Research

NT-3 facilitates hippocampal plasticity and learning and memory by regulating neurogenesis

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In the adult brain, the expression of NT-3 is largely confined to the hippocampal dentate gyrus (DG), an area exhibiting significant neurogenesis. Using a conditional mutant line in which the *NT-3* gene is deleted in the brain, we investigated the role of NT-3 in adult neurogenesis, hippocampal plasticity, and memory. Bromodeoxyuridine (BrdU)-labeling experiments demonstrated that differentiation, rather than proliferation, of the neuronal precursor cells (NPCs) was significantly impaired in DG lacking NT-3. Triple labeling for BrdU, the neuronal marker NeuN, and the glial marker GFAP indicated that NT-3 affects the number of newly differentiated neurons, but not glia, in DG. Field recordings revealed a selective impairment in long-term potentiation (LTP) in the lateral, but not medial perforant path-granule neuron synapses. In parallel, the NT-3 mutant mice exhibited deficits in spatial memory tasks. In addition to identifying a novel role for NT-3 in adult NPC differentiation *in vivo*, our study provides a potential link between neurogenesis, dentate LTP, and spatial memory.

One of the two regions where adult neurogenesis occurs is at the dentate gyrus (DG) of the hippocampus (Gage 2000). The generation of new neurons in the adult dentate is a multistep process. Initially, stem cell-derived progenitor cells proliferate in the subgranular zone of the DG. These cells then migrate into the granule cell layer, where they differentiate into neurons and glial cells (Kempermann et al. 2004a). During the process, some of the differentiated and undifferentiated cells die. A subset of surviving new neurons, however, gradually develop functional neuronal properties, form new synapses, and integrate into the existing neural network (Song et al. 2002; van Praag et al. 2002). Defining diffusible factors that regulate this process constitutes one of the most active areas in neural stem cell biology (Gage 2000; Gould and Gross 2002).

Neurotrophin-3 (NT-3, also known as Ntf3) may serve as an excellent candidate as a diffusible factor that regulates dentate neurogenesis. The expression of the *NT-3* gene in the adult brain is highly confined to the dentate gyrus (Maisonpierre et al. 1990; Friedman et al. 1991; Lauterborn et al. 1994). Elevated neuronal activity, either by LTP-inducing stimuli or ischemia, enhances the expression of NT-3 mRNA in the dentate (Lindvall et al. 1992; Bramham et al. 1996). Cell culture experiments showed that NT-3 antagonizes the proliferative effects of basic fibroblast growth factor (bFGF) on progenitor cells, and enhances the differentiation of newborn neurons (Ghosh and Greenberg 1995; Vicario-Abejon et al. 1995; Barnabe-Heider and Miller 2003). In addition, dietary restriction enhanced the expression of NT-3 in dentate gyrus, paralleling the increase in the number of newborn neurons (Lee et al. 2000). However, direct evidence for the role of

endogenous NT-3 in the proliferation, survival or differentiation of hippocampal progenitor cells *in vivo* is lacking.

The functional significance of adult neurogenesis remains one of the key unsolved issues in neuroscience (Fuchs and Gould 2000; Kempermann et al. 2004b). Although there may be a correlation between neurogenesis and memory, the relationship remains controversial (Gould et al. 1999b; Lu and Chang 2005). Activities known to enhance learning and memory, such as exercise or living in an enriched environment, promotes neurogenesis in the adult hippocampus (Kempermann et al. 1997; Nilsson et al. 1999; van Praag et al. 1999a,b; Cotman and Berchtold 2002). Learning itself has also been shown to enhance the survival of newly generated neurons in the dentate (Gould et al. 1999a). The most direct evidence so far was demonstrated in an experiment in which inhibition of neurogenesis using a mitotic inhibitor impaired hippocampal-dependent, but not hippocampal-independent memory (Shors et al. 2001). Inhibition of neurogenesis by radiation also impaired hippocampus-dependent, place-recognition task, and spatial memory retention, but not object-recognition task (Madsen et al. 2003; Rola et al. 2004). An attractive hypothesis is that at least some forms of hippocampus-dependent memory are mediated by the generation of new neurons (Gould et al. 1999b; Kempermann 2002). How does this model reconcile the conventional belief that hippocampal LTP is a cellular model for hippocampus-dependent learning and memory? It is possible that newly generated neurons could contribute to the formation of new synapses, leading to alterations of LTP in the dendritic network of the dentate granule neurons. Indeed, running-enhanced neurogenesis is accompanied by a selective increase in dentate LTP (van Praag et al. 1999a). Newly generated granule neurons exhibit a lower threshold for LTP induction, but the magnitude of LTP is smaller when stronger stimulation is used (Schmidt-Hieber et al. 2004). The causal relationship between neurogenesis and LTP was implied in one study showing that inhibition of NPC proliferation drastically reduced LTP in the medial perforant path-dentate synapses (Snyder et al. 2001). It is unclear, however, whether inhibition of neurogenesis

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Figure 1. Cre-mediated recombination and ablation of the *NT-3* gene in adult hippocampus. (A) Nestin-Cre mediated recombination in adult hippocampus (2-mo old). Coronal section from P2.5 animal [Nes-Cre/+ (male) X floxedROSA-26^{ret/+} (female)] stained by X-gal (dark blue) to detect cre-mediated recombination at the *ROSA-26* locus and counterstained with Neutral red. Note the widespread and complete or near-complete recombination in hippocampus and cerebral cortex. (B) Southern blot showing Cre-mediated recombination in the hippocampus and cortex of adult mutant mice. DNA samples were prepared from brain tissues of mice of different genotypes. (Lane 1) *NT-3*^{1lox/2lox}, *Nes-cre*^{+/0}; (lane 2) *NT-3*^{2lox/+}, *Nes-cre*^{+/0}; (lane 3) *NT-3*^{1lox/2lox}, *Nes-cre*^{0/0}; (lane 4) *NT-3*^{1lox/2lox}, *Nes-cre*^{+/0}; (lane 5) *NT-3*^{2lox/+}, *Nes-cre*^{+/0}. Arrows: +: Wild-type allele, or *NT-3*⁺; f: Floxed allele, or *NT-3*^{lox}; R: knockout allele, or *NT-3*^{lox-}. (C) Northern blot analysis of mRNAs isolated from adult hippocampi of different genotypes. (Lane 1) *NT-3*^{+/+}, *Nes-cre*^{0/0}; (lane 2) *NT-3*^{+/+}, *Nes-cre*^{+/0}; (lane 3) *NT-3*^{1lox/2lox}, *Nes-cre*^{+/0}; (lane 4) *NT-3*^{1lox/2lox}, *Nes-cre*^{0/0}; (lane 5) *NT-3*^{+/+}, *Nes-cre*^{0/0}; (lane 6) *NT-3*^{2lox/+}, *Nes-cre*^{+/0}. (Bottom) rRNA loading control is shown below. Arrows point to the positions of 18S and 28S rRNAs.

could alter synaptic plasticity in the existing synaptic network, and if so, whether such alterations have behavioral consequences.

In the present study, the role of neurogenesis in hippocampal LTP and learning and memory was examined using a line of *NT-3* conditional knockout mice, in which the *NT-3* gene is selectively deleted in the brain throughout development. We tested whether *NT-3* is involved in the proliferation or differentiation of progenitor cells in the dentate. We further investigated the role of neurogenesis in synaptic plasticity of specific synaptic pathways of dentate neurons and the underlying mechanisms. Finally, we examined the behavior deficits of the mutant mice. Our results suggest that *NT-3* specifically regulates the differentiation, but not the proliferation of neuronal precursor cells in the dentate. Such regulation appears to be important for LTP in the lateral, but not medial perforant path-dentate synapses. These results may help us to understand how neurogenesis could contribute to long-term synaptic plasticity and memory.

Results

An *NT-3* conditional mutant mouse, created using the cre/loxP system as previously described (Bates et al. 1999, 2002; Akbarian et al. 2001), was used. To ensure that Nes-Cre is expressed in the hippocampus, we crossed Nes-Cre transgenic mice with a Rosa-26 reporter line that expresses β -galactosidase upon Cre-mediated removal of a stop codon (Soriano 1999). Strong expression was detected in the granule cell layer of the dentate gyrus and CA1–CA3 regions of the hippocampus (Fig. 1A). Southern blot was performed using cortical and hippocampal tissues from various genotypes. We generated mice with different genotypes by mat-

ing as previously described (Bates et al. 1999). We used the following nomenclature for the *NT-3* alleles: Recombined: *NT-3*^{1lox}; Floxed: *NT-3*^{2lox}; Wild-type: *NT-3*⁺ (Bates et al. 1999). Thus, the genotype of conditional mutant is *NT-3*^{1lox/2lox}, *Nes-cre*^{+/0}. We used two types of mice as controls, i.e., *NT-3*^{+/+}, *Nes-cre*^{0/0}, which is the wild-type and *NT-3*^{2lox/2lox}, *Nes-cre*^{0/0}, which is the floxed animals. In the hippocampus, the homologous recombination was more than 80% completed in the mutant mice (Fig. 1B). Moreover, Northern blot revealed that *NT-3* transcripts in the hippocampus from the *NT-3*^{1lox/2lox}, *Nes-cre*^{0/0} mice were essentially undetectable (Fig. 1C). *NT-3* ELISA was used to measure the levels of *NT-3* protein in adult hippocampus. We detected 5.35 ± 0.28 pg/20 μ g, and 15.50 ± 1.17 pg/100 μ g *NT-3* protein from the wild-type tissues, but none from the mutant tissues.

Proliferation and survival of BrdU-positive cells in dentate gyrus

Brains from our conditional mutant mice appeared normal (data not shown) except for a defect in cerebellar foliation, which is consistent with previous reports (Bates et al. 1999, 2002; Akbarian et al. 2001). Examination of hippocampal sections did not reveal any gross morphological abnormality (Fig. 2A). Since the endogenous *NT-3* gene expression in adult was relatively confined to the dentate gyrus of the hippocampus (Maisonpierre et al. 1990; Lauterborn et al. 1994), we examined cytoarchitectural organization of this region in some detail. The volumes of the granule cell layer (GCL), hilus, the whole dentate gyrus (DG), or

Figure 2. Normal morphology of adult hippocampus in *NT-3* mutant mice. (A) Low power ($4\times$) images of hippocampus from an *NT-3* mutant mouse. H&E staining reveals no abnormality of morphology of the hippocampus. (B) Quantitative measurement of the volumes of the subregions of hippocampus. The volumes of granule cell layer (GCL), the hilar region, the whole dentate gyrus (DG), and the whole hippocampus were measured. $n = 6$ pairs of male mice; 2-mo old. (C) Summary of cell counts. Total number of granule cells in DG is presented. $n = 5$ pairs of 2-mo-old male mice.

the whole hippocampal region (Hippo) in the mutant mice were almost identical to that of wild-type control (Fig. 2B). For cell counting, hippocampal sections were stained by the H&E method. Stereological analyses showed that there was no statistical difference in the number of granule neurons per dentate gyrus between wild-type and mutant mice (Fig. 2C). Thus, inactivation of the *NT-3* gene does not result in obvious anatomical changes in the adult hippocampus.

Since NT-3 has been implicated in neurogenesis (Ghosh and Greenberg 1995; Vicario-Abejon et al. 1995), we asked whether deletion of the *NT-3* gene could affect the proliferation or survival of neuronal precursor cells (NPCs) in adult dentate. To examine NPC proliferation, we pulse labeled the proliferating NPCs by injecting intraperitoneally bromodeoxyuridine (BrdU) 3 h before the brains were processed for BrdU immunohistochemistry. In this method, only a fraction of dividing cells were labeled, but the labeled cells would not have sufficient time to undergo more than one round of mitosis. BrdU(+) cells were found in both wild-type and mutant dentate, mostly beneath the granule cell layer with a few also in the hilus (Fig. 3A). There was no difference in the number of proliferating cells between wild-type and mutant mice (Fig. 3B). To examine the survival of the BrdU-labeled NPCs, we injected BrdU twice daily for 10 d so that a large proportion of dividing NPCs were labeled. In the remaining 2 wk after the last BrdU injection, many NPCs underwent apoptosis. The surviving NPCs were detected by BrdU immunohistochemistry. We found that there was a small decrease in the number of BrdU(+) cells in the granule cell layer of the mutant mice, albeit barely significant compared with the wild-type mice (Fig. 3C) ($P = 0.08$, t -test). No difference was detected in the hilus area. Taken together, these results suggest that NT-3 may regulate the survival, but not the proliferation, of NPCs in the dentate.

To determine whether NT-3 differentially affects the differentiation of the surviving NPCs into neurons and glia, we performed triple staining using the same sections derived from the

NPC survival study described above. BrdU was detected by a rat anti-BrdU antibody (green). A mouse anti-NeuN antibody (red) was used to label mature neurons, while a rabbit anti-GFAP antibody (purple) was used to label astrocytes. Fluorescence microscopy using three different wavelengths showed that BrdU, NeuN, and GFAP could be clearly detected in the same section (Fig. 4A). Cell counting using stereological techniques showed that the number of BrdU(+) neurons was significantly reduced, while the number of BrdU(+) astroglia was not changed in the R/f, + mutant mice (Fig. 4B). Pie plots indicated that in the R/f, + mutant, the decrease in the number of neurons was accompanied by an increase in the number of undifferentiated cells (labeled "Neither" in Fig. 4C). These results suggest that in addition to NPC survival, NT-3 may affect maturation, or fate determination (to become a neuron or a glia) of NPCs.

Synaptic transmission and plasticity

NT-3 has been shown to both induce differentiation of hippocampus-derived NPCs and to facilitate synaptogenesis in cultured hippocampal neurons (Vicario-Abejon et al. 1998, 2000). We therefore examined whether the deficits in the survival and/or differentiation of dentate NPC in NT-3 mutant mice would result in impairments in LTP. Two major sets of excitatory synapses on the dendrites of dentate granule neurons were studied, i.e., one made by lateral perforant path (LPP) and the other by medial perforant path (MPP). By carefully positioning the stimulating and recording electrodes, we could induce LTP at LPP and MPP synapses separately (Fig. 5A). Application of tetanic stimulation (4×0.5 sec, 50 Hz, 30-sec interval) to MPP induced a robust LTP in both wild-type and mutant MPP synapses, with very similar magnitudes (Fig. 5B, bottom). In contrast, the LTP recorded at LPP synapses in mutant mice exhibit a severe deficit, as compared with that seen in wild-type mice (Fig. 5B, middle). The magnitude of LTP measured 60 min after tetanus (4×0.5 sec, 50 Hz, 30-sec interval) was $156.3 \pm 9.6\%$ in wild-type mice, but $121.2 \pm 9.1\%$ in R/f, + mice, respectively ($P < 0.001$, t -test). As a control, we also recorded LTP at the Schaffer collateral-CA1 synapses. Tetanic stimulation (2×1 sec, 100 Hz, 20-sec interval) induced identical LTP in wild-type and mutant mice (Fig. 5B, top). Thus, deletion of *NT-3* gene selectively impairs LPP-granule cell synapses in hippocampal dentate.

LTP at dentate synapses is facilitated by inhibiting GABA_A receptor-mediated transmission (Hanse and Gustafsson 1992). Since NT-3 has been shown to attenuate GABAergic transmission in relatively mature neurons (Kim et al. 1994), one potential mechanism by which NT-3 enhances LTP is to suppress GABAergic inputs to the granule neurons. If the LPP-LTP deficit seen in NT-3 mutant mice was due to an increased GABAergic inhibition, one would expect no difference between wild-type and mutant mice when GABAergic transmission is completely blocked. To test this possibility, we recorded LPP-LTP in the presence of GABA_A antagonist bicuculline (10 μ M). Unlike MPP synapses recorded in the submerged chamber (Snyder et al. 2005), application of bicuculline did not seem to dramatically in-

Figure 3. Effects of NT-3 deletion on proliferation and survival of NPC. (A) Medium-power (20 $\times$) images of BrdU-labeled cells in the wild-type (top) and mutant (bottom) dentate gyrus. The sections were processed for BrdU immunohistochemistry followed by Nissl counter stain. (B) Effect on proliferation of NPCs. Three hours after BrdU was injected intraperitoneally, the brains of the injected animals were fixed and processed for BrdU histochemistry. The number of BrdU(+) cells in the hilus and granule cell layer (GCL) were counted using stereological techniques. $n = 3$ pairs of male mice; 4-mo old. (C) Effect on survival of NPCs. BrdU was injected twice daily for 10 d, and BrdU immunohistochemistry was performed 2 wk after the last BrdU injection. Cells were counted the same way as B. $n = 6$ pairs of male mice; 2-mo old.

Figure 4. Effect of NT-3 deletion on NPC-derived neurons and glia. Wild-type and mutant mice were injected with BrdU twice/day for 10 d. Two weeks later, the animals were processed for triple labeling for BrdU (green), NeuN (red), and GFAP (blue), using confocal microscopy. (A) Confocal images of BrdU-positive neurons (left) and astroglia (right). (B) Quantitative analysis of the number of NPC-derived neurons (left) and that of glia (right) in the dentate of control and mutant mice. $n = 6$ pairs of male mice; 2-mo old. (*) Significantly different between control and mutant mice, $P < 0.01$, Student's t -test, two-tailed. (C) Relative distribution of cells derived from NPCs. $n = 6$ pairs of male mice; 2-mo old.

crease the magnitude of LTP in LLP synapses recorded in the interface chamber (Fig. 5C). Surprisingly, the difference in LTP between the wild-type and mutant mice was much bigger when slices were treated with bicuculline, as compared with that without bicuculline (Fig. 5B,C). As an additional control, we recorded LTP in the floxed mice, which contains floxed NT-3, but not Cre-recombinase ($NT-3^{2lox/2lox}$, $Nes-cre^{0/0}$). The LTP in these mice were almost identical to that in wild-type mice (Fig. 5C). Given that NT-3 has also been shown to enhance GABAergic transmission, which in developing neurons is excitatory rather than inhibitory (Gao and van den Pol 1999), deletion of the NT-3 gene may reduce the excitatory effects of GABA, leading to impairments of LTP at the LPP synapses made by the NPC-derived cells.

We performed a series of experiments to investigate the potential mechanisms underlying the LTP deficit seen at LPP synapses. First, we examined the basal synaptic transmission. Field EPSPs were evoked by stimulating LPP with increasing stimulus intensity. The slope of EPSPs was plotted against stimulus intensity to establish input-output relationships. No difference was observed in the input-output relationships in wild-type and mutant synapses (Fig. 6A), suggesting that the deletion of the NT-3 gene does not alter basal synaptic transmission. Next, we examined post-tetanic potentiation (PTP), a phenomenon thought to be due to enhanced presynaptic transmitter release (Zucker 1989). PTP was induced by tetanic stimulation in the presence of NMDA receptor antagonist APV. The magnitude of PTP at 1 min after tetanus, defined as a percentage increase of the average EPSP slope over baseline, was significantly reduced in slices from R/f, + mice ($129.1 \pm 6.0\%$), as compared with the ones from +/+ animals ($148.1 \pm 6.9\%$, Fig. 6C). Since changes in PTP reflect pre-synaptic problems, we further examined paired-pulse facilitation (PPF), a form of plasticity that reflects changes in the probability of transmitter release (Zucker 1989). PPF was measured as the ratio of two consecutive EPSP slopes. At the LPP synapses, the

ratio is usually greater than one. PPFs at interpulse intervals of 10, 20, and 50 msec were markedly reduced in the R/f, + mice as compared with wild-type mice (Fig. 6B), suggesting an alteration in pre-synaptic properties. This result is consistent with previous reports, which used $NT-3^{+/-}$ mice (Kokaia et al. 1998; Asztely et al. 2000). Finally, to determine whether NT-3 gene deletion also affects the number of synaptic vesicles in the reserved and readily releasable pools, we measured synaptic responses to a prolonged, low-frequency stimulation (LFS) and a brief, high-frequency stimulation (HFS), respectively (Cabin et al. 2002). As shown in Figure 6, D and E, no differences were observed between wild-type and mutant mice in either response. These results suggest that the deletion of the NT-3 gene does not alter synaptic vesicle distribution.

Behavioral test

To determine whether the impaired dentate neurogenesis and deficits in LPP-LTP described above are associated with behavioral consequences, we subjected NT-3 mutant mice to the Morris water-maze test. Briefly, mice were allowed to swim in a circular pool filled with opaque water and trained to find a hidden platform, which was kept at a constant location, using external visual cues. To test the learning capability, we measured escape latency (time to find the hidden platform), which became progressively shortened during a 7-d training period (twice daily) in wild-type mice (Fig. 7A). In marked contrast, the NT-3 mutant mice exhibited significantly longer latency times to find the hidden platform (Fig. 7A), indicating that they had a severe problem learning. Overall, the escape latency of NT-3 mutant mice improved much slower relative to wild-type mice during the 7 d of training.

At the end of the learning period, the platform was removed and the animals were given a 60-sec probe trial to test their memory, which was measured by the percentage of time they spent in the platform quadrant (Fig. 7B). When the probe trial was administered 3 d after the end of training, wild-type mice spent more time in the correct quadrant (platform, 43%) than all other quadrants. In contrast, NT-3 mutant mice spent only 9.1% of their time in the platform quadrant (Fig. 7B), indicating that their ability to remember the correct quadrant was severely compromised. Interestingly, the mutant mice also spent more time in the left quadrant (40%). It is unclear whether this reflects a trend in which the mutant animals tend to stay in areas closer to the platform. The memory capacity can also be reflected by the number of times the animals crossed the platform area and the distance the animals traveled in the correct quadrant after the platform was removed. Figure 7C depicts examples of the swimming tracks by a wild-type and a mutant mouse. The wild-type mouse swam a significantly longer distance in the correct quadrant than the mutant mouse. Moreover, the wild-type mouse crossed the platform areas about six times in a minute, while the mutant crossed only once per minute.

Recently, NT-3 mutant mice generated by a different cre/loxP system were shown to have defects in geniculocortical projections, leading to impairments in visual function (Ma et al.

Figure 5. NT-3 regulates synaptic plasticity at a subset of hippocampal synapses. (A) Schematic showing the arrangement of stimulating and recording electrodes in CA1 (top) and DG (bottom). (B) LTP recordings in the CA1 region (top), the lateral perforant path (LPP) (middle), and the medial perforant path (MPP) (bottom). Tetanic stimulation was applied at time "0". Data from multiple slices (n) were pooled and expressed as mean $\pm$ SEM. (C) LTP deficit in LPP synapses in the presence of GABA_A antagonist bicuculline (10 μ M). Mice with the NT-3 gene floxed (NT-3^{2lox/2lox}, Nes-cre^{0/0}, indicated as flox/flox) were studied as an additional control. Number of mice (N) and slices (n) are indicated in the figure. All mice used in electrophysiology and behavior experiments were between 1.5 and 2.5-mo old.

2002). Using the same visual cliff avoidance test, we found no such impairment in visual function in our mutant mice. Both wild-type and mutant mice spent most of their time on the covered (bench) side of the visual cliff box without crossing the cliff edge. The time spent on the open (suspended) side was $17.8 \pm 15.3\%$ for NT-3 mutant and $27.7 \pm 12.8\%$ for control mice ($n = 5$ pairs, $P > 0.05$). These results suggest that our NT-3 mutant mice have relatively normal visual function. We also measured locomotor activity of the mice using a Digiscan activity monitor (a 16-inch square chamber equipped with two rows of 16×16 infrared sensors). These sensors were used to localize the animal's floor position on an x - y plane, and automatically measure their horizontal and vertical movements. As shown in Figure 7D, there was no statistical difference in any of the parameters measured between the wild-type and NT-3 mutant mice. Specifically, the mutant mice did not show any abnormality in total distance traveled, horizontal and vertical beam breaks, or time spent at the edge and center of the box. Thus, the cognitive deficits in NT-3 mutant mice are not likely to be accounted for by motor or visual deficits.

Discussion

NT-3 stimulates NPCs to differentiate into neurons in culture (Ghosh and Greenberg 1995; Vicario-Abejon et al. 1995). Deletion of the NT-3 receptor TrkC reduces the number of entorhinal afferents and the density of synaptic contacts in postnatal dentate (Martinez et al. 1998). However, since BrdU-labeling experiments were not performed, it is unclear whether the synaptic effects were due to facilitation of differentiation of NPC-derived neurons by NT-3. Our study demonstrates that the number of BrdU-labeled dentate cells in the NT-3 mutant mice are reduced.

More importantly, of the surviving BrdU cells in the NT-3 mutants, the number of NeuN-labeled neurons, but not GFAP-labeled glia, was significantly decreased, while the number of undifferentiated cells was increased. These results suggest that NT-3 is required to stimulate NPCs to differentiate into new neurons in the adult dentate in vivo.

An important, yet unresolved issue in the neural stem-cell field is the biological function of adult neurogenesis (Kempermann 2002; Kempermann et al. 2004b). It has long been speculated that adult neurogenesis is involved in learning and memory (Gould et al. 1999b), but how newly generated neurons contribute to learning and memory is not known. Inhibition of NPC proliferation in the adult dentate by either acute radiation (Madsen et al. 2003; Rola et al. 2004) or mitotic inhibition using MAM (Shors et al. 2001), leads to significant impairments in hippocampus-dependent memory. Interestingly, spatial learning, as assessed by the escape latency in the water-maze training, was not affected. Two caveats are noted. First, drugs such as MAM may interfere with protein synthesis or signaling mechanisms required for memory, or cause cytotoxic effects, and radiation may damage the stem-cell niche in the subgranular zone (Monje et al. 2002). Second, the mitotic inhibitor or radiation affects only the proliferation, but not differentiation, of the NPCs. In this study, we show that deletion of the NT-3 gene affects the differentiation, but not proliferation of NPCs. This apparently affects both learning and memory, as assessed by spatial navigation tests. Similarly, mice lacking methyl-CpG-binding protein 1 (MBD1) show deficits in NPC differentiation but not proliferation, as well as impairments in both learning and memory in water-maze tests (Zhao et al. 2003). These results raised the interesting possibility that the differentiation, but not proliferation of NPCs might somehow be involved in the spatial learning process.

Figure 6. Impairments in presynaptic function of LPP-dentate synapses in NT-3 mutant mice. Numbers of slices recorded are indicated in the respective figures. (A) Input-output curves. No difference was observed between control and mutant mice. (B) Paired pulse facilitation (PPF). NT-3 mutant mice exhibit a substantial reduction in PPF at shorter interstimulus intervals. (C) Post-tetanic potentiation (PTP). PTP was induced by a tetanus (100 Hz, 1 sec) in the presence of NMDA receptor blocker (APV, 100 μ M). There was a small, but significant decrease in PTP in the NT-3 mutant mice. (D) Synaptic fatigue. Synaptic responses to a brief, high-frequency stimulation (HFS, 100 Hz, 20 pulses) were recorded. The slopes of all EPSPs were normalized to that of the 2nd EPSP. (E) Synaptic response to a prolonged, repetitive stimulation (LFS, 300 stimuli at 14 Hz, 280 pulses).

The present study also provides a potential connection between adult neurogenesis and dentate LTP, and therefore a possible mechanism by which neurogenesis could contribute to hippocampus-dependent learning and memory. Since dentate LTP is believed to be critical for hippocampus-dependent memory (Richter-Levin et al. 1995; Abraham and Williams 2003), it is conceivable that adult neurogenesis participates in hippocampus-dependent learning and memory by regulating LTP at synapses in the dentate gyrus. Consistent with this idea, Wojtowicz and colleagues have shown that inhibition of neurogenesis by radiation selectively blocks ACSF-LTP in MPP, a form of LTP attributable to newborn young neurons (Snyder et al. 2001). The MBD1 mutant mice exhibit a parallel reduction in neurogenesis and an impairment in LTP at the MPP synapses (Zhao et al. 2003). We have now shown that deficits in NPC differentiation caused by a deletion of the *NT-3* gene in the brain impairs LTP selectively at the LPP synapses. While we do not know whether the LPP-LTP is also impaired in the MBD1^{-/-} mice, the selective impairment of LTP at the lateral, but not medial perforant path in the NT-3 mutant mice could be explained by a number of mechanisms. One possibility is that NT-3 may be required for dendritic growth and branching. Deletion of the *NT-3* gene may therefore prevent newborn granule cells from extending their apical dendrites beyond MPP into LPP. Alternatively, NT-3 may selectively regulate the growth of LPP afferents. The deficits in PPF and PTP at the LPP synapses in the NT-3 mutant mice support this notion. Regardless of the mechanism, the normal LTP

seen at the CA1 synapses in the NT-3 mutant mice suggest that NT-3 does not have a general effect on synapse formation in vivo. Consistent with this, LTP at the CA1 synapses was normal in another line of NT-3 conditional mutant mice that were generated by crossing NT-3 floxed mice with Cre transgenic mice, where the Cre gene was controlled by the synapsin I promoter to restrict expression to postnatal neurons (Ma et al. 1999). Finally, it should be pointed out that our results, albeit more specific than radiation or drug treatment, are still correlative in nature. DG-specific deletion of the *NT-3* gene in adult mice should provide further insights into the relationship between neurogenesis, dentate plasticity, and memory.

While we interpret our results as indicating that neurogenesis plays an important role in dentate LTP, an alternative is that NT-3 facilitates LPP-LTP directly. The effect of NT-3 on hippocampal plasticity has been examined by a number of groups. NT-3^{+/-} mice exhibit essentially normal short- and long-term plasticity at the dentate synapses (Asztely et al. 2000; Olofsdotter et al. 2000). The only deficit observed was a reduction in PPF at the LPP synapses (Kokaia et al. 1998; Asztely et al. 2000). An important observation is that acute application of recombinant NT-3 or anti-NT-3 antibodies had no effect on LTP at the CA1 synapses (Figurov et al. 1996; Chen et al. 1999). These results suggest that NT-3 does not modulate synaptic transmission or plasticity directly.

Although we cannot rule out a developmental effect of NT-3 on NPCs, since the *NT-3* gene is deleted during development, the fact that the DG volume and the total number of granule neuron were not changed suggests that NT-3 is not essential for embryonic neurogenesis or the survival of the DG granule neurons during development.

Assuming adult neurogenesis does regulate dentate LTP, how do newly generated neurons contribute to hippocampus-dependent memory? An attractive hypothesis is that new neurons have special properties that allow them to integrate into the existing neural network, leading to an alteration of synaptic plasticity. It has been shown that young neurons form immature synapses at MPP (Wang et al. 2000; Snyder et al. 2001). These synapses exhibit lower threshold for LTP induction, but the magnitude of LTP is smaller when stronger stimulation is used (Snyder et al. 2001; Schmidt-Hieber et al. 2004). Newly generated neurons form new synapses, which are integrated into the existing neural network (Kempermann 2002). Our field-recording experiments showed that LTP in the LPP, but not MPP, was selectively impaired in the NT-3 mutant mice. Why alteration of NPC differentiation affects only LPP but not MPP synapses is not known. An important part of the neuronal differentiation program is to grow elaborate dendritic trees. We speculate that the newly generated neurons in the mutant mice may grow limited dendritic trees that fail to appropriately contact the LPP. In addition, the synaptic connections made by the lateral entorhinal afferents may also be weak. It is conceivable that impairments in

Figure 7. Deficits in spatial learning and memory in NT-3 mutant mice. (A) Deficits in acquisition. The animals were trained twice a day for 7 d to find the hidden platform. The latency to find the platform is plotted against the training days. The wild-type mice rapidly learned the location of the submerged platform, whereas the mutant mice showed a much slower learning curve (ANOVA, $P < 0.05$). (B) Deficits in spatial memory. The probe test conducted at the end of training period. The platform was removed and the animals were allowed to swim freely in the pool for 60 sec. The percentage of time the animals spent in each of the four quadrants was measured. The mutant mice spent less time in the platform quadrant than wild-type mice (ANOVA followed by post hoc test, $P < 0.002$). (C) Representative swim paths illustrate the impairment of spatial cognition during the probe trial in the mutant mice. (D) Open field activity. Locomotor activities were measured every 10 msec for 10 min. Unit of measurements: time, sec; distance, cm; beam break, number of times.

the formation of new synapses contribute to the deficits in field LPP-LTP. Further experiments are necessary to test these ideas.

Materials and Methods

NT-3 conditional knockout mice

An NT-3 loxP line was crossed with a transgenic line, in which Cre gene expression was under the control of the Nestin promoter, as previously described (Bates et al. 2002). The following nomenclature was used in the present study. Wild-type, NT-3^{+/+} = +; Floxed, NT-3^{2lox} = f; Recombined, NT-3^{1lox} = R. Cre positive: +; Cre negative, 0. For mating, we crossed NT-3^{1lox/2lox}, *Nes-cre*^{+/0} males with NT-3^{2lox/2lox}, *Nes-cre*^{0/0} females to generate NT-3^{1lox/2lox}, *Nes-cre*^{+/0} offspring. We also crossed NT-3^{2lox/2lox}, *Nes-cre*^{0/0} males with NT-3^{2lox/2lox}, *Nes-cre*^{0/0} females to generate more NT-3^{2lox/2lox}, *Nes-cre*^{0/0} offspring. A third type of mating used animals of purely wild types, with the same genetic background as the first two. In general, NT-3^{1lox/2lox}, *Nes-cre*^{+/0} (MT) and wild-type (WT, in the

same genetic background) mice were used for experiments. For some experiments, NT-3^{2lox/2lox}, *Nes-cre*^{0/0} (floxed, in the same genetic background) mice were used as further control. Only male animals were used for experiments. Genotyping, Southern and Northern blotting, and X-gal staining were performed as described (Bates et al. 2002). In all experiments, male, 2–4-mo-old mice were used.

BrdU labeling

BrdU (Sigma) was dissolved in 0.9% NaCl and sterile filtered through 22- μ m filters. The mice received a single dose of BrdU at 100 μ g/gm of body weight from a concentration of 10 mg/mL, one intraperitoneal injection per day for 10 consecutive or for 1 d. Three hours or 2 wk after the last injection of BrdU, the mice were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were post-fixed overnight, followed by transferring to 30% sucrose solution. The brain was mounted on a pedestal using Tissue-Tek O.C.T. compound and cut at 40- μ m sections using a cryostat. The sections were stored at -80°C until use. The 3-h time point was selected because it is sufficient for BrdU uptake but not for completion of mitosis or migration.

Immunohistochemistry

All staining was done on free-floating 40- μ m sections that were derived from brains of BrdU-injected animals. To count the number of BrdU-positive cells, BrdU staining was performed using the ZYMED BrdU staining protocol (Zymed Laboratories Inc.) as described previously (Zhou et al. 2003). Antibodies used for immunofluorescent triple labeling of BrdU, NeuN, and GFAP, were rat anti-BrdU (Accurate, Harlan Sera-Lab), 1:100; mouse anti-NeuN (Chemicon), 1:200; and rabbit anti-GFAP (Chemicon), 1:200, respectively. The fluorescent secondary antibodies used were anti-rat Alexa Fluor 488, anti-mouse Alexa Fluor 568, and anti-rabbit Alexa Fluor 647 (Molecular Probe), 1:250.

Cell counting

Images were acquired using an Olympus IX 70 microscope equipped with a video camera and the IPlab software (Scanalytics). A 40- μ m 1-in-6 series of section from mouse was used to quantify the area of hippocampus and the number of positive cells using stereological methods combined with appropriate correction procedures (Gundersen et al. 1988; Coggeshall and Lekan 1996; Guillery and Herrup 1997; Kempermann et al. 1998). The granule cell areas of each section were carefully traced in granule cell layer (GCL), hilus, dentate gyrus, and whole hippocampus. The total areas multiplied the sampled distance to determine the reference volume. For cell counting, histological staining was performed on sections using hematoxylin and eosin (H&E) staining method. The total granule cell number was estimated by the mean positive cell number multiplied by the reference volume.

Triple-labeled fluorescence images were acquired using BioRad MRC 1024 confocal microscopy and Lasersharp 2000 software (Bio-Rad Laboratories). In total, 100–200 BrdU-positive cells per animals were analyzed for coexpression of BrdU

and NeuN for neuronal phenotype and GFAP for glial phenotype.

Electrophysiological recording

Transverse hippocampal slices were prepared from 2-mo-old animals, maintained in an interface chamber for both recovery (>2 h) and recording, as described previously (Bramham and Sarvey 1996; Pozzo-Miller et al. 1999). Field EPSPs were evoked in CA1 stratum radiatum by stimulating Schaffer collaterals in CA1 region, or in molecular layer by stimulating perforant pathways in DG with Teflon-insulated monopolar electrodes. A brief, high-frequency stimulation (HFS, 100 Hz, 20 pulses) was used to study docked vesicles, and a prolonged, low-frequency stimulation (LFS, 14 Hz, 280 pulses) to study reserved pool vesicles. Tetanus used to induce LTP: CA1 synapses, 100 Hz, 1 sec, two times with a 20-sec interval; DG synapses, 100 Hz, 0.5 sec, four times, with a 30-sec interval. Two points are worth mentioning with regard to LTP in the dentate. First, unlike recordings obtained from the submerged chamber (Snyder et al. 2005), the magnitude of LTP from either lateral (LPP) or medial (MPP) perforant pathway in the interface chamber was much bigger. We adjusted the positions of stimulating and recording electrodes to record fEPSPs for MPP or LPP and intensity of tetanus to induce DG LTP, as described previously (Dahl and Sarvey 1989). Second, unlike that in the submerged chamber, the LTP recorded from LPP in the interface chamber is not sensitive to inhibition of GABAergic transmission (see Fig. 5C).

Behavior tests

1. Morris water maze. A circular pool (diameter: 0.9 m) filled with cloudy water was placed in a quiet room with easily identifiable objects on the wall. A circular platform (12 cm in diameter) was submerged 1.5 cm below the water surface and placed in a specific location in the pool. Animals were subjected to four trials a session, two sessions a day. A total of 6 d of training were given. In each of the four trials, animals were placed at four different starting positions equally spaced around the perimeter of the pool in a random order. Animals were allowed to find the platform within 120 sec, or else were guided to the platform. At the end of training, each mouse was given a probe trial (60 sec. with no platform). Memory function was evaluated by the amount of time spent on the correct (platform) quadrant, the distance traveled, and the number of times the animal crossed the platform area.
2. Visual cliff test. An open-topped box (60 × 60 × 30 cm³, with clear Perspex bottom) was positioned on the edge of a bench such that half of its base was suspended 1 meter above the ground, creating a visual cliff. Mice were placed at the edge of the cliff, and their activity monitored for 5 min by a video camera. The percentage of time each mouse spent at the edge, bench side, and open (suspended) area, and the direction in which the first step outside of the edge, were measured.
3. Open-field activity. Animals were individually placed in a Digiscan open-field box (RXYZCM, AccuScan Instruments, Inc.) equipped with two rows of infrared beams that automatically detected and quantified their activities every 10 msec for 10 min. This equipment took measurements of total distance traveled, horizontal and vertical beam breaks, horizontal and vertical time spent, resting time, edge and center time, edge and center distance traveled, and time spent in the left-front, left-rear, right-front, and right-rear areas.

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NT-3 facilitates hippocampal plasticity and learning and memory by regulating neurogenesis

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