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Mutant α -synuclein exacerbates age-related decrease of neurogenesis

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Abstract

In Parkinson disease, wild-type α -synuclein accumulates during aging, whereas α -synuclein mutations lead to an early onset and accelerated course of the disease. The generation of new neurons is decreased in regions of neurogenesis in adult mice overexpressing wild-type human α -synuclein. We examined the subventricular zone/olfactory bulb neurogenesis in aged mice expressing either wild-type human or A53T mutant α -synuclein. Aging wild-type and mutant α -synuclein-expressing animals generated significantly fewer new neurons than their non-transgenic littermates. This decreased neurogenesis was caused by a reduction in cell proliferation within the subventricular zone of mutant α -synuclein mice. In contrast, no difference was detected in mice overexpressing the wild-type allele. Also, more TUNEL-positive profiles were detected in the subventricular zone, following mutant α -synuclein expression and in the olfactory bulb, following wild-type and mutant α -synuclein expression. The impaired neurogenesis in the olfactory bulb of different transgenic α -synuclein mice during aging highlights the need to further explore the interplay between olfactory dysfunction and neurogenesis in Parkinson disease.

Keywords

Cell death; Neural stem/progenitor cells; Neurogenesis; Olfactory bulb; Parkinson's disease; Synucleinopathy

1. Introduction

In Parkinson disease (PD), as well as in other synucleinopathies, the accumulation of misfolded α -synuclein within neuronal cell bodies, axons and synapses has been proposed to be one of the crucial pathogenic hallmarks (Hashimoto and Masliah, 1999; Takeda et al., 1998). α -Synuclein is a 140-amino acid synaptic molecule that was originally identified as the non-A β component precursor protein of Alzheimer disease amyloid plaques (Iwai et al., 1996). A physiological role of α -synuclein has been proposed in developmental plasticity and synaptic remodeling (Chandra et al., 2004; George et al., 1995; Hsu et al., 1998). Point mutations in α -

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synuclein (A53T, A30P, E46K) have been identified in rare Mendelian forms of familial PD designated as PARK1 (Kruger et al., 1998; Polymeropoulos et al., 1997; Zarranz et al., 2004).

These α -synuclein mutations accelerate its aggregation and oligomerization (Conway et al., 1998; Narhi et al., 1999). As a result, in patients affected by the α -synuclein A53T mutation, the age of onset is much earlier than for patients presenting with sporadic PD (Kruger et al., 1998; Polymeropoulos et al., 1997; Zarranz et al., 2004). In PD, accumulation of α -synuclein aggregates has not only been observed in the midbrain and brain stem nuclei, but also in the olfactory bulb at a very early stage of the disease (Braak et al., 2003). Accumulation of α -synuclein in the olfactory bulb is particularly of clinical relevance, because olfactory dysfunction is one of the early symptoms in PD (Berendse et al., 2001; Sobel et al., 2001). Moreover, other neurodegenerative diseases such as Alzheimer disease or dementia with Lewy bodies result in olfactory dysfunction too (for review see Hawkes, 2006). The pathology in the olfactory bulb correlates strongly with the disease progression in dementia (Jellinger and Attems, 2005; Ohm and Braak, 1987; Tsuboi et al., 2003).

The olfactory bulb is one of the regions, where new neurons are constantly added to the olfactory circuitry throughout lifetime. The proliferating neural stem cells (NSCs) residing in the basal forebrain subventricular zone (SVZ) give constantly rise to neuronal precursors and therefore constitute the keystone of the olfactory bulb neurogenesis (Reynolds and Weiss, 1992). Newly generated neuronal precursors leave the SVZ and migrate along the rostral migratory stream (RMS) towards the olfactory bulb (Lois and Alvarez-Buylla, 1994). Upon arrival, newly generated cells mature and integrate as interneurons into the granule cell and the glomerular layer of the olfactory bulb (Betarbet et al., 1996; Winner et al., 2002). Furthermore, our previous work showed that continuous overproduction and turnover of newly generated neurons is an important regulatory feature of the adult olfactory bulb neurogenesis (Winner et al., 2002). The functional integration of the newly generated interneurons into the olfactory neuronal circuitry (Carlen et al., 2002; Carleton et al., 2003) strongly suggests a relevance of adult neurogenesis in olfaction (Magavi et al., 2005).

To further elucidate the α -synuclein-associated pathological mechanisms, animal models for synucleinopathies have been established using various promoters to drive expression of α -synuclein. The neuropathological changes observed in these models included protein accumulation in different areas of the brain according to a promoter-dependent topography (Masliah et al., 2000; Rockenstein et al., 2002). For example, PDGF-promoter driven expression of mutant A53T α -synuclein has resulted in a transgenic mouse model with a severe motor impairment (Hashimoto et al., 2003). α -Synuclein is endogenously expressed in NSCs and their progeny in the adult SVZ/olfactory bulb system as well as in the hippocampus (Li et al., 2002; Winner et al., 2004). Recently, using a transgenic mouse line that expresses high levels of wild-type human α -synuclein (WTS) we reported that increased levels of WTS affected adult neurogenesis. In particular, increased α -synuclein, obtained via transgenic expression of human α -synuclein led to the death of immature neurons in the brain of adult animals, indicating that α -synuclein has an impact on adult neurogenesis (Winner et al., 2004).

Clinical manifestations of synucleinopathies occur to a large extent in advanced age. We therefore examined the impact of α -synuclein on olfactory bulb neurogenesis during aging using the models of (i) transgenic mice overexpressing WTS and (ii) transgenic mice expressing the mutant A53T α -synuclein (MTS), a mutation accelerating the α -synuclein aggregation.

2. Methods

2.1. Animals and paradigm

For the present study, aged human wild-type α -synuclein (aged WTS) and A53T mutant α -synuclein transgenic mice (aged MTS) were compared to littermate controls (aged CTR). The aged animals were 15 month-old at the beginning of the experiment ($n = 6$ per group) and their genetic background was C57BL6/DBA. The synuclein transgenic mice (WTS and MTS) expressed α -synuclein under the regulatory control of the PDGF-promoter and the WTS group belongs to the human WTS high expresser line D (Masliah et al., 2000). The MTS group carrying the A53T mutation was described earlier (Hashimoto et al., 2003). To determine the impact on neurogenesis, the mice received daily intraperitoneal injections of bromodeoxyuridine (BrdU, 50 mg/kg) for 5 consecutive days and were sacrificed 1 month after the BrdU injections (details of experimental design: see Table 1A). In a second experiment a detailed analysis of proliferation was performed in adult A53T mutant α -synuclein transgenic mice (adult MTS, aged 5 months) (Hashimoto et al., 2003) and littermate non-transgenic controls (adult CTR, aged 5 months). These animals received a single injection of BrdU (200 mg/kg) and were perfused 24 h after the injection (Table 1B) (Cameron and McKay, 2001). All mice were kept in normal light dark cycle (12 h light/12 h dark) and had free access to food and water.

All mice were sacrificed following National Institutes of Health guidelines for the humane treatment of animals. Therefore the animals were deeply anesthetized with chloral hydrate and perfused transcardially with 4% paraformaldehyde in 100 mM phosphate buffer (PB), pH 7.4. The brains were removed, post-fixed in 4% paraformaldehyde/PB for 24 h, and placed in 30% sucrose/PB solution. The brains were cut sagittally into 25 μ m sections using a sliding microtome on dry ice. The sections were stored in cryoprotectant (ethylene glycol, glycerol, 0.1 M phosphate buffer pH 7.4, 1:1:2 by volume) at -20°C until further processed for immunohistochemistry or -fluorescence.

2.2. Histology

2.2.1. Antibodies and immunochemicals

2.2.1.1. Primary antibodies and final dilutions: Rat α -BrdU 1:500 (Oxford Biotechnology, Oxford, UK); mouse α -NeuN 1:500, rabbit α -tyrosine hydroxylase (TH) 1:1000 (both Chemicon, Temecula, CA, USA), goat α -DCX 1:500, mouse α -proliferating cell nuclear antigen (PCNA) 1:1000 (both Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse α -human α -synuclein 1:500 (Zymed, South San Francisco, CA, USA). Secondary antibodies for immunofluorescence were donkey anti-goat, -mouse, -rabbit or -rat conjugated with fluorescein (FITC), rhodamine X (RHOX), CY5 or biotin 1:500 (Jackson Immuno Research, West Grove, PA, USA). For immunohistochemistry donkey anti-mouse, -rabbit or -goat biotinylated 1:500 (Jackson Immuno Research, West Grove, PA, USA) and avidin–biotin peroxidase complex 1:100 (Vectastain Elite, Vector Laboratories, Burlingame, CA, USA) were used.

2.2.2. Immunohistochemistry—Free floating sections were treated with 0.6% H_2O_2 in Tris-buffered saline (TBS: 0.15 M NaCl, 0.1 M Tris–HCl, pH 7.5) for 30 min. Thereafter, incubation in TBS/0.25% Triton-X100/3% normal donkey serum for 30 min was followed by incubation with primary antibodies in TBS/donkey serum overnight at 4°C . Sections were incubated for 1 h with biotinylated secondary antibodies directed against mouse, goat or rabbit. Following rinses in TBS, avidin–biotin peroxidase complex was applied for 1 h, and then peroxidase detection for 10 min was performed (25 mg/ml diaminobenzidine (DAB), 0.01% H_2O_2 , 0.04% NiCl in TBS). For detection of BrdU-labeled nuclei the following DNA denaturation steps preceded the incubation with anti-BrdU antibody: 2 h incubation in 50%

formamide/2 × SSC (2 × SSC: 0.3 M NaCl, 0.03 M sodium citrate) at 65 °C, 5 min rinse in 2 × SSC, 30 min incubation in 2 M HCl at 37 °C, and 10 min rinse in 0.1 M boric acid, pH 8.5.

2.2.3. Immunofluorescence—Free-floating sections were treated to denature the DNA as described above. Afterwards the combination of antibodies was applied in TBS-donkey serum for 48 h at 4 °C, followed by incubation with secondary fluorochrome antibodies as described above. After several washes in TBS, sections were mounted on gelatin-coated glass slides and coverslipped using Prolong (Molecular Probes, Eugene, USA).

2.2.4. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)—Histological detection of cell death was performed using the ApopTag *in situ* Apoptosis Detection Kit (Chemicon, Temecula, CA, USA) in a modified procedure for free floating sections as described recently (Biebl et al., 2005). DNA degradation detected by TUNEL staining reflects a late stage in cell death. Hence, TUNEL staining may underestimate the total number of dying cells. The use of this method determines rather a proportional than an absolute number of cell death (Biebl et al., 2005).

2.3. Counting procedures

For quantification, a systematic, random counting procedure, similar to the optical dissector (Gundersen et al., 1988), was used as described by Williams and Rakic (1988). To determine the number of BrdU-, DCX-, PCNA- or TUNEL-positive cells, every sixth section (150 µm interval) of the left hemisphere was selected from each animal and processed for immunohistochemistry. Sections were analyzed for BrdU-, DCX-, or TUNEL-positive cells in the olfactory bulb. PCNA-, BrdU- and TUNEL-positive cells were quantified in the SVZ.

The reference volume was determined by tracing the areas using a semi-automatic stereology system (Stereoinvestigator, MicroBrightField, Colchester, VT, USA). In the granule cell layer of the olfactory bulb, BrdU- or DCX-positive cells were counted within a 30 µm × 30 µm counting frame, which was spaced in a 300 µm × 300 µm counting grid. Positive profiles that intersected the uppermost focal plane (exclusion plane) or the lateral exclusion boundaries of the counting frame were not counted. The total counts of positive profiles were multiplied by the ratio of reference volume to sampling volume in order to obtain the estimated number of positive cells for each structure.

Cells expressing cell death markers are comparatively rare in the SVZ and the olfactory bulb glomerular layer. Therefore, no counting frames were used here, but these regions were exhaustively counted on each section under exclusion of the uppermost focal plane.

To determine the frequency of neuronal and dopaminergic differentiation of newborn cells, a series of every 6th section (150 µm interval) was examined using the confocal laser microscope (Leica TCS-NT, Bensheim, Germany) equipped with a 40 × PL APO oil objective (1.25 numeric aperture) and a pinhole setting that corresponded to a focal plane of 2 µm or less. On average 50 BrdU-positive cells were analyzed in each region in each animal (20 in the glomerular layer of the old group) for neuronal differentiation. BrdU-positive cells were randomly selected and analyzed by moving through the z-axis of each cell, in order to exclude false double labeling due to an overlay of signals from different cells (Kuhn et al., 1997). Colabeling was analyzed sequentially at each wavelength and in its z-axis to ensure that the fluorophores were present in the identical focal plane.

BrdU-positive (newborn cells) cells were distinguished from BrdU/NeuN positive cells (newborn neurons) and BrdU/TH double-positive cells (newborn dopaminergic neurons).

2.4. Statistical analysis

The data are expressed as mean values $\pm$ standard deviation (S.D.). One-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test was used for the aged WTS, MTS and CTR groups. Furthermore the unpaired, two-sided *t*-test was used to compare adult MTS and adult CTR group. A two-way ANOVA (age $\times$ genotype) was performed to assess the influence of age (adult versus aged) and genotype (CTR versus MTS, Prism Graph Pad Software, San Diego, CA, USA). The significance level was set at $P < 0.05$.

3. Results

3.1. Synuclein expression in regions of adult neurogenesis

The transgenic animal models used in this study express human wild-type α -synuclein (WTS) or A53T mutant allele (MTS) in the olfactory bulb and the hippocampus, as well as in other regions of the adult brain (Hashimoto et al., 2003; Masliah et al., 2000). In adult WTS, human α -synuclein is coexpressed in DCX-immunopositive neuroblasts (Couillard-Despres et al., 2005; Winner et al., 2004). This expression pattern is also observed in the SVZ/rostral migratory stream of aged WTS mice (Fig. 1A–C, arrow). In contrast, a lower expression level of α -synuclein is detected in aged MTS mice (Fig. 1D). Here, α -synuclein immunofluorescence appears in part more condensed in DCX-expressing neuroblasts (Fig. 1D–F, arrow). Note, that the morphology of DCX-neuroblasts differs between WTS and MTS mice. In WTS mice, DCX expressing cells show long processes (Fig. 1B) whereas only few processes are present in MTS mice (Fig. 1E).

3.2. Decreased neurogenesis in aged WTS mice due to decreased survival in the olfactory bulb

Our previous study showed that neurogenesis was decreased in adult WTS mice without changes in NSC proliferation (Winner et al., 2004). Substantiating this finding, no significant differences in SVZ cell proliferation using PCNA labeling, a marker for proliferating cells (Takasaki et al., 1981), could be detected in the aged WTS and CTR groups (approximately 1900 PCNA-immunopositive cells in both groups, Table 1C, Fig. 2A, B, E). This suggests, that proliferation is not affected in aged WTS mice.

Detailed analysis of newly generated BrdU-labeled cells integrating into the olfactory bulb was further performed separately for the granule cell and the glomerular layer.

In the olfactory bulb granule cell layer, the number of BrdU-positive cells was reduced significantly (-29%) to 13.0×10^3 cells in the aged WTS mice, as compared to the aged CTR group (18.4×10^3 cells, Table 1D, Fig. 3A–C). Adult neurogenesis was further studied by determining cell fate of BrdU-labeled cells. In the granule cell layer for the colocalization of BrdU and NeuN no difference in the percentage of neuronal differentiation of BrdU-positive cells between aged WTS and CTR was present (89% in both groups, Table 1D, Fig. 3I–K). Notwithstanding the fact that cell-fate of newly generated cells remained unchanged, but taking into account a decrease in BrdU-cell numbers in the WTS group, neurogenesis in the olfactory bulb granule cell layer was significantly reduced in the aged WTS group (approximately -30% , Table 1D, Fig. 3M). A reduced neurogenesis in the granule cell layer was also indicated by a lower number of DCX-expressing neuroblasts in the aged WTS group (-33% , Table 1D, Fig. 3E–G).

In the olfactory bulb glomerular layer a significant reduction of BrdU-positive cells (-66%) as well as newly generated neurons (-69%) and newly generated dopaminergic neurons (-64%) were detected in the aged WTS mice as compared to the aged CTR mice (Table 1E, Fig. 4). Similar to our previous observation in adult WTS mice (Winner et al., 2004), an increase

in TUNEL-positive cells (approximately 2.5-fold) was detected in the glomerular layer. Taken these data together, this indicates that neurogenesis was reduced in aged WTS mice due to less survival of newly generated cells integrating in their target region, i.e. the olfactory bulb.

3.3. Pronounced reduction of neurogenesis in A53T MTS mice due to a decreased proliferation

We next examined the effects of A53T mutation on neurogenesis. In contrast to aged WTS mice, aged MTS mice show a massive decrease in cell proliferation within the SVZ. A 44% reduction in PCNA-expressing cells was observed in the aged MTS group compared to the aged WTS (Table 1C, Fig. 2A–C). To further test whether the massive reduction of SVZ proliferation in A53T mutant mice is present in adult MTS animals, a second experiment using a single BrdU pulse was performed.

This single dose of BrdU, injected systemically 24 h before perfusion revealed also a decrease (–40%) in proliferation in the adult MTS group (Table 1C) compared to the adult CTR group.

To delineate, whether the decrease in proliferation in both adult and aged MTS mice is accompanied by an increased cell death in the SVZ, we quantified the number of TUNEL-positive cells in this structure. A significant increase in TUNEL-positive cell number of the SVZ could be indeed detected in adult and aged MTS mice compared to their age-matched controls (Table 1C: 2-fold increase in adult and 3-fold increase in aged mice). This finding suggests that the increased cell death within the SVZ parallels the reduced proliferation in the SVZ. Furthermore, this implies that the vulnerability of newly generated neurons in MTS mice does increase with the age of the animals (15-month-old).

In order to study the arrival of immature neuroblasts in the olfactory bulb, the total DCX-positive cells was estimated in the olfactory bulb granule cell layer. We measured a significant reduction (–67%) of DCX-expressing neuroblasts in the aged MTS compared to the aged CTR group (Table 1D, Fig. 3E, F, H). A similar reduction of DCX-neuroblasts (–61%) could also be observed between adult controls and adult MTS animals. To independently determine the impact of aging in the decrease of DCX-expressing cells, a two-way ANOVA (age × genotype) was performed. A significant age-dependent decrease was present in controls (58% decrease between young and aged controls) and MTS mice (65% decrease between young and aged MTS mice). These findings together indicate that the decrease of neuroblasts in the olfactory bulb is both a phenomenon related to mutant synuclein as well as to aging.

Consecutively, we quantified TUNEL-positive cells in the granule cell and glomerular layer of the olfactory bulb in the aged animals. Here, we observed a significant increase of apoptotic profiles in the glomerular layer of the aged MTS mice compared to aged controls (Table 1E, 50% increase of TUNEL-positive cells). A two-way ANOVA (age × genotype) revealed no age-dependent effect on TUNEL labeled profiles in the granule cell layer of the CTR and MTS. However, an age-dependent decrease of TUNEL positive profiles was present in the glomerular layer of CTR (43% less positive cells in aged controls). This age-dependent decrease of TUNEL positive profiles observed in the controls is not present in the glomerular layer of MTS animals. This finding indicates, that cell death within the olfactory bulb may underlie different mechanisms in the aged MTS group.

Programmed cell death is a prominent feature of adult neurogenesis: a large proportion of neural progenitors and young neurons are eliminated by programmed cell death unless they receive synaptic input or trophic support (Biebl et al., 2000; Winner et al., 2002). Cell death occurs in immature neurons supported by double labeling of TUNEL and DCX in the granule cell layer of the olfactory bulb (Fig. 5).

To examine the survival of newly generated cells in the olfactory bulb granule cell and glomerular layer, we performed a stereological analysis of BrdU-labeled cells in the aged MTS group (Table 1A: BrdU analysis 4 weeks after BrdU injection). The number of BrdU-labeled cells in the granule cell layer was diminished to about 9.4×10^3 in the aged MTS group (Table 1D, 49% reduction compared to aged CTR). This reduction was paralleled by a 50% decrease in the total number of BrdU/NeuN colabeled cells (Fig. 3J, L, M). Therefore, cell survival of the newly generated cells targeted to the olfactory bulb granule cell layer was more affected in the aged MTS group (28% less) compared to the aged WTS group.

In the glomerular layer of the aged MTS group on the other side, survival of new cells was even further reduced than in the granule cell layer and was decreased by 74% as compared to the aged CTR group (Table 1E). Since the frequency of neuronal differentiation was comparable in all three aged groups (76% in the aged CTR, 77% in the aged WTS and 73% in the aged MTS group, Table 1E), neurogenesis in the glomerular layer was thus severely decreased in the aged MTS group. These findings indicate that mutations in α -synuclein exacerbate its deleterious impact on NSCs and progenitor cells, leading to a severe disruption of neurogenesis in the olfactory bulb of aged MTS mice.

The fraction of newly generated cells adopting a dopaminergic phenotype did not differ between aged MTS transgenics and the aged CTR (Table 1E). However, due to the decreased survival of newly generated cells in the glomerular layer the net number of newly generated TH-expressing neurons decreased in the aged MTS group (Table 1E). Thus, we observed 3-fold less newly generated TH-positive cells in the aged MTS mice, compared to the aged CTR group indicating a reduced dopaminergic neurogenesis in these mice (Table 1E).

4. Discussion

Considering the limited success for supplying exogenously cells for the treatment of PD (Freed et al., 2001), the interest in understanding the characteristics of endogenous neural precursor raised considerably over the past years (for examples, see Baker et al., 2004, Hoglinger et al., 2004). To further explore the potential of these cells, it is important to characterize the biology of endogenous NSCs populations in models of PD. Recently, we reported that adult neurogenesis is decreased in adult WTS mice, expressing the human α -synuclein wild-type allele under the control of the PDGF-promoter, due to a lower survival of newly generated cells in neurogenic regions (Winner et al., 2004).

Here, we analyzed neurogenesis and more specifically neuronal precursor generation and survival in aged mouse models of synucleinopathies. We analyzed two different aged transgenic models, one expressing the human wild-type α -synuclein protein (WTS) another expressing A53T mutant human α -synuclein (MTS), both under the control of the PDGF-promoter.

In adult rodents, immunoreactivity of endogenous α -synuclein has been described in regions of the fore- and hindbrain, including regions of neurogenesis, namely the olfactory bulb and the hippocampus (Li et al., 2002; Winner et al., 2004). In accordance with our previous report (Winner et al., 2004), we found that the human α -synuclein transgene was expressed in neural progenitor cells in neurogenic regions of both adult and aged WTS and MTS mice, thus making it feasible to study age-dependent effects of α -synuclein on olfactory bulb neurogenesis.

4.1. Differential expression of α -synuclein in WTS and MTS mice

Previous studies have shown, that the α -synuclein load in the A53T mice was about a third of that found in the WTS mice (referred to as high expresser line D in the original publication). With respect to the endogenous α -synuclein levels, A53T was estimated to be expressed at

approximately 1.5:1 ratio (Hashimoto et al., 2003). Despite the lower expression level of the mutant α -synuclein, A53T mice develop more pronounced neurogenesis deficits and motor-related impairments. This discrepancy between α -synuclein expression and functional impairment has been contributed to a greater toxicity of α -synuclein oligomers compared to the wild-type line (Hashimoto et al., 2003).

α -Synuclein typically accumulates in synapses and neurons; however, very few inclusions could be detected in aged animals expressing A53T either under the PDGF or the PrP promoter (Hashimoto et al., 2003; Lee et al., 2002). In this respect, it has been proposed, that certain α -synuclein species are localized within the nucleus and interact with histones (Goers et al., 2003; Specht et al., 2005). In addition, we hypothesize that the severely impaired neurogenesis in aged MTS mice may be related rather to toxic oligomer species than to α -synuclein accumulation (Hashimoto et al., 2003). Our finding that even adult animals already show a massive negative impact of mutant α -synuclein on olfactory neurogenesis further supports this hypothesis.

4.2. Mutant α -synuclein aggravate reduction of adult neurogenesis

Changes in neurogenesis in adult WTS mice occurred due to a lower survival of neuronal precursors within their target regions during the phase of neuronal differentiation and integration (Winner et al., 2004). In the aged WTS mice a lower survival was also observed within the granule cell and glomerular cell layer of the olfactory bulb, hence, resulting in a lower rate of neurogenesis. Nevertheless, proliferation was not affected by the transgenic expression of aged WTS. It is important to note, that both proliferation and cell death were not altered in the SVZ, indicating that the WTS impact on newly generated neuroblasts survival occurred late, i.e. during the integration and differentiation.

Reduction of neurogenesis was more pronounced in aged MTS mice as compared to aged WTS mice. Additional mechanisms contributed to the decreased neurogenesis measured in the aged MTS mice. We observed both a decrease in cell proliferation and an increase in apoptotic profiles within the SVZ. Our data suggest that cell death occurs more frequently within the SVZ of A53T mutant α -synuclein mice. It is well known that programmed cell death is an important characteristic observed within neurogenic regions of the adult brain (Biebl et al., 2000; Kuhn et al., 2005). Furthermore, the apoptotic elimination of neural progenitor cells was shown by colabeling of DCX neuroblasts with TUNEL (Fig. 5 and Kuhn et al., 2005, Winner et al., 2002). In conjunction with a previous study, the adult and aged control mice showed an age-dependent decrease in cell death in the olfactory bulb (Mirich et al., 2002). TUNEL labeling in MTS animals is not decreased in aged animals, thus indicating that the olfactory bulb circuits underlay a constant pathological challenge due to mutant α -synuclein. Whether the effect of mutant α -synuclein on the generation of new neurons for the olfactory bulb is cell intrinsic due to the toxicity of α -synuclein or induced by an adverse micro-environment needs further studies. One potential candidate molecule might be transforming growth factor β 1 (TGF- β 1) since we delineated an inhibiting effect of TGF- β 1 on adult neurogenesis (Wachs et al., 2006). Furthermore, increased expression levels of cyclin-dependent kinase inhibitors result in a reduced progenitor proliferation in the SVZ (Molofsky et al., 2006).

In the olfactory bulb of control mice, different newly generated populations were analyzed separately and cell death did not occur preferentially in a selective neurotransmitter phenotype such as GABA, Calretinin or TH-expressing newborn cells (Enwere et al., 2004). In acute lesion models of PD, a shift in cell fate determination has been described, leading to an increased dopaminergic neurogenesis in the olfactory bulb (Winner et al., 2006; Yamada et al., 2004). Interestingly, in parallel with these findings, more TH-expressing neurons were determined in the glomerular layer of PD patients' olfactory bulbs (Huisman et al., 2004).

However, the decrease of dopaminergic and non-dopaminergic olfactory bulb neurogenesis appears not to be different among the α -synuclein animals.

An enhanced α -synuclein toxicity could be detected in the adult and the aged MTS group. Thus, whereas an excess of WTS diminished the survival of integrating new neurons, A53T mutant α -synuclein concomitantly inhibits the generation of new neurons and decreases the survival of the latter at a very early stage in the SVZ.

Similar to tau, α -synuclein induces polymerization of purified tubulin into microtubules (Alim et al., 2004). Due to increased α -synuclein levels and accumulation during aging, axonal transport and neuritic outgrowth may be perturbed thus dysregulating synaptic integration (Li et al., 2004a,b; Takenouchi et al., 2001). Opposed to this important role in synaptic plasticity, recent studies implicated that the accumulation and aggregation of misfolded α -synuclein is also linked to exacerbated oxidative stress and p53-dependent apoptosis (Dawson and Dawson, 2003; Tatton et al., 2003). The differential effect of wild-type and mutant synuclein on intrinsic cell-survival promoting pathways may depend also on the altered capacity to bind to synphilin-1, which displays an antiapoptotic function (Giaime et al., 2006).

One might argue, that motor deficits in MTS mice contribute to the decreased olfactory bulb neurogenesis. Although increased physical exercise has been conclusively shown to increase hippocampal neurogenesis (van Praag et al., 1999), olfactory neurogenesis appears to be independent of this stimuli (Brown et al., 2003).

4.3. Disease-related modulation of adult neurogenesis

Adult neurogenesis is modulated in several models of CNS diseases (e.g. depression, stroke, epilepsy) (Jin et al., 2001; Malberg et al., 2000; Parent et al., 1997). The capacity of the diseased brain to recruit endogenous NSCs, as demonstrated following striatal ischemic lesions, is of particular clinical interest (Arvidsson et al., 2002). Importantly, the capacity of the brain to mobilize endogenous neural progenitors persists in the aging brain (Darsalia et al., 2005) is thus of particular interest for age-dependent neurodegenerative disorders. Physical activity and odor enrichment resulted in differential effects modulating hippocampal and olfactory adult neurogenesis (Brown et al., 2003; Rochefort et al., 2002; van Praag et al., 1999). Whether in general the observed differences in neurogenesis between transgenics and wild-living rodents are partially due to the deprived housing conditions of laboratory animals is still an open debate.

Several toxic and transgenic PD-related animal models have revealed decreased proliferation and/or survival of newly generated neurons in regions of neurogenesis (Baker et al., 2004; Hoglinger et al., 2004; Winner et al., 2004, 2006). A recent study also support the hypothesis that neurogenesis is also affected in human PD, as suggested by the decrease in the number of mitotic cells in the SVZ (Hoglinger et al., 2004).

4.4. Olfactory bulb neurogenesis and its potential link to PD

The SVZ/rostral migratory stream/olfactory bulb system provides an ideal model system to study early events in PD. Lewy body and neurite formation occur in the olfactory bulb at a very early stage of the disease (Braak et al., 2003). Moreover, dysfunction of the olfaction has also proven to be a reliable predictive marker for motor impairment, appearing later in the course of PD (Berendse et al., 2001). Functionally, odor enrichment selectively enhances olfactory neurogenesis and improves olfactory memory (Rochefort et al., 2002) whereas on the other hand olfactory deprivation (Corotto et al., 1994) leads to less newly generated interneurons in the olfactory bulb. Interestingly, in the aged olfactory system, deficits in fine olfactory discrimination have been attributed to diminished olfactory neurogenesis (Enwere et

al., 2004). Hence, evidence available supports the notion that the abnormal olfactory dysfunction detected in PD patients may be linked to an impaired neurogenesis.

The present study examined aged transgenic animal models of PD, mimicking more closely the clinical phenotype of PD, usually (1) affecting an aging population and (2) presenting olfactory deficits early in the course of the disease (Berendse et al., 2001). We report here that increased content of α -synuclein is deleterious for adult neurogenesis, especially when a mutant form of α -synuclein (A53T) is present. Whereas WTS expression reduced the survival of newly generated neurons, the A53T mutant allele diminished furthermore the number of proliferating NSCs located in the SVZ, the source for new olfactory bulb neurons. Both dopaminergic and non-dopaminergic new neuron generation and survival were severely affected by the expression of MTS. This interrelation between α -synuclein mutation reducing neurogenesis and the reduced proliferation in the SVZ of PD patients highlights the need to better understand the biology of endogenous NSCs during the course of PD in order to stimulate endogenous cell populations in PD patients.

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Fig. 1.

Human wild-type α -synuclein expression and A53T α -synuclein expression in the SVZ.

Human α -synuclein is expressed in the subventricular zone/rostral migratory stream of the aged WTS mice (A–C) and MTS mice (D–F). A and D show the immunoreactivity of human α -synuclein within this region (green staining (488 nm): α -human α -synuclein, A, D). Color separated recorded images show co-labeling with DCX (568 nm, red staining; B, C; arrow). A lower expression level of α -synuclein is detected in aged MTS (D). Here, α -synuclein appears more condensed (D, arrow) and DCX-expressing neuroblasts show less processes (E, F). Scale bar represents 20 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 2.

Proliferation within the SVZ in human α -synuclein overexpressing and A53T transgenic mice. Cell numbers of PCNA-positive cells in the SVZ are depicted for aged (A–C) and adult (D, F) controls (CTR), human wild-type α -synuclein (WTS) and A53T (MTS) mice. Note the significant decrease of PCNA-expressing cells in the aged (C) and adult (F) MTS compared to age-matched controls (A, D) and aged WTS (B). E shows the quantification of PCNA-positive cells in the SVZ. Note the significant reduction of PCNA-positive cells between adult/aged MTS mice and their corresponding CTR and WTS mice. Adult MTS mice show a significant reduction of mitotically active cells within the SVZ (D–F). Scale bar (F) represents 50 μ m. Error bars (E) represent S.D.; # indicates significance comparing MTS with CTR; *, significance comparing MTS with WTS; $P < 0.05$.

Fig. 3.

Less neurogenesis in the olfactory bulb granule cell layer of aged synuclein transgenic mice. Reduction of newly generated cells (BrdU-labeled nuclei are visualized in black) in the granule cell layer was present in the aged WTS mice, but even more pronounced in the aged MTS group (A: graph; B: CTR (control), C: WTS (h-wt α -synuclein), D: MTS (A53T)).

Complementary to the assessment of newly generated cells immature DCX-labeled neuroblasts are quantified (E–H). Here a decrease in DCX-expressing profiles is also present in the WTS (G) and MTS (H) group compared to controls (F), which is significantly more pronounced in the MTS group. Using colocalization of BrdU and NeuN there is no difference in the percentage of neuronal differentiation of BrdU-positive cells between aged CTR, WTS and MTS (89% in

all groups, I–L). The number of newly generated mature neurons (total number of BrdU/NeuN coexpressing cells) was decreased in the granule cell layer of the WTS and MTS animals (M). Separate confocal analysis of the fluophores red (568 nm: NeuN; O) and green (488 nm: BrdU; P). Merged in the identical focal plane (N). Scale bars: 50 μ m B–D, F–H; 25 μ m J–L, N–P. Error bars (A, E, I, M) represent S.D.; + indicates significance comparing WTS with CTR; #, significance comparing MTS with CTR; *, significance comparing MTS with WTS; $P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 4.

Decreased numbers of newly generated cells in the glomerular layer of the olfactory bulb in aged synuclein transgenic mice. A decrease in newly generated cells (BrdU-labeled nuclei are visualized in black) was present in the glomerular layer of aged WTS, but even more pronounced in the aged MTS group A: graph; B: CTR (control); C: WTS (h-wt α -synuclein); D: MTS (A53T). The number of newly generated TH-expressing neurons was also decreased in the glomerular layer of the aged α -synuclein transgenic animals (E). F: Confocal microscopy of a glomeruli in MTS mouse, depicting a newly generated dopaminergic neuron (F) and the separate channels in the z-axis for BrdU (green, G, I), TH (red, H, J). Scale bars 50 μ m in B–D; 20 μ m in F–I. Error bars (A, E) represent S.D.; + indicates significance comparing WTS with CTR; #, significance comparing MTS with CTR; *, significance comparing MTS with WTS; $P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 5. Cell death of neuroblasts in adult neurogenesis in the olfactory bulb. xyz -reconstruction and color separation of a confocal image depicting colabeling (A) of a cell double-labeled for TUNEL (green, C) and DCX (red, B), a marker for neuronal progenitor cells. Hairline cross shows the level at which the y - z reconstruction and the x - z reconstruction were performed. Scale bar represents 10 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1

	A (aged)	B (aged)
Groups:	CTR (n=6) WTS (n=6) MTS (n=6)	CTR (n=6) WTS (n=6) MTS (n=6)
Treatment:	Brd U i.p. (50 mg/kg)	Brd U i.p. (50 mg/kg)

The diagram shows five vertical brain sections, each with a black arrow pointing downwards from the top, labeled 'perfusion'. The arrows are positioned at the top of each section, and the sections are arranged in a row. The arrows are black and point downwards, indicating the direction of perfusion. The sections are represented by black outlines of brain slices.

A (aged)		B (aged)	
Experimental day	0		32
Animal age (months)	15		16
(C) Cell number and cell fate of newborn cells in the subventricular zone			
SVZ	Control (CTR)	h-wt- α -synuclein (WTS)	A53T
Proliferation (PCNA+) adult	2331 $\pm$ 523		10
Proliferation (PCNA+) aged	1934 $\pm$ 300	1852 $\pm$ 347	10
Proliferation (BrdU+) adult	2356 $\pm$ 899		14
Cell death (TUNEL+) adult	54 $\pm$ 7		10
Cell death (TUNEL+) aged	76 $\pm$ 23	73 $\pm$ 29	2
(D) Cell number and cell fate of newborn cells in the olfactory bulb (granule cell layer)			
GCL	Control (CTR)	h-wt- α -synuclein (WTS)	A53T
BrdU + cells aged	18.4 $\times 10^3 \pm 2.2 \times 10^3$	13.0 $\times 10^3 \pm 2.1 \times 10^3$	9.4 $\times 10^3 \pm 2.1 \times 10^3$
% NeuN + cells aged	89 $\pm$ 2	89 $\pm$ 3	89 $\pm$ 3
BrdU/NeuN total cell number aged	16.4 $\times 10^3 \pm 1.9 \times 10^3$	11.6 $\times 10^3 \pm 2.3 \times 10^3$	8.2 $\times 10^3 \pm 1.5 \times 10^3$
Neuroblasts (DCX+) adult	103 $\times 10^3 \pm 16 \times 10^3$		40 $\times 10^3 \pm 16 \times 10^3$
Neuroblasts (DCX+) aged	43 $\times 10^3 \pm 7 \times 10^3$	29 $\times 10^3 \pm 7 \times 10^3$	14 $\times 10^3 \pm 7 \times 10^3$
Cell death (TUNEL+) adult	148 $\pm$ 17		14 $\pm$ 17
Cell death (TUNEL+) aged	115 $\pm$ 22	190 $\pm$ 33	115 $\pm$ 22
(E) Cell number and cell fate of newborn cells in the glomerular layer			
GLOM	Control (CTR)	h-wt- α -synuclein (WTS)	A53T
BrdU + cells aged	4508 $\pm$ 652	2957 $\pm$ 384	12
% NeuN + cells aged	76 $\pm$ 5	77 $\pm$ 5	7
%TH + cells aged	19 $\pm$ 5	17 $\pm$ 6	2
BrdU/NeuN total cell number aged	3298 $\pm$ 279	2290 $\pm$ 348	87
BrdU/TH total cell number aged	612 $\pm$ 123	392 $\pm$ 195	17
Cell death (TUNEL+) adult	66 $\pm$ 11		7
Cell death (TUNEL+) aged	38 $\pm$ 7	92 $\pm$ 21	8

(A) Experimental design for the control (aged CTR), A53T α -synuclein (aged MTS) and human wild-type α -synuclein overexpressing (aged WTS) groups. BrdU was injected on 5 consecutive days. Animals were perfused 1 month after the BrdU injections. (B) Experimental design for the adult CTR and adult MTS group. A single BrdU pulse was injected 24 h before perfusion. (C) Cell numbers of mitotic cells within the subventricular zone (SVZ) of transgenic animals (adult and old MTS and old WTS) and controls (adult and old CTR) are presented as the mean number of PCNA-positive and BrdU-positive cells $\pm$ S.D. Moreover, cell numbers of TUNEL-positive cells $\pm$ S.D. are depicted. (D) The cell numbers of newly generated cells $\pm$ S.D. (BrdU-positive) and new neurons $\pm$ S.D. (BrdU/NeuN-positive total cell number) in the granule cell layer (GCL) of the olfactory bulb are shown for all groups. Moreover, percentages $\pm$ S.D. of new neurons colabeling for BrdU and NeuN (%NeuN + cells) are presented. An additional analysis estimates total cell numbers for doublecortin (DCX) expressing neuroblasts $\pm$ S.D. in this region. A two-way ANOVA (age $\times$ genotype) for DCX expressing neuroblasts revealed a significant difference for the genotype ($P < 0.001$) and the age ($P < 0.0001$). TUNEL-positive profiles ($\pm$ S.D.) were counted. Here, a two-way ANOVA (age $\times$ genotype) for TUNEL-positive profiles in the GCL revealed no significant differences both for age

and genotype. (E) The quantification of the glomerular layer (GLOM) of the olfactory bulb comprises the following measures: newly generated cells (BrdU-positive), percentage of newly generated neurons (%NeuN-positive cells), percentages of newly generated dopaminergic neurons (%TH-positive cells), total number of new neurons (BrdU/NeuN total cell number) and total number of new dopaminergic neurons (BrdU/TH total cell number), and the number of TUNEL-positive cells. A two-way ANOVA (age $\times$ genotype) revealed for controls only an age-dependent decrease ($P < 0.05$). All measures are given as mean $\pm$ S.D.

⁺ Indicates significance comparing WTS with CTR;

[#] significance comparing MTS with CTR.

^{*} significance comparing MTS with WTS;

Δ an age-dependant effect for CTR and MTS;

Φ an age-dependant effect for CTR.