

Characterization of hippocampal Cajal-Retzius cells during development in a mouse model of Alzheimer's disease (Tg2576)

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doi:10.4103/1673-5374.128243
<http://www.nrronline.org/>

Accepted: 2014-01-22

Abstract

Cajal-Retzius cells are reelin-secreting neurons in the marginal zone of the neocortex and hippocampus. The aim of this study was to investigate Cajal-Retzius cells in Alzheimer's disease pathology. Results revealed that the number of Cajal-Retzius cells markedly reduced with age in both wild type and in mice over-expressing the Swedish double mutant form of amyloid precursor protein 695 (transgenic (Tg) 2576 mice). Numerous reelin-positive neurons were positive for activated caspase 3 in Tg2576 mice, suggesting that Cajal-Retzius neuronal loss occurred via apoptosis in this Alzheimer's disease model. Compared with wild type, the number of Cajal-Retzius cells was significantly lower in Tg2576 mice. Western blot analysis confirmed that reelin levels were markedly lower in Tg2576 mice than in wild-type mice. The decline in Cajal-Retzius cells in Tg2576 mice was found to occur concomitantly with the onset of Alzheimer's disease amyloid pathology and related behavioral deficits. Overall, these data indicated that Cajal-Retzius cell loss occurred with the onset and development of Alzheimer's disease.

Key Words: nerve regeneration; neurodegeneration; Alzheimer's disease; Cajal-Retzius cells; hippocampus; development; neuronal apoptosis; reelin; Tg2576 mice; NSFC grant; neural regeneration

Funding: This work was supported by the National Natural Science Foundation of China, No. 31070952, 81071029; the Joint Funds of the NSFC with Henan Province Government for Fostering Talents, No. U1204809, and the Henan Province Science Research Project, No. 132102310111.

Yu DM, Fan WJ, Wu P, Deng JX, Liu J, Niu YL, Li MS, Deng JB. Characterization of hippocampal Cajal-Retzius cells during development in a mouse model of Alzheimer's disease (Tg2576). *Neural Regen Res.* 2014;9(4):394-401.

Introduction

Cajal-Retzius cells are a class of neurons located in the marginal zones of the neocortex and hippocampus^[1-2]. They derive from the cortical hem and ganglionic eminence and then migrate tangentially to the cortical layer I^[1-3]. At the molecular level, contact repulsion controls the dispersion and final distribution of Cajal-Retzius cells^[4-5]. During cortical and hippocampal development, Cajal-Retzius cells synthesize and secrete the glycoprotein, reelin^[1,6]. Reelin acts as a stop signal to regulate neuronal migration, and binds to the very low-density lipoprotein receptor and the apolipoprotein E receptor 2^[7]. Reelin also plays an important role in normal synaptic plasticity, dendritic morphogenesis, and learning and memory^[8-10]. Loss of reelin activity in the brain occurs in the reeler mouse and in rare human neurological cases, causing severe cortical and hippocampal malformations^[11-14]. Reelin-dependent activation of neuronal adhesion to the extracellular matrix is crucial for the eventual birth-date-dependent layering of the neocortex^[15-17]. Cajal-Retzius cells and reelin are essential for the

formation of layer-specific hippocampal connections^[18-19]. Cajal-Retzius cell-mediated guidance of entorhinal axons has also been confirmed^[20]. In the normal adult cortex, most Cajal-Retzius cells undergo apoptosis following formation of the cortical layers and a small number persist where they continue to produce reelin^[21]. Reelin is known to modulate synaptic plasticity by enhancing the induction and maintenance of long-term potentiation^[9,22]. In addition, reelin has recently been implicated in some neurological diseases, such as temporal lobe epilepsy and Alzheimer's disease^[23-25]. However, the relationship between Cajal-Retzius cells and Alzheimer's disease is unknown. Alzheimer's disease is a neurodegenerative disorder characterized clinically by progressive memory and cognitive impairment^[26]. Pathological features include the deposition of amyloid plaques, which are composed of beta amyloid (A β), and intracellular accumulation of neurofibrillary tangles consisting of microtubule-associated protein tau. These phenomena are accompanied by neuronal and synaptic loss, and dysfunction of several neurotransmitter systems^[26-28]. Cajal-Retzius cells and

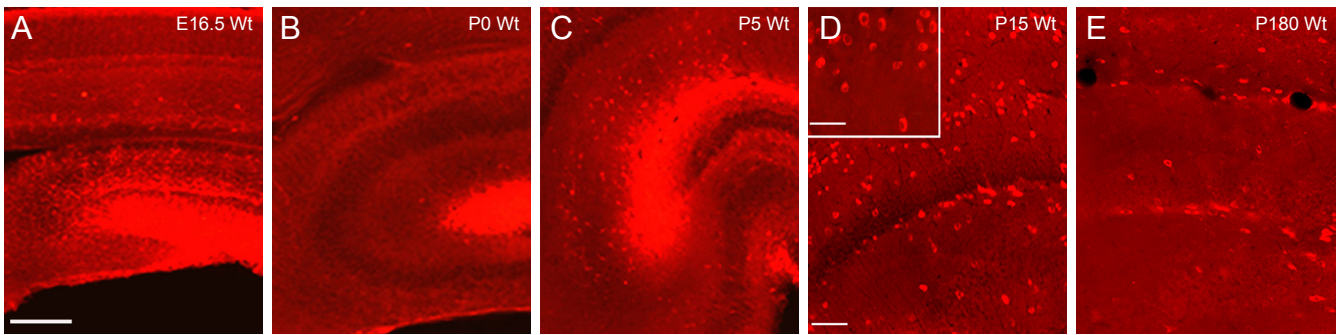


Figure 1 The distribution of Cajal-Retzius cells in normal (wild-type (Wt)) hippocampus at different developmental stages (thioflavin S staining).

(A–C) The distribution of reelin-immunoreactive Cajal-Retzius cells at embryonic day 16.5 (E16.5), postnatal day 0 (P0), and P5. A large number of Cajal-Retzius cells are located in the marginal zone of the neocortex and molecular layer of the hippocampus. Because of extremely dense packing of Cajal-Retzius cells in the molecular layer, the individual morphologies of individual cells could not be distinguished. (D) Cajal-Retzius cells in the molecular layer of the dentate gyrus at P15. Cajal-Retzius cells are seen in the molecular layers of both the hippocampus proper and dentate gyrus. The hippocampal fissure can be used to differentiate between the molecular layer of dentate gyrus and that of the hippocampus proper. Inset, Cajal-Retzius cells at high magnification. (E) Very few Cajal-Retzius cells are observed in the molecular layer of the dentate gyrus at P180. Scale bars: 100 μ m in A–C, 50 μ m in D, E and 30 μ m in D-inset.

reelin have been shown to be involved in the pathogenesis of Alzheimer's disease^[25, 29-30]. Furthermore, altered expression of cerebral reelin in Alzheimer's disease and blockage of reelin signaling *via* apolipoprotein E receptor 2 can enhance tau phosphorylation and increase the formation of intracellular neurofibrillary tangles^[31-32]. However, further studies are required to determine their causality and relationship with Alzheimer's disease. Aging animals, and electrical and chemical lesions (including the administration of excitatory amino acids or A β injection), are used as animal models of Alzheimer's disease^[23, 32]. However, these models do not completely recapitulate the pathological features of Alzheimer's disease, and their phenotypes are unstable. Some cases of Alzheimer's disease are caused by mutations in the gene encoding amyloid precursor protein (APP), and a double mutation in the APP gene is believed to be the cause of the Swedish (Swe) type of familial Alzheimer's disease^[26]. The human mutant APP (hAPP) transgenic (Tg) mouse model over-expressing the Swedish double mutant form of APP695 (Tg2576) has been established^[33]. Heterozygous Tg2576 Tg mice express high levels of hAPP in different brain regions, including the hippocampus. This region is affected early in Alzheimer's disease pathology and shows extensive amyloid deposition associated with cognitive dysfunction resembling Alzheimer's disease^[34]. Therefore, this model may be used to study the relationship between Cajal-Retzius cells and Alzheimer's disease.

Very little is known about the morphometric alterations and the relevance of Cajal-Retzius cells in the pathogenesis of Alzheimer's disease. A β deposits and behavioral deficits are observed in Tg2576 adult mice^[32]. Therefore, we aimed to examine the expression of Cajal-Retzius cells in the hippocampus of Tg2576 mice from embryonic age (16.5 days) to 12 months of age (adult).

Results

Quantitative analysis of experimental animals

Tg2576 mice and their wild-type littermates were randomly

assigned to eight groups by age: embryonic day 16.5, and postnatal days 0, 5, 7, 15, 30, 180, and 360. A total of 128 animals ($n = 64$ Tg2576 and 64 wild type) were used for this study. For each age group, at least eight mice (five for histological analysis and three for western immunoblot assay) were used for the Alzheimer's disease model (Tg2576 mouse) or as controls (wild type).

Developmental presence of Cajal-Retzius cells in the normal wild-type hippocampus

Thioflavin S staining analysis revealed reelin-positive Cajal-Retzius cells in the stratum lacunosum-moleculare of the hippocampus proper and in the outer molecular layer of the dentate gyrus. Cajal-Retzius cells were observed in the molecular layer of the dentate gyrus as early as embryonic day 16.5, and were densely concentrated in the molecular layer of the dentate gyrus (Figure 1A). Because of their compact distribution, the morphology of single Cajal-Retzius cells was not seen until postnatal day 7 and continued thereafter (Figure 1A–C). Cajal-Retzius cells gradually decreased in the molecular layers of both the hippocampus proper and dentate gyrus. In mice older than 6 months, the presence of Cajal-Retzius cells was extremely low (Figure 1A–E). To address whether this cell loss was due to neuroapoptosis, cells in the molecular layer of the dentate gyrus at postnatal day 30 were double-labeled with antibodies against activated caspase 3 and reelin. Results revealed that a large number of reelin-positive Cajal-Retzius cells were also co-labeled with activated caspase 3, indicating extensive Cajal-Retzius cell apoptosis (Figure 2A).

Gamma-aminobutyric acid (GABA) and glutamate are specific markers for interneurons and excitatory neurons, respectively^[35-36]. To address the neurotransmitter types associated with Cajal-Retzius cells, reelin-positive cells were co-stained with GABA- or glutamate-specific antibodies. Before postnatal day 30, reelin-positive Cajal-Retzius cells in the molecular layer of the dentate gyrus were negative. However, from postnatal day 30 these cells were co-localized with reelin and GABA (Figure 2B). Approximately 14%

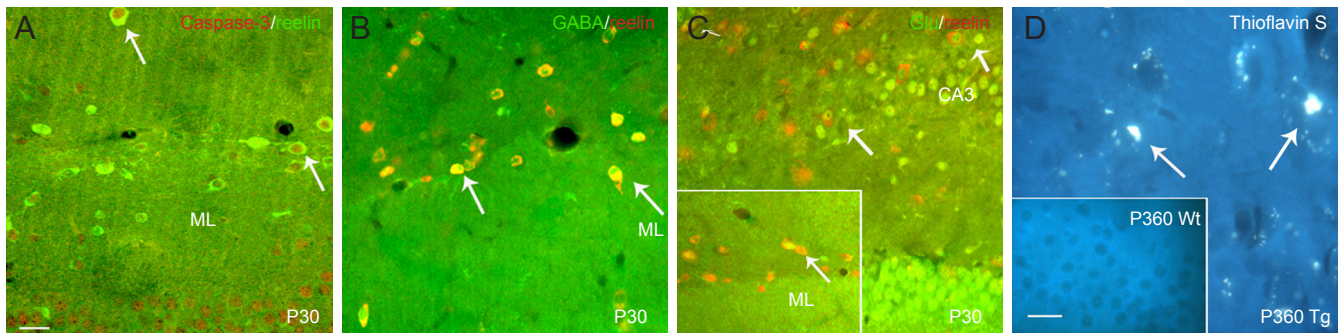


Figure 2 Immunohistochemical characteristics and pathological alterations of Cajal-Retzius cells in the hippocampus of transgenic (Tg)2576 mice (immunofluorescence staining).

(A) Cajal-Retzius cells (green) double-labeled with caspase-3 (red) indicate Cajal-Retzius cell (arrows) apoptosis. (B) Reelin (red) and gamma-aminobutyric acid (GABA) (green) are co-localized in some Cajal-Retzius cells (arrows). (C) Reelin (red) and glutamate are co-localized in some Cajal-Retzius cells (arrows), and these cells are observed in the hilus and molecular layer of the dentate gyrus (inset). (D) Amyloid plaques (arrows) in the hippocampus of P360 Tg2576 mice revealed by thioflavin S staining showing that plaques are absent from age-matched WT mice (inset). Scale bars: 30 μ m in A–C & C-inset, 10 μ m in D and 30 μ m in D-inset. P: Postnatal day; Wt: wild-type.

of Cajal-Retzius cells were GABA-positive at postnatal day 30, and this number increased to about 80% by postnatal day 180. From postnatal day 360 and thereafter, almost all reelin-positive Cajal-Retzius cells in the molecular layer of dentate gyrus were GABA-positive. Before postnatal day 180, glutamate-positive Cajal-Retzius cells were almost entirely absent in the molecular layer of dentate gyrus, although double labeling for reelin and glutamate occurred in some mossy cells in the hilus (Figure 2C). After postnatal day 180, some reelin-positive Cajal-Retzius cells in the molecular layer of the dentate gyrus were also positive for glutamate (Figure 2C-inset). After postnatal day 360, the majority of Cajal-Retzius cells were positive for both GABA and glutamate, suggesting that Cajal-Retzius cells can function as both interneurons and excitatory neurons.

Pathology of Tg2576 mice

By monitoring the development of heterozygous Tg2576 mice, behavioral deficits were already present at 3 months of age. Based on previous studies^[37-38], we found that Tg2576 mice exhibited slow reaction times and cognitive decline compared with wild-type littermates (data not shown). Early death was common in these Tg mice, typically dying before 12 months of age. The survival rate at 6 months of age was 82% for Tg mice compared with 95% for wild type. At 12 months, the survival rate of Tg mice was < 10% compared with 91% for wild-type mice. To confirm Alzheimer's disease-type pathology, brain sections of Tg and wild-type mice were analyzed for thioflavin-positive amyloid. Heterozygous and wild-type mice at postnatal days 90, 180, and 360 were used for pathological analyses. Results revealed the presence of widespread amyloid plaques in the cortex and hippocampus of Tg mice from 6 months of age and onwards. Amyloid plaques appeared to have a dense core with fibrillar corona. No amyloid plaques were detected in wild-type mice (Figure 2D).

Developmental distribution of Cajal-Retzius cells in Tg2576 mice

In Tg2576 mice, the number of Cajal-Retzius cells in the molecular layer of the dentate gyrus gradually decreased

with increasing age (Figure 3A–F), which was also seen in wild-type mice. However, quantitative differences were found between both groups at postnatal day 90. Before postnatal day 90, no significant differences in the distribution of Cajal-Retzius cells in dentate gyrus of wild-type and transgenic mice were observed. However, after postnatal day 90, the number of Cajal-Retzius cells in the Tg2576 mice was significantly ($P < 0.05$) reduced compared with age-matched wild-type mice (Figure 4A). Amyloid plaques in the hippocampus were also observed at this time point, suggesting a link between the onset of Alzheimer's disease pathology and Cajal-Retzius cell loss. Western blot analysis revealed that expression of reelin was significantly ($P < 0.05$) reduced in Tg2576 mice compared with wild-type mice (Figure 4B–D). This finding was confirmed by quantitative immunocytochemistry (Figure 4A).

Discussion

Developmental distribution of Cajal-Retzius cells

Reelin (produced by Cajal-Retzius cells) is crucial for neural migration and cortical lamination because its deficiency has been associated with disorders of cortical plate development and some forms of lissencephaly^[10, 39]. During hippocampal and cortical development, the majority of Cajal-Retzius cells disappear following the immediate postnatal period, thus few Cajal-Retzius cells survive into adulthood. Cajal-Retzius cells in the postnatal period continue to express reelin and other markers, such as GABA and glutamate^[35, 40-41]. A large number of Cajal-Retzius cells are GABAergic neurons, while others are glutamatergic. Both GABAergic Cajal-Retzius cells and glutamatergic Cajal-Retzius cells interact with each other to regulate neural migration and the formation of the neural network in the cortex and hippocampus. For instance, glutamate is released from glutamatergic Cajal-Retzius cells and facilitates the migration of GABAergic Cajal-Retzius cells and interneurons, which in turn releases GABA and facilitates the migration of glutamatergic neuroblasts^[36-40].

The present study first investigated the developmental distribution of Cajal-Retzius cells in the molecular layer of the normal wild-type dentate gyrus. In the fetus, reelin-positive

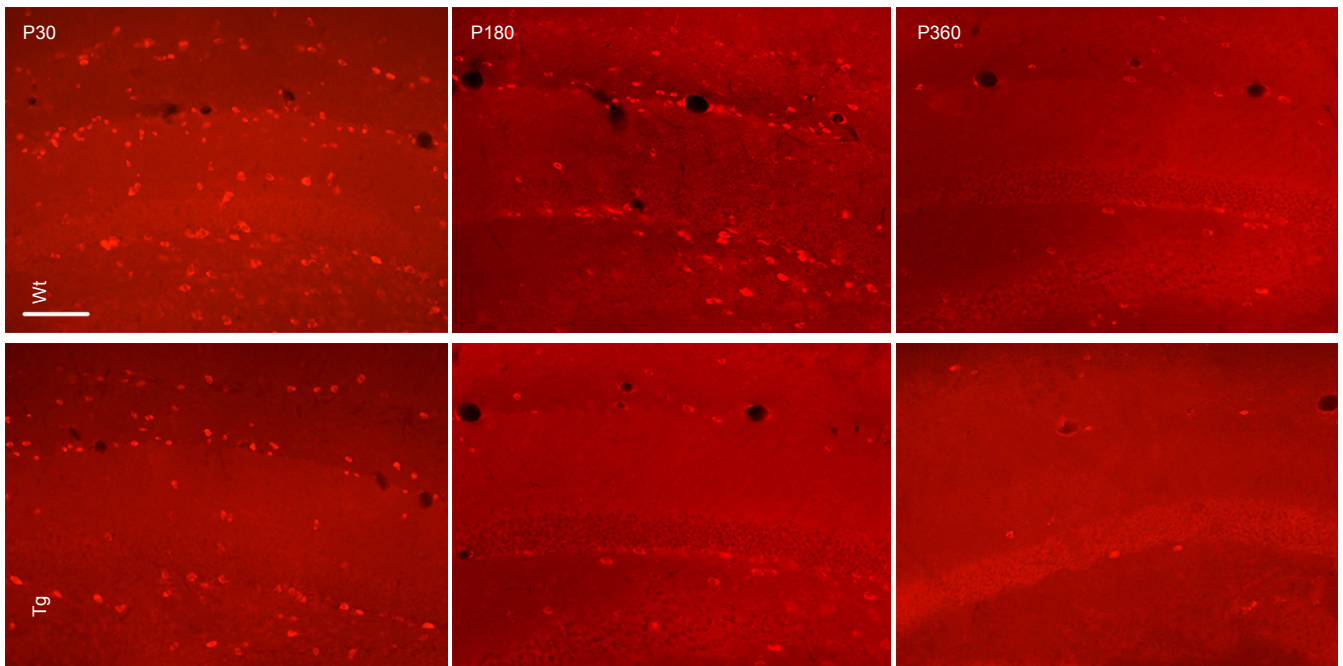


Figure 3 Developmental distribution of Cajal-Retzius cells in the molecular layer of the dentate gyrus in Wt and Tg2576 mice. The number of Cajal-Retzius cells decreases with increasing age in Tg2576 and Wt mice. At P30, many reelin-positive Cajal-Retzius cells are present in the molecular layer of the dentate gyrus but at P360, these cells are only present in low numbers in the same region. The number of reelin-positive cells in the molecular layer of the dentate gyrus are notably reduced in Tg2576 mice compared with Wt mice. Scale bar: 20 μ m. Wt: Wild-type; Tg: transgenic; P: postnatal day.

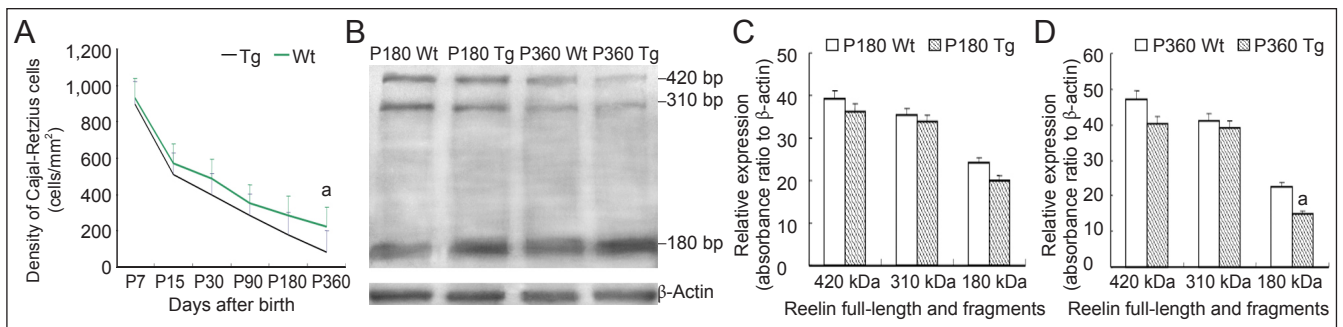


Figure 4 Cajal-Retzius cell density and expression of reelin in Tg2576 and Wt mice at different ages. (A) The density of Cajal-Retzius cells is gradually reduced with increasing age, and the mean density in Tg2576 mice is significantly less than Wt mice after P180. (B) Western blot of reelin levels from the cerebral cortex and hippocampus of Tg2576 and Wt mice, and (C, D) quantification of reelin expression. Reelin is significantly reduced in P360 Tg2576 mice compared with age-matched wild types. Wt: Wild-type; Tg: transgenic; P: postnatal day. Data are expressed as mean \pm SD. The difference between Tg2576 and wild-type mice in the density of Cajal-Retzius cells was analyzed by one-way analysis of variance followed by the Student-Newman-Keuls multiple range test. ^a $P < 0.05$, vs. P360 Wt.

Cajal-Retzius cells were widely distributed in the molecular layer of hippocampus proper and dentate gyrus. Because developmental establishment of the neocortex and hippocampus occurs *in utero* and in the immediate neonatal period, the Cajal-Retzius-mediated stop signal likely played an important functional role at this time in the present study. At later time points, reduced numbers of Cajal-Retzius cells in the dentate gyrus and neocortex may have accompanied the establishment of cortical circuitry. Our results revealed that reelin-positive cells in the hippocampus and dentate gyrus decreased gradually after birth, with a low number of Cajal-Retzius cells in the adult. The decrease in the number of Cajal-Retzius cells may have been due to neuroapoptosis

because many cells were positive for activated caspase 3. There is continuing debate on which neurotransmitter is released from Cajal-Retzius cells and whether these cells are interneurons or excitatory neurons. Results of the present study showed that reelin-positive cells that were GABAergic or glutamatergic increased in density with increasing age. Moreover, these cells were both GABAergic and glutamatergic. Reelin-positive mossy cells in the dentate hilus were predominantly glutamatergic, but in the molecular layer of the dentate gyrus, reelin-positive cells that were GABAergic and glutamatergic showed a spatiotemporal pattern. After postnatal day 90, almost all reelin-positive cells in the molecular layer of dentate gyrus were GABAergic. These GABA-pos-

itive cells with horizontal processes are thought to form a dense GABA fiber network layer^[36, 42]. In the present study, most reelin-positive mossy cells in the hilus were glutamatergic before postnatal day 180. However, few glutamatergic Cajal-Retzius cells are located in the molecular layer of the dentate gyrus^[43-45]. After postnatal day 360, all Cajal-Retzius cells in the molecular layer of dentate gyrus were also glutamate-positive, a characteristic of projection neurons or excitatory neurons. According to our findings, Cajal-Retzius cells in the molecular layer of the dentate gyrus may have been glutamatergic after postnatal day 180, suggesting that these cells in this region were both GABAergic and glutamatergic neurons. GABA is synthesized by glutamate decarboxylase from glutamate, thus providing a reason why GABAergic neurons contained glutamate after postnatal day 180. Therefore, GABAergic Cajal-Retzius cells may change into glutamatergic neurons. The equilibrium between the activity of GABAergic and glutamatergic neurons is central to the generation of behavioral relevant patterns^[42, 46-47]. In adult neural networks, glutamatergic neurons modulate the migration of GABAergic interneurons, in addition to GABAergic neurons modulating the migration of glutamatergic pyramidal cells^[48-50]. Therefore, by secreting excitatory or inhibitory neurotransmitters at different stages of development, Cajal-Retzius cells may play an important role in the regulation of synapse formation and synaptic plasticity in the postnatal period.

Cajal-Retzius cell development in Tg2576 mice

Cajal-Retzius cells have recently been suggested to be involved in cognition^[6]. Cajal-Retzius cells may play a role in cognition, such as learning and memory, because alterations in the number of Cajal-Retzius cells have been suggested as a cause of neuronal dysfunction in Alzheimer's disease^[51]. Cajal-Retzius cells and reelin have been implicated in neurological and psychiatric diseases, including Alzheimer's disease^[25, 52], temporal lobe epilepsy^[18, 53] and autism^[54-55]. Chin et al.^[23] have reported that reelin expression and reelin-positive pyramidal cells are decreased in the entorhinal cortex of Tg2576 mice. Abnormalities of reelin expression have been associated with the accumulation of proteins involved in APP trafficking and processing^[32, 52, 56]. However, the relationship between the progression of Alzheimer's disease and Cajal-Retzius cell development is not well understood and thus formed the basis for our present investigation.

Our findings indicated that reelin-positive cells were abundant in the molecular layer of the hippocampus proper and dentate gyrus in both Tg2576 and wild-type mice at embryonic and neonatal ages. No significant difference in the density of Cajal-Retzius cells between Tg2576 and wild-type mice was found before postnatal day 90. However, the density of these cells in Tg2576 mice was significantly reduced after postnatal day 90. Immunohistochemistry revealed that neurons were positive for reelin and activated caspase-3, indicating that Cajal-Retzius cells had undergone neuroapoptosis, a finding that has also been suggested by previous studies^[17, 57]. Neuronal loss in Tg2576 mice has been suggested to result from neuroapoptosis as a consequence of A β

toxicity^[54]. Our findings showed thioflavin S-positive amyloid plaques in the cortex and hippocampus of Tg2576 mice after 6 months. Interestingly, the reduction in Cajal-Retzius cell number and the appearance of extracellular amyloid plaques in the hippocampus occurs at 6 months, which is the same age as the onset of behavioral changes^[58], suggesting that there may be a causal relationship between Alzheimer's disease pathology and Cajal-Retzius cell loss. In the present study, Cajal-Retzius cells were observed in both Tg2576 and wild-type mice; however, the number of these cells decreased as age increased. Furthermore, no amyloid plaques were found in wild-type mice, suggesting that the reduction of Cajal-Retzius cells was probably caused by presence of amyloid plaques in the hippocampus rather than their absence. Unlike Cajal-Retzius cells, which were detected in the molecular layer, thioflavin S-positive amyloid plaques were found in the cortex and hippocampus. Therefore, in the present study, the A β from amyloid plaques may have moved to the molecular layer, resulting in the pathological effects. Because Cajal-Retzius cells and reelin play key roles in the guidance of neuronal migration and maturation, small changes in Cajal-Retzius cell numbers in Tg2576 mice may induce large alterations in neurogenesis, neuronal migration, and neuronal pathfinding. Therefore, dysregulation of neurogenesis, neuronal migration, and neuronal pathfinding may contribute to cognitive impairments in Alzheimer's disease patients^[52, 59].

In conclusion, our results suggested that reelin signaling pathways may have been involved in neuroapoptosis and in Alzheimer's disease pathological changes, including amyloid plaque deposition. Therefore, Cajal-Retzius cell dysfunction may have contributed to Alzheimer's disease progression. Overall, these findings may provide a better understanding of the pathology of Alzheimer's disease for possible future clinical management of this disease.

Materials and Methods

Design

Neurodevelopmental observation experiment.

Time and setting

The experiments were performed at a laboratory in the Institute of Neurobiology, Henan University, China from September 2009 to June 2012.

Materials

Tg2576 mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). All experiments involved the progeny of hemizygous males harboring human APP containing the Swedish familial Alzheimer's disease double mutation, hAPP695Swe, under the control of a hamster prion protein promoter, crossed with wild-type female mice on a hybrid C57BL/6-SJL background^[35]. Wild-type littermates were used as controls. All experiments were carried out with the approval of and in accordance with the guidelines of the Animal Welfare and Use Committees of Henan University in China. Animals were housed in standard breeding cages with a 12-hour light/dark cycle. Females were checked each

morning for the presence of a vaginal plug. A positive plug was defined as embryonic day 0.5 (postnatal day 0 was defined as the first 24 hours after birth). Offspring were produced from timed pregnancies. Tail DNA was extracted and genotyped by PCR to detect hTg2576 using the following hAPP-specific primers: 5'-GTG GAT AAC CCC TCC CCC AGC CTA GAC CA-3' and 5'-CTG ACC ACT CGA CCA GGT TCT GGG T-3'. Offspring genotypes were identified as wild type (-/-, control mice lacking a PCR band) and heterozygous (+/-, mice giving rise to a 466 bp PCR band). A total of 160 animals (Tg2576 = 64 and wild type = 64, with equal numbers of each gender) were used for this study.

Methods

Thioflavin S staining

After anesthesia (sodium pentobarbital, 30 mg/kg, intraperitoneal (i.p.) injection), pups were perfused transcardially with 4% paraformaldehyde in PBS (0.1 mol/L, pH 7.2). Brains were then dissected and post-fixed in 4% paraformaldehyde for further 1–2 days at 4°C, then dehydrated in graded ethanol and embedded in paraffin. Hippocampi were cut into coronal sections (thickness of 5 µm). These sections were dewaxed with xylene and alcohol, followed by water, and incubated in 0.25% potassium permanganate solution for 20 minutes. After rinsing in distilled water, sections were incubated in bleaching solution for 30 seconds. A drop (~50 µL) of 1% thioflavin S staining solution was applied to each section and incubated for 3–5 minutes. After washing several times in 50% ethanol and distilled water, the sections were mounted under 65% glycerol in 0.1 mol/L PBS. The stained slide was examined using a fluorescence microscope (BX53, Olympus, Tokyo, Japan), resulting in a very bright blue ultraviolet excitable stain.

Immunofluorescence staining

Postnatal mice were anesthetized with sodium pentobarbital (20 mg/kg, i.p.), then transcardially perfusion-fixed with 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.2) and post-fixed in the same fixative for 1–2 days at 4°C. For embryonic mice, pregnant dams were also anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and fetuses were harvested by Cesarean section. Embryonic brains were fixed with 4% paraformaldehyde for 2–3 days at 4°C. Coronal vibratome sections (thickness of 50 µm) were rinsed in 0.1 mol/L PBS and preincubated in blocking solution (5% normal goat serum, 0.2% Triton-X100 in PBS) for 30 minutes at room temperature. After rinsing in 0.1 mol/L PBS, sections were stained using single or double immunolabeling. The following primary antibodies were used: mouse anti-reelin monoclonal antibody (1:1,000; Chemicon, Billerica, MA, USA), goat anti-caspase3 (1:1,000; Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-glutamate polyclonal antibodies (1:500; Sigma-Aldrich, St. Louis, MO, USA), and rabbit anti-GABA polyclonal antibodies (1:100; Chemicon). The corresponding secondary antibodies were Alexa 568 goat anti-mouse IgG (1:600; Invitrogen, Carlsbad, CA, USA), Alexa 488 goat anti-rabbit IgG (1:300; Invitrogen), and Alexa 568 donkey anti-goat IgG (1:600; Invitrogen). Sections were incubated with

the appropriate dilutions of primary antibodies overnight at 4°C, washed, and then incubated with secondary antibodies at room temperature for 3 hours. After washing three times in PBS, coverslips were applied to the sections in mounting medium, and viewed under fluorescence microscopy.

Western blot analysis

Proteins were extracted from the hippocampus of wild-type and heterozygous Tg2576 mice at postnatal days 180 and 360. Proteins were resolved by 4–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred (semi-dried: 40 minutes at 10 V; wet: overnight at 15 V) to polyvinylidene difluoride membranes. Non-specific binding was blocked with 5% skim milk powder in Tris-buffered saline containing 0.2% Tween 20. Membranes were incubated (overnight at 4°C) with the primary antibody, mouse anti-reelin monoclonal antibody (1:1,000; Chemicon, Billerica, MA, USA). After washing, membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000; Millipore, Billerica, MA, USA) in conjunction with an enhanced chemiluminescence system (ECL Plus Western Blotting Detection System; GE Healthcare)^[60]. Mouse anti-β-actin monoclonal antibody (Sigma-Aldrich) served as the internal control. The absorbance ratio of reelin-positive bands to β-actin represented the relative expression level of reelin protein.

Statistical analysis

Data are expressed as mean ± SD. The difference between Tg2576 and wild-type mice in the density of Cajal-Retzius cells was analyzed by one-way analysis of variance followed by the Student-Newman-Keuls multiple range test. SPSS17.0 software (SPSS, Chicago, IL, USA) was used to perform all analyses. A value of $P < 0.05$ was considered statistically significant. Image J software (ImageJ 1.47, NIH, Bethesda, MD, USA) was used to measure the area of the molecular layer, the number of Cajal-Retzius cells in the molecular layer, and the number of these cells per unit area (Reelin-positive cells/mm²) in this region.

Author contributions: Yu DM and Fan WJ provided, analyzed, integrated experimental data and wrote the manuscript. Wu P participated in statistical processing. Deng JX, Niu YL and Li MS provided technical assistance, data support, and study guidance. Liu J authorized the manuscript. Deng JB designed this study. All authors approved the final version of this paper.

Conflicts of interest: None declared.

Peer review: This study has investigated the changes and expression characteristics of Cajal-Retzius cells in the hippocampus of Tg2576 mice throughout the whole development from embryos to postnatal phase by using morphological technique and western blot assay. This study analyzed the potential relationship between the Cajal-Retzius cell growth rules and the genesis of Alzheimer's disease and their possible mechanisms based on literature reviews.

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Copyedited by Farso M, Zhang B, Bi J, Yu J, Qiu Y, Li CH, Song LP, Zhao M