Early locus coeruleus degeneration and olfactory dysfunctions in Tg2576 mice

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Abstract

Olfactory deficiency has been reported in the early stages of Alzheimer’s disease (AD) in humans but is very poorly understood due to the lack of investigations in animal models of AD. Recent studies point to the noradrenergic system as an important target of the AD pathological process. In addition, noradrenalin has been shown to influence adult neurogenesis which is implicated in cognitive functions. We have therefore investigated the olfactory neurogenesis and cognitive performances in young transgenic Tg2576 mice in relation with the status of the noradrenergic and the cholinergic systems. Tg2576 showed a deficit in neurogenesis in the olfactory bulb evidenced by an increased death of newborn cells and a reduced expression of PSA-NCAM. The locus coeruleus degenerated in Tg2576 between the age of 6.5 and 8 months. These changes were associated with olfactory memory impairments. Our findings indicate that a noradrenergic deficiency could play a role in the early stages of the pathological process in this transgenic model and induce olfactory cognitive impairments through an alteration of olfactory neurogenesis.

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1. Introduction

The locus coeruleus (LC) is the main source of cortical noradrenaline (NA), and the LC/NA system has been shown to be functionally involved in selective attention, arousal, learning and more generally in the adaptive behavioral responses (Berridge and Waterhouse, 2003; Bouret and Sara, 2005). In rodents, about 40% of LC/NA neurons project towards the olfactory bulb (OB) (McLean et al., 1989; Shipley et al., 1985) where NA is involved in different types of olfactory learning (Brennan et al., 1998; Levy et al., 1990; Sullivan et al., 1992). In the OB, α2-adrenoreceptor antagonists which stimulate NA release, promote survival of newly generated granular neurons (Bauer et al., 2003; Veyrac et al., 2005) indicating that LC/NA afferents could regulate the permanent neurogenesis that characterizes this structure. Indeed, proliferating cells in the subventricular zone (SVZ) of the anterior forebrain migrate among the rostral migratory stream (RMS) and reach the OB where they differentiate into neurons (Kaplan et al., 1985; Lledo et al., 2006; Ming and Song, 2005).

Neurodegeneration of the LC has been recognized in brains of patients deceased from Alzheimer’s (AD) disease (German et al., 1992; Marien et al., 2004; Szot et al., 2006). A dysfunction of the noradrenergic system has thus been proposed to be involved in the progression of dementia in AD (Colpaert, 1994; Marien et al., 2004). This view has recently been supported by the demonstration that a lesion of the noradrenergic system in a transgenic model of AD, the APP 23 mice, increased simultaneously β-amyloid deposition and cognitive deficits (Heneka et al., 2002, 2006; Kalinin et al., 2006). Finally, in AD, important functional olfactory deficits have been described (Hawkes, 2003; Mesholam et al., 1998;
Murphy, 1999) that appear very early in this human pathological process (Christen-Zaech et al., 2003; Peters et al., 2003).

Because neurogenesis has recently been shown to be involved in olfactory discrimination and memory performances (Alonso et al., 2006; Gheusi et al., 2000; Mandairon et al., 2006; Rochefort et al., 2002), functional olfactory deficits associated with the earliest symptoms of AD might be related to an alteration of neurogenesis induced by a dysfunction of the LC/NA system. In order to assess this hypothesis, we investigated the possible disruptions of the noradrenergic system, the rate of bulbar neurogenesis, and the olfactory memory performances in young Tg2576 mice, a model of AD. Tg2576 mice express the Swedish mutation of the human Amyloid Precursor Protein (Hsiao et al., 1996). This well-characterized transgenic strain develops an age-dependent elevation of Amyloid-beta protein (Aβ) brain levels (soluble Aβ1-40 and Aβ1-42) with an onset at 8 months of age and with Aβ-containing neuritic plaques occurring in the neocortex and hippocampus by 10–16 months (Hsiao et al., 1996; Jacobsen et al., 2006; Kawarabayashi et al., 2001). Transgenic mice show impairments in various spatial and fear conditioning memory tests starting at the age of 9–10 months (Barnes and Good, 2005; Corcoran et al., 2002; Dong et al., 2005; Hale and Good, 2005; Hsiao et al., 1996; King and Arendash, 2002; Ognibene et al., 2005; Savonenko et al., 2005; Westerman et al., 2002). Despite the growing interest in olfactory alterations as a potential help for early diagnosis of AD (Christen-Zaech et al., 2003; Peters et al., 2003), the olfactory status of animal models of AD has not been yet investigated.

In this study, olfactory neurogenesis and cognitive performances were assessed in 6.5–8 month old animals as well as the status of the cholinergic and noradrenergic systems. Results indicated a reduction in the volume of the LC in Tg2576 compared to control mice which occurred between 6.5 and 8 months. This reduction in the volume of the LC was associated with a deficit in the number of newborn neurons in the granular cell layer (GrL) of the OB, a lower expression of PSA-NCAM, and subtle olfactory memory deficits. In the horizontal diagonal band (HDB), a transient increase in choline acetyl transferase (ChAT) expression was measured at 6.5 months that returned to control levels at 8 months.

Our data are consistent with the hypothesis that an early disruption of the LC noradrenergic system could lead to a deficit in bulbar neurogenesis and to early alterations of the OB homeostasis and olfactory memory.

2. Materials and methods

2.1. Animals and housing

Twenty-nine Tg2576 (Tg) and twenty-two wild type (WT) male mice were used in this study. They were purchased at the age of 2.5–3 months (Taconic, Germantown, NY, USA) and were housed single at a temperature of 20–24 °C and under a constant light–dark cycle (12:12 h) in the laboratory animal facility. Mice had free access to food and water except during the associative learning period (see below). Upon arrival, each animal was assigned a code number and the experimenter was blind to the genotype during all the experimentations. All efforts were made to minimize both the number of animals used and their suffering during the experimental procedure in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC), and the French Ethical Committee.

2.2. Behavioral testing

Behavioral experiments began when mice were 5.5–6 month old. All behavioral experiments were conducted in the afternoon (13:00–18:00).

2.2.1. Experimental set-up

All mice (Tg and WT) were tested in a computer-assisted 2-hole board apparatus (40 cm × 40 cm) administered by specific software. The trial started by placing the mouse on the board facing the holes, and the sequence and duration of nose poking into the holes (3 cm diameter, 4.5 cm deep) were measured. The holes contained a polypropylene swab embedded in fine plastic mesh and covered by fresh bedding. The polypropylene swab was impregnated with mineral oil or, when needed, it was odorized with the odorant Proprionic acid (PA, 20 μl of the odorant diluted 1:100 in mineral oil). The odorant position was randomized between the two holes for each mouse. Between each trial, the mice were put back in their home cage.

2.2.2. Odorant detection

To ensure that mice could detect PA, they were submitted to a preliminary detection test session. The session consisted of 4 successive trials (2-min duration, 15 min inter-trial interval (ITI)). During the first 3 trials, no odor was presented; the holes contained only mineral oil. For the fourth trial, one of the two holes was odorized with PA. Time spent exploring each of the holes was measured. Detection was recognized as a significant change in the exploration time of the odorized hole when compared to the mean exploration time of the no-odor trials (Student t-tests).

2.2.3. Habituation

Each training session consisted of 3 successive trials of 2-min duration with an ITI of 5 or 15 min. During each trial, one of the two holes was odorized with PA and the time spent exploring each of the two holes was analyzed. Inter-trial comparisons were made by Wilcoxon signed rank tests on the exploration times. Data are represented as percentage of the exploration time during the first trial.

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2.2.4. Olfactory associative learning

Five days before the onset of training, mice were food-deprived and received 80% of the normal daily ration. Their weight decreased by 8–10%. Each training session consisted of 4 successive trials (ITI 5 min) and sessions were organized according to a two-step paradigm (Mandairon et al., 2006).

- Shaping: Mice were first trained to retrieve a reward (small bit of sweetened cereal, Kelloggs, Battle Creek, MI) by digging through the bedding. Each mouse was put in the start area and was allowed to dig for 2 min. During the first few trials, the reward was placed on the top of the bedding of one of the holes. After several successful retrievals, the reward was buried deeper into the bedding. Shaping was considered to be complete when a mouse could successfully retrieve a reward that was deeply buried into the bedding (8–12 trials).

- Conditioning: It consisted of 8 sessions (one per day). During the first 4 sessions, the hole odorized with PA was reinforced with the food reward hidden in the bedding. From days 5 to 9 of training, the rule was reversed and the reward was placed in the non-odorized hole. The trial was recorded as successful when the first visit (nose poke) was in the reinforced hole (hole containing the reward). Once the mice found the reward, they were allowed to eat it and then were returned to their home-cage until the next trial.

During shaping and conditioning, the position of the reinforced hole was randomized to prevent any spatial learning.

The percentage of successful trials was calculated each day and the data were analyzed by ANOVA for repeated measures followed by Bonferroni post hoc tests.

2.3. 5-Bromo-2′-deoxyuridine (BrdU) protocol

The thymidine analogue BrdU (Sigma) was used as a marker of neurogenesis. It was administered to 10 WT and 10 Tg mice 1 week after the end of behavioral testing. At this time, mice were 6.5–7 months and received 3 intraperitoneal injections of BrdU at the dose of 50 mg/kg, 2 h apart. Five Tg2576 and 5 WT mice were killed the same day, 2 h after the last BrdU injection to assess proliferation of progenitors in SVZ. The remaining animals were killed 45 days later (at the age of 8–8.5 months) to assess long term survival of the newborn cells in the OB.

2.4. Tissue preparation and sectioning

At the end of the experimentations, mice were deeply anesthetized (Pentobarbital, 5 mg/kg) and perfused transcardially with 50 ml of fixative (4% paraformaldehyde in phosphate buffer, pH 7.4). Brains and nasal cavities were removed, kept in paraformaldehyde for one night and then cryoprotected by a 5-day immersion in a 20% sucrose solution in phosphate buffer. Brains were divided in two parts at the level of the colliculi before being snap frozen in isopentane, cooled with liquid nitrogen (−45 °C) and stored at −80 °C. Serial frontal sections (thickness 14 μm) of the anterior part of the brain (from the OB to the SVZ) and of the brainstem (Fig. 1) were cut at −20 °C on a cryostat (Jung). The sections were mounted onto glass slides coated with poly-l-lysine (0.05%; Sigma–Aldrich, Saint Quentin Fallavier, France) and stored at −20 °C. Adjacent sections were collected on 5 series of slides in order to be able to use different stainings on homologous sections covering the antero-posterior forebrain or brainstem sections.

One series was Nissl-stained with cresyl-violet to allow morphometric measurements and the proper visualization of anatomical landmarks.

Nasal cavities were similarly frozen and serially cut at 14 μm. Sections were stained by cresyl-violet and mounted with DPX (Fluka, Sigma).

2.5. Immunohistochemistry (IHC)

Neurogenesis was assessed by BrdU and PSA-NCAM IHC performed on OB and SVZ-containing sections. In these regions, we also revealed cell death by the expression of the activated (clived) form of caspase-3, an executive caspase of the apoptotic process (Krantic et al., 2005) previously used to detect cell death in the OB (Giachino et al., 2005; Yamaguchi and Mori, 2005). Cholinergic neurons in the HDB were labelled by Choline-acetyl-transferase (ChAT) IHC. Finally, Dopamine-β-hydroxylase (DBH) IHC on sections of the brainstem was used to visualize noradrenergic LC neurons (Fig. 1).
2.5.1. BrdU staining

As previously described (Mandairon et al., 2003, 2006), brains sections were first incubated in Target Retrieval Solution (Dako, Trappes, France) for 20 min at 98 °C. After cooling for 20 min, they were treated with Triton 0.5% (Sigma X100) in Phosphate buffered saline (PBS) for 30 min then for 3 min with pepsin (0.43 U/ml in 0.1 N HCl, Sigma). Endogenous peroxidases were blocked with a solution of 3% H2O2 in 0.1 M PBS. Then, sections were incubated for 90 min in 5% normal horse serum (Vector Laboratories, Burlingame, CA, USA) in 5% bovine serum albumine (BSA, Sigma) and 0.125% Triton X-100 to block non-specific binding, and then incubated overnight at 4 °C in a mouse anti-BrdU primary antibody (1/100, Chemicon, Temecula, CA). Sections were then incubated in a horse biotinylated anti-mouse secondary antibody (1/200, Vector) for 2 h. Sections were then processed with avidin-biotin-peroxydase complex (ABC Elite Kit, Vector) for 30 min and followed by three rinses of 5 min in PBS. Finally, sections were reacted in 0.05% 3,3′-diaminobenzidine-tetra-hydrochloride (DAB, Sigma), 0.03% NiCl2 and 0.03% H2O2 in Tris–HCl buffer (0.05 M, pH 7.6), dehydrated in graded ethanol, and covered-slipped in DPX.

2.5.2. ChAT and DBH staining

Brains sections were incubated in Target Retrieval Solution for 20 min at 98 °C, and allowed to cool down for 20 min. Sections were incubated for 90 min in 5% normal serum in 5% BSA and 0.125% Triton X-100 to block non-specific binding, and incubated overnight at 4 °C in goat anti-ChAT primary antibody (Chemicon, 1/100 dilution) or in rabbit anti-DBH primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1/200 dilution) for a minimum of 40 h at 4 °C. Sections were then incubated in an appropriate biotinylated secondary antibody (1/200 dilution) for 2 h. The remaining treatments were similar to those used for BrdU labelling.

2.5.3. Caspase-3 and β-amyloid staining

Brain sections were hydrated 10 min in PBS and then incubated 20 min in 3% H2O2. Sections were then processed for 60 min in 2–5% normal goat serum in 2% BSA and 0.5% Triton X-100 to block non-specific binding. They were then and incubated in rabbit anti-Caspase-3 primary antibody (1/500 dilution, Cell signaling, Ozyme, Saint Quentin Yvelines, France) for 48 h at 4 °C (Giachino et al., 2005) or in rabbit anti-β amyloid primary antibody (Chemicon, 1/200 dilution). Sections were then incubated in a goat biotinylated anti-rabbit secondary antibody (1/200 dilution) for 90 min. The remaining treatments were similar to those used for BrdU labelling.

2.5.4. PSA-NCAM staining

Brain sections were incubated 30 min in 0.1% Triton in PBS. They were then processed for 60 min in 5% normal horse serum in 2% BSA and 0.1% Triton X-100 to block non-specific binding and incubated in mouse anti-PSA-NCAM primary antibody (1/200 dilution, Abcys, Paris France) overnight at 4 °C. Sections were then incubated in a horse anti-mouse secondary antibody FITC (1/200 dilution, Vector) for 2 h. After rinsing in phosphate buffer, the sections were mounted in Vectashield medium (Vector).

2.5.5. BrdU-NeuN double labellings

For double-labelling experiments, BrdU labelling was performed as described above except that the primary antibody used was made in rat (1/100, Oxford Biotechnology, Kidlington, UK) and a goat anti-rat antibody coupled to Texas red (Vector) was used in order to obtain fluorescent nuclei. After three rinses in PBS, non-specific antigenic sites were blocked for 1 h 30 min in 2% BSA (Sigma), 0.1% Triton X-100, 2% normal horse serum (Vector) and 2% normal goat serum (Vector). The sections were then incubated overnight at 4 °C with mouse monoclonal antibody against NeuN (1/500, Chemicon), washed in PBS and incubated for 2 h at room temperature with a horse biotinylated anti-mouse secondary antibody (Vector). They were then incubated for 90 min with Avidin DN FITC 1/100 in Carbonate buffer. After rinsing in phosphate buffer, the sections were mounted in Vectashield medium (Vector). The double-labelled cells (10–20 cells/animal, n = 5 Tg and n = 4 WT) were observed and analyzed by confocal scanning microscopy using a Leica microscope (Centre Commun de Quantiömétrie, Université Lyon 1).

2.6. Quantifications

Quantifications were conducted with the aid of a computer-assisted image analysis system (Mercator Pro, Explora Nova, La Rochelle, France) coupled to a Zeiss microscope.

2.6.1. Morphometric measurements of the OB

The different layers of the OB (Fig. 2a) were delineated on 6 Nissl-stained (cresil-violet) sections equally distributed along the antero-posterior axis of the OB with an interval of 280 μm. The volume of the different layers was approximated by the Cavalieri equation:

\[
\sum_{i=0}^{n} A_i d_i = V
\]

where \(A_i\) equals the area of the considered layer on the \(i\)th section, \(d_i\) is the distance between traced sections and \(n\) is the total number of measured sections (Howard and Reed, 1998). The volume of the Grl was used as the reference volume to estimate the number of labelled cells in the GrL (see below).

2.6.1.1. Measurements of epithelial thickness. For each animal, 6 sections of the olfactory epithelium distributed along the antero-posterior axis and stained with cresyl-violet were selected. For each of these sections, the thickness of...
the epithelium was measured along the septum (Mercator, Explora Nova). Mean thickness obtained in Tg and WT were compared by Student t-tests.

2.6.1.2. In situ labelling. BrdU-positive cells: Every BrdU-positive cell was counted in 2 sections of the granule cell layer (GrL) of the OB that were randomly selected in front of the accessory olfactory bulb. In this anterior part of the OB, the density of BrdU-positive cells is homogenous when measured using a 70 μm interval between sections, and adding more sections to the analysis would not affect the value of cell density (personal data). BrdU-positive cells were also counted in 3 sections of the SVZ that were spaced by 140 μm starting at the opening of the lateral ventricles.

Caspase-3 positive cells: These cells were counted in the GrL, RMS-OB and SVZ using a similar method as for BrdU cell counts except that in the OB, 8–10 sections were examined.

ChAT-positive cells: Sections were selected from the stereotaxic coordinates Bregma 0.86 to Bregma −0.34 (Franklin and Paxinos, 1997) (inter-section interval 140 μm). On these sections, ChAT positive cells were counted in the HDB delineated with the aid of the atlas.

DBH-positive cells: All sections (inter-section interval of 140 μm) on which DBH-positive cells in the LC could be visualized were analyzed. In addition, the sectional areas of the labelled profiles were recorded to assess the size of the LC neurons.

For the different labellings, the surface of the regions of interest in which labelled cells were counted was recorded on each section analyzed. Based on the number of labelled cells and area of the region of interest, the profile density (number of labelled profiles/μm²) was calculated. The volumes of the SVZ, HDB and LC were estimated by the Cavalieri method (Eq. (1)) and used to extrapolate the total number of labelled cells (T) estimated using the formula:

\[ T = \frac{N \times V}{t} \]

where \( V \) is the volume of the region of interest, \( N \) the profile density and \( t \) the thickness of the sections.

PSA-NCAM labelling: Microphotographs (3–5 microphotographs per animal and per region of interest, \( n = 5 \) Tg and 3 WT) were taken under controlled camera settings, from the SVZ, the RMS and the RMS-OB (the latter designates the most anterior section of the RMS located in the centre of the OB). The labelled area was delineated and the mean OD in the green wave lengths was measured within this area (MorphoExpert, Explora Nova, La Rochelle, France). OD values were averaged for the different regions within each group (Tg or WT aged 6.5 or 8 months).

2.6.1.3. Data analysis. Comparisons between Tg and WT and age differences within each genotype were done by Student t-tests.

3. Results

3.1. The volume of the GrL is reduced in the OB in Tg2576 in absence of β-amylloid accumulation

The volume of the GrL was reduced by \( \approx 22\% \) in 8 month-old Tg compared to WT mice (\( p < 0.03 \)) (Fig. 2b); whereas no difference could be observed at 6.5 months (not shown). This reduction was specific to this layer. Indeed, the volumes of the other 4 layers of the OB were similar in WT and Tg. The rate of cell death in the GrL was estimated using the IHC detection of the cleaved form of caspase-3 (Fig. 3A) since a stimulation of cell death could take part to the decrease in volume. The level of cell death was similar in young (6 months) WT and Tg. At 8 months, the number of apoptotic cells was higher in Tg than in WT but this difference remained marginally significant (\( p = 0.07 \)) (Fig. 3B). The same trend was found when apoptotic cells were counted in the RMS-OB (1.04 ± 0.5 in WT; 2.15 ± 1.3 in Tg, mean ± S.E.M. \( \times 10^{-6} \), t-test \( p = 0.06 \), not shown). When looking at individual levels of cell death in the GrL, in animals of the two age groups, we found that the level of cell death was significantly more variable in Tg than in WT mice (test F, \( p = 0.04 \)). This increased dispersion of the caspase-3-positive cell densities was mostly due to individuals with a rate of cell death higher than the mean (Fig. 3C).
Fig. 3. Apoptosis in the GrL. (A) An example of activated caspase-3 labelling in the GrL of the Ob. Bar = 20 μm. (B) Density in caspase-3 expressing cells in the GrL of Tg and WT aged 6.5 or 8 months. (C) Individual values of caspase-3-positive cell density for WT and Tg. The range of values is significantly larger for Tg than WT. (■) 6.5 months mice; (▲) 8 months mice.

Immunohistochemical detection of β-amyloid in Tg and WT mice at 8 months of age revealed the lack of labelling in the OB as in other adjacent cortical areas (not shown).

3.2. The rate of neurogenesis is reduced in Tg2576

The rate of newborn neuron survival in the OB was assessed by counting the BrdU-positive cells in the GrL 45 days following BrdU administration. The labelled cell density was higher in WT than in Tg (4.13 ± 0.3 versus 2.93 ± 0.5 × 10^{-5} cell/μm², respectively, mean ± S.E.M., p = 0.04, not shown). This decrease in labelled cell density in Tg mice, combined to the reduced volume of the GrL as described above, resulted in a significant decrease in the total number of BrdU positive cells in the GrL of Tg compared to WT mice (Fig. 4A).

Confocal analysis of BrdU-NeuN double labelling (Fig. 4B) indicated that the rate of neuronal differentiation of newly formed cells was not different between the two genotypes (Fig. 4C). To address the possibility that the reduced number of BrdU-positive cells retrieved in the GrL of the OB was due to a reduced proliferation of progenitors, BrdU-positive cells were counted in the SVZ in the group of animals killed 2 h after the last injection of BrdU. No difference was found between Tg and WT which suggests similar rates of proliferation (Fig. 4D). The density of cells expressing the activated caspase-3 in the SVZ was also similar in Tg and WT (1.08 ± 0.9 in WT; 0.63 ± 0.7 in Tg, ×10^{-6} cell/μm², mean ± S.E.M., p = 0.35, not shown). Finally, we looked at the expression of the adhesion molecule PSA-NCAM, a key factor of the migration process expressed by migrating neu-
roblasts, in the SVZ, RMS and RMS-OB (Alvarez-Buylla, 1997). Regardless of their age, Tg mice showed a reduced expression of PSA-NCAM compared to WT animals in the 3 regions investigated (Fig. 5 A and B).

3.3. LC, but not HDB, degenerates in Tg2576

At the age of 6.5 months, the volume of the LC was similar in WT and Tg mice. At 8 months, Tg mice showed a reduced volume of the LC compared to WT. An abrupt decrease in the volume of the LC thus occurred in Tg between the age of 6.5 and 8 months (Fig. 6A). Within the same time-window, Tg showed an increase in DBH-positive cell density (Fig. 6B) which partially compensated for the decrease in volume so that the total number of DBH-positive cells in the LC of Tg mice remained stable between 6.5 and 8 months. However, it did not prevent an overall decrease of the total number of DBH-positive cells in Tg compared to WT at 8 months (Fig. 6C). At 8 months, the size of the LC neurons was similar in WT and Tg mice (104 ± 10 and 98.75 ± 9.4 μm², mean ± S.E.M., respectively, not shown).

In contrast to the LC, the volume of the HDB was similar in WT and Tg mice (104 ± 10 and 98.75 ± 9.4 μm², mean ± S.E.M., respectively, not shown).

3.4. Normal organization of the olfactory epithelium and odorant detection in Tg2576

We verified that the olfactory epithelium of Tg2576 did not bear alterations which could induce a deficit in odor detection. Its thickness was measured as an index of olfactory neuron loss (Costanzo, 1984). No difference between Tg and WT was found (WT: 47.8 ± 3.34 μm; Tg: 57.4 ± 10.45, mean ± S.E.M., n = 4 WT and n = 4 Tg, p = 0.26). In addition, the gross cellular organization of the OE was preserved. Detection of the odorant also was assessed behaviorally (see Section 2). Both Tg and WT explored the odorant at levels above 50%, when presented in one of the 2 holes (p < 0.005 in both groups), indicating that Tg and WT easily detected the odorant used in the memory tests.

3.5. Short term memory impairments in Tg2576

The animals used for the histo-pathological analysis had been submitted beforehand to two tests of olfactory memory, a habituation task (implicit memory) and an associative learning task.

In the habituation test, mice faced two holes, only one being odorized with PA, for 3 trials (2 min duration, ITI 15 min). The exploration time of the odorized hole decreased with trials for WT but not for Tg (p = 0.007 and 0.65, respectively, for difference between habituation trials 1 and 3) suggesting that Tg forgot the odor between two trials. When the ITI was reduced to 5 min, Tg mice showed habituation (p = 0.035 between trials 1 and 3) (Fig. 7). To confirm that the decrease in exploration time, when present, was due to odor habituation and not to a non specific loss of interest of the animals towards exploration of the holes, it was verified that the time spent in the non odorized hole did not evolve with trials in any of the three experiments described above (p > 0.05). In addition, the time spent exploring the holes during the first trial was similar for the three conditions suggesting that there is no difference in the overall exploration capabilities or motivational state between Tg and WT mice.

From days 1 to 4 of the associative learning task, the mice had to associate PA with a food reward placed in the odorized hole. From days 5 to 9 of training, the association was reversed and the reward was systematically placed in the non-odorized hole. A global ANOVA analysis indicated that the performance evolved with session (F(8, 320) = 10.19, p < 0.0001) but did not reveal any effect of the group (F(1, 40) = 0.323, p = 0.573). The overall performances of Tg and WT should thus be viewed as very similar in this task (Fig. 8). However, the comparison of the two groups on days 4 and 5 indicated that the significant drop of the performance (F(1, 49) = 55.28, p < 0.0005) was influenced by the group (group effect, F(1, 49) = 4.56, p = 0.03). Indeed, while performances dropped below chance (50%) in WT (one sample t-test, p = 0.02), it remained at chance level in Tg (same test, p = 0.44).
Fig. 6. Noradrenergic and cholinergic neuromodulatory systems. The volumes of the LC (A) and the HDB (D) and the densities of DBH-positive cells in the LC (B) and ChAT-positive cells in the HDB (E) were measured. Stereological methods were used to calculate the total number of labelled cells in the LC (C) and HDB (F) of WT and Tg mice aged 6.5 or 8 months. G: Pearson correlation between DBH- and ChAT-positive cell densities in 8 months Tg mice. *p < 0.05; **p < 0.005; ***p < 0.0005.
Fig. 7. Olfactory habituation task. Time spent exploring the odorized hole during 3 successive trials, expressed as percentage of the first trial. The inter-trial interval was 15 min in WT (WT 15') and Tg (Tg 15') or 5 min in Tg (Tg 5'). *p < 0.05 for difference between habituation trials 1 and 3.

Fig. 8. Olfactory associative task. Percentage of correct responses across days of training for WT (A) and Tg (B). The arrow indicates the day of reversal. *p < 0.05 for difference from 50%.

To ensure that these effects were not due to genotype-dependent differences in the exploration capabilities of the animals, the time spent exploring the holes was measured on the first day of training and was found to be similar in Tg and WT as well as the number of nose pokes, despite a trend towards more exploration in Tg compared to WT (p = 0.07 and 0.08, respectively).

4. Discussion

The main result of the present study is the finding of a degeneration of the LC associated with a decrease in olfactory neurogenesis in young Tg2576 mice. This reduction of neurogenesis in the OB was not due to a deficit in proliferation of stem cells or progenitors because similar numbers of BrdU-positive cells were retrieved 2 h following BrdU injections in the SVZ of Tg and WT mice. The reduced number of newborn neurons in the OB of Tg2576 mice could thus be due to a compromised survival of these cells as indicated by the decrease of 45 day-old BrdU-labelled cells in the OB of Tg mice. However, the number of apoptotic cells in the GrL labelled for activated caspase-3 was only marginally raised up in Tg compared to WT while being significantly more variable. A larger number of animals could provide the power to achieve significant differences between WT and Tg. However, a possible explanation of this partial discrepancy may relate to the fact that these measures of cell death were obtained from relatively young animals in which the cell death may just begin to increase with an onset which may vary from one animal to the other. It should also be considered that the process of apoptosis is very fast so that methods revealing a specific phase of this process, like Caspase-3 activation, may capture only a small fraction of the cells engaged in the apoptotic process at the time of sacrifice and cannot label cells which died in the hours and days before sacrifice (Bursch et al., 1990).

In summary, although an increased number of apoptotic cells could not be firmly demonstrated in the GrL at the time of sacrifice, a reduced survival of newborn cells in the GrL of Tg mice most likely occurred as indicated by the decrease in 45 day-old BrdU-labelled newborn cells. Such a reduction of neurogenesis due to an increased death of newborn cells has been described in the Dentate Gyrus (DG), the second main neurogenetic area of the adult brain, of Tg2576 mice (Dong et al., 2005) or of other transgenic models of AD (Donovan et al., 2006; Haughey et al., 2002; Zhang et al., 2006).

Another possible mechanism leading to a reduced number of BrdU-positive neurons in the OB is related to the reduced labelling for PSA-NCAM in the SVZ and RMS. PSA-NCAM is expressed by neuroblasts (Alvarez-Buylla, 1997; Peretto et al., 1997) and is involved in the migration of neuroblasts along the RMS (Chazal et al., 2000). A decreased expression of PSA-NCAM could thus lead to a defect in their migration. Alternatively, despite the lack of detectable increase in cell death in the SVZ, it cannot probably be excluded, due the methodological limitations of cell death detection discussed above, that the reduced PSA-NCAM labelling in Tg reflected death of neuroblasts. A deficit in the expression of Doublecortin, another marker of neuroblasts, was also reported recently in the Dentate Gyrus (DG) (Zhang et al., 2006). The deficit in PSA-NCAM expression was already detected in 6.5 months old Tg and may thus be a very precocious sign of pathology in this model. Taken together, our data on neurogenesis suggest that similar mechanisms could play a role in the OB and GD and underlie the alterations of neurogenesis in AD-like mice.

In a second part of our study we investigated the possible alterations of the neuromodulatory cholinergic and noradrenergic systems. First, we found a reduction in the volume of the LC that occurred in Tg mice between the age of 6.5 and 8 months. This reduction was not due to shrinkage of LC neurons because their size was not affected in Tg compared
to WT mice at 8 months. It could thus arise from an actual cell loss or from a decrease in DBH expression and then in the staining of noradrenergic neurons. The latter hypothesis can be discarded because concomitantly to the reduction of the LC volume, we observed an increase in labelled cell density and a net decrease in the number of DBH-positive neurons in the LC of 8 month-old Tg mice. These concomitant observations suggest that DBH expression was enhanced in the surviving neurons and may represent a compensatory increase in DBH expression in response to cell loss. This finding is consistent with the increased TH expression recently described in the LC from AD patients (Szot et al., 2006). Our data are also in accordance with the previous demonstration of an increased binding to alpha-1 noradrenergic receptors in forebrain regions of old Tg2576 mice (Klingner et al., 2003). These receptors are primarily post-synaptic and their enhanced expression could thus represent a long-term compensatory response of LC targets due to reduced noradrenergic inputs.

Interestingly, in Tg2576 mice in which the time course of β-Amyloid accumulation have been fully characterized (Jacobsen et al., 2006; Kawarabayashi et al., 2001), β-Amyloid levels become elevated between the ages of 6.5 and 8 months simultaneously with the decrease in LC volume observed in our study, suggesting a possible role of β-Amyloid in LC degeneration. In addition, β-Amyloid have been reported to impair neurogenesis in the DG (Haughey et al., 2002). A toxic action of β-Amyloid as a common origin of the reduction of newborn neurons survival and of cell loss in the LC may thus be considered as a relevant hypothesis. However, the different APP mutant strains used as models of AD and which accumulate β-Amyloid in their brains, seem to differ significantly with regards to the status of the LC; with variations from neuronal shrinkage in PDAPP (German et al., 2005) to no alteration in old APP23 mice showing extensive β-Amyloid accumulation (Heneka et al., 2006). These differences suggest that the degeneration of the LC may not depend exclusively on the β-Amyloid load. In line with this, other deficits such as behavioral and synaptic alterations have been described in young transgenic animals preceding significant increase in extracellular β-Amyloid levels and plaque deposition (Jacobsen et al., 2006; Lanz et al., 2003; Wu et al., 2004). Regarding the origin of the reduced neurogenesis, not only β-Amyloid but also the noradrenergic deficit may contribute to the reduction of newborn cell survival. This hypothesis is supported by our previous studies showing the neuroprotective action of drugs that enhanced NA release on granule cells in the OB, including newborn cells (Bauer et al., 2003; Veyrac et al., 2005). Experimentally induced NA deficits have already been shown to increase neuroinflammation, β-Amyloid load, and to aggravate cognitive impairments in an APP mutant mice with no spontaneous LC degeneration (Heneka et al., 2002, 2003, 2006; Kalinin et al., 2006). Our data further suggest that an impairment of neurogenesis could be another consequence of NA deficit which may contribute to AD progression.

Interactions between the noradrenergic and cholinergic systems are suggested by the correlation reported here between DBH- and ChAT-positive cell densities in 8 month-old transgenic animals. The cellular basis for such interactions is exemplified by the presence of cholinergic receptors on noradrenergic cortical terminals (Marien et al., 2004). Consistent with this localization and the loss of NA neurons that we report here, a reduced binding of H Hemicholinium to cortical cholinergic receptors has been described in young Tg2576 (Apelt et al., 2002). In accordance with a previous study (Apelt et al., 2002), we found no difference in ChAT-positive cell density at 8 months between Tg and WT. However, we measured in Tg mice at the age of 6.5 months a transient increase in ChAT-positive cell density which may reflect an increase in ChAT expression. Similarly, an increase in ChAT activity has been described in the early stages of AD in humans and proposed as a mechanism of synaptic scaling, compensating for cholinergic neuronal loss (Frolich, 2002). In Tg2576 mice, no cell loss is observed and increased ChAT expression may be related to the noradrenergic deficit. Indeed, cholinergic terminals express alpha-2 adrenergic receptors which are inhibitory on Ach release. A reduced NA input may thus increase Ach release by putting more demand on Ach synthesis and ChAT, accounting for its increased expression. This interpretation would also be consistent with the reported increase in the expression of the vesicular transporter for Ach in young Tg2576 (Klingner et al., 2003). The return to control level of the number of ChAT-positive cells by the age of 8 months could be due to the increase in β-Amyloid as the pathology develops. Indeed, a deleterious effect of β-Amyloid on cholinergic transmission has been extensively described (Kar et al., 2004).

Finally, we were able to characterize some olfactory memory deficits in Tg2576 mice. The main deficit was found in the habituation task when the ITI was set at 15 min. Since habituation was obtained in Tg mice when the ITI was reduced to 5 min, the alteration observed when the ITI was 15 min is most likely related to an impairment of short-term memory rather than of the habituation process itself (alteration of novelty recognition) in line with a study of LC-lesioned animals (DSP4 treated rats) (Guan et al., 1993). In the associative task, differences between Tg and WT appeared on the day of reversal of the task; the WT mice explored the previously reinforced hole more than the Tg mice. This difference may reveal a stronger conditioning in WT. Finally, the control of the peripheral olfactory epithelium, which showed no alteration in Tg mice along with the lack of deficit in odor detection, indicates that the altered performances of Tg mice cannot be assigned to a peripheral degenerative process. These performances most likely reflect deficits in short-term implicit memory and in associative memory to a lesser extent. Because olfactory signs are very precocious in AD, we have conducted our study in young mice before any senile plaques can be detected in the OB. This strategy allowed us to identify a turning point in the progression of the pathological process.
in Tg2576 mice in the early stages of ageing, between the ages of 6.5 and 8 months, marked by noradrenergic degeneration and concomitant alteration of bulbar neurogenesis and occurrence of memory deficits.

5. Conflict of interest

The authors declare no actual or potential conflicts of interests.

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