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Human Wild-Type α-Synuclein Impairs Neurogenesis

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Abstract. Neurodegenerative diseases classified as synucleinopathies are characterized by α-synuclein inclusions. In these disorders, α-synuclein accumulates within glial or neuronal cells in the brain including regions of adult neurogenesis. We hypothesized a pathophysiological role for α-synuclein in newly generated cells of the adult brain and in this study examined regions of neurogenesis in adult mice overexpressing human wild-type α-synuclein under the control of the platelet-derived growth factor promoter. The number of proliferating cells and the fate of newly generated cells were analyzed in the olfactory bulb system and in the hippocampal dentate gyrus. There were no effects on proliferation detectable; however, significantly less neurogenesis and fewer neurons were observed in the olfactory bulb as well as in the hippocampus of adult human α-synuclein mice compared to control littermates. This effect was almost exclusively due to diminished survival of neuronal precursors in the target regions of neurogenesis. Our data imply that the finely tuned equilibrium of neuronal cell birth and death in neurogenic regions may be altered in human α-synuclein-overexpressing mice. We hypothesize that reduced adult neurogenesis in the olfactory bulb may contribute to olfactory deficits in neurodegenerative disorders associated with α-synuclein inclusions.

Key Words: Cell death; Hippocampus; Neuronal stem/progenitor cells; Olfactory bulb; Parkinson disease; Synucleinopathy.

INTRODUCTION

Numerous neurodegenerative diseases with Lewy body and Lewy neurite pathology are associated with accumulation of wild-type α-synuclein protein and have therefore been categorized as synucleinopathies (i.e. Parkinson disease, dementia with Lewy bodies, and multiple system atrophy) (1–6). Human α-synuclein abnormally accumulates and aggregates as intracellular filamentous deposits in Lewy bodies and Lewy neurites. Transgenic mice, overexpressing human wild-type α-synuclein under the control of the platelet-derived growth factor (PDGF) promoter, represent an animal model for the role of α-synuclein in the CNS (7). In these animals, human α-synuclein-immunopositive intracytoplasmic inclusions resemble Lewy bodies and accumulate in neurons of the neocortex, substantia nigra, hippocampus, and the olfactory bulb. The intraneuronal inclusions, both nuclear and cytoplasmic, lack fibrillar components; however, they are accompanied by a reduction of dopaminergic markers in the substantia nigra, as well as early-onset of disease with a progressive decline of motor performance (7).

Under physiological conditions, α-synuclein is a presynaptic protein that participates in embryonic neuronal development (8) in synaptic remodeling and maintenance (9). In adult rats, immunoreactivity of endogenous α-synuclein is described in regions of the fore- and hindbrain, including the olfactory bulb and the hippocampus (10).

Neurogenesis in the adult brain occurs in the dentate gyrus of the hippocampal formation and in the subventricular zone (SVZ) and is preserved into old age (11–16). SVZ-derived neural progenitor cells migrate towards the olfactory bulb along a well-defined pathway, the rostral migratory stream (17). In the olfactory bulb, the newly generated cells ascend radially, differentiate into interneurons in the granule and glomerular layer (12, 18, 19), and functionally integrate into olfactory circuitries (20). Within the hippocampus, new neurons are generated at the border between hilus and granule cell layer (15, 21) and show electrophysiological characteristics indistinguishable from the surrounding granule cells (22). Neurogenesis is regulated at the level of cell proliferation and programmed cell death of progenitor cells and young neurons (19, 23, 24), which is similar to the processes taking place during brain development (25, 26).

In this study we analyzed cell dynamics in prototypal regions of adult neurogenesis in a transgenic model for synucleinopathies. We investigated cell proliferation, neurogenesis, and cell death in adult brains of mice overexpressing human wild-type α-synuclein under the PDGF promoter, focusing on regions of adult neural stem cell proliferation, migration, and differentiation. This was accomplished by systemic BrdU injection into transgenic human wild-type α-synuclein mice, followed by quantitative and qualitative analysis of cell numbers and the

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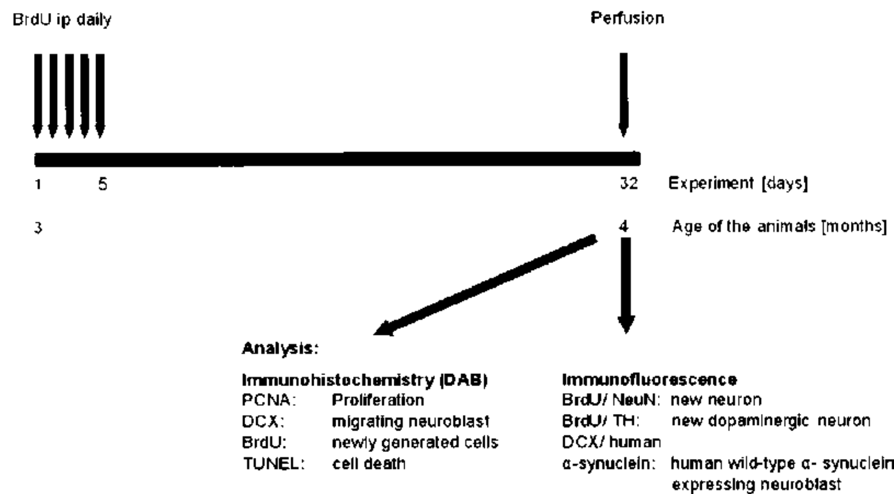

Fig. 1. Experimental design. Injection paradigm and antibodies used for the experiment.

fate of the newly generated cells. We also addressed the time point of elimination of the newly generated neurons.

MATERIALS AND METHODS

Animals and BrdU Labeling

Human wild-type α-synuclein transgenic mice were compared to littermate controls (genetic background C57BL6/DBA). All mice were kept in normal light-dark cycle (12 hours light/12 hours dark) and had free access to food and water. The transgenic mice expressed α-synuclein under the regulatory control of the PDGF promoter and belong to the human wild-type α-synuclein high expresser line D (7). At 3 months of age, all mice received daily intraperitoneal injections of BrdU (50 mg/kg) for 5 consecutive days. The animals were killed 1 month after the BrdU injections (n = 5 per group). The animals were deeply anesthetized and perfused transcardially with 4% paraformaldehyde in 100 mM phosphate buffer (PB), pH 7.4. The experiment was performed following NIH guidelines for the treatment of animals. The brains were removed, post-fixed in 4% paraformaldehyde/PB for 24 hours, and placed in 30% sucrose/PB solution. The brains were cut sagittally into 40-μm 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 immunofluorescence (Fig. 1).

Histology

Antibodies and Immunochemicals: The following antibodies and final dilutions were used: Primary antibodies: rat α-BrdU 1:250 (Oxford Biotechnology, Oxford, UK); mouse α-NeuN 1:500, rabbit α-tyrosine hydroxylase (TH) 1:1,000, sheep α-α-synuclein 1:250 (all from Chemicon, Temecula, CA), goat α-doublecortin (DCX) 1:500, mouse α-proliferating cell nuclear antigen (PCNA) 1:1,000 (both from Santa Cruz Biotechnology, Santa Cruz, CA), mouse α-human α-synuclein 1:500 (Zymed, South San Francisco, CA). Secondary antibodies for immunofluorescence: donkey α-rat and α-goat fluorescein (FITC) 1:500, donkey α-mouse and donkey α-rabbit rhodamine X 1:300, donkey α-rabbit and donkey α-rat CY5 1:500 (all from Jackson Immuno Research, West Grove, PA). For immunohistochemistry, donkey α-mouse biotinylated, donkey α-rabbit biotinylated, donkey α-goat biotinylated 1:500 (Jackson Immuno Research) and avidin-biotin-peroxidase complex 1:100 (Vectastain Elite, Vector Laboratories, Burlingame, CA) were used.

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 (mouse α-BrdU, mouse α-PCNA, mouse α-NeuN, goat α-DCX, rabbit α-TH) 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 intermittent rinses in TBS, avidin-biotin-peroxidase complex was applied for 1 h, 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 α-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 2M HCl at 37°C; and 10-min rinse in 0.1M boric acid, pH 8.5.

Immunofluorescence: Free-floating sections were treated to denature the DNA as described above. Then a combination of rat α-BrdU, goat α-DCX, mouse α-NeuN, rabbit α-TH, or mouse α-human α-synuclein antibodies was applied in TBS-donkey serum for 48 h at 4°C. Donkey α-mouse, donkey α-rabbit, and donkey α-sheep rhodamine X, donkey α-rat and donkey α-goat FITC, and donkey α-rabbit-Cy5 were used as secondary antibodies. After several washes in TBS, sections were mounted on gelatin-coated glass slides and coverslipped using Prolong (Molecular Probes, Eugene, OR).

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Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL): Histological detection of cell death was performed using the Apoptag In Situ Cell Death Detection Kit (Intergene, Purchase, NY) in a modified procedure for free-floating sections as previously described (23, 27).

Counting Procedures

For quantification, a systematic, random counting procedure, similar to the optical dissector (28), was used as described by Williams and Rakic (29). To determine the number of BrdU-, DCX-, NeuN-, or PCNA-positive cells, every sixth section (240-μm interval) of the left hemisphere was selected from each animal and processed for immunohistochemistry. Sections were analyzed for BrdU- or NeuN-positive cells in the olfactory bulb and dentate gyrus. In the glomerular layer, TH-positive cells were also counted. Additionally, DCX- and PCNA-positive cells were counted in the dentate gyrus, as were PCNA-positive cells in the SVZ.

The reference volume was determined by tracing the areas using a semi-automatic stereology system (Stereoinvestigator, MicroBrightField, Colchester, VT). In the granule cell layer of the olfactory bulb, BrdU- or NeuN-positive cells were counted within a 60- × 60-μm counting frame, which was spaced in a 200- × 200-μm counting grid. TH-positive cells in the glomerular layer were determined within a 20- × 20-μm counting frame that was spaced in a 100- × 100-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.

BrdU-, DCX-, and PCNA-labeled cells are comparatively rare in the SVZ, the olfactory bulb glomerular layer, and dentate gyrus granule cell 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 sixth section (240-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, 100 BrdU-positive cells were analyzed in each region in each animal 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 (30). BrdU-positive (newborn cells) cells were distinguished from BrdU/NeuN-positive cells (newborn neurons) and BrdU/TH double-positive cells (newborn dopaminergic neurons).

Statistical Analysis

The data are expressed as mean ± SEM. Statistical analysis was performed using the unpaired, two-sided *t*-test comparison Student *t*-test between human α-synuclein transgenic and control groups (StatView Software, Cary, NC). The significance level was set at $p < 0.05$.

RESULTS

Human Wild-Type α-Synuclein Expression in Neurogenic Regions of the Adult Brain

The transgenic mice used in this study have previously been described to overexpress human wild-type α-synuclein under the PDGF promoter in different regions of the adult brain, including the olfactory bulb and the hippocampus (7). In a more detailed analysis of the neurogenic areas, we used an antibody specific for human α-synuclein and detected immunopositive cells in the SVZ and in the rostral migratory stream (Fig. 2). In the SVZ, human α-synuclein expressing cells had short processes and were organized in chain-like structures (Fig. 2A). In the rostral migratory stream, human α-synuclein expressing cells had an elongated morphology with a leading process (Fig. 2B) reminiscent of migrating neuronal precursors (17). In addition, human α-synuclein-expressing cells were found in small numbers within the olfactory bulb (Fig. 2C, D). The cell morphology of human α-synuclein-expressing cells is reminiscent of neuroblasts proliferating in the SVZ, migrating through the rostral migratory stream and integrating into the olfactory bulb. Within the hippocampus, human α-synuclein-positive cells were dispersed throughout the dentate gyrus (Fig. 2E). Human α-synuclein expression was not found in control littermates; however, endogenous mouse α-synuclein expression was detected in regions of neurogenesis of control littermates (e.g. SVZ, dentate gyrus, Fig. 3A, B).

To further demonstrate human wild-type α-synuclein expression in neuronal progenitor and precursors cells, we localized DCX expression in human α-synuclein expressing cells. DCX is a microtubule binding protein (31, 32) that is transiently expressed in adult proliferating neuronal progenitor and neuronal precursor cells (33). Most of the α-synuclein-positive cells within the SVZ and rostral migratory stream co-labeled with DCX (Fig. 2). In addition, human wild-type α-synuclein/DCX double-labeled cells could be identified within the olfactory bulb granule cell and glomerular layer as well as within the dentate gyrus granule cell layer (Fig. 2C–E).

Reduction of Neurogenesis in Human Wild-Type α-Synuclein Mice

Since the human wild-type α-synuclein transgene is expressed in neuronal progenitors and precursors of transgenic mice, we analyzed adult neurogenesis in these transgenic animals by determining cell proliferation and cell numbers and fate of BrdU-incorporating cells. We studied mitotic activity and proliferation in the SVZ and dentate gyrus by counting cells positive for the cell cycle marker PCNA, which is synthesized in early G1 and S phases of the cell cycle (34). There was no significant difference in the number of PCNA-positive cells in the

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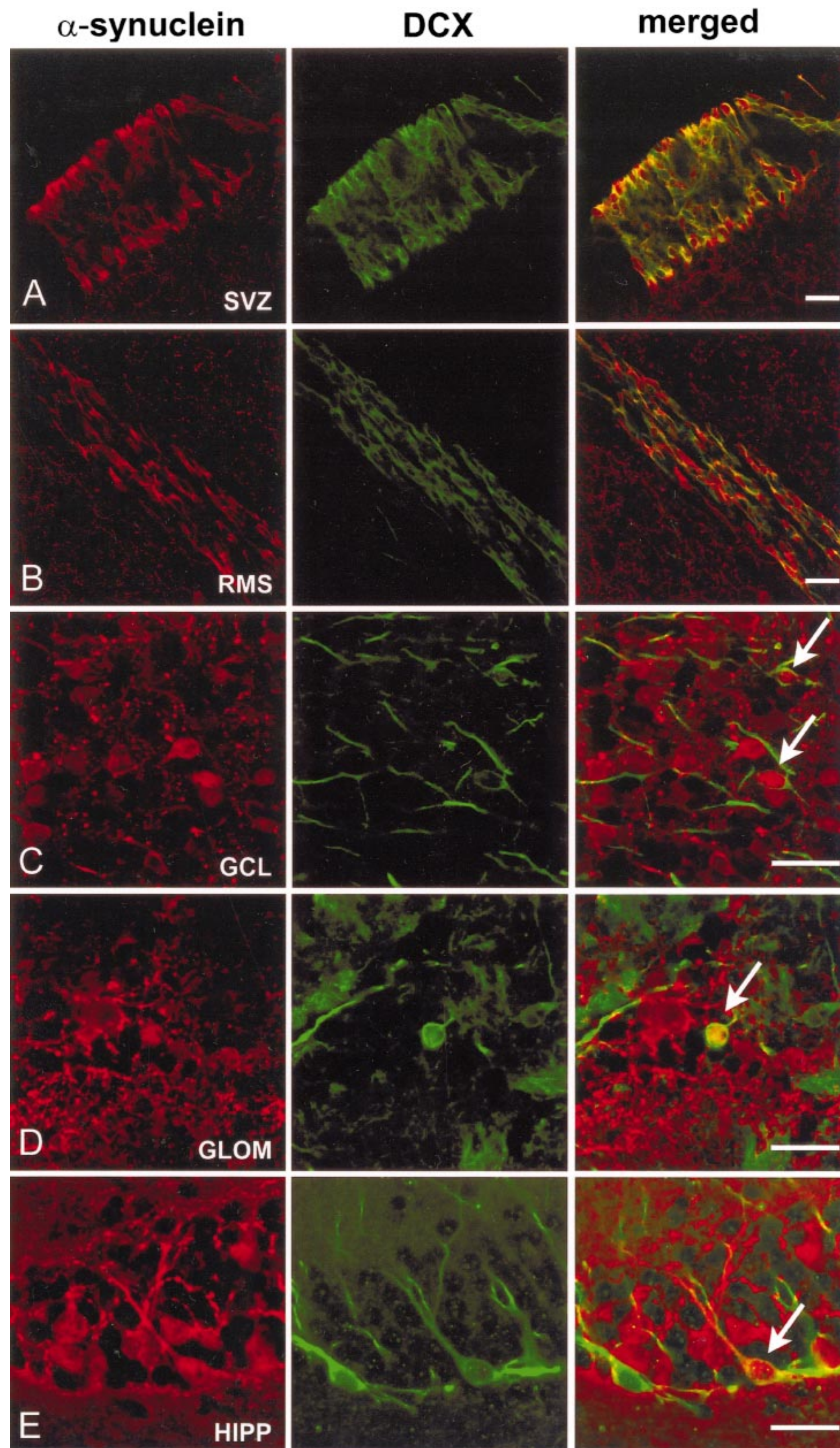

Fig. 2. Human wild-type α-synuclein expression in regions of adult neurogenesis. Human α-synuclein is expressed in the SVZ/rostral migratory stream/olfactory bulb system and in the dentate gyrus. Note the morphology of human α-synuclein-positive cells in immunofluorescence stainings (red staining, rhodamine X): α-human α-synuclein, **A–E)**, with short processes in the SVZ **(A)**. In the rostral migratory stream, α-synuclein-positive cells have an elongated morphology with a leading process **(B)**. Within the olfactory bulb (**C:** granule cell layer, **D:** glomerular layer) and the hippocampal dentate gyrus granule cell layer **(E)** α-

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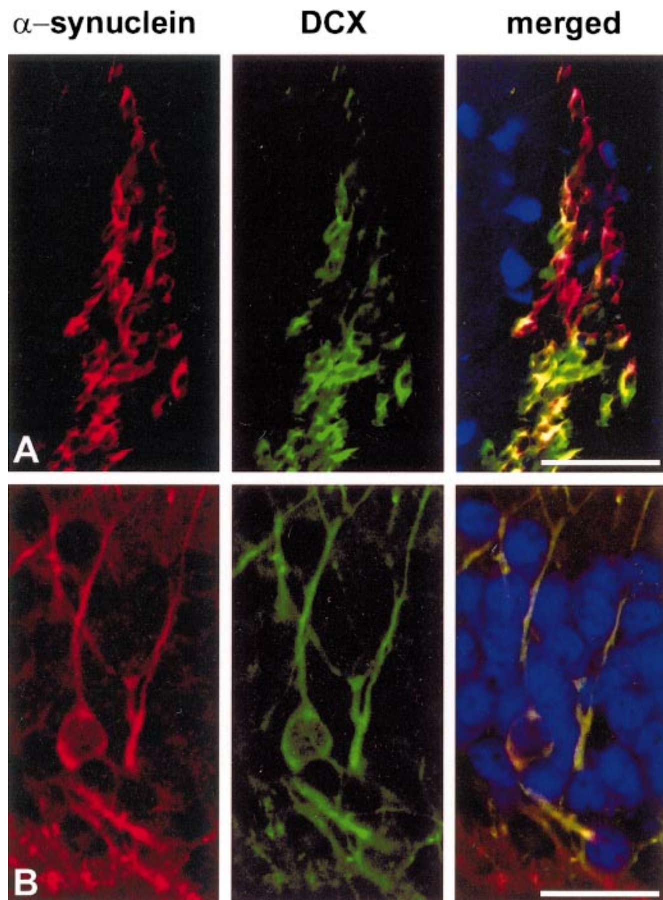

Fig. 3. Endogenous α-synuclein expression in littermate controls. **A, B:** Endogenous mouse α-synuclein-positive cells are found in the SVZ and the dentate gyrus of the hippocampus in littermate controls. Scale bars: A = 50 μm; B = 25 μm.

SVZ and the granule cell layer of the dentate gyrus between the human α-synuclein and control littermates (Fig. 4). This finding indicates that the number of cycling cells is not altered in neurogenic regions of human α-synuclein-overexpressing adult mice.

A cell fate analysis of BrdU-incorporating cells 1 month after BrdU injection indicated a significant reduction of BrdU-positive cells present in adult human α-synuclein mice in the target areas of neurogenesis, the hippocampus, and the olfactory bulb. This corresponds to a time point when most of the newly generated cells have differentiated (18, 19, 24). In the olfactory bulb, the number of BrdU-positive cells was reduced by approximately 50% in human α-synuclein mice (Fig. 5A–D). A significant difference between controls and human α-synuclein mice was also observed in the granule cell layer of the hippocampus: human α-synuclein mice contained 54% fewer newly generated cells in the granule cell layer of the dentate gyrus (Fig. 5E, F).

The ratio of neuronal (NeuN-positive) profiles among the BrdU-positive cells was not significantly altered: neurons represented the majority of newly generated cells (93% in the olfactory bulb granule cell layer, 74% in the glomerular layer, and 76% in the hippocampal dentate gyrus). Moreover, the TH-positive profiles in the glomerular layer of the olfactory bulb (17% of all BrdU-positive cells) were not significantly different between both groups. As a consequence of reduced BrdU-positive cells and unchanged neuronal ratio, neurogenesis was significantly reduced in human α-synuclein mice. Only half of the number of new neurons was present in human α-synuclein mice in the granule cell layer of the olfactory bulb (about 29,000 ± 3,300 BrdU/NeuN double-labeled cells), compared to 59,500 ± 7,100 in the control group (Fig. 6A, B). The total number of neurons in the granule cell layer decreased significantly by 34% (0.83×10^6 ± 50,000 NeuN-positive cells in the human α-synuclein mice, compared to about 1.25×10^6 ± 83,000 in the control group). Fewer new neurons were also observed in the glomerular layer of the olfactory bulb of the α-synuclein mice (2,400 ± 200 vs 4,500 ± 1,000 newly generated neurons in the control group). In particular, dopaminergic neurogenesis measured by coexpression of TH and BrdU was reduced by 59% in the glomerular layer of the olfactory bulb (Fig. 6C, D). The total number of TH-positive cells significantly decreased by 47% (35,000 ± 3,000 in the human α-synuclein mice compared to 66,000 ± 3,000 in the control group). In the granule cell layer of the hippocampus, neurogenesis was decreased by 38% (Fig. 6E, F). Here the total number of NeuN-positive cells was lower as well (70,000 ± 6,600 in the human α-synuclein mice compared to 89,000 ± 6,300 in the control mice).

Increased Cell Death Due to Human α-Synuclein

Because the number of dividing (PCNA-positive) cells in human α-synuclein mice was unchanged compared to controls, we hypothesized that the reduction of neurogenesis in the presence of human α-synuclein is due to decreased survival of neuronal committed progenitors and young neurons. In the presence of human α-synuclein we observed increased cell death in neurogenic regions. There was a more than 50% increase of TUNEL-positive cells in the granule cell layer of the dentate gyrus of

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synuclein-positive cells are observed as well. Co-labeling with DCX (FITC, green; arrows in **C–E**), a marker for neuroblasts and neuronal precursors, depicts newly generated cells expressing α-synuclein in all regions of neurogenesis. It is important to note that no human α-synuclein-positive cells were found in the control group. Scale bars: 25 μm.

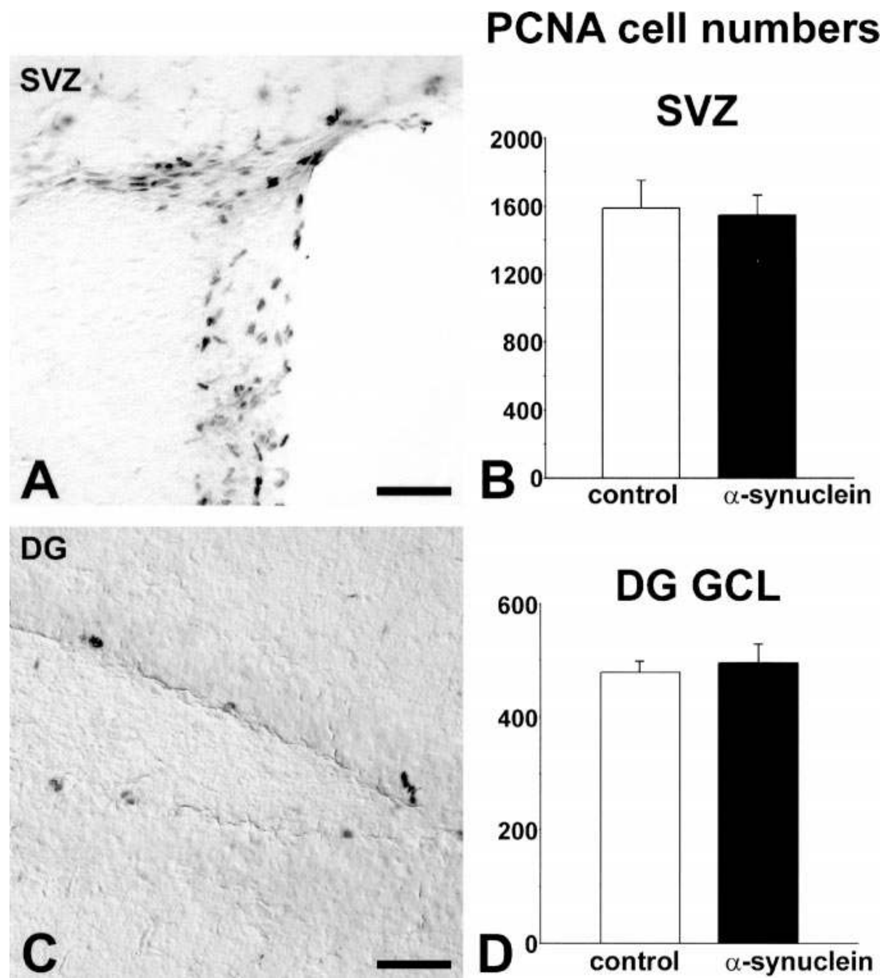

Fig. 4. Same amount of proliferation in human α-synuclein-overexpressing mice. Panels **(A)** and **(C)** depict PCNA-positive cells in human α-synuclein mice. The number of PCNA-positive cells (DAB staining) in the SVZ **(B)** and dentate gyrus granule cell layer (**D;** DG GCL) was not statistically different in both groups. Scale bar: 50 μm.

human α-synuclein mice (Fig. 7A, B). Significant increases of apoptotic profiles were also found in the target regions of the olfactory bulb (Fig. 7C–F).

In order to determine at what phase during neural progenitor maturation cells are eliminated, we made use of a morphological distinction between ''early'' and ''mature'' DCX-positive cells. The first category (''early'') was defined as DCX-positive, which form clusters in the subgranular zone of the hippocampus with either no process or processes oriented parallel to the granule cell layer (Fig. 8C). These cells are found within the first days after BrdU labeling and co-label for proliferative markers such as Ki-67 (33). The second category (''mature'') are DCX-positive cells (present from about 7 days after BrdU labeling) that integrated into the granule cell layer and displayed dendritic processes spanning the entire granule cell layer and frequently reaching further into the molecular layer (Fig. 8A–C). According to this distinction we classified cell numbers and morphologies of DCX-positive cells in α-synuclein transgenic animals. Concordant with less neurogenesis, as measured by BrdU and NeuN, the cell counts of DCX-positive cells in the dentate gyrus granule cell layer of the hippocampus revealed a significant difference (i.e. 48% fewer DCX-positive cells in human α-synuclein transgenic animals, Fig. 8D). Interestingly, there was no difference in cell numbers of ''early'' DCX-positive neuronal progenitors detectable in both groups. However, ''mature'' DCX-positive cells with processes spanning the entire layer were significantly reduced by 64% in the human α-synuclein group (Fig. 8D).

DISCUSSION

This study demonstrates that the PDGF promoter directs the expression of human wild-type α-synuclein not only to mature neurons but also to progenitor cells in neurogenic regions of the adult brain. The number of dividing cells in the SVZ and granule cell layer of the hippocampus did not differ between transgenic mice and controls as measured by PCNA expressing profiles. However, there was a significant reduction of hippocampal

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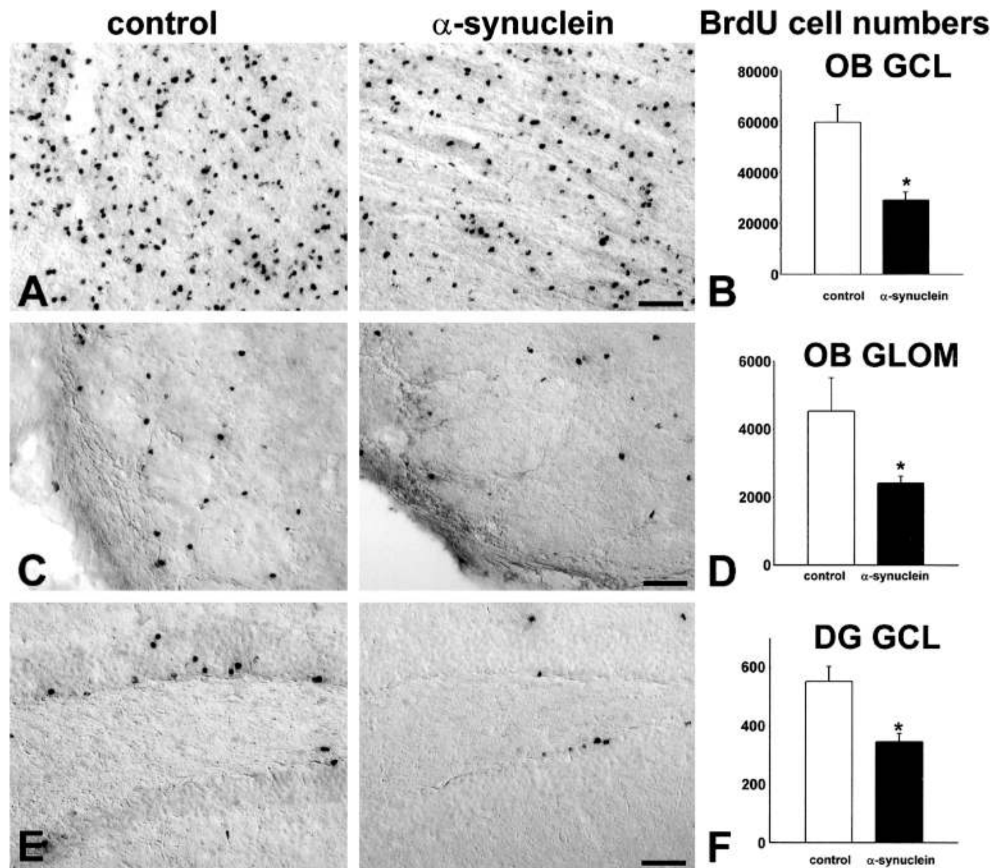

Fig. 5. Proliferation 1 month after BrdU injection is decreased in human α-synuclein-overexpressing mice. The number of BrdU-positive cells counted in a BrdU DAB staining is significantly lower in the human α-synuclein group in the olfactory bulb granule cell layer (**A, B;** OB GCL) and glomerular layer (**C, D;** OB GLOM), as well as the granule cell layer of the dentate gyrus of the hippocampus (**E, F;** DG GCL). Scale bars: 50 μm, *$p < 0.05$.

and SVZ/olfactory bulb neurogenesis in adult human α-synuclein animals due to an increase in cell death in the neurogenic regions of human α-synuclein transgenic animals. We determined that transgene expression caused a decreased survival rate of newborn cells in the target regions in the human α-synuclein mice at the stage of neuronal differentiation. This is supported by the reduction of ''mature'' newly generated neurons in the α-synuclein mice, whereas the number of ''early'' stage neuronal progenitors was not altered. This indicates that neuronal precursors are eliminated during the time they reach the target region and differentiate into interneurons. Moreover, fewer neurons are observed in the neurogenic regions. No obvious abnormalities in hippocampal or SVZ derived neurogenesis have been described during development either in α-synuclein knockouts or in α-synuclein-overexpressing animals (7, 35–38). Therefore, the finding of a decreased neurogenesis in adult human α-synuclein-overexpressing mice is quite surprising.

Human α-Synuclein under the PDGF Promoter

Interpretations of results using transgenic mice overexpressing human α-synuclein under the PDGF promoter require a detailed understanding of the transgene expression pattern in the mouse line used. This is especially true when considering the pathophysiological role of α-synuclein and in comparing endogenous with wild-type human α-synuclein expression. Endogenous rodent α-synuclein is present in several areas of the brain (i.e. olfactory bulb, neocortex, basal ganglia, hippocampus, and substantia nigra [10]). In the transgenic α-synuclein mice used in this study, the pattern of α-synuclein expression under the PDGF promoter mimics the pattern observed for endogenous α-synuclein (37). Previous data on the human wild-type-overexpressing mice under the PDGF promoter show prominent human α-synuclein-immunopositive intraneuronal inclusions both in the olfactory bulb and hippocampus starting during late adulthood, indicating age-dependent changes induced by overexpression of human wild-type α-synuclein. However, no fibrillar elements have been found in any of these regions (7). Interestingly, α-synuclein expression in the olfactory bulb has also been observed in early stages of patients with Parkinson disease (39). Whether the PDGF promoter itself may alter neurogenesis has to be determined by

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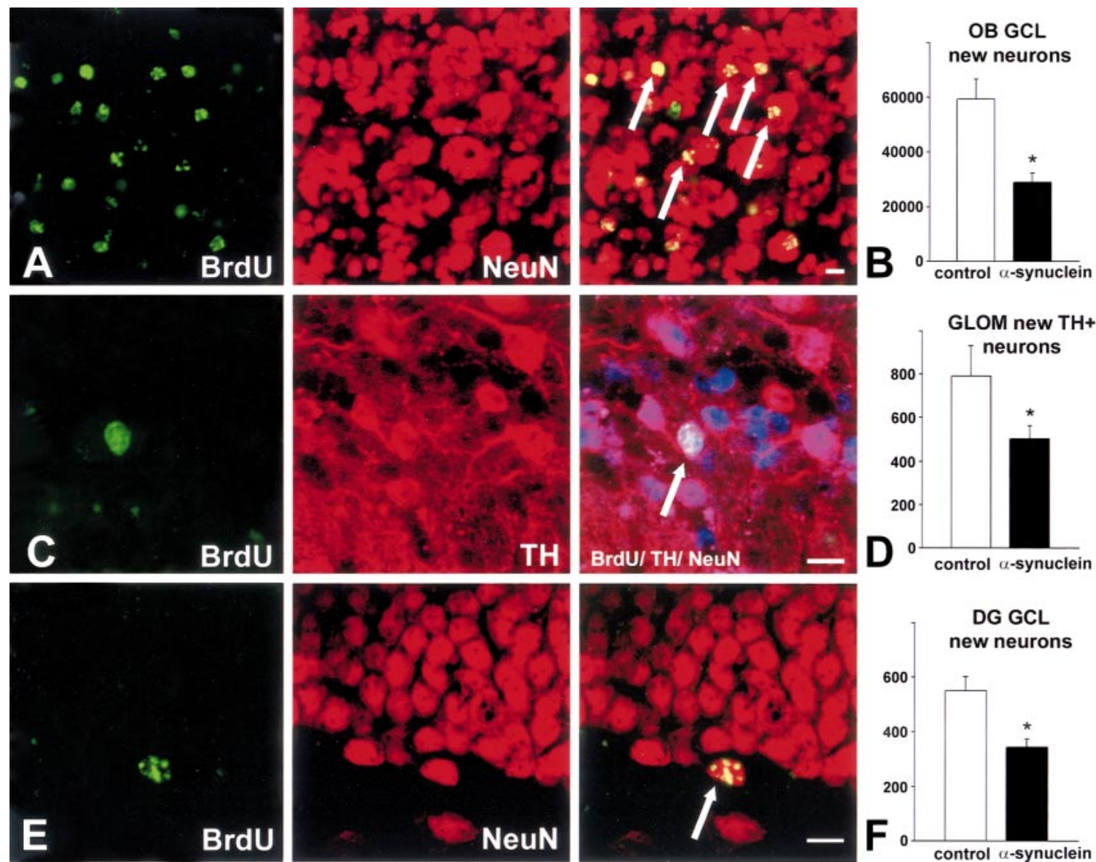

Fig. 6. Neurogenesis is decreased in α-synuclein-overexpressing mice. Neurogenesis is revealed by immunofluorescence labeling for BrdU (green) in combination with NeuN (red in **A, E,** blue in **C**), TH (red in **C**) for new neuronal cells (arrows in **A, E**)/new dopaminergic neurons (arrow in **C**). Double labeling was detected by confocal laser microscopy using a pinhole setting, which reflects an optical thickness of less than 2 μm. Scale bar: 10 μm. Quantification: The number of new neurons was determined by multiplying neuronal percentages with the absolute number of BrdU-positive cells. Note the statistical differences in neurogenesis in the olfactory bulb **(B, D)** as well as in the hippocampus **(F)**, $*p < 0.05$. (**A, B:** OB GCL; **C, D:** OB GLOM; **E, F:** DG GCL).

using transgenic mice overexpressing a protein not related to α-synuclein pathology.

Validity of Using BrdU for Labeling Dividing Cells

The generation of new neurons and apoptosis of newly generated cells occur simultaneously in neurogenic regions of the adult brain. Therefore, a frequent concern when using BrdU for the detection of dividing cells is whether BrdU incorporation may reflect mitotic events or DNA damage/repair. For instance, brain irradiation drastically increased the ratio of SVZ cells undergoing DNA repair and apoptosis (40, 41). However, a dose-dependent decline in proliferation and BrdU-positive cells was detected (42, 43). Moreover, other labeling techniques such as retroviral labeling and ultrastructural analysis led to similar results (22, 44–46). Using an extended time course analysis, studies have shown that BrdU-positive cells in neurogenic regions go through distinct phases: the BrdU-positive cells co-label on day 1 with cell cycle markers (27, 47–49), followed at day 2 to 10 by early markers for neuronal progenitor cells (27, 46, 50). Finally, after more than 10 days, the BrdU-positive cells begin to co-label with mature neuronal or glial markers (19).

The data of the present study showing a decreased number of DCX-positive cells in the dentate gyrus of human α-synuclein mice (Fig. 8) provide further evidence that the decrease of BrdU-positive cells is paralleled by fewer DCX-positive neuroblasts in the human α-synuclein mice. Taken together, previous data and the present study strongly argue that BrdU labeling is a robust and reliable method for detecting neurogenesis in the adult brain.

Neurogenesis and Apoptosis in Neurogenic Regions of Human Wild-Type α-Synuclein Animals

The balance of proliferation and cell death was shifted towards increased apoptosis in human α-synuclein ani-

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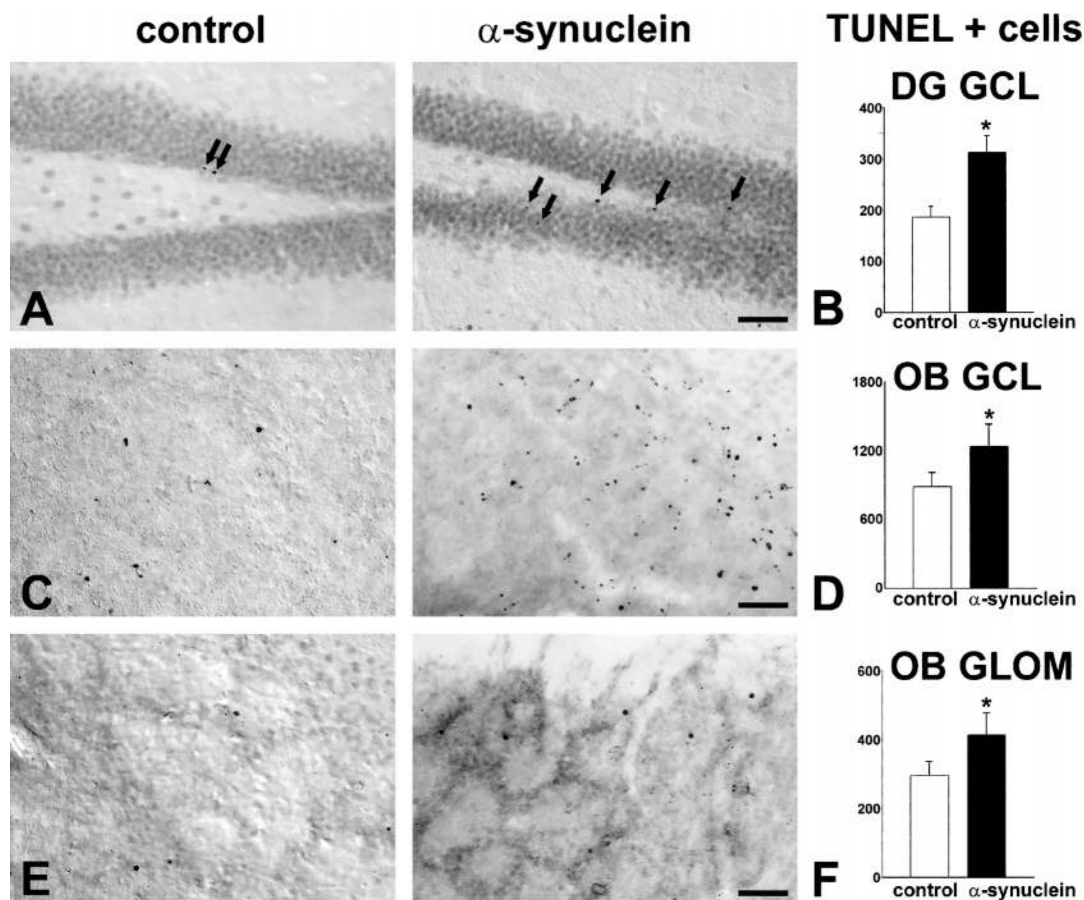

Fig. 7. Cell death is increased in α-synuclein mice. TUNEL-positive cells are depicted (DAB staining). There is a more than 50% increase of dUTP-nick end labeling (TUNEL)-positive cells in the granule cell layer of the hippocampal dentate gyrus (**A, B:** DG GCL) as well as a significant difference in TUNEL cell numbers in the granule cell layer (**C, D:** OB GCL) and glomerular layer (**E, F:** OB GLOM) of the olfactory bulb. Scale bar: 50 μm, $*p < 0.05$.

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mals, resulting in decreased neurogenesis. In rodent models we have previously shown that elimination of new cells via cell death is a very important regulatory mechanism during adult neurogenesis, and that apoptotic cells occur at up to 100-fold higher frequency in neurogenic regions of the adult brain (19, 23, 24). Programmed cell death is a prominent feature of both embryonic and adult neurogenesis: a large proportion of neural progenitors and young neurons are eliminated by programmed cell death unless cells receive synaptic input or trophic support during development (25). Similar cellular dynamics exist in areas of adult neurogenesis (19, 23). Immature neurons might be eliminated by apoptotic cell death; their survival depends on the level of activity and trophic support they receive. However, once adult newborn cells survive an early critical period they have escaped endogenous cell death mechanisms (19, 24, 51).

Whether a cell-autonomous effect of human α-synuclein is directly involved in a decreased neurogenesis in human α-synuclein mice cannot be determined by the present study, but our data of transgene expression in SVZ precursors and an increase in cell death in newly generated neurons suggest that an accumulation of α-synuclein might be involved in an increase of cell death and consecutively the impairment of neurogenesis.

It is also important to note that the number of TH-positive newly generated neurons in the olfactory bulb glomerular layer is reduced. This area is strongly immunopositive for endogenous α-synuclein in adult rats (10). Currently, dopaminergic neurogenesis in the substantia nigra of acutely lesioned animals is under debate (52, 53); but if so, it is assumed to occur in very low numbers (52). Our study focused on the large pool of TH-positive neurons, which are continually generated in the glomerular layer of the olfactory bulb (19). We therefore propose that overexpression of α-synuclein can affect dopaminergic neurogenesis; however, whether the substantia nigra is also affected by this mechanisms needs further study.

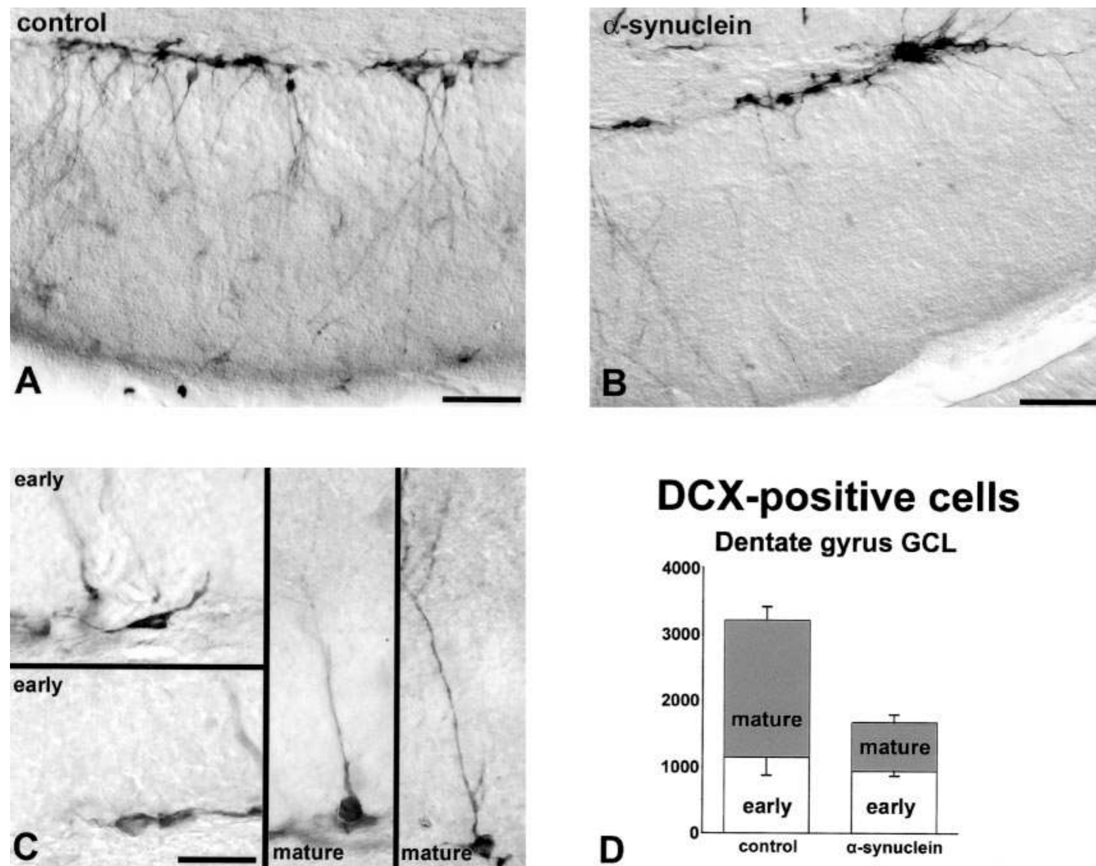

Fig. 8. Characterization of DCX-positive neuroblasts. Morphologies of DCX expressing cells were further characterized for control littermates **(A)** and human α-synuclein mice **(B)**. Two different cellular morphologies were distinguished: The first class were ''early'' DCX-positive cells forming clusters in the subgranular zone of the hippocampus with either no process or processes oriented parallel to the granule cell layer (**C;** left half). The second category consists of ''mature'' DCX-positive cells that integrated into the granule cell layer and displayed processes spanning the entire layer and reached further into the molecular layer (**C;** right half). According to this DCX subdivision we classified the DCX-positive cells in human α-synuclein transgenic animals and control littermates. Cell numbers of ''early'' DCX neuroblasts did not differ between the groups (**D**). A significant reduction to 36% of ''mature'' DCX-positive cells with processes spanning the entire layer was found in the human α-synuclein group (**D**). Scale bars: A, B = 50 μm; C = 25 μm. *$p < 0.05$.

Determination of Time Point of Decrease in Neurogenesis: Survival of Integrating Neuroblasts

The reduction of neuroblasts, measured by determining morphological characteristics of DCX-expressing cells further defined the time point of decreased survival of newly generated cells. The total number of DCX-positive cells decreased in the α-synuclein mice. By performing a subclass analysis we observed that cell numbers of ''early'' stage DCX neuronal progenitors were unchanged in α-synuclein mice compared to controls.

Human Wild-Type α-Synuclein Expression in Neurogenic Regions Is Reminiscent of Embryonic Development

The immunoreactivity of adult neuroblasts for human α-synuclein in the adult neurogenic regions as well as the distribution of endogenous α-synuclein in neuronal precursor cells during embryonic CNS development resembles the spatio-temporal distribution and colocalization with genes involved in neuronal migration, for example DCX (31, 32). In the cell bodies of E15 to E18 mice, neuronal precursors are prominently immunoreactive for α-synuclein in growth plate areas of the cortex as determined by localization of the non-Aβ component of Alzheimer disease amyloid precursor protein, which is identical to human α-synuclein (8). This indicates that α-synuclein plays a dual role during CNS development: it may be involved during neuronal development before synaptogenesis in the embryo, and at later stages of embryonic development α-synuclein is intracellularly redistributed to the nerve terminals to play an important role in synaptic plasticity (8, 9). It might be speculated from our data that overexpressed human α-synuclein, like the

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microtubule-associated protein DCX (31, 32), is involved in the integration of neuroblasts in the adult system.

Neurogenesis and Olfaction in Parkinson Disease

Interestingly, neuropathological examinations of Parkinson disease brains revealed α-synuclein-immunopositive Lewy neurites and Lewy bodies in the olfactory bulb at very early stages of the disease (39). In accordance with these neuropathological findings, a decreased sense of smell was found in asymptomatic patients who subsequently developed clinical parkinsonism (54).

α-Synuclein-immunoreactive inclusions lead to the formation of Lewy bodies and cell death of the affected cells (55). Lewy bodies have also been demonstrated within the olfactory bulb and the hippocampus, which might be correlated with the increase of cell death in the α-synuclein transgenic mice (56). Acute lesion models of Parkinson disease are known to induce astrogenesis; however, the rate of neurogenesis in the basal ganglia and substantia nigra is controversial and reported to be very low (52), or nonexistent in the striatum (57) and the substantia nigra (53).

Experimentally, odor deprivation leads to reduced neurogenesis in the SVZ/rostral migratory stream/olfactory bulb system (58, 59), whereas odor enrichment increases neurogenesis in these regions (60). Also anosmic mice have less olfactory bulb neurogenesis (24). In the olfactory bulb, functional integration of newly generated neurons into adult neuronal circuits is finally achieved in a unique sequence of events (20).

We present experimental data of a model for synucleinopathies, which suggest that not only an increase in apoptosis but also a decrease in neurogenesis in the SVZ/olfactory bulb system may contribute to the impairment of olfactory function at an early stage of neurodegenerative diseases.

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