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CX3 chemokine receptor 1 deficiency leads to reduced dendritic complexity and delayed maturation of newborn neurons in the adult mouse hippocampus

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Abstract

Previous studies have shown that microglia impact the proliferation and differentiation of neurons during hippocampal neurogenesis *via* the fractalkine/CX3 chemokine receptor 1 (CX3CR1) signaling pathway. However, whether microglia can influence the maturation and dendritic growth of newborn neurons during hippocampal neurogenesis remains unclear. In the present study, we found that the number of doublecortin-positive cells in the hippocampus was decreased, and the dendritic length and number of intersections in newborn neurons in the hippocampus were reduced in transgenic adult mice with CX3CR1 deficiency ($CX3CR1^{GFP/GFP}$). Furthermore, after experimental seizures were induced with kainic acid in these CX3CR1-deficient mice, the expression of c-fos, a marker of neuronal activity, was reduced compared with wild-type mice. Collectively, the experimental findings indicate that the functional maturation of newborn neurons during hippocampal neurogenesis in adult mice is delayed by CX3CR1 deficiency.

***Key Words:** nerve regeneration; fractalkine; CX3 chemokine receptor 1; neuronal maturation; dendrites; doublecortin; synaptic maturation; newborn neurons; neural regeneration*

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Introduction

Microglia are the resident immune cells in the central nervous system and act as macrophages (Aloisi, 2001). Microglia can rapidly respond to homeostatic disturbances by secreting inflammatory molecules (Hu et al., 1995; Inoue, 2006) and neurotrophic factors (Batchelor et al., 1999; O'Donnell et al., 2002; Nakajima and Kohsaka, 2004). Microglia are restrained by numerous microenvironmental factors, many of which are produced by neurons (Neumann, 2001). Fractalkine is a chemokine that is constitutively expressed by healthy neurons and functions as a neuroimmune regulatory protein. By binding to its receptor on microglia, CX3 chemokine receptor 1 (CX3CR1), fractalkine inhibits microglial activity under inflammatory conditions (Harrison et al., 1998; Ransohoff et al., 2007). In the brain, CX3CR1 is primarily expressed by microglia (Harrison et al., 1998). Only a few types of neurons express CX3CR1. Consequently, targeting fractalkine/CX3CR1 signaling has been used to modulate the neurotoxicity of microglia in diverse models of neurological disorders, such as neuropathic pain (Sun et al., 2007), age-related macular degeneration (Combadière et al., 2007), peripheral lipopolysaccharide injection-induced intracranial inflammation, Parkinson's disease and amyotrophic lateral sclerosis (Cardona et al., 2006). Accumulating evidence indicates a major role of fractalkine/CX3CR1 signaling in the central nervous system. Bachstetter et al. (2011) showed that the functional disruption of CX3CR1 in $CX3CR1^{GFP/GFP}$ mice results in the impaired proliferation and differentiation of neurons during hippocampal neurogenesis. Furthermore, another study from the same laboratory showed that the impaired hippocampal neurogenesis resulted in cognitive impairments, including behavioral deficits in the Morris water maze and contextual fear conditioning tests (Rogers et al., 2011). Interestingly, CX3CR1 signaling in microglial cells was shown to be necessary for the survival of layer V cortical neurons during development (Ueno et al., 2013). Additionally, microglia engulf synaptic material and play a major role in synaptic pruning. When not challenged by inflammatory factors, microglia play a critical role in synaptic maturation in the central nervous system (Paolicelli et al., 2011). These and other studies have shown that, in the intact central nervous system, microglia in the hippocampus impact the proliferation and differentiation of neurons during hippocampal neurogenesis, and consequently affect cognitive functioning. However, whether fractalkine/CX3CR1 signaling plays a role in the synaptic maturation of newborn neurons during hippocampal neurogenesis remains unclear. Therefore, in the present study, we investigated the role of fractalkine/CX3CR1 signaling during hippocampal neurogenesis.

Materials and Methods

Animals

$CX3CR1^{GFP/GFP}$ mice, backcrossed to the C57BL/6 background for greater than 10 generations

were provided by JAX Laboratories (Bar Harbor, ME, USA). Colonies of the CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ mice were maintained at Central South University (China). In CX3CR1$^{+/GFP}$ and CX3CR1GFP/GFP mice, the receptor was knocked out and replaced with the *GFP* gene. Hence, in CX3CR1$^{+/GFP}$ mice, CX3CR1 was partially inactivated, while in CX3CR1$^{GFP/GFP}$ mice, the receptor was completely inactivated (Jung et al., 2000). Clean 12-week-old male CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ littermates and 12-week-old male C57BL/6 mice, of specific pathogen-free grade, were used in the experiments. Animals were correspondingly grouped into CX3CR1$^{+/GFP}$, CX3CR1$^{GFP/GFP}$ and wild-type groups. Four animals in each group were used for the counting of doublecortin (DCX)-positive cells, and another four animals in each group were used for dendritic analysis. Three animals in each group were selected at each time point for cell maturation analysis. The protocols were approved by the Institutional Animal Care Committee of Central South University in China.

Sample collection and tissue processing

All animals were anesthetized by peritoneal injection of an overdose of 2% sodium pentobarbital and prefixed with 4% paraformaldehyde transcardially. All the brains were post-fixed for 48 hours in 4% paraformaldehyde and dehydrated in 30% sucrose solution until they sank to the bottom of the vial. Serial coronal frozen sections (40-μm thick) were cut on a freezing microtome (Norton, Trenton, NJ, USA) and prepared for immunohistochemistry.

Immunohistochemistry for DCX-positive cells

In the dentate gyrus, DCX is exclusively expressed in immature neurons from postnatal 1 day to 4 weeks (Couillard-Despres et al., 2005), and thus has been widely used as a marker of immature neurons, and it reliably reflects the level of dendritic growth in these cells. Brain sections were blocked with a solution containing 0.01 M PBS, 0.1% Triton X-100 and 10% normal goat serum for 30 minutes, and subsequently incubated at 4°C overnight with rabbit anti-DCX polyclonal antibody (1:500; Cell Signaling Technology, Beverly, MA, USA). After thorough washes, sections were then incubated with biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA, USA) and avidin-biotin complex (1:200; Vector Laboratories) at room temperature for 60 minutes. Sections were then reacted with diaminobenzidine (Sigma, St. Louis, MO, USA) and 0.1% H_2O_2 for 10 minutes. The sections were dehydrated in graded alcohol and cleared in toluene, and then coverslipped. Sections were observed using an Olympus CHBS light microscope (New York Microscope Company Inc., New York, NY, USA).

Immunohistochemistry for the maturation of newborn neurons

The maturation and activation marker expression of newborn neurons were assessed using triple fluorescence labeling for BrdU, NeuN and c-fos (Deng et al., 2009). One of the six serial sections was treated with 2 M HCl for 30 minutes at 37°C and subsequently incubated simultaneously in three primary antibodies, diluted in PBS containing 0.5% Triton-X and 3% donkey serum, for 48 hours at 4°C. Primary antibodies included rat anti-BrdU polyclonal antibody (1:1,000; Accurate Chemical & Scientific Corporation Company, Philadelphia, PA, USA), goat anti-NeuN antibody (1:400; Cell Signaling Technology Inc.) and rabbit anti-c-fos polyclonal antibody (1:500; Cell Signaling Technology Inc.). After incubation of primary antibodies, donkey anti-rat Alexa 568, donkey anti-goat Alexa 647 and donkey anti-rabbit Alexa 488 antibodies (1:250; Invitrogen Life Technologies, Carlsbad, CA, USA) were added onto the slides for 90 minutes at room temperature. After three rinses, the slides were mounted with florescence mounting media (DAKO, Carpinteria, CA, USA) and coverslipped.

Analysis of the number of DCX-positive cells and dendritic complexity of newborn neurons

DCX-positive cells in the dentate gyrus in the dorsal part of the hippocampus (Yau et al., 2011) were counted using Stereo Investigator software (Micro-Bright Field Biotechnology, New York, NY, USA) by a skilled and blinded experimenter. The software randomly selected 30–50 visual fields under a 40× light lens, and the number of labeled cells was counted. The dendritic outline, dendritic length, and the number of intersections and branch points of DCX-positive cells in the granule cell layer of the hippocampal dentate gyrus were traced using Filament tracer software (Bitplane Inc., South Windsor, CT, USA) and then analyzed with ImarisTrack software (Bitplane Inc.). A qualified neuron for analysis displayed a comparatively independent dendritic tree with at least tertiary branches. Tracings were analyzed by Sholl analysis (Sholl and Uttley, 1953) in 10–200-μm concentric circles centered in the cell body. In brief, the dendritic length counted as the length of the trace between every two concentric circles. The intersection number was the number of traces crossing each circle. A higher dendritic length value or greater number of intersections indicates a neuron with more complex dendritic branching.

Seizures and c-fos analysis

Kainic acid was used to induce seizures in mice and induce the expression of immediate early genes in the dentate gyrus (Deng et al., 2009). Although c-fos is upregulated in very few granule cells under physiological conditions, including novelty exposure or learning, all granule cells upregulate immediate early genes such as *c-fos* in response to electrical activity, such as seizures (Dragunow and Faull, 1989; Kempermann et al., 2003). c-fos expression in newborn neurons after seizure indicates that they are fully mature and integrated into the existing neuronal circuitry (Snyder et al., 2009). BrdU was injected intraperitoneally six times (50 mg/kg, twice per day; Sigma) to mice. Kainic acid (35 mg/kg; Tocris Bioscience, Minneapolis, MO, USA) was injected intraperitoneally 1, 2, 3, 4 and 10 weeks after the last injection of BrdU. Seizures were stopped by injection of the GABA agonist sodium pentobarbital (50 mg/kg, intraperitoneally; Sigma) 30 minutes after

the onset of stage 5 seizure activity. One hour later, animals were sacrificed for the cell maturity analysis.

Statistical analysis

All data are presented as the mean ± SEM. Comparisons of multiple groups were done by one-way analysis of variance followed by Student-Newman-Keuls test for two-group comparisons within the multiple groups using SPSS 16.0 statistical software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered to indicate a significant difference.

Results

Fewer neurons were produced in the brain of CX3CR1$^{GFP/GFP}$ mice than in CX3CR1$^{+/GFP}$ mice

DCX is expressed in newly formed neurons, and is associated with the migration and differentiation of neuronal progenitor cells (Brown et al., 2003). Therefore, DCX can be used to label newborn neurons in the subgranular zone of the hippocampus. As shown in **Figure 1**, most of the DCX-positive cell bodies were located at or just beneath the bottom of the granular layer, and had short or long processes. The number of DCX-positive cells in the subgranular zone was substantially lower in CX3CR1$^{GFP/GFP}$ mice than in CX3CR1$^{+/GFP}$ mice ($P < 0.01$). A decrease in the number of DCX-positive cells in the subgranular zone was also observed in the CX3CR1$^{+/GFP}$ mice, compared with wild-type mice ($P < 0.05$; data not shown).

CX3CR1 deficiency reduced the dendritic complexity of newborn hippocampal neurons

To evaluate the impact of CX3CR1 deficiency on the morphology of newborn neurons, we examined the morphology of DCX-positive cells in the dentate gyrus. DCX-positive cells were divided into class I and class II. Class II DCX-positive cells were characterized as cells with a primary dendrite that was oriented perpendicular to the subgranular zone (Plümpe et al., 2006; Oomen et al., 2011). Class I DCX-positive cells were located in the subgranular zone, without a dendrite or with only a short dendrite. Every selected class II DCX-positive cell with at least tertiary branches was traced using Filament tracer software and analyzed by Sholl analysis. Compared with wild-type mice, the dendritic length of class II DCX-positive cells was greatly reduced in CX3CR1$^{GFP/GFP}$ mice ($P < 0.05$; **Figure 2A**). In addition, the number of intersections was reduced in CX3CR1$^{GFP/GFP}$ mice compared with wild-type mice ($P < 0.05$; **Figure 2B**). No significant dendritic defect in class II DCX-positive cells was observed in CX3CR1$^{+/GFP}$ mice (**Figure 2**). The dendritic length and the number of intersections are two parameters reflecting the complexity of dendritic trees in Sholl analysis. Our findings show that CX3CR1 deficiency reduces the dendritic complexity of newborn neurons.

Neuronal functional maturation was delayed in CX3CR1$^{GFP/GFP}$ mice

Mature granule cells functionally integrated into the neural circuitry can be activated by very strong stimuli, such as kainic acid. To assess the functional maturation of newborn neurons, changes in c-fos expression in BrdU$^+$ cells in the dentate gyrus in response to kainate-induced seizures were measured in 1- to 10-week-old mice. Ten weeks after the final BrdU injection, more than 95% of BrdU$^+$ cells in all groups strongly expressed c-fos. However, the expression of c-fos was significantly decreased in CX3CR1$^{GFP/GFP}$ mice with kainate-induced seizures 3 weeks after the last BrdU injection, compared with wild-type mice ($P < 0.05$; **Figure 3**). This reduction in expression of c-fos indicates that CX3CR1 signaling contributes to the maturation of newborn neurons in the hippocampus.

Discussion

In the present study, we found that the number of DCX-positive cells in the subgranular zone was dramatically lower in CX3CR1$^{GFP/GFP}$ mice compared with CX3CR1$^{+/GFP}$ mice. Furthermore, in CX3CR1$^{GFP/GFP}$ mice, the dendritic length and the number of intersections were greatly reduced. Notably, the disruption of fractalkine/CX3CR1 signaling resulted in a temporary reduction in c-fos expression after kainic acid-induced seizures. These findings indicate that the disruption of fractalkine/CX3CR1 signaling results in the impairment of dendritic morphology and a delayed maturation of newborn neurons during hippocampal neurogenesis.

Recently, the role of microglia in the uninjured brain has attracted increasing attention (Lu et al., 2013). Microglia are highly dynamic cells with extremely motile processes and protrusions, and they continually survey their microenvironment (Nimmerjahn et al., 2005). In the current study, we found that disruption of the fractalkine/CX3CR1 signaling pathway caused a reduction in the number of DCX-positive cells in both homozygous and heterozygous mutant mice. These results are similar to previous reports (Bachstetter et al., 2011; Rogers et al., 2011). Impaired hippocampal neurogenesis eventually leads to cognitive decline. Hence, it is intriguing that CX3CR1$^{+/GFP}$ mice suffering from impaired hippocampal neurogenesis exhibit only mild cognitive deficits. The proliferation of neural progenitor cells and neuronal differentiation are only two stages of hippocampal neurogenesis. The successful migration and maturation of newborn neurons is necessary for learning and memory function in experimental animals. Based on this premise, we analyzed the changes in the dendritic morphology of immature neurons in the dentate gyrus. The dendritic length and the number of intersections in young neurons, as assessed by Sholl analysis, was significantly reduced in CX3CR1$^{GFP/GFP}$ mice, but not in CX3CR1$^{+/GFP}$ mice. The findings show that the complexity of the dendritic tree in newborn neurons in CX3CR1$^{+/GFP}$ mice was similar to that in wild-type mice. This similarity might account for the observation that CX3CR1$^{+/GFP}$ mice displayed a comparatively normal performance in cognitive tasks.

DCX is a protein that promotes microtubule polymerization, and is abundantly expressed in migrating neuroblasts and young neurons (Gleeson et al., 1998). DCX labeling reveals the processes of immature neurons and is useful for investigating the development of dendrites morphologically. Dendritic

development in immature neurons is closely associated with the efficacy of antidepressant drugs (Wang et al., 2008), as well as seizures (Overstreet-Wadiche et al., 2006). Our data here show that in the CX3CR1$^{GFP/GFP}$ mice, CX3CR1 deficiency impairs cell proliferation and neuronal differentiation during hippocampal neurogenesis, and also affects the dendritic development of immature neurons

Another critical stage in hippocampal neurogenesis is the maturation of young neurons and their subsequent integration into the existing hippocampal circuitry (Deng et al., 2009, 2010). This requires the ability to form synapses with hilar interneurons, mossy cells or CA3 pyramidal cells (Toni et al., 2008). A recent study showed that mice with a transient deficit in granular neuron maturation is unable to display robust, long-term spatial memory and exhibits impaired performance in extinction tasks (Deng et al., 2009). Therefore, we examined c-fos expression in newborn neurons following seizure. The expression of c-fos was reduced in 3-week-old, but not in 10-week-old, CX3CR1$^{GFP/GFP}$ mice. c-fos is an immediate early gene that is rapidly and transiently induced in response to certain stimuli in various types of cells (Hu et al., 1994). Acute expression of c-fos in neurons indirectly reflects the firing of action potentials (Dragunow and Faull, 1989). Here, the expression of c-fos in BrdU^{+}/NeuN^{+} cells indicates that they are newborn neurons that have integrated into the existing neural circuitry (Kempermann et al., 2003; Snyder et al., 2009).

To our knowledge, we provide the first evidence that fractalkine/CX3CR1 signaling participates in the maturation of newborn neurons during hippocampal neurogenesis. The finding that the expression level of c-fos was similar in wild-type and CX3CR1$^{GFP/GFP}$ mice is in keeping with the observation that the CX3CR1$^{+/GFP}$ mice displayed a comparatively normal cognitive phenotype in the previous study. Moreover, a postponed maturation of newborn neurons in the CX3CR1$^{GFP/GFP}$ mice was observed. Thus, disruption of fractalkine/CX3CR1 signaling only delays the maturation of immature neurons in the brain.

Previous studies have shown that microglia regulate the maturation of synapses during the embryonic stage, and the first wave of microglial ancestors enter neural tissue very early during embryonic development. This entry precedes both astrogliogenesis and oligodendrogliogenesis during the perinatal period (Ginhoux et al., 2010) and coincides with the first wave of embryonic synaptogenesis (Kettenmann et al., 2013). The promotion of synaptic maturation by microglia was also recently shown during the postnatal stage (Paolicelli et al., 2011). Microglia engulf synaptic debris and actively monitor the surrounding environment. A transient increase in spine density was observed during development in mice with CX3CR1 deficiency, suggesting that microglia participate in synaptic pruning through fractalkine/CX3CR1 signaling. Our data suggest that CX3CR1 deficiency delays the maturation of neurons, and thereby postpones synaptic integration. Whether microglia function in synaptic pruning during hippocampal neurogenesis through fractalkine/CX3CR1 signaling is unknown and will require further study.

In conclusion, disruption of fractalkine/CX3CR1 signaling results in dendritic defects and the delayed maturation of neurons in the subgranular zone. Our findings provide insight into the role of the fractalkine/CX3CR1 pathway during hippocampal neurogenesis in the normal brain.

Author contributions: *FX designed and completed the experiment, as well as analyzed the data and wrote the paper. JMX and XHJ sponsored the experiment and revised the paper. XHJ assisted to design the experiment and perform part of the animal feeding. All authors approved the final version of the paper.*

Conflicts of interest: *None declared.*

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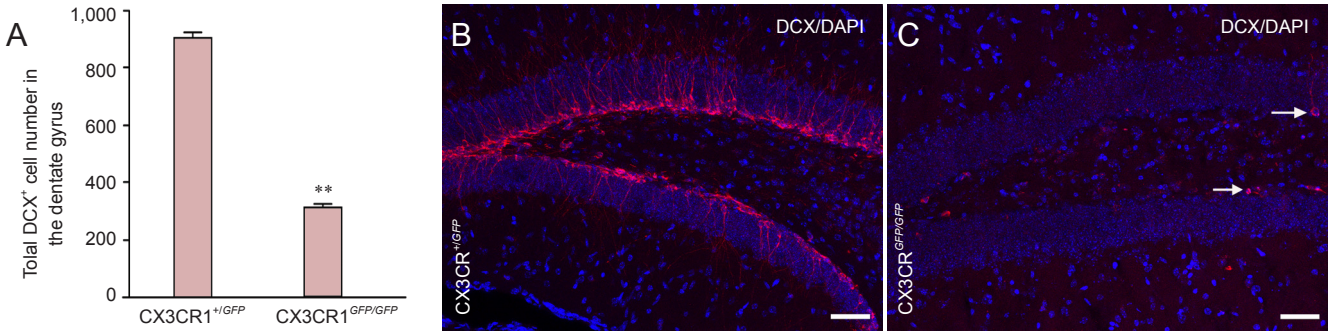

Figure 1 Expression of doublecortin (DCX) in the dentate gyrus of CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ mice.
(A) Number of DCX-positive cells in the dentate gyrus of CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ mice. All data are presented as the mean ± SEM (n = 4 mice in each group). Comparisons of multiple groups were done by one-way analysis of variance followed by Student-Newman-Keuls test. **P < 0.01, *vs.* CX3CR1$^{+/GFP}$ mice. (B, C) Expression of DCX in the dentate gyrus of CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ mice (immunohistochemical staining). Arrows indicate DCX expression. Scale bars in B and C: 50 μm.

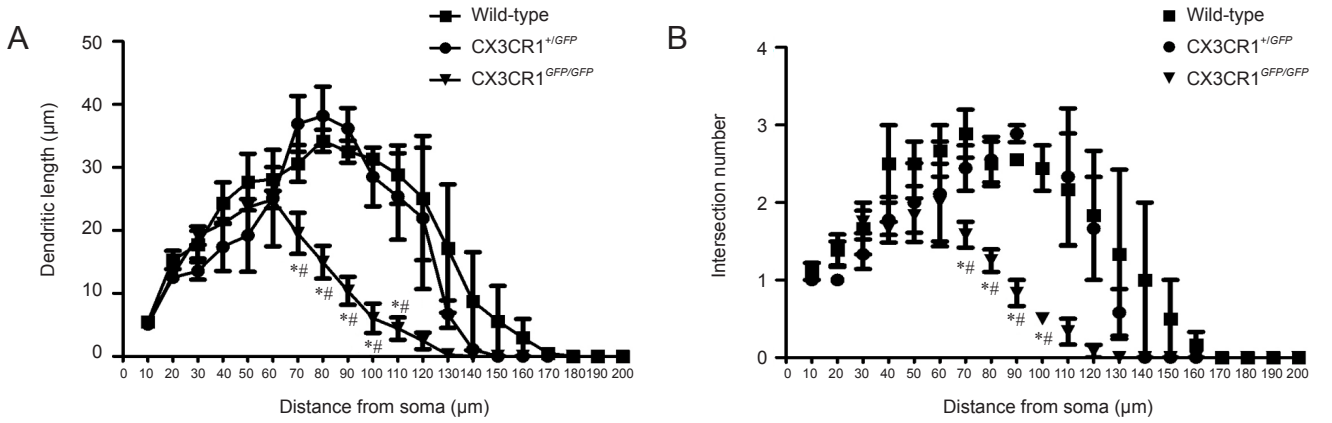

Figure 2 CX3CR1 deficiency reduces the dendritic complexity of newborn hippocampal neurons.
(A) Dendritic length; (B) number of intersections. All data are presented as the mean ± SEM (n = 4 mice in each group). Comparisons of multiple groups were done using one-way analysis of variance followed by Student-Newman-Keuls test. *P < 0.05, *vs.* CX3CR1$^{+/GFP}$ mice; #P < 0.05, *vs.* wild-type mice.

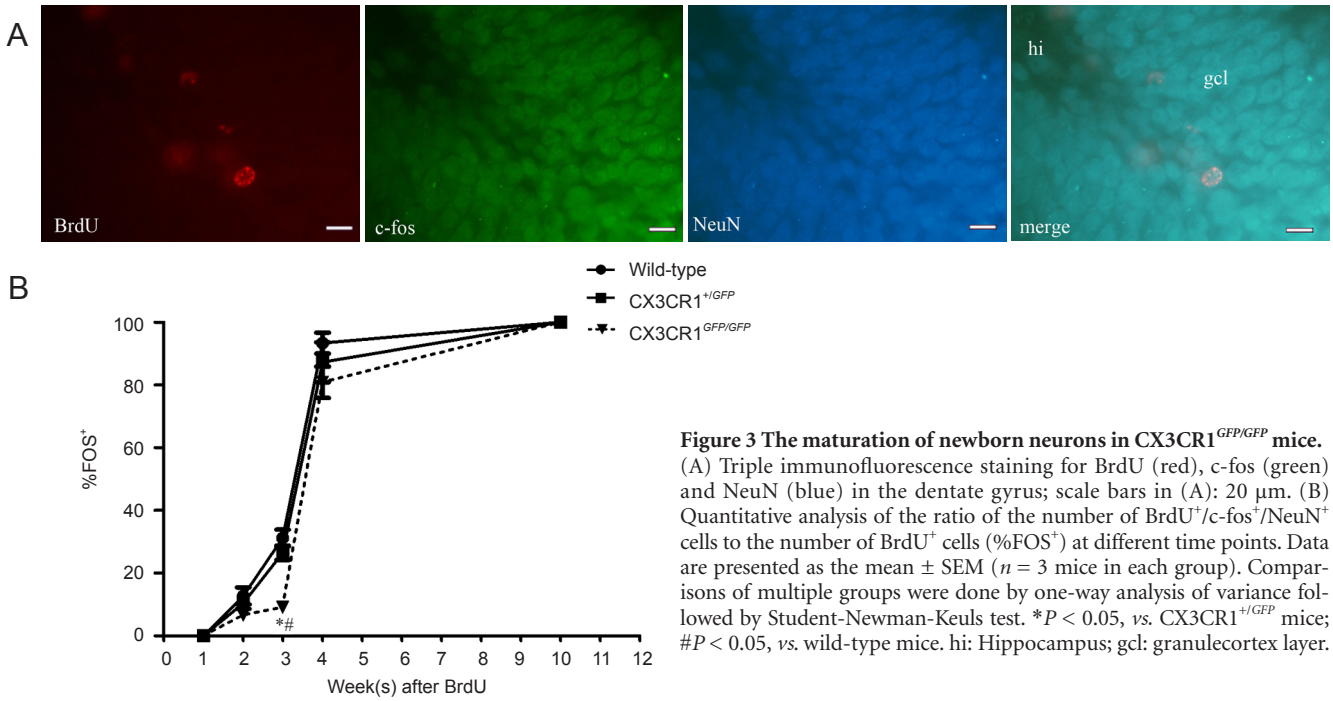

Figure 3 The maturation of newborn neurons in CX3CR1$^{GFP/GFP}$ mice.
(A) Triple immunofluorescence staining for BrdU (red), c-fos (green) and NeuN (blue) in the dentate gyrus; scale bars in (A): 20 μm. (B) Quantitative analysis of the ratio of the number of BrdU$^+$/c-fos$^+$/NeuN$^+$ cells to the number of BrdU$^+$ cells (%FOS$^+$) at different time points. Data are presented as the mean ± SEM (n = 3 mice in each group). Comparisons of multiple groups were done by one-way analysis of variance followed by Student-Newman-Keuls test. *P < 0.05, *vs.* CX3CR1$^{+/GFP}$ mice; #P < 0.05, *vs.* wild-type mice. hi: Hippocampus; gcl: granulecortex layer.

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