellular pool of Aβ42 (Sannerud et al., 2016). Furthermore, if conversion of monomeric to aggregated/prion-like Aβ is a stochastic process (Goedert, 2015), one would expect the biological conversion to happen where concentrations are high as they are in subcellular compartments (Hu et al., 2009); the acidic environment of the late endosome/lysosome also favors Aβ aggregation (Burdick et al., 1992). Thus, if Aβ has prion-like properties, one might expect intracellular prion-like conversion as is the case for prion protein and other prion-like proteins (Aguzzi and Lakkaraju, 2016; Luk et al., 2009; Sanders et al., 2014).

In this study we induce human APP expressing N2a neuroblastoma cell lines to stably form intracellular inclusions of Aβ by treating them with AD transgenic mouse brain extracts. We characterize these cell lines biochemically and with infrared (IR) absorbance spectroscopy and conclude that the inclusions are oligomers. We show that lysates of the inclusion bearing cells, a purely cellular source of Aβ, can be used to induce naive APP-expressing cell lines to also form inclusions. In summary, we provide a cellular model of seeded nucleation of Aβ, where the inclusion forming Aβ can be propagated both vertically (the phenotype is stable over time) and horizontally (naive cells can be infected). These data are consistent with prion-like conversion of intracellular Aβ.

## 2. Materials and methods

### 2.1. Preparation of mouse brain material

The forebrains of 21-month-old APP/PS1 (The Jackson laboratory, B6C3-Tg(APPswe,PSEN1dE9)85Dbo/Mmjax), Tg19959 harboring the Swedish and London mutations in APP (Li et al., 2004) or wild type (WT) mice were collected and immediately frozen on dry ice and stored at −80 °C. The forebrains were homogenized in 10% weight/volume sterile PBS, sonicated 3 times for 5 s at 80% amplitude with a Branson SLPe model 4C15 sonifier and centrifuged at 3000*g* for 5 min. The resulting supernatant was sonicated 3 times for 20 s each at 80% amplitude as described in Langer et al., 2011 (Langer et al., 2011). The supernatant was then aliquoted and kept at −80 °C until use.

### 2.2. Cell culture

N2a cells were grown in media containing 47% high glucose DMEM, 47% Optimem, 5% FBS and 1% penicillin/streptomycin at 37 °C in a humid 5% $CO_2$ incubator. Single cell cloning was done *via* serial dilution in 96 well plates.

### 2.3. Cell pellet collection

Cells were washed twice on ice with ice-cold PBS and collected with a cell scraper. Cells were then pelleted at 10,600*g* for 2 min at 4 °C, snap-frozen in liquid nitrogen and stored at −80 °C.

### 2.4. Treatment with brain supernatant

N2a cells expressing human APP with the Swedish mutation (APPswe) were passaged and the following day treated with brain supernatant at a concentration of 0.5% from either aged APP/PS1 or WT mice in Optimem with 1% penicillin/streptomycin and 0.5% FBS; low serum media was used to inhibit cell growth. The supernatant was kept on the cells for 4 days at a time and then passaged and treated again or single cell cloned to isolate cells with aggregates.

### 2.5. Immunocytochemistry

Cells were grown on glass coverslips (VWR 12 mm microscope cover glasses). Cells were washed 3 times on ice with ice cold PBS, fixed with cold 4% PFA in PBS for 15 min at room temperature (RT) and then washed 3 times with PBS at RT. Blocking, to reduce unspecific antibody binding, was done with 2% NGS, 1% BSA and 0.1% saponin in PBS for 1 h. Cells were stained with primary Aβ/C99 N-terminus-specific antibody 82E1 (IBL) at 1:200 and fibrillar oligomer-specific Aβ antibody OC (Millipore) 1:1000 in PBS 2% NGS overnight at 4 °C. Cells were then washed with PBS-T for 5 min, 15 min and 15 min. Then incubated with secondary antibodies at 1:500; AF488 anti-mouse and Cy3 anti-rabbit (Jackson ImmunoResearch) for 1 h at RT in the dark. Cells were again washed with PBS-T 3 times for 5 min each and during the second wash 0.1% DAPI was added. Coverslips were then mounted on glass slides with slow fade gold anti-fade reagent (Life Technologies) dried in the dark overnight and then sealed with Covergrip Coverslips Sealant (Biotum).

### 2.6. Confocal microscopy

Images were obtained using a Leica TCS SP8 confocal microscope (Leica Microsystems) equipped with Diode 405/405 nm and Argon (405, 488, 552, 638 nm) lasers with an HP PL APO 63x/NA1.2 water immersion objective. Autoquant (MediaCybernetics) was used for image deconvolution. Two-dimensional images obtained by confocal microscopy were reconstructed using Imaris (Bitplane).

### 2.7. Blue native (BN) PAGE

N2a cell pellets were triturated and incubated on ice for 30 min in a buffer solution of 20 mM Tris, 50 mM NaCl, 1% triton X pH adjusted to 7.4 with HCl and with 1:100 protease inhibitor cocktail (Thermo Fisher Scientific). The cells were then centrifuged at 10,600*g* at 4 °C for 20 min, mixed with NativePAGE sample buffer 4× (Thermo Fisher Scientific) and loaded onto 3–12 or 4–16% Bis Tris protein gels (Thermo Fisher Scientific). Native-Mark unstained protein standards (Invitrogen) were used as molecular weight markers with the addition of a 14 kDa marker (Sigma-Aldrich). Before protein transfer, the gels were washed with running buffer with 0.1% added SDS (Sigma) for 10 min. Transfers for all protein electrophoresis gels were done onto polyvinylidene difluoride (PVDF) membranes (iBlotR, NovexR, Life Technologies). Membranes were then boiled in PBS for 5 min and blocked in 5% skim milk in PBS-T for 30 min at RT. Primary antibody incubation was done at 4 °C overnight in 5% skim milk PBS-T; for BN-PAGE, antibody 82E1 was used at 1:700. The membranes were then washed 4 times for 20 min each in PBS-T and incubated with secondary HRP antibody 1:2000 for 1 h in 5% skim milk in PBS-T at RT. The membranes were then washed 3 times for 15 min each in PBS-T and developed with Clarity western ECL blotting substrate (Bio-Rad).

### 2.8. Dot blot

Cell Samples were prepared as for BN-PAGE. 1 µl of sample was put on a nitrocellulose membrane and allowed to dry and then washed with PBS-T for 15 min 3 times and blocked for 30 min in 5% skim milk PBS-T. Primary antibody incubation was done at 4 °C overnight in 5% skim milk PBS-T and A11 or OC was used at a concentration of 1:5000. After primary incubation the procedure is identical to that of BN-PAGE.

### 2.9. Semi denaturing detergent (SDD) PAGE

The cells were either lysed as for BN PAGE or as 20% wt/vol