Reduction of brain stem pathology and transient amelioration of early cognitive symptoms in transgenic mice treated with a monoclonal antibody against α-synuclein oligomers/protofibrils

S. Ekmark-Lewén [a], A. Aniszewska [a], A. Molisak [a], A. Gumucio [a], V. Lindström [a], P.J. Kahle [b], E. Nordström [c], C. Möller [c], J. Fälting [c], L. Lannfelt [a,c], J. Bergström [a], M. Ingelsson [a,d,e,*]

[a] Department of Public Health and Caring Sciences, Molecular Geriatrics, Uppsala University, Uppsala, Sweden
[b] Department of Neurodegeneration, Hertie Institute for Clinical Brain Research and German Center for Neurodegenerative Diseases, Tübingen, Germany
[c] BioArctic AB, Stockholm, Sweden
[d] Krembil Brain Institute, University Health Network, Toronto, Ontario, Canada
[e] Tanz Centre for Research in Neurodegenerative Diseases, Departments of Medicine and Laboratory Medicine & Pathobiology, University of Toronto, Toronto, Ontario, Canada

ARTICLE INFO

ABSTRACT

Immunotherapy against alpha-synuclein (α-syn) is a promising novel treatment strategy for Parkinson's disease (PD) and related α-synucleinopathies. We have previously shown that systemic treatment with the monoclonal oligomer/protofibril-selective antibody mAb47 targeting cytotoxic α-syn leads to reduced central nervous system levels of such species as well as an indication of reduced late-stage symptoms in aged (Thy-1)-h[A30P] α-syn transgenic mice.

Here, we performed an early-onset long-term treatment study with this antibody to evaluate effects on brain pathology and behavioral outcomes in the same mouse model. Compared to the placebo group, the treatment strongly reduced phosphorylated α-syn (pS129 α-syn) pathology in the upper brain stem. Moreover, a preserved recognition memory and risk assessment behavior could be seen in antibody-treated mice at six months of age, even although these effects were no longer significant at eleven months of age. Importantly, no evidence of inflammatory responses or other potential toxic effects was seen with the treatment. Taken together, this study supports the strategy to target α-syn oligomers/protofibrils with monoclonal antibodies to counteract early symptoms and slow down the progression of PD and other α-synucleinopathies.

Introduction

Parkinson's disease (PD) and dementia with Lewy bodies (DLB) are common neurodegenerative disorders causing progressive nerve cell loss in various brain regions. The pathogenesis of these disorders involves fibrillization and deposition of the presynaptic protein alpha-synuclein (α-syn) into Lewy bodies and Lewy neurites, the pathological hallmarks of PD and DLB [1–3].

Insoluble forms of α-syn are formed via a number of intermediate steps, with the monomeric protein undergoing a conformational

shift followed by formation of dimers, oligomers and protofibrils [4]. Whereas Lewy bodies were previously believed to be the only disease-causing pathological entity [5], both *ex vivo* and *in vivo* studies have indicated that soluble α-syn oligomers and protofibrils are more prone to exert toxic effects, such as disruption of cellular membranes [6,7] and inflammatory reactions [8,9].

In addition to sporadic disease forms, mutations and/or multiplications in the α-syn gene (*SNCA*) can lead to familial PD and DLB, supporting the disease-related significance of α-syn [10]. The dominantly inherited A30P and A53T mutations are believed to be pathogenic by promoting self-oligomerization of α-syn [11]. The (Thy-1)-h[A30P] α-syn transgenic (tg) mouse model overexpresses human A30P α-syn under the Thy-1 promoter and features aggregated α-syn accumulation in neuronal cell bodies and neurites [12,13]. Further, these mice display early behavioral disturbances, such as memory impairment, as well as late-life motor symptoms, including paralysis and impaired coordination with a variable age of onset [14,15].

Novel treatment strategies for PD and DLB are aimed at reducing and limiting the propagation of α-syn pathology in the central nervous system (CNS). Immunotherapy, passive or active, targeting α-syn has been shown to decrease α-syn levels, improve behavioral symptoms and reduce cell-to-cell transfer of α-syn [16–19]. Masliah et al. demonstrated a significant reduction in α-syn burden and prevention of cell loss in tg mice actively immunized against α-syn for eight months [18]. Due to the lack of excessive immune reactions, it was suggested that the mechanism of α-syn clearance is dependent on antibody internalization and lysosomal degradation, rather than activation of the cell-mediated immune response. Additional α-syn immunization strategies have yielded similar results (reviewed in [20,21]).

We developed several α-syn oligomer/protofibril selective antibodies that were proven to be internalized by cells via the Fcγ receptor and reduce α-syn pathology *ex vivo* [22–24]. In addition, we have demonstrated that short term systemic treatment with one of these, mAb47, reduced levels of pathogenic α-syn species in the CNS and indicated a decreased appearance of severe end-stage symptoms in aged (Thy-1)-h[A30P] α-syn tg mice [25]. In the present study, we aimed at evaluating if mAb47 also has a preventive effect on neuropathological and behavioral abnormalities in these mice. Mainly, we found that weekly intraperitoneal antibody administrations, from one and a half to eleven months of age, ameliorated α-syn brain stem pathology as well as early cognitive symptoms, without causing any neuroinflammatory responses.

Materials and methods

Animals

All experiments involving mice were approved by the Uppsala County Animal Ethics Board (5.8.18–08038/2018) and in compliance with the European Communities Council Directive for animal experiments (2010/63/EU). Homozygous (Thy-1)-h[A30P] α-syn tg mice [12], expressing human α-syn with the A30P mutation under the Thy-1 promoter, were used for the study. All animals were housed at the National Veterinary Institute (Uppsala, Sweden) in open cages on a 12:12 h reversed dark: light cycle, with food and water available *ad libitum*. The study was blinded for the researchers performing injections, behavioral assessment and data analyses.

Passive immunization

In total, 63 mice were included in the study. From six weeks of age, (Thy-1)-h[A30P] α-syn tg mice (n = 22, twelve males and ten females) received weekly intraperitoneal injections with 20 mg/kg of the oligomer/protofibril-selective version of the recombinant mouse IgG1, Igh^c haplotype, monoclonal antibody mAb47 (mAb47-group) or the corresponding volume of sterile PBS (n = 23, twelve males and eleven females, placebo-treated group). One group of C57bl6/*J* wild type (Wt) mice, the same background line as (Thy-1)-h [A30P] α-syn tg mice, born and housed in the same animal facility as the tg mice, received PBS in order to control for the effect of injections only (n = 18, ten males and eight females, Wt group). All mice received weekly injections for ten months, until eleven months of age, when they were sacrificed.

Sample size

The sample size was calculated based on results from our previous paper, analyzing behavior in (Thy-1)-h[A30P] α-syn tg mice [14]. A power analysis showed that the sample size of 16 mice/group had an 80% power to detect an effect size of 18 % change, assuming a 5 % significance level and a two-sided test. Considering the long study period, the group size was increased slightly from the calculated number of mice.

Perfusion and tissue sampling

Within 24 h from the last behavioral test, at eleven months of age, mice were anesthetized with isoflurane before being transcardially perfused with 0.9% saline, followed by removal of the brain and spinal cord. The left brain hemispheres and the spinal cords were frozen on dry ice and stored at −70 °C while the right hemispheres were fixed in 4 % PFA, followed by a brief exposure to 70% ethanol and paraffin embedding.

Assessment of antibody concentration in plasma and brain

Antibody concentration was measured in plasma and brain. Plasma was collected from the heart before perfusion of the eleven

months old mice. 96-well MSD standard plate (Mesoscale) were coated with 0.5 µg/ml of α-syn monomers in PBS overnight at 4 °C. Plates were blocked with 1% Blocker A (Mesoscale) for 1 h, after which standards and samples were added in duplicates and incubated while shaking (900 rpm) for 2 h at room temperature. The TBS-Triton brain tissue extracts were diluted 1:200 and plasma samples were diluted 1:40 000 in 1% Blocker A. For detection, biotinylated goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL, USA) was used, followed by Streptavidin SULFO-Tag (Mesoscale). Both incubations steps lasted for 1 h at room temperature at 900 rpm. Thereafter 2x Read buffer (Mesoscale) was added to each well and the signal was determined using MSD Sector Imager (Mesoscale). Wells were washed three times in TBS-Tween (0.05%) between each step.

Immunohistochemistry

Immunohistochemical (IHC) detection was used to analyze the impact of the treatment on α-syn pathology as well as assessment of potential detrimental effects of long-term mAb47 treatment on brain inflammation.

Paraffin embedded right hemispheres from 27 mice (n = 9 for males and females, randomly selected from each treatment group) were sagitally sectioned (5 µm) and mounted on glass slides. Four sections from each animal were probed for pS129 α-syn, GFAP, Iba-1 and Mac-2. In addition, six sections from each of the PBS-treated animals (n = 9) (three sections for pK treatment and three control sections) were used to investigate resistance to proteinase K (pK, Invitrogen, Waltham, MA).

Prior to IHC, sections were deparaffinized by subsequent incubation with xylene (2x 10 min), followed by rehydration with decreasing ethanol concentrations (100% x2, 96%, 70%, x10 min) and water (2x 10 min) and a standard epitope retrieval procedure in 25 mM sodium citrate. The tissue sections analyzed for pK resistance were thereafter incubated for 1 min with 50 µg/ml of pK. For immunodetection, sections were pretreated with DAKO peroxidase blocking solution (DAKO), blocked in 5% NGS or M.O.M. blocking solution (Vector, Burlingame, CA) and incubated with primary antibodies overnight in 4 °C. The following day, sections were incubated with appropriate secondary antibodies. All antibodies and dilution used in IHC procedures are listed in Table 2.

Antibody binding was visualized using HRP conjugated streptavidin (Mabtech, Nacka Strand, Sweden), diluted 1:250 in PBS and NovaRed Chromogen Peroxidase Substrate kit (Vector, Burlingame, CA). Subsequently, sections were dehydrated and fixed in increasing ethanol concentrations (70–100%) and xylene, then mounted with Pertex (Histolab, Askim, Sweden).

All IHC pictures were acquired using a Nikon Eclipse 80i microscope and NIS Elements BR 4. 20.00 software. The phosphorylated α-syn (pS129 α-syn) staining was automatically analyzed on four brain sections/animal in two pictures/region taken at 40x, in three selected areas with widespread pS129 α-syn pathology: the upper brain stem region, hippocampal dentate gyrus (DG) and prefrontal cortex, using Image J (NIH). For the tissues used to assess pK digestion six tissue sections per animal (three with and three without prior pK-treatment) were analyzed in two brain regions (upper brain stem and prefrontal cortex). The numbers of Mac2 positive cells were quantified by a blinded researcher on four brain sections for each animal and cells were counted manually on the whole section area.

Assessment of astrocyte activation

To determine if treatment with mAb47 causes upregulation of the astrocyte activation marker glial fibrillary acidic protein (GFAP), a sandwich ELISA was used. High-binding 96 well plates were coated overnight in 4 °C with 0.5 µg/mL of mouse anti-GFAP (Sigma Aldrich, G3893) antibody, followed by blocking in blocking buffer containing 1% BSA and incubation with brain homogenates diluted in TBS. Rabbit anti-GFAP (DAKO, Z0334) in 0.96 µg/mL was used as a detection antibody and 0.4 µg/mL of HRP conjugated anti-rabbit IgG (ThermoFisher, 31460) was used as a secondary antibody. The reaction was visualized using K-Blue Aqueous TMB substrate (Neogen, 331177), and 1 M sulfuric acid was added to stop the colorimetric reaction. Plates were read at 450 nm on Tecan Infinite M200 PRO reader (Tecan Group Ltd, Männedorf, Switzerland). The experiment was repeated three times, using n = 17 of mAb47 treated and n = 14 of placebo-treated animals, compared with n = 14 wt animals to assess baseline astrocyte activity. To measure astrocyte hyperactivity, relative OD values were used to compare between mAb47-treated and placebo-treated mice.

Behavioral evaluation

For behavioral analyses, animals were tested at three different time-points during the study: at two months (after two weeks treatment, for baseline behavior), six months (after 20 weeks of treatment) and eleven months (after 42 weeks of treatment). Each behavioral assessment included a handling of mice the first week to reduce stress, followed by the multiple concentric square field (MCSF) test for behavioral profiling and the novel object recognition (NOR) test for memory recognition. The weight of the mice was measured at each time-point they were handled. All animals were placed in the test room for at least 45 min to acclimatize to conditions before behavioral testing started.

Novel object recognition test

The novel object recognition test is a test to explore learning, memory and exploratory activity. The first day animals were habituated to the arena for 10 min and the second day, during the familiarization session, two identical objects were placed in the arena after which the animals were allowed to explore them for 10 min. The third day, 24 h after the familiarization session, one object was replaced by a new object and the time exploring the new object compared to the familial object was analyzed. The central zone of the MCSF test arena (40x40 cm) was used for testing and objects were attached to the floor (10 cm from the wall) using tape. The objects used were yellow rectangular Duplo and a dish brush head, which were cleaned with dish soap and 70% ethanol and left to dry

between trials. The animals were randomly familiarized with one of the two objects and during the test session, the new object was placed either on the right or left side. For half of the mice, the sample object was the Duplo and the novel object was the brush, while for the other half, the sample object was the brush and the novel object was the Duplo. These modifications were made to reduce object and place preference effects. All trials were video recorded and the nose contact with the object, distance moved and velocity in the arena were analyzed by Ethovision XT 13.0.

Multivariate concentric square field (MCSF) test

The multivariate concentric square field (MCSF) test is a test for behavioral profiling and general locomotor activity. The same setup as in previous experiments was used [14]. Briefly, the arena (70x70 cm) is divided into different zones in order to enable analysis of different behavioral strategies in risk taking, risk assessment and exploratory behavior. From the central zone (40x40 cm) animals can enter three different corridors (Corr A-C) leading to a safe dark corner room (DCR), a hole board for exploratory measures or a slope leading to an illuminated bridge area, perceived as a risky environment for rodents. Animals were tested in the MCSF test for 20 min. All trials were video recorded and analyzed by an automatic software (Ethovision XT 13.0, Noldus Technology, Wageningen, Netherlands) in combination with manual scoring of rearing and grooming behaviors using Score version 2.2 (Copyright Solids, Uppsala, Sweden).

Statistical analyses

Shapiro–Wilk's W test was used to determine normal distribution and such data were analyzed with a parametric t-test. In case of non-normally distributed data, appropriate non-parametric tests were instead chosen (Mann–Whitney U test for group wise comparisons). Results of the GFAP ELISA and Mac2 scoring were analyzed with one-way ANOVA. All statistical analyses were made between the antibody-treated and the placebo-treated groups. In the immune response assessments Wt mice are shown to represent baseline parameters of brain immune response. Statistica 13.4.0.14 software (StatSoft Inc., Tulsa, OK) was used for statistical analyses, and a p-value < 0.05 was considered statistically significant. All data are shown as mean ± SEM. GraphPad Prism (v6.07) was used for the preparation of graphs.

Results

Animal health and survival

During the course of the treatment, the mice were carefully monitored and sacrificed if any neurological or other adverse symptoms occurred. None of them displayed any overt motor disturbances, such as ataxia or loss of coordination. However, eye symptoms (cataract), tumors and weight loss led to a reduction in the number of mice over time, but these events did not differ between the groups (n = 3/23 in placebo group, n = 3/22 in mAb47 group and n = 2/18 in Wt group) and no group difference in survival was observed (not shown). Moreover, the weight of the mice was monitored weekly as a measure of general health. There was a gain in weight with age, but with no differences between the groups at any time point (not shown).

Antibody concentration

To evaluate the transfer of treatment antibody across the blood-brain barrier (BBB) and blood spinal cord barrier (BSCB), the levels of mAb47 in plasma, spinal cord and brain were measured. The antibody levels in spinal cord and brain were then found to be 0.8–1.9‰ of the corresponding levels in plasma, with the highest antibody passage seen for spinal cord (Table 1).

Effects of mAb47-treatment on α-syn pathology

Immunohistochemistry with the 211 antibody, detecting total human (tg) α-syn, resulted in a diffuse staining pattern with somal α-syn inclusions in the upper brain stem and round-shaped nuclear staining in the prefrontal cortex. In the hippocampus, α-syn staining was mainly observed in the outer boundaries of the cell soma (Supplementary Fig. 1). Due to the diffuse staining pattern with the 211 antibody, immunoreactivity was examined in the microscope by two blinded researchers and no difference in immunoreactivity was observed between the mAb47-treated and the placebo-treated mice.

Table 1
Concentration of the treatment antibody mAb47 in plasma (μg/ml), spinal cord and brain (ng/g tissue) at the end of the study, i.e. when the mice were sacrificed at eleven months of age, together with an estimate of antibody passage into spinal cord and brain. Mean ± SEM is shown.

Compartment	Concentration	Penetrance
Plasma (μg/ml)	231±30	
Spinal cord (ng/g)	446±62	1.9%
Brain (ng/g)	184±23	0.8%

Table 2

Primary and secondary antibodies used in IHC experiments.

| IHC	211 α-syn	P129 α-syn	GFAP	Iba1	Mac2
Primary antibodies	anti- α-synuclein 211; Abcam, ab80627; diluted 1:250	anti- α-synuclein phospho-Ser129 (EP1536Y); Abcam, ab51253; diluted 1:250	anti-GFAP DAKO, Z0334; diluted 1:500	anti-Iba1 WAKO, 019–19741; diluted 1:500	anti-Mac2 (Clone M3/38) CEDARLANE, CL8942B; diluted 1:250
Secondary antibodies	anti-mouse IgG (Vector, MKB-2225–1)	goat anti-rabbit IgG (H + L) (Vector, BA-1000)			–
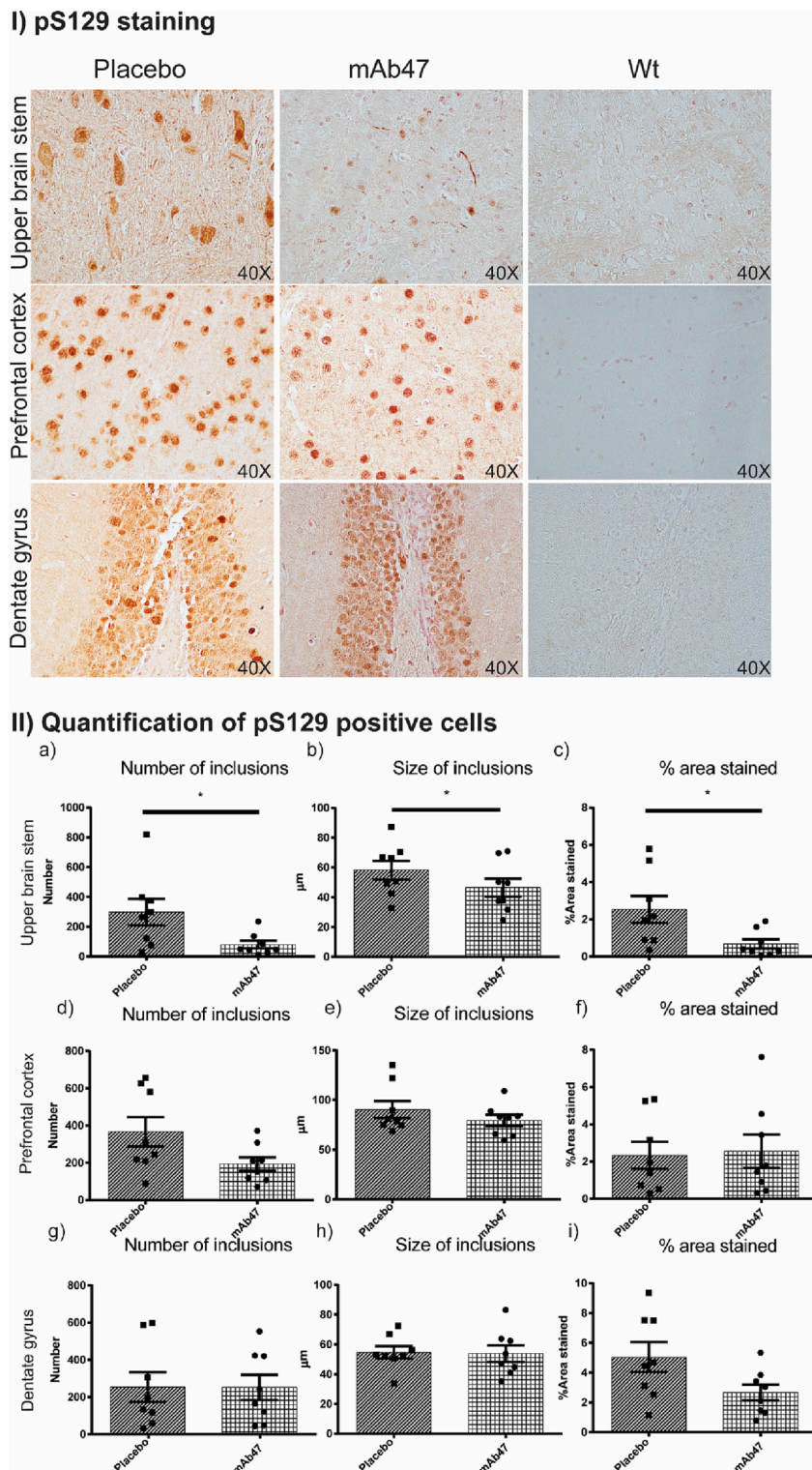
Fig. 1. Immunostaining of α-syn pathology with the p129 α-syn antibody. Brain sections from eleven months old tg mice, 40x magnification, were used. Immunopositive cells were observed in the upper brain stem (top panel), prefrontal cortex (middle panel) and hippocampus dentate gyrus (DG) (bottom panel). No immunoreactivity was detected in wild type (Wt) mice. (I). Quantification of α-syn pathology (pS129 α-syn positive cells) showed that mAb47 treatment reduced the number, size and area of pS129 α-syn stained inclusions in the upper brain stem. Data shown as mean ± SEM, *$p < 0.05$ (II). Placebo = tg mice treated with PBS, mAb47 = tg mice treated with the antibody.

Staining against pathological pS129 α-syn revealed neuronal inclusions mainly in cell bodies in the brain stem, hippocampus and neocortex. In the upper brain stem, pale body like α-syn somal inclusions were seen together with some staining of processes, whereas in hippocampus and neocortex a nuclear staining pattern was observed (Fig. 1I).

In the upper brain stem, staining against pS129 α-syn revealed a significantly reduced pathology in mAb47-treated mice compared to placebo-treated mice (Fig. 1I, upper panel), both with respect to the number and average size of the pS129 α-syn ($p = 0.03$, $U = 11$, $Z = -2.2$) and size of inclusions ($p = 0.03$, $U = 11$, $Z = -2.2$). Also the percentage of the area stained with the pS129 α-syn antibody was reduced in mAb47-treated compared to placebo-treated mice (Fig. 1IIc) (Mann-Whitney *U* test, $p = 0.01$, $U = 8$, $Z = -2.47$). In the prefrontal cortex the number of pS129 α-syn deposits showed a trend towards reduction after mAb47 treatment (Fig. 1I, middle panel), but the difference did not reach statistical significance (Mann-Whitney *U* test $p = 0.10$, $U = 16$, $Z = -1.6$). No difference between groups was seen for the size of these nuclear pS129 α-syn profiles or for the percentage of area stained in this region (Fig. 1IId-f). No differences in α-syn pathology between mAb47- and placebo-treated mice could be seen in the hippocampus, neither in dentate gyrus (DG) (Fig. 1 I lower panel, Fig. 1IIg-i) nor in CA1 (not shown).

Pre-treatment of the tissue with PK significantly reduced the number ($p = 0.0017$) and the size ($p = 0.0017$) of the pS129 α-syn deposits in the upper brain stem. In contrast, PK treatment neither reduced the number nor the size of the nuclear pS129 α-syn deposits in the prefrontal cortex (Supplementary Fig. 2).

Neuroinflammation

To determine if treatment with mAb47 induces abnormal brain inflammation, glial cell activities were assessed. The distribution and abundance of glial cells in mAb47-treated brains versus placebo-treated and Wt brains were analyzed by immunostaining of activated astrocytes (glial fibrillary acidic protein, GFAP) and activated microglia (ionized calcium binding adaptor molecule 1, Iba1). Additionally, to compare astrocyte activation in antibody and placebo treated mice, GFAP was measured with a sandwich ELISA. To assess the presence of macrophages, galectin 2 (Mac2) positive cells were quantified individually based on IHC.

Iba1 is a well-established marker of mature microglial cells and it can be expressed on resting and activated microglia [26,27]. We did not observe any differences in the numbers of such cells in the analyzed brain regions between mAb47 and placebo treated brains (Fig. 2).

Upregulation of GFAP expression and astrocytosis in the affected area are well established indicators of immune response in the brain. We compared brains from mice treated with mAb47 with brains from placebo-treated mice, as well as with Wt brains. In all brain regions included, i.e. upper brain stem, prefrontal cortex and hippocampus, a similar pattern of GFAP positive cells was observed
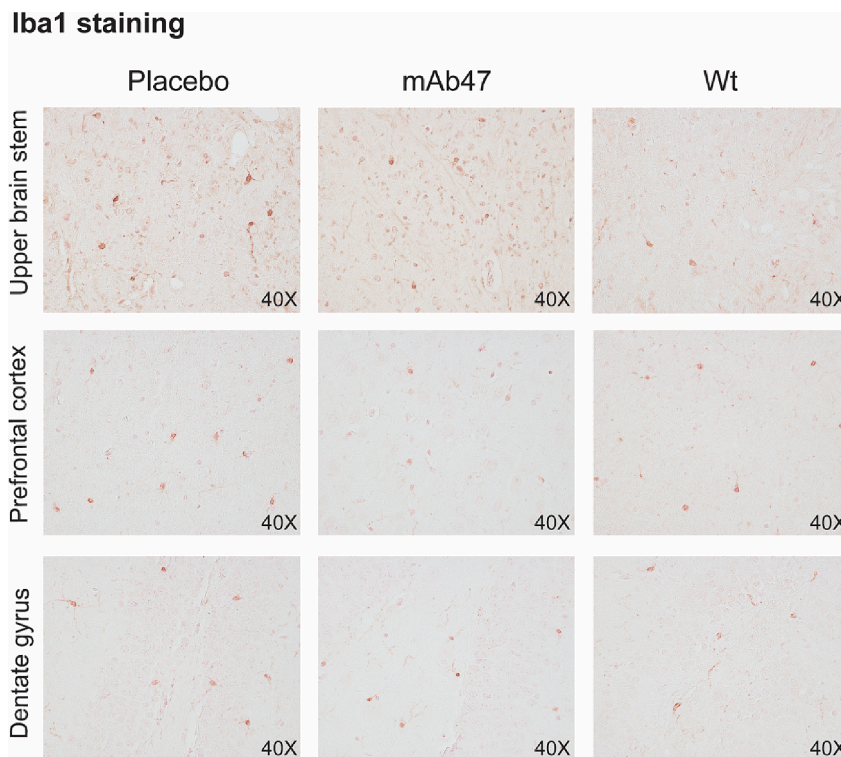

Fig. 2. Treatment with mAb47 did not induce any activation of microglia. Immunodetection of the microglial activation marker Iba-1 was performed on placebo-treated, mAb47 and Wt brain sections. Iba1 positive cells were analyzed in upper brain stem, prefrontal cortex and dentate gyrus. 40x magnification. Placebo = tg mice treated with PBS, mAb47 = tg mice treated with the antibody, Wt = non-transgenic, wild type mice.

irrespective of treatment and strain (Fig. 3). To verify these results we quantified GFAP expression with a sandwich ELISA and could not observe any differences between the three groups of mice (Supplementary Fig. 3).

In order to further assess whether long-term treatment with mAb47 induces an excessive immune response, we analyzed the expression of the macrophage marker Mac2. Quantification of the number of Mac2 positive cells in four sagittal sections from the whole hemispheres of mAb47-treated, placebo-treated and Wt mice showed that the number was generally very low, two to eight cells per hemisphere, and that long term treatment with mAb47 did not lead to any increase in the number of Mac2 positive cells (Fig. 4).

Taken together, based on the analysis of inflammatory responses, we did not observe any increased such reactions in the brains of mice treated with mAb47 compared to placebo-treated mice.

Behavioral evaluation

Antibody-treated and placebo-treated (Thy-1)-h[A30P] α-syn tg mice were evaluated for behavioral impairments at two, six and eleven months of age. The results for males and females were combined, as there were no sex differences in the performed tests (not shown).

Novel object recognition test

In order to analyze recognition memory, the novel object recognition (NOR) test was used. In the habituation phase, the time spent investigating the two different objects (brush and Duplo) was measured. In the first trial all groups of mice spent more time investigating the brush compared to the Duplo whereas at the second and last trials, no differences were observed in the investigation time between the two objects (not shown).

The NOR test showed no difference in time investigating the objects at two and eleven months of age (Fig. 5a and c). At six months of age, the antibody-treated mice spent less time investigating objects compared to placebo-treated animals (Fig. 5b). Both groups of mice were able to discriminate between the old and the new object at two months of age, as shown as the percentage of time the mice were investigating old versus new objects (Fig. 5d and g). However, only the mAb47 treated mice were able to discriminate between the objects at six months (paired t-test, $p < 0.001$, $t = 6.166$, $df = 16$), which at eleven months was still seen as a non-significant trend (Fig. 5e and f). The discrimination index, measured as the time mice were investigating the new object divided by the time they were investigating the old object, also showed a difference at six months (un-paired t-test, $p = 0.0005$, $t = 3.857$, $df = 32$) and a non-
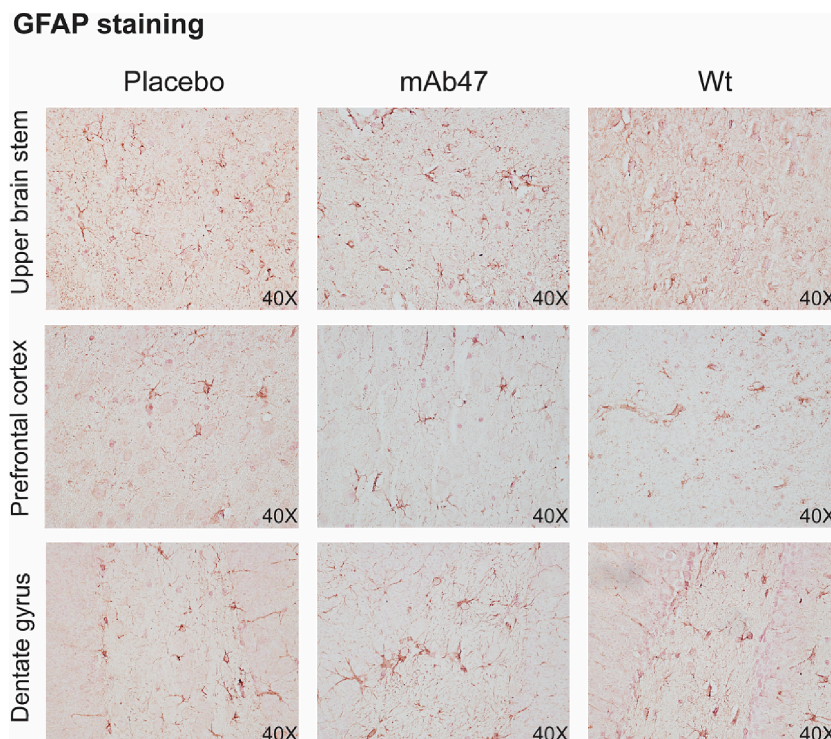

Fig. 3. Treatment with mAb47 did not trigger any excessive activation of astrocytes. Immunodetection of glial fibrillary acidic protein (GFAP) was performed on placebo-treated, mAb47 and Wt brain sections. GFAP positive cells were analyzed in the upper brain stem, prefrontal cortex and hippocampus dentate gyrus. 40x magnification. Placebo = tg mice treated with PBS, mAb47 = tg mice treated with the antibody, Wt = non-transgenic, wild type mice.
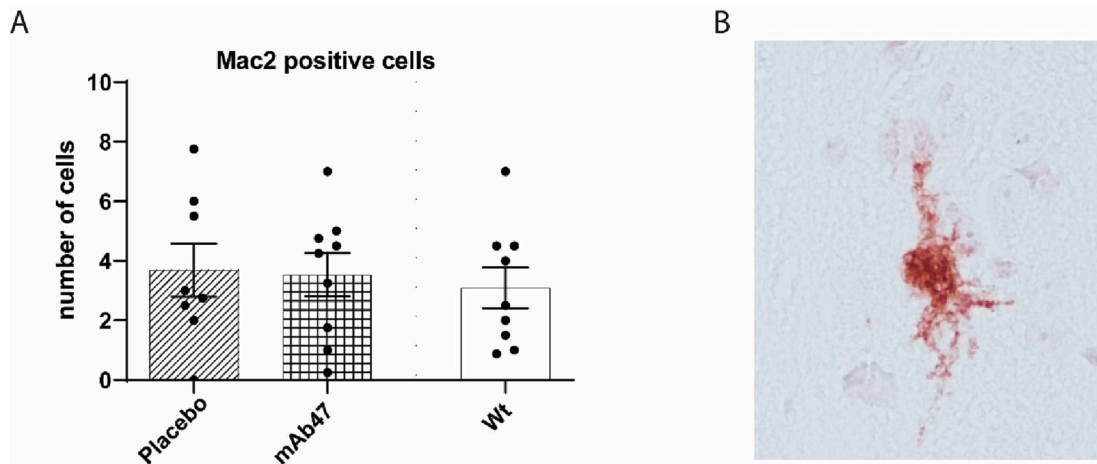
Fig. 4. Treatment with mAb47 did not lead to any increase in cells positive for the macrophage marker Mac 2. The number of Mac2 positive cells was calculated in placebo-treated (n = 7) and mAb47- (n = 9) treated mice and compared to Wt animals (A). Example of a Mac2 positive cell from the brain of mAb47-treated mice is presented in B. Data shown as mean ± SEM, p < 0.05. Placebo = tg mice treated with PBS, mAb47 = tg mice treated with the antibody, Wt = non-transgenic, wild type mice.

significant trend at eleven months (Fig. 5h).

Multivariate concentric square field test

To test for risk assessment, risk taking behavior, locomotor activity and exploratory behavior the multivariate concentric square field (MCSF) test was used.

Risk assessment, the animals' safety evaluation ability, was measured as latency to their first entry to the slope leading to the illuminated bridge (bridge slope latency), time to enter the bridge grid (bridge grid latency) and the total percentage of time spent in the corridors (% total corridor duration). There was no difference in risk assessment behavior in the first trial at two months of age, but at six months of age the mAb47-group showed an improved risk assessment behavior compared to placebo-treated mice (Mann-Whitney *U* test, bridge grid latency, p = 0.036, U = 83, Z = 2.1; bridge slope latency, p = 0.046, U = 86, Z = 1.99) (Fig. 6d and e). At eleven months of age, however, there were no longer any statistically significant changes with respect to risk assessment behavior between the two groups (Fig. 6g and h).

Risk taking behavior, the visits to zones that are considered unsafe in the MCSF test, was measured as the number of entries to and time spent on the bridge slope (bridge slope frequency and latency) and time spent in the center of the arena (center duration). No difference in risk taking behavior was observed between the antibody-treated and placebo-treated tg mice (not shown).

As for locomotor activity, there were no differences in activity measures including velocity, distance moved and number of passages through the center (center frequency) between the groups at any time point (Supplementary Fig. 4Aa-i). Both groups of mice were active in the MCSF test at all ages and moved more than 6000 cm during each test session (Supplementary Fig. 4Aa, d, g).

Exploratory behavior was assessed by measuring the amount of rearing, including wall rearing and free rearing behavior. Both antibody-treated and placebo-treated mice were wall rearing to the same extent at all time-points evaluated (Supplementary Fig. 4Ba, d, g). In the first trial, at two months of age, there was no difference in free rearing behavior between the two treatment groups (Supplementary Fig. 4Bb). At later time points, there was a slight decrease in free rearing activity in the antibody-treated group compared to placebo-treated mice, although this behavior showed high inter-group variability, especially in the placebo-treated group (p = 0.079 at eleven months of age, Supplementary Fig. 4Be and h).

Discussion

Immunotherapy targeting α-syn can be considered as one of the most promising treatment strategies aimed to slow the progression of PD. The efficacy of such therapies are currently being assessed in clinical trials run by Roche (prasinezumab, phase II), Astra Zeneca (MEDI1341, phase I) and Lundbeck (LuAF82422, Phase I). Biogen recently reported results from their phase II anti α-syn immunotherapy trial, in which the primary endpoints were not met [28]. Also, AbbVie has recently concluded a phase I study on ABBV-0805, based on the murine mAb47 antibody which was used in our current study. Interestingly, a recent interim analysis of the phase II PASADENA study showed that, although prasinezumab failed to meet the primary endpoint, there were evidence that one year of treatment had slowed some of the deterioration of motor symptoms, as measured by the MDS UPDRS scale [29].

Preclinical evaluation of anti α-syn antibodies designed to measure treatment efficacy on behavior and pathology, as well as analyses of inflammatory reactions and other potential side effects, are particularly relevant. Since most neurodegenerative disorders include a presymptomatic phase, it is important to assess these therapies before severe cell loss has occurred. Although animal models
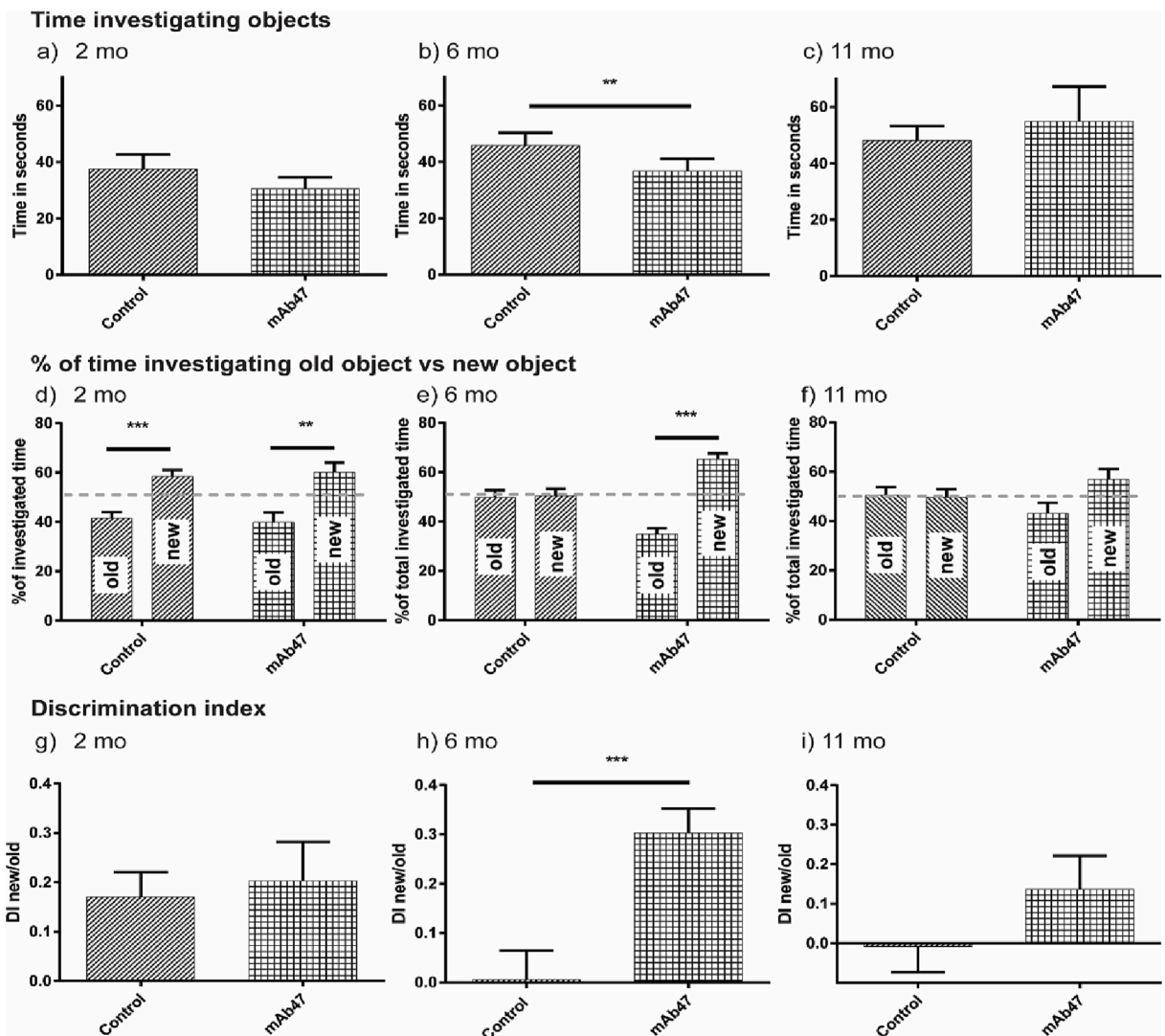
Fig. 5. The novel object recognition test showed no difference in the time that mice were investigating the objects at two and eleven months of age (a, c). At six months of age, however, the mAb47 treated mice spent less time investigating objects compared to placebo-treated mice (b). Both groups of mice were able to discriminate between the old and the new object at two months of age, as shown as the percentage of time that they were investigating the old versus the new object (d,g). At six months only the mAb47 treated mice were able to discriminate between the objects (e, f), supported by the discrimination index (h, i). At eleven months a non-significant trend of this difference remained. Data shown as mean ± SEM. Placebo = tg mice treated with PBS, mAb47 = tg mice treated with the antibody.

of PD have limitations and do not quite mimic the human condition, α-syn overexpressing mice are used to study α-syn pathology, a central event in the pathogenesis of PD. However, the variable onset of pathology in most of the mouse models may affect study outcomes. Similarly, preclinical *in vivo* studies related to Alzheimer's disease have also demonstrated a great variability in tg models. Whereas some models have responded promptly to pharmacological therapies (reviewed in [30]), others have turned out to be far more resistant to treatment [31,32].

In spite of these general animal model-related difficulties, we could here demonstrate that early and long-term administration with the α-syn oligomer/protofibril-selective antibody mAb47 both ameliorates upper brain stem pS129 α-syn pathology and transiently reduces some of the behavioral dysfunctions in Thy-1-h[A30P] α-syn tg mice. In an earlier study we showed that anti-α-syn immunotherapy with the same antibody lowers the CNS levels of α-syn oligomers/protofibrils in these mice, when the treatment was initiated later in life [25]. Moreover, our previous study indicated that anti α-syn immunotherapy also can reduce late-stage symptoms in the mice [25].

The main objective of the present study was to investigate if an early-onset and long-term treatment with mAb47 also can prevent CNS pathology and behavioral deficits in the mice. Hence, we started the therapy at six weeks of age and sacrificed the animals at eleven months of age. Thus, the treatment window was preceding the one used in our previous investigation, in which the mice were
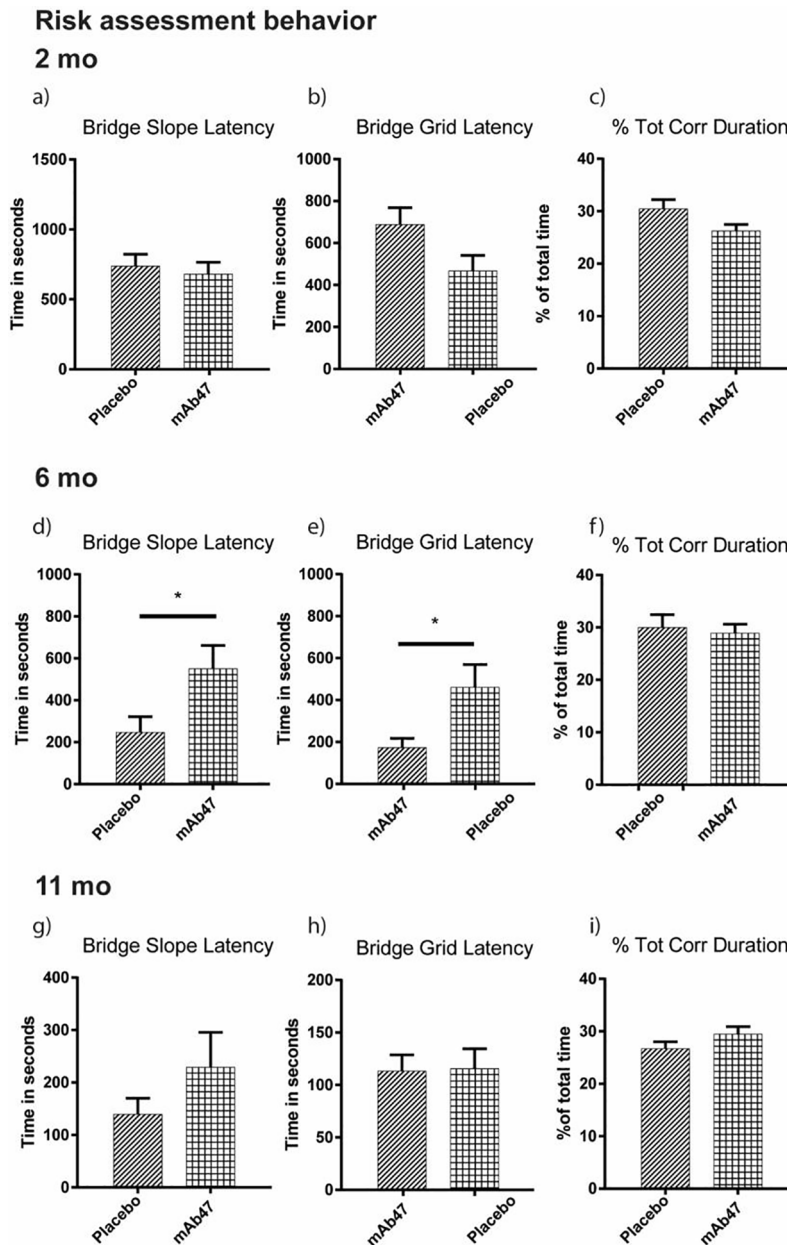
Risk assessment behavior

Fig. 6. Risk assessment, according to the MCSF test, was measured as time to enter the bridge slope, bridge grid (bridge slope/grid latency) or the percentage of time spent in the corridors (percentage of total corridor duration). No difference between placebo-treated and antibody-treated mice was seen at two months of age (a-c). At six months of age, the antibody-treated mice showed increased risk assessment behavior compared to placebo-treated mice (d,e), but at eleven months of age again no statistically significant difference could be observed. Data shown as mean ± SEM. Placebo = tg mice treated with PBS, mAb47 = tg mice treated with the antibody.

treated between 14 and 17 months of age. We thus initiated treatment from a time point that was expected to precede the expected onset of behavioral impairments [14], and administered weekly intraperitoneal injections until a time point that precedes the onset of overt motor impairment - staggering gait, loss of coordination and paralysis of hind limbs - which these mice have been reported to develop from 14 months of age [13].

Treatment with mAb47 reduces α-syn brain stem pathology

Alpha-synuclein in Lewy bodies is typically phosphorylated at serine 129 (pS129 α-syn) [33,34] and it has been suggested that phosphorylated α-syn species might be crucial for the spreading and progression of PD pathology [35]. As previously reported, pS129

α-syn pathology in Thy-1-h[A30P] α-syn tg mice is mainly located to the hippocampus, prefrontal cortex and brain stem [13,36]. For that reason, these regions were chosen for our analyses.

Interestingly, the number and size of pS129 α-syn deposits in the upper brain stem were markedly reduced with mAb47 treatment, while staining for total α-syn did not reveal any differences between treated and untreated animals. Moreover, we found a reduction in the total area stained for pS129 α-syn in the upper brain stem of antibody-treated mice, as compared to placebo-treated mice. In addition, there was a slight pS129 α-syn reduction, albeit not statistically significant, in the prefrontal cortex of mAb47-treated mice compared to mice that had received placebo. In the upper brain stem, we could mainly observe somal and neuritic deposits which may indicate the presence of pre-inclusion α-syn pathology in this area. In the hippocampus and prefrontal cortex, on the other hand, the pS129 α-syn staining was instead nuclear, which may represent a pathology that is more resistant to mAb47 treatment. These observations correspond well to what has been previously reported on Thy-1-h[A30P] α-syn tg mice [36]. In that prior study, it was found that pS129 α-syn accumulates in the cell soma of brain stem neurons whereas a nuclear localization dominates in cells of the hippocampus and neocortex. Moreover, these features could be seen already from four months of age and the differences remained as the mice grew older [36].

As our current data suggest that mAb47 targets on-pathway species in the brain stem but not nuclear pS129 α-syn in prefrontal cortex, we conducted a proteinase K (PK) digestion experiment (Supplementary Fig. 2). At 11 mo of age pS129 α-syn immunoreactivity in brain stem sections was found to be more PK-sensitive compared to that in prefrontal cortex. The more occluded nuclear localization and possible differences in aggregate structures may thus make pS129 α-syn profiles in the prefrontal cortex and regions with similar pathology less susceptible to clearance.

Further emphasizing its distinct quality, nuclear pS129 α-syn was reported to be more susceptible to inhibition by polo-like kinase 2 [37], and has also been found at increased levels in human α-synucleinopathy brains [38]. Thus, subcellular and brain-region related differences in the properties of α-syn pathology need to be taken into account when designing clinical trials and may have important implications for which effects of therapeutic antibodies against α-syn that can be obtained.

Treatment with mAb47 does not cause any adverse neuroinflammatory reactions

Passive immunotherapy against α-syn most likely induces autophagy-dependent protein clearance and may elicit inflammatory responses [17–19]. Since immunotherapy against α-syn mostly will have to be performed over a long time, it is crucial to assess inflammatory effects and other potential side effects [39].

Microglial and astrocytic responses were carefully evaluated in the present study. We compared astrocyte and microglia morphology, GFAP levels and number of infiltrating Mac2 positive cells between mice treated with mAb47 and placebo. Taken together, similar to what was shown in our previous short-term treatment study [25], a 42 week long chronic administration of mAb47 did not lead to any signs of increased brain inflammation.

Treatment with mAb47 has a transient effect on cognition

Cognitive dysfunction is a common feature of PD, with about 80% of the patients eventually developing dementia and with 20–55% of the non-demented PD patients displaying mild cognitive impairment [40]. Therefore, measures of cognitive function are of great importance for the evaluation of new PD therapies.

In the present study, we were able to show that the mAb47 antibody treatment had a transient effect on some of the cognitive functions. The effects on behavioral outcomes were most profound in the NOR test for memory function, a widely used model for the investigation of retention alterations. Typically, when animals are exposed to a familiar and a novel object, they approach the novel object more frequently than the familiar one [41]. The preference for a novel object means that the familiar object is already represented in the animals' memory [41].

In the first trial mice from both the mAb47-treated and the placebo group were, as expected, spending more time with the novel object on the test day. However, as the study proceeded, only the mAb47-treated mice were able to distinguish between the two objects at six months of age. Whereas these group differences were no longer statistically different at the eleven month assessment, there was still a trend for an effect on this cognitive measure with treatment. These results indicate that the tg mice display impaired memory function, likely associated with α-syn pathology, and that antibody treatment can counteract such a cognitive decline.

The results of the NOR test depend on cognitive as well as non-cognitive traits, such as attention, working and long-term memory, anxiety level and preference for novelty [42,43]. Performance depends on both hippocampal and cortical areas [44,45] (and reviewed in [46]). Treatment with Mab47 did not result in any significant α-syn changes in these regions (Fig. 1 and Supplementary Fig. 1). However, the obvious beneficial effects on NOR might arise from antibody clearance of α-syn species that we are currently unable to detect. Moreover, assessment of memory formation depends on neural activity from multiple brain regions. Novelty is considered rewarding for rodents and exploration of novel objects has been related to dopamine release [47]. Considering that dopamine is involved in novelty processing and can enhance hippocampal synaptic plasticity [47–50], spreading of α-syn pathology throughout regions interconnected with dopaminergic areas most likely contributes to abnormalities in dopamine-dependent neuromodulation of cognitive processes. However, as we previously found that the levels of dopamine or its metabolites are unchanged in Thy-1-h[A30P] α-syn tg mice [51], such an effect may possibly instead be the result of a harmful interaction between α-syn and either dopamine itself or its receptors.

In this study, the clearest reduction of pathology with treatment was found in the brain stem, a region crucial for integrative brain functions by modulating dopamine and other neurotransmitter signaling pathways that project to higher order brain structures

[52–55]. Thereby it serves as a crossing point between ascending and descending pathways [56]. The reticular formation, mainly located in the brain stem, is considered particularly important for cognition due to its role in maintaining a basic level of arousal [57]. Accordingly, lesions in the upper brain stem and pons are often causing serious cognitive and behavioral abnormalities, in addition to severe motor impairments [58,59]. Therefore, an abundance of α-syn pathology in these areas, even without significant levels of neurodegeneration, might contribute to abnormalities in upstream signaling, subsequently causing impairments in higher brain functions, including cognition.

Considering that Braak staging in PD implicates a low-to-high brain area pattern of α-syn spreading during disease progression [60,61], treatment strategies aimed at reducing brain stem α-syn pathology might be beneficial to prevent progression of pathology and cognitive deficits at early disease stages.

The MCSF test has a multivariate and ethoexperimental approach, assessing several naturally occurring behaviors and different aspects of evolutionarily conserved strategies for survival in rodents. With repeated testing, a memory effect can be measured depending on how well the mice remember the first visit to the MCSF arena [62,63]. The second trial is generally characterized by fewer visits to the various areas (i.e. lower activity and less performance of risk assessment and risk-taking behavior) [62,64] and indicates how well the memory of the first trial was consolidated, which may be influenced by the perceptive and cognitive abilities of the animal during its stay in the MCSF in the first trial.

Importantly, in the second trial of this study, the antibody-treated mice showed preserved risk assessment behavior compared to placebo-treated mice. Risk assessment is considered a behavioral strategy to evaluate the potential risks vs. benefits deriving from exploration [65] which in the MCSF means visits to, and behaviors performed in relation to, areas associated with risk. Risk assessment behavior is rather complex and depends on many different brain regions, including prefrontal cortex and the limbic system, and the positive effect on this behavior indicates a preservation of high-order brain functions with antibody treatment.

Conclusions

In conclusion, we could show that treatment with the oligomer/protofibril-selective α-syn antibody mAb47 results in reduced α-syn pathology in the upper brain stem and a transient beneficial effect on some cognitive functions in Thy-1-h[A30P] α-syn tg mice. In addition, no evidence of treatment-related neuroinflammation could be found. Immunotherapy with antibodies targeting α-syn oligomers/protofibrils should thus hold promise as a safe future disease-modifying treatment for PD and related disorders, also with respect to counteracting some of the associated cognitive symptoms.

Declaration of Competing Interest

Eva Nordström, Christer Möller, Johanna Fälting and Lars Lannfelt are employees and shareholders at BioArctic AB. Martin Ingelsson is a paid consultant to BioArctic AB.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbas.2023.100086.

References

[1] Burre J, Sharma M, Tsetsenis T, Buchman V, Etherton MR, Sudhof TC. Alpha-synuclein promotes SNARE-complex assembly in vivo and in vitro. Science 2010; 329(5999):1663–7.

[2] Chadchankar H, Ihalainen J, Tanila H, Yavich L. Decreased reuptake of dopamine in the dorsal striatum in the absence of alpha-synuclein. Brain Res 2011;1382: 37–44.

[3] Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. Nature 1997;388(6645):839–40.

[4] Uversky VN, Li J, Fink AL. Evidence for a partially folded intermediate in alpha-synuclein fibril formation. J Biol Chem 2001;276(14):10737–44.

[5] Schulz-Schaeffer WJ. The synaptic pathology of alpha-synuclein aggregation in dementia with Lewy bodies, Parkinson's disease and Parkinson's disease dementia. Acta Neuropathol 2010;120(2):131–43.

[6] Danzer KM, Haasen D, Karow AR, Moussaud S, Habeck M, Giese A, et al. Different species of alpha-synuclein oligomers induce calcium influx and seeding. J Neurosci 2007;27(34):9220–32.

[7] Winner B, Jappelli R, Maji SK, Desplats PA, Boyer L, Aigner S, et al. In vivo demonstration that alpha-synuclein oligomers are toxic. Proc Natl Acad Sci U S A 2011;108(10):4194–9.

[8] Wilms H, Rosenstiel P, Romero-Ramos M, Arlt A, Schafer H, Seegert D, et al. Suppression of MAP kinases inhibits microglial activation and attenuates neuronal cell death induced by alpha-synuclein protofibrils. Int J Immunopathol Pharmacol 2009;22(4):897–909.

[9] Zhang W, Wang T, Pei Z, Miller DS, Wu X, Block ML, et al. Aggregated alpha-synuclein activates microglia: a process leading to disease progression in Parkinson's disease. FASEB J 2005;19(6):533–42.

[10] Lee VM, Trojanowski JQ. Mechanisms of Parkinson's disease linked to pathological alpha-synuclein: new targets for drug discovery. Neuron 2006;52(1):33–8.

[11] Conway KA, Lee SJ, Rochet JC, Ding TT, Williamson RE, Lansbury Jr PT. Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy. Proc Natl Acad Sci U S A 2000;97(2):571–6.

[12] Kahle PJ, Neumann M, Ozmen L, Muller V, Jacobsen H, Schindzielorz A, et al. Subcellular localization of wild-type and Parkinson's disease-associated mutant alpha -synuclein in human and transgenic mouse brain. J Neurosci 2000;20(17):6365–73.

[13] Neumann M, Kahle PJ, Giasson BI, Ozmen L, Borroni E, Spooren W, et al. Misfolded proteinase K-resistant hyperphosphorylated alpha-synuclein in aged transgenic mice with locomotor deterioration and in human alpha-synucleinopathies. J Clin Invest 2002;110(10):1429–39.

[14] Ekmark-Lewén S, Lindstrom V, Gumucio A, Ihse E, Behere A, Kahle PJ, et al. Early fine motor impairment and behavioral dysfunction in (Thy-1)-h[A30P] alpha-synuclein mice. Brain Behav 2018;8(3):e00915.

[15] Freichel C, Neumann M, Ballard T, Muller V, Woolley M, Ozmen L, et al. Age-dependent cognitive decline and amygdala pathology in alpha-synuclein transgenic mice. Neurobiol Aging 2007;28(9):1421–35.

[16] Bae EJ, Lee HJ, Rockenstein E, Ho DH, Park EB, Yang NY, et al. Antibody-Aided Clearance of Extracellular alpha-Synuclein Prevents Cell-to-Cell Aggregate Transmission. J Neurosci 2012;32(39):13454–69.

[17] Mandler M, Valera E, Rockenstein E, Weninger H, Patrick C, Adame A, et al. Next-generation active immunization approach for synucleinopathies: implications for Parkinson's disease clinical trials. Acta Neuropathol 2014;127(6):861–79.

[18] Masliah E, Rockenstein E, Adame A, Alford M, Crews L, Hashimoto M, et al. Effects of alpha-synuclein immunization in a mouse model of Parkinson's disease. Neuron 2005;46(6):857–68.

[19] Masliah E, Rockenstein E, Mante M, Crews L, Spencer B, Adame A, et al. Passive immunization reduces behavioral and neuropathological deficits in an alpha-synuclein transgenic model of Lewy body disease. PLoS One 2011;6(4):e19338.

[20] Lindstrom V, Ihse E, Fagerqvist T, Bergstrom J, Nordstrom E, Moller C, et al. Immunotherapy targeting alpha-synuclein, with relevance for future treatment of Parkinson's disease and other Lewy body disorders. Immunotherapy 2014;6(2):141–53.

[21] Valera E, Spencer B, Masliah E. Immunotherapeutic Approaches Targeting Amyloid-beta, alpha-Synuclein, and Tau for the Treatment of Neurodegenerative Disorders. Neurotherapeutics 2016;13(1):179–89.

[22] Fagerqvist T, Lindstrom V, Nordstrom E, Lord A, Tucker SM, Su X, et al. Monoclonal antibodies selective for alpha-synuclein oligomers/protofibrils recognize brain pathology in Lewy body disorders and alpha-synuclein transgenic mice with the disease-causing A30P mutation. J Neurochem 2013;126(1):131–44.

[23] Gustafsson G, Eriksson F, Moller C, da Fonseca TL, Outeiro TF, Lannfelt L, et al. Cellular Uptake of alpha-Synuclein Oligomer-Selective Antibodies is Enhanced by the Extracellular Presence of alpha-Synuclein and Mediated via Fcgamma Receptors. Cell Mol Neurobiol 2017;37(1):121–31.

[24] Nasstrom T, Gonçalves S, Sahlin C, Nordstrom E, Screpanti Sundquist V, Lannfelt L, et al. Antibodies against alpha-synuclein reduce oligomerization in living cells. PLoS One 2011;6(10):e27230.

[25] Lindstrom V, Fagerqvist T, Nordstrom E, Eriksson F, Lord A, Tucker S, et al. Immunotherapy targeting alpha-synuclein protofibrils reduced pathology in (Thy-1)-h[A30P] alpha-synuclein mice. Neurobiol Dis 2014;69:134–43.

[26] Ito D, Imai Y, Ohsawa K, Nakajima K, Fukuuchi Y, Kohsaka S. Microglia-specific localisation of a novel calcium binding protein, Iba1. Brain Res Mol Brain Res 1998;57(1):1–9.

[27] Ito D, Tanaka K, Suzuki S, Dembo T, Fukuuchi Y. Enhanced expression of Iba1, ionized calcium-binding adapter molecule 1, after transient focal cerebral ischemia in rat brain. Stroke 2001;32(5):1208–15.

[28] Lang AE, Siderowf AD, Macklin EA, Poewe W, Brooks DJ, Fernandez HH, et al. Trial of Cinpanemab in Early Parkinson's Disease. N Engl J Med 2022;387(5): 408–20.

[29] Pagano G, Taylor KI, Anzures-Cabrera J, Marchesi M, Simuni T, Marek K, et al. Trial of Prasinezumab in Early-Stage Parkinson's Disease. N Engl J Med 2022;387 (5):421–32.

[30] Blennow K, de Leon MJ, Zetterberg H. Alzheimer's disease. Lancet 2006;368(9533):387–403.

[31] Philipson O, Hammarstrom P, Nilsson KP, Portelius E, Olofsson T, Ingelsson M, et al. A highly insoluble state of Abeta similar to that of Alzheimer's disease brain is found in Arctic APP transgenic mice. Neurobiol Aging 2009;30(9):1393–405.

[32] Lord A, Gumucio A, Englund H, Sehlin D, Sundquist VS, Soderberg L, et al. An amyloid-beta protofibril-selective antibody prevents amyloid formation in a mouse model of Alzheimer's disease. Neurobiol Dis 2009;36(3):425–34.

[33] Fujiwara H, Hasegawa M, Dohmae N, Kawashima A, Masliah E, Goldberg MS, et al. alpha-Synuclein is phosphorylated in synucleinopathy lesions. Nat Cell Biol 2002;4(2):160–4.

[34] Anderson JP, Walker DE, Goldstein JM, de Laat R, Banducci K, Caccavello RJ, et al. Phosphorylation of Ser-129 is the dominant pathological modification of alpha-synuclein in familial and sporadic Lewy body disease. J Biol Chem 2006;281(40):29739–52.

[35] Karampetsou M, Ardah MT, Semitekolou M, Polissidis A, Samiotaki M, Kalomoiri M, et al. Phosphorylated exogenous alpha-synuclein fibrils exacerbate pathology and induce neuronal dysfunction in mice. Sci Rep 2017;7(1):16533.

[36] Schell H, Hasegawa T, Neumann M, Kahle PJ. Nuclear and neuritic distribution of serine-129 phosphorylated alpha-synuclein in transgenic mice. Neuroscience 2009;160(4):796–804.

[37] Elfarrash S, Jensen NM, Ferreira N, Schmidt SI, Gregersen E, Vestergaard MV, et al. Polo-like kinase 2 inhibition reduces serine-129 phosphorylation of physiological nuclear alpha-synuclein but not of the aggregated alpha-synuclein. PLoS One 2021;16(10):e0252635.

[38] Koss DJ, Erskine D, Porter A, Palmoski P, Menon H, Todd OGJ, et al. Nuclear alpha-synuclein is present in the human brain and is modified in dementia with Lewy bodies. Acta Neuropathol Commun 2022;10(1):98.

[39] Vaikath NN, Hmila I, Gupta V, Erskine D, Ingelsson M, El-Agnaf OMA. Antibodies against alpha-synuclein: tools and therapies. J Neurochem 2019;150(5): 612–25.

[40] Goldman JG, Litvan I. Mild cognitive impairment in Parkinson's disease. Minerva Med 2011;102(6):441–59.

[41] Ennaceur A. One-trial object recognition in rats and mice: methodological and theoretical issues. Behav Brain Res 2010;215(2):244–54.

[42] Goulart BK, de Lima MN, de Farias CB, Reolon GK, Almeida VR, Quevedo J, et al. Ketamine impairs recognition memory consolidation and prevents learning-induced increase in hippocampal brain-derived neurotrophic factor levels. Neuroscience 2010;167(4):969–73.

[43] Silvers JM, Harrod SB, Mactutus CF, Booze RM. Automation of the novel object recognition task for use in adolescent rats. J Neurosci Methods 2007;166(1): 99–103.

[44] Buckmaster CA, Eichenbaum H, Amaral DG, Suzuki WA, Rapp PR. Entorhinal cortex lesions disrupt the relational organization of memory in monkeys. J Neurosci 2004;24(44):9811–25.

[45] Clark RE, Zola SM, Squire LR. Impaired recognition memory in rats after damage to the hippocampus. J Neurosci 2000;20(23):8853–60.

[46] Cohen SJ, Stackman Jr RW. Assessing rodent hippocampal involvement in the novel object recognition task. *A review* Behav Brain Res 2015;285:105–17.

[47] Duzel E, Bunzeck N, Guitart-Masip M, Duzel S. NOvelty-related motivation of anticipation and exploration by dopamine (NOMAD): implications for healthy aging. Neurosci Biobehav Rev 2010;34(5):660–9.

[48] Lisman J, Grace AA, Duzel E. A neoHebbian framework for episodic memory; role of dopamine-dependent late LTP. Trends Neurosci 2011;34(10):536–47.

[49] D'Ardenne K, Eshel N, Luka J, Lenartowicz A, Nystrom LE, Cohen JD. Role of prefrontal cortex and the midbrain dopamine system in working memory updating. Proc Natl Acad Sci U S A 2012;109(49):19900–9.

[50] Lisman JE, Grace AA. The hippocampal-VTA loop: controlling the entry of information into long-term memory. Neuron 2005;46(5):703–13.

[51] Behere A, Thornqvist PO, Winberg S, Ingelsson M, Bergstrom J, Ekmark-Lewen S. Visualization of early oligomeric alpha-synuclein pathology and its impact on the dopaminergic system in the (Thy-1)-h[A30P]alpha-syn transgenic mouse model. J Neurosci Res 2021;99(10):2525–39.

[52] Kitahama K, Nagatsu I, Geffard M, Maeda T. Distribution of dopamine-immunoreactive fibers in the rat brainstem. J Chem Neuroanat 2000;18(1–2):1–9.

[53] Lester DB, Miller AD, Pate TD, Blaha CD. Midbrain acetylcholine and glutamate receptors modulate accumbal dopamine release. Neuroreport 2008;19(9): 991–5.

[54] Forster GL, Blaha CD. Pedunculopontine tegmental stimulation evokes striatal dopamine efflux by activation of acetylcholine and glutamate receptors in the midbrain and pons of the rat. Eur J Neurosci 2003;17(4):751–62.

[55] Miller AD, Blaha CD. Nigrostriatal dopamine release modulated by mesopontine muscarinic receptors. Neuroreport 2004;15(11):1805–8.

[56] Hurley RA, Flashman LA, Chow TW, Taber KH. The brainstem: anatomy, assessment, and clinical syndromes. J Neuropsychiatry Clin Neurosci 2010;22(1):iv–7.

[57] Arguinchona JH, Tadi P. Neuroanatomy, Reticular Activating System. In: StatPearls 2023: Treasure Island (FL).

[58] Fu X, Lu Z, Wang Y, Huang L, Wang X, Zhang H, et al. A Clinical Research Study of Cognitive Dysfunction and Affective Impairment after Isolated Brainstem Stroke. Front Aging Neurosci 2017;9:400.

[59] Lee TM, Cheung CC, Lau EY, Mak A, Li LS. Cognitive and emotional dysfunction after central pontine myelinolysis. Behav Neurol 2003;14(3–4):103–7.

[60] Braak H, Del Tredici K, Rub U, de Vos RA, Jansen Steur EN, Braak E. Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging 2003; 24(2):197–211.

[61] McCann H, Cartwright H, Halliday GM. Neuropathology of alpha-synuclein propagation and braak hypothesis. Mov Disord 2016;31(2):152–60.

[62] Meyerson BJ, Augustsson H, Berg M, Roman E. The Concentric Square Field: a multivariate test arena for analysis of explorative strategies. Behav Brain Res 2006;168(1):100–13.

[63] Roman E, Colombo G. Lower risk taking and exploratory behavior in alcohol-preferring sP rats than in alcohol non-preferring sNP rats in the multivariate concentric square field (MCSF) test. Behav Brain Res 2009;205(1):249–58.

[64] Roman E, Meyerson BJ, Hyytia P, Nylander I. The multivariate concentric square field test reveals different behavioural profiles in male AA and ANA rats with regard to risk taking and environmental reactivity. Behav Brain Res 2007;183(2):195–205.

[65] Blanchard DC, Blanchard RJ. Ethoexperimental approaches to the biology of emotion. Annu Rev Psychol 1988;39(1):43–68.