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CORRESPONDENCE

Formaldehyde-fixed brain tissue from spontaneously ill α-synuclein transgenic mice induces fatal α-synucleinopathy in transgenic hosts

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Compelling evidence suggests that in a variety of neurodegenerative diseases the induction and spreading of proteinaceous lesions involve a prion-like seeding mechanism [5]. Experimentally and for Alzheimer's disease (AD), it has been shown that cerebral β-amyloidosis can be instigated in susceptible hosts [i.e., young amyloid precursor protein (APP) transgenic (tg) mice] by the intracerebral injections of diluted extracts from β-amyloid-laden brains of aged APP tg mice or AD patients. The β-amyloid-inducing agent in the inoculate is an aggregated form of the amyloid-β peptide (Aβ) [8]. Remarkably, we recently reported that extracts of formaldehyde-fixed brains of aged APP tg mice or AD patients also induces cerebral β-amyloidosis [2]. Thus, Aβ seeds share one of the most remarkable attributes of prions, namely the resistance to inactivation by formaldehyde.

The histopathological hallmarks of α-synucleinopathies such as Parkinson's disease (PD) and dementia with Lewy bodies (DLB) are intracellular Lewy bodies and Lewy neurites, comprised primarily of hyperphosphorylated α-synuclein [3]. In PD brain, α-synuclein lesions progress in a stereotypic manner [1]. The underlying mechanism is hypothesized to be cell-to-cell transmission of aggregated α-synuclein that initiates a cascade of progressive α-synuclein misfolding and aggregation reminiscent of prion disorders [4, 5]. Experimentally similar to Aβ inoculations, α-synuclein lesions can be induced in susceptible hosts by intracerebral inoculation of extracts from human brains affected by α-synucleinopathies or brains from spontaneously ill α-synuclein tg mice containing aggregated α-synuclein [6, 7]. Given the astonishing findings of formaldehyde-resistant Aβ seeds [2], we asked whether this is also true for α-synuclein seeds.

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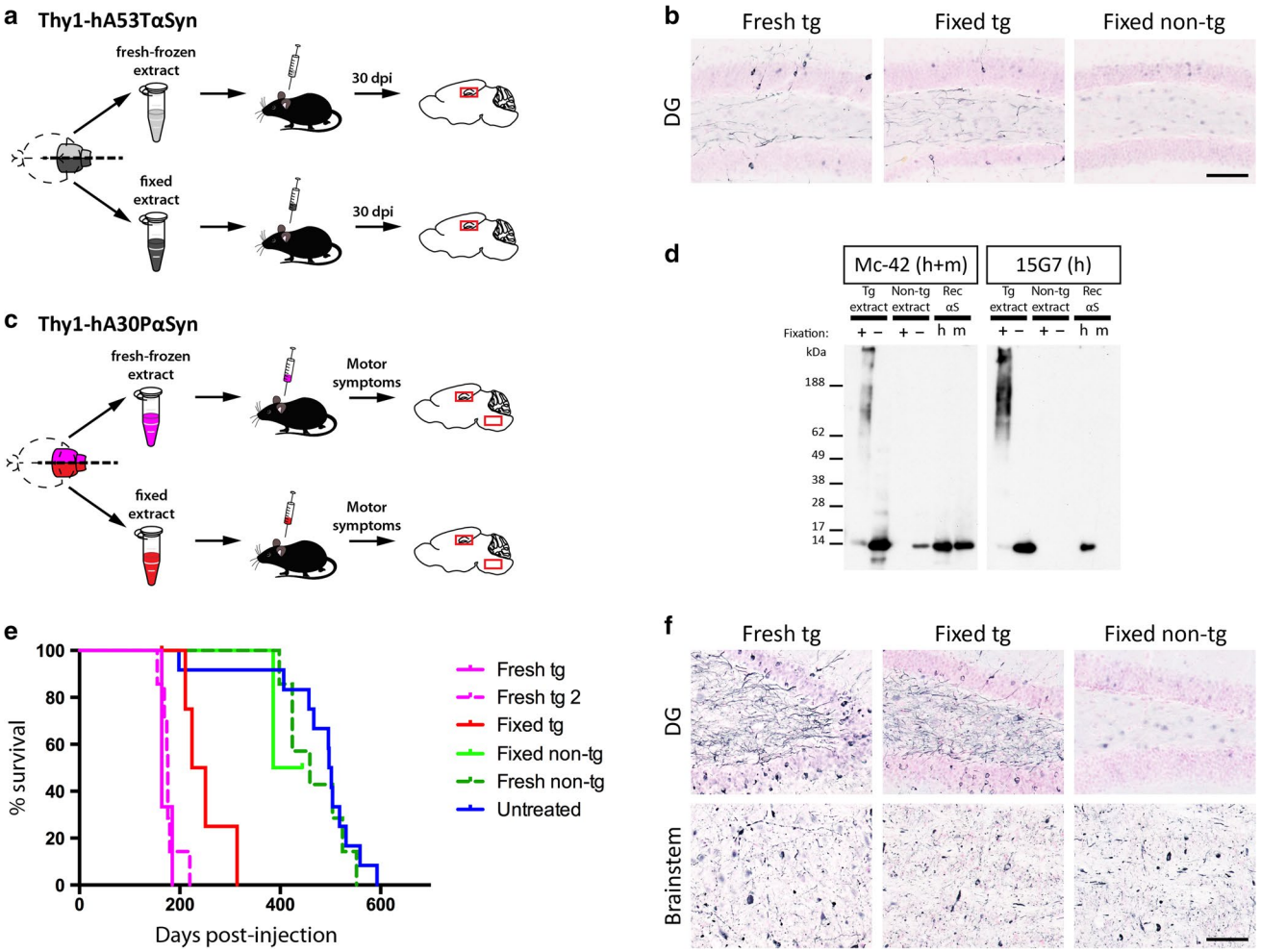

Fig. 1 **a**, **c** Illustration of tissue preparation and experimental setup. **b** Immunohistochemical analysis of DG (25 µm sagittal section) with antibodies against pSer129 α-synuclein (Epitomics) and nuclear fast red counterstain of Thy1-hA53TαSyn mice that have been injected 30 days prior with extracts from fixed and fresh-frozen α-synuclein lesion-containing tg brainstem tissue ($n = 3$ each). Extract from fixed non-tg tissue is also shown. **d** Immunoblot of 3,000×*g* extracts of fixed and fresh-frozen brainstem. Antibody Mc-42 recognizes human (h) and murine (m) α-synuclein while 15G7 recognizes only human α-synuclein. 100 ng recombinant (rec) α-synuclein was loaded as control. **e** Survival of Thy1-hA30PαSyn mice inoculated with extracts from fixed tg brainstem (median survival 238 dpi; $n = 4$) and fresh-frozen tg brainstem (164 dpi; $n = 3$) in comparison to untreated mice (median survival 500 dpi; $n = 12$; $p < 0.01$, log-rank test with Bonferroni correction). At the time of reporting the study, one mouse injected with fixed non-tg extract is still alive (444 dpi). For comparison, survival curves from mice injected with extracts from fresh-frozen tg (176 dpi, fresh tg 2) and non-tg brainstem (459 dpi) were added ($n = 7$ each). **f** Immunohistochemistry (pSer129) of inoculated Thy1-hA30PαSyn tg mice; shown are DG and brainstem. *Scale bar* 100 µm

In a first experiment, we used Thy1-hA53TαSyn tg mice [10]. As donor tissue brainstem from a symptomatic 8-month-old tg and an age-matched non-tg mouse was divided with one half immersion-fixed in formaldehyde (4 % in PBS) at 4 °C for 48 h and then cryoprotected in 30 % sucrose for 48 h before freezing. The other half was immediately fresh-frozen (Fig. 1a). Subsequently, tissues were homogenized at 10 % [w/v] in PBS at 4 °C (Precellys, 4 × 10 s at 5,500 rpm) and centrifuged at 3,000×*g* for 5 min. The supernatant is referred to as "Extract". Extracts (2.5 µl) were injected bilaterally into the dentate gyrus (DG; from Bregma AP −2.5, ML ±2.0, DV −1.8) of young, 2- to 3-month-old Thy1-hA53TαSyn mice (for methodological details see [2]). Analysis 30 days post-injection (dpi) revealed that both fixed and fresh-frozen tg extracts induced phosphorylated α-synuclein pathology in the DG (Fig. 1b). In contrast, mice injected with fixed or fresh-frozen non-tg extract did not exhibit any pS129-positive α-synuclein aggregates (Fig. 1b).

In contrast to Aβ seed-inoculated APP tg mice, α-synuclein tg mice inoculated with brain extracts from spontaneously ill α-synuclein tg mice or DLB brain exhibit a progressive and terminal motor phenotype [6, 7]. Thus, to assess if formaldehyde-fixed tissue from aged

symptomatic α-synuclein tg mice would induce fatal end-stage α-synucleinopathy in the host mice, we used brainstem from a spontaneously ill 20-month-old Thy1-hA30PαSyn tg mouse [9] and an age-matched non-tg control (Fig. 1c). Estimation of α-synuclein levels in the extracts was done using NuPAGE SDS-PAGE (Life Technologies) with antibodies against both mouse and human α-synuclein, with qualitatively similar results (Fig. 1d). The fresh-frozen tg extract revealed the expected 14-kDa monomeric α-synuclein band and some higher molecular weight bands indicative for multimeric α-synuclein while the fixed tg brainstem extract revealed primarily high molecular weight bands indicative of cross-linking due to formaldehyde fixation. Subsequently, extracts (2.5 µl) were injected again into the DG of 4- to 6-month-old (presymptomatic) female Thy1-hA30PαSyn mice. Untreated female Thy1-hA30PαSyn animals served as additional control. All mice were analyzed at end-stage displaying severe motor symptoms (i.e., clinical endpoint). Both, Thy1-hA30PαSyn mice that received extracts from fixed and fresh-frozen tg brainstem revealed a significantly reduced median survival (238 and 164 dpi, respectively) compared to untreated mice (500 dpi) and controls injected with extract from non-tg fixed material (Fig. 1e). Although not statistically significant, results indicate that the extract from the fresh-frozen material is more potent in inducing end-stage α-synucleinopathy compared to the extract from the fixed tissue (Fig. 1e). Immunohistochemical analysis revealed severe (+++) and similar phosphorylated α-synuclein pathology (pS129) in the brainstem of all groups (Fig. 1f). On average, the level of induced α-synuclein pathology in DG of mice injected with the fresh-frozen extract was greater (+++) compared to mice injected with the fixed extract (++; blinded assessment). No α-synuclein pathology was observed in DG injected with the extract from fixed non-tg tissue (−). The same results were found when sections were stained with thioflavin S (supplementary Fig. 1). Because fixation was limited to 48 h, it is possible that prolonged fixation would result in some further deactivation of α-synuclein seeds although in our previous study Aβ seeds resisted at least 2 years of formaldehyde fixation [2].

In summary, we find that α-synuclein seeds in brain resist formaldehyde fixation as previously reported for Aβ [2]. It is likely, albeit to be proven, that this is also true for aggregated tau and other self-propagating pathogenic protein aggregates. These findings can now be exploited to further establish the relationship between the molecular architecture of α-synuclein lesions and individual pathogenesis and thereby exploit archived formalin-fixed brain material.

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