

Intramuscular injection of α -synuclein induces CNS α -synuclein pathology and a rapid-onset motor phenotype in transgenic mice

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It has been hypothesized that α -synuclein (α S) misfolding may begin in peripheral nerves and spread to the central nervous system (CNS), leading to Parkinson disease and related disorders. Although recent data suggest that α S pathology can spread within the mouse brain, there is no direct evidence for spread of disease from a peripheral site. In the present study, we show that hind limb intramuscular (IM) injection of α S can induce pathology in the CNS in the human Ala53Thr (M83) and wild-type (M20) α S transgenic (Tg) mouse models. Within 2–3 mo after IM injection in α S homozygous M83 Tg mice and 3–4 mo for hemizygous M83 Tg mice, these animals developed a rapid, synchronized, and predictable induction of widespread CNS α S inclusion pathology, accompanied by astrogliosis, microgliosis, and debilitating motor impairments. In M20 Tg mice, starting at 4 mo after IM injection, we observed α S inclusion pathology in the spinal cord, but motor function remained intact. Transection of the sciatic nerve in the M83 Tg mice significantly delayed the appearance of CNS pathology and motor symptoms, demonstrating the involvement of retrograde transport in inducing α S CNS inclusion pathology. Outside of scrapie-mediated prion disease, to our knowledge, this finding is the first evidence that an entire neurodegenerative proteinopathy associated with a robust, lethal motor phenotype can be initiated by peripheral inoculation with a pathogenic protein. Furthermore, this facile, synchronized rapid-onset model of α -synucleinopathy will be highly valuable in testing disease-modifying therapies and dissecting the mechanism(s) that drive α S-induced neurodegeneration.

amyloid | Parkinson disease

Synucleinopathies are a group of diseases defined by the presence of amyloidogenic α -synuclein (α S) inclusions that can occur in neurons and glia of the central nervous system (CNS) (1–4). In Parkinson disease (PD), a causative role for α S has been established via the discovery of mutations in the α S gene *SNCA* resulting in autosomal-dominant PD (4–11). Although α S inclusions (e.g., Lewy bodies) are the hallmark pathology of PD, how they contribute to disease pathogenesis remains controversial (1, 3, 4, 12).

Postmortem studies have suggested that α S pathology may spread following neuroanatomical tracts (13–15) and between cells (16–18). α S pathology has also been found in the peripheral nervous system (PNS): for example, in the enteric and pelvic plexus (19, 20). And it has been suggested that α S pathology might originate in the nerves of the PNS and spread to the CNS (14). Experimentally, it has been reported that intracerebral injections of preformed amyloidogenic α S fibrils in nontransgenic (nTg) and α S transgenic (Tg) mice induce the formation of intracellular α S inclusions that appear to progress from the site of injection (21–26). Collectively, these studies support the notion that α S inclusion pathology may propagate via a prion-like conformational self-templating mechanism (27, 28). A caveat of

the direct intracerebral injection of α S is that this CNS invasive surgical procedure directly alters brain homeostasis that could influence or facilitate the formation of brain pathologies, especially because incidents such as traumatic brain injury can promote the formation of α S pathology (29). Here, we report that the intramuscular (IM) injection of fibrillar (fib) α S in M83 Tg mice expressing human Ala53Thr (A53T) α S can result in the rapid and synchronized development of hind limb motor weakness and robust widespread CNS α S pathology. Additionally, similar injection into M20 Tg mice expressing human wild-type α S, which do not intrinsically develop pathology, leads to the induction of CNS α S pathology as early as 4 mo postinjection.

Results

Native M83^{+/+} Tg homozygous mice show signs of motor impairment from 8 to 16 mo of age, with an arched back as the initial disease presentation that progresses to quadriplegia and a moribund state requiring euthanasia within 2 wk (30). The presentation of this phenotype is associated with the formation of α S inclusion pathology throughout most of the spinal cord and brainstem, but this phenotype never occurs spontaneously before 7 mo of age, with a median age of onset of ~12 mo of age (30, 31). We performed IM injections with various α S preparations into hind-limb muscles of 2-mo-old M83^{+/+} Tg mice and monitored them for disease (Fig. 1 and Fig. S1). Initially, 10 μ g of recombinant full-length mouse fibrillar α S (mfib), 21–140 fibrillar human α S (hfib21–140), human Δ 71–82 α S, or lipopolysaccharide

Significance

α -Synuclein (α S) inclusions are a hallmark of many progressive neurodegenerative disorders. Previously, intracerebral injection of exogenous preformed fibrillar α S in mouse models was shown to induce neuronal α S aggregation—a finding that has been interpreted as a prion-like mechanism. We now show that α S inclusion pathology can be induced in the brain and spinal cord of α S transgenic mice by a single peripheral intramuscular injection of α S. The formation of α S inclusions occurred concurrently with the presentation of a motor impairment in mice expressing mutant Ala53Thr human α S. This new model of robust and predictable induction of α S pathology will be especially valuable to further study the pathogenic mechanisms and assessment of therapeutic interventions.

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(LPS; 25 μ g) were injected bilateral in an upper hind limb muscle (biceps femoris muscles). Both m α S and h α S21-140 α S are amyloidogenic, seeding α S aggregation in vitro, in culture, and in vivo. The N-terminal truncated 21–140 α S h α S21-140 protein was used because it enables assessment of aggregation by endogenous α S by detection with N-terminal-specific α S antibodies

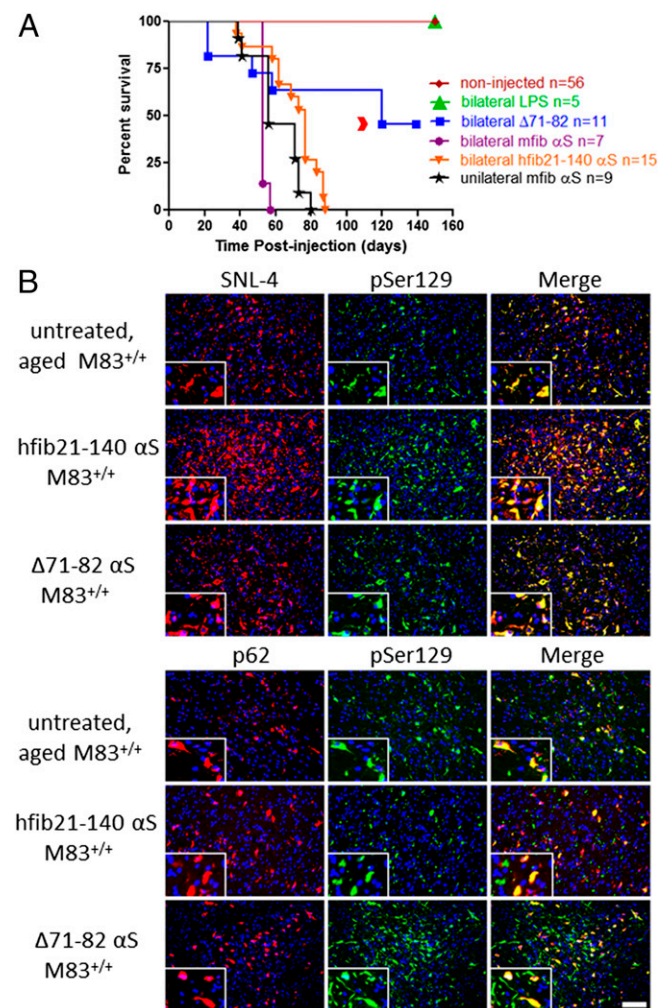


Fig. 1. α S IM injection reduces survival in $M83^{+/+}$ Tg mice. (A) Kaplan–Meier survival plot shows decreased survival time (due to death or euthanasia because of paralysis) for $M83^{+/+}$ Tg mice bilaterally IM-injected (biceps femoris muscle) with h α S21-140 α S compared with identical IM injection with $\Delta 71$ –82 α S or LPS, and noninjected age-matched $M83^{+/+}$ Tg mice. Median times to moribund state were as follows: within 88 dpi (median 62 dpi) for mice IM-injected bilaterally with fibrillar (m α S and h α S21-140) α S ($n = 22$); within 80 dpi (median 56 dpi) for those injected unilaterally with m α S ($n = 9$); and within 120 dpi for phenotypic mice injected with $\Delta 71$ –82 α S ($n = 6$); the red arrowhead indicates time to moribund state for the two mice with foot drop/paralysis. Mice IM-injected with LPS ($n = 5$) and noninjected $M83^{+/+}$ Tg mice ($n = 56$) remained disease free ($P < 0.0001$; χ^2 , 123.7; df, 5). All mice IM-injected bilaterally (biceps femoris muscle) with m α S died or had to be killed due to paralysis within 57 dpi ($n = 7$; median 53 dpi) and with h α S21-140 α S within 88 dpi ($n = 14$; median 77 dpi) ($P < 0.01$; χ^2 , 10.62; df, 1). (B) Double immunofluorescence analyses of midbrain region from an untreated, aged (11-mo-old) movement-impaired $M83^{+/+}$ Tg mouse, an $M83^{+/+}$ Tg mouse that is 2 mo postinjection with 10 μ g of h α S21-140 α S, and an $M83^{+/+}$ Tg mouse that is 4 mo postinjection with 10 μ g of $\Delta 71$ –82 α S. Anti-pSer129-labeled α S inclusions (green) were also detected with the anti-N-terminal α S antibody SNL-4 (red, Upper) and with an antibody to p62, a nonspecific intracellular inclusion marker (red, Lower). Tissue sections were counterstained with DAPI (blue). (Scale bar: B, 100 μ m; Insets, 25 μ m.)

(32–35). Nonamyloidogenic human $\Delta 71$ –82 α S has a deletion of α S required for amyloid formation and lacks the ability to form or seed α S amyloid in vitro and in cultured cells under relatively physiologic conditions (32, 33, 36, 37). IM injection of human $\Delta 71$ –82 α S was used to test the hypothesis that induction of α S pathology occurs through amyloidogenic conformational templating. LPS was used as a control for potential inflammatory effects induced by α S (38–40). Forty to 90 d post IM injection (dpi) with h α S21-140 or m α S, $M83^{+/+}$ Tg mice developed a unilateral foot drop that progressed rapidly to a bilateral foot drop followed by full hind limb paralysis within a week of onset (Fig. 1 and Movie S1). Mice reached a terminal state at a median of 53 d and 77 d postinjection for m α S and h α S21-140 α S, respectively. Although $M83^{+/+}$ Tg mice injected with m α S showed an early disease onset (Fig. 1 and Table S1), there was no detectable difference in disease progression. Unilateral IM injection of m α S in a lower hind limb muscle (gastrocnemius) of $M83^{+/+}$ Tg mice also resulted in the same induction of phenotype, albeit delayed compared with the bilateral IM-injected mice (Fig. 1 and Table S1).

Mice injected with fibrillar α S developed α S inclusion pathology that was nearly indistinguishable morphologically in anatomic distribution from that seen in aged (>8 mo old) untreated $M83^{+/+}$ Tg mice (Fig. 2 and Fig. S1) (30). In contrast to IM injection of fibrillar α S, no α S inclusion pathology or any overt behavioral changes were observed in mice injected with LPS (Fig. 1 and Table S1). α S inclusion pathology in mice injected with preformed fibrillar α S was robust in the spinal cord, brainstem, and midbrain structures, but sparse in the cortex (Fig. 2). There was a high density of α S pathology in the midbrain, but inclusions were rarely observed in dopaminergic neurons (Fig. S2). The α S inclusions were composed of endogenous α S, as shown by reactivity with anti-N-terminal α S antibodies Syn506 and SNL-4, and they are also strongly reactive with p62 antibodies, a nonspecific marker of intracellular protein aggregates (Fig. 1 and Fig. S1) (41). α S inclusions were not found in the sciatic nerve or muscle injection site (Fig. S3). It is possible that the paucity of α S aggregates at these sites is due to only a small quantity of α S accumulating at these compartments, or that they have been degraded or even truncated by the time of examination. Nevertheless, these data indicate that a peripheral IM h α S21-140 or m α S injection dramatically accelerates onset of disease in the $M83^{+/+}$ Tg model, with complete penetrance in this study.

Compared with results with amyloidogenic forms of α S, IM injection of the nonamyloidogenic $\Delta 71$ –82 resulted in delayed onset of disease and incomplete penetrance of the accelerated pathology. Six of 11 $\Delta 71$ –82 α S IM-injected $M83^{+/+}$ Tg mice became moribund, with 2 of these 6 developing a motor phenotype at 120 dpi indistinguishable from that observed in the fib- α S-injected mice. The phenotype in the additional 4 moribund mice was distinct as these mice had a more generalized muscle weakness but without hind leg drop foot. The 2 mice with the hind leg paralysis plus foot drop and 3 other mice killed at 139 dpi without motor signs showed α S pathology similar to untreated, aged $M83^{+/+}$ Tg mice (Figs. 1 and 2, Fig. S1, and Table S1). In contrast, the additional 4 moribund $M83^{+/+}$ Tg mice IM injected with $\Delta 71$ –82 α S did not present with any detectable α S inclusion pathology.

To compare induction of a motor phenotype and α S pathology in a model with lower endogenous expression of α S, we performed the same IM injections in $M83^{+/-}$ Tg hemizygous mice. The $M83^{+/-}$ Tg mice express A53T human α S throughout the neuroaxis similarly to $M83^{+/+}$ Tg mice but at $\sim 60\%$ the expression level, and they do not endogenously develop α S inclusion pathology or motor impairments before 18 mo of age (30). We initially performed bilateral IM injections of fibrillar α S in $M83^{+/-}$ Tg mice to test that the same motor phenotype could be induced (Fig. 3 and Table S1). Bilateral or unilateral injection of fib α S in $M83^{+/-}$ Tg mice resulted in an identical progressive degenerative motor phenotype to that seen in injected

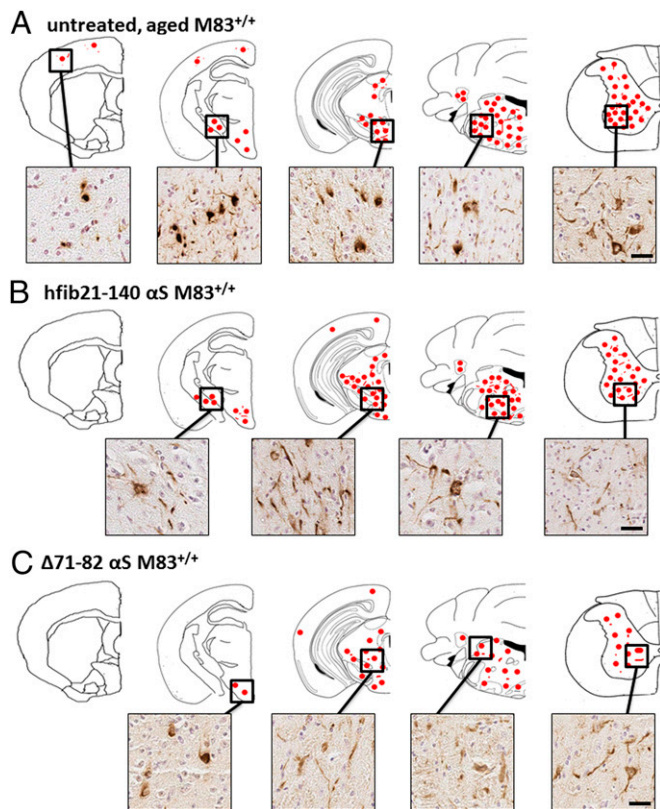


Fig. 2. α S pathology in $M83^{+/+}$ Tg mice IM-injected with α S is similar to that seen in untreated, aged $M83^{+/+}$ Tg mice. Schematic representation of the distribution of CNS α S inclusion pathology (depicted as red dots) detected by pSer129 staining for hyperphosphorylated α S and that was also confirmed with Syn506 staining, an anti-N-terminal α S antibody that conformationally detects α S inclusions (57) and p62, an antibody that nonspecifically recognizes intracellular protein aggregates (41) (Fig. S1). Representative immunohistochemical (IHC) images of α S inclusion pathology from various regions are shown. (A) α S inclusion pathology distribution in untreated, aged (>11 mo old) movement-impaired $M83^{+/+}$ Tg mice. α S pathology is abundant in the spinal cord, brainstem, and midbrain areas and rare in the cortex and absent in the hippocampus (30). $M83^{+/+}$ Tg mice IM-injected with 10 μ g of hfib21-140 α S (B) or 10 μ g of Δ 71-82 α S (C) at 4 mo of age and 6 mo of age, respectively, depicted similar bilateral pathology (also see Fig. S1). (Scale bars: 50 μ m).

$M83^{+/+}$ Tg mice, but with a delayed onset (Fig. 3A and Table S1). The distribution and density of α S pathology induced in all $M83^{+/+}$ Tg mice at the time of demise was also identical to that seen in $M83^{+/+}$ Tg mice.

To determine whether induction of α S pathology in the brain and spinal cord postinjection was due to neuroinvasion of the injected α S via retrograde transport through the sciatic nerve (the major nerve that innervates lower leg muscles), we performed complete transection of the left sciatic nerve 3 d before injection of 10 μ g of mfb α S in the left gastrocnemius muscle in $M83^{+/+}$ Tg mice. Sciatic nerve transection significantly delayed and, in some mice, perhaps completely prevented the CNS neuroinvasion of the IM-injected α S (Fig. 3 and Table S1). Three of the seven mice on which we performed this procedure still developed motor impairment, with CNS α S pathology identical to mice without nerve transection, but the onset was dramatically delayed (Fig. 3 and Table S1). Four of the seven mice that had sciatic nerve transection followed by fibrillar α S muscle injection showed no motor deficits 200 d post IM injection. These data implicate retrograde axonal transport as the predominant mechanism for CNS neuroinvasion of the IM-injected α S.

IM α S injection studies were also conducted in $M20$ Tg mice that overexpress human wild-type α S but never intrinsically develop a phenotype or α S inclusion pathology (30, 31). Because $M20^{+/+}$ Tg hemizygous mice express human α S at similar levels and neuroanatomical distribution as $M83^{+/+}$ Tg mice (30), we performed all these studies with $M20^{+/+}$ Tg mice for more appropriate comparison. In contrast to the $M83$ Tg mice, IM-injected $M20^{+/+}$ Tg mice did not develop any overt motor phenotype; however, beginning at 4 mo postinjection, rare α S inclusions were detected in the spinal cord α S antibodies, but these aggregates were not detected with antibodies to the general inclusion marker, p62 (41). At 8 and 12 mo after IM injection of hfib21-140 α S, induction of α S inclusion pathology was observed in the CNS, albeit less robustly than in $M83$ Tg mice (Fig. 4, Fig. S1, and Table S2). α S pathology in hfib21-140 α S IM-injected $M20^{+/+}$ Tg mice was predominantly found in neuronal processes and some cell bodies in the periaqueductal gray area of the midbrain and in white and gray matter at all levels of the spinal cord. Although α S pathology was found in the midbrain brain, inclusions were rarely found in dopaminergic neurons

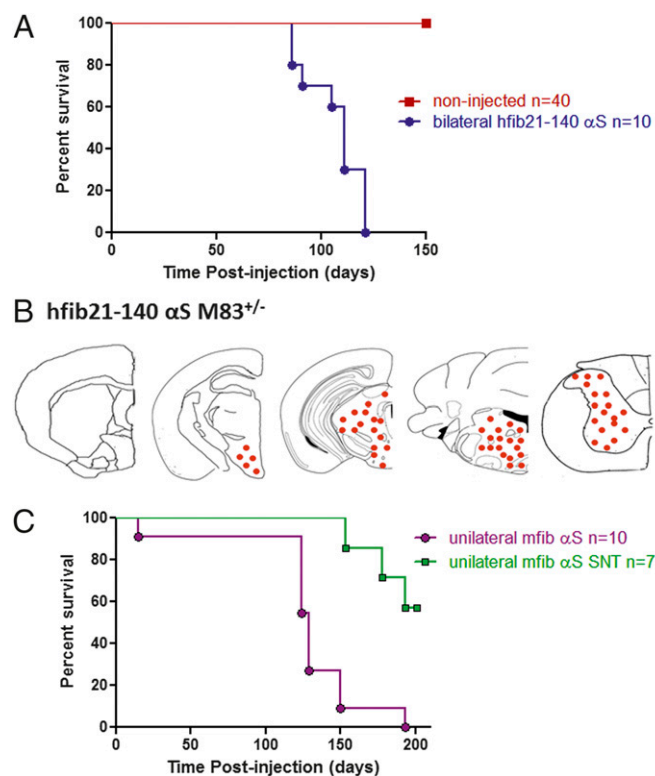


Fig. 3. α S IM injection reduces survival in $M83^{+/+}$ Tg mice. (A) Kaplan-Meier survival plot shows a decreased time to terminal state (killed due to paralysis) for $M83^{+/+}$ Tg mice injected bilaterally (gastrocnemius muscle) with 10 μ g of hfib21-140 α S ($n = 10$) compared with noninjected $M83^{+/+}$ Tg mice ($n = 40$). All injected mice reached a terminal state (paralysis) within 121 d postinjection (median 111 d; $P < 0.0001$; χ^2 , 67.58; df, 1). (B) Schematic representation of the distribution of CNS α S inclusion pathology (depicted as red dots) in $M83^{+/+}$ Tg mice detected by pSer129 staining for hyperphosphorylated α S and that was also confirmed with Syn506 staining, an anti-N-terminal α S antibody that conformationally detects α S inclusions (54) and p62, an antibody that nonspecifically recognizes intracellular protein aggregates (41). (C) All $M83^{+/+}$ Tg mice unilaterally injected (gastrocnemius muscle) with 10 μ g of mfb α S reached a terminal state (killed due to paralysis) within 193 d postinjection (median 129 d). However, in $M83^{+/+}$ Tg mice in which the sciatic nerve was transected (3 d) preinjection, the time to terminal state was delayed past the window seen with the nontransected controls ($P < 0.001$; χ^2 , 12.01; df, 1).

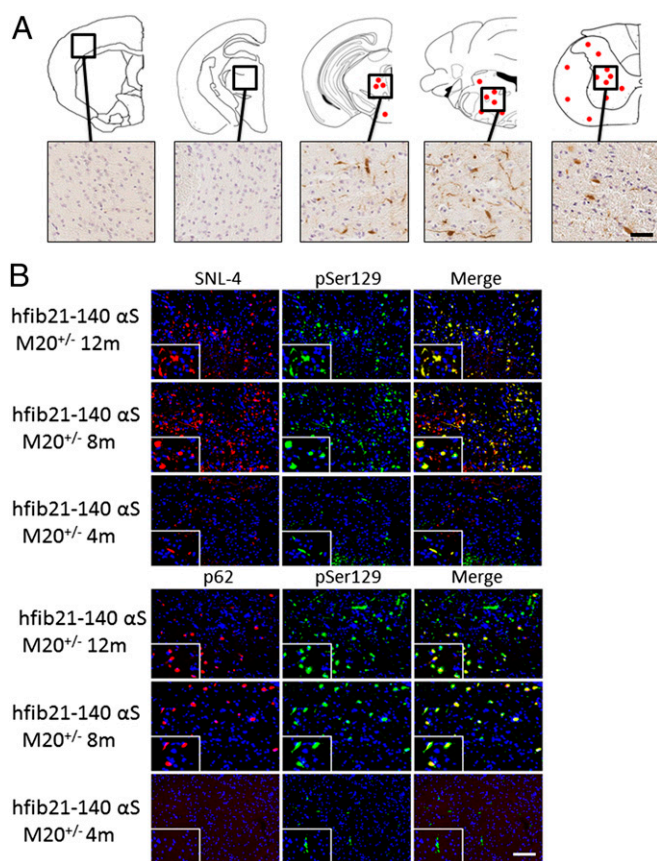


Fig. 4. Induction of α S pathology post IM injection of 10 μ g of hfib21-140 α S in M20^{+/-} Tg mice. (A) Schematic summary of rostral-caudal α S pathology distribution in M20^{+/-} Tg mice 12 mo after bilateral IM injection with hfib21-140 α S that is detected by a panel of α S inclusion markers. A moderate density of α S pathology was seen throughout the spinal cord, brainstem, and midbrain, with no pathology in the striatum, hippocampus, or cortex. The hyperphosphorylated α S inclusions were also detected by Syn506, an anti-N-terminal α S antibody that conformationally detects α S inclusions (57), and p62, an antibody that nonspecifically recognizes intracellular protein aggregates (41). Equivalent density and distribution of α S pathology was observed bilaterally. (B) Double immunofluorescence analyses of lumbar spinal cord sections from M20 Tg mice that are 12 mo, 8 mo, and 4 mo postinjection with 10 μ g of hfib21-140 α S. pSer129 α S inclusions (green) are also labeled with SNL-4, an anti-N-terminal α S antibody (red, *Upper*), and with an antibody to p62, a nonspecific intracellular inclusion marker (red, *Lower*), at 12 mo and 8 mo postinjection. At 4 mo postinjection, pSer129-labeled α S inclusions were also detected with SNL-4, but they were not detected with p62 antibodies. Tissue sections were counterstained with DAPI (blue). (Scale bars: A, 50 μ m; B, 100 μ m; *Insets*, 25 μ m.)

(Fig. S2). α S inclusions were not found in the sciatic nerve near the muscle injection site (Fig. S3).

We performed similar IM injections of fibrillar α S in non-transgenic (nTg) mice (C3H/C57BL6 strain background that is the same as M83 and M20 Tg mice) as well as in *SNCA*^{-/-} mice (Table S3). No mice from these cohorts developed a motor phenotype or α S pathology at 12 mo postinjection (Table S3). The paucity of induction of CNS α S pathology in nTg mice is consistent with retrograde axonal transport as a major mode transmission/induction and the low level of expression of endogenous α S in the sciatic nerve and the spinal cord (Fig. S3) (42).

To try to understand the physiological changes associated with α S inclusion pathology and the motor impairments in α S Tg mice IM injected with α S, we investigated alterations in the neuroinflammatory state. Tissue sections from all M83^{+/-} Tg injected mice were stained for the astrocyte marker, GFAP, and the

microglia marker, Iba-1 (Fig. S4). In untreated, aged M83^{+/-} Tg mice with CNS α S inclusion pathology, there was concurrent astrogliosis in the areas with α S pathology; however, there was little increase in microgliosis. Interestingly, M83^{+/-} Tg mice IM injected with LPS or Δ 71–82 α S that did not develop α S pathology even up to 4 mo postinjection did not show elevated levels of astrogliosis or microgliosis. On the other hand, M83^{+/-} Tg mice injected with Δ 71–82 α S or fibrillar α S (no differences seen between mfib or hfib21-140, bilateral or unilateral injections) that developed α S pathology also showed massive astrogliosis in the areas with α S inclusion pathology as seen in aged M83^{+/-} Tg mice that develop a motor phenotype, as well as elevated microgliosis in the areas with α S pathology. Comparatively, no elevated neuroinflammatory response in M20^{+/-} Tg mice was observed at any time point postinjection compared with PBS-injected M20^{+/-} Tg mice (Table S2) and naive M20^{+/-} Tg mice controls. Collectively, these data indicate that IM injections in M83^{+/-} Tg mice leading to the development of α S pathology are associated with increased microgliosis and astrogliosis.

Interestingly, α S pathology in the brain and spinal cord of α S-IM-injected M20 and M83 Tg mice was found in both astrocytes and neurons; however, although α S pathology was readily found in motor neurons in the ventral horn of the spinal cord in M83 Tg mice, they were less frequently seen in M20 Tg mice with α S pathology (Fig. S5). As the development of α S pathology in M83 Tg motor neurons could contribute to the development of the progressive motor phenotype seen in these mice, we quantified the number of motor neurons and found that there is a 76% loss of motor neurons in injected M83 Tg mice (M83^{+/-} Tg mice average number of motor neurons per section was 1.8 ± 0.5 compared with an average of 7.7 ± 1.0 motor neurons per section in age-matched, naive nTg mice; $P < 0.0001$). In a previous characterization of the M83 Tg mice, it has been shown that untreated, motor-impaired aged M83 Tg mice do not have statistically different levels of motor neurons compared with nTg mice (30); therefore, this loss of motor neuron is related to the injection of exogenous α S. We additionally found that the loss of motor neurons in phenotypic M83 Tg mice corresponded to a 72% decrease in the innervation of the neuromuscular junction (NMJ) in the digitorum brevis muscles, which are solely innervated by a branch of the sciatic nerve (average ratio of innervated NMJ in injected M83^{+/-} Tg mice is 0.25 ± 0.09 compared with age-matched, naive M83^{+/-} Tg mice with 0.92 ± 0.07 ; $P < 0.0001$) (Fig. S6). Collectively, these data point to the progressive induction of α S pathology in ventral horn motor neurons, with the demise of these cells as a causative factor for inciting the motor dysfunction seen in M83 Tg mice IM injected with α S.

Discussion

Our findings show that α S pathology in the CNS can be efficiently induced with peripheral injections of amyloidogenic α S and less efficiently with “non-amyloidogenic” α S in M83 Tg mice. In both cases, α S pathology is associated with a rapidly progressive motor phenotype that is subtly distinct from that in untreated, aged M83 Tg mice due to the initial foot drop presentation and the heightened neuroinflammatory response. From our findings, IM injections appear to induce a more rapid lethal motor phenotype (50–120 d incubation time) than reported in some studies where either homozygous or hemizygous M83 Tg were intracerebrally inoculated with CNS extracts from symptomatic M83 Tg (~200 d incubation time) (23, 24). However, Luk et al. (21) reported that, in similar M83 Tg CNS extract transmission studies or with intracerebral injection of recombinant fib α S in homozygous M83 Tg mice, the incubation time to cause motor impairments was closer to what we observed resulting from IM injection of fib α S (i.e., ~100 d) (21). Nevertheless, the relative rapid induction of widespread CNS α S pathology from the peripheral injection of fib α S in our IM experimental paradigm is in contrast to the studies of peripheral inoculations of scrapie prions where the incubation times are

always longer than those found after intracerebral inoculation (43). It is possible that some unique properties of α S, such as vesicle binding and more rapid retrograde transport, facilitate this process. However, more rigorously controlled parallel intracerebral versus IM inoculation studies are needed before definite comparison of injection sites on incubation lag time to disease state can be established. All of the α S transmission studies performed by different groups using M83 Tg mice were conducted with different forms and amounts of inoculums. Thus, varying amounts of transmissible α S likely used in these different studies confounds the direct comparison of incubation time needed to lead to disease from different sites of injections.

α S IM-injected M83 Tg mice also have a large decrease in spinal cord motor neurons and reductions in innervated NMJs in muscle innervated by the sciatic nerve. The delay and attenuation of disease-induction results observed in the sciatic nerve transection studies provide strong support that retrograde transport of exogenous α S to the spinal cord is a primary mechanism of CNS neuroinvasion. It is not completely clear how α S CNS pathology was still induced in a subset of mice with sciatic nerve transection. The sciatic nerve is the major nervous system connection between the spinal cord and the lower hind leg muscles, but there are other minor nerves that may also lead to retrograde transport. The site of muscle injection (gastrocnemius muscle) is far from the site of transection at the level of the thigh, but it is possible that some injected α S was able to remain in the muscle long enough for some nerve sprouting to occur. Furthermore, it is possible that some of the injected α S was able to enter the CNS via other systemic circulation mechanism(s) or that other secondary induction mechanism(s) can indirectly contribute to the induced CNS α S pathology.

The finding that α S inclusion pathology was also induced in glial cells in the spinal cord indicates that induction/spread does not in all cases follow neuroanatomical pathways. It is also intriguing that no α S inclusions were observed in the sciatic nerve of any of the injected mice. This finding suggests that (i) the retrograde transport in this nerve may be too rapid for inclusion formation to occur, (ii) α S being transported via retrograde transport is in a state such that it is not available to polymerize into inclusions, or (iii) the concentration of α S in the sciatic nerve is not sufficient for inclusion formation.

We have additionally shown that similar injections of exogenous α S into M20 Tg mice induce the formation of CNS α S inclusion pathology. However, this process is much slower than in M83 Tg mice. The specific factors that account for the difference between these two lines remain enigmatic but these components may hold important clues to how to halt disease progression in humans. The M20 Tg mice express wild-type human α S whereas the M83 Tg mice express A53T human α S, which have a greater propensity to aggregate into amyloid in vitro (44–46). This difference may contribute to the increased vulnerability of M83 Tg mice, but other still unknown synergistic mechanisms may also be more pronounced in M83 Tg mice.

Peripheral injections of α S aggregates into nTg mice did not produce any obvious neuropathological or behavioral abnormality. This finding is similar to another study of intranasal administration of α S (25). Recently, Rey et al. (47) have shown that injection of exogenous monomeric or aggregated α S in the mouse olfactory bulb can result in the transport to neurons of neuroanatomically connected brain regions, but the injected proteins had a short half-life (less than 72 h) and no induction of α S pathology was observed (47). Thus, it appears that the higher endogenous levels of α S in primed animals greatly accentuate the experimental peripheral induction of α S pathology. Indeed, it has been recently shown that i.p. administration of exogenous tau into transgenic mice overexpressing human mutant P301L tau results in the induction of an intracerebral tauopathy at 9 mo postinjection (48). Similarly, i.p. injections of brain extracts laden with A β aggregates into transgenic mice expressing the amyloid precursor protein also induced cerebral β -amyloidosis (49, 50). Overall, our experience has been that using transgenic mice in

exogenous seeding experiments greatly enhances the probability of transmitting pathogenic protein aggregation.

Our findings also highlight the fact, as we have noted elsewhere (51, 52), that there seem to be significant differences in the susceptibility of different models to induction of pathology. Mice expressing A53T α S are susceptible to rapid onset of a neurodegenerative phenotype associated with motor neuron death and a decrease in NMJ integrity in the affected limbs. The time-course studies in M20 Tg mice show a progressive spread of α S, albeit less efficient, from the spinal cord to the brain in the absence of a neuroinflammatory response. Studies using this model may in turn provide more direct evidence for the prion-like spread of α S pathology, without the exacerbating effects of multiple synergistic mechanisms.

Previous experimental studies have provided evidence for in vivo prion-like spread of α S pathology (21–26); however, several findings here, and in other recent studies from our group, suggest that additional nonexclusive mechanisms inducing or promoting α S inclusion pathology formation should be considered (33, 51–53). Indeed, (i) the distribution of α S pathology in M83 Tg mice IM injected with various forms of α S was identical to that seen in untreated, aged M83 Tg mice, (ii) IM injection of a non-amyloidogenic form of α S (Δ 71–82) induced CNS α S inclusion pathology, albeit less efficiently than fibrillar α S, and (iii) there were significant increases of both astrogliosis and microgliosis associated with the regions where abundant α S pathology was induced.

Our finding using of Δ 71–82 α S suggests that the amyloidogenic form of α S is not required for the induction of α S pathology and a motor phenotype and that mechanism(s) besides prion-like conformational templating may contribute to induction of disease. Under experimental native and physiological conditions, Δ 71–82 α S is refractory to amyloid formation, and it does not promote or influence amyloid formation of full-length α S (32, 33, 36, 37); however, under nonphysiological conditions (i.e., the presence of SDS), it can be artificially induced to form amyloid fibrils (54). At present, we cannot completely exclude the possibility that a small amount of soluble Δ 71–82 α S may spontaneously form amyloid seeds in vivo. It is possible that additional factors, analogous to the Protein X in prion disease (55), absent in in vitro systems could facilitate the conversion of the nonamyloidogenic α S to an amyloidogenic form in vivo. Furthermore, amyloidogenicity is not a prerequisite for all forms of self-templating propagation of protein aggregation, and it may be that Δ 71–82 α S is directly inducing structural changes in endogenous α S, leading to aggregation by a nonamyloidogenic mechanism.

Our ability to now synchronously and rapidly induce a motor phenotype and α S pathology by a peripheral injection of α S may prove invaluable in future studies exploring mechanisms of pathology induction and α S toxicity and may extend recent studies showing rapid induction following intracerebral inoculation (21–26). Our finding that disease onset in M83 Tg mice can be shortened, predicted, and synchronized through a simple manipulation provides a valuable model to accelerate studies designed to fully understand the mechanisms underlying induction of the inclusion pathology and motor phenotype, but also to enable much more rapid and cost-effective preclinical testing of novel PD therapies.

Materials and Methods

Mice Husbandry and Procedures. All procedures were performed according to the National Institutes of Health Guide for the Care and Use of Experimental Animals and were approved by the University of Florida Institutional Animal Care and Use Committee. M83 Tg mice expressing human α S with the A53T mutation or M20 Tg mice expressing wild-type human α S drive by the mouse prion protein promoter were previously described (30, 31). *SNCA*^{-/-} mice (56) were obtained from The Jackson Laboratory, and C3H/C57BL6 nTg mice were obtained from Harlan Labs. All animals were maintained on ad libitum food and water with a 12 h light/dark cycle.

Intramuscular Injection into M83 Tg or M20 Tg Mice. Bilateral injection of α S proteins and lipopolysaccharide (LPS; Sigma-Aldrich) or PBS control was performed by inserting the needle ~1 mm deep into the biceps femoris or gastrocnemius muscle (as summarized in Tables S1–S3). Mice at 2 mo of age were anesthetized with isoflurane (1–5%) inhalation. Injections were made using a 10- μ L Hamilton syringe with a 25-gauge needle. Different syringes were used for each type of inoculum to prevent any contamination. Post-injection, mice were placed on a heating pad for recovery before being returned to their home cage. Some M83^{+/+} α S Tg mice and M83^{+/-} α S Tg mice were also unilaterally injected with mouse α S fibrils in the left gastrocnemius

muscle. For a subset of these M83^{+/-} α S Tg mice, the left sciatic nerve was completely severed at the level of the thigh 3 d before muscle injection.

Additional information on other materials and methods are included in *SI Materials and Methods*.

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