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### Publication Date

2017-08-01

### DOI

10.1016/j.nbd.2017.05.002

Peer reviewed



Published in final edited form as:

*Neurobiol Dis.* 2017 August ; 104: 85–96. doi:10.1016/j.nbd.2017.05.002.

## Differential effects of immunotherapy with antibodies targeting $\alpha$ -synuclein oligomers and fibrils in a transgenic model of synucleinopathy

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### Abstract

Disorders with progressive accumulation of  $\alpha$ -synuclein ( $\alpha$ -syn) are a common cause of dementia and parkinsonism in the aging population. Accumulation and propagation of  $\alpha$ -syn play a role in the pathogenesis of these disorders. Previous studies have shown that immunization with antibodies that recognize C-terminus of  $\alpha$ -syn reduces the intra-neuronal accumulation of  $\alpha$ -syn and related deficits in transgenic models of synucleinopathy. These studies employed antibodies that recognize epitopes within monomeric and aggregated  $\alpha$ -syn that were generated through active immunization or administered via passive immunization. However, it is possible that more specific effects might be achieved with antibodies recognizing selective species of the  $\alpha$ -syn aggregates. In this respect we recently developed antibodies that differentially recognized various oligomers (Syn-O1, -O2, and -O4) and fibrillar (Syn-F1 and -F2) forms of  $\alpha$ -syn. For this purpose wild-type  $\alpha$ -syn transgenic (line 61) mice were immunized with these 5 different antibodies and neuropathologically and biochemically analyzed to determine which was most effective at reducing  $\alpha$ -syn accumulation and related deficits. We found that Syn-O1, -O4 and -F1 antibodies were most effective at reducing accumulation of  $\alpha$ -syn oligomers in multiple brain regions and at

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Available online on ScienceDirect ([www.sciencedirect.com](http://www.sciencedirect.com)).

**Conflict of interest:** The authors declare no competing financial interests.

preventing neurodegeneration. Together this study supports the notion that selective antibodies against  $\alpha$ -syn might be suitable for development new treatments for synucleinopathies such as PD and DLB.

## Keywords

$\alpha$ -synuclein; Oligomers; Fibrils; Passive immunization; Dementia with Lewy bodies; Parkinson's disease

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## 1. Introduction

Synucleinopathies (Galvin et al., 2001) of the aging population, including dementia with Lewy bodies (DLB) (McKeith et al., 2004), Parkinson disease (PD), and multiple system atrophy (MSA), are characterized by behavioral alterations, cognitive impairment, sleep disorders, olfactory deficits, and gastrointestinal dysfunction (Savica et al., 2013).  $\alpha$ -Synuclein ( $\alpha$ -syn) is a 140 amino acid synaptic protein (Iwai et al., 1995) involved in neurotransmitter release (Liu et al., 2004; Nemani et al., 2010) that accumulates in synaptic terminals (Bellucci et al., 2012; Kramer and Schulz-Schaeffer, 2007; Roy et al., 2007), axons (Dickson et al., 1994; Games et al., 2013), neuronal soma (Spillantini et al., 1997; Takeda et al., 1998), and oligodendrocytes (Papp and Lantos, 1992; Wakabayashi et al., 2000). Under physiological conditions,  $\alpha$ -syn is a relatively unstructured monomer (Lashuel et al., 2013; Tsigelny et al., 2007; Tsigelny et al., 2008; Tsigelny et al., 2012) that adopts a  $\beta$ -helical structure when associated with membranes (Ulmer et al., 2005).  $\alpha$ -Syn can also adopt a tetramer conformation (Bartels et al., 2011) that is important for vesicular function (Wang et al., 2014) and accumulates as aggregated species (oligomers, protofibrils and fibrils) (Conway et al., 1998; Hashimoto and Masliah, 1999; Iwatsubo et al., 1996; Lansbury, 1999; Lashuel et al., 2013; Oueslati et al., 2010; Taschenberger et al., 2012; Trojanowski et al., 1998; Tsigelny et al., 2008; Winner et al., 2011). Aggregated species trigger neurodegeneration and can propagate from neuron-to-neuron and neuron-to-glial cells via prion-like fashion (Lee et al., 2010; Prusiner et al., 2015). The mechanisms through which  $\alpha$ -syn might trigger degeneration are complex and might include autophagy, endoplasmic reticulum (ER) stress, and mitochondrial alterations (Nakamura et al., 2011; Oliveira et al., 2011; Plotegher et al., 2014; Song et al., 2004) among others.

Currently, there are no effective treatments available for these disorders that affect over 1 million in the US alone (NIA, 2015) and probably over 10 million worldwide (NIA, 2015). Experimental treatment strategies include reducing  $\alpha$ -syn expression with anti-sense or miRNA; decreasing  $\alpha$ -syn aggregation with small molecules, increasing the clearance of  $\alpha$ -syn with drugs that promote autophagy and preventing the seeding and prion-like spreading of  $\alpha$ -syn (Lashuel et al., 2013; Valera and Masliah, 2016). Alternatively, previous immunotherapy studies have demonstrated that vaccination against  $\alpha$ -syn protected against neurodegeneration and reduced  $\alpha$ -syn accumulation by triggering clearance via autophagy (Mandler et al., 2014; Masliah et al., 2005; Masliah et al., 2011) and microglial pathways (Mandler et al., 2015). Similarly, immunization with monoclonal antibodies that recognize epitopes in the non-amyloid  $\beta$  component (NAC) and C-terminus of  $\alpha$ -syn ameliorated

behavioral deficits, reduced neurodegeneration, and  $\alpha$ -syn accumulation in neurons (Masliah et al., 2011) and glial cells reducing inflammation (Bae et al., 2012). Moreover, these antibodies reduced  $\alpha$ -syn dissemination (Bae et al., 2012; Valera and Masliah, 2013), by blocking  $\alpha$ -syn C-terminal (CT) truncation (Games et al., 2014; Valera and Masliah, 2013) and prion-like propagation (Tran et al., 2014).

However, most of these studies utilized antibodies recognizing monomeric and aggregated  $\alpha$ -syn. Therefore, we developed single chain antibodies targeting  $\alpha$ -syn oligomers and fibrils expressed from lentiviral vectors (Price et al., 2016; Spencer et al., 2014; Spencer et al., 2016). Although promising, there were some limitations, which led to the development of highly specific monoclonal antibodies differentially targeting oligomers versus fibrils. These antibodies demonstrated high specificity utilizing inhibition ELISA, as well as preabsorption of the monoclonal antibodies with  $\alpha$ -syn fibrils or monomers, which was further confirmed using surface plasmon resonance (Vaikath et al., 2015). None of the antibodies cross-reacted with monomeric or fibrillar forms of  $\beta$ - or  $\gamma$ -syn, or with other amyloidogenic proteins and peptides (Vaikath et al., 2015). Further characterization, indicated that monoclonal antibodies (mAbs) Syn-O1, -O2, and -O4 recognized both early “oligomers” and late aggregates “amyloid fibrils”, whereas Syn-F1 and -F2 preferentially recognized late aggregates (Vaikath et al., 2015). In this context, for the present study, we immunized mThy1- $\alpha$ -syn mice that mimic some aspects of DLB and PD with these 5 different antibodies (Syn-O1, -O2, -O4, -F1, and -F2) against oligomers and fibrils. We found that the Syn-O1, Syn-O4 and Syn-F1 antibodies were most effective at ameliorating neurodegeneration by preventing of  $\alpha$ -syn accumulation and related mitochondrial alterations. Together, this study supports the notion that selective antibodies against  $\alpha$ -syn oligomers might be suitable for development of new treatments for synucleinopathies such as DLB and PD.

## 2. Materials and methods

### 2.1. Mouse model of $\alpha$ -syn accumulation and passive immunization

We utilized 6 month old female mice over-expressing human  $\alpha$ -syn under the mThy1 promoter (mThy1- $\alpha$ -syn, Line 61) (Rockenstein et al., 2002) and female non-transgenic (tg) mouse littermates. The line 61  $\alpha$ -syn tg model was selected because these mice develop behavioral motor deficits (Fleming et al., 2004), axonal pathology and accumulation of CT-cleaved  $\alpha$ -syn and aggregates in neocortex, limbic system and subcortical regions (Games et al., 2013). In this model, accumulation of  $\alpha$ -syn in the hippocampus results in Lewy body neurites and degeneration of CA3 neurons similar to DLB (Overk et al., 2014; Rockenstein et al., 2007; Rockenstein et al., 2002). Female mice were utilized since the transgene integration site is on the X chromosome; therefore, female mice display higher levels of  $\alpha$ -syn and pathology that is more consistent.

For the immunotherapy experiments, 6 non-tg and 36  $\alpha$ -syn tg mice were included in this randomized and double-blind study. Groups of 6 mice each were immunized with either vehicle or Syn-O1, -O2, -O4, -F1 and -F2 antibodies. Mice received weekly intraperitoneal injections at 30 mg/kg for a period of 3 months (Fig. 1A). The antibodies were generated with recombinant  $\alpha$ -syn where Syn-O1, -O2, and -O4 antibodies preferentially recognize

early “oligomers” and late aggregates “amyloid fibrils”, while Syn-F1 and -F2 antibodies preferentially recognizes late aggregates (Vaikath et al., 2015). We have previously shown that comparable FITC-tagged monoclonal antibodies traffic into the CNS and recognize  $\alpha$ -syn aggregates (Masliah et al., 2011).

## 2.2. Behavioral analysis

Mice were tested in the open field (25.5 × 25.5 cm) for 15 min using an automated system (TruScan system for mice; Coulbourn Instruments, Allentown, PA). Time spent in-motion (total activity) was automatically collected for 15 min using the TruScan software. This test reflects adaptation of the animals to the novel environment, as well as anxiety behavior.

Upon sacrifice, the whole brain was removed and split in the sagittal plane. The right hemisphere was post-fixed in phosphate-buffered 4% paraformaldehyde (pH 7.4) at 4 °C for 48 h for neuropathological analysis, and the right hemisphere was flash-frozen and stored at -80°C for ELISA assays. All experiments described were approved by the animal use and care committee of the University of California San Diego (UCSD) and were performed according to NIH guidelines for animal use.

## 2.3. Antibodies for immunotherapy and in vitro cell based characterization

The detailed generation and characterization of the antibodies were reported previously, (Vaikath et al., 2015). In brief, for the generation of the antibodies, mice were immunized with  $\alpha$ -syn fibrils. Following booster immunization, mice with high antibody titers were sacrificed, and the spleen cells isolated and fused with myeloma cells. Positive hybridomas were selected by ELISA and stable clones selected by limiting dilution. The antibody was purified by protein G affinity and characterized extensively. The specificity of the antibodies was detected by dot blot, affinity of the antibodies was determined by inhibition ELISA and surface plasmon resonance. The epitope mapping was carried out by pepscan.

To further characterize the differential ability of the 5 monoclonal antibodies at reducing  $\alpha$ -syn oligomerization, a cell based  $\alpha$ -syn assay based on bimolecular fluorescence complementation (BiFC) was utilized (Bae et al., 2014). The assay consists with two different SH-SY5Y cell lines expressing  $\alpha$ -syn fused to either the N-terminus (V1S) or C-terminus (SV2) fragment of Venus, a variant of yellow fluorescence protein (Fig. 1A). For co-culture, V1S and SV2 stable cells (125,000 cells each) were mixed and seeded on to poly-L-lysine coated coverslips. After a 3-day co-culture, the interaction of V1S and SV2 fused  $\alpha$ -synuclein was visualized and analyzed by venus protein complementation analysis (Fig. 1A).

## 2.4. ELISA for total, human, oligomers and p- $\alpha$ -synuclein

As previously described (Vaikath et al., 2015), frozen hemi-brains from mice passively immunized using one of five different monoclonal antibodies (Syn-O1, -O2, -O4, -F1 and -F2) as well as vehicle-treated non-tg and  $\alpha$ -syn tg mice were homogenized in CelLytic buffer (Sigma-Aldrich, USA), containing a cocktail of protease inhibitors, including AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride), aprotinin, E-64, EDTA and leupeptin (Calbiochem, Germany) and centrifuged at 100,000×g for 3 h at 4 °C. The

supernatant was collected, total protein concentration was measured, and all samples were adjusted to 0.1 mg/ml and stored at  $-80^{\circ}\text{C}$ . Total-, human total-, oligomeric- and phosphorylated at Ser129- (pS129)  $\alpha$ -syn levels were measured using recently developed ELISA assays (Majbour et al., 2016a; Majbour et al., 2016b). Briefly, to capture total-, human total-or pS129- $\alpha$ -syn, a 384-well ELISA microplate was coated by overnight incubation at  $4^{\circ}\text{C}$  with 0.1  $\mu\text{g/ml}$  Syn-140 (sheep anti- $\alpha$ -syn polyclonal antibody) in 200 mM  $\text{NaHCO}_3$ , pH 9.6 (50  $\mu\text{l/well}$ ), while 0.2  $\mu\text{g/ml}$  of 5G4 (anti-aggregated  $\alpha$ -syn mouse monoclonal antibody, EMD Millipore, MA, USA), was used to capture  $\alpha$ -syn oligomers. After incubation with 100  $\mu\text{l/well}$  of blocking buffer (PBS-T containing 2.25% gelatin) for 2 h at  $37^{\circ}\text{C}$ , 50  $\mu\text{l/well}$  of the brain lysates (5  $\mu\text{g/ml}$  in CellLytic buffer, SigmaAldrich, USA) and serial dilutions of recombinant human-, pS129-or oligomeric- $\alpha$ -syn (50  $\mu\text{l}$ ) were dispensed in each well and the plate was incubated at  $37^{\circ}\text{C}$  for 2.5 h. After washing with PBS-T, 50  $\mu\text{l/well}$  of FL-140 (rabbit polyclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA), 11D12 (mouse anti- $\alpha$ -syn monoclonal antibody) and PS129 (mouse anti-pS129- $\alpha$ -syn monoclonal antibody) for measuring total-, oligomeric-, total human- $\alpha$ -syn or pS129- $\alpha$ -syn, respectively, were added to the corresponding wells, and incubated at  $37^{\circ}\text{C}$  for 2 h. Next, the plates were washed and incubated for 2 h at  $37^{\circ}\text{C}$  with 50  $\mu\text{l/well}$  of species-appropriate secondary antibodies (goat anti-rabbit IgG HRP or donkey anti-mouse IgG HRP, Jackson ImmunoResearch Laboratories Inc., USA, 1:20 K). After washing, the plates were incubated with 50  $\mu\text{l/well}$  of an enhanced chemiluminescent substrate (SuperSignal ELISA Femto, Pierce Biotechnology, Rockford, Illinois, US). The chemiluminescence, expressed in relative light units, was immediately measured using VICTOR™ X3 multi-label plate reader (PerkinElmer, Turku, Finland). Samples were measured in a blinded fashion and tested randomly. The results were confirmed with at least two independent experiments. A series of internal controls was also run to check for run-to-run variations. The concentrations of  $\alpha$ -syn species in the samples were calculated using the corresponding standard curve.

## 2.5. Slot blot experiment

The amount of aggregated  $\alpha$ -syn present in the brain lysates was assessed by filter retardation assay. Briefly, 2  $\mu\text{g}$  (50  $\mu\text{l}$ ) of the transgenic brain lysates from the antibody treated, vehicle and 0.5  $\mu\text{g}$  of recombinant human  $\alpha$ -syn fibrils and monomers in duplicates were filtered through nitrocellulose membrane (0.45  $\mu\text{m}$  pore size, Amersham Protran, GE Healthcare Life science) using a 48-slot slot-blot filtration apparatus (GE Healthcare), and washed with 1000 $\mu\text{l}$  of PBSpH7.4. The nitrocellulose membrane was air dried and then incubated with 5% skim milk before probed with 0.2  $\mu\text{g/ml}$  of 5G4 antibody (anti-aggregated  $\alpha$ -syn mouse monoclonal antibody, EMD Millipore, MA, USA). The membrane was washed and incubated with secondary antibody (goat anti-mouse IgG HRP, Jackson ImmunoResearch Laboratories Inc., USA, 1:100 K) and developed with enhanced chemiluminescent substrate (SuperSignal West Femto Maximum sensitivity substrate, Pierce Biotechnology, Rockford, Illinois, USA).

## 2.6. Immunohistochemistry, neuropathological and image analysis

Analysis of  $\alpha$ -syn accumulation was performed using vibratome-cut free-floating coronal brain sections (40  $\mu\text{m}$ ). Brain sections were incubated overnight at  $4^{\circ}\text{C}$  with a polyclonal

antibody against total  $\alpha$ -syn (1:500, affinity-purified rabbit polyclonal, Millipore) (Games et al., 2013; Masliah et al., 2000) in the presence or absence of proteinase K (PK; 10  $\mu$ g/ml for 15 min) or non-immune IgG controls (background levels), a neuronal marker (NeuN, mouse monoclonal, Millipore 1:2500), an astroglial cell marker (glial fibrillary acidic protein [GFAP], mouse monoclonal, Millipore 1:2500), a microglial cell marker (Iba1; goat polyclonal, Abcam, 1:5000). followed by incubations with secondary antibodies biotinylated (1:100, Vector Laboratories, Inc., Burlingame, CA), Avidin D-HRP (1:200, ABC Elite, Vector) and detection with 3,3'-diaminobenzidine (Masliah et al., 2011). All sections were processed simultaneously under the same conditions and experiments were performed in triplicate in order to assess the reproducibility of results.

Sections were analyzed with an Olympus BX41 digital video microscope equipped with a Optronics magna fire SP camera, Lucis<sup>TM</sup> DHP unique contrast enhancement software and Image Pro Express<sup>TM</sup> for live image acquisition and processing at 630 $\times$  magnification. For each section 4 areas of interest (1024  $\times$  1024 pixels) within the neocortex, hippocampus, striatum and *S. nigra* were analyzed in real time using the Image Pro Plus program.  $\alpha$ -Syn and GFAP levels of immunoreactivity were expressed as corrected optical density (arbitrary units). As per our previous studies, while total  $\alpha$ -syn antibodies label cell bodies and neuropil, PK-resistant aggregates are primarily found in the synapses (Games et al., 2014). Sections labeled with Iba-1, were analyzed utilizing the Image-Pro Plus program (Media Cybernetics, Silver Spring, MD) (10 digital images per section at 400 $\times$  magnification) and analyzed in order to estimate the average number of immuno-labeled cells per unit area (100  $\mu$ m<sup>2</sup>). Ramification of Iba-1 positive microglia was evaluated as previously described (Morrison and Filosa, 2013). Sections were scanned with a digital Olympus bright field digital microscope (BX41).

Briefly as previously described (Overk et al., 2014), the numbers of NeuN-immunoreactive neurons in the hippocampus were estimated utilizing unbiased stereological methods. The CA3 region of the hippocampus was outlined using an Olympus BX51 microscope running StereoInvestigator 8.21.1 software (Micro-BrightField, Cochester, VT) (grid sizes 150  $\times$  150  $\mu$ m and the counting frames were 30  $\times$  30  $\mu$ m). The average coefficient of error for each region was <0.09. Sections were analyzed using a 100  $\times$  1.4 PlanApo oil-immersion objective. A 5  $\mu$ m high disector allowed for 2  $\mu$ m top and bottom guard-zones.

## 2.7. Double immunolabeling and laser scanning confocal microscopy

To evaluate the effects of immunization at the synaptic site, sections were immunolabeled with antibodies against the synaptic vesicle protein, synapsin I (1:100 rabbit polyclonal, Millipore) (Scott et al., 2010) and synaptophysin (mouse monoclonal, Millipore, 1:100), or  $\alpha$ -syn (mouse monoclonal LB5091:1000). Vibratome sections were single and double labeled as previously (Scott et al., 2010). Then reacted with secondary antibodies tagged with FITC (vector, horse anti mouse, 1:200) to detect synaptophysin and with the tyramide Red amplification system (Perkin-Elmer, 1:100, NEN Life Sciences, Boston, MA) to detect synapsin I or  $\alpha$ -syn. Sections were mounted on superfrost slides (Fisher) and cover-slipped with media containing DAPI. Sections were imaged with a Zeiss 63X (N.A. 1.4) objective on an Axiovert 35 microscope (Zeiss) with an attached MRC1024 laser scanning confocal



microscope system (BioRad, Hercules, CA). All experiments were conducted blind-coded, code was broken after analysis was performed. For each animal approximately 100 presynaptic terminals were in an area of 100  $\mu\text{m}^2$ . Digitized images were analyzed with Image J as previously described to estimate the co-localization of  $\alpha$ -syn with synaptophysin and synapsin I and synaptophysin (Scott et al., 2010).

## 2.8. Statistical analysis

All experiments were done blind-coded and in duplicate. Values in the figures were expressed as means  $\pm$  SEM. To determine the statistical significance, values were compared using one-way analysis of variance (ANOVA) with Dunnett's post-hoc test comparing each group to vehicle-treated  $\alpha$ -syn tg mice, as indicated in each figure legend. The adjusted p-values are reported in the results section, and the means were considered significantly different if the adjusted p-values were  $<0.05$ .

## 3. Results

### 3.1. Immunotherapy with antibodies against oligomers and fibrils differentially reduces accumulation of PK resistant $\alpha$ -syn in various brain regions of the $\alpha$ -syn tg mice

The five different monoclonal antibodies selected for this in vivo study has been previously characterized utilizing cell free assays (Vaikath et al., 2015). To further verify the activity of these antibodies in a cell based system, the  $\alpha$ -syn BiFC assay was utilized. With this system,  $\alpha$ -syn dimerization or oligomerization was detected as a fluorescent inclusion in SH-SY5Y cells each expressing half of the tag (Fig. 1A). As expected in cultured single cells (V1S) no fluorescent aggregates were detected (Fig. 1B), in contrast in the vehicle treated with the two cell system (V1S/SV2) abundant fluorescent aggregates were detected compared to vehicle treated V1S single cell system (Fig. 1B, C; p-value = 0.0001). Treatment with Syn-O1 (p-value = 0.0001) and -O4 (p-value = 0.0001) significantly reduced fluorescent aggregates. Syn-O2 also reduced fluorescent aggregates, albeit to a lesser extent (Fig. 1B, C; p-value = 0.0078) and no effects were detected with Syn-F1 or -F2 (Fig. 1B, C). In order to determine the effects of passive immunization using 5 different monoclonal antibodies (Syn-O1, -O2, -O4, -F1 and -F2) on the accumulation of  $\alpha$ -syn in the cortex, hippocampus, striatum, and *substantia nigra* (*S. nigra*), 6-month old female mice (6 month old, N = 6/group) were immunized weekly ip for 3 months, along with vehicle controls in both  $\alpha$ -syn tg and non-tg mice. Vibratome sections were immunoreacted with anti-total-(t)- $\alpha$ -syn antibody (Fig. 2A) and semi-quantitated using computer-aided analysis of the optical density (Fig. 2B–E). As expected, non-tg mice injected with the vehicle control showed mild punctae immunolabeling of the neuropil (Fig. 2A), while  $\alpha$ -syn tg mice injected with the vehicle control showed strong immuno-reactivity for t- $\alpha$ -syn in the both neuronal cell bodies as well as in neurites across all brain regions (Fig. 2; p-value = 0.0001 for each brain region comparison). Compared to vehicle-injected  $\alpha$ -syn tg mice, mice immunized with Syn-O1 showed a statistically significant reduction in t- $\alpha$ -syn immunoreactivity across all brain regions (Fig. 2; p-value = 0.0001 for neocortex, hippocampus, and striatum; p-value = 0.0154 for *S. nigra*), while Syn-O2 weakly reduced t- $\alpha$ -syn in the striatum (Fig. 2A, B–E; p-value = 0.0381). Interestingly, Syn-O4 showed region-specific reduction of t- $\alpha$ -syn in the neocortex (p-value = 0.0001) and hippocampus (Fig. 2A–C; p-value = 0.0001). Syn-F1



immunization resulted in a reduction of t- $\alpha$ -syn in the neocortex (Fig. 2A, B; p-value = 0.0005), striatum (Fig. 2A, D; p-value = 0.0005), and *S. nigra* (Fig. 2A, E; p-value = 0.0002), but not in the hippocampus (Fig. 2A, C), while immunization with Syn-F2 resulted in a region-specific reduction of t- $\alpha$ -syn in the striatum (Fig. 2A, D; p-value = 0.0280) and *S. nigra* (Fig. 2A, E; p-value = 0.0001).

Next, to determine the effects of the immunotherapy on the accumulation of aggregated  $\alpha$ -syn vibratome brain sections from the immunized mice were pretreated with PK prior to being immunoreacted with anti- $\alpha$ -syn antibody (Fig. 3A). As expected, non-tg mice injected with the vehicle control showed only light background staining (Fig. 3A), while  $\alpha$ -syn tg mice injected with the vehicle control showed strong PK resistant  $\alpha$ -syn immunoreactivity in dystrophic neurites in the neuropil across all brain regions (Fig. 3A), as well as accumulation in neuronal cell bodies in the *S. nigra* (Fig. 3A). The significant increase in immunoreactivity in vehicle-treated  $\alpha$ -syn tg mice compared to vehicle-treated non-tg mice was confirmed by computer-aided densitometry in the neocortex (p-value = 0.0001), hippocampus (p-value = 0.0001), striatum (p-value = 0.0001), and *S. nigra* (p-value = 0.0001) (Fig. 3A, B–E). Immunization with Syn-O1 lead to a significant reduction in PK-resistant  $\alpha$ -syn immunoreactivity in the neocortex (Fig. 3A, B; p-value = 0.0070), hippocampus (Fig. 3A, C; p-value = 0.0381) and striatum (Fig. 3A, D; p-value = 0.0001), but not the *S. nigra* (Fig. 3A, E), while immunization with Syn-O2 weakly reduced PK-resistant  $\alpha$ -syn immunoreactivity in the hippocampus (Fig. 3A, C; p-value = 0.0222) and striatum (Fig. 3A, D; p-value = 0.0476). Similar to immunization with Syn-O1, Syn-O4 immunization also lead to a significant reduction in PK-resistant  $\alpha$ -syn immunoreactivity when compared to vehicle-treated  $\alpha$ -syn tg mice in the neocortex (Fig. 3A, B; p-value = 0.0034), hippocampus (Fig. 3A, C; p-value = 0.0001) and striatum (Fig. 3A, D; p-value = 0.0001), but not the in the *S. nigra* (Fig. 3A, E). Syn-F1 (p-value = 0.0001) and -F2 (p-value = 0.0001) immunization lead to a selective reduction in PK-resistant  $\alpha$ -syn immunoreactivity only in the hippocampus (Fig. 3A, C).

### 3.2. Analysis of the effects of immunotherapy with antibodies against oligomers and fibrils on $\alpha$ -syn by ELISA in $\alpha$ -syn tg mice

In order to further characterize the effects of passive immunization with monoclonal antibodies (Syn-O1, -O2, -O4, -F1 and -F2) on  $\alpha$ -syn levels in  $\alpha$ -syn tg mice, brain homogenates were evaluated using ELISAs for total-, human-, oligomer-, and pS129- $\alpha$ -syn (Fig. 4). Compared to vehicle-treated  $\alpha$ -syn tg mice, immunization with Syn-O2 reduced total  $\alpha$ -syn in  $\alpha$ -syn tg mice (Fig. 4A; p-value = 0.0033), while passive immunization with Syn-O4 significantly decreased the levels of human  $\alpha$ -syn in  $\alpha$ -syn tg mice when compared to vehicle-treated  $\alpha$ -syn tg mice (Fig. 4B; p-value = 0.0146). Immunization with each of the five monoclonal antibodies: Syn-O1 (p-value = 0.0002), -O2 (p-value = 0.0001), -O4 (p-value = 0.0001), -F1 (p-value = 0.0098), and -F2 (p-value = 0.0011) was able to significantly reduce the levels of oligomeric  $\alpha$ -syn in  $\alpha$ -syn tg mice compared to vehicle-treated  $\alpha$ -syn tg mice (Fig. 4C). Levels of pS129  $\alpha$ -syn were elevated in the vehicle-treated  $\alpha$ -syn tg mice compared to vehicle-treated non-tg mice (p-value = 0.0001) while none of the monoclonal antibodies reduced pS129  $\alpha$ -syn levels in the  $\alpha$ -syn tg mouse line (Fig. 4D). In order to further confirm the effects of passive immunization with monoclonal antibodies on the levels

of  $\alpha$ -syn oligomers in  $\alpha$ -syn tg mice, brain homogenates were evaluated by Slot Blot using 5G4 antibody specific for aggregated  $\alpha$ -syn (Fig. 4E). Aggregated  $\alpha$ -syn was significantly increased in vehicle-treated  $\alpha$ -syn tg mice compared to vehicle-treated non-tg mice (Fig. 4E, F; p-value = 0.0001).  $\alpha$ -Syn tg mice immunization with Syn-O1 (p-value = 0.0006), -O2 (p-value = 0.0153), and -O4 (p-value = 0.005) significantly reduced the amount of aggregated  $\alpha$ -syn compared to vehicle-treated  $\alpha$ -syn mice (Fig. 4E, F). Although not considered statistically significant, Syn-F1 (p-value = 0.0559) and -F2 (p-value = 0.0954) trended toward also decreasing the levels of aggregated  $\alpha$ -syn.

### 3.3. Passive immunotherapy with antibodies against $\alpha$ -syn oligomers and fibrils differentially ameliorates neurodegeneration in the $\alpha$ -syn tg mice

We have previously shown that in the  $\alpha$ -syn tg there is an age-dependent neuronal loss in the hippocampus (Overk et al., 2014) accompanied by astrogliosis (Kim et al., 2015) and microgliosis (Watson et al., 2012). We chose to focus on these brain regions as we have previously shown that in addition to the vulnerability of the striato-nigral system,  $\alpha$ -syn oligomers also accumulates in the CA3 neurons and promote selective degeneration mimicking DLB (Overk et al., 2014; Rockenstein et al., 2007; Rockenstein et al., 2002). Therefore, to evaluate the effects of passive immunization using five different monoclonal antibodies on rescuing neurons from neurodegeneration associated with abnormally aggregated  $\alpha$ -syn, the number of NeuN-positive pyramidal cells in the CA3 region of the hippocampus was estimated (Fig. 5). Compared to vehicle-treated non-tg mice, vehicle-treated  $\alpha$ -syn tg mice had a significant decrease in the number of NeuN-positive neurons in CA3 (Fig. 5A, B; p-value = 0.0222). Immunization with Syn-O1 (p-value = 0.0013), Syn-O4 (p-value = 0.0003), and Syn-F1 (p-value=0.0004) antibodies, prevented the loss of neurons in the hippocampal CA3 regions (Fig. 5A, B); while immunization with the Syn-O2 and Syn-F2 antibodies failed to ameliorate the loss of NeuN-positive neurons compared to vehicle-treated non-tg mice (Fig. 5A, B).

Next, we investigated the effects of passive immunization with the five different monoclonal antibodies at reducing the neuroinflammation associated with the accumulation of  $\alpha$ -syn in the hippocampus. Compared to vehicle-treated non-tg mice,  $\alpha$ -syn tg mice had a significant increase in immunoreactivity for the astroglial marker, GFAP in the hippocampus (Fig. 6A, B; p-value = 0.0001). Interestingly, only Syn-O1 (p-value = 0.0001) and Syn-O4 (p-value = 0.001) were effective at reducing GFAP-immunoreactivity levels in the  $\alpha$ -syn tg mice, comparable to vehicle-treated non-tg mice (Fig. 6A, B). While,  $\alpha$ -syn tg mice immunized with Syn-O2, -F1, and -F2 showed increases in the GFAP-immunoreactivity (Fig. 6A, B).

Finally, to evaluate the effects of immunization with the five monoclonal antibodies on levels of microgliosis immunocytochemistry and image analysis with an antibody against Iba-1 was performed. Compared to vehicle-treated non-tg mice, the vehicle treated  $\alpha$ -syn tg displayed an increase in the number of Iba-1-positive cells in the hippocampus (Fig. 7A, B; p-value = 0.0005). Interestingly, only mice immunized with Syn-O4 antibody displayed levels of Iba-1-positive cells in the hippocampus that were comparable to the vehicle treated non-tg mice and significantly reduced compared to vehicle treated  $\alpha$ -syn tg mice (Fig. 7A, B; p-value = 0.0001), while mice immunized with Syn-O1, -O2, -F1, and -F2 antibodies

failed to reduce the numbers of Iba-1-positive cells in the hippocampus compared to the vehicle treated  $\alpha$ -syn-tg mice (Fig. 7A, B). Additionally, microglia ramification was quantified. Similarly to the increase in the number of Iba-1 positive cells, there was also an increase in ramification in vehicle-treated  $\alpha$ -syn tg mice compared to vehicle-treated non-tg mice (Fig. 7A, C; p-value = 0.0385).  $\alpha$ -Syn tg mice immunized with Syn-O1 (p-value = 0.0003) and -O4 (p-value = 0.0118) showed significantly reduced microglial ramification compared vehicle-treated  $\alpha$ -syn tg mice (Fig. 7A, C), while treatment with Syn-O2, -F1, and -F2 failed to reduce Iba-1 ramification.

#### **3.4. Immunotherapy with antibodies against $\alpha$ -syn oligomers and fibrils differentially prevent alterations in the presynaptic terminals of $\alpha$ -syn tg mice**

Previous studies have shown that among other mechanisms,  $\alpha$ -syn oligomers might trigger neurodegenerative pathology by interfering with the distribution of synaptic vesicle proteins in the presynaptic bouton (Scott et al., 2010). In experimental models of synucleinopathy and in patients with PD/DLB levels of synapsin I, SNAP25, and other vesicle proteins are reduced (Scott et al., 2010). Therefore, sections from the mice immunized with the five antibodies were immunolabeled with synapsin I (red channel) and synaptophysin (FITC channel), and analyzed with the laser scanning confocal microscope. Compared to the vehicle-treated non-tg mice, the vehicle-treated  $\alpha$ -syn tg mice displayed decreased colocalization between synapsin I and synaptophysin (Fig. 8A, B; p-value < 0.0001). Immunotherapy with the Syn-O1 (p-value < 0.0001), -O4 (p-value < 0.0001), and -F1 (p-value = 0.0048) rescued the presynaptic alterations in hippocampal neurons in the  $\alpha$ -syn tg mice (Fig. 8A, B). In contrast,  $\alpha$ -syn tg mice treated with Syn-O2 and F2 displayed persistent colocalization of synapsin I and synaptophysin (Fig. 8A, B). Next, sections double labeled with synaptophysin (FITC channel) and  $\alpha$ -syn (red channel) were analyzed with the laser scanning confocal microscope. Compared to the vehicle treated non-tg mice, vehicle treated  $\alpha$ -syn tg mice displayed increased co-localization of  $\alpha$ -syn with synaptophysin (Fig. 9A, B; p-value = 0.0001). Immunotherapy with the Syn-O1 (p-value = 0.0001), -O4 (p-value = 0.0001), -F1 (p-value = 0.0055), and F2 (p-value = 0.0001) reduced the colocalization of  $\alpha$ -syn immunoreactivity with synaptophysin in the  $\alpha$ -syn tg mice compared to vehicle-treated  $\alpha$ -syn tg mice (Fig. 9A, B).

#### **3.5. Immunotherapy with antibodies against $\alpha$ -syn oligomers and fibrils differentially decreases hyperactivity in $\alpha$ -syn tg**

We have previously shown that  $\alpha$ -syn tg mice displayed increase activity in the open field test (Wrasidlo et al., 2016). These alterations appear related to anxiety and might be consistent with the hippocampal alterations. In order to evaluate the functional effects of the five different monoclonal antibodies mice were tested using total activity in an open field, rearing, and thigmotaxis. Total activity in  $\alpha$ -syn tg mice has been well characterized as being abnormally increased, which was recapitulated in vehicle-treated tg mice compared to vehicle-treated non-tg mice (Fig. 10A; p-value = 0.0063). Interestingly, passive immunization with Syn-O1 (p-value = 0.0064), -O4 (p-value = 0.0036), and -F1 (p-value = 0.0142) reduced the aberrant increase in total activity to levels found in vehicle-treated  $\alpha$ -syn-tg mice (Fig. 10A). There were no significant alterations in the rates of rearing (Fig. 10B) or thigmotaxis (Fig. 10C).

## 4. Discussion

Recent studies suggest that diverse species of  $\alpha$ -syn aggregates might be responsible for the phenotypical differences in synucleinopathies of the aging population (Guo et al., 2013; Peelaerts et al., 2015; Prusiner et al., 2015). Therefore, developing antibodies that selectively target specific conformers might be relevant toward developing more effective therapies for PD, DLB and MSA. For this reason, for the present study we evaluated the differential effects of passive immunization with three different monoclonal antibodies against  $\alpha$ -syn oligomers and two monoclonal antibodies against  $\alpha$ -syn fibrils on deficits associated with abnormal  $\alpha$ -syn accumulation in the neocortex, limbic system, and subcortex in  $\alpha$ -syn tg mice. The limbic system alterations mimic some aspects of DLB. We showed that Syn-O1, -O4 and -F1 antibodies were effective at reducing accumulation of  $\alpha$ -syn oligomers in multiple brain regions and at ameliorating deficits associated with the accumulation of abnormal  $\alpha$ -syn aggregates in  $\alpha$ -syn tg mice (Table 1). Specifically, Syn-O1 and -O4 reduced levels of total and PK-resistant  $\alpha$ -syn in at least three of the four evaluated regions of interest. Syn-F1 similarly reduced total  $\alpha$ -syn levels in three of the four evaluated regions, and reduced PK-resistant  $\alpha$ -syn aggregate accumulation in the striatum. Moreover, Syn-O1 and -O4 and -F1 antibodies prevented neurodegeneration and reduced astrogliosis in the CA3 region of the hippocampus (Table 1). Additionally, Syn-O4 also reduced levels of the neuroinflammatory marker, Iba-1 in the hippocampus (Table 1). Importantly, passive immunization with Syn-O1, -O4 and -F1 antibodies resulted in improved the total activity behavioral outcome measure (Table 1).

These results are in agreement with our recent studies utilizing brain penetrant single chain antibodies that showed that clones capable of detecting  $\alpha$ -syn oligomers were more effective at reducing synucleinopathy in tg when compared to single chain antibodies that had higher affinity for  $\alpha$ -syn monomers (Spencer et al., 2014; Spencer et al., 2016). The advantage of the single chain antibodies in this study was the specificity and the ability to cross the blood brain barrier and as such they represent an important proof of principle. However, single chain antibodies are short lived, moreover delivery in our experimental models required production of the single chain from lentiviral vectors, which could makes clinical development more difficult. For these reasons, development of comparable, (humanized) monoclonal antibodies targeting selected  $\alpha$ -syn aggregates is important for therapeutical development.

It is worth noting that while for previous studies we developed active and passive immunization with linear peptides (Mandler et al., 2014; Masliah et al., 2005) and antibodies recognizing the C-terminus of  $\alpha$ -syn (Games et al., 2014; Masliah et al., 2011) that appear effective, these antibodies not only recognized  $\alpha$ -syn monomers but also aggregates including oligomers and fibrils found in Lewy bodies. In contrast, the five new monoclonal antibodies selected for this study are different in that they specifically target aggregates but not monomers. These antibodies were generated by immunizing animals with  $\alpha$ -syn fibrils rather than linear peptides or  $\alpha$ -syn monomers. Syn-O1, -O2, and -O4 recognize  $\alpha$ -syn oligomers and amyloid fibrils while Syn-F1 and -F2 have a preference for  $\alpha$ -syn fibrils (Vaikath et al., 2015). Syn-O1 and -O4 appear to target early oligomers, while -O2 might recognize later stage oligomers. Moreover, another advantage of these antibodies is that they

have higher IC<sub>50</sub> values compared to other reports of affinities for monoclonal antibodies against  $\alpha$ -syn oligomers/protofibrils (Vaikath et al., 2015). In addition, Syn-O1, -O2, -O4, -F1, and -F2 are highly specific to  $\alpha$ -syn and do not cross react with  $\beta$ - or  $\gamma$ -syn, nor do they cross react with A $\beta$ , Tau40, IAPP or ABri, which is of interest since most antibodies with specific conformational epitopes are less selective (Vaikath et al., 2015). These antibodies all exhibited very weak reactivity for C-terminal portion of  $\alpha$ -syn (amino acids 127–140). The superior performance of antibodies against oligomers such as Syn-O1 and -O4 compared to antibodies against fibrils might in part be related to our model, which mimics aspects of early synucleinopathy. In our Line 61 mice, abundant formation and accumulation of oligomers is detected but fibrils are less common (Rockenstein et al., 2014). An alternative explanation for the different effects of the antibodies might be related to affinity. While we do not have a direct comparison of the antibodies to determine affinity; however, in vitro assay results indicated that Syn-O1, -O2 and -O4 recognize both oligomers and fibrils while Syn-F1 and -F2 preferentially recognize amyloid fibril (late aggregates) of  $\alpha$ -syn (Vaikath et al., 2015). More recently, novel models have been developed involving the use of protofibrils and fibrils that seeds the aggregation of endogenous  $\alpha$ -syn (Luk et al., 2012), in future studies it will be of interest to compare in vivo the activities of Syn-O1, -O2, -O4, -F1, and -F2 in such models.

One of the advantages of targeting  $\alpha$ -syn oligomers and fibrils include selectively reducing the toxic species of  $\alpha$ -syn while minimizing disruption of the physiological functions of  $\alpha$ -syn. While the exact physiological role of  $\alpha$ -syn is still under investigation, it appears to be involved in neurotransmission and vesicular function (Wang et al., 2014). Aggregated  $\alpha$ -syn accumulation as oligomers and fibrils (Conway et al., 1998; Hashimoto and Masliah, 1999; Iwatsubo et al., 1996; Lansbury, 1999; Lashuel et al., 2013; Oueslati et al., 2010; Taschenberger et al., 2012; Trojanowski et al., 1998; Tsigelny et al., 2008; Winner et al., 2011) might trigger neurodegeneration by interfering with  $\alpha$ -syn function at the synapse and by gain of toxicity. Several mechanisms for gain of toxicity has been invoked including defective axonal transport, endosomal and lysosomal activity, ER stress, alterations in autophagy, mitochondria (Plotegher et al., 2014), microtubules and calcium regulation among others. Moreover, recent studies have linked accumulation of  $\alpha$ -syn with alterations in the synapse (Scott et al., 2010). In experimental models or synucleinopathy and in patients with DLB and PD, synapsin I is a marker of synaptotoxicity (Scott et al., 2010). In agreement with this possibility, we observed that in the vehicle treated  $\alpha$ -syn tg mice, the colocalization between synapsin I and synaptophysin is dramatically decreased while the presence of pathological  $\alpha$ -syn is increased. Immunotherapy with Syn-O1 -O4 and -F1 time decreased the colocalization of pathological  $\alpha$ -syn with synaptophysin and concomitantly increased the colocalization of synapsin I with synaptophysin thus improving the markers of synaptotoxicity. These results are in agreement with the neuropathological and biochemical studies and support the notion that among others, one novel potential mechanism for immunotherapy against  $\alpha$ -syn is rescuing neurons from synaptotoxicity by reducing pathological  $\alpha$ -syn in the presynaptic bouton. Taken together, immunotherapy with anti- $\alpha$ -syn oligomer antibodies might rescue the phenotype in the tg mice by ameliorating alterations in synaptic function; however, additional studies will be needed to clarify the molecular mechanisms involved.

Therefore identifying antibodies that specifically recognize  $\alpha$ -syn aggregates but not the monomer, such as Syn-O1, -O4, and -F1, which decreased aggregated and oligomeric  $\alpha$ -syn, and ameliorated neurodegeneration and mitochondrial alterations are of scientific interest in treating synucleinopathies. In this context, this study supports the notion that selective antibodies against  $\alpha$ -syn might be suitable for development new treatments for synucleinopathies such as DLB and PD.

## Acknowledgments

This work was funded by NIH grants AG18840, NS044233, BX003040, AG0051839, and AG10483. The funding sources had no role in study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

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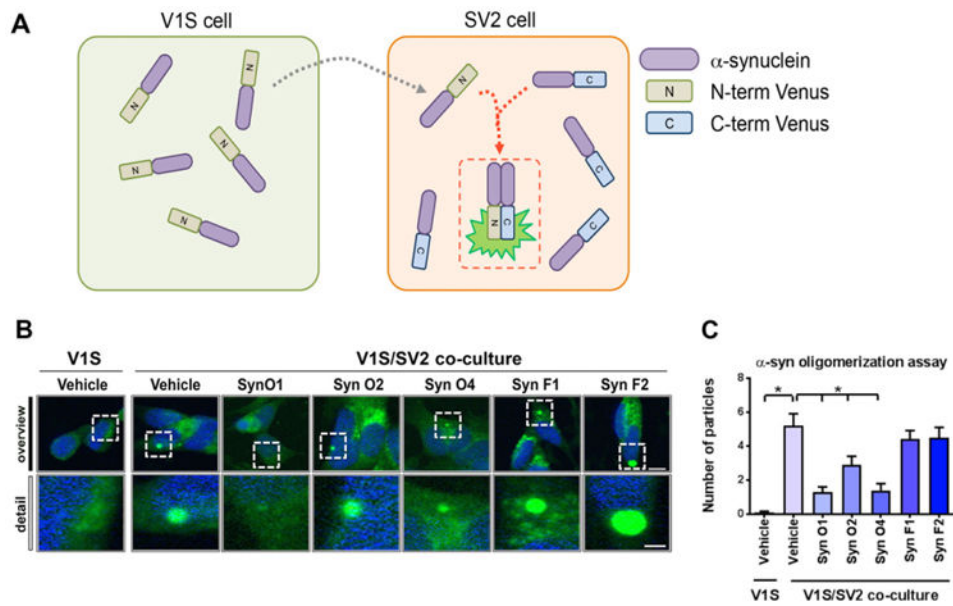


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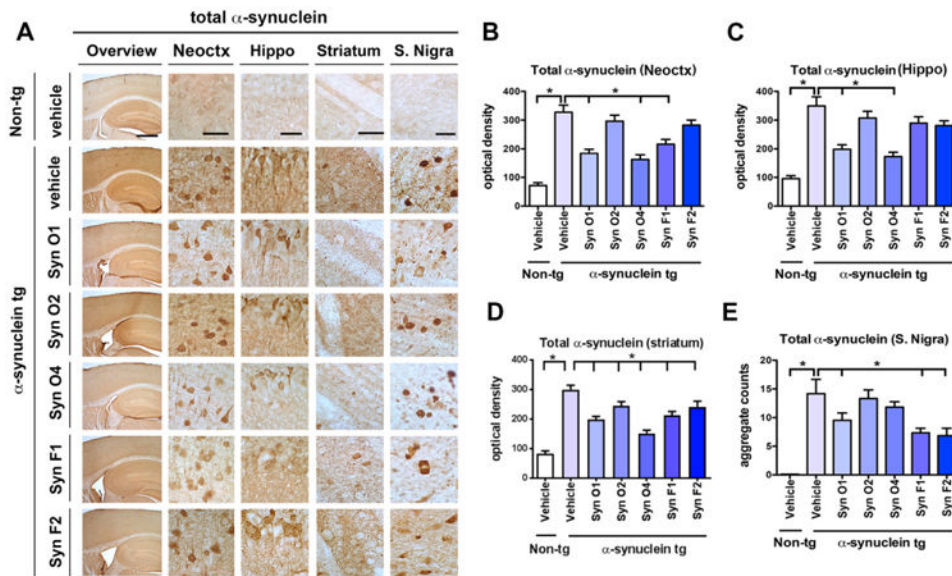
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## Abbreviations

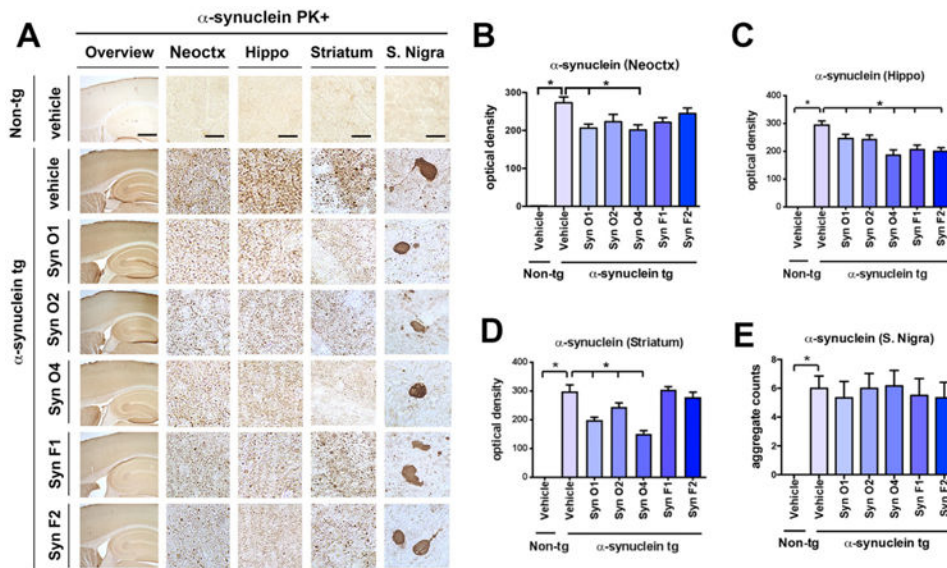
|                                  |  |
|----------------------------------|--|
| <b>AEBSF</b>                     | 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride |
| <b><math>\alpha</math>-syn</b>   | $\alpha$ -synuclein                                    |
| <b>BiFC</b>                      | bimolecular fluorescence complementation               |
| <b>CT</b>                        | C-terminal   |
| <b>DLB</b>                       | dementia with Lewy bodies                              |
| <b>ER</b>                        | endoplasmic reticulum                                  |
| <b>mAbs</b>                      | monoclonal antibodies                                  |
| <b>MSA</b>                       | multiple system atrophy                                |
| <b>NAC</b>                       | non-amyloid $\beta$ component                          |
| <b>PD</b>                        | Parkinson disease                                      |
| <b>PK</b>                        | proteinase K   |
| <b>pS129</b>                     | phosphorylated Ser129                                  |
| <b>tg</b>                        | transgenic   |
| <b>t-<math>\alpha</math>-syn</b> | total- $\alpha$ -syn                                   |

**Fig. 1.**

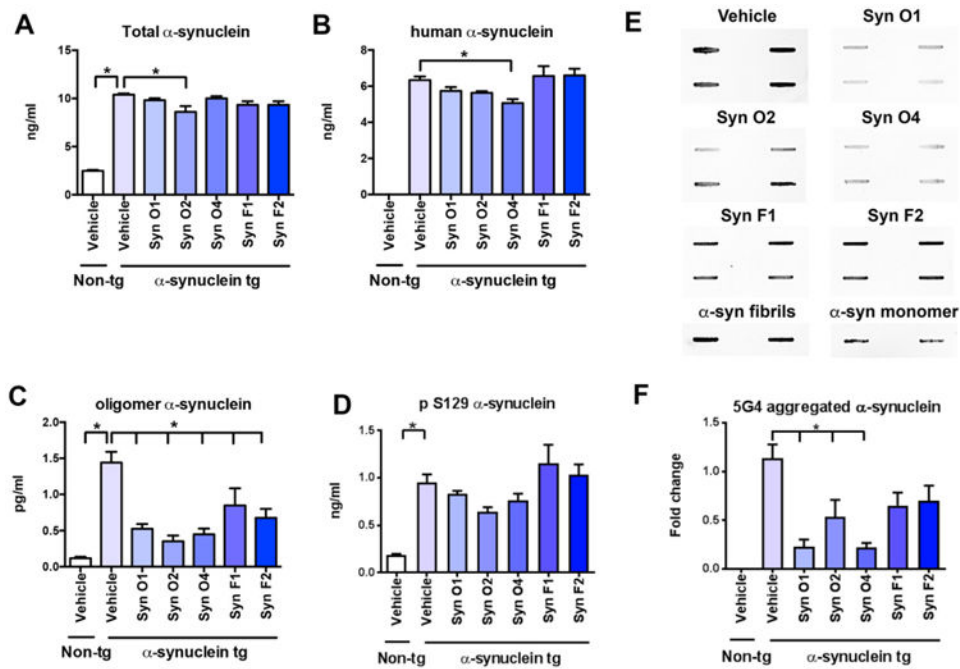
In vitro testing of the differential ability of the  $\alpha$ -syn antibodies at blocking oligomerization in a cell-based fluorescent complementation assay. (A) Schematic diagram of cell-based  $\alpha$ -synuclein oligomerization monitoring assay. The assay consists with two different SH-SY5Y cell lines expressing N-terminal venus (V1S) or C-terminal venus (SV2) conjugated  $\alpha$ -synuclein, respectively. To visualize fluorescence complementation, V1S and SV2 cells were co-cultured for 3 days. (B) Representative images were taken from SV2 cells after a 3-day co-culture in the presence of various  $\alpha$ -synuclein antibodies. (C) Quantification of the average numbers of fluorescence particles was analyzed in SV2 cells. Scale bar upper panel = 10  $\mu$ m; lower panel = 5  $\mu$ m. \* = p-value < 0.05 using One-way ANOVA followed by Dunnett's multiple comparisons post hoc test comparing each group with vehicle-treated  $\alpha$ -syntg mice.



**Fig. 2.** Effects of passive immunization on total  $\alpha$ -syn accumulation in  $\alpha$ -syn tg mice. Female  $\alpha$ -syn tg mice were passively immunized once a week for 3 months starting at 6 months of age using one of five different monoclonal antibodies (Syn-O1, -O2, -O4, -F1, and -F2) or vehicle control. Vehicle-treated non-tg mice were included as a control group. (A) Overview and high magnification of total  $\alpha$ -syn immunoreactivity in the frontal cortex, hippocampus, striatum, and *S. nigra* of vehicle-treated non-tg control mice, vehicle-treated  $\alpha$ -syn tg mice, and passively immunized  $\alpha$ -syn tg mice. (B) Computer-aided quantitation of the optical density of total  $\alpha$ -syn immunoreactivity in the (B) frontal cortex, (C) hippocampus, (D) striatum, and (E) aggregate counts in the *S. nigra*. Low magnification scale bar = 250  $\mu$ m and high magnification scale bar = 25  $\mu$ m; N = 6 per group. \* = p-value < 0.05 using one-way ANOVA followed by Dunnett's post-hoc test comparing each group and vehicle-treated  $\alpha$ -syn tg mice.

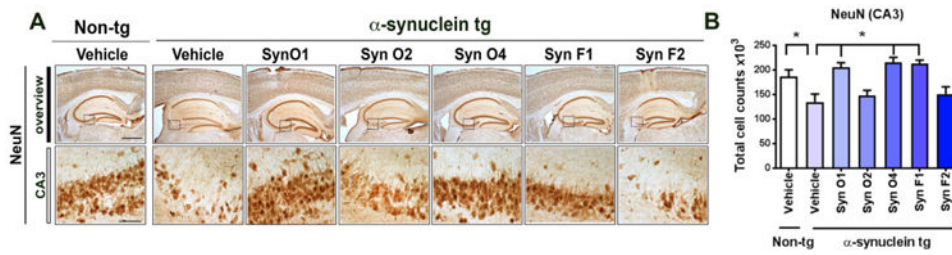


**Fig. 3.** Effects of passive immunization on PK-resistant  $\alpha$ -syn aggregate accumulation in  $\alpha$ -syn tg mice. Female  $\alpha$ -syn tg mice were passively immunized once a week for 3 months starting at 6 months of age using one of five different monoclonal antibodies (Syn-O1, -O2, -O4, -F1, and -F2) or vehicle control. Vehicle-treated non-tg mice were included as a control group. (A) Overview and high magnification of PK-resistant  $\alpha$ -syn immunoreactivity in the frontal cortex, hippocampus, striatum, and *S. nigra* of vehicle-treated non-tg control mice, vehicle-treated  $\alpha$ -syn tg mice, and passively immunized  $\alpha$ -syn tg mice. (B) Computer-aided quantitation of the optical density of PK-resistant  $\alpha$ -syn immunoreactivity in the (B) frontal cortex, (C) hippocampus, (D) striatum, and (E) aggregate counts in the *S. nigra*. Low magnification scale bar = 250  $\mu$ m and high magnification scale bar = 25  $\mu$ m; N = 6 per group. \* = p-value < 0.05 using one-way ANOVA followed by Dunnett's post-hoc test compared to vehicle-treated  $\alpha$ -syn tg mice.

**Fig. 4.**

ELISA analysis of the effects of passive immunization with anti- $\alpha$ -syn antibodies on total, human, oligomer and p- $\alpha$ -syn in tg mice. Female  $\alpha$ -syn tg mice were passively immunized once a week for 3 months starting at 6 months of age one of five different monoclonal antibodies (Syn-O1, -O2, -O4, -F1, and -F2) or vehicle control. Vehicle-treated non-tg mice were included as a control group. (A) Quantitative ELISA analysis of total  $\alpha$ -syn indicated a significant increase across all immunization groups in the wt  $\alpha$ -syn tg mice compared to the non-tg mice. Passive immunization with Syn-O2 significantly reduced total  $\alpha$ -syn in wt  $\alpha$ -syn tg mice compared to vehicle-treated wt  $\alpha$ -syn tg mice. (B) Quantitative ELISA analysis of human  $\alpha$ -syn indicated that non-tg mice are devoid of human  $\alpha$ -syn, and that passive immunization with Syn-O4 significantly decreased the levels of human  $\alpha$ -syn in wt  $\alpha$ -syn tg mice compared to vehicle-treated wt  $\alpha$ -syn tg mice. (C) Quantitative ELISA analysis of oligomer  $\alpha$ -syn revealed a statistically significant increase in vehicle-treated wt  $\alpha$ -syn mice compared to vehicle-treated non-tg mice. Passive immunizations with each of the five monoclonal antibodies significantly reduced the levels of oligomeric  $\alpha$ -syn in wt  $\alpha$ -syn tg mice compared to vehicle-treated  $\alpha$ -syn mice. (D) Quantitative ELISA analysis of pS129  $\alpha$ -syn indicated a significant increase in all wt  $\alpha$ -syn tg mouse groups and that none of the monoclonal antibodies were able to reduce pS129  $\alpha$ -syn levels in the wt  $\alpha$ -syn tg mouse line. (E) Slot blot images and (F) quantification of the band intensity for the brain lysates. \*p-value < 0.05 using ANOVA followed by Dunnett's post-hoc test comparing each group with vehicle-treated  $\alpha$ -syn tg mice N = 6 per group.

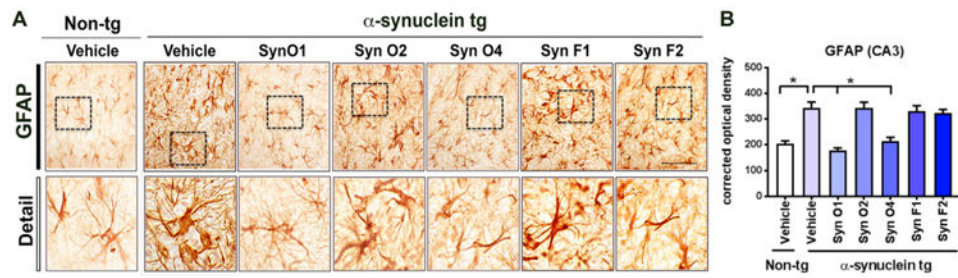




**Fig. 5.**

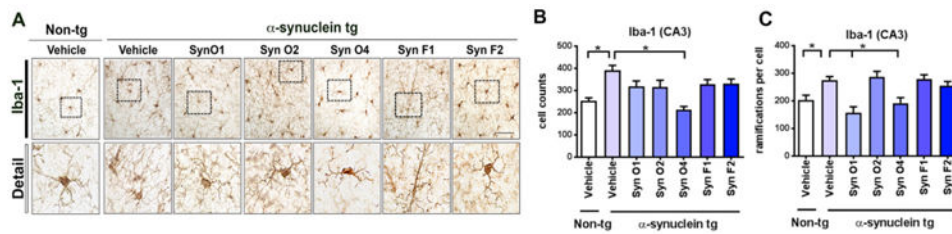
Immunohistochemical analysis of the effects of passive immunization with anti- $\alpha$ -syn antibodies in a neuronal marker (NeuN) in  $\alpha$ -syn tg mice. Female  $\alpha$ -syn tg mice were passively immunized once a week for 3 months starting at 6 months of age using one of five different monoclonal antibodies (Syn-O1, -O2, -O4, -F1, and -F2) or vehicle control. Vehicle-treated non-tg mice were included as a control group. (A) Representative low and high photomicrographs and (B) computer-aided analysis of CA3 hippocampus immunostained with the neuronal antibody marker NeuN. \*p-value < 0.05 using ANOVA followed by Dunnett's post-hoc test comparing each group with vehicle-treated  $\alpha$ -syn tg mice. Low magnification scale bar = 250  $\mu$ m, high magnification scale bar 50  $\mu$ m. N = 6 mice/group.





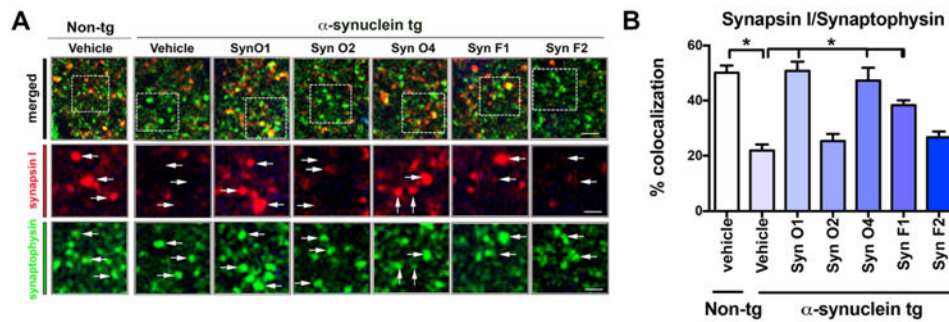
**Fig. 6.**

Immunohistochemical analysis of the effects of passive immunization with anti- $\alpha$ -syn antibodies on an astroglial cell marker (GFAP) in  $\alpha$ -syn tg mice. Female  $\alpha$ -syn tg mice were passively immunized once a week for 3 months starting at 6 months of age using one of five different monoclonal antibodies (Syn-O1, -O2, -O4, -F1, and -F2) or vehicle control. Vehicle-treated non-tg mice were included as a control group. (A) Representative low and high photomicrographs and (B) computer-aided analysis of CA3 hippocampus immunostained with the neuroinflammation antibody marker GFAP. \*p-value < 0.05 using ANOVA followed by Dunnett's post-hoc test comparing each group with vehicle-treated  $\alpha$ -syn tg mice. Scale bar = 50  $\mu$ m; N = 6 mice/group.

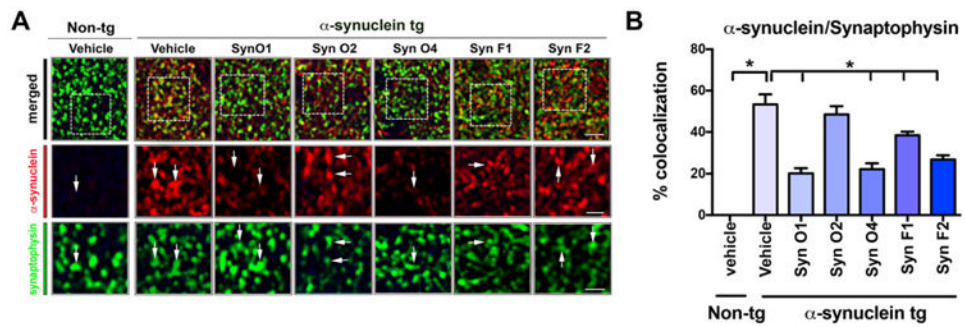


**Fig. 7.**

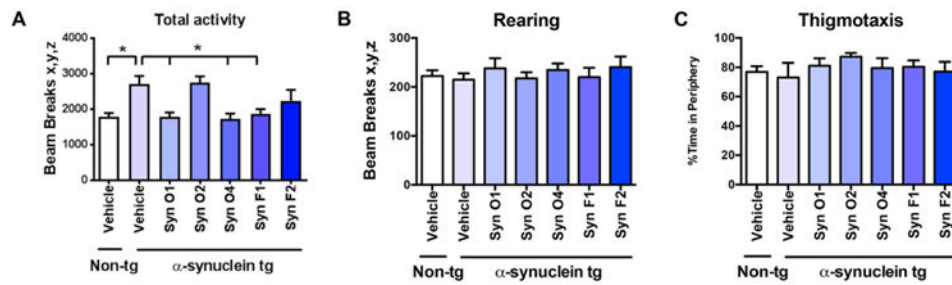
Immunohistochemical analysis of the effects of passive immunization with anti-oligomer antibodies on a microglial cell marker (Iba-1) in  $\alpha$ -syn tg mice. Female  $\alpha$ -syn tg mice were passively immunized once a week for 3 months starting at 6 months of age using one of five different monoclonal antibodies (Syn-O1, -O2, -O4, -F1, and -F2) or vehicle control. Vehicle-treated non-tg mice were included as a control group. (A) Representative low and high magnification photomicrographs and (B) computer-aided analysis of CA3 hippocampus immunostained with the neuroinflammation antibody marker Iba-1. \*p-value < 0.05 using ANOVA followed by Dunnett's post-hoc test comparing each group with vehicle-treated  $\alpha$ -syn tg mice. Scale bar = 50  $\mu$ m; N = 6 mice/group.



**Fig. 8.** Double immunocytochemical and laser scanning confocal microscopy analysis of the effects of passive immunization with anti- $\alpha$ -syn antibodies on the synaptic vesicle marker synapsin I in  $\alpha$ -syn tg mice. Female  $\alpha$ -syn tg mice were passively immunized once a week for 3 months starting at 6 months of age using one of five different monoclonal antibodies (Syn-O1, -O2, -O4, -F1, and -F2) or vehicle control. Vehicle-treated non-tg mice were included as a control group. Vibratome sections containing the hippocampal CA3 region immunoreacted with an antibody against the synaptic vesicle marker, synapsin I, and synaptophysin and analyzed by confocal microscopy. (A) Representative confocal images of synapsin I (red channel) and synaptophysin (FITC channel) staining (B) quantitative analysis. \*p-value < 0.05 using ANOVA followed by Dunnett's post-hoc test comparing each group with vehicle-treated  $\alpha$ -syn tg mice. Lower magnification scale bar = 5  $\mu$ m, higher magnification scale bars = 2  $\mu$ m; N = 6 per group.



**Fig. 9.** Double immunocytochemical and laser scanning confocal microscopy analysis of presynaptic terminals and  $\alpha$ -syn in mice passively with anti- $\alpha$ -syn antibodies. Female  $\alpha$ -syn tg mice were passively immunized once a week for 3 months starting at 6 months of age using one of five different monoclonal antibodies (Syn-O1, -O2, -O4, -F1, and -F2) or vehicle control. Vehicle-treated non-tg mice were included as a control group. Vibratome sections containing the hippocampal CA3 region immunoreacted with an antibody against the presynaptic marker, synaptophysin (green), and  $\alpha$ -syn (red) and analyzed by confocal microscopy. (A) Representative low- and high-magnification (inset) confocal images of colocalization between synaptophysin-immunoreactive punctuate staining and  $\alpha$ -syn and (B) quantitative analysis. \*p-value < 0.05 using ANOVA followed by Dunnett's post-hoc test comparing each group with vehicle-treated  $\alpha$ -syn tg mice. Lower magnification scale bar = 5  $\mu$ m, and high magnification scale bar = 2  $\mu$ m; N = 6 per group.

**Fig. 10.**

Functional analysis of the effects of immunization with  $\alpha$ -syn oligomer and fibril antibodies in the open field in  $\alpha$ -syn tg mice. Female  $\alpha$ -syn tg mice were passively immunized once a week for 3 months starting at 6 months of age using one of five different monoclonal antibodies (Syn-O1, -O2, -O4, -F1, and -F2) or vehicle control. Vehicle-treated non-tg mice were included as a control group. (A) Total activity, as measured by the number of beam breaks, was significantly increased in vehicle-treated  $\alpha$ -syn tg mice compared to vehicle-treated non-tg mice. Passive immunization with Syn-O1, -O4, and -F1 significantly decreased total activity in  $\alpha$ -syn tg mice compared to vehicle-treated  $\alpha$ -syn tg mice. (B) Rearing and (C) thigmotaxis were unaffected in any of the treatment groups. \*p-value < 0.05 using ANOVA followed by Dunnett's post-hoc test comparing each group with vehicle-treated non-tg mice. N = 6 mice/group.

Table 1

Summary of mAb improvements in tested outcome measures.

| Outcome measure                                       | Syn-O1                   | Syn-O2 | Syn-O4 | Syn-F1 | Syn-F2 |
|---|--------------------------|--------|--------|--------|--------|
| Total $\alpha$ -syn levels improved vs. vehicle       | ✓                        | ✓      | ✓      | ✓      | ✓      |
|   | Neocortex                |        |        |        |        |
|   | Hippocampus              |        |        |        |        |
|   | Striatum                 |        |        |        |        |
|   | <i>S. nigra</i>          |        |        |        | ✓      |
| PK-treated $\alpha$ -syn levels improved vs. vehicle  | ✓                        | ✓      | ✓      | ✓      | ✓      |
|   | Neocortex                |        |        |        |        |
|   | Hippocampus              |        |        |        |        |
|   | Striatum                 |        |        |        |        |
|   | <i>S. nigra</i>          |        |        |        |        |
| $\alpha$ -Syn levels improved vs. vehicle (ELISA)     | ✓                        | ✓      | ✓      | ✓      | ✓      |
|   | t- $\alpha$ -syn         |        |        |        |        |
|   | h- $\alpha$ -syn         |        |        |        |        |
|   | Oligo- $\alpha$ -syn     |        |        |        |        |
|   | pS129- $\alpha$ -syn     |        |        |        |        |
| Rescue neurodegeneration                              | ✓                        | ✓      | ✓      | ✓      | ✓      |
| Rescue neuroinflammation                              | ✓                        | ✓      | ✓      | ✓      | ✓      |
|   | Aggregated $\alpha$ -syn |        |        |        |        |
|   | NeuN                     |        |        |        |        |
|   | GFAP                     |        |        |        |        |
|   | Iba-1                    |        |        |        |        |
|   | Iba-1 ramification       |        |        |        |        |
| Increase colocalization synapsin I and synaptophysin  | ✓                        | ✓      | ✓      | ✓      | ✓      |
| Reduce colocalization synaptophysin and $\alpha$ -syn | ✓                        | ✓      | ✓      | ✓      | ✓      |
| Normalizing behavior                                  | ✓                        | ✓      | ✓      | ✓      | ✓      |
|   | Total activity           |        |        |        |        |
|   | Rearing                  |        |        |        |        |
|   | Thigmotaxis              |        |        |        |        |