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# Effects of α-Synuclein Immunization in a Mouse Model of Parkinson's Disease

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## Summary

**Abnormal folding of α-synuclein (α-syn) is thought to lead to neurodegeneration and the characteristic symptoms of Lewy body disease (LBD). Since previous studies suggest that immunization might be a potential therapy for Alzheimer's disease, we hypothesized that immunization with human (h)α-syn might have therapeutic effects in LBD. For this purpose, hα-syn transgenic (tg) mice were vaccinated with hα-syn. In mice that produced high relative affinity antibodies, there was decreased accumulation of aggregated hα-syn in neuronal cell bodies and synapses that was associated with reduced neurodegeneration. Furthermore, antibodies produced by immunized mice recognized abnormal hα-syn associated with the neuronal membrane and promoted the degradation of hα-syn aggregates, probably via lysosomal pathways. Similar effects were observed with an exogenously applied FITC-tagged hα-syn antibody. These results suggest that vaccination is effective in reducing neuronal accumulation of hα-syn aggregates and that further development of this approach might have a potential role in the treatment of LBD.**

## Introduction

Lewy body disease (LBD) is a heterogeneous group of disorders that includes Parkinson's disease (PD) and dementia with Lewy bodies (DLB) (Hansen and Galasko, 1992; Kosaka et al., 1984; McKeith, 2000), characterized by degeneration of the dopaminergic system (Shastry, 2001), motor alterations (Braak et al., 2002), cognitive impairment (Salmon et al., 1996), and formation of Lewy bodies (LBs) in cortical and subcortical regions (Trojanowski and Lee, 1998). The number of patients affected by these devastating conditions continues to climb as the population ages, creating a serious public health problem. The cause for LBD is controversial, and multiple factors probably play a role, including various neurotoxins and genetic susceptibility factors (Betarbet et al., 2000; Coleman et al., 1996; D'Amato et al., 1986; Forno et al., 1996; Jenner, 1998; Veldman et al., 1998).

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In recent years, new hope for understanding the pathogenesis of this disease has emerged. Specifically, several studies have shown that the synaptic protein α-synuclein (α-syn) (Iwai et al., 1994) plays a central role in LBD pathogenesis since (1) this molecule accumulates in LBs (Spillantini et al., 1997; Takeda et al., 1998b; Wakabayashi et al., 1997), (2) mutations and multiplication in the α-syn gene are associated with rare familial forms of parkinsonism (Kruger et al., 1998; Polymeropoulos et al., 1997; Singleton et al., 2003), and (3) its expression in transgenic (tg) mice (Lee et al., 2002; Lee et al., 2004b; Masliah et al., 2000) and *Drosophila* (Feany and Bender, 2000) mimics several aspects of PD. Thus, the fact that accumulation of α-syn in the brain is associated with similar morphological and neurological alterations in species as diverse as humans, mice, and flies suggests that this molecule contributes to the development of LBD.

The mechanisms by which accumulation of α-syn leads to neurodegeneration and the characteristic symptoms of LBD are unclear. However, recent studies suggest that abnormal accumulation of α-syn oligomers in the synaptic terminals and axons plays an important role (Hashimoto and Masliah, 1999; Iwatsubo et al., 1996; Lansbury, 1999; Trojanowski et al., 1998).

Although experimental therapies utilizing neurotrophic factors (Kirik et al., 2004) and grafting of dopaminergic cells (Kim, 2004; Yoshizaki et al., 2004) have yielded promising results, alternative approaches directed at reducing the neuronal accumulation of α-syn are necessary. Viable strategies might include the use of viral vectors expressing antiaggregation molecules (Burton et al., 2003; Hashimoto et al., 2004) or factors such as immunotherapy, which promote the degradation or clearance of α-syn. For example, recent studies in a tg mouse model of Alzheimer's disease (AD) have shown that antibodies against β-amyloid 1-42 (Aβ) promote the removal of amyloid from the brain, resulting in improved cognitive performance (Janus et al., 2000; Morgan et al., 2000; Schenk et al., 1999). Moreover, a vaccination approach has been shown to be effective experimentally in tg mice at reducing the accumulation of prion protein (Sigurdsson et al., 2002) and huntingtin (Luthi-Carter, 2003; Miller et al., 2003), molecules that, like α-syn, accumulate intracellularly. In this context, we postulate that immunization of human (h)α-syn tg mice (a mouse model of LBD) with purified recombinant hα-syn might activate an immune response that will reduce hα-syn accumulation and, more importantly, neurotoxicity. This approach may be a suitable target for the development of an alternative immune therapy for PD and other disorders with LBs, parkinsonism, and dementia.

## Results

### Characterization of Antibody Titers, Relative Affinity, and Epitope Mapping

Three-month-old tg mice for group I and six-month-old tg mice for group II were immunized for 8 months with

Table 1. Summary of hα-Syn Antibody Titers and Relative Affinity, Corrected for Titer

| Group | Antibody Relative Affinity by Miniblot | Antibody Affinity to Synapses | Antibody Affinity to Inclusions | Antibody Titers (First Bleed) | Antibody Titers (Second Bleed) | Antibody Titers (Third Bleed) |
|---|---|---|---|---|---|---|
| Group I/α-syn | 109,147 ± 2700 | 1.9 ± 0.73 | 1.2 ± 0.4 | 2332 ± 500 | 2772 ± 1176 | 3644 ± 2365 |
| Group I/CFA | 113 ± 113 | 0.4 ± 0.1 | 0 | 19 ± 6.7 | 30 ± 12 | 7 ± 4 |
| Group II/α-syn | 235,747 ± 74,000 | 4.1 ± 0.9 | 2.8 ± 1.0 | 3813 ± 1200 | 2926 ± 976 | 1468 ± 641 |
| Group II/CFA | 400 ± 358 | 0.3 ± 0.2 | 0.1 ± 0.1 | 23 ± 9 | 21 ± 14 | 0.6 ± 0.6 |

recombinant hα-syn or Complete Freund's adjuvant (CFA) alone. Antibody titers in animals belonging to group I ranged from 200 to 20,000 (Table 1), and in mice from group II, titers ranged from 200 to 13,000 (Table 1). Levels of antibody relative affinity by immunoblotting were higher in mice from group II compared to immunized mice from group I (Table 1). By immunocytochemistry (ICC), sera from mice vaccinated with hα-syn showed either labeling of neurons (Figure 1A), intraneuronal inclusions (Figure 1B), or presynaptic terminals (Figure 1C) in hα-syn tg mice, but only displayed mild background staining in nontg mice (Figure 1D). Sera from mice treated with CFA alone showed nonspecific background staining in both hα-syn tg (Figure 1E) and nontg (Figure 1F) mice. By Western blot (WB) analysis, sera from mice vaccinated with hα-syn recognized hα-syn in tg mice (Figure 1G). In contrast, no immunoreactivity (IR) was observed with sera from mice treated with CFA alone (Figure 1H). Epitope mapping studies showed that in the vaccinated mice, antibodies recognized epitopes within the C terminus region of hα-syn, including amino acids (aa) 85–99, 109–123, 112–126, and 126–138.

### Immunization Reduces hα-Syn Accumulation and Preserves Synaptic Density in tg Mice

To determine the effects of immunotherapy and antibody relative affinity on hα-syn accumulation, sections were analyzed by ICC with antibodies against hα-syn (Figure 2). Since antibody relative affinity (Figure 3A) and titers (Table 1) show a wide range of variability and linear regression analysis shows that antibody relative

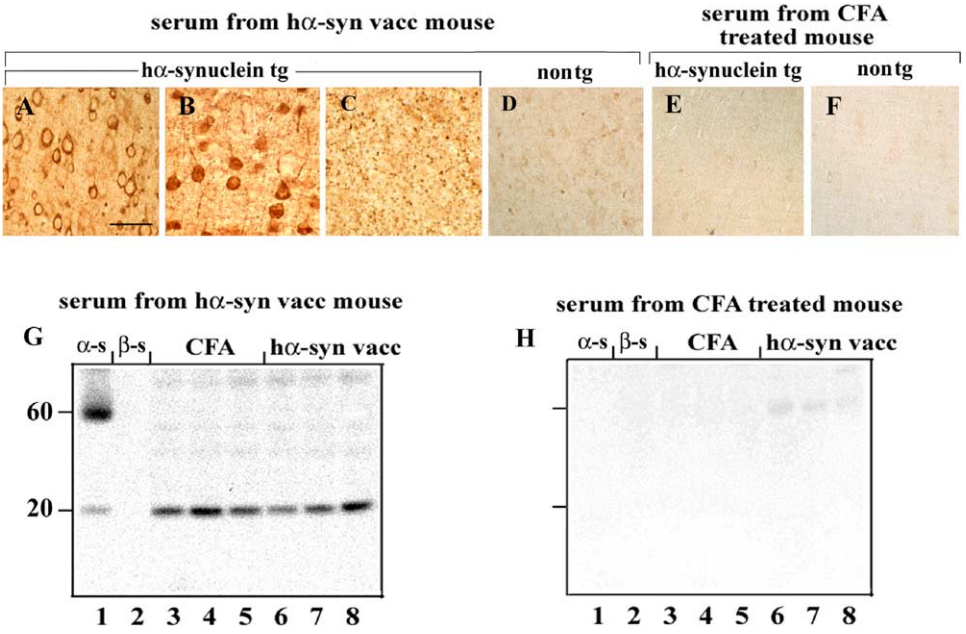


Figure 1. Immunocytochemical and Immunoblot Analysis with the Sera from Vaccinated Mice in the Brains of nontg and hα-Syn tg Mice

For panels (A)–(F), serial vibratome sections from nonimmunized hα-syn tg (Line D, 6-month-old) and nontg mice were immunostained with sera (normalized to 1:1000) from CFA and hα-syn-vaccinated (vacc) animals. All panels are from the temporal cortex. (A–C) Sections from hα-syn tg mice immunolabeled with the sera from immunized mice displayed neuronal staining (A), inclusion body staining (B), and granular neuropil immunolabeling suggestive of presynaptic terminals (C). (D) Section from a nontg mouse immunostained with the sera from immunized mice shows only mild background staining. (E and F) Sections from hα-syn tg (E) and nontg (F) mice immunolabeled with sera from CFA-treated mice showed only nonspecific background staining of the neuropil. Scale bar, 40 μm. For panels (G) and (H), homogenates from the brains of hα-syn tg mice treated with CFA or immunized with hα-syn were resolved by SDS-PAGE, and blots were probed with the sera from treated mice. (G) Immunoblot analysis with the sera from vaccinated mice shows a prominent band at approximately 19 kDa corresponding to recombinant hα-syn (lane 1, α-s), hα-syn in the brains of tg animals treated with CFA (lanes 3 through 5) or vaccinated with hα-syn (lanes 6 through 8). The sera did not recognize recombinant β-syn (lane 2, β-s). (H) Immunoblot analysis with the sera from CFA-treated animals shows no IR to recombinant hα-syn (lane 1, α-s), recombinant β-syn (lane 2, β-s) or in the brains of tg animals treated with CFA (lanes 3 through 5) or vaccinated with hα-syn (lanes 6 through 8).

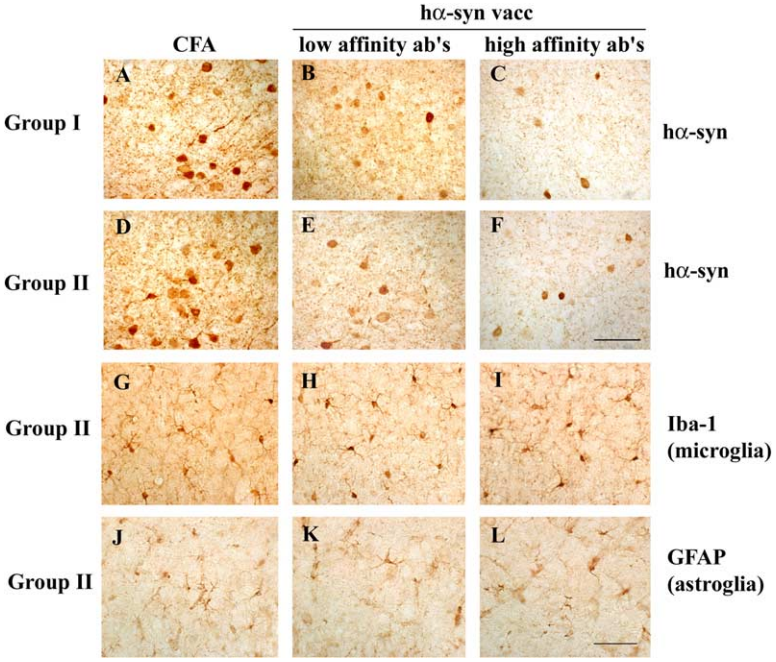


Figure 2. Patterns of hα-Syn IR and Glial Response in the Brains of Immunized hα-Syn tg Mice

Panels are from the temporal cortex of CFA or hα-syn-vaccinated mice reacted with antibodies against hα-syn, Iba1, or GFAP, developed with DAB, and analyzed by bright field microscopy. Panels (A)–(C) are representative images from the brains of group I mice (11-month-old), and panels (D)–(L) are from the brains of group II mice (14-month-old). Mice from both groups were divided into subsets of animals that produced antibodies with low (<100,000 units) or high (>100,000 units) relative affinities to hα-syn. (A and D) CFA-treated mice showed abundant hα-syn IR in intraneuronal inclusions as well as the neuropil. (B, E, C, and F) Immunized mice that produce low relative affinity (B and E) antibodies display a modest decrease in hα-syn-immunoreactive inclusions and neuropil labeling, and those that produce high relative affinity antibodies (C and F) demonstrate a more significant decrease in hα-syn-immunoreactive inclusions and neuropil labeling. (G–L) Compared to CFA-treated mice (G and J), animals vaccinated with hα-syn show only a mild increase in microglial IR (Iba-1, [H and I]) and mild astroglial activation (GFAP, [K and L]). Scale bar, 50 μm.

affinity was one of the strongest predictors for the effects on reducing the neuropathology on the vaccinated hα-syn tg mice (Table 2), animals were divided into subgroups consisting of those that show low (<100,000 units; Figures 2B, 2E, 2H, and 2K) and high (>100,000 units; Figures 2C, 2F, 2I, 2L, and 3A) relative affinity to hα-syn (expressed as units of intensity volume). In tg mice treated with CFA alone, abundant hα-syn IR was observed in the neuropil as well as in intraneuronal inclusions (Figures 2A and 2D). In contrast, immunized mice from both groups showed a reduction in the number of inclusions in the temporal cortex (Figure 3B). Compared to mice producing low relative affinity antibodies (Figures 2B and 2E), this effect was more pronounced in mice producing antibodies with high relative affinity to hα-syn (Figures 2C and 2F). Moreover, immunization resulted in a decrease in hα-syn IR in the neuropil of tg mice producing antibodies with high relative affinity to hα-syn (Figures 2C and 2F), with a greater effect in mice from group II (Figure 2F) than group I (Figures 2C and 3D), compared to CFA-treated tg mice (Figures 2A and 2D). To investigate whether the effects of the immunization were accompanied by a neuroinflammatory response, immunocytochemical analysis with antibodies against the microglial marker (Iba-1) and the astroglial marker (glial fibrillary acidic protein [GFAP]) were performed. These studies showed that in both CFA-treated (Figures 2G and 2J) and hα-syn-vaccinated (Figures 2H, 2I, 2K, and 2L) animals, there was a mild increase in Iba-1 and GFAP IR; however, no overt differences were detected between the groups.

To further investigate the effects of the vaccination on the synapses, higher-resolution analysis with the laser scanning confocal microscope (LSCM) was performed. These studies confirmed that compared to CFA controls (Figures 3D, 4A, and 4C) the reduction in the neuropil immunostaining was associated with decreased accumulation of hα-syn in presynaptic terminals (Figures 3D, 4B, and 4D). To ascertain the effects of the immunotherapy on neuropil integrity, sections were immunostained with an antibody against synaptophysin (Figures 4E–4H). Compared to nontg mice, hα-syn tg mice treated with CFA alone (Figures 4E and 4G) showed an average of 20% decrease in the number of synaptophysin-immunolabeled terminals (Figure 3C). In contrast, immunized mice from both groups I and II showed levels of synaptophysin IR comparable to nontg controls (Figures 3C, 4F, and 4H). This effect was more prominent in mice that produced antibodies with high relative affinity to hα-syn (Figure 3C).

To rule out immunization masking effects, control experiments were performed by comparing the levels of murine (m)α-syn. Similar to hα-syn, mα-syn IR was abundant in the neuropil in association with nerve terminals, but was absent from the neuronal cell bodies and in the inclusions (Figures 4I–4L). Both in CFA (Figures 4I and 4K) and hα-syn immunized mice (Figures 4J and 4L), patterns and levels of mα-syn were comparable. Additional analysis of the immunization specificity was performed by comparing levels of β-syn IR, a close α-syn homolog (Iwai et al., 1994). Abundant β-syn IR was observed in the neuropil in association with the presynaptic terminals, and mild immunolabeling was detected in the neuronal cell bodies, but not in the inclusions (Figures 4M–4P). Compared to tg mice treated with CFA alone (Figures 4M and 4O), no differences in the patterns and levels of β-syn were found in mice immunized with hα-syn (Figures 4N and 4P). These studies suggest that vaccination specifically affects hα-syn but not other related synaptic molecules.

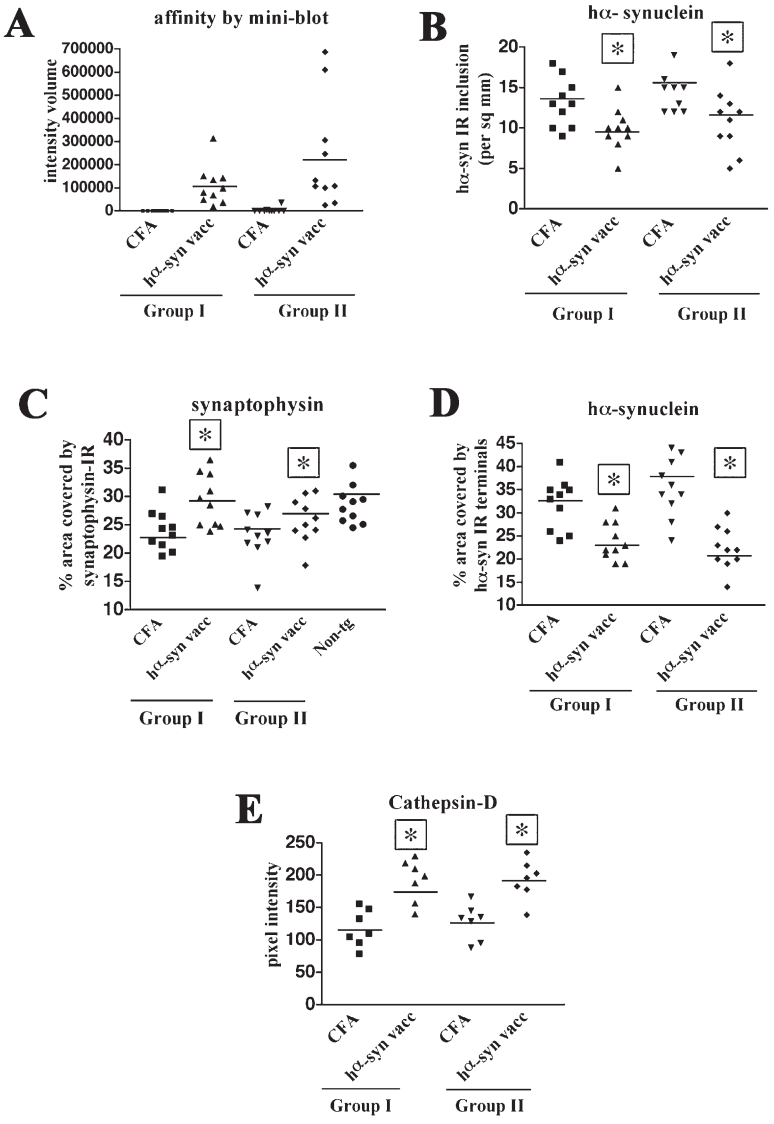


Figure 3. Image Analysis of the Levels of hα-Syn IR and Other Markers of Neurodegeneration

(A) Relative affinity by mini-blot of hα-syn antibodies produced by mice treated with CFA alone or immunized with hα-syn was assessed. (B) Scatterplot of number of hα-syn-positive inclusions in the temporal cortex of mice treated with CFA alone or immunized with hα-syn. Vaccination resulted in a significant decrease in the number of inclusions in both groups compared to controls, an effect more pronounced in group II mice (14-month-old) as opposed to group I (11-month-old). (C) Percent area of the neuropil occupied by synaptophysin-immunoreactive terminals in the temporal cortex. Compared to nontg controls, in tg mice treated with CFA alone, the number of synaptophysin-immunolabeled terminals decreased by an average of 20%. In contrast, tg mice from both groups showed levels of synaptophysin IR comparable to nontg controls. (D) Percent area of the neuropil occupied by hα-syn-immunoreactive terminals in the temporal cortex. In tg mice from both groups vaccinated with hα-syn, there was a decrease in the accumulation of hα-syn in synaptophysin-immunoreactive terminals. (E) Levels of cathepsin D IR (measured as pixel intensity) in CFA-treated and hα-syn-vaccinated tg mice. In vaccinated tg mice, there were significantly higher levels of cathepsin D IR in both groups compared to CFA controls. * = significant difference compared to CFA-treated hα-syn tg mice ($p < 0.05$, unpaired, two-tailed Student's t test).

To better characterize the effects of vaccination on hα-syn aggregation in the synapses, double immunocytochemical analysis with antibodies against hα-syn and the presynaptic terminal marker synaptophysin and WB analysis with synaptosomal preparations were performed. Under physiological conditions, hα-syn is localized primarily to the presynaptic boutons (Iwai et al., 1994), and in LBD and the tg mice, increased accumulation of hα-syn in the synapses is associated with functional deficits and synapse loss (Hashimoto et al., 2001). Confocal imaging showed that in comparison to CFA-treated hα-syn tg mice (Figure 3D), vaccinated mice displayed a decrease in the proportion of synaptophysin-immunoreactive nerve terminals in the neocortex that show hα-syn immunolabeling (Figure 3D). Immunoblot analysis with synaptosomal preparations (Figure 5A, upper panel) showed that immunization decreased the accumulation of hα-syn higher molecular weight bands (Figure 5A, upper panel, and Figure 5B), but no effects were observed on mα-syn (data not shown). Furthermore, compared to CFA-treated tg mice, levels of synaptophysin IR were higher in the synaptosomal preparations from immunized mice (Figure 5A, lower panel, and Figure 5B). These results suggest that immunotherapy might ameliorate the neuronal damage in the brains of tg mice by reducing the accumulation of potentially toxic hα-syn in the synapses.

### Immunization Effects Are Dependent on the Relative Affinity of Antibodies to Recognize Membrane-Associated Aggregated hα-Syn

Since abnormal accumulation of hα-syn is associated with translocation of hα-syn from the cytosol to the membrane, it is possible that this might explain the antibodies' ability to recognize abnormally accumulated hα-syn in the immunized tg mice. WB analysis showed abundant monomeric hα-syn in both the cytosol (Figure 5C) and membrane (Figure 5D). However, oligomeric hα-syn was more abundant in the membrane fractions where, compared to CFA controls, immunization with hα-syn resulted in decreased accumulation of hα-syn oligomers (Figure 5D).

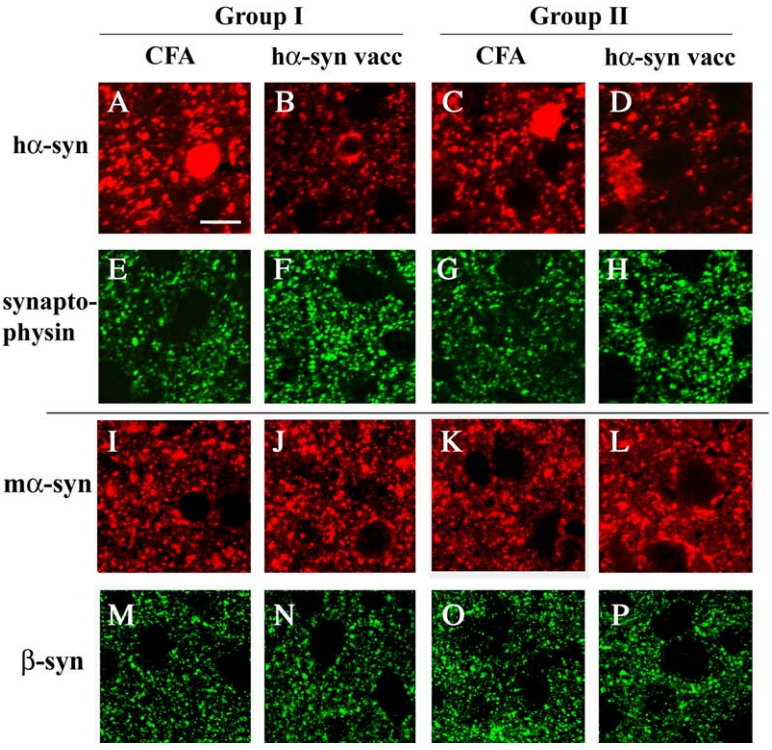


Figure 4. Immunocytochemical Analysis of hα-Syn, Synaptophysin, mα-Syn, and β-Syn IR in the Brains of hα-Syn tg Mice from Groups I and II

All panels are from the temporal cortex of CFA-treated or vaccinated mice. Group I, 11-month-old; group II, 14-month-old. (A–D) Sections were reacted with an antibody against hα-syn, labeled with Tyramide Red and imaged with the LSCM. Compared to tg mice treated with CFA alone (A and C), immunized mice (B and D) showed a reduction in the number of inclusions in the temporal cortex as well as a decrease in hα-syn IR (presynaptic terminals) in the neuropil. (E–H) Sections were immunostained with an antibody against synaptophysin and FITC-labeled for imaging with the LSCM. In tg mice treated with CFA alone (E and G), levels of synaptophysin IR were reduced, whereas immunized mice showed levels of synaptophysin IR comparable to nontg controls. (I–P) Sections were reacted with an antibody against mα-syn and labeled with Tyramide Red (I–L) or with an antibody against and β-syn and FITC-labeled (M–P), and imaged with the LSCM. (I–L) Levels of endogenous mα-syn remain unchanged in tg mice treated with CFA alone (I and K) and immunized mice (J and L). All display mα-syn IR in the neuropil, but there is no labeling of the intraneuronal inclusions as is found with antibodies against hα-syn. (M–P) Both hα-syn tg mice treated with CFA alone (M and O) and tg mice vaccinated with hα-syn (N and P) display abundant β-syn IR in the neuropil and mild immunolabeling in the neuronal cell bodies, but there is no labeling of the intraneuronal inclusions as is found with antibodies against hα-syn. Scale bar, 10 μm.

To better understand which factors might predict the effectiveness of the immunotherapy, linear regression analysis was performed between the neuropathological markers of hα-syn accumulation and the antibody titers and relative affinity (Table 2). A significant correlation was observed between relative antibody affinity by immunoblot and levels of hα-syn IR in the synapses, but not with the numbers of neuronal inclusions. Similarly, relative antibody affinity to recognize synapses by ICC was inversely correlated with levels of hα-syn in the synapses and directly correlated with the percent area occupied by synaptophysin-labeled nerve terminals, but not with the numbers of neuronal inclusions. Antibody titers correlated with the percent area of the neuropil labeled with the anti-hα-syn antibody, but negatively correlated with neuronal inclusions (Table 2). These results suggest that the relative immunoblot reactivity of the anti-hα-syn antibodies and to some extent the antibodies' titers correlate with the reduction of neuronal hα-syn accumulation.

### Anti-hα-Syn Antibodies Are Internalized and Trigger Clearance of hα-Syn Aggregates via Lysosomal Activation

To determine if anti-hα-syn antibodies generated by the immunized mice recognize hα-syn aggregates in the tg mice, immunocytochemical analysis was performed with horse anti-mouse IgG antibodies. Double immunostaining experiments showed that compared to CFA-treated tg mice (Figures 6A–6C), in hα-syn vaccinated mice, the anti-mouse IgG and the hα-syn IR were colocalized in the periphery of the cell bodies (Figures 6D–6F), the neuritic processes and synapses (Figures 6G–6I). In hα-syn containing neurons, the two markers were detected in granular subcellular structures averaging 0.4–0.8 μm in diameter (Figures 6G–6L). Furthermore, these granular structures displayed cathepsin D IR, suggesting that the internalized anti-hα-syn antibodies reacted with hα-syn within lysosomes (Figures 6M–6O).

To corroborate that vaccination resulted in lysosomal activation, cathepsin D IR was analyzed. Compared to

Table 2. Summary of Correlations between Antibody Relative Affinity, Neuropathology, and Titers

| Neuropathological Markers | Antibody Relative Affinity by Miniblot | Antibody Affinity to Synapses | Antibody Affinity to Inclusions | Antibody Affinity to Neurons | Antibody Titers (First Bleed) |
|---|---|---|---|---|---|
| Number of α-syn (+) inclusions | −0.11 | 0.04 | 0.12 | −0.21 | 0.1 |
| % area of neuropil α-syn (+) synapses | −0.46 (p = 0.003) | −0.41 (p = 0.009) | −0.43 (p = 0.005) | 0.06 | −0.47 (p = 0.007) |
| % area of neuropil synaptophysin (+) synapses | 0.06 | 0.35 (p = 0.04) | 0.01 | 0.04 | 0.12 |
| Antibody relative affinity by miniblot | — | 0.74 (p = 0.0001) | 0.70 (p = 0.0001) | −0.16 | 0.85 (p = 0.0001) |
| Antibody titers (first bleed) | 0.85 (p = 0.0001) | 0.62 (p = 0.0001) | −0.18 | 0.81 (p = 0.0001) | — |

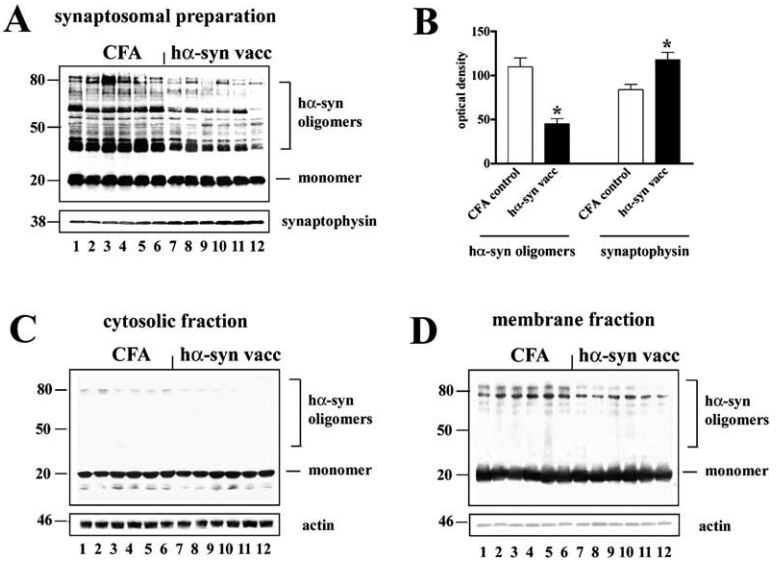


Figure 5. WB Analysis of the Levels of hα-Syn IR in Synaptosomal, Membrane, and Cytosolic Preparations from Vaccinated Animals

(A and B) Compared to brains of tg mice treated with CFA alone (lanes 1 through 6), in hα-syn-vaccinated tg mice (lanes 7 through 12), levels of hα-syn oligomers were decreased (upper panel), while levels of synaptophysin IR increased in the immunized animals (lower panel). (C and D) Compared to the cytosolic fraction of the brains of tg mice (C), a majority of hα-syn high molecular weight oligomers are present in the membrane fraction (D), where compared to mice treated with CFA alone (lanes 1 through 6), there is a decrease in hα-syn oligomers in the fractions from vaccinated animals (lanes 7 through 12). * = significant difference compared to hα-syn tg mice treated with CFA alone ($p < 0.05$, unpaired, two-tailed Student's t test). Animals were 14 months old.

CFA controls (Figures 7A–7C), in vaccinated mice there was increased cathepsin D IR (Figures 3E and 7D–7F). Similar increases in levels of cathepsin D IR were detected in the hα-syn-vaccinated mice from both groups when compared to the CFA-treated group (Figure 3E). In vaccinated tg mice, the cathepsin D-immunolabeled lysosomes were colocalized with discrete hα-syn-immunoreactive granular aggregates (Figure 7F), but no colocalization was observed in CFA-treated tg mice (Figure 7C).

To determine if exogenously applied antibodies recognize hα-syn aggregates in tg mice and activate lysosomal pathways, purified monoclonal anti-hα-syn antibodies were tagged with fluorescein isothiocyanate (FITC) and injected into tg and nontg animals. The FITC-tagged anti-hα-syn recognized hα-syn aggregates in the neuronal cell bodies and synapses of tg animals (Figure 7G–I), but not the endogenous mα-syn in the nontg controls (Figures 7J–7L). Furthermore, in tg mice treated with the FITC-tagged antibody, there was an increase in neuronal cathepsin D IR and colocalization with hα-syn (Figures 7M–7O) compared to nontg controls (Figures 7P–7R). No specific labeling of hα-syn or increase in cathepsin D IR was detected in control experiments where tg and nontg mice were treated with a nonimmune FITC-tagged IgG (data not shown). These studies suggest that circulating antibodies might recognize abnormally aggregated hα-syn associated with the neuronal membrane, which in turn might lead to clearance via lysosomal activation.

## Discussion

The present study showed that hα-syn antibodies generated in vaccinated mice reduced the abnormal accumulation of this protein in the neuronal cell bodies and synapses and ameliorated the loss of synaptophysin-immunoreactive nerve terminals in hα-syn tg mice. This is consistent with recent in vitro studies showing that intracellular antibodies can inhibit α-syn aggregation (Emadi et al., 2004; Zhou et al., 2004). Moreover, our findings are consistent with studies showing that immunotherapy might reduce the accumulation of extracellularly deposited proteins such as Aβ (Games et al., 2000; Lemere et al., 2003; Morgan et al., 2000) and of intracellular proteins such as huntingtin (Luthi-Carter, 2003), as well as membrane-associated molecules such as prion proteins (Bainbridge and Walker, 2003; White et al., 2003; White and Hawke, 2003). More recently, a study showed that immunotherapy with copolymer-1-immune cells might reduce neurodegeneration in the MPTP model of PD (Benner et al., 2004). While this immunotherapy approach focused on protecting via a general anti-inflammatory mechanism, the present study sought to elicit a specific response to promote degradation of toxic hα-syn. The mechanisms through which the antibodies generated in the vaccinated mice might recognize and promote the clearance of intracellular hα-syn aggregates and other neuronal proteins are less clear. One possibility is that circulating antibodies in the CNS might recognize and cross-link abnormally conformed proteins in the neuronal cell surface (Figure 8). For this, at least a limited amount of the target antigen must be present in the neuronal cell membrane (Luthi-Carter, 2003). A similar mechanism has been described in the antibody-mediated clearance of viruses from cells (Garzon et al., 1999; Ubol et al., 1995). Interestingly, in the case of α-syn, under basal conditions the monomeric forms of this molecule are almost exclusively present in the cytosolic fraction (Eliezer et al., 2001; Iwai, 2000). In contrast, under pathological circumstances leading to α-syn aggregation, oligomers and protofibrils can be found in the plasma membrane (Eliezer et al., 2001; Lansbury, 1999; Dixon et al., 2005), where they could potentially be recognized by circulating antibodies. Supporting this possibility, immunoblot and immunocytochemical analysis showed that vaccination reduced hα-syn accumulation in the membrane and that mouse IgG and hα-syn were colocalized in the periphery of the neurons, suggesting

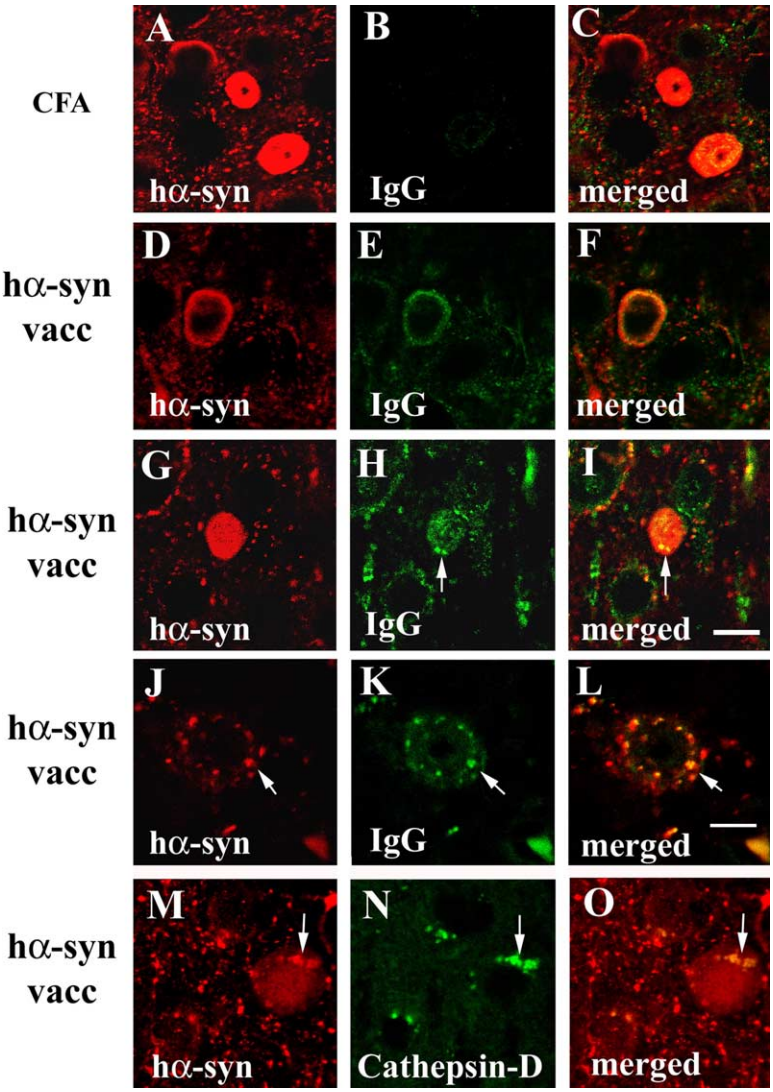


Figure 6. Double Immunocytochemical Analysis of the Patterns of hα-Syn and anti-IgG or Cathepsin D IR in Vaccinated Animals

All images are from the temporal cortex. Sections from CFA-treated and hα-syn vaccinated animals from group II were double labeled with antibodies against hα-syn (panels to the left, red) and FITC-tagged anti-mouse IgG or cathepsin D (central panels, green) and imaged with the LSCM. The images to the right represent the merged panels (yellow indicates colocalization of the two markers), demonstrating that neuronal cell bodies labeled by the anti-mouse IgG and the intraneuronal granular structures labeled by cathepsin D displayed hα-syn IR (arrows). (A–C) Tg mice treated with CFA show characteristic inclusions that react strongly with hα-syn antibodies and show background levels of reactivity with anti-mouse IgG. (D–F) Tg mice vaccinated (vacc) with hα-syn show a close colocalization between hα-syn and anti-mouse IgG in the periphery of the neuronal cell bodies. (G–L) Additionally, the anti-mouse IgG immunostaining is colocalized with hα-syn in small granular structures within the cell bodies (arrows). (M–O) These small granular structures also displayed lysosomal cathepsin D IR that colocalized with hα-syn immunolabeling. Scale bar, 10 μm (A–I), 5 μm (J–L). Animals were 14 months old.

that such interactions might take place in the neuronal surface.

Since circulating antibodies might be able to recognize membrane bound α-syn, there are several possibilities as to how they might promote the clearance of intracellular aggregates. Antibodies in close opposition with the neuronal surface might enter the neurons alone via surface receptors or in association with membrane bound α-syn and promote lysosomal degradation (Figure 8). This process may be mediated by receptor-dependent or receptor-independent endocytosis of the antibody or antibody-antigen complex, followed by fusion with lysosomes and incorporation of α-syn aggregates (Figure 8). Supporting this possibility, anti-mouse IgG, an exogenously applied FITC-tagged anti-hα-syn antibody, and cathepsin D colocalized with hα-syn aggregates. Consistent with the possibility that internalization of the antibody complex might play a role in clearance of α-syn aggregates, previous studies have shown that neurons can uptake immunoglobulins (Fabian and Petroff, 1987) and internalize some through the Thy 1.1 receptor in the neuronal and synaptic plasma membrane (Fabian, 1990). Other receptors that might be involved in this process and mediate the endocytosis of antibodies or antibody-antigen complexes include lipoprotein receptor-related protein (LRP) (Herz et al., 1990; Kounnas et al., 1995). This receptor is highly abundant in neurons (Schneider and Nimpf, 2003; Hussain, 2001), and macromolecules endocytosed via LRP target the lysosomal pathway (Gonias et al., 2004), suggesting that clearance of intracellular hα-syn aggregates might involve lysosomal degradation. In support of this mechanism, the present study showed that in the neurons of vaccinated mice, granular hα-syn-immunoreactive structures were also labeled with antibodies against the lysosomal marker cathepsin D. Similarly, previous studies have shown that immunoglobulins internalized by neurons form granular cytoplasmic structures that display lysosomal-like activity (Meeker et al., 1987). Moreover, recent studies have shown that lysosomally dependent autophagy might facilitate the clearance of α-syn oligomers (but not fibrils) and there-

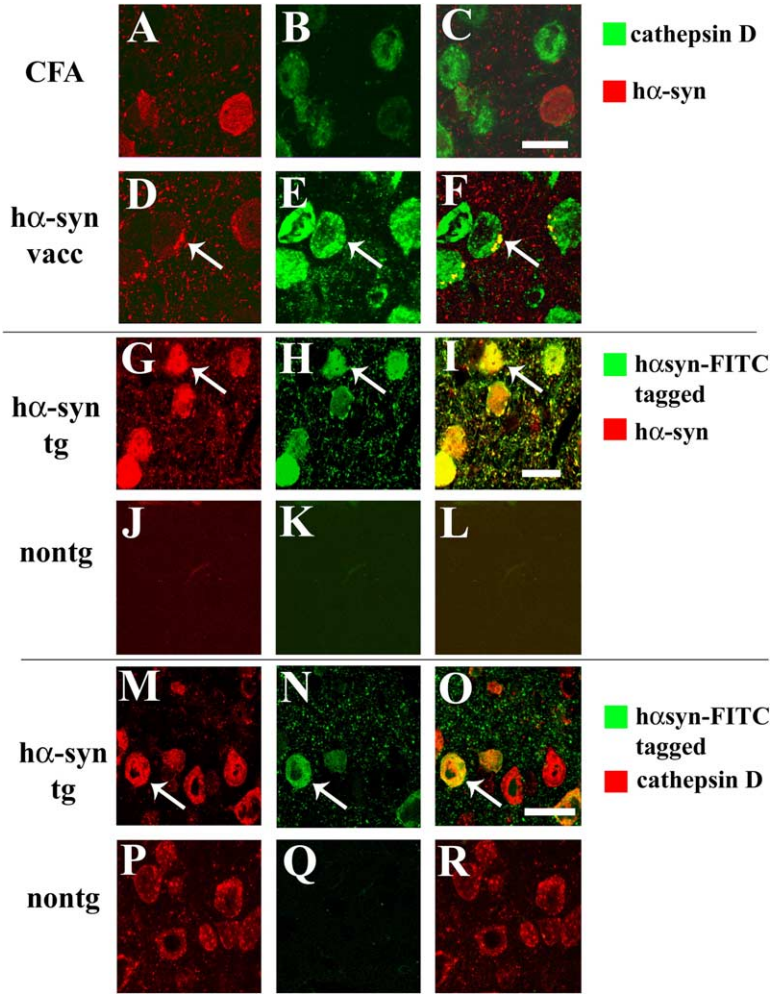


Figure 7. Antibody Recognition of hα-Syn and Lysosomal Activation in Vaccinated hα-Syn tg Animals

All panels are LSCM images from the temporal cortex. Panels (A)–(F) are from CFA-treated or immunized hα-syn tg mice from group II (14-month-old), and panels (G)–(R) are from non-immunized nontg and hα-syn tg animals (5-month-old) that received intracerebral injections of an FITC-tagged hα-syn antibody. (A–C) Images from sections of a hα-syn tg mouse treated with CFA alone double immunolabeled with antibodies against hα-syn (red) and the lysosomal marker cathepsin D (green). (D–F) Images from sections of a vaccinated hα-syn tg mouse double immunostained with antibodies against hα-syn (red) and cathepsin D (green). Yellow indicates colocalization between granular hα-syn-immunoreactive structures and cathepsin D (arrows). Note the higher levels of cathepsin D IR in these images. (G–I) Images from sections of a hα-syn tg mouse that received intracerebral injections of an FITC-tagged antibody against hα-syn (green) coimmunolabeled with a different antibody against hα-syn (red). Yellow indicates colocalization between injected and immunolabeled anti-hα-syn antibodies. (J–L) Images from sections of a nontg mouse that received intracerebral injections of an FITC-tagged antibody against hα-syn (green) coimmunolabeled with a different antibody against hα-syn (red). (M–O) Images from sections of a hα-syn tg mouse that received intracerebral injections of an FITC-tagged antibody against hα-syn (green) coimmunolabeled with an antibody against cathepsin D (red). Yellow indicates colocalization between hα-syn and cathepsin D (arrows). (P–R) Images from sections of a nontg mouse that received intracerebral injections of an FITC-tagged antibody against hα-syn (green) coimmunolabeled with an antibody against cathepsin D (red). Scale bar, 15 μm.

fore may play a protective role (Cuervo, 2004; Cuervo et al., 2004; Lee et al., 2004a). These studies suggest that in the immunized mice, internalized antibodies might promote degradation of hα-syn aggregates via activation of lysosomal pathways. Alternatively, active vaccination might also promote the clearance of α-syn aggregates via an immune-mediated cellular response. This is unlikely, because in the present study, no apparent infiltration by lymphocytes was noted and only mild microglial activation was detected. However, it is important to consider this possibility because active immunization in other neurodegenerative disorders has been associated in some cases with vasculitis and autoimmune response (Ferrer et al., 2004), and caution should be exercised when considering the use of active immunization as a potential therapy in patients with PD. For this reason we are currently investigating the effects of passive immunization with antibodies against various regions of α-syn.

Antibodies against α-syn not only decreased accumulation in neuronal cell bodies, but also in neuropil, suggesting that the immunotherapy also reduced the accumulation of hα-syn aggregates in the synapses. This effect was associated with amelioration of the neurodegenerative pathology. It is unclear whether this might be accompanied by the improvement of behavioral deficits; experiments are currently underway to assess this possibility. The ability of the antibodies to recognize neuronal cell bodies and synapses was a good predictor of their ability to reduce hα-syn aggregation and neurodegeneration at least as determined by synaptic integrity. This is consistent with studies in AD, where antibodies with high affinity for plaques displayed a better response (Hock et al., 2002). While for Aβ, the most effective antibodies recognize the N terminus (Bard et al., 2003), for hα-syn, high relative affinity antibodies recognized epitopes within the C terminus. Recent studies have shown that C-terminal fragments of α-syn assemble into protofibrils and associate with the membrane (McLean et al., 2000; Kim et al., 2002; McLean et al., 2000). Deletion of key amino acids 125–140 within the C-terminal domain greatly alters α-syn aggregation (Kim et al., 2002), and in the brains of patients with LBD as well as in tg animal models, there is abundant accumulation of C-terminal α-syn fragments (Iwatsubo et al., 1996; Takeda et al., 2000; Takeda et al., 1998a). These studies suggest that the antibodies' ability to recognize the C-terminal region might be important for the therapeutic effects.

Considerable effort has recently been directed to-

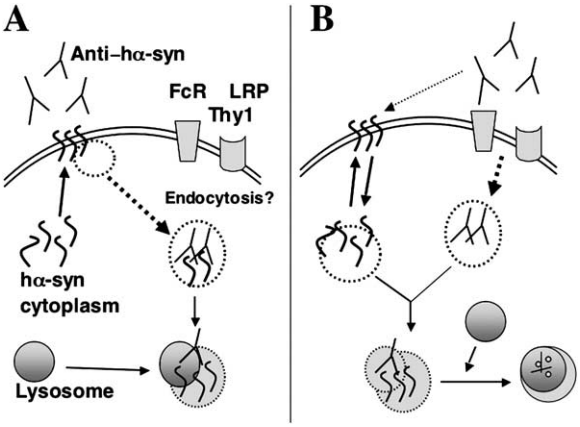


Figure 8. Potential Mechanisms of Antibody-hα-Syn Recognition and Targeting to the Lysosomal Pathway

(A) Antibodies might recognize hα-syn bound to the membrane. This complex might in turn be endocytosed and targeted for lysosomal degradation. (B) Receptor-mediated endocytosis of the antibodies and hα-syn aggregates that in turn could be targeted for lysosomal degradation.

ward the development of immunization therapies for neurodegenerative disorders, including the use of active and passive immunization strategies (Frenkel and Solomon, 2001; Morgan et al., 2000; Schenk et al., 1999), DNA vaccination (Luthi-Carter, 2003), and, more recently, adoptive transfer of immune cells to protect nigrostriatal neurons (Benner et al., 2004). Adding to this growing body of evidence supporting a role of immunization in the management of neurodegenerative disorders, the present study suggests that vaccination is effective in reducing the neuronal accumulation of toxic hα-syn aggregates and that further development of this approach might have a potential place in the treatment of LBD.

## Experimental Procedures

### Vaccination of hα-Syn tg Mice

For this study, heterozygous tg mice (Line D) expressing hα-syn under the regulatory control of the platelet-derived growth factor-β (PDGFβ) promoter (Masliah et al., 2000) were used. These animals were selected because they display abnormal accumulation of detergent-insoluble hα-syn and develop hα-syn-immunoreactive inclusion-like structures in the brain. Although some nuclear staining has been observed in this model, distinct cytoplasmic inclusion-like structures have been consistently identified by confocal and electron microscopy (Masliah et al., 2000; Masliah et al., 2001; Rockenstein et al., 2002). Furthermore, these animals also display neurodegenerative and motor deficits that mimic certain aspects of LBD. Experimental animals were divided into two groups. For the first group, a total of 20 young (3-month-old) tg mice were immunized for 8 months with purified recombinant hα-syn expressed in *E. coli* from sequence-verified hα-syn cDNA (n = 10) or CFA alone (n = 10). For the second group, a total of 20 young adult (6-month-old) tg mice were immunized for 8 months with recombinant hα-syn (n = 10) or CFA alone (n = 10). The immunization protocol consisted first of an injection with recombinant hα-syn (80 μg/ml; 100 μl) with CFA (Sigma-Aldrich, St. Louis, MO). Two weeks later, mice received another injection of hα-syn (80 μg/ml; 100 μl) with incomplete Freund's Adjuvant (FA), followed by reinjection once a month (for the subsequent 7 months) with hα-syn (80 μg/ml; 100 μl) in phosphate-buffered saline. Recombinant hα-syn was prepared and purified and tested for endotoxins at Elan following a modified version of a previously described protocol (Hashimoto et al., 1998).

### Determination of Antibody Titers and Relative Affinity to hα-Syn

Antibody levels in plasma were determined using 96-well microtiter plates coated with 0.4 μg per well of purified full-length hα-syn. Samples were incubated overnight followed by goat anti-mouse IgG alkaline phosphatase-conjugated antibody (1:7500, Promega, Madison, WI). The plate was read at wavelengths of 450 nm and 550 nm. Results were plotted on a semi-log graph with relative fluorescence units versus serum dilution. Antibody titer was defined as the dilution at which there was a 50% reduction from the maximal antibody binding.

To determine the relative affinity of the antibodies for hα-syn, three assays were performed. In the first, brain homogenates from nonimmunized hα-syn tg mice were run in a minigel, multichannel apparatus (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes. Each channel on the membrane was separated and incubated individually with diluted serum from each of the vaccinated mice. Serum dilution was adjusted to 1:1000 for all samples based on serum titers. Purified recombinant hα-syn was used as a standard, and all channels were normalized to this as a control. Channels were then incubated with secondary rabbit anti-mouse antibody (Vector Laboratories, Burlingame, CA) followed by $I^{125}$-tagged protein A (ICN Pharmaceuticals, Costa Mesa, CA) (Alford et al., 1994). Blots were imaged and analyzed with the PhosphorImager (Molecular Dynamics, Piscataway, NJ). For the second, to determine the specificity of the antibodies, brain homogenates from nontg animals, CFA-treated hα-syn tg mice, and immunized hα-syn tg mice were analyzed by WB with serum from CFA-treated mice and high relative affinity serum from hα-syn vaccinated tg mice. Blots were imaged and analyzed on a VersaDoc XL imaging apparatus (BioRad, Hercules, CA). For the third, serial vibratome sections from an untreated hα-syn tg mouse were incubated in diluted serum from each of the treated mice (adjusted to 1:1000) followed by biotinylated horse anti-mouse IgG (1:100, Vector), Avidin D-horseradish peroxidase (HRP, 1:200, ABC Elite, Vector), and reacted with diaminobenzidine tetrahydrochloride (DAB) containing 0.001% $H_2O_2$. After microscopic examination, sections were scored according to the cellular compartment labeled (neuronal cell bodies, synapses, and inclusions) and the degree of IR (0 = none; 1 = very mild, 2 = mild, 3 = moderate, 4 = intense).

### Epitope Mapping of hα-Syn Antibodies

The epitopes recognized by hα-syn antibodies were determined by an ELISA that measures the binding of an antibody to overlapping linear peptides that covered the entire hα-syn sequence. C-terminally biotinylated peptides with sequences of hα-syn (Mimotopes, San Diego, CA) were prepared as 15 aa long peptides with an overlap of 12 residues and a step of 3 residues per peptide. To run the assay, these biotinylated peptides were coated down overnight at 5 nM onto ELISA plates precoated with streptavidin (Pierce, Rockford, IL), followed by washing and incubation for 1 hr with diluted serum samples. Serum samples with titers lower than 5000 were diluted 1:1000 for this incubation. After another washing step, the bound antibodies were detected using species-specific secondary antibodies conjugated to HRP in a colorimetric ELISA format.

### Tissue Processing

Following NIH guidelines for the humane treatment of animals, mice were anesthetized with chloral hydrate and flush-perfused transcardially with 0.9% saline. Briefly, the right hemibrain was frozen and homogenized for determinations of hα-syn IR by WB (Masliah et al., 2000). The left hemibrain was fixed in 4% paraformaldehyde (PFA) and serially sectioned with the vibratome (Leica, Wetzlar, Germany) for ICC analysis.

### Synaptosomal Preparation, Membrane and Cytosolic Fractionation, and Immunoblot Analysis

Synaptosomal fractions were prepared essentially as previously described (Dodd et al., 1981a; Dodd et al., 1981b). After resuspension in 0.32 M sucrose, synaptosomal fractions were analyzed by SDS-PAGE on a 10% tris-acetate polyacrylamide gel (NuPAGE, In-

vitrogen) and transferred onto Immobilon membranes (Millipore). For further analysis, total brain homogenates were separated into membrane and cytosolic fractions, prepared essentially as previously described (Hashimoto et al., 2002). The cytosolic and membrane fractions were analyzed by SDS-PAGE on 4%–12% Bis-Tris gels and transferred onto Immobilon membranes (Millipore).

Immunoblots with synaptosomal, cytosolic, and membrane fractions were probed with an affinity-purified rabbit polyclonal antibody against hα-syn (72-10, 1:5000) (Masliah et al., 2000) or with primary antibodies against synaptophysin (1:500, Chemicon, Temecula, CA) or actin (1:1000, Chemicon), followed by secondary goat anti-rabbit or anti-mouse IgG tagged with HRP (1:5000, Santa-Cruz Biotechnology, Inc., Santa Cruz, CA). Blots were visualized by ECL and analyzed with a VersaDoc XL imaging apparatus (BioRad).

### Neuropathological and Immunocytochemical Analysis

Briefly, as previously described (Masliah et al., 2000), serially sectioned, free-floating, blind-coded vibratome sections were incubated overnight at 4°C with an anti-hα-syn-specific antibody (72-10, 1:500). To analyze the effects of the immunization in glial cell activation, sections were labeled with a mouse monoclonal antibody against the astroglial marker GFAP (1:500, Chemicon) or a rabbit polyclonal antibody against the microglial marker Iba-1 (1:1000, Wako Chemicals, Richmond, VA). Incubation with the primary antibodies was followed by biotinylated goat anti-rabbit IgG or horse anti-mouse IgG (1:100, Vector), Avidin D-HRP (1:200, ABC Elite, Vector), and reaction with DAB tetrahydrochloride containing 0.001% $H_2O_2$. Sections were analyzed with the Quantimet 570C (Leica) in order to determine the number of hα-syn-immunoreactive inclusions, astroglia, or microglia in the temporal cortex, a brain region often affected in patients with LBD (Hansen et al., 1990). For each case, three sections were analyzed, and the results were averaged and expressed as numbers per square millimeter.

Double-immunocytochemical analysis was performed as previously described (Hashimoto et al., 2004; Hashimoto et al., 2001) to determine the effects of vaccination on nerve terminal density and hα-syn accumulation in synapses. Vibratome sections were double-labeled with rabbit polyclonal antibodies against hα-syn (72-10, 1:5000) detected with Tyramide Red (1:2000, Roche, Switzerland) and with the mouse monoclonal antibody against synaptophysin (1:15, Chemicon), detected with a horse anti-mouse IgG FITC-tagged secondary antibody (1:75, Vector). For each case, sections were immunolabeled in duplicate and analyzed by LSCM and NIH Image 1.43 software to calculate the percent area of the neuropil covered by synaptophysin-immunoreactive terminals in the temporal cortex (Mucke et al., 2000) and the proportion of synaptophysin-immunoreactive terminals that were hα-syn positive (Hashimoto et al., 2004).

Control experiments were performed where sections were immunolabeled with an antibody against mα-syn (Masliah et al., 2000), detected with Tyramide Red (1:2000, Roche), or with a rabbit polyclonal antibody against β-syn (1:1000, Chemicon), detected with FITC-tagged goat anti-rabbit secondary antibody (1:75, Vector). In order to confirm the specificity of the primary antibodies, control experiments were performed where sections were incubated overnight in the absence of primary antibody (deleted), with the primary antibody preadsorbed for 48 hr with 20-fold excess of the corresponding peptide or with preimmune serum. To identify if antibodies generated by the vaccinated mice detected hα-syn in the tg animals, sections were double labeled with a horse anti-mouse FITC-tagged IgG (1:75, Vector) and a rabbit polyclonal antibody against hα-syn (72-10, 1:5000), detected with Tyramide Red (1:2000, Roche). To determine if hα-syn colocalized to lysosomes, sections were double labeled with an antibody against hα-syn (72-10, 1:5000), detected with Tyramide Red (1:2000, Roche), and a rabbit polyclonal antibody against cathepsin D (1:500, Calbiochem, San Diego, CA), detected with goat anti-rabbit FITC-tagged secondary antibody (1:75, Vector). All sections were processed simultaneously under the same conditions, and experiments were performed twice in order to assess the reproducibility of results. Sections were imaged with a Zeiss 63X (N.A. 1.4) objective on an Axiovert 35 microscope (Zeiss, Germany) with an attached MRC1024 LSCM system (BioRad, Wattford, UK) (Masliah et al., 2000). Images were analyzed with the image program NIH Image 1.43 to determine levels of mα-syn, β-syn, or cathepsin D IR.

### Determination of In Vivo Antibody Recognition of Intracellular hα-Syn

To further validate whether antibodies against hα-syn recognized intracellular hα-syn, we generated an FITC-tagged antibody for injection into the brains of nontg and hα-syn tg (Line D) mice. For this purpose, a monoclonal antibody that recognizes aa 118–126 of hα-syn was generated in mice using recombinant hα-syn (clone 9E4, Elan Pharmaceuticals). This antibody was concentrated with a 10 kDa cutoff concentrator centrifuge tube (Millipore, Billerica, MA) and linked to the FITC molecule utilizing a FluoroTag FITC conjugation kit (Sigma) according to the manufacturer's instructions. The FITC-tagged hα-syn antibody or a control nonimmune FITC-tagged IgG was injected into the brains of these nontg (n = 5) and hα-syn tg (n = 5) mice (5-month-old). Briefly, as previously described (Marr et al., 2003), mice were placed under anesthesia on a Koft stereotaxic apparatus, and each mouse was injected with 3 μl of FITC-tagged anti-hα-syn antibody (approximately 0.5 μg/μl) or anti-mouse FITC-tagged IgG into the temporal cortex (using a 5 μl Hamilton syringe, 0.25 μl/min). After 3 days, mice were anesthetized with chloral hydrate and flush-perfused transcardially with 0.9% saline. Brains were fixed in 4% PFA for 48 hr and vibratomed. Sections from the brains of these mice were then analyzed by direct fluorescence or were coimmunolabeled with antibodies against hα-syn (72-10, 1:1000) or cathepsin D (1:500, Calbiochem), detected with secondary anti-rabbit Texas Red-tagged IgG (1:75, Vector). All sections were imaged with the LSCM as described before.

### Statistical Analysis

After all results were obtained, the code was broken and statistical comparisons between groups were performed utilizing the two-tailed unpaired Student's t test. Linear regression analysis was performed to ascertain the relationship among variables. The Bonferroni correction was applied to account for multiple comparisons.

### Supplemental Data

The authors' conflict of interest statement and Supplemental Data can be found online at http://www.neuron.org/cgi/content/full/46/6/857/DC1/.

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