

Cell proliferation and total granule cell number in dentate gyrus of transgenic Tg2576 mouse

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This report provides *in vivo* evidence of adult neurogenesis and the total granule cell count in the dentate gyrus of the Tg2576 mouse model of Alzheimer's disease. Mice were deeply anaesthetised and perfused with 4% buffered paraformaldehyde. Brains were removed and post-fixed in the same fixative overnight. Following equilibration in 30% sucrose, 30 µm sections were cut in sagittal plane in freezing microtome for immunohistochemistry and 20 µm from plastic embedded brains. Thioflavin-S confirmed the presence of amyloid plaques in the Tg2576 mice. Cell proliferation in the subventricular zone and dentate gyrus of hippocampus were observed with Ki-67 and doublecortin markers. Using optical fractionator, total granule number was estimated to be 445,280 per hemisphere in the 18-month-old Tg2576 mouse. Cell proliferation tends to end in the dentate gyrus but continues in the SVZ and the total granule cell number was less compared to normal laboratory and wild rodents.

Key words: Alzheimer's disease, dentate gyrus, granule cell number, neurogenesis, subventricular zone, Tg2576 mouse

The core factors driving neurogenesis in Alzheimer's disease (AD) have not been fully elucidated. The factors that control neurogenesis, hence regenerative potential in the adult central nervous system, are complex, and it is likely that the cholinergic system plays a key role since acetylcholine acts as a growth-regulatory signal in the brain. It promotes the proliferation of neural stem cells (Ma et al. 2000) and rescues synaptic plasticity deficits in nerve growth factor knockout models (Pesavento et al. 2002). In addition, experience-dependent plasticity in the human cortex is modulated by acetylcholine (Thiel et al. 2002). Cooper-Kuhn and coworkers (2004) showed that immunotoxic lesions to the cholinergic basal forebrain neurons led to decreased neurogenesis in the dentate gyrus and in the olfactory bulb of adult rats, and to increased apoptosis specifically in the subventricular zone (SVZ) of the lateral ventricle, the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus, and within

the periglomerular layer of the olfactory bulb, thus enhancing the concept of a correlation between neurogenesis and the cholinergic system. Based on transgenic animal studies and cell culture work, β-amyloid may also play a role in regulating neurogenesis, although the results of experimental studies are inconsistent, with different studies reporting that amyloid protein either reduces (Haughey et al. 2002) or induces (Lopez-Toledano and Shelanski 2004) neurogenesis in adult transgenic animals.

Several mouse models of AD have been found to have decreased hippocampal neurogenesis (Feng et al. 2001 in presenilin-1 knockout mice; Haughey et al. 2002 in presenilin-1; Wen et al. 2002a, b in FAD mutant presenilin-1; Dong et al. 2004 in APP^{sw} following isolation stress; Wang et al. 2004 in presenilin-1; Donovan et al. 2006 in PDAPP). These mice all have distinct genetic mutations that underlie their AD pathology (German and Eisch 2004), yet the similarity of these findings suggests that normalization of decreased hippocampal neurogenesis might be a therapeutic goal for AD treatments. Surprisingly, neurogenesis in post-mortem AD brain (Jin et al. 2004b) and in

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one AD mouse model (APPSw, Ind; Jin et al. 2004a) was reportedly increased, not decreased. Although these latter studies were qualitative in nature, they raised doubt about the actual relationship between AD and hippocampal neurogenesis (Donovan et al. 2006).

The Tg2576 presents an age dependent onset in AD pathology such as plaque deposition (Apelt and Schliebs 2001) which could be from moderate to high cortical beta-amyloid plaque load at about 17 months (Klinger et al. 2003). Demars and coauthors (2010) suggested that impaired neurogenesis is an early critical event in the course of Alzheimer's disease that may underlie memory impairments, at least in part, and exacerbate neuronal vulnerability in the hippocampal formation and olfaction circuits.

We therefore set out to investigate and establish the existence of adult neurogenesis especially in the SVZ-Rostral Migratory Stream (RMS) and the dentate gyrus of the hippocampus in the aged Tg2576 mouse. The strategy for identification of proliferating cells was detecting the endogenous protein Ki-67 expressed in mitotically dividing cells and doublecortin for immature neurons. The total granule cell number in the dentate gyrus was also established.

Transgenic mice Tg2576 (n=2) and wild-type littermate control (n=2) aged 16-18 months were used in this study. The transgenic mice contained the human APP695 with the double mutation (K670N, M671L), which was found in a large Swedish family with early onset of Alzheimer's disease, inserted into a hamster prion protein (PrP) cosmid vector in which the PrP open reading frame was replaced by that for the variant APP (Tg(HuAPP695.K670N-M671L)2576), as developed and described previously by Hsiao and coworkers in 1995 and 1996. The transgene is expressed in C57B6/SJL F1 mice (kindly provided by Dr. Karen Hsiao, University of Minnesota), backcrossed to C57B6 breeders. N2 generation mice were studied at ages of 16 months. The transgenicity was determined in 2-month-old animals in tail biopsy material by PCR (Hsiao et al. 1995, 1996), and further cross-checked after sacrifice. Both transgenic and wild type were housed in plastic cages and maintained under standard conditions with a 12-h light/12-h dark cycle and free access to food and water. The animal treatment was according to the procedure approved by the University of Leipzig.

Mice were deeply anaesthetised (5 mg per kg body weight ketamine/50 mg per kg body weight xylazine) and transcardially perfused with saline followed by

fixative (4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4). Brains were removed from the skull and post-fixed in the same fixative overnight (for 20 h) at room temperature. Following equilibration in 30% sucrose in PB, 30 µm sections were cut in the coronal plane (between bregma -1.0 and -2.3 mm; Franklin and Paxinos 1997) and sagittal plane (between L1.1 and L2.0; Slotnick and Leonard 1975) and immersed in 0.1 M PB (pH 7.4).

Immunohistochemical staining for amyloid plaques and proliferating cells were performed on free-floating brain sections. Sections were incubated with 1% thioflavin-S (Sigma) for 30 min, followed by 35 minutes incubation in 85% alcohol and a final rinse in distilled water, dehydrated in toluene and covered with coverslip using Enthelan.

Sections were washed three times for 10 minutes in Tris-buffered saline (TBS; pH 7.4) and pretreated with 0.6% hydrogen peroxide for 10 min at room temp to quench endogenous peroxidases. Then sections were incubated in 3% normal goat serum in TBS containing 0.3% Triton-X 100 (TBS-NGS-T) for 1 h. Sections were immunoreacted with primary antibody (anti Ki-67 rabbit monoclonal 1:200 dilution, Epitomics, California #K1700-05D) overnight at 4°C. Thereafter, the sections were rinsed in TBS, incubated for 1 h at room temperature with HRP-conjugated goat-anti-rabbit IgG (1:200; Jackson Immuno Research Lab Inc.). The sections were washed and then incubated with 3,3'-diaminobenzidine (DAB, 0.025%; Sigma, Munich, Germany); in the presence of 0.001% hydrogen peroxide diluted in 0.05 M Tris-HCl buffer (pH 7.6) until suitable staining developed. Finally, the sections were mounted onto gelatine-coated glass slides, air-dried, followed by counterstaining in Haematoxylin, dehydrated in 90% and 100% alcohol, and rinsed in toluene before being covered with Enthelan.

Endogenous peroxidase activity was blocked by incubation of the sections in 0.6% hydrogen peroxide for 30 minutes, rinsed again and incubated for 1 hour in TBS containing 0.25% Triton, 2% normal serum of the animal the secondary antibody was raised in, and 1% bovine serum albumin (BSA). Primary antibodies DCX (polyclonal goat antibody, Santa Cruz Biotechnology, 1:1000) were diluted in the same diluent, and sections were incubated at 4°C over night. Incubation with secondary antibodies (rabbit anti goat, goat anti rabbit Vectastain Elite ABC kit) was followed by avidin-biotin complex according to manufacturer's

TABLE I

Stereological parameters for optical fractionators analysis (adapted from Amrein et al. 2004). *Present study.				
Species	No. of sections analyzed	a(x,y step; μm)	Total granule cells ($\times 10^6$)	Gundersen Coefficient of Error (CE)
Wood mice	31 (28-33)	210 x 210	1.53	
Bank voles	27 (25-30)	210 x 210	1.97	
Pine voles	17.5 (16-19)	150 x 150	0.64	
Laboratory mice	19.5 (18-20)	110 x 110	0.50	
*Tg2576 mice	33	110 x 110	0.45	0.05

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instructions and stained with DAB as chromogen. Sections were mounted, embedded and coverslipped with Eukitt. For each marker and animal, between 5 and 12 sections containing the hippocampal structure were analyzed qualitatively.

The left hemispheres were dehydrated for a total of 10 hours in alcohol (4 h in 70%, 4 h in 96%, 2 h in 99%) and embedded in glycolmethacrylate (Technovit 7100, Kulzer GmbH & Co., Wehrheim, Germany) according to the manufacturer's instructions and described by Amrein and his group in 2004. Sagittal 20 μm sections were cut with a metal knife on a Leitz Rotary Microtome. Every fourth section was mounted on slides and dried immediately in an oven at 70°C for 1 hour. Following the protocol of Iniguez and colleagues (1985), slides were incubated in Giemsa staining solution (Giemsa stock solution 1.09204.0500, Merck, Darmstadt, Germany) diluted 1:10 in buffer (67 mmol KH_2PO_4) at 60°C for 10 min, rinsed in buffer for 1 min and differentiated in 3 min in 99% alcohol, cleared in Xylol and mounted with Eukitt.

Brain sections stained were analyzed and photomicrographs taken using a Zeiss Axioplan 2 light microscope including a Sony DXC-930P colour video camera system. For stereology, the total granule cell number in the dentate gyrus was estimated using the

Optical Fractionator principle (West et al. 1991) with StereoInvestigator software 7.0 (MicroBrightField Inc., Williston, USA) on a Zeiss Axioplan 2 light microscope and a 63 $\times$ oil-immersion lens. From the sum of the areas of the dentate gyrus on all sections, x-y-steps of approximately 20 μm provided 483 disector sampling sites. At these sites, optical disectors, with a counting frame measuring 10 $\times$ 10 μm and a height (h) of 10 μm , and stepping size of 110 μm were used to count granule cell nuclei in a known fraction of the dentate gyrus of the hippocampus (West et al. 1991). Section thickness was measured at every 4th sampling site. This sampling scheme was applied to all of the sagittal series (Table I). Cell counts were performed using an $\times 63$ oil immersion lens.

Thioflavin-S staining confirmed the absence of beta-amyloid plaques in the wild-type non-transgenic control and its presence in the transgenic Tg2576 mouse (Fig. 1A). The plaques deposits were present in both the cerebral cortex and hippocampus (Fig. 1B).

Both the 16 month old transgenic Tg2576 and wild-type non-transgenic mice showed Ki-67 positive cells in the SVZ (not shown). A visual analysis does indicate more of the proliferating cell groups in the Tg2576. In addition, we did demonstrate Ki-67 positive cell along the rostral migratory stream (RMS) and towards the

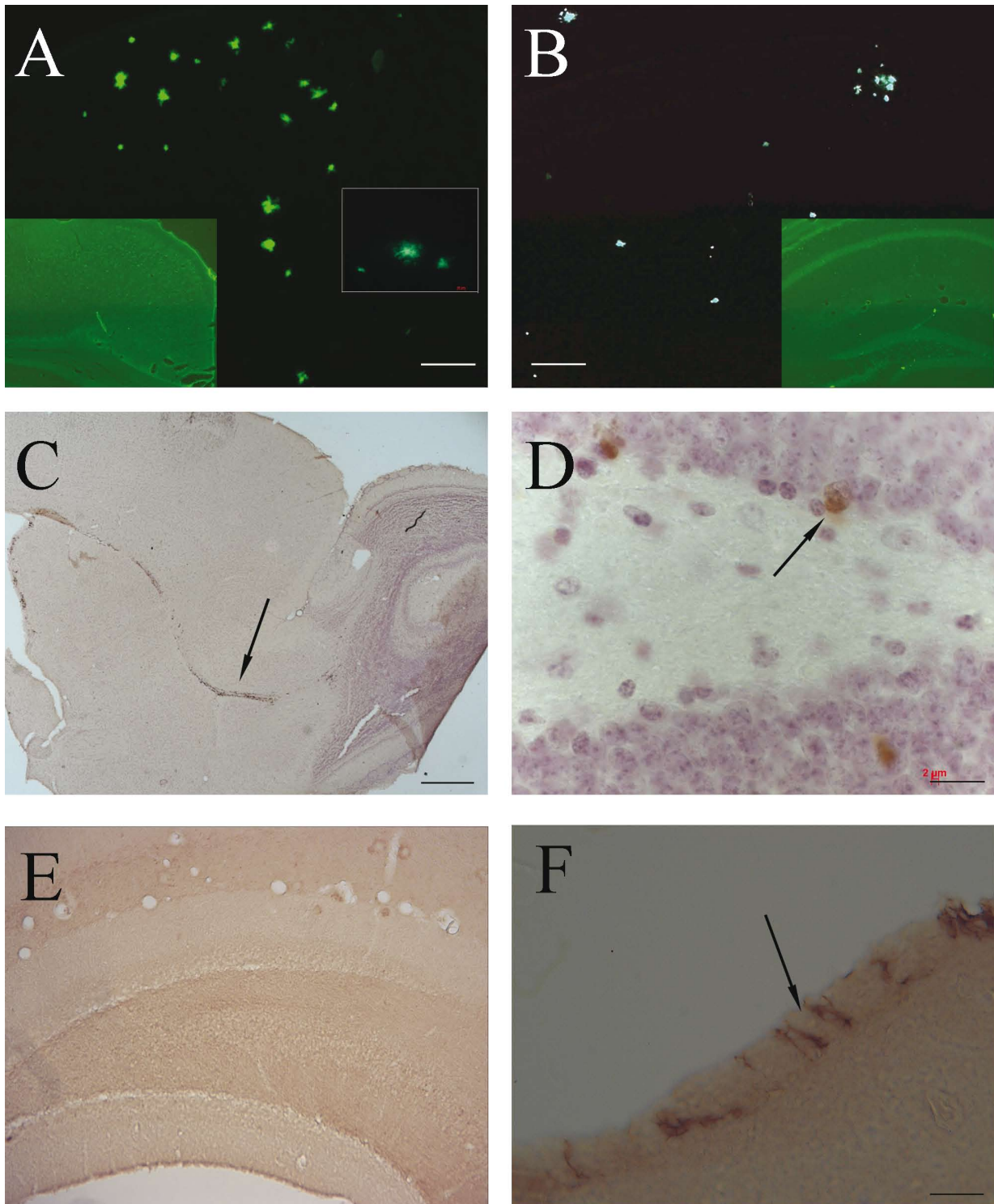


Fig. 1. Representative photomicrographs of immunostaining for amyloid plaques, Ki-67 and doublecortin in the Tg2576 mouse. **(A)** Thioflavin-S staining for amyloid plaques in the cerebral cortex of Tg2576 mouse (left insert is the cerebral cortex of non transgenic control mouse with no plaques; right insert is higher magnification of a plaque). **(B)** Amyloid plaques in the hippocampus of Tg2576 mouse. Insert is hippocampus of non transgenic control mouse with no plaques **(C)** The rostral migratory stream (arrow) in the Tg2576 mouse. **(D)** The arrow indicates a Ki-67 positive cell in the subgranular zone of the dentate gyrus of the hippocampus. **(E, F)** DCX immunostaining in the hippocampus (negative) and subventricular zone (positive) respectively in the 18 month old Tg2576 mouse. Scale bar; **(A, B)** = 10µm; **(C)** = 2.5µm; **(D)** = 5µm; **(E, F)** = 10µm.

olfactory bulb in the 16 month old Tg2576 mice (Fig. 1C). No proliferating cells were observed in the dentate gyrus of the hippocampus in the wild-type non-transgenic control compared to the Tg2576 which demonstrated neuronal progenitor cells in the subgranular zone in the 16 month old (Fig. 1D). However, in the 18-month-old Tg2576 mice, no Ki-67 positive cell was observed in the dentate gyrus. In the absence of Ki-67 positive cells in DG of the hippocampus in the 18-month-old mice, we stained for Doublecortin, a marker for immature neurons and displaying the microtubules associated with cell division. While it was completely negative in the DG of the hippocampus (Fig. 1E), few positive cells were observed in the SVZ of the lateral ventricle (Fig. 1F).

The total granule cell in the DG of the hippocampus in the 18-month-old transgenic Tg2576 mouse model of AD was estimated to be 445,280 per hemisphere (Table I). The Gundersen coefficient of error (CE) was 0.05 which is considered to be excellent. The estimated total granule cell is lower than in the normal laboratory mouse and also wild rodents (Fig. 2).

Adult neurogenesis has been investigated in many AD models with inconsistent findings. The reasons that led to this discrepancy among those different transgenic or knock-in mouse strains or between those studies are not clear (Chen et al. 2008). Using 16-18 month old Tg2576 mouse model of AD we observed that there is an age dependent response to cell proliferation as in normal rodents. The effect of neurodegeneration on neurogenesis being neurodegenerative stage-dependent was therefore visible from our findings in the Tg2576 mouse.

In the present study we established adult neurogenesis in the Tg2576 mouse model of Alzheimer's disease in addition to providing an estimate of the total granule cell number in the dentate gyrus of the hippocampus using the optical fractionator. We chose 16-18 month old mice knowing that the deposition of senile and diffuse amyloid plaques in the neocortex and hippocampus is well established from the postnatal ages of 10 month onwards (Apelt and Schliebs 2001). Bi-Tg mice, like pPDGF-APP^{Sw}, Ind Tg mice, develop an age-dependent β -amyloid plaque formation and accumulation that are associated with neurocognitive dysfunction, which recapitulates some etiological and pathological features of AD patients (Hsia et al. 1999). Gan and coworkers (2008) observed a decrease of neural progenitor cells (NPCs) in the hippocampus at the beta-amyloid plaque onset and progression stages. Since

they did not detect significant cell death of NPCs and gliogenesis from NPCs in the hippocampus, they reason that the decreased NPCs are most likely due to the elevation of migration, which leads to NPC distribution and reorganization, and the increase of neurogenesis, which turns NPCs into neuron-like cells.

For the Tg2576 mouse model, it appears that the 18 month old time point is about when cell proliferation begins to cease in the hippocampus as no Ki-67 positive cells were observed in the outer portion of the granule cell layer as opposed to the report in the PDAPP mice (Donovan et al. 2006). Even though adult dentate neurogenesis is prominent in young rodents, the amount of neuronal progenitors rapidly decreases over the following months (Kuhn et al. 1996, Bondolfi et al. 2004, Heine et al. 2004, Kronenberg et al. 2006, Montaron et al. 2006) and is present at much lower levels in adult and particularly aged animals (Kuhn et al. 2007).

However, the subventricular zone still showed indications of some adult neurogenesis. In the 16 month-old Tg2576, committed progenitor cells migrated via the rostral migratory stream (RMS) into the olfactory bulb where they differentiate into interneurons. The staining for doublecortin also confirmed cell proliferation in the SVZ in the 18 month-olds.

At early stages of neurodegeneration, the effects on all neurogenic stages including proliferation, differen-

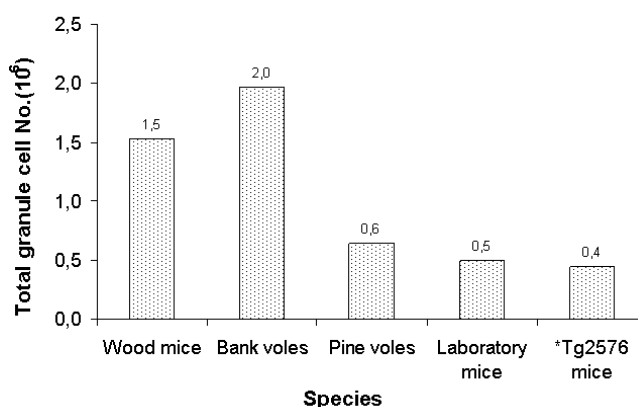


Fig. 2. Estimate of total granule cells (in millions) in the dentate gyrus in different rodent species and the Tg2576 mouse (adapted from Amrein et al. 2004, *present study). Reproduced by permission from Granule cell number, cell death and cell proliferation in the dentate gyrus of wild-living rodents, Amrein et al, Eur J Neurosci 20(12)(c) 2004 [John Wiley & Sons].

tiation, new neuron generation, and survival were robust. At late stages of neurodegeneration, however, these effects were dramatically decreased, indicating that the overall effect of the neurodegeneration on neurogenesis is decreased following neurodegenerative progression (Chen et al. 2008). As neurogenesis is down regulated by aging (Amrein et al. 2004, Bondolfi et al. 2004, Verret et al. 2007), it is not clear whether this shift is or is not purely due to the effect of aging on neurogenesis.

Animal models are a critical and important tool in the examination of neurogenesis in the postnatal brain, insofar as they allow a temporal and spatial analysis of the course of neurogenesis in relation to the progression of neuropathology (Demars et al 2010). Most studies which examined hippocampal or SVZ neurogenesis in transgenic mice expressing one or two APP mutation show impaired proliferation of progenitor cells and/or impaired neuronal differentiation in these mice (Lazarov and Marr 2010). However, the exact time of absence of cell proliferation remains variable. Our findings do indicate absence of cell proliferation in the hippocampus but persisting in the subventricular zone in the brain of 18 month old Tg2576 mouse model of AD.

The total granule cell number in the Tg2576 mouse was far less compared to the normal laboratory mouse and also in several wild rodents as reported (Amrein et al. 2004). The likely explanation will be to ascribe this to the aging process as in normal rats of the same age. However, the amyloid plaque burden must be playing a role in the process. According to Slomianka and West (2005) the coefficient of error (CE) of an estimation procedure is an expression of the reproducibility of the result that one obtains from the procedure. The Gundersen–Jensen coefficient of error estimator or the ‘Split-Sample’ coefficient of error estimator can provide useful information about the precision of stereological estimates. With the Gundersen CE obtained, the reproducibility of the total granule cell count obtained is not in doubt.

The hippocampus remains one of the most vulnerable brain regions to AD, and the degeneration in this brain structure may directly underlie memory deficit, the earliest symptom of AD (Barber et al. 2001, Thompson et al. 2004). Therefore, to attenuate or stop neuronal loss in the brain is a fundamental strategy that may eventually cure this disease. In order to investigate this mechanism it is necessary to further establish an

estimate of the total granule cell number in the Tg2576 mouse model of Alzheimer’s disease in early adult.

In conclusion we can say that cell proliferation continues in the subventricular zone up to the age of 18 months but absent in the dentate gyrus in the Tg2576 mouse. The total cell count is also less than that in the laboratory mouse. The increasing load of the deposition of amyloid plaque in the hippocampus gyrus may have a role which warrants further investigations.

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