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Cognitive deficits in a mouse model of pre-manifest Parkinson's disease

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Abstract

Early cognitive deficits are increasingly recognized in patients with Parkinson's disease (PD), and represent an unmet need for the treatment of PD. These early deficits have been difficult to model in mice, and their mechanisms are poorly understood. α -Synuclein is linked to both familial and sporadic forms of PD, and is believed to accumulate in brains of patients with PD before cell loss. Mice expressing human wild-type α -synuclein under the Thy1 promoter (Thy1-aSyn mice) exhibit broad overexpression of α -synuclein throughout the brain and dynamic alterations in dopamine release several months before striatal dopamine loss. We now show that these mice exhibit deficits in cholinergic systems involved in cognition, and cognitive deficits in domains affected in early PD. Together with an increase in extracellular dopamine and a decrease in cortical acetylcholine at 4–6 months of age, Thy1-aSyn mice made fewer spontaneous alternations in the Y-maze and showed deficits in tests of novel object recognition (NOR), object–place recognition, and operant reversal learning, as compared with age-matched wild-type littermates. These data indicate that cognitive impairments that resemble early PD manifestations are reproduced by α -synuclein overexpression in a murine genetic model of PD. With high power to detect drug effects, these anomalies provide a novel platform for testing improved treatments for these pervasive cognitive deficits.

Keywords

α -synuclein; novel object recognition; novel place recognition; operant-reversal learning; Y-maze

Introduction

Only a subset of patients with the neurodegenerative disorder Parkinson's disease (PD) develop major cognitive disturbances (Aarsland *et al.*, 2001); however, minor cognitive deficits are frequent (Elgh *et al.*, 2009; Mamikonyan *et al.*, 2009), and they can occur before motor symptoms emerge, as shown in siblings of patients with familial PD (Kéri *et al.*,

2010). These early cognitive deficits, which can predict the development of dementia in later stages (Janvin *et al.*, 2005, 2006), do not respond well to dopamine therapies, and affect quality of life (Klepac *et al.*, 2008; Cools *et al.*, 2010). They include impairments in set-shifting (Monchi *et al.*, 2004), in task-switching (Cameron *et al.*, 2010), in probabilistic reversal learning (Peterson *et al.*, 2009), in a delayed win–stay task related to both striatal and prefrontal cortex dysfunction (Partiot *et al.*, 1996), in recognition memory (Higginson *et al.*, 2005; Whittington *et al.*, 2006), and in implicit memory (Knowlton *et al.*, 1996; Wang *et al.*, 2009).

Mutations in α -synuclein cause familial forms of PD (Polymeropoulos *et al.*, 1997), and widespread α -synuclein pathology is present in both pre-motor and motor stages of sporadic PD (Braak *et al.*, 2003). Whereas dementia may be associated with cortical α -synuclein pathology in late disease stages (Kalaitzakis *et al.*, 2009), the mechanisms of early cognitive deficits in PD remain poorly understood.

Previous studies have examined cognitive function in α -synucleinoverexpressing mice, but have primarily used the Morris water maze test and found deficits only at ages older than 12 months (Freichel *et al.*, 2007; Nuber *et al.*, 2008; Zhou *et al.*, 2008). We sought to determine whether α -synuclein overexpression can cause a broader range of cognitive deficits similar to the human deficits occurring early in the course of the disease, both to elucidate their mechanisms and to provide a model with which to test novel treatments.

Mice overexpressing full-length, wild-type (WT), human α -synuclein under the Thy1 promoter (Thy1-aSyn mice) develop a 40% loss of striatal dopamine and L-DOPA responsive motor deficits by 14–15 months of age (Lam *et al.*, 2011). Earlier in life, these mice exhibit non-motor deficits similar to those observed in the pre-manifest phase of PD (Fleming *et al.*, 2008; Magen & Chesselet, 2010) and an increase in extracellular dopamine (Lam *et al.*, 2011) reminiscent of observations made with brain imaging techniques in carriers of the *LRRK2* mutation, a cause of familial PD (Sossi *et al.*, 2010). As both dopaminergic and cholinergic mechanisms may be implicated in early cognitive deficits in PD (Lange *et al.*, 1993), we investigated whether these mice also present alterations in cholinergic neurons and deficits in: (i) tasks similar to tests in which PD patients were impaired, such as reversal learning (Peterson *et al.*, 2009); (ii) striatal-dependent tasks such as habit learning in the holeboard (Packard & McGaugh, 1996); and (iii) tasks mediated by dopaminergic and muscarinic mechanisms – the Y-maze, object–place recognition, and novel object recognition (NOR) (Wall & Messier, 2002; Wall *et al.*, 2003; Barker & Warburton, 2008; Botton *et al.*, 2010).

Materials and methods

Mice

Animal care was conducted according to the US Public Health Service *Guide for the Care and Use of Laboratory Animals*, and the procedures were approved by the Institutional Animal Care and Use Committee at the University of California, Los Angeles. Thy1-aSyn mice were created and maintained on a hybrid (C57BL/6J $\times$ DBA/2N)F1 background (Rockenstein *et al.*, 2002). Animals were maintained on this background by mating N5 females hemizygous for the transgene with male WT mice on the hybrid background obtained from Charles River Laboratories (Wilmington, MA, USA) (Fleming *et al.*, 2004, 2006). Male and female littermates were never bred together. Litter sizes ranged from four to 11 mice per litter. The genotypes of all Thy1-aSyn and WT mice were determined prior to the beginning of the experiment with polymerase chain reaction amplification analysis of DNA obtained from tail tissue, and confirmed by re-genotyping each mouse at the end of the experiment.

Only male mice were used in the study, because gender can influence cognitive behavior. In addition, the transgene in Thy1-aSyn mice is inserted in the X chromosome, leading to potential random inactivation in somatic cells of hemizygous females. Hemizygous Thy1-aSyn and WT littermate mice on a mixed (C57BL/6J $\times$ DBA/2N)F1 background from a total of 52 litters were used in the study for behavioral testing. To conserve animals, mice were tested on one or more of five different tests, including the NOR, object-place recognition, Y-maze, holeboard and operant reversal learning tasks. The distribution of cohorts across the various tests was performed to avoid systematic training effects, and data from each experiment/cohort were carefully analyzed to detect any possible effect of training in cohorts that were exposed to more than one test, to conserve animals. We also took great care to avoid possible effects of food restriction by making sure that tests involving food restriction were performed after those that do not, or that the mice had ample time to recover from previous food restriction. A total of 126 mice were used for the behavioral experiments, and Table 1 summarizes the number of mice used for each behavioral test performed on each cohort at any given age. The first eight cohorts of mice (from 38 litters) were maintained on a reverse light/dark cycle with lights off at 10:00 h and on at 22:00 h. The last two cohorts of male mice (from 14 litters) were maintained on a regular light/dark cycle with lights on at 06:00 h and off at 18:00 h. All tests were performed in the dark phase, except for the Y-maze at 7–9 months of age for cohort 10, and operant reversal learning at 3–4 months for cohort 9. All tests were performed under *ad libitum* feeding conditions, except for the operant reversal learning and holeboard tests. Mice in cohort 10 were tested on the Y-maze at 7–9 months under food restriction, but by 11–13 months of age they were fed *ad libitum*.

Separate cohorts were used for histological and biochemical analyses. For histology, five mice per genotype from six litters were perfused at 5 months of age, and brain sections were used for double-labeling experiments with choline acetyltransferase (ChAT) and asynuclein (both human and human + mouse) in the basal nucleus of Meynert. For biochemical analyses, nine WT and seven Thy1-aSyn mice from 11 litters were decapitated at 6 months of age, and fresh frozen cerebral cortices were taken for acetylcholine (ACh) measurements. All mice from cohorts 1 and 2 that were used in the behavioral tests (Table 1) were also decapitated at 7.5 months of age, and fresh frozen hippocampi were taken for ACh measurements.

Y-maze

Spontaneous alternations in the Y-maze were assessed in 3–4-, 5–6, 7–9-, 11–13- and 15–18-month-old mice. Two cohorts were repeatedly tested in the Y-maze – cohort 5 at 3–4 months and then at 5–6 months, and cohort 10 at 7–9 months and 11–13 months (Table 1). The apparatus was a three-arm horizontal maze in which the arms were arranged at 120° angles to each other. Two arms (B and C) were 15 cm in length, and one arm (A) was 20 cm in length. All arms were 5 cm in width, and the walls were 12 cm in height. The maze was made of clear Plexiglas, and was attached to a rectangular floor of dimensions 45 $\times$ 60 cm. The maze walls and floor were made opaque with 0.5-cm-thick poster board. The poster board was attached to the outside walls of the maze, and a separate piece was placed under the maze base, making the floor opaque as well. Mice were habituated to the room for 1 h prior to the test, in the dark phase. The maze was placed in a dimly lit room. Mice were initially placed in the long arm (A) with their heads facing the maze arms (towards the point at which both arms meet). The mice were given 7 min to explore the maze, while an overhead video camera tracked their behavior. The experimenter could not be seen by the mice during the 7 min. The mouse was removed from the maze and returned to its home cage. The maze was thoroughly cleaned with a 70% alcohol solution after each trial. An experimenter made sure that the maze was dry before the next trial was run. An alternation

was defined as consecutive entries into all three arms (i.e. ABC, CAB, or BCA, but not BAB). Alternations were counted for all of the overlapping sequences of three consecutive entries by an observer who was blind to genotype. The percentage of alternations was calculated as follows: $[\text{number of alternations}/(\text{total number of arm visits} - 2)] \times 100$ (modified from Yang *et al.*, 2009).

One-trial object–place recognition

Mice were tested in this task at 4–5 months of age. The one-trial object–place recognition test was performed in the same Y-maze described above, following the protocol of Zlomuzica *et al.* (2008), with modifications. Two identical versions of a plastic giraffe figurine were used as objects. In order to control for scent-marking behavior, the objects were thoroughly cleaned with 70% ethanol between trials. Each mouse was first subjected to a sample phase of 10 min duration, in which two identical copies of the giraffe figurine were placed at the ends of two randomly selected arms of the Y-maze and the mouse was placed in the long arm of the maze; the mouse was allowed to explore the maze freely during the trial. Upon completion of trial 1, a 15-min delay was observed, during which time the mouse was returned to its home cage. During the 5-min test phase, one of the objects was moved from the arm in which it was placed during the sample phase to the arm not used in the sample phase. For each mouse, the time spent exploring the two objects during the sample and test phases was scored off-line from videotapes by a rater who was blind to genotype. A discrimination index (DI) was calculated as: $(t_{\text{novel}} - t_{\text{familiar}})/(t_{\text{novel}} + t_{\text{familiar}})$, and was used as a measure of place recognition in the test trial, wherein DIs higher than zero indicate good place recognition memory. The total exploration time of the objects in both locations was used as a measure of activity to rule out any confounding effects of differences in activity on differences in the discrimination ability between WT and Thy1-aSyn mice.

NOR test

Mice were 4–5 months of age at the time of testing. The test was conducted in the dark phase, under dim light. Mice were habituated to the testing room for 1 h prior to testing. The apparatus consisted of a circular, white and opaque tank (55 cm in diameter; 60 cm in height); a tripod-mounted camera was used to record behavior. Training/testing was conducted over 3 days. On the first 2 days, mice were habituated to the tank for 5 min. On the third day, two identical objects (plastic pig figurines) were placed in the center of the tank. The mouse was placed in the tank, and its behavior was recorded over one 5-min trial. Thirty to forty minutes later, one of the objects was replaced at random with a novel object (plastic dog figurine), and the mouse's behavior was again recorded over one 5-min trial. The apparatus and objects were cleaned between trials, with disinfectant and 70% ethanol, respectively, to remove any traces of odor on the objects. Upon review of the video footage, an experimenter blinded to mouse genotype measured the duration of investigation of each object (touching or sniffing it). The DI was calculated as follows: $(N - F)/(N + F)$, where F and N represent exploration times of familiar and novel objects, respectively (Ennaceur & Delacour, 1988). Novel object location (left or right) was counterbalanced. As in the object–place recognition, the total exploration time of the familiar and novel objects was also used to assess the absence of confounding motor effects. Mice with a total exploration time of <7 s during the second trial were discarded from the analyses (de Bruin & Pouzet, 2006).

Operant learning task

Thy1-aSyn mice and their WT littermates were trained in the operant learning task, starting from the age of 3–4 months and completing the test at the age of 5 months.

Apparatus

Standard extra tall aluminum and Plexiglas operant conditioning chambers with a curved panel fitted with a horizontal array of five nose-poke apertures on one side and a photocell-equipped pellet receptacle on the other side (Medical Associates, Mt Vernon, VT, USA) were used. The boxes were housed inside a sound-attenuating cubicle with ambient white noise (85 dB) broadcast to mask external noise; the environment was illuminated with a house light diffuser that was positioned within the testing chamber, directly above the food delivery magazine.

Food restriction

Standard chow was withheld for the first 2 days of food restriction, although the mice did receive ~0.5 g of the reinforcer pellets (Dustless precision pellets; BioServ, Frenchtown, NJ, USA) in the home cage during this time. Chow amounts provided to the mice each day after testing were adjusted daily in order to maintain the subjects at no <80% of their pre-restriction body weight.

Habituation

Habituation and pre-training were conducted precisely as described earlier (Laughlin *et al.*, 2011).

Left–right bin discrimination

Mice were initially trained to perform a series of nose-poke responses to obtain a food pellet. Each trial began with illumination in the center nose-poke aperture (hole 3 of 5). After the mouse made a response at the center hole, lights in the apertures to the left and right (holes 2 and 4 of 5) were illuminated. For half of the animals in each genotype group, the left hole was designated ‘correct’, and the other half had the opposite contingency (the right hole was ‘correct’). If the mouse responded into the correct aperture, a pellet was dispensed. If the nose poke was into the incorrect aperture, the mouse received a time-out. In either case, the next trial commenced 5 s later. To demonstrate competency on this task, the mouse had to successfully choose the correct aperture in at least 17 of 20 consecutive trials. The mouse was tested in daily sessions of up to 60 min until it achieved this criterion in a single session.

Reversal training

After reaching the criterion on the initial acquisition of the operant learning task, the subjects were assessed for reversal learning. These trials were the same as above, except that the correct aperture was switched so that the mouse now had to nose-poke into the hole that was opposite to the one that was reinforced in the initial learning stage. The session was finally ended when the mouse chose the new correct hole in 17 of 20 consecutive trials. The mouse was tested in daily sessions of up to 60 min until it achieved this criterion in a single session.

Holeboard

Mice were 3–4 months old at the beginning of the test. A holeboard with an array of 16 holes in a 4 × 4 grid was placed in the open field chambers (Truscan system for mice; Coulbourn Instruments, Whitehall, PA, USA). Mice that were food-restricted to 90% of their free-feeding body weight were habituated over 5 days (15 min/day) to the nose-poke floor, with all holes baited (20-mg chocolate reward; BioServ). The test was conducted under red light conditions, and no visual cues from outside the apparatus were visible to the mice. Following habituation, all 16 holes were still baited, but only four of them were

accessible to the mice, the other 12 being blocked by a meshed wire. This prevented the mice from identifying the accessible holes from the smell of the food, which was important in order to rule out any confounding effect of the olfactory deficits that have been previously shown in Thy1-aSyn mice (Fleming *et al.*, 2008). Mice were then tested over 5 days (three 3-min trials on the first day; thereafter, three 2.5-minutes trials/day) for their ability to retrieve the rewards from the four open holes. Forty-eight hours after the fifth day, a probe trial was conducted in complete darkness. One day after the probe trial, a switch session of three 2.5-min trials was carried out; the holes used were the same as those open during the initial learning phase, but the mouse was placed in the maze in a different position (rotated 90°) so that it had to turn in the opposite direction in order to find the nearest open hole, and its ability to retrieve rewards from four open holes was assessed again. Nose-poke behavior was recorded automatically with TRUSCAN V 1.012.00 software. The number of pellets retrieved was confirmed visually after each trial. The order of testing for mice was pseudorandomized, such that the first mouse to be tested every day was the last to be tested on the next day. After completion of the switch phase, the cardboard surrounding the open field was removed so that mice could be seen by the experimenter, and the cumulative time spent retrieving the pellets from the four open holes was measured with a stopwatch, in order to account for a possible effect of motor dysfunction on the differences detected in the time spent in the open holes during the learning phase (see Results for details). Mice that were poorly motivated to perform the task (i.e. consuming fewer than four pellets in any of the three trials on every single day in the learning phase) were excluded from all of the analyses.

Tissue processing

Mice were anesthetized with 100 mg/kg intraperitoneal pentobarbital, and intracardially perfused with 0.1 M phosphate-buffered saline followed by ice-cold 4% paraformaldehyde. Brains were quickly removed, postfixed overnight in 4% paraformaldehyde, cryoprotected in 30% sucrose in 0.1 M phosphate-buffered saline until they sank to the bottom of tube, frozen on powdered dry ice, and stored at -80 °C.

Immunohistochemistry – double labeling of ChAT and α -synuclein

Forty-micrometer free-floating coronal sections were cut on a Leica CM 1800 cryostat (Leica, Deerfield, IL, USA), and collected for immunohistochemical analysis. Sections were washed in Tris-buffered saline, blocked in 10% goat serum, and then incubated with rabbit anti-ChAT antibody (1 : 1000, Cat. no. AB143; Chemicon, Temecula, CA, USA) and mouse anti- α -synuclein antibody (1 : 250; Cat. no. 610787; BD Biosciences, San Diego, CA, USA), which detects both mouse and human α -synuclein, or rat anti-human α -synuclein (1 : 5, Cat. no. S-1114; AG Scientific, San Diego, CA, USA) at 4 °C overnight. Sections incubated with the mouse anti- α -synuclein antibody were also blocked for 1 h with mouse IgG blocking reagent prior to blocking with goat serum. After washes in Tris-buffered saline, sections were incubated with Alexa Fluor 555 goat anti-rabbit at 1 : 1000 (Cat. no. A-21428; Invitrogen, Carlsbad, CA, USA) and goat anti-mouse dylight 649 at 1 : 500 (Cat. no. 115-495-166; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or goat anti-rat IgG Cy5 at 1 : 500 (Cat. no. AP136S; Chemicon) for 2 h. Sections were mounted in Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (Cat. no. H-1200; Vector Laboratories, Burlingame, CA, USA). Sections exposed to the secondary antibody only were also included in each experiment, as negative controls.

Image acquisition and processing

Images were serially scanned through the Z-plane with a Leica TCS-SP MP confocal microscope and Leica CONFOCAL software version 2.5 (Leica, Heidelberg, Germany). All

images were saved and processed with Photoshop 6.0 (Adobe Systems, San Jose, CA, USA).

ACh measurements

Mice were killed by decapitation at 6 months of age, and the portion of the cortex above the striatum, including the cingulate, somatosensory and motor regions, was dissected and snap-frozen in dry ice, and then stored at -80°C . Mice aged 7.5 months were also decapitated, and their hippocampi were dissected and stored at -80°C . The tissues were then homogenized in ice-cold RIPA buffer containing 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% sodium dodecylsul-fate, 50 mM Tris, and a 1 : 25 cocktail of Protease inhibitors (Cat. no. 1697498, Roche tablet; Roche, Indianapolis, IN, USA), and centrifuged at 621 g for 10 min at 4°C ; the supernatant was then collected. ACh was measured in the supernatant with the Amplex red kit (Cat. no. A12217; Invitrogen) according to the manufacturer's instructions, and was normalized to the protein concentration determined in the supernatant by the Bradford assay with a Bradford reagent (Cat. no. 500-0205; Bio-Rad, Hercules, CA, USA) and bovine serum albumin standard set (Cat. no. 500-0207; Bio-Rad). ACh levels are presented as nmol/mg protein.

Statistical analysis

Data are presented as mean $\pm$ standard error of the mean. Repeated-measure ANOVAS followed by Fisher's LSD test were used to analyze data from the operant learning and holeboard tasks, which were performed repeatedly on the same mice. A two-tailed unpaired Student's *t*-test was used to analyze the data from the NOR, object–place recognition and Y-maze tests, which were not repeated in the same mice, and to analyze ACh levels; in the two former tests, a single-sample two-tailed *t*-test was also used to compare the DIs in each genotype with zero (chance level), in order to determine the absence or presence of discrimination ability, and Fisher's exact test was used to compare the percentage of performers (mice with $\text{DI} > 0$) between the genotypes in the NOR test. Student's *t*-test was also used to compare the cumulative time spent in four open holes of the holeboard when the pellets were eaten. The statistical tests for significance of difference between groups and the power analysis to determine the minimal number of mice required to obtain a 30 or 50% drug effect with 80% power were performed with GB-Stat v8.0 (Dynamic Microsystems, Silver Spring, MD, USA) and SigmaStat 12.0 (Systat Software, Chicago, IL, USA). The level of significance was set at $P < 0.05$. Statistically significant outliers were excluded according to Grubb's test (Grubbs, 1969). Mice tested in different conditions were pooled into age groups when statistics showed them to be equivalent.

Results

We have previously shown that striatal dopamine loss in Thy1-aSyn mice is preceded by a transient increase in extracellular dopamine, with a 160% increase at 6 months, returning to normal levels by 9.5 months. Changes in extracellular dopamine have been associated with alterations in the Y-maze (Li *et al.*, 2010). Therefore, we first examined Thy1-aSyn mice and their WT littermates in this task at several ages corresponding to the observed changes in extracellular striatal dopamine (see Fig. 6).

Spontaneous alternations in the Y-maze

The Y-maze test was performed at 3–4, 5–6, 7–9, and 11–13 months. A previous study has shown that repeated testing in the Y-maze does not have training or habituation effects (Senechal *et al.*, 2008), and we have also found this to be true, after comparing the first and second Y-maze performances of mice that were repeatedly tested (data not shown). Thus, we excluded any possible effects of repeated testing on the results. Student's *t*-test revealed

significant differences between the groups at ages 5–6 months ($t_{26} = 2.26$, $P = 0.032$) and 7–9 months ($t_{27} = 2.8$, $P = 0.009$), with 18 and 20% deficits as compared with WT littermates, respectively, but not at 3–4 months ($t_{25} = 0.98$, $P = 0.34$), or 11–13 months ($t_{21} = 1.05$, $P = 0.31$) (Table 2). Although most of the mice at 7–9 months of age were tested in the light phase (Table 1), the overall activity level, reflected by total number of arm visits, was not different between these mice and mice tested in the dark phase at other ages, or between Thy1-aSyn mice and age-matched WT mice at any time point [$P > 0.05$, not significant (NS)]. In addition, the percentage of spontaneous alternations is normalized to the number of arm visits, and therefore takes into account differences in activity levels. Power analysis revealed that minimum numbers of 10 and four mice are sufficient to detect a 30 and 50% drug effect on percentage of alternations at 5–6 months, respectively, with 80% power ($P < 0.05$), whereas 19 and seven mice are sufficient to detect the respective drug effects at 7–9 months with 80% power ($P < 0.05$), indicating the robustness of the effect. An additional group of mice was tested at 15–18 months, but was excluded from the analysis because of severe hypokinesia in the Thy1-aSyn group, which was previously shown in the open field at 14 months (Hean *et al.*, 2010). Thy1-aSyn mice and their WT littermates made similar numbers of total arm visits at both 5–6 and 7–9 months (Table 2), and Thy1-aSyn mice were shown to be hyperactive at 7.5 months (Lam *et al.*, 2011). Therefore, akinesia is unlikely to have caused the deficits observed at the earlier ages.

One-trial object–place recognition

On the basis of the time course of deficits observed in the Y-maze, we focused our further analysis on 4–5-month-old mice in the hope of detecting the earliest deficits. At 4–5 months, the DI of Thy1-aSyn mice ($n = 10$) was markedly lower than that of WT mice ($n = 12$) (Fig. 1A; -0.11 ± 0.11 vs. 0.28 ± 0.1 , respectively; unpaired t -test, $t_{20} = 2.65$, $P = 0.02$). In addition, the DI was significantly different from chance levels in WT mice (single-sample t -test, $t_{11} = 2.68$, $P = 0.02$) but not in Thy1-aSyn mice ($t_9 = 0.99$, $P = 0.35$). The total exploration times of the objects in the familiar and novel locations in the test trial did not differ between WT and Thy1-aSyn mice (Fig. 1B; $P = 0.56$), similar to previous data on different tasks (Fleming *et al.*, 2008) and ruling out any confounding effect of motor deficits. Power analysis revealed that minimum numbers of 28 and nine mice are needed to detect a 30 and 50% drug effect, respectively, on the DI [expressed as novel/(novel + familiar) to reduce variability] with 80% power ($P < 0.05$).

NOR

To control for potential preference for one of the figurines used for the test, seven naïve WT mice and six naïve Thy1-aSyn mice were exposed to the pig and dog figurines. Both groups displayed DIs (calculated with dog as ‘novel object’) that were not significantly different from the chance level of zero (Fig. 2A; $P > 0.05$, NS), indicating that they had no baseline preference for either figurine [protocol modified from Simmons *et al.* (2009)]. A different set of mice was used for the actual test. Within this group of mice, one mouse was excluded from the WT group because of low exploration time (< 7 s) in trial 2, and an additional mouse in this group was a significant outlier after performance of Grubb's test (Grubbs, 1969). Neither WT nor Thy1-aSyn mice showed any preference for either one of the identical objects presented in trial 1, the training trial (data not shown). However, in the test trial, 4–5-month-old WT mice ($n = 18$) showed a clear preference for the novel object, as reflected by a DI that was significantly different from zero (Fig. 2B; 0.32 ± 0.05 , one-sample t -test, $t_{17} = 6.08$, $P < 0.001$). Although the DI of age-matched Thy1-aSyn mice ($n = 17$) was also different from zero (0.14 ± 0.06 , $t_{16} = 2.17$, $P = 0.045$), it was significantly reduced by 56% as compared with WT mice (Fig. 2B; $t_{33} = 2.13$, $P = 0.04$). Indeed, the percentage of mice that explored the novel object more than the familiar object was significantly lower in the Thy1-aSyn group (52.8%, nine of 17) than in the WT group (94.4%, 17 of 18) (Fig. 2C;

Fisher's exact test, $P = 0.007$). Furthermore, in WT mice, the difference between the time spent exploring the novel and familiar objects was highly significant (paired t -test, $t_{17} = 5.1$, $P = 0.002$, data not shown), but it was reduced to a non-significant trend in the transgenic mice (paired t -test, $t_{16} = 2.06$, $P = 0.055$, data not shown). The deficits observed in transgenic mice in this test are unlikely to result from visual impairments, because naïve Thy1-aSyn mice showed no baseline preference for either one of the objects, suggesting that they could see and visually explore both (Fig. 2A). Furthermore, the total exploration time of the objects (novel + familiar) did not differ between the genotypes ($P = 0.44$, data not shown), again ruling out a confounding effect of motor deficits. Power analysis revealed that minimum numbers of 11 and four mice are sufficient to detect a 30 and 50% drug effect, respectively, on the DI [novel/(novel + familiar)] with 80% power ($P < 0.05$), again indicating the robustness of the effect and its suitability for drug testing.

Operant learning task

PD patients are impaired in reversal learning early in the course of the disease (Peterson *et al.*, 2009). Therefore, we tested 4–5-month-old mice in an operant learning task. Mice from both genotype groups were first trained to learn a simple response rule (e.g. epoke into either the left or the right hole to obtain access to a food pellet); this represents a simple form of feedback-based learning. They were then trained to reverse this rule, necessitating both new learning and response inhibition. Repeated-measures ANOVA with genotype as between-subject factor and stage as within-subject factor was used to analyze the primary dependent measure – namely, the trials to criterion required to achieve competent performance at each stage. A genotype $\times$ stage interaction ($F_{1,18} = 5.04$, $P = 0.04$) was revealed, and *post hoc* tests indicated that this interaction stemmed from a lack of group difference during the initial learning stage (Fig. 3A: WT, $n = 11$, 46.7 ± 8.7 ; Thy1-aSyn, $n = 9$, 43.9 ± 9.7 ; Fisher's LSD, $P = 0.88$), along with a significant effect of genotype under reversal conditions, with a 66% difference in the trials to criterion (Fig. 3A: WT, 76.5 ± 12.79 ; Thy1-aSyn, 127 ± 20 , $P = 0.01$). Measures of the time required for the mouse to respond to the left or right hole in cases when a correct response was made (a measure of motor response times) were not modulated by genotype during either the acquisition or reversal phases of the experiment (Fig. 3B, main genotype effect, $F_{1,18} = 0.14$, $P = 0.71$), ruling out a potential confounding effect of motor deficits. In addition, the operant/reversal learning task used in this study is based on nose poke, not fine motor skills in the paws, which are impaired in these mice (Fleming *et al.*, 2004). The lack of genotype difference in the initial learning stage also supports the absence of motor deficits in this task, and in addition argues against the possibility that the deficits in the reversal learning resulted from an inability to detect light because of blindness in Thy1-aSyn mice, as the mice had to nose-poke in response to light in the initial acquisition stage. Power analysis revealed that minimum numbers of 39 and 14 mice are needed to detect a 30 and 50% drug effect, respectively, on the trials to criterion in the reversal phase, with 80% power ($P < 0.05$).

Holeboard

Habit learning is affected in manifest PD (Knowlton *et al.*, 1996), and was assessed in mice in the present study in the version of the holeboard requiring response learning, which is striatum-dependent (Packard & McGaugh, 1996). By the end of the habituation phase, all mice, regardless of genotype, learned to consume 14–16 pellets in a 15-min trial in the holeboard (data not shown). However, three WT mice and one Thy1-aSyn mouse were excluded from analysis of the learning phase, owing to poor motivation (see Materials and methods). In addition, of the remaining mice, one mouse from each group was excluded from the analysis of time spent in open holes after performance of Grubb's test for outliers (see below). Repeated-measure ANOVA was performed, with genotype as between-subject factor and day as within-subject factor. In the learning phase, no main effects of genotype

were found on either reference memory ratio, the ratio of visits to open holes out of total visits (Fig. 4A; repeated-measure ANOVA, $F_{1,19} = 0.0003$, $P = 0.98$), latency to complete the task (Fig. 4B; repeated-measure ANOVA, $F_{1,19} = 0.4$, $P = 0.53$), distance traveled during the task (Fig. 4C; repeated-measure ANOVA, $F_{1,19} = 0.33$, $P = 0.57$), or latency to enter the first open hole in the learning phase (Fig. 4D; repeated-measure ANOVA, $F_{1,19} = 0.25$, $P = 0.62$). Both genotypes (WT, $n = 10$; Thy1-aSyn, $n = 11$) improved their performance over the 5 testing days ($P < 0.01$ for main effect of day for all parameters). In contrast, a main effect of genotype was found on the cumulative time spent in the open holes in the learning phase averaged across three trials a day, which was longer in Thy1-aSyn mice ($n = 10$, one outlier excluded) than in WT mice ($n = 9$, one outlier excluded) (Fig. 4E; repeated-measure ANOVA, $F_{1,17} = 31.55$, $P < 0.001$), and also on the time spent in the open holes in the first trial of each day (Fig. 4F; repeated-measure ANOVA, $F_{1,17} = 19.21$, $P < 0.001$). Fisher's LSD revealed differences between the genotypes in the average time spent in the open holes, when it was compared individually for each day ($P < 0.01$ for all days), and in the time spent in the open holes in the first trials on days 1–4 ($P < 0.01$) and day 5 ($P < 0.05$). A probe trial was performed in order to determine whether mice rely on the location of the red lamp as a spatial cue, or the different appearance of the open holes and the screened holes, by removing the red lamp and performing the task in complete darkness. Neither group declined in performance in the probe trial, as reflected by latency to complete the task, latency to enter the first open hole, and reference memory ratio, as compared with the first trial on the fifth day, which was the last before the probe test, indicating that mice might have learned the turning response from the release position (data not shown). However, in both groups, the distance traveled during the task in the probe trial increased in comparison to the first trial on the fifth day, which could mean that both genotypes had to work harder to perform the task with similar accuracy as on the previous days, or that they became more active because of the elimination of the red light (Fig. 4G; main genotype effect, $F_{1,19} = 0.79$, $P = 0.38$; main time effect, $F_{5,100} = 12.13$, $P < 0.0001$; Fisher's LSD, $P < 0.01$ vs. trial 1 on day 5 in both genotypes). The switch phase, which was performed for 1 day, did not reveal any significant differences in any of the three trials (data not shown).

The increased time spent in the open holes could reflect impairments in retrieving the pellets from these holes, as we have previously reported fine motor skill impairments in young Thy1-aSyn mice (Fleming *et al.*, 2004). If this is the case, then 'baited' visits to open holes (i.e. those accompanied by pellet consumption) should have been longer in the Thy1-aSyn mice. However, as the software could not differentiate between 'baited' and 'unbaited' visits (those not accompanied by pellet consumption), we measured and calculated the cumulative time spent in four 'baited' visits to the open holes, when mice were visible to the experimenter, using a stopwatch. The cumulative time spent in the open holes was almost twice as great in the Thy1-aSyn mice than in the WT mice (Fig. 4H; WT, 2.83 ± 0.2 s, $n = 9$; Thy1-aSyn, 5.58 ± 0.74 s, $n = 10$; unpaired *t*-test, $t_{17} = 3.39$, $P = 0.0035$), indicating that Thy1-aSyn mice are impaired in their fine motor skills and therefore take longer to 'dig' the pellet from the hole. This may be similar to their deficits in the nest building test that we have previously observed (Fleming *et al.*, 2004). The values measured by a human experimenter were slightly lower than the respective values measured by the software and averaged across days and trials (3.53 ± 0.27 s for WT mice and 5.99 ± 0.33 s for Thy1-aSyn mice), because the former values do not include time spent in open holes when pellet is not consumed. Power analysis revealed that minimum numbers of 31 and 11 mice are needed to detect a 30 and 50% drug effect, respectively, with 80% power ($P < 0.05$) on the time spent in open holes measured by a human experimenter.

Table 3 summarizes the results of the different tests performed on the mice, including the endpoint measures used, the direction of change in Thy1-aSyn mice, the number of mice

used, conditions of housing/testing, and number of animals needed to detect a 30 and 50% drug effect with 80% power and $P < 0.05$.

Biochemical and histological analysis of cholinergic cortical systems in Thy1-aSyn mice

Several of the tasks that we found to be impaired in the young Thy1-a Syn mice are known to depend on the integrity of cortical cholinergic neurotransmission (Barker & Warburton, 2008; Botton *et al.*, 2010). To determine whether alterations in this system could contribute to the deficits that we observed, we first confirmed that cholinergic neurons of the basal nucleus of Meynert overexpress human α -synuclein in our model. Figure 5A–C show ChAT labeling in green, human α -synuclein labeling in red and the overlay in the basal nucleus of a WT mouse, and Fig. 5D–F show the respective labeling in a Thy1-aSyn mouse. As expected, ChAT was expressed in both genotypes (Fig. 5A and D) but human α -synuclein was expressed only in Thy1-aSyn mice (Fig. 5E), and not in WT mice (Fig. 5B). Human α -synuclein was expressed in ChAT-positive neurons, as seen in the overlay of red and green staining yielding yellow labeling (Fig. 5F) in the cytoplasm, nucleus, and processes. Double labeling was also performed with an antibody detecting both human and mouse α -synuclein. Colocalization of ChAT and α -synuclein was observed in only one of the WT mice. In contrast, colocalization was found in ChAT-positive neurons of all Thy1-aSyn mice ($n = 5$ per genotype, data not shown). These data confirm the overexpression of α -synuclein in the cholinergic neurons of the basal nucleus of Meynert, whereas the level of endogenous α -synuclein in these neurons is low, below the level of detection of our immunostaining in most WT mice. We then measured the level of ACh in the cerebral cortex of 6-month-old mice, to determine whether α -synuclein overexpression resulted in alterations in the levels of this neurotransmitter in a region known to play a critical role in the cognitive tasks that showed deficits in our model. ACh levels decreased by 30% in the cerebral cortex of Thy1-aSyn mice (Fig. 5G; $t_{14} = 2.23$, $P = 0.043$; WT, $n = 9$; Thy1-aSyn, $n = 7$). Hippocampal ACh levels were not affected by genotype (data not shown, $P > 0.05$, NS). These data indicate that, together with alterations in striatal dopamine release, deficits in cortical ACh may contribute to the cognitive deficits that we observed in young Thy1-aSyn mice.

Discussion

The present study reveals that a range of cognitive deficits reminiscent of those observed in early-stage PD can be detected at an early age in a mouse model with broad overexpression of α -synuclein (Rockenstein *et al.*, 2002), reproducing the pathological accumulation of this protein in the brains of PD patients (Braak *et al.*, 2003). This indicates that α -synuclein pathology can produce a range of cognitive deficits similar to those observed in early PD in the absence of striatal dopamine loss (Lam *et al.*, 2011) or changes in tyrosine hydroxylase immunoreactivity (Fernagut *et al.*, 2007), and provides a battery of tests with high power to detect drug effects on early cognitive deficits in a genetic mouse model of PD.

The early cognitive deficits of PD, although mild, are pervasive (Elgh *et al.*, 2009; Mamikonyan *et al.*, 2009). Although some impairment may be related to the progressive loss of striatal dopamine in these patients, other mechanisms are probably involved, because these impairments are poorly reversed by L-DOPA (Jubault *et al.*, 2009; Cools *et al.*, 2010). For example, performance in tasks that are normally linked to frontostriatal circuitry have been associated with an increase in blood flow to the prefrontal cortex, possibly because of compensation in response to nigrostriatal dysfunction (Cools *et al.*, 2002; Beauchamp *et al.*, 2008). Thus, these mild cognitive deficits may be related to overactivity in brain regions not affected by the primary pathology in PD, perhaps explaining the difficulties in multi-tasking that are observed in these patients (Brown *et al.*, 2010; LaPointe *et al.*, 2010).

The decrease in spontaneous alternations in the Y-maze appeared after 5 months of age, and showed a progressive worsening from 5–6 to 7–9 months, although there was no longer any significant genotype effect at 11–13 months; this could be attributable to smaller sample sizes or to the fact that these deficits relate to the hyperdopaminergic tone found in young (6 months), but not old (>14 months), Thy1-aSyn mice (Lam *et al.*, 2011). This suggests that a hyperdopaminergic state that may precede the development of parkinsonism, as seen in asymptomatic *LRK2* carriers (Sossi *et al.*, 2010), could contribute to early cognitive deficits in patients (see Fig. 6 for visualization).

Deficits in the Y-maze and object–place recognition and NOR tests may be related to a compromised cholinergic system, as both tasks are impaired by administration of scopolamine, a muscarinic antagonist (Wall & Messier, 2002; Barker & Warburton, 2008; Botton *et al.*, 2010). The cholinergic system is impaired in PD, with cholinergic cell loss in the basal nucleus of Meynert being found in patients (Zarow *et al.*, 2003) and Lewy body pathology being present in this region as early as stage 3 of pre-manifest PD (Braak *et al.*, 2003). ChAT activity is decreased in the prefrontal cortex of PD patients (Mattila *et al.*, 2001), and reduced nicotinic ACh receptor binding has been found in many cortical regions (Meyer *et al.*, 2009). We have found an accumulation of human α -synuclein in the cholinergic neurons of the basal nucleus of Meynert at 5 months, in association with a 30% reduction in ACh levels in the cerebral cortex at 6 months in the line of Thy1-aSyn mice that revealed the presence of cognitive deficits in the present study. As the cholinergic neurons in the basal nucleus project to the cerebral cortex (Mesulam *et al.*, 1983), it is possible that the α -synuclein overexpression in these neurons has caused the loss of cortical ACh, as it is known to affect mechanisms such as synthesis, packing into vesicles, and release of neurotransmitters (Di Rosa *et al.*, 2003).

It is important to note that the NOR and object–place recognition tests reflect different cognitive functions. In the former test, mice are exposed to two novel, but identical, objects, are allowed to explore them and, after a delay, one of these objects is switched to an object different in shape and color. Thus, successful discrimination between the familiar and the novel object would indicate an intact recognition memory – memory for the appearance and features of the object. On the other hand, in the object–place recognition test, the same two objects are used in both the first and second trials; however, one of them is displaced from its original location in the second trial, and therefore this test examines spatial memory – memory for the location of the objects. Despite the dissimilarities between these two tests, and the fact that they are mediated by different brain regions (Barker & Warburton, 2011), functions similar to the ones implicated in both tests were impaired in PD patients (Mollion *et al.*, 2011), supporting their relevance in modeling PD-related cognitive deficits in our mouse model.

Reversal learning, which measures the ability to update reinforced motor actions, was also impaired in Thy1-aSyn mice, consistent with previous reports of reversal learning impairments in PD patients (Peterson *et al.*, 2009) or after D2 antagonism (Lee *et al.*, 2007). Indeed, young Thy1-aSyn mice have profoundly abnormal responses to D2 agents in the striatum (Lam *et al.*, 2011). Interestingly, the impairments were not apparent in the acquisition phase of the task, in which mice had to achieve the initial criterion before reversing the contingency (data not shown), but only in the reversal phase. Thus, Thy1-aSyn mice could see and learn a rule as efficiently as WT mice, but had difficulties in switching to a new, reversed rule. This is similar to findings in human patients, who made more errors in a sequential learning task only when switched from an already learned sequence to a new one, but learned the initial task as accurately as normal controls (Mochizuki-Kawai *et al.*, 2010).

Striatal dysfunction is often assessed with a 'striatal' version of the T-maze, in which spatial cues are masked. However, this test could not be used here, because only a few mice were motivated to perform it. Therefore, we used a modified version of the holeboard (Dodart *et al.*, 2002) with no visual cues present. Importantly, the array of holes was symmetrical, and enabled the learning of a turning response, which is striatal-dependent (Packard & McGaugh, 1996), in search for the food reward. No differences were found between the WT and Thy1-aSyn mice in most aspects of the task, even in the switch phase, but group differences were found in the time spent visiting baited holes during the task. The longer visit times of Thy1-aSyn mice could be explained by impaired fine motor skills, necessitating more time spent in retrieving pellets from the holes, similar to deficits in nesting behavior (Fleming *et al.*, 2004). Similar impairments in fine motor skills were found in PD patients (Pradhan *et al.*, 2010). The high power for detecting a drug effect on this measurement will make it a useful addition to preclinical testing of drugs that may alter the course of PD (Lemesre *et al.*, 2010; Richter *et al.*, 2010; Fleming *et al.*, 2011).

An interesting distinction can be made between behavioral tests that showed deficits or abnormalities in the Thy1-aSyn mice (NOR, Y-maze, object-place recognition, and operant learning) and those that did not detect any cognitive deficits (holeboard) – with the exception of the operant learning task, the former tests assess spontaneous behavior, which does not involve any reward, whereas the latter test uses a reward. A recent study showed that PD patients and healthy elderly subjects performed an antisaccade task as efficiently as normal young subjects when anticipating a monetary reward (Harsay *et al.*, 2010), and hyperdopaminergic mice work harder for food reward than normal mice (Beeler *et al.*, 2010). As Thy1-aSyn mice also display an increase in extracellular dopamine in the striatum at 6 months of age (Lam *et al.*, 2011), it is conceivable that this might help them to overcome and compensate for any cognitive deficits that they have through modulation of the neural pathways involved in cognition. However, this was not the case in the operant learning task, which was successful in detecting switching deficits, despite the involvement of food reward. This could reflect higher cognitive demands in this task, which could prevail over the higher motivation for reward in the Thy1-aSyn mice. In all of the tests used, hyperactivity in the Thy1-aSyn mice at the young ages examined here (Lam *et al.*, 2011) was not a confounding factor, because the endpoint measures used do not reflect motor activity. We also have ruled out possible confounding effects of the fine motor skill deficits seen previously (Fleming *et al.*, 2004) and in the holeboard (this study) in the operant learning task by using a nose poke and by verifying the absence of time differences in accomplishing the task. In addition, blindness of the Thy1-aSyn mice, which could also be a confounding factor, can be ruled out, because these mice could detect light as equally well as WT mice, as indicated by the similar learning of the initial rule in the operant learning task, which involved response to light.

In summary, we have shown that α -synuclein overexpression is sufficient to elicit progressive cognitive deficits in a wide range of tasks, with similarities to the early cognitive deficits observed in patients with PD. Rather than a model of PD with dementia, our data provide a model of deficits that affect a large number of PD patients at a time in disease progression when they are professionally active, with often well-controlled motor symptoms. Therefore, identifying the neurochemical and neuropathological mechanisms underlying these cognitive deficits in Thy1-aSyn mice may point to much needed targets for cognitive enhancers in PD, and the high power to detect drug effects of the tests that we describe will be most useful for preclinical studies of drugs that may alleviate these early and pervasive cognitive impairments.

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Abbreviations

ACh	acetylcholine
ChAT	choline acetyltransferase
DI	discrimination index
NOR	novel object recognition
NS	not significant
PD	Parkinson's disease
Thy1-aSyn mice	mice overexpressing human wild-type α -synuclein under the Thy1 promoter
WT	wild-type

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Fig. 1.

(A) DI in one-trial object–place recognition in 4–5-month-old mice. (B) Total exploration time of objects in both locations. WT, gray bars; Thy1-aSyn, black bars. # $P < 0.05$ vs. zero (chance level), single-sample t -test. * $P < 0.05$ vs. WT, unpaired t -test. WT, $n = 12$; Thy1-aSyn, $n = 10$.

Fig. 2. NOR performance. (A) Naïve mice were exposed to the two figurines later used in the NOR test. The DI for dog vs. pig is not significantly different between genotypes (t -test, $P > 0.05$, NS; WT, $n = 7$; Thy1-aSyn, $n = 6$). (B) Mice (4–5 months old) previously exposed to two pig figurines were subsequently exposed to pig and dog figurines. DI with dog as a novel object: WT, $n = 18$; Thy1-aSyn, $n = 17$. WT, gray bars; Thy1-aSyn, black bars. * $P < 0.05$ vs. WT, unpaired t -test; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. zero (chance level), single-sample t -test. (C) Percentage of performers [mice exploring the novel object (dog) longer than the familiar object (white portion of bars)] vs. non-performers [mice exploring the familiar object (pig)]

longer than the novel object (dog) (hatched portion of bars)] in the experiment illustrated in B. $**P < 0.01$ vs. WT, Fisher's exact test.

Fig. 3.

(A) Number of trials to criterion in the acquisition and reversal phases of the operant learning task in 4–5-month-old mice. (B) Time required to respond to the left or right hole when a correct response was made, in both phases of learning. WT, gray bars; Thy1-aSyn, black bars. $*P < 0.05$ vs. WT at the same phase, repeated-measures ANOVA followed by Fisher's LSD. WT, $n = 11$; Thy1-aSyn, $n = 9$.

Fig. 4.

Holeboard performance in 3–4-month-old mice. (A–E) Parameters averaged over three trials per day. (A) Reference memory ratio. (B) Latency to complete task. (C) Distance traveled during task. (D) Latency to visit first open hole. (E) Time spent in open holes. (F and G) Parameters measured only for the first trial on each day. (F) Time spent in open holes. (G) Distance traveled during task. (H) Cumulative time needed to retrieve pellets from four open holes. WT, gray circles or bar; Thy1-aSyn, black circles or bar. * $P < 0.05$, ** $P < 0.01$ vs. WT on same day, Fisher's LSD or t -test. $\Delta\Delta P < 0.01$ vs. same genotype on day 5, trial 1 (repeated-measure ANOVA followed by Fisher's LSD). WT, $n = 9$ –10; Thy1-aSyn, $n = 10$ –11.

Fig. 5. Double labeling for ChAT and human α -synuclein in the basal nucleus of Meynert. (A–C) ChAT, human α -synuclein and overlay labeling, respectively, in a 5-month-old WT mouse. (D–F) ChAT, human α -synuclein and overlay labeling, respectively, in a 5-month-old Thy1-aSyn mouse. Scale bar: 50 μ m. (G) ACh levels in the cortex decrease in Thy1-aSyn mice at 6 months of age. WT, gray bar; Thy1-aSyn, black bar. * $P < 0.05$ vs. WT, unpaired t -test. WT, $n = 9$; Thy1-aSyn, $n = 7$.

Fig. 6.

Diagram illustrating the relationship between striatal dopamine (DA) levels and cognitive deficits in the Thy1-aSyn model, on a timeline. The black dots represent time points when measurements were performed. '=' indicates no change in the parameter measured as compared with the wild type; '↑' indicates an increase; and '↓' indicates a decrease.

Table 1

Summary of the tests performed on the same cohort of mice indicated by the ‘X’ symbol

Cohort	Test/age											
	Number of WT mice	Number of Thyl-aSyn mice	Number of litters	Holeboard 3-4 months	Y-maze 3-4 months	Operant learning 4-5 months	Object-place recognition 4-5 months	NOR 4-5 months	Y-maze 5-6 months	Y-maze 7-9 months	Y-maze 11-13 months	Y-maze 15-18 months
1	6	4	3					X				
2	6	5	4					X	X*			
3	10	6	9									X
4	9	8	8	X	X			X*				
5	10	9	8	X*	X*		X		X*			
6	1	3	2				X		X			
7	3	3	2				X*		X			
8	1	1	2								X	
9	11	9	7			X						
10	13	8	7							X		X

* Only a subset of mice in the cohort was tested.

Table 2

Summary of Y-maze results - percentage of spontaneous alternations and total visits in arms

	<u>Age (months)</u>							
	<u>3-4</u>		<u>5-6</u>		<u>7-9</u>		<u>11-13</u>	
	Wild type (n = 14)	Thy1-aSyn (n = 13)	Wild type (n = 14)	Thy1-aSyn (n = 14)	Wild type (n = 16)	Thy1-aSyn (n = 13)	Wild type (n = 14)	Thy1-aSyn (n =9)
% Alternations	56.80 ± 2.84	52.29 ± 3.7	62.56 ± 2.84	51.49 ± 3.25 *	58.71 ± 1.88	46.66 ± 4.22 **	58.68 ± 3.01	53.75 ± 3.55
Total visits	27.86 ± 4.31	20.88 ± 1.88	31.64 ± 3.52	30.21 ± 2.50	25.93 ± 2.43	27.54 ± 3.84	27.75 ± 3.24	23.72 ± 3.47

Data are presented as mean ± standard error of the mean.

* $P < 0.05$ ** $P < 0.01$ vs. wild type, Student's *t*-test.

Table 3

Summary of the results of the different tests performed in the mice, including mouse numbers, ages, housing conditions, endpoint measures, and the number of animals needed to detect a 30 and 50% drug effect with 80% power ($P < 0.05$)

Behavioral test/measurement	Age of mice (months)	<u>N (after outlier exclusion)</u>		Endpoint measure(s)	Results (direction of change in Thyl-aSyn mice)	<u>Animals needed to detect drug effect with 80% power ($P < 0.05$)</u>		Conditions of test
		Wild	Thy1-aSyn			30% effect	50% effect	
Object-place recognition	4-5	12	10	DI	↓	28	9	Dark phase, <i>ad libitum</i> food
NOR	4-5	18	17	DI	↓	11	4	
Y-maze	3-4	14	13	Percentage of alternations	0	NA		Dark phase, <i>ad libitum</i> food
	5-6	14	14		↓	10	4	
	7-9	11	8		↓↓	19	7	Light phase, FR
	7-9	5	5		Trend to ↓			Dark phase, <i>ad libitum</i> food
	11-13	14	9		0	NA		
Holeboard	3-4	9-10	10-11	Reference memory ratio, latency to complete task, time spent in open holes, latency to first baited hole	↑ time in open holes, 0 in other measures	31/5 [*] (for time in open holes)	11/2 [*] (for time in open holes)	Dark phase, FR
Operant learning task	4-5	11	9	Trials to criterion in reversal phase	↑	39	14	Light phase, FR

↑, increase; ↓, decrease (number of arrows symbolize magnitude of change); 0, no change (Thyl-aSyn vs. WT); FR, food restriction; NA, not applicable.

^{*} Number of animals calculated depends on data used (see Results section). Varying *N*-values mean that outliers were excluded only in some of the multiple endpoint measures used for the test.