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Prion-like transmission of α -synuclein pathology in the context of an NFL null background

Nicola J Rutherford^{a,b}, Mieu Brooks^{a,b}, Cara J Riffe^{a,b}, Kimberly-Marie M Gorion^{a,b}, Jasie K Howard^{a,b}, Jess-Karan S Dhillon^{a,b}, and Benoit I Giasson^{a,b,c,*}

^aCenter for Translational Research in Neurodegenerative Disease, University of Florida, Gainesville, FL 32610, USA

^bDepartment of Neuroscience, University of Florida, Gainesville, FL 32610, USA

^cMcKnight Brain Institute, College of Medicine, University of Florida, Gainesville, FL 32610, USA

Abstract

Neurofilaments are a major component of the axonal cytoskeleton in neurons and have been implicated in a number of neurodegenerative diseases due to their presence within characteristic pathological inclusions. Their contribution to these diseases are not yet fully understood, but previous studies investigated the effects of ablating the obligate subunit of neurofilaments, low molecular mass neurofilament subunit (NFL), on disease phenotypes in transgenic mouse models of Alzheimer's disease and tauopathy. Here, we tested the effects of ablating NFL in α -synuclein M83 transgenic mice expressing the human pathogenic A53T mutation, by breeding them onto an NFL null background. The induction and spread of α -synuclein inclusion pathology was triggered by the injection of preformed α -synuclein fibrils into the gastrocnemius muscle or hippocampus in M83 versus M83/NFL null mice. We observed no difference in the post-injection time to motor-impairment and paralysis endpoint or amount and distribution of α -synuclein inclusion pathology in the muscle injected M83 and M83/NFL null mice. Hippocampal injected M83/NFL null mice displayed subtle region-specific differences in the amount of α -synuclein inclusions however, pathology was observed in the same regions as the M83 mice. Overall, we observed only minor differences in the induction and transmission of α -synuclein pathology in these induced models of synucleinopathy in the presence or absence of NFL. This suggests that NFL and neurofilaments do not play a major role in influencing the induction and transmission of α -synuclein aggregation.

*Corresponding author: Benoit I. Giasson, BMS J483/CTRND, 1275 Center Drive, Gainesville, FL 32610, USA, Tel: 352-273-9367, Fax: 352-273-9370, bgiasson@ufl.edu.

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Disclosures

Conflicts of interest: none.

Authors' contributions

NJR prepared total protein lysates and performed immunoblotting analyses, produced and purified recombinant α S proteins, prepared fibrils for injection, performed intramuscular and hippocampal injections, performed immunohistochemistry analyses and was a major contributor in writing the manuscript. MB set up breeding, maintained the mouse colony, and harvested and processed tissue. CJR maintained the mouse colony, and harvested and processed tissue. KMMG analyzed stained tissue. JKH performed hippocampal injections. JKSD performed hippocampal injections. BIG participated in the design of the study and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Keywords

Neurofilaments; α -synuclein; NFL gene (*NEFL*) knockout; Parkinson's disease; transgenic mice; synucleinopathy

Introduction¹

Neurofilaments (NFs) are the major type of intermediate filament that constitute the axonal cytoskeleton in neurons of both the central and peripheral nervous systems [1,2]. NFs provide structural support and scaffolding for organelle organization in addition to maintaining axon caliber [1–3]. NFs are composed of three proteins: the light (NFL), medium (NFM) and heavy (NFH) molecular mass subunits [1,2]. NFL is the obligate subunit of NFs forming either homopolymers, or heteropolymers with NFM or NFH, whereas NFM and NFH are not able to self-assemble and polymerize into mature filaments [4,5]. Despite the neuronal cytoskeletal importance of NFs, both the naturally occurring Japanese quail (quiver), which has a nonsense mutation in the NFL gene (*NEFL*), and mice genetically engineered to ablate the *NEFL* gene are viable [6–8]. Quiver quail display reduced axonal diameter and conduction velocity associated with intermittent quivering [7,9]. On the other hand, NFL null mice do not display any overt neurological phenotype, perhaps due to a compensatory increase in tubulin expression however, they display reduced axonal diameter and impaired sprouting following injury [8].

NFs are known to accumulate into inclusions in a number of neurodegenerative diseases including neurofilament inclusion disease (NFID)[10–14], amyotrophic lateral sclerosis (ALS)[15,16], neuroaxonal dystrophy [17–19], Charcot Marie Tooth (CMT) disease [20], and within Lewy bodies of Parkinson's disease (PD)[21–24] and neurofibrillary tangles (NFTs) of Alzheimer's disease (AD)[25,26]. Whether NFs generally play a causative role in disease, or if they accumulate solely as a result of neuronal impairment or proteostatic imbalance is unknown in most cases. However, in some cases mutations in the *NEFL* and *NEFH* genes can directly cause CMT subtype 2E/1F [2,27–29] or ALS, respectively [2,30,31].

The involvement of NFs in neurodegeneration is an active area of research and has been investigated by breeding transgenic mouse models of AD and tauopathy onto an NFL null background in order to assess the contribution of NFs to the pathogenesis of disease in these particular models [32,33]. Ishihara and colleagues observed delayed development of the typical behavioral phenotype and reduced pathology in mice transgenic for human tau (T44 mice)[34] when they lacked NFs compared to T44 mice with NFs [33] suggesting that NFs,

¹**List of Abbreviations:** A β : amyloid β , AD: Alzheimer's disease, ALS: amyotrophic lateral sclerosis, α S: α -synuclein, BCA: bicinchoninic acid, BSA: bovine serum albumin, CMT: Charcot Marie Tooth, DAB: 3, 3' diaminobenzidine, *E. coli*: *Escherichia coli*, FBS: fetal bovine serum, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, K114: (trans, trans)-1-bromo-2, 5-bis-(4-hydroxy)styrylbenzene, M83: A53T human α S transgenic mice, *NEFH*: heavy molecular mass neurofilament subunit gene, *NEFL*: low molecular mass neurofilament subunit gene, NF: neurofilament, NFH: heavy molecular mass neurofilament subunit, NFID: neurofilament inclusion disease, NFL: low molecular mass neurofilament subunit, NFM: medium molecular mass neurofilament subunit, NFT: neurofibrillary tangle, PBS: phosphate buffered saline, PCR: polymerase chain reaction, PD: Parkinson's disease, pSer129: phosphorylated serine 129, SDS: sodium dodecyl sulfate, *SNCA*: α -synuclein gene, T44: human tau transgenic mice, TBS: Tris buffered saline.

at least in part, contribute to the onset of tauopathy in these mice. However, Fernandez et al observed a worsening of the pathological phenotype in their APP/PS1 (APP^{swe}/PSEN1^{dE9}) transgenic mice on an NFL null background [32,35]. They detected an increase in cortical amyloid- β (A β) deposition and dystrophic neurite pathology along with increased synaptic vulnerability [32], which suggests that NFs may play a protective role in this mouse model. These two studies show opposing effects of the deficiency of NFL, and therefore NFs, on the disease phenotype in their models of neurodegeneration.

A role for NFs in influencing the induction and prion-like spread of α -synuclein (α S) inclusion pathology is anticipated since 1) although α S is the major component of Lewy pathology in PD and Lewy body dementia [36–38], NFs are also a component of Lewy pathology [21–24], 2) phosphorylation of α S at Ser129, which is a sensitive marker of α S inclusion formation, shares close molecular homology with the major in vivo phosphorylation site in NFL [39], and 3) NFs play a critical role in maintaining axonal diameter and conductivity [6–9]. Herein, we investigate the role of NFs in the context of synucleinopathies by assessing the effect of NFL paucity on the onset and severity of disease in α S transgenic mouse models of synucleinopathies in which we induced α S prion-like aggregation through the injection of preformed α S fibrils.

Materials and Methods

Mouse Lines

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. M83 transgenic mice express human α S with the pathogenic A53T mutation on the C57BL/6 background and were previously described [40]. NFL null mice on the C57BL/6 background were a kind gift from Dr. Janice Robertson and were previously described [8]. In order to generate M83 hemizygous NFL null mice, male M83 homozygous mice were bred with female NFL null mice to produce M83^{+/-}/NFL^{+/-} offspring (F1). Male F1 mice were then mated with female NFL null mice, to produce M83^{+/-}/NFL null mice (M83/NFL null; predicted 25% of F2). Genotypes were determined by PCR of mouse tail snips using the Extract-N-Amp Tissue PCR kit according to manufacturer's protocol and specific primers. Primers were designed to *SNCA* (Forward: 5' GTGTAGCAGAAGCAGCAGGAAAGACA 3', Reverse: 5' GGCTTCAGGTTTCGTAGTCTTGA 3') and *NEFL* (Forward: 5' GAAGCCGAGCTGTTGGTGCTG 3', Reverse: 5' TGGATCTGAGCCTGCAGCTCG 3').

Antibodies

SNL1 is a rabbit polyclonal antibody raised against residues 104–119 of α S [41]. NR4 is a mouse monoclonal NFL antibody (Sigma-Aldrich Cat# N5139, RRID:AB_477276). Mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Meridian Life Science Cat# H86504M, RRID:AB_151542) and anti-actin clone C4 (Millipore Cat# MAB1501, RRID:AB_2223041) were used as loading controls. LS4-2G12 is a mouse monoclonal antibody that detects α S phosphorylated at Ser129 (pSer129), and does not cross-react with phosphorylated NFL [39]. 9C10 is a mouse monoclonal antibody raised

against the N-terminus of α S. Rabbit polyclonal antibody, p62 (SQSTM1) is a general inclusion marker (Proteintech Group Cat# 18420-1-AP, RRID:AB_10694431).

Immunoblotting of Total Protein Lysates of Mouse Spinal Cord

Mice were humanely euthanized and spinal cords were harvested. Tissues were homogenized in 2 % sodium dodecyl sulfate (SDS)/50 mM Tris, pH 7.5 using a pestle, followed by probe sonication. Samples were then incubated at 100°C for 10 mins. Protein concentrations were determined by bicinchoninic acid (BCA) assay using bovine serum albumin (BSA; Pierce) as the protein standard. SDS sample buffer was added to tissue homogenates and equal amounts of protein (5 μ g) were loaded onto 13 % polyacrylamide gels. Protein samples were resolved and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked with 5 % dry milk/Tris buffered saline (TBS) and incubated in primary antibody diluted in 5 % dry milk/TBS overnight at 4°C. Following washes, membranes were incubated in horse radish peroxidase conjugated goat anti-mouse or donkey anti-rabbit secondary antibodies for 1 hour. Protein bands were visualized using Western Lightning-Plus ECL reagents (PerkinElmer) and imaged on a GeneGnome XRQ system using GeneTools software (Syngene).

Recombinant α S Expression, Purification and Fibril Formation

Bacterial expression vector, pRK172, containing cDNA encoding human or mouse full-length α S was transformed into BL21 (DE3)/RIL *Escherichia coli* (*E. coli*; Agilent Technologies)[42,43]. Recombinant α S was purified from *E. coli* by size exclusion chromatography and subsequent anion exchange as previously described [42,43]. Protein concentrations were determined by BCA assay using BSA as the protein standard. Recombinant α S (5 mg/mL in sterile phosphate buffered saline (PBS)) was incubated at 37°C with constant shaking at 1050 rpm (Thermomixer R, Eppendorf) for >48 hours. Fibril formation was monitored by (trans, trans)-1-bromo-2, 5-bis-(4-hydroxy)styrylbenzene (K114) fluorometry as previously described [44]. Fibrils were diluted to 2 mg/mL in sterile PBS and gently sonicated in a water bath for 2 hours, prior to injection.

Mouse Injection Procedures

Hemizygous M83 mice do not intrinsically develop α S pathology until 22–28 months of age [40] therefore, injections were performed on mice that were ~3 months of age. For stereotaxic injection, M83 and M83/NFL null mice (2 – 3 months of age) were anesthetized with 1–5 % isoflurane and bilaterally injected in the hippocampus (coordinates from Bregma: anterior/posterior –2.2, lateral $\pm$ 1.6, dorsal/ventral –1.2) with 2 μ L of human α S fibrils (2 mg/mL; n=10/group) or PBS (n=5; M83/NFL null only) at a rate of 0.2 μ L/min. Mice were sacrificed at 4 months post-injection.

For muscle injection, M83 and M83/NFL null mice (2 – 2.5 months of age) were anesthetized with 1–5 % isoflurane and bilaterally injected in the gastrocnemius muscle with 5 μ L of mouse α S fibrils (2 mg/mL; n=10/group) or PBS (n=10; M83/NFL null only). The muscle injected mice were monitored for the development of paralysis before being sacrificed. PBS muscle injected mice were sacrificed at 6 months post-injection. Mice were humanely euthanized by CO₂, followed by cardiac perfusion of PBS/heparin. Brain and

spinal cord were harvested and fixed in 70 % ethanol/150 mM NaCl. Tissues were dehydrated and infiltrated with paraffin, then cut into 5 µm sections using a microtome.

Immunohistochemistry Analyses

Sections were deparaffinized with xylene then rehydrated with a descending series of ethanols (100 – 70 %). For antigen retrieval, sections were incubated in a steam bath in 0.05 % Tween-20 for 30 mins, and then endogenous peroxidase activity was quenched with 1.5 % hydrogen peroxide/0.005 % Triton X-100/PBS for 20 mins. Sections were blocked with 2 % fetal bovine serum (FBS)/100 mM Tris, pH 7.6, and then incubated in primary antibody diluted in 2 % FBS/100 mM Tris, pH 7.6 overnight at 4°C. The following day, sections were incubated in biotinylated horse anti-mouse or anti-rabbit secondary antibodies (Vector Laboratories) diluted in 2 % FBS/100 mM Tris, pH 7.6 for 1 hour at room temperature. Sections were then incubated in tertiary antibody (VECTASTAIN ABC kit, Vector Laboratories) diluted in 2 % FBS/100 mM Tris, pH 7.6, for 1 hour and staining was developed using the chromogen 3, 3' diaminobenzidine (DAB). Sections were counterstained with Mayer's hematoxylin then dehydrated using an ascending series of ethanols (70 – 100 %) followed by xylene. Sections were then coverslipped using cytooseal. When dry, sections were scanned using an Aperio ScanScope CS (40x magnification; Aperio Technologies Inc.).

Results

In this study, we aimed to assess the involvement of NFs in the induction and progression of αS inclusion pathology using a mouse model of synucleinopathy. To accomplish this, we utilized mice that are transgenic for A53T human αS (M83 line)[40] and bred them with NFL null mice [8] to produce hemizygous M83 mice on an NFL null background (M83/NFL null). As NFL is an obligate subunit of NFs, NFL null mice display a paucity of NFs [8]. To determine the genotype of these mice we performed PCR analysis using primers designed against *SNCA* and *NEFL* (Figure 1A). We confirmed the absence of NFL and the expression of the αS transgene in the M83/NFL null mice using immunoblot analysis of total protein lysates from spinal cord (Figure 1B). The M83/NFL null mice appeared normal, although they were smaller than their M83 littermates (at 2–2.5 months of age; overall: 21.0 g versus 29.8 g, males: 24.1 g versus 37.3 g, females: 18.2 g versus 24.2 g).

We next wanted to investigate whether the induction and transmission of αS pathology following injection of αS fibrils were altered in M83/NFL null versus M83 mice. The intramuscular and intracerebral injection of αS fibrils to induce early αS pathology are well established experimental paradigms [45–48]. For muscle injection, mice were injected bilaterally in the gastrocnemius muscle with mouse αS fibrils and aged until the appearance of motor impairment leading to paralysis. Of the 10 mice per group, 1 from the M83/NFL null group and 3 from the M83 group had to be sacrificed early due to fight wounds, leaving 9 and 7 mice for analysis in each respective group. The period of time until endstage for the M83/NFL null mice intramuscularly injected with mouse αS fibrils did not differ from M83 mice (Figure 2A; Log rank test, $p=0.662$), with all mice succumbing between 80 and 100 days post-injection. Immunohistochemical analysis of nervous tissue revealed a very similar

pattern and amount of α S inclusion pathology in both groups of mice (Figure 2B and C), with pSer129- α S (LS4-2G12) immunopositive inclusions predominantly in the spinal cord, brainstem, midbrain, periaqueductal grey region, and thalamus. The authenticity of α S inclusions was confirmed by immunostaining with anti-N-terminal α S antibody 9C10 and inclusion marker p62. M83/NFL null mice injected with PBS exhibited no motor symptoms and did not develop α S pathology by 6 months post-injection (Supplemental Figure 1). Our previous study demonstrated that hemizygous M83 mice do not develop α S pathology up to 6 months post muscle injection of control proteins including keyhole limpet hemocyanin and β -synuclein [49].

For intracerebral injection, mice were injected bilaterally in the hippocampus and sacrificed at 4 months post-injection. Immunohistochemical analysis revealed more abundant LS4-2G12 immunopositive α S inclusions in the hippocampus of M83/NFL null mice compared to M83 mice (Figure 3). The majority of pathology was neuritic in the hippocampus of M83/NFL null mice whereas a higher proportion of inclusions in the M83 mice were perikaryal. In both groups of mice, both 9C10 and p62 antibodies also stained α S inclusions in the hippocampus, but less robustly than LS4-2G12, though this was more evident in M83/NFL null mice, due to the greater density of α S inclusions. Looking at the global induction of α S pathology, α S inclusions were present at the same locations in both groups of mice however, the amount of inclusions varied (Figure 3B and 4). In M83 mice, pathology was abundant in the brainstem, thalamus and spinal cord, where it was more modest in M83/NFL null mice. In M83/NFL null mice there were greater numbers of inclusions in the hippocampus and cortex than in the M83 mice. All M83/NFL null mice injected in the hippocampus with PBS showed no α S pathology at 4 months post-injection (Supplemental Figure 2).

Discussion

In this study we sought to investigate the contribution of NFLs to the induction of disease in a transgenic mouse model of synucleinopathy by ablating NFL. Previous studies have used this experimental approach to investigate the role of NFLs in different transgenic mouse models of neurodegenerative disease however, the resulting phenotype alterations have been disparate [32,33]. Interestingly, tau transgenic mice (T44) showed a delay in disease onset [33] whereas APP/PS1 transgenic mice displayed increased levels of pathology and synaptic vulnerability when NFL was absent [32]. In the present study, we observed no major differences in the onset of disease or α S pathology in human A53T α S transgenic (M83) mice [40] with or without NFL following α S fibril seeding from intramuscular injection.

We employed two well characterized α S fibril injection models to induce α S aggregation in these mice [45–48]. For the muscle injection study, we allowed the mice to age and develop a motor phenotype as an indicator of disease. The time from injection to paralysis did not differ between the M83 and M83/NFL null groups, and the resulting α S pathology was indistinguishable. The detection of pathological α S was performed using our recently developed pSer129 antibody, LS4-2G12 [39]. This antibody does not cross-react with NFL, like the commonly used clone 81A [47], allowing us to distinguish between the α S and NFL in the M83 mice. At 4 months post-intrahippocampal injection of α S fibrils, both groups of

mice displayed α S pathology throughout the neuraxis however, inclusions were more concentrated in the brainstem and thalamus of M83 mice while M83/NFL null mice had a greater proportion of α S inclusions in the hippocampus and cortex. Additionally, neurites appeared to be the favored morphology of α S inclusion in the hippocampus for M83/NFL mice however, this preference did not continue throughout the rest of the nervous system.

Altogether, the 3 studies of transgenic mice of neurodegenerative disease lacking NFs have yielded very different results, suggesting that the overexpressed proteins in these models cause disease, or interact with NFL, in different ways. The APP/PS1 transgenic mice [35], overexpressing A β and presenilin 1 primarily develop A β plaques, and suffered from a worse phenotype when on an NFL null background [32]. T44 mice, transgenic for tau [34], develop hyperphosphorylated tau inclusions and are a model for tauopathy, but showed a later onset and less severe disease phenotype when NFL was absent [33]. Finally, the M83 line of α S transgenic mice [40] showed the same motor impairment and similar α S pathology following induction by α S fibril injection, independent of the presence of NFL.

These results suggest that NFL, or NFs, may contribute to tauopathy, acting as pathological chaperones for tau aggregation [33], but may actually be protective when it comes to A β , somehow preventing the accumulation of pathological A β [32]. Regardless of NFs' role(s) in these models of disease, we did not see a similar result in our experiments, signifying that NFs do not influence the aggregation of α S. This was surprising, as NFs are a major component of the Lewy pathology that is characteristic of synucleinopathies [21,24]. Even if NFs do not directly contribute to the formation of α S inclusions, the vast amount of NFs in neurons puts a huge burden on the proteostasis machinery of the cell, merely to maintain the routine turnover of the NF triplet proteins. Therefore, removing this encumbrance should render the neurons better able to deal with the continuous presence of misfolding proteins, preventing the buildup and aggregation of said proteins within inclusions. In addition, NFL null mice have significantly reduced axonal diameters which may indicate slower axonal transport [8], but this does not seem to have a major impact on the seeding and transmission of α S pathology, as intramuscular injection of α S fibril seeds for the induction of disease in M83 mice resulted in paralysis with the same time of onset with or without NFL.

Despite the presence of NFs in many hallmark inclusions of different neurodegenerative diseases, their contribution to the pathogenesis in disease models is not equivalent. In addition, increased levels of NFL in peripheral fluids (i.e. blood and cerebrospinal fluid) has emerged as a biomarker for some synucleinopathies and other neurodegenerative diseases [50–52], although the mechanism responsible for this increase is poorly understood. The identification of factors that either cause/help or protect against the progression of disease is important, as they may be suitable targets for therapeutic intervention, by either downregulating or upregulating respectively to enhance the positive effects. In the case of M83 α S transgenic mice, the lack of NFL, and therefore NFs, did not alter the onset or severity of α S pathology or motor phenotype from peripheral induction, indicating that NFs are not a critical modulator of synucleinopathy prion-like transmission, although more subtle differences in the distribution of pathology was observed in the direct brain injection model.

Conclusions

In conclusion, even though removing NFs through the ablation of NFL has been shown to modify phenotypes in other mouse models of neurodegenerative disease, this is not the case for M83 α S transgenic mice. These results suggest that NFL and NFs are not likely to be a suitable target for therapeutic intervention of synucleinopathies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Seeding of α S pathology in A53T human α S transgenic mice on an NFL null background
- Cerebral prion-like transmission of α S is maintained in an NFL null environment
- Paucity of neurofilaments does not influence peripheral to CNS transmission of α S

Figure 1. Generation of M83 transgenic mice on an NFL null background

(A) Genotyping analyses to confirm the presence of the *SNCA* transgene and absence of *NEFL*. Sizes are in base pairs. (B) Western blot analysis of total protein lysates from spinal cord of wild-type (WT), M83, NFL null and M83/NFL null mice. Antibody NR4 shows the presence/absence of NFL. Antibody SNL1 shows the overexpression of α S. GAPDH and β -actin are shown as loading controls. Molecular weights are in kDa.

Figure 2. Assessment of motor impairment and αS inclusion pathology of M83/NFL null mice versus M83 mice following intramuscular injection of αS fibrils

(A) Survival curve of M83 and M83/NFL null mice following intramuscular injection of mouse αS fibrils (n=7 and 9, respectively) or PBS (n=10; M83/NFL null mice only). Curves of mouse αS fibril injected mice are not significantly different (Log-rank test; p=0.662). (B) Distribution maps of LS4-2G12 immunopositive αS pathology in mouse αS fibril muscle injected M83 and M83/NFL null mice. (C) Representative images of immunohistochemistry staining of αS pathology in the spinal cord, brainstem and thalamus of mouse αS fibril muscle injected M83 and M83/NFL null mice. LS4-2G12 detects pSer129 αS, 9C10 detects the N-terminus of αS and p62 is a general inclusion marker. Scale bars = 100μm.

Figure 3. Hippocampal αS pathology in M83/NFL null mice versus M83 mice following intrahippocampal injection of αS fibrils

(A) Representative images of immunohistochemistry staining of hippocampi of M83 and M83/NFL null mice 4 months following intrahippocampal injection of human αS fibrils. Scale bar = 300μm. (B) Distribution maps of LS4-2G12 immunopositive αS inclusion pathology in hippocampal injected M83 and M83/NFL null mice (n=10/group).

Figure 4. Representative α S inclusion pathology in M83/NFL null mice versus M83 mice following intrahippocampal injection of α S fibrils

(A) Representative images of immunohistochemistry staining of the entorhinal cortex, thalamus, brainstem and spinal cord of M83 and M83/NFL null mice 4 months following intrahippocampal injection of human α S fibrils. Scale bars = 100 μ m.