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Amyloidogenic α -synuclein seeds do not invariably induce rapid, widespread pathology in mice

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Abstract

To further evaluate the parameters whereby intracerebral administration of recombinant α -synuclein (α S) induces pathological phenotypes in mice, we conducted a series of studies where α S fibrils were injected into the brains of M83 (A53T) and M47 (E46K) α S transgenic (Tg) mice, and non-transgenic (nTg) mice. Using multiple markers to assess α S inclusion formation, we find that injected fibrillar human α S induced widespread cerebral α S inclusion formation in the M83 Tg mice, but in both nTg and M47 Tg mice, induced α S inclusion pathology is largely restricted to the site of injection. Furthermore, mouse α S fibrils injected into nTg mice brains also resulted in inclusion pathology restricted to the site of injection with no evidence for spread. We find no compelling evidence for extensive spread of α S pathology within white matter tracts, and we attribute previous reports of white matter tract spreading to cross-reactivity of the α S pSer129/81A antibody with phosphorylated neurofilament subunit L (NFL). These studies suggest that with the exception of the M83 mice which appear to be uniquely susceptible to induction of inclusion pathology by exogenous forms of α S there are significant barriers in mice to widespread induction of α S pathology following intracerebral administration of amyloidogenic α S.

Keywords

amyloid; Parkinson's disease; pathology; α -synuclein; transgenic mice

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Author Contribution

ANS designed the study, performed the injections, analyzed the data, and drafted the manuscript. MB collected the samples and analyzed the data. MAT and ABM performed the injections and analyzed the data. NHM, NLR, and CCD analyzed the data. JR participated in the design of the study. TEG and BIG participated in the design and coordination of the study, analyzed the data, and helped to draft the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Introduction

α -Synucleinopathies, a spectrum of neurodegenerative disorders, most notably Parkinson's disease (PD), are characterized by the presence of intracellular α -synuclein (α S) inclusions. These inclusions are formed from the amyloidogenic aggregation of the normally soluble presynaptic protein α S [13,37,93]. Mature α S inclusions are also labeled with other markers of intracellular protein aggregates including ubiquitin and p62 [58,65]. A direct causal role for α S in neurodegeneration is supported by missense mutations or increased copy number of the α S gene (*SNCA*) in patients with familial PD [5,25,52,57,61,75,76,85,101].

α -Synucleinopathies are progressive neurodegenerative disorders and multiple experimental and pathologic studies have been interpreted as supportive of a "prion-like" spread mechanism where amyloidogenic α S serves as a template to drive the conversion of soluble, natively unfolded α S to a conformationally-altered, aggregated form which transmits from cell-to-cell [40,48,56,62,63,66,67,70,74,77,83,90,91]. For example, in post-mortem studies of PD patients who had received therapeutic striatal transplants of fetal dopaminergic neurons, neurons subsequently developed α S pathology [56,62,63], and a similar phenomenon has also been reported in wild type mouse neurons transplanted into an α S Tg mouse model [41]. However, other mechanisms could also explain this phenomenon (reviewed in [9]). In recent studies, it has also been shown that intracerebral injections of extracts from brains of humans with α S pathology or amyloidogenic fibrillar forms of human α S can induce pathology in M83 (A53T) α S transgenic (Tg) mice [67,70,91]. In addition, amyloidogenic murine α S was reported to induce widespread pathology and neurodegeneration within 1 month in non-transgenic (nTg)/native mice [66]. In cell culture studies, it has been reported that the addition of preformed exogenous α S amyloid seeds induces intracellular α S aggregates in both α S overexpressing and naïve primary mouse neuronal cultures [19,40,90], with evidence in these studies for both strain like conformational templating, passaging, and possibly cell-to cell transmission [83].

Despite the growing evidence for prion like induction of α S pathology both in culture and *in vivo* [40,48,56,62,63,66,67,74,77,83,90,91], alternative and perhaps synergistic mechanisms of pathology induction have not been excluded. Indeed, we have observed that non-amyloidogenic form of α S (human 71–82 α S) that does not form amyloid *in vitro* or seed α S inclusion formation in culture can induce inclusion formation *in vivo* [82]. Moreover, the extent of pathology induction *in vivo* and the time course of induction that have been reported are also quite variable. Given the potential importance of these induced models for mechanistic and therapeutic studies, we conducted a series of *in vivo* studies in which different forms and types of α S fibrils were injected into the brains of M83 (A53T) and M47 (E46K) α S transgenic (Tg) mice, and non-transgenic (nTg) mice. At post-injection times similar to what has been previously reported, we demonstrate that fibrillar human α S induced widespread cerebral α S inclusion formation only in the M83 Tg mice, but not in nTg and M47 Tg mice. In both nTg and M47 mice α S pathology is induced but it is largely restricted to the site of injection and in the nTg mice actually diminishes over time, confirming that there are significant barriers to widespread induction of pathology except in the M83 mice. Finally, we find that the α S pSer129/81A antibody used in many of the studies to report widespread pathology induction *in vivo* (and also white matter pathology in

human samples) shows significant cross reactivity with phosphorylated neurofilament subunit L (NFL). Use of this antibody to track α S pathology can result in a marked overestimation of pathology induction, and thus raises additional questions about the mechanisms of pathology induction that have been reported to occur through white matter tracts.

Results

Pathological changes following intrahippocampal injection of preformed α S fibrils in M83 Tg mice

Recent studies have shown robust induction of widespread CNS α S pathology using M83 Tg mice challenged with adult brain injection of human fibrillar (hfb) α S [67]. M83 Tg mice intrinsically develop a severe motor phenotype associated with flame-like α S inclusions. On the current genetic background, the motor phenotype develops over a wide range of time from 7–15 months. Inclusion pathology occurs throughout the neuroaxis, but largely spares the hippocampus, striatum, and the cortex [33]. To limit confounds from intrinsic pathology formation, intrahippocampal injection of 21–140 hfb α S was performed in young 2 month-old M83 Tg mice, which were analyzed 2 months later, a time when no intrinsic pathology is observed (Table 1). We injected N-terminally truncated protein 21–140 α S, as we and others previously have shown that it can seed α S inclusions in cultured cells as efficiently as full-length α S [68,83,95,96], and it enables monitoring of the aggregation of endogenous α S with N-terminal specific antibodies such as SNL-4 and Syn506 [21,34,92]. 21–140 hfb α S intrahippocampal injection resulted in widespread α S inclusion formation that was found in all the regions where intrinsic α S pathology would form. Abundant inclusions were also noted in areas where inclusion formation does not intrinsically occur in these mice including the hippocampus, where the protein was injected, and the striatum and cortex, though the striatal/cortical inclusions were less frequent (Fig. 1; Supp. Figs. 1–3). Intrahippocampal injection of PBS resulted in no α S pathology formation. At the site of injection, 71–82 α S, which has a deletion in the middle of the hydrophobic region of α S that lacks the ability to form or seed α S amyloid *in vitro* and in cultured cells [35,68,83,98], resulted in sparse altered pSer129/81A staining of perikaryal cell bodies and structures that resembles dystrophic synaptic terminals or that could be Jucker bodies (Supp. Fig. 4; data not shown). Similar to the α S inclusions seen in symptomatic M83 Tg mice (Fig. 1B), those seen post-injection of 21–140 hfb α S are predominantly flame-like, filling the somatodendritic compartment, and are robustly recognized by the α S antibody Syn506 and by an antibody to p62, which is specific for inclusion formation [58] (Fig. 1; Supp. Figs. 1–3).

Although our findings are consistent with those of Luk and colleagues in the ability of intracerebral injection of exogenous α S fibrils to rapidly induce α S inclusion pathology M83 Tg mice, using multiple α S antibodies including Syn506, we were not able to observe robust evidence of inclusion pathology within brain white matter tracts as previously reported [67]. The vast majority of the white matter staining by pSer129/81A antibody was not reactive with other α S antibodies (Supp. Fig. 5). This difference is significant as it was used to support the concept of axonal spread of inclusion pathology from the site of injection [67]. Only sparse α S inclusions that could be labeled with other α S antibodies were observed in

the brain white matter tracts of hfib α S injected M83 Tg mice. pSer129/81A readily labeled α S inclusions as shown by double-labeling; however, it did not stain areas abundant in normal α S such as the neuropil areas of the cerebellum (Supp. Fig. 5), consistent with previous findings that α S is only modestly phosphorylated at Ser129 under normal conditions but hyperphosphorylated in aggregates [4,27,94]. However, the intense pSer129/81A white matter tract staining was still present throughout the CNS in *SNCA*^{-/-} mice as shown by immunofluorescence microscopy and IHC (Supp. Figs. 5–6). This finding is consistent with our recent studies that also revealed robust pSer129/81A staining of cultured neuronal processes from *SNCA*^{-/-} mice [83], indicating that this staining is due to cross-reactivity of the pSer129/81A antibody with a non- α S target.

Analyses of intrahippocampal injection of preformed α S fibrils in nTg mice

To further study the pathological changes resulting from cerebral challenge of exogenous preformed α S fibrils, we performed similar intrahippocampal injection of 21–140 hfib α S in nTg mice (Table 1). This treatment resulted in the accumulation of neuronal pSer129 immunostaining localized to the site of injection with very limited pathology in the cortex (Fig. 2A). Intrahippocampal injections also resulted in the formation of some α S inclusions that could be detected with Syn506 and p62 antibodies (Fig. 2B). pSer129/81A, Syn506, and p62 immunoreactive perikaryal and neuritic inclusions in the hippocampus of nTg mice challenged with 21–140 hfib α S persisted for up to 4 months, although with decreasing abundance (Fig. 2B). During this time period there was no evidence of spread beyond the initial sites of pathology.

The limited level of α S pathology induced after intrahippocampal injection of 21–140 hfib α S prompted us to examine whether there is a cross-species barrier inhibiting inclusion formation. We performed intrahippocampal injection of full-length mouse fibrillar (mfib) α S. At 2 months post-injection of mfib α S, there were α S inclusions readily detected with pSer129/81A, Syn506, and p62 antibodies at the site of injection, and the amount of pathology was more robust than for 21–140 hfib α S (Fig. 2B); however, there was no difference in the distribution pattern of pathology. The more robust induction of local pathology by mfib α S could simply be due to the fact that mouse α S intrinsically have the A53T substitution that promotes α S aggregation and seeding [12,36,79]. Therefore, our findings are consistent with those recently reporting that mfib α S can induce the formation of some α S inclusions in nTg mice [67,69], but in our studies α S pathology was highly localized to the site of injection.

Analyses of intrahippocampal injection of preformed α S fibrils in M47 Tg mice

Since we observed rapid and widespread induction of α S pathology in M83 Tg mice, but not in nTg mice, we next examined induction of α S inclusion formation following stereotactic brain injection of fib α S in a Tg mouse model expressing human α S with the E46K missense mutation (line M47, Table 1). These mice typically develop Lewy body-like α S inclusions with a distribution similar to M83 Tg mice but not before 15 months of age [23]. At 4 months post hippocampal injection of hfib α S, α S inclusions were predominantly found at the site of injection in the hippocampus, with modest amounts in the cortex, midbrain, and brainstem (Fig. 3A). The rounded, perinuclear and neuritic inclusions similar to those seen

in symptomatic M47 Tg mice were also detectable by Syn506 and p62 (Fig. 3B–C). We further investigated whether α S fibrils with pathogenic mutants such as A53T or E46K could be more potent at inducing α S pathology in M47 Tg mice. The hippocampal injection of fibrils comprised of E46K α S in M47 Tg mice also recapitulates the condition where the same protein is injected as is expressed, alleviating concerns of cross-species differences. The location and formation of α S pathology in M47 Tg mice injected with A53T or E46K α S fbs was similar to the mice injected with the 21–140 hfb α S (Fig. 4).

Analyses of intracortical injection of preformed α S fibrils in nTg mice

Since we were unable to induce widespread α S inclusion pathology by the hippocampal injection of exogenous α S fibrils in nTg mice as previously reported [66], we explored the possibility that the site of injection (cortical vs hippocampal) may have contributed to this difference although Luk and colleagues have already reported that similar induction of α S should result from the injection in various brain regions including cortex, striatum, and hippocampus [66]. We performed cortical injections of 21–140 hfb α S or 71–82 α S in nTg mice with analyses conducted at 1, 2, and 4 months post-injection (Table 1). At 1 month post-injection, we observed a robust increase in pSer129/81A perikaryal, neuronal immunoreactivity that radiates throughout the cortex from the site of injection following both types of injections (Fig 5. and Supp. Figs. 7–8). The intense induced pSer129/81A cortical staining was not observed in PBS injected nTg mice (Fig. 5 and Supp. Figs. 7–8).

In nTg mice injected with 21–140 hfb α S, only some scattered more compacted pSer129/81A immunoreactive perikaryal or neuritic profiles persisted up to 4 months post-injection, but the initial induced cortical pSer129/81A immunoreactivity that radiated from the injection had subsided (Fig. 5). Similarly at 2 and 4 months post-injection of 71–82 α S, the initially induced perikaryal pSer129/81A staining was no longer detected (Fig. 5). Staining of these perikaryal neuronal cell bodies as seen with the anti-pSer129/81A antibody at 1 month post injection was not observed with a panel of other α S antibodies (Syn506, Syn303, Syn505, Syn514, SNL-1, SNL-4, and HuA) or p62 (Fig. 5, Supp. Fig. 9, and data not shown) even after systematically evaluating various antigen retrieval methods [21,34,92]. In comparison, bona fide mature α S inclusions in symptomatic M83 A53T α S Tg mice were readily detected with multiple α S antibodies (Fig. 1, Supp. Figs. 2, 9, and data not shown).

Identification of the major axonal pSer129/81A cross-reactive protein as NFL phosphorylated at Ser473

The antibody pSer129/81A has been used in many seminal reports to track α S pathology [40,66,67,90]. Previous findings [83,94] and the data shown here (Fig. 5 and Supp. Figs 5–6, 7–8) indicate that this antibody cross-reacts with a non- α S pSer129 target with increased immunoreactivity following exogenous α S treatment. Therefore we conducted studies to identify this non- α S pSer129 target. We first performed biochemical fractionations from CNS areas enriched in grey matter (cortex) and white matter (spinal cord and brain stem) from nTg and *SNCA*^{−/−} mice. Western blot analysis of these fractions demonstrated that α S is predominantly present in the HS fractions, and the presence of modestly pSer129 phosphorylated α S in the HS-soluble fraction of only nTg mice (Supp. Fig. 9). This is consistent with previous characterizations of normal α S protein demonstrating that it is

largely soluble and only modestly phosphorylated at Ser129 [4,18,27,94,99]. As previously reported, endogenous α S is also more highly expressed in the cortex than the spinal cord [32,33]. Importantly, this analysis demonstrates that pSer129/81A also strongly reacts with a ~70 kDa non- α S target that is still present in *SNCA*^{-/-} mouse brain and enriched in the white matter. Biochemically, this protein is enriched in the SDS-urea fraction, but is also present in lesser amounts as a soluble form in the HS-fraction (Supp. Fig. 9). This finding is also consistent with our previous characterization, where we showed this antibody can cross-react with a detergent insoluble ~70 kDa non- α S target enriched in white matter brain tissue, like cerebellar white matter [94], and we hypothesized that the reactivity with this protein was largely responsible for the intense pSer129/81A staining observed in neuronal processes still present in *SNCA*^{-/-} mice [83].

We found that the ~70 kDa pSer129/81A reactive protein was soluble in either 4M urea or 2% SDS and that it was similarly abundant in white matter from human cortex or cerebellum (Fig. 6A). Compared to total protein Coomassie stained gel, we found that the pSer129/81A reactive ~70 kDa band aligned with the low-molecular-mass neurofilament subunit (NFL) (Fig. 6A). To ascertain that pSer129/81A could be reacting with NFL, we performed immunoprecipitation (IP) assays with pSer129/81A and showed by immunoblotting that pSer129/81A antibody could immunoprecipitate NFL from both human and mouse white matter (Fig. 6B). We then performed dephosphorylation studies to show that the reactivity of the pSer129/81A antibody with the ~70 kDa protein band was completely dependent on phosphorylation (Fig. 6C). To confirm that the ~70 kDa protein recognized by pSer129/81A antibody was NFL, we analyzed extracts from NFL^{-/-} mice and showed that it was completely absent in tissue from NFL^{-/-} mice (Fig. 6D). To further confirm that pSer129/81A was reacting with phosphorylated NFL we purified recombinant NFL from bacteria and phosphorylated the protein with casein kinase II *in vitro*. We found that the pSer129/81A antibody only reacted with recombinant NFL phosphorylated *in vitro* (Fig. 6E). The major *in vivo* phosphorylation site in NFL is Ser473 (normally modified at ~73%), which corresponds to Ser472 in bovine NFL [88,100]. Like the Ser129 phosphorylation site in α S, this site in NFL is also an excellent substrate for casein kinase II [45,72,94] and analysis of the sequence revealed homology between each site: DEPPpSEGEAE in NFL versus AYEMPpSEEGYQ in α S (the sequence used to make the pSer129/81A antibody) [94]. Furthermore, the amino acid residues surrounding Ser473 in NFL are conserved across all species [30,49,50,88,100]. Therefore, we generated recombinant NFL with the Ser473Ala mutation and showed that this mutation ablated the reactivity of pSer129/81A with NFL incubated with casein kinase II (Fig. 6E). Collectively, these studies show that the ~70 kDa protein recognized by pSer129/81A antibody is NFL phosphorylated at Ser473.

Analysis of pSer129/81A detection of phosphorylated NFL in white matter tracts of nervous tissue

Since it has been previously suggested that α S pathology spreads from the site of intracerebral injection of fibrillar α S via white matter tracts based on pSer129/81A staining [67], we investigated the extent to which pSer129/81A detects phosphorylated NFL in the white matter tracts of nTg mice. In untreated nTg mice, IHC can readily detect NFL in the white matter tracts of the corpus callosum, hippocampus, striatum, brain stem, and

cerebellum (Fig. 7). IHC staining with pSer129/81A shows a similar neuroanatomical staining pattern of white matter tracts as NFL antibody staining. Conversely, IHC with anti- α S antibody Syn506 does not stain white matter tracts since α S is normally a presynaptic protein [31,46,47](Fig. 7). Immunofluorescent analysis also demonstrated that pSer129/81A immunoreactivity co-localizes with phosphorylated NFL in the majority of white matter tracts and also in a subset of cell bodies (Supp. Fig. 10). For comparison, we stained nervous tissue from NFL $^{-/-}$ mice and show that the loss of the NFL staining pattern is accompanied by a similar loss of white matter pSer129/81A staining, but the pSer129/81A and NFL staining of white matter tracts are unaltered in *SNCA* $^{-/-}$ mice (Supp. Fig 10). This data indicates that pSer129/81A can readily detect phosphorylated NFL in white matter tracts and some cell bodies.

To further investigate if pSer129/81A could react with inclusions comprised of neurofilaments (NFs), we then stained the nervous tissue from NFH-LacZ Tg mice, which develop intracellular NF inclusions throughout the brain and brain stem [24,89]. We found that pSer129/81A strongly detects the Lewy body-like perikaryal NF inclusions and these inclusions do not contain α S, as they are not detected by the α S antibody SNL-4 (Supp. Fig 11). The NF inclusions in NFH-LacZ Tg mice were also not stained with more than 10 other α S antibodies [21,34].

Expanding upon our findings in murine nervous tissue, we next tested the specificity of pSer129/81A staining in human nervous tissue and in murine primary mixed neuronal cultures. In nervous tissue from a control human subject, we found that pSer129/81A also stains phosphorylated NFL in the white matter tracts in the absence of accumulated endogenous α S (Supp. Fig. 12). Similar to staining in mouse CNS tissue, anti-pSer129/81A can robustly cross-react with the non- α S target in white matter tracts in human nervous system tissue that can be easily misinterpreted as α S pathology (Fig. 8). Having identified phosphorylated NFL as the major non- α S target recognized by antibody pSer129/81A, we performed double-label immunofluorescence analysis of the tissue sections from nTg mice that were cortically injected with α S and demonstrated robust pSer129/81A increased immunoreactivity that could not be detected with other non-phospho-specific α S antibodies. These studies revealed that the majority of the robust induced pSer129/81A immunoreactivity co-localized with NFL staining (Supp. Fig 13). These data indicate that most of this induced pSer129/81A immunoreactivity was likely due to hyperphosphorylation of NFL and/or accumulation of NFL.

We and others have used primary neuronal cultures as *in vitro* modeling systems for α S pathology [83,90]. We have previously shown in mixed neuronal cultures from untreated nTg and *SNCA* $^{-/-}$ mice that pSer129/81A antibody demonstrated robust cross-reactivity with an unknown non- α S target in the cell bodies and processes [83]. We show that in these cultures the pSer129/81A antibody also can almost completely co-localize with the NFL profile indicating that the majority of the pSer129 staining in native neuronal cultures is due to reactivity with Ser473 phosphorylated α S (Supp. Fig 14).

Discussion

Our studies demonstrate that direct intracerebral injection of fib α S can reproducibly and rapidly induce widespread α S inclusion pathology in M83 α S Tg mice but not in nTg or M47 α S Tg mice. Although these studies confirm the primary observation reported in several recent studies [66,67,69] that α S pathology can be induced by exogenous challenge with fibrillar forms of α S, they indicate that except in a line of mice that is most intrinsically vulnerable to α S pathology, induction of α S pathology in focal areas does not invariably lead to rapid widespread dissemination of the pathology within the brain. This observation is important as it suggests that there are biological barriers, which can regulate the rate and extent of α S pathology induction. These data may be viewed as controversial, but we believe that a reappraisal of previous data that relied heavily on pSer129/81A antibody and even other pSer129 antibody staining to track pathology induction is essential. We have now shown that pSer129/81A antibody recognizes pSer473 in NFL as a cross-reactive epitope. Indeed, our data suggests that the phospho-NFL cross-reactivity likely accounts for the majority of staining within white matter tracts and neuronal processes that has been used to support the hypothesis that α S pathology spreads *in vivo* via axonal connections [67]. Furthermore, we show that in nTg mice cortical injection of amyloidogenic (fibrillar) and non-amyloidogenic (71–82) forms of α S can induce robust, but transient pSer129/81A staining that is predominantly due to NFL phosphorylation and not α S aggregation. Although it could be argued that the time frame (up to 4 months) for our studies was not sufficiently long to result in “prion-like” spread of α S pathology in nTg and M47 Tg mice, the 4 month time point is longer than most time points that were reportedly demonstrated to result in widespread α S pathology in nTg mice [66]. Furthermore, we find that while hfb α S induces some focal α S pathology at the injection site, the pathology actually diminishes over time rather than progressively spreads.

Though data from numerous laboratories, including our own, suggested that α S, like almost all amyloids, can behave like a prion in some experimental conditions [40,66–68,77,83,91,95], several findings in this study and previous studies suggest that it is premature to conclude that the spread of pathology observed *in vivo* is attributable, or at least solely attributable, to prion-like mechanisms. The observation that soluble 71–82 α S, which is non-amyloidogenic *in vitro* and in cell culture studies, can induce α S pathology *in vivo*, albeit less efficiently than fib α S [82], is challenging to reconcile with a prion-like only replication hypothesis. It is possible that additional factors *in vivo* can convert the soluble non amyloidogenic 71–82 α S into a form capable of conformationally templating endogenous α S. However, it is also possible that other mechanism such as the pro-inflammatory properties of α -synuclein, direct toxicity, or disrupted proteostasis could induce α S pathology [3,7,10,14,15,17,22,26,51,53–55,60,64,78,80,86,87,102]. In addition, our data and that of others, rather than being indicative of “spread” could be attributable to initial diffusion of the injected α S within the brain, which then induces pathology at sites distal to the site of injection. A gradient of injected α S away from the injection site, possibly along normal anatomical connections, could lead to the progressive appearance of α S pathology. In this scenario there could be progressive “seeding” but no true spread except for distribution of the exogenous seeds. Finally, we would like to point out that in no study in

which an α S aggregate containing brain homogenate has been used to induce robust α S pathology in mice, has an α S depleted control homogenate been shown to not induce α S pathology. In a recent report by Recasens and colleagues, it was shown that the nigral injection of LB-enriched extracts from PD brains could result in progressive dopaminergic neuronal demise in mice and monkeys and this activity could be immune-depleted with α S antibodies, but only subtle accumulations of α S and not formation of authentic α S pathological inclusions was reported [77]. These findings are in fact in line with an alternative hypothesis that exogenous α S may lead to progression of disease due to neuronal toxicity as has been reported in many studies [15,17,22,51,64].

As noted above, our data support the concept that biological barriers exist that regulate the induction of α S pathology *in vivo* by exogenous α S. This extends observations made in various lines of unmanipulated α S Tg mice that clearly demonstrate a differential intrinsic susceptibility to inclusion formation and neurodegenerative phenotypes between the various models expressing human WT and PD-linked mutant forms of α S, despite similar levels of expression [23]. There are several non-exclusive hypotheses that could explain both the differential intrinsic susceptibility of these various α S Tg models and the differential vulnerability observed here following exogenous α S Tg challenge. One hypothesis would be that α S can assume different conformations and that these different conformers or “strains” may i) initially aggregate with different kinetics and ii) propagate through conformational templating with differing kinetics, cell-to-cell transmissibility, and mutability. α S conformers most efficient at conformational templating and transmissibility would thus be most likely to induce pathology both intrinsically and in seeding paradigms. There is growing evidence that ultrastructural differences in α S polymers, which can in some cases be attributed to PD-linked mutations in α S and could be referred to as conformers, may have distinct biological activities [8,11,36,39,43,73,83]. There is more limited evidence that distinct conformers might be associated with different levels of toxicity and capacity to induce inclusion formation *in vivo*. Indeed, it may be speculated that this may account for the heterogeneity of clinical disease associated with α -synucleinopathy in humans. Perhaps the most provocative findings in this regard are that homogenates from brains of humans with multiple systems atrophy are capable of seeding pathology in M83 Tg mice, but again this was only shown in this primed animals and no immune-depletion of brain extracts were reported [91]. Nevertheless, in humans there is no evidence that an α -synucleinopathy would begin through an amyloid seeded mechanism; it more likely begins through an intrinsic disruption of mechanisms that maintain normal proteostasis. In any case, it will be very challenging to dissociate subsequent prion like spreading from progressive disruptions of proteostasis *in vivo*, as the two may be inexorably linked. The second hypothesis is that the M83 Tg mice are uniquely primed to develop α S pathology, and that exogenous α S results in a stress signal that simply accelerated the development of the intrinsic phenotype. Indeed, exogenous stressors such as lipopolysaccharide can trigger α S pathology in the M83 Tg mouse line [28,29], and there is growing evidence that exogenous amyloidogenic proteins like α S, especially when aggregated, act as danger associated molecular patterns to activate innate immunity [38,81]. Alternatively, exogenous α S that is internalized by cells may also create a proteotoxic stress that leads to a more generalized disruption of proteostasis. Notably, strain like conformational templating in which distinct morphology of inclusions is

induced by different mutant fibrils could be used in future studies to distinguish between some of these mechanisms. One would predict that if prion-like mechanisms predominate then, as we have observed in primary cell culture systems [83], the exogenous α S strains will dictate the conformation and morphology of the α S pathology.

The lengthy time course of these *in vivo* studies and the numerous experimental variables including but not limited to different conditions of α S protein purification, fibril preparation, and different inoculation paradigms, could clearly contribute to differential results obtained by various groups. We have taken great care in these studies to insure that the fibrillar and non-fibrillar α S we use in these studies are well characterized. For the fibrillar and soluble α S we insure they are capable of efficiently inducing or do not induce pathology, respectively, in our primary cell culture systems. As opposed to PrP derived prions, which once conformationally-templated, are highly immutable in terms of their properties, α S conformers like many other amyloids can be heterogeneous. We believe that in the future it will be essential for researchers to exchange α S preparations between laboratories to establish how much “strain-like” differences in α S conformers contribute to the differential results reported to date. One key observation in the prion field is that different strains of prions can require different incubation times; thus, at this time we cannot exclude that injection of exogenous α S in M47 and nTg may lead to more extensive delayed induction of α S inclusion pathology [2,69,82].

pSer129 immunostaining with antibody 81A and other similar antibodies not only has been used as a major method to assess α S pathology in recent mouse studies of “seeding”; it has also been used to assess α S pathology in human studies [6,42,44,59,66,67,69,71,84,91]. Anti-pSer129 reactivity is a sensitive marker of α S pathology, because α S is normally only marginally phosphorylated while it is hyperphosphorylated in inclusions [4,27,94]. However, we attribute the pSer129/81A immunoreactivity that we have observed in brain sections from control patients that mimics LB pathology to cross-reactivity with NFL. Indeed, this antibody can readily label the NF inclusions in NFH-LacZ Tg mice. Thus, the conclusions drawn from any study using this antibody as a sole marker of α S pathology must be viewed cautiously until the pathology is confirmed with other α S antibodies. Although other pSer129 antibodies are available commercially and some have been used to document peripheral α S inclusion pathology [6,42,44,59,71,84], it is likely that some preparations of rabbit polyclonal pSer129 can also react with NFL phosphorylated at Ser473 due to the homology between both epitopes, but each preparation of pSer129 α S antibodies will need to be specifically tested. We have tried to test the specificity of several pSer129 antibodies from several manufacturers and have discovered that some of the antibodies such as EP1536Y are either no longer available due to lack of specificity or are of low quality giving very low signal (i.e. not detecting α S) or reacting with non-phosphorylated α S. We have only specifically shown cross-reactivity with the pSer129/81A antibody, which is the most commonly used antibody for seminal reports [19,40,66,67,90]. We do not know if pSer129 antibody 1175 that has been used in one study to report delayed induction of α S pathology following cerebral injection of α S fibrils cross-reacts with phosphorylated NFL [69], but this antibody was not biochemically characterized for specificity, and Masuda-Suzukake and colleagues did not document α S inclusion formation with any other α S antibodies [69].

In conclusion, as we have hypothesized in a recent review, the “spread” of α S and other pathologies is likely to be complex and not mediated by a single mechanism [38]. “Prion-like” seeding, induction of a toxic environment, and intrinsic disruptions of proteostasis may all synergistically contribute to the induction and spread of α S inclusion pathology as well as the proteinopathies that underlie other neurodegenerative CNS diseases. Additional studies will be needed to more conclusively determine the relative contribution of these various mechanisms to induction and propagation of α S pathology. Resolving the mechanism(s) of disease progression is important for biological, experimental, medical, and social reasons, but it also has direct therapeutic implications. Indeed, if we can identify the barriers to induction and spread of α S, then we may be able to harness these barriers to therapeutically intervene in PD and other α -synucleinopathies.

Materials and Methods

Expression and purification of recombinant α S proteins

The pRK172 cDNA constructs expressing human α S with amino acid 71–82 deleted (71–82), N-terminal truncated wild-type 21–140 human α S (with a Met codon added before amino acid 21), and full-length murine α S or full-length human α S with the A53T or E46K mutations were previously described [35,95,97,98]. Recombinant α S proteins were expressed in *E. coli* BL21 (DE3) and purified to homogeneity as previously described [35,39].

Fibril preparation of recombinant α S for mouse brain injection

21–140 human α S, murine α S, and A53T or E46K human α S proteins were assembled into filaments by incubation at 37°C at 5 mg/ml in sterile phosphate buffered saline (PBS, Invitrogen) with continuous shaking at 1050 rpm (Thermomixer R, Eppendorf, Westbury, NY) for 2 days, and then transferred to a new tube for testing and sonication. α S amyloid fibril assembly was monitored as previously described with K114 fluorometry [16,98]. α S fibrils were diluted to a concentration of 1–2 mg/mL in sterile PBS and treated by mild water bath sonication for 2 hours that fragments the typical elongate α S fibrils into smaller polymers and fibrils (see Supp. Fig 15). These fibrils were tested for robust induction of intracellular amyloid inclusion formation as previously described [83,95,96].

Mice husbandry and stereotactic injections

All procedures were performed according to the NIH Guide for the Care and Use of Experimental Animals and were approved by the University of Florida Institutional Animal Care and Use Committee or the Canadian Council on Animal Care and approved by the Faculty of Medicine Animal Care Committee at the University of Toronto, Canada. M47 and M83 Tg mice expressing human α S with the E46K mutation or the A53T mutation, respectively, were previously described [23,33]. *SNCA*^{−/−} mice [1] were obtained from The Jackson Laboratory (Bar Harbor, MA). NFH-LacZ Tg mice that accumulate widespread brain NF inclusions were previously described [24,89]. NFL null mice [103] were a kind gift from Dr. Jean-Pierre Julien (Laval University, Canada) and maintained on a C57BL/6J background. nTg, *SNCA*^{−/−}, and M83 and M47 Tg mice at 2 months of age were bilaterally stereotactically injected with 2 μ L of 1mg/mL α S proteins or PBS in the hippocampus

(coordinates from Bregma: A/P -1.7, L +/-1.6, D/V -2.0) or the cortex (coordinates from Bregma: A/P -1.7, L +/-1.6, D/V -1.0) as indicated in the text (Table 1). The inoculum was injected at a rate of 0.2 μ L per min (total volume of 2 μ L per hemisphere) with the needle in place for 15 min at each site. Mice n = 4 for all cohorts, and without gender bias.

Antibodies

pSer129, also known as clone 81A, is a mouse monoclonal antibody that reacts with α S phosphorylated at Ser129 [94]. SNL-4 and SNL-1 are rabbit polyclonal antibodies raised against synthetic peptides corresponding to amino acids 2–12 and 104–119 of α S, respectively [34]. Syn506 is an anti- α S mouse monoclonal antibody that recognizes the N-terminus of α S [21,92]. Anti-p62 (SQSTM1; Proteintech; Chicago, IL) is a rabbit polyclonal antibody. Mouse anti-actin (clone C4) monoclonal antibody reacts with all forms of vertebrate actin (Millipore, Billerica, MA). NR4 is a mouse monoclonal antibody specific for NFL (Sigma-Aldrich). Chicken and rabbit polyclonal antibodies for NFL were generously provided by Dr. Gerry Shaw (Encor Biotechnology Inc.). Rabbit anti-NFL monoclonal antibody C28E10 was purchased from Cell Signaling Technology.

Immunohistochemical analysis

Mice were sacrificed with CO₂ euthanization and perfused with PBS/heparin, followed by perfusion with 70% ethanol/150mM NaCl. The brain and spinal cord were then removed and fixed for at least 24 hours. Tissues were dehydrated and infiltrated with paraffin as previously described [20]. The tissues were cut into 7 μ m sections. Immunostaining of the sections was performed using previously described methods [20] using avidin-biotin complex (ABC) system (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA) and immunocomplexes were visualized with the chromogen 3,3'-diaminobenzidine. Sections were counterstained with hematoxylin. All slides were scanned using an Aperio ScanScope CS (40 $\times$ magnification; Aperio Technologies Inc., Vista, CA) and images of representative areas of α S pathology were taken using the ImageScope™ software (40 $\times$ magnification; Aperio Technologies Inc.).

Double-labeling immunofluorescence analysis of mouse brain tissue

Paraffin-embedded tissue sections were deparaffinized and hydrated through a series of graded ethanol solutions followed by 0.1M Tris, pH 7.6. The sections were blocked with 5% dry milk/0.1M Tris, pH 7.6 and were incubated simultaneously with combinations of primary antibodies diluted in 5% dry milk/0.1M Tris, pH 7.6. After extensive washing, sections were incubated with secondary antibodies conjugated to Alexa 594 or Alexa 488 (Invitrogen, Eugene, OR). Sections were post-fixed with formalin, incubated with Sudan Black, and stained with 4',6-diamidino-2-phenylindole (DAPI)(Invitrogen, Eugene, OR). The sections were coverslipped with Fluoromount-G (SouthernBiotech, Birmingham, AL) and visualized using an Olympus BX51 microscope mounted with a DP71 Olympus digital camera to capture images.

Biochemical fractionation and immunoblotting analysis of mouse brain tissue

Cortex and brainstem/spinal cord from 4 month-old nTg, *SNCA*^{-/-} and *NFL*^{-/-} mice were biochemically fractionated as previously described [33]. Briefly, tissue samples were dissected, weighed, and homogenized in 3mL/g of high-salt (HS) buffer (50mM Tris, pH 7.5, 750mM NaCl, 20mM NaF, 5mM EDTA) with protease inhibitors and sedimented at 100,000 x g for 20 min. Pellets were re-extracted in HS buffer, followed by subsequent extractions in HS/1% Triton, HS/1M sucrose, RIPA buffer (50mM Tris, pH 8.0, 150mM NaCl, 5mM EDTA, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS with the protease inhibitors), and 2%SDS/4M urea. SDS/urea fractions were then sonicated. 2% SDS was added to all the fractions, which were heated to 100°C for 10 minutes, except for those fractions containing urea. For some experiments the RIPA homogenate was separated in half and the subsequent pellets were re-suspended in 4M urea or 2% SDS by sonication. Protein concentrations were quantified using the bicinchoninic acid (BCA) assay and bovine serum albumin as a standard (Pierce Biotechnology, Rockford, IL).

SDS-PAGE and immunoblotting analysis

Protein was resolved by SDS-PAGE on 7% (w/v), 8% or 13% polyacrylamide gels, as indicated, followed by electrophoretic transfer onto nitrocellulose membranes. Some gels were directly stained with Coomassie R-250 or transferred by electrophoresis onto nitrocellulose membranes. For Western blotting, membranes were blocked in Tris buffered saline (TBS) with 5% dry milk, and incubated with primary antibodies in TBS/5% dry milk or TBS/5% BSA for pSer129/81A overnight. Following washes, membranes were incubated with a goat anti-mouse antibody conjugated to horseradish peroxidase (HRP) or a goat anti-rabbit antibody conjugated to HRP. Protein bands were detected using chemiluminescent reagent (NEN, Boston, MA) and a FluorChem E and M Imager (ProteinSimple, San Jose, California).

In situ nitrocellulose protein dephosphorylation

For dephosphorylation of protein directly on the membrane, following transfer from SDS-polyacrylamide gel, the membrane was blocked with 5% BSA in TBS overnight. The membrane was then rinsed in phosphatase buffer (50mM Tris, pH 9.2, 0.5mM MgCl₂) and incubated in phosphatase buffer with or without 250U/mL bovine intestinal mucosa alkaline phosphatase (Sigma-Aldrich) overnight. The membranes were rinsed with TBS and blocked for 1 hour with 5% skimmed milk in TBS and processed for immunoblotting as described above.

Immunoprecipitation

pSer129/81A antibody was pre-absorbed with protein A/G agarose beads (Santa Cruz Biotechnology, Inc) and exchanged in CSK buffer (50mM Tris, 150mM NaCl, pH 7.4, 20mM NaF, 1mM EDTA, 1% Triton X-100). The SDS fraction from human cortical white matter was diluted in 20 volumes of CSK buffer with protease inhibitors and incubated with the pSer129/81A-protein A/G agarose beads overnight at 4°C with rotation. After extensive washing with CSK buffer, the immuno-precipitated complex was eluted with SDS sample buffer and heating to 100°C for 5 minutes.

Expression, purification, and phosphorylation of murine NFL from bacteria

Recombinant NFL was expressed in *Escherichia coli* BL21 (DE3) using mouse NFL cDNA cloned into the pET-23d expression vector (Novagen, Inc. Madison, WI). The same construct with the Ser473Ala mutation was generated using the QuickChange Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and specific oligonucleotides.

Following transformation, bacteria were grown to an OD₆₀₀ of 0.6 and the expression of the recombinant protein was induced with 1mM isopropyl-β-D-thiogalactopyranoside for 2 hours. NFL was purified using inclusion bodies procedure. Cells were pelleted, re-suspended into lysis buffer (25% sucrose, 50mM Tris, pH 8.0, 1mM EDTA, 2mg/mL lysozyme, and a cocktail of protease inhibitors) and incubated on ice for 30 minutes. Ten mM MgCl₂, 10μg/mL DNase1 and 1μg/mL RNaseA were added to the homogenate, which was incubated on ice for another 30 minutes. Ten mL of detergent buffer (0.2M NaCl, 1% deoxycholic acid, 1% IGEPAL CA630, 20mM Tris, pH 8.0, 2mM EDTA) per mL of lysis buffer were added and, after vigorous mixing, the insoluble material was sedimented at 5,000 × *g* for 30 minutes. The supernatant was discarded and the pellet was repeatedly washed with 1mM EDTA/0.5% Triton to generate a highly compact pellet, which was solubilized in 8M urea, 10mM Tris, pH 8.0, 1mM EDTA. Protein concentration was determined using Bradford assay (Thermo-Fisher) and bovine serum albumin as the standard.

Recombinant NFL (1mg/mL) was dialyzed overnight in 20mM Tris pH 7.5, 50mM KCl. Kinase reaction was performed by adding 10mM MgCl₂, 200μM ATP and 10000U casein kinase II/mL (control reaction had no kinase added). Reactions were incubated at 30°C for 1 hour. Sample buffer was added to the reactions and incubated at 100°C for 10 minutes.

Mixed neuronal-glia primary cultures

Primary cultures were prepared from P0 C3HBL/6 mouse brains (Harlan Labs). Cerebral cortices were dissected from P0 mouse brains and dissociated in 2mg/mL papain (Worthington) and 50 μg/mL DNAase I (Sigma) in sterile Hank's Balanced Salt Solution (HBSS, Life Technologies) at 37°C for 20 minutes. They were then washed three times in sterile HBSS to inactivate the papain and switched to 5% fetal bovine serum (HyClone) in Neurobasal-A growth media (Gibco), which includes 0.5mM L-glutamine (Gibco), 0.5mM GlutaMax (Life Technologies), 0.01% antibiotic-antimycotic (Gibco), and 0.02% SM1 supplement (Stemcell). The tissue mixture was then triturated three times using a 5 mL pipette followed by a Pasteur pipette, and strained through a 70 μm cell strainer. The cell mixture was then centrifuged at 200 × *g* for 3 minutes, and re-suspended in fresh Neurobasal-A media. They were then plated onto poly-D lysine coated chamber slides (Life Technologies) or dishes at around 100,000–200,000 cells/cm². Cells were maintained in the Neurobasal-A growth media without fetal bovine serum at 37°C in a humidified 5% CO₂ chamber.

Negative Staining Electron Microscopy

αS filaments were absorbed to 300 mesh carbon coated copper grids and stained with 1% uranyl acetate as described previously [36]. Images were captured with a JEOL 1010

transmission electron microscope (Peabody, MA) mounted with a Hamamatsu digital camera (Bridgewater, MA) using AMT software (Danvers, MA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1. Induction of αS pathology at 2 months post intrahippocampal injection of 21–140 hfib αS in M83 Tg mice

(A) Schematic map showing rostral-caudal distribution of αS pathology in M83 Tg mice following hippocampal injection of 21–140 hfib αS. In these mice there was robust induction of αS pathology at the site of injection, but also throughout the CNS. Red dots indicate locations of intrinsic αS pathology observed when M83 Tg mice become symptomatic with aging [33] and blue dots indicate locations of additional αS pathology uniquely induced by hippocampus injection with 21–140 hfib αS. Therefore, in M83 Tg mice injected with 21–140 hfib αS, αS neuronal inclusions were observed in the location indicated by both red and blue dots. Similar density and distribution of αS pathology was seen bilaterally. This distribution of αS inclusions was assessed with both pSer129/81A and Syn506 antibodies. (B) IHC of brainstem tissue sections from a symptomatic 15 month-old M83 Tg mouse and a M83 Tg mouse at 2 months post intrahippocampal injection of 21–140 hfib αS showing pSer129/81A+ inclusions that fill the cell bodies and extend out into the processes along with the presence of dystrophic neurites. These αS inclusions are also readily detected by staining with Syn506 and p62 antibodies. Tissue sections were counterstained with hematoxylin. (C) Double immunofluorescence analysis of the hippocampal region of a M83 Tg mouse at 2 months post intrahippocampal injection of 21–140 hfib αS for pSer129 (green) and SNL-4 (upper panels) or p62 (lower panels) shows that all pSer129/81A+ perikaryal inclusions are also SNL-4+ and that the majority of pSer129/81A+ inclusions are also p62+. Cell nuclei were counter stained with DAPI. Scale bar = 100 μm and 25 μm (inset).

Fig 2. Induction of α S inclusions in nTg mice after intrahippocampal injection of 21–140 hfib α S or full-length mfib α S

(A) Schematic map showing rostral-caudal distribution of α S in nTg mice at 4 months post-injection. Similar density and distribution of α S pathology was seen bilaterally. Inclusions were mainly localized around the site of injection (hippocampus) with sparse pathology seen in the cortex for both human and mouse derived α S amyloidogenic fibrils. (B)

Representative images of IHC in the hippocampal region surrounding the site of injection showing decreasing pSer129/81A pathology at 1 month, 2 months, and 4 months post-injection of hfib α S. Some inclusions reactive with Syn506, a mouse monoclonal antibody that conformationally detects α S aggregates, and p62, a rabbit polyclonal antibody that nonspecifically recognizes protein aggregates, were also observed. Similar staining also shows α S inclusion formation resulting from the hippocampal injection of mfib α S at 2 months post-injection. Some of these α S inclusions are also detected by Syn506 and p62. Tissue sections were counterstained with hematoxylin. Scale bar = 50 μ m.

Fig 3. Characterization and distribution of αS inclusion pathology at 4 months post intrahippocampal injection of 21–140 hfib αS in M47 Tg mice

(A) Diagram shows rostral/caudal distribution of αS inclusion pathology in M47 Tg mice. Tissue sections were stained with antibodies pSer129/81A and Syn506 to detect αS inclusion pathology. Intrahippocampal injections resulted in the induction of αS pathology at the site of injection with some additional pathology in the midbrain and brainstem. Similar density and distribution of αS pathology was seen bilaterally. (B) Brain tissue sections with representative regions of the hippocampus in injected M47 Tg mice show rounded, perinuclear inclusions and neuritic pathology stained with pSer129/81A. Inclusions were also recognized by Syn506, a mouse monoclonal antibody that conformationally recognizes αS inclusions, and p62, a rabbit polyclonal antibody that non-specifically recognizes protein aggregates. (C) Double immunofluorescence analysis of the hippocampal region stained for pSer129/81A (green) and SNL-4 (red; upper panels) or p62 (red; lower panels) shows that most hyperphosphorylated αS inclusions are SNL-4+ and that a large portion are also p62+ in M47 Tg mice injected with 21–140 hfib αS. There is also significant p62 immunoreactivity that does not co-localize with pSer129 indicating the formation of more than one type of protein aggregate. Tissue sections were counterstained with hematoxylin (B) and DAPI (C). Scale bar = 50 μm (B), and 100 μm and 25 μm (C; insets).

Fig 4. Induction of α S induction pathology in M47 Tg mice following hippocampal injection of wild-type, E46K, or A53T hfib α S

(A) Schematic summary of α S pathology distribution at 4 months post intrahippocampal injection of wild-type 21–140 (WT), or full-length E46K or A53T hfib α S in M47 Tg mice. Diagrams show rostral/caudal distribution of α S pathology in M47 Tg mice. Tissue sections were stained with pSer129 and Syn506 to detect α S pathology. (B–D) Immunohistochemistry showing similar α S pathology induced by intrahippocampal injection of WT, E46K, or A53T hfib α S in M47 Tg mice at 4 months post-injection. Brain tissue sections with representative regions of the hippocampus surrounding the injection site in M47 Tg mice injected with WT (B), E46K (C), or A53T (D) hfib α S show rounded, perinuclear inclusions and neuritic pathology regardless of the type of inoculum detected with pSer129/81A antibody. Inclusions were also recognized by Syn506, a mouse monoclonal antibody that conformationally recognizes α S inclusions, and p62, a rabbit polyclonal antibody that non-specifically recognizes protein aggregates. Tissue sections were counterstained with hematoxylin. Scale bar = 50 μ m.

Fig 5. Immunohistochemical analysis of α S immunoreactivity in nTg mice after intracerebral injection with 21–140 hfib α S or 71–82 α S

At one month post-injection of both 21–140 hfib α S (A) and 71–82 α S (D) robust staining with pSer129/81A was seen at the site of injection in the cortex; however, the increased pSer129 immunoreactivity was not detected by other markers of α S pathology, Syn506 or p62. At 2 and 4 months post-injection of 21–140 hfib α S (B, C) and 71–82 α S (E, F) almost all the intense but diffuse perikaryal pSer129 immunoreactivity had subsided. At 4 months post injection of 21–140 hfib α S (C), scattered dense cortical pSer129 reactive inclusions were present in the cortex and rarely in the hippocampus, but most of these were not detected with either Syn506 or p62 antibodies. Tissue sections were counterstained with hematoxylin. Scale bar = 50 μ m.

Fig 6. The ~70 kDa protein recognized by pSer129/81A antibody is NFL phosphorylated at Ser473

(A) Immunoblotting with pSer129/81A and anti-NFL antibody NR4 or Coomassie R250 stained 8% polyacrylamide gels of 2% SDS or 4M urea fractions from the cortical (CX) or cerebellar (CB) white matter of human brain. (B) pSer129/81A antibody immunoprecipitates NFL from human cortical white matter or mouse spinal cord/brain stem.

Immunoprecipitation was performed as described in Material and Methods. Input, unbound, and immunoprecipitated fractions were analyzed by immunoblotting with pSer129/81A and anti-NFL antibody NR4. (C) In situ alkaline phosphatase treatment showing that the pSer129/81A-reactive ~70 kDa protein is a phospho-protein in both human and mouse brain. Nitrocellulose membranes with SDS fractions from human cortical white matter or murine SDS/urea spinal cord and brain stem were incubated without (–) or with (+) alkaline phosphatase (AP) as described in Material and Methods and immunoblotted with pSer129 or anti-NFL NR4 antibodies. (D) Immunoblotting of SDS/urea fractions from nTg/wild-type (WT) and NFL^{–/–} mice with pSer129/81A or anti-NFL NR4 antibodies showing that the ~70 kDa protein band is NFL. (E) *In vitro* casein kinase II phosphorylation of recombinant wild-type (WT) and Ser473Ala NFL followed by immunoblotting with either anti-NFL antibody NR4 or pSer129/81A antibody demonstrating that pSer129/81A antibody

recognized NFL phosphorylated at Ser473. GFAP, glial fibrillary acid protein; NFL, low molecular-mass neurofilament subunit; NFM, mid-sized molecular-mass NF subunit; NFH, heavy molecular-mass NF subunit.

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Fig 7. Immunocytochemical pSer129/81A staining of the white matter tracts in nTg/native mice is absent in NFL^{-/-} mice

(A) White matter tracts of the corpus callosum and internal capsule in nTg mice are detected by staining for NFL. Areas within those tracts are also detected with pSer129/81A, but not by α S marker Syn506, indicating that pSer129/81A is detecting a non- α S target in the tracts. In NFL^{-/-} mice, there is no staining for NFL or pSer129/81A in these white matter tracts. (B) Similarly, white matter tracts in the cerebellum of nTg mice, which are detected by NFL, also stain with pSer129/81A and not Syn506. These are also unstained for NFL and pSer129/81A in NFL^{-/-} mice. Tissue sections were counterstained with hematoxylin. Scale bar = 150 μ m and 50 μ m (inset) for A and B.

Fig 8. pSer129/81A antibody can intensely detect white matter tracts that is not due to α S in human nervous tissue sections

IHC (A) and immunofluorescence (B–D) analyses of fixed CNS tissue from a control subject or a dementia with LBs (DLB) patient stained with pSer129/81A and SNL-4. White matter tracts in the pons of the control subject and DLB patient were detected with pSer129/81A by both IHC (A) and IF (B) staining; however, this reactivity was not detectable when staining for α S with SNL-4 (C). pSer129/81A staining in the gray matter area of the cingulate cortex of a DLB patient depicting cortical LBs (indicated by arrows) detected by both IHC (A) and immunofluorescence analysis (B) that was also detectable with SNL-4 (C, D). Cell nuclei were counter stained with hematoxylin (A) and DAPI (B–D). Scale bar = 100 μ m and 25 μ m (insets).

Table 1

Summary of intracerebral injections of mice.

Strain	Number of mice	Injection site	Inoculum	Time post-injection	Induced pSer129/81A staining	Authentic αS pathology ^a	
						Detection by multiple markers	Spread of αS pathology outside of injection site
M83 (A53T αS)	4	HC	2μL 2mg/mL 21-140WT hfib αS	2 months	yes	yes	yes
M83 (A53T αS)	4	HC	2μL 2mg/mL 71-82 αS	2 months	yes, but limited ^b	no	no
M83 (A53T αS)	4	HC	2μL PBS	2 months	no	no	no
M47 (E46K αS)	6	HC	2μL 2mg/mL 21-140 WT hfib αS	4 months	yes	yes	minimal ^b
M47 (E46K αS)	6	HC	2μL 2mg/mL E46K FL hfib αS	4 months	yes	yes	minimal ^b
M47 (E46K αS)	5	HC	2μL 2mg/mL A53T FL hfib αS	4 months	yes	yes	minimal ^b
C57BL6/C3H	4	HC	2μL 2mg/mL 21-140WT hfib αS	1 month	yes	yes	no
C57BL6/C3H	4	HC	2μL 2mg/mL 21-140WT hfib αS	2 months	yes	yes	no
C57BL6/C3H	4	HC	2μL 2mg/mL 21-140WT hfib αS	4 months	yes	yes	no
C57BL6/C3H	5	HC	2μL 2mg/mL FL WT mfib αS	2 months	yes	yes	no
C57BL6/C3H	4	CTX	2μL 2mg/mL 21-140WT hfib αS	1 month	yes	yes, sparse	no
C57BL6/C3H	4	CTX	2μL 2mg/mL 21-140WT hfib αS	2 months	yes	yes, sparse	no
C57BL6/C3H	4	CTX	2μL 2mg/mL 21-140WT hfib αS	4 months	yes	yes, sparse	no
C57BL6/C3H	4	CTX	2μL 2mg/mL 71-82 αS	1 month	yes	no	no
C57BL6/C3H	4	CTX	2μL 2mg/mL 71-82 αS	2 months	no	no	no
C57BL6/C3H	4	CTX	2μL 2mg/mL 71-82 αS	4 months	no	no	no
C57BL6/C3H	4	CTX	2μL PBS	1 month	no	no	no
C57BL6/C3H	4	CTX	2μL PBS	2 months	no	no	no
C57BL6/C3H	4	CTX	2μL PBS	4 months	no	no	no

^a αS pathology confirmed with multiple αS antibodies and aggregate markers.^b See text.