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Depletion of central BDNF in mice impedes terminal differentiation of new granule neurons in the adult hippocampus

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Abstract

Granule neurons generated in the adult mammalian hippocampus synaptically integrate to facilitate cognitive function and antidepressant efficacy. Here, we investigated the role of BDNF in facilitating their maturation *in vivo*. We found that depletion of central BDNF in mice elicited an increase in hippocampal cell proliferation without affecting cell survival or fate specification. However, new mutant neurons failed to fully mature as indicated by their lack of calbindin, reduced dendritic differentiation and an accumulation of calretinin⁺ immature neurons in the BDNF mutant dentate gyrus. Furthermore, the facilitating effects of GABA_A receptor stimulation on neurogenesis were absent in the mutants, suggesting that defects might be due to alterations in GABA signaling. Transcriptional analysis of the mutant hippocampal neurogenic region revealed increases in markers for immature neurons and decreases in neuronal differentiation facilitators. These findings demonstrate that BDNF is required for the terminal differentiation of new neurons in the adult hippocampus.

Introduction

The hippocampus is a site of active neurogenesis throughout life in mammals, including humans (Kempermann et al., 1997; Eriksson et al., 1998; Gould et al., 1999). New granule neurons generated through this form of brain plasticity functionally integrate into the dentate gyrus to facilitate cognitive function (van Praag et al., 2002; Ramirez-Amaya et al., 2006). Indeed, interfering with this process hinders some forms of hippocampal-dependent learning, long-term potentiation and antidepressant efficacy (Santarelli et al., 2003; Snyder et al., 2005; Saxe et al., 2006). Adult neurogenesis is dynamically regulated by environmental manipulations, neurotransmitters and endocrine and growth factors (Warner-Schmidt and Duman, 2006). However, the signaling mechanisms that influence the proliferation, survival and differentiation of progenitors in this brain area have not been fully elucidated. Because of their established roles in the developing nervous system, brain-derived neurotrophic factor

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(BDNF) and its receptor TrkB quickly emerged as candidate modulators of adult neurogenesis. However, the intricacies of their actions remain unknown.

BDNF appears to contribute to the neurogenic milieu that supports precursors in the adult hippocampus. Evidentiary is the finding that BDNF^{+/-} mutant mice exhibited reduced survival of new cells in this region (Sairanen et al., 2005). Furthermore, BDNF haploinsufficiency suppressed the enhancing effects of dietary restriction and antidepressant treatment on hippocampal neurogenesis (Lee et al., 2002; Santarelli et al., 2003). The subgranular zone (SGZ) of the dentate gyrus contains two distinct populations of cycling progenitors (type 1 and 2 cells) and newly post mitotic cells in different stages of differentiation (Ambrogini et al., 2004; Garcia et al., 2004). Type 1 precursors are putative stem cells that express glial fibrillary acidic acid (GFAP) and give rise to type 2 progenitors, which are neuronal lineage-restricted precursors and contain GABA_A receptors (Seri et al., 2001; Fukuda et al., 2003; Garcia et al., 2004). Because of their high intracellular levels of chloride, GABA exerts a depolarizing effect on these cells, promoting their exit from the cell cycle and differentiation into granule neurons (LoTurco et al., 1995; Wang et al., 2000; Ge et al., 2005; Tozuka et al., 2005; Wang et al., 2005).

It remains unclear at which stages BDNF might act to modulate development of neural precursors in the adult hippocampus and whether its actions influence cell fate specification and terminal differentiation of these cells *in vivo*. It was recently reported that TrkB expression in adult-generated hippocampal neurons increases as these cells develop (Donovan et al., 2008). As BDNF mediates neuronal differentiation during early development (Patapoutian and Reichardt, 2001), it is plausible that it acts on newly-post mitotic cells to direct their maturation into functional granule neurons in the adult hippocampus. Moreover, because this neurotrophin plays a critical regulatory role in GABAergic signaling in the hippocampus and other brain areas (Brunig et al., 2001; Elmariah et al., 2004; Henneberger et al., 2005), its actions could involve facilitating the effects of GABA. To test this, we examined adult hippocampal neurogenesis in mutant mice with depletion of central BDNF. We found that they exhibited deficits in the terminal differentiation, dendritic complexity and migration of new granule neurons and blunted GABA_A-mediated effects on neurogenesis. These findings demonstrate that BDNF is an essential constituent of the neurogenic microenvironment in the adult hippocampus.

Results

Proliferating and post-mitotic neuronal precursors in the adult hippocampus contain TrkB receptors

Little is known regarding the effects of BDNF on actively dividing cells in the adult dentate gyrus or on new cells that recently exited the cell cycle to follow a neuronal differentiation pathway. To gain insight into the actions of BDNF, we examined TrkB expression in cells at different developmental stages in the adult hippocampus as an indicator of their ability to respond to this neurotrophin. To distinguish proliferating cells in wild type mice, we administered BrdU, a thymidine analog, to mark cells in the S-phase of the cell cycle. Twenty-four hours later, brains were extracted and processed for BrdU and TrkB co-localization analysis. We found that only 30% (18/61, n=3) of BrdU⁺ cells contained TrkB receptors (Fig. 1A–C). Next, we examined hippocampal precursors 3 days after BrdU administration and found that 50% of BrdU⁺ cells contained TrkB receptors (34/68, n=3). Consistent with the findings of Donovan et al., (2008), our studies indicate that TrkB expression increases at later stages of cell development in the adult hippocampus.

To examine TrkB expression in post-mitotic cells in early stages of neuronal differentiation, we performed triple-localization studies of TrkB, BrdU and neuron-specific nuclear protein

(NeuN), a marker for post mitotic cells committed to a neuronal fate, five days following BrdU treatment. Confocal analysis of immunolabeled tissues revealed co-localization of TrkB, NeuN and BrdU signals in the dentate gyrus of adult wild type mice (Fig. 1D–F). Previous studies showed that five days following BrdU administration, most marked proliferating neuronal precursors exit the cell cycle and express NeuN but exhibit immature morphology and do not express calbindin (Kempermann et al., 2004), a marker for mature granule neurons (Sloviter, 1989). Therefore, we conclude that immature granule neurons in the adult hippocampus contain TrkB receptors. This finding is consistent with a recent report demonstrating that DCX⁺ cells in the adult dentate gyrus contain TrkB receptors (Donovan et al., 2008).

BDNF mutants exhibit increased cell proliferation in the dentate gyrus

As a fraction of cycling precursors in the adult hippocampus contain TrkB receptors, it is possible that BDNF regulates their proliferation. Conflicting reports exist in the literature in regards to the role of BDNF in this cellular process. Increased hippocampal proliferation was described in adult BDNF^{+/-} mutant mice and transgenic mice expressing a dominant negative form of the TrkB receptor (Sairanen et al., 2005). In contrast to those findings, Lee et al. (Lee et al., 2002) reported a 21% reduction in proliferation in BDNF^{+/-} mutant mice, which contain 50% of the normal levels of this neurotrophin. We investigated whether our previously reported line of BDNF conditional mutants (BDNF^{2L/2LCK-cre}) exhibited any of the previously described alterations in proliferation. Using the cre recombinase-loxP system, BDNF expression was terminated across the postnatal brain of mutant mice, the cerebellum exempted (Rios et al., 2001). Depletion of BDNF in this line of mutants begins during the first postnatal week and is completed two weeks later. Therefore, BDNF expression is preserved during prenatal development. Previous analysis demonstrated that only 5% of the normal levels of BDNF mRNA are retained in the hippocampi of BDNF^{2L/2LCK-cre} mutants (Rios et al., 2001; Chan et al., 2006). Before examining hippocampal proliferation, we investigated whether early postnatal depletion of BDNF resulted in gross developmental defects in the hippocampus. Volumetric measurements of dentate gyrus, hilus and the CA3 region failed to uncover any significant differences between wild type and BDNF mutant mice (Fig. 2A, B and C). Additionally, NeuN immunolabeling indicated normal cellular organization in the dentate gyrus and CA3 region of BDNF^{2L/2LCK-cre} mutants (Fig. 2D and E and data not shown). Finally, similar levels and patterns of synaptophysin immunolabeling were observed in the wild type and BDNF^{2L/2LCK-cre} mutant CA3 region (Fig. 2F and G), consistent with normal synaptic input from the granule cell layer. In summary, these results suggest that BDNF^{2L/2LCK-cre} mutants lack gross developmental defects that might interfere with adult hippocampal neurogenesis.

Next, we conducted immunohistochemical analysis of tissue sections obtained from wild type and mutant mice that received BrdU 24 hours earlier. It revealed a significant increase (43%) in BrdU⁺ cells in the mutant dentate gyrus (Fig. 3A and B). Whereas 2198 ± 246 BrdU⁺ cells were present in the wild types, 3152 ± 217 were observed in the mutants (n = 8; P = 0.01). Upon further examination, we found that wild types and mutants contained the same proportion of BrdU⁺ cells that expressed GFAP (38.7 ± 6% in controls; n = 4 and 47.5 ± 7% in mutants; n = 4; P = 0.3) and the type 2b progenitor marker doublecortin (DCX) (47 ± 3% in controls; n = 4 and 40 ± 7% in mutants; n = 4; P = 0.4). These findings indicate that BDNF^{2L/2LCK-cre} mutants have an increase in the total number of proliferating cells in the hippocampus but maintain similar proportions of type 1 and 2b cells as the wild types.

Lack of BDNF does not alter survival or fate choices of new hippocampal cells but hinders terminal differentiation of new granule neurons

Following exit from the cell cycle, new cells in the adult hippocampus are subjected to a selection process and many undergo programmed cell death (Biebl et al., 2000; Gould et al.,

2001). Whereas the majority of surviving cells follow a neuronal differentiation pathway, some commit to a glial fate (Kempermann et al., 2004). The effect of BDNF on these cell fate choices *in vivo* has not been explored. Because BDNF mediates neuronal survival and differentiation during fetal and early postnatal development (Hofer and Barde, 1988; Patapoutian and Reichardt, 2001), we investigated whether it served similar functions in the adult dentate gyrus. BrdU was administered to wild type and BDNF^{2L/2LCK-cre} mutant mice and hippocampal BrdU-containing cells were examined 28 days later. During this time period, most of the cells that incorporated BrdU will exit the cell cycle, differentiate and migrate into the dentate gyrus (Zhao et al., 2006). The number of BrdU⁺ cells in the BDNF mutant hippocampus was increased to 150% over wild type levels (data not shown). However, in contrast with findings in BDNF^{+/-} mice (Sairanen et al., 2005), we found that the survival rate of new cells was similar in wild type and BDNF^{2L/2LCK-cre} mutant mice (Fig. 4A; $n = 12$). The number of surviving new cells was also comparable in wild types and mutants at 8 weeks post BrdU administration (wild types: 32%; mutants: 38%; $n = 4$; $P = N/S$), ruling out the possibility that the rate of cell death increased in the mutants at a later stage. As an independent assessment of hippocampal cell death, we measured apoptosis in the adult wild type and mutant dentate gyrus using the terminal-dUTP nick-end labeling (TUNEL) method. Wild type and mutant dentate gyri exhibited comparable amounts of TUNEL⁺ cells (Figure 4B). We conclude that survival of new cells in the adult hippocampus of BDNF^{2L/2LCK-cre} mutant mice is normal.

Next, we asked whether depleted BDNF stores led to altered cell fate specification of new cells in the adult hippocampus. Four weeks following BrdU administration, we measured co-localization of BrdU with NeuN or GFAP, neuronal and glial markers, respectively. BDNF^{2L/2LCK-cre} mutant and wild type mice had similar proportions of BrdU⁺ cells that expressed NeuN (Figure 5A; $72 \pm 5\%$ in mutants versus $71 \pm 5\%$ in controls; $n = 4$; $P = n/s$) or GFAP (Figure 5B; $14 \pm 2\%$ in mutants versus $14 \pm 1\%$ in controls; $n = 4$; $P = n/s$). These proportions of new neurons and glia are consistent with previous findings (Kempermann et al., 1997). These results indicate that new hippocampal cells do not require BDNF to follow neuronal or glial differentiation pathways and that BDNF mutants exhibit a net increase of neurogenesis and gliogenesis in the adult hippocampus.

To further explore whether neuronal precursors in the BDNF mutant hippocampus differentiate normally, we examined expression of calbindin, a calcium binding protein expressed in mature granule neurons (Sloviter, 1989). Because calbindin is thought to be an important element in calcium buffering, its expression is considered to be a feature of fully developed and functional new granule neurons (Lledo et al., 1992). We found that whereas $62 \pm 2\%$ (74/117) of wild type BrdU⁺ cells contained calbindin, only $42 \pm 4\%$ (146/332) of the new mutant cells expressed this terminal differentiation marker at 4 weeks post BrdU administration (Fig. 5C) ($P = 0.008$).

Because many new granule neurons in the BDNF mutants failed to acquire a calbindin peptidergic phenotype by 4 weeks following BrdU administration, we hypothesized that in the absence of this neurotrophin, new neurons experience developmental delay or arrest. To distinguish these, expression of calbindin in BrdU⁺ cells was examined 8 weeks following BrdU administration. Similar to our observations at 4 weeks post treatment, a significant number of mutant new cells failed to acquire a calbindin peptidergic phenotype (Fig. 5D). However, the deficit was more pronounced at this time point. Whereas $77.3 \pm 2.2\%$ (74/95) of BrdU⁺ cells expressed calbindin in the wild type dentate gyrus, only $37.3 \pm 2.3\%$ (98/260) contained this calcium binding protein in the BDNF mutants ($P < 0.0001$; $n = 4$). These results indicate that between 4 and 8 weeks post BrdU administration, additional wild type cells transitioned to a mature stage denoted by calbindin expression whereas many mutant cells remained developmentally arrested. It is worth noting that there were no significant differences in the total number of calbindin⁺ cells between controls and mutants (data not shown) at this time

point, perhaps reflective of the increased levels of cell proliferation observed previously in the mutants. These alterations could be part of a homeostatic response to the deficient development of new neurons lacking BDNF signal.

To further investigate the possibility that adult-born granule neurons that lack BDNF signal become developmentally arrested, we measured the number of cells that expressed calretinin in the wild type and BDNF mutant dentate gyrus. This calcium binding protein marks a transient stage in early neuronal development in the adult dentate gyrus that precedes expression of calbindin (Brandt et al., 2003). A dramatic 3.5-fold increase ($P = 0.02$; $n = 4$) in the number of calretinin⁺ cells was observed in the mutant SGZ (Fig. 5E and F). To further assess the failure of mutant cells to transition to full maturity, co-localization of BrdU and calretinin was examined in wild type and mutant mice that received BrdU 4 or 8 weeks earlier. It was previously reported that whereas at 7 days post BrdU administration, 77% of new cells contain calretinin, less than 5% continue to express it at 4 weeks post treatment (Brandt et al., 2003). We found that at 4 weeks post BrdU administration only 2% of BrdU⁺ cells in the wild type SGZ had persistent expression of calretinin, indicating that most new cells had already transitioned to a more mature stage (Fig. 5G). In contrast, 14% of newly generated granule neurons in the mutant SGZ had continued expression of this marker of immature neurons (Fig. 5G; $P = 0.01$; $n = 4$). At 8 weeks after BrdU treatment, none of the BrdU⁺ cells in the wild type SGZ contained calretinin in all of the animals examined ($n = 4$). In contrast, $12.4 \pm 3\%$ of BrdU⁺ cells in the mutants had persistent expression of calretinin ($n = 4$; $P = 0.008$; data not shown). We also investigated whether there was an accumulation of doublecortin (DCX)-containing immature neurons in the BDNF mutant dentate gyrus. DCX is a marker for immature neurons in the adult hippocampus expressed earlier than calretinin (Brandt et al., 2003). As shown in Fig. 5H, there was a 70% increase in DCX⁺ cells in the BDNF mutants ($P = 0.02$; $n = 3$). The cumulative data indicate that in the absence of BDNF signaling, new granule neurons become developmentally arrested.

New granule neurons in adult BDNF mutants exhibit decreased dendrite branching, length, and complexity

Dendritic development is an essential feature of normal granule cell maturation and is critical for functional integration into the hippocampus. BDNF can both induce and inhibit dendritic growth depending on neuronal cell type and brain region (McAllister et al., 1997; Lom and Cohen-Cory, 1999). To assess what effect BDNF might have on adult-born granule neurons, we examined their dendritic morphology in BDNF mutants. For this, new granule cells in wild type and BDNF^{2L/2LCK-cre} mice were marked by stereotaxic delivery of a retrovirus encoding green fluorescent protein (GFP) to hippocampi of adult wild type and BDNF^{2L/2LCK-cre} mutant mice. As only proliferating cells are infected by retrovirus, only newly-generated cells express GFP. Twenty days later, when granule cells have normally undergone extensive dendrite arborization (Zhao et al., 2006), we measured dendritic branching, length, and complexity of GFP⁺ cells.

GFP⁺ granule cells from both BDNF^{2L/2LCK-cre} mice and control mice displayed dendrite processes spanning into the molecular cell layer. However, GFP⁺ cells from BDNF^{2L/2LCK-cre} mice showed a 31% decrease in the total number of dendrite branches compared to GFP⁺ cells of wild type animals (BDNF Mutants: 3.8 ± 0.3 , $n = 40$; Wild types: 5.5 ± 0.4 , $n = 30$; $P = 0.0007$) (Fig 6A, B, C, D and E). Additionally, BDNF mutant cells displayed a 21% decrease in total dendrite length compared to those from control mice (BDNF Mutants: $567.1 \mu\text{m} \pm 40.2$, $n = 40$; Wild types: $449.5 \mu\text{m} \pm 30.5$, $n = 30$; $P = 0.02$) (Fig. 6F). Sholl Analysis for dendrite complexity revealed that GFP⁺ neurons from BDNF^{2L/2LCK-cre} mice were less complex than those from control mice at $50 \mu\text{m}$ from the soma ($P = 0.02$) (Fig. 6G). There was a trend towards a significant reduction in complexity in the mutants at $60 \mu\text{m}$

from the soma but this did not reach statistical significance ($P = 0.068$). Collectively, these results indicate that BDNF is required for normal dendritic differentiation of developing granule cells in the adult hippocampus.

Migration of new granule neurons is impaired in adult BDNF mutants

Newly born granule neurons migrate from the subgranular zone of the dentate gyrus to different locations throughout the whole thickness of the granule cell layer (Kempermann et al., 2003). As BDNF facilitates migration of cerebellar granule neurons (Borghesani et al., 2002), we investigated whether migration of new granule cells in the adult hippocampus also required BDNF. To better label and visualize newly generated cells, we deliver GFP-encoding retrovirus to the hippocampus of adult wild type and $BDNF^{2L/2LCk-cre}$ mutant mice. Their migration patterns were analyzed 28 days after surgery. To ensure neuronal identity, only cells expressing both GFP and NeuN were examined (Fig. 6A). Consistent with previous findings (Kempermann et al., 2003), we found that the majority of new granule neurons in both wild types and BDNF mutants were located in the inner third of the granule cell layer (Fig. 6B). In wild type mice, $11 \pm 2\%$ of new granule neurons reached the outermost region of the granule cell layer. In contrast, only $4 \pm 1\%$ (Figure 6B; $9/177$; $n = 6$; $P < 0.05$) of new cells in the $BDNF^{2L/2LCk-cre}$ mutants migrated to this area. A concomitant and significant increase in $GFP^+/NeuN^+$ cells in the middle segment of the granule cell layer of the mutants was observed (Fig. 6B; mutants, $19 \pm 2\%$, $34/177$ versus wild types, $15 \pm 1\%$, $16/101$, $P < 0.05$). It is worth noting that previous cell fate data were also confirmed; that is, a similar proportion of GFP^+ cells expressed NeuN in wild types ($79 \pm 3\%$; $106/135$, $n = 6$) and $BDNF^{2L/2LCk-cre}$ mutants ($78 \pm 4\%$; $78/100$; $n = 6$). We also confirmed that the mutant SGZ contained 150% more new cells than that of wild types. In summary, our analysis indicates that a reduced proportion of new neurons migrate to the outer segment of the DG in BDNF conditional mutants.

BDNF facilitates the effects of GABA on adult hippocampal neurogenesis

GABA plays an integral part in the regulation of adult hippocampal neurogenesis. GABAergic terminals from local interneurons can be found in close apposition to type 2 cells (Tozuka et al., 2005), which contain $GABA_A$ receptors (Ambrogini et al., 2004; Overstreet Wadiche et al., 2005; Wang et al., 2005). Stimulation of those receptors elicits calcium influx via voltage-gated calcium channels, exit from the cell cycle and neuronal differentiation. Blocking $GABA_A$ receptors induces type 2 cell accumulation as progression through the cell cycle is prevented (Tozuka et al., 2005). Conversely, stimulation of $GABA_A$ receptors with pentobarbital increases the number of calbindin⁺ granule neurons. BDNF is known to influence $GABA_A$ R expression and clustering and GABA release probability (Brunig et al., 2001; Elmariah et al., 2004; Henneberger et al., 2005). These previous findings led us to investigate whether attenuated $GABA_A$ -mediated responses might underlie the abnormal neurogenesis in $BDNF^{2L/2LCk-cre}$ mutants. First, we studied the effect of the $GABA_A$ receptor antagonist picrotoxin on the proliferation of hippocampal cells in wild type and BDNF mutant mice. As expected, picrotoxin treatment elicited a 68% increase in the number of BrdU⁺ cells in wild type hippocampus (Fig. 7A, 4548 ± 524 for picrotoxin treated and 2700 ± 123 for saline control; $n = 4$; $P = 0.01$). In contrast, $GABA_A$ receptor blockade had no significant effect in the BDNF mutant hippocampus (Fig. 7A, 4415 ± 569 for saline treated and 5082 ± 1451 for picrotoxin treated; $n = 4$; $P = n/s$). The failure of picrotoxin treatment to further increase the size of the cycling pool of cells in the BDNF mutants suggests reduced activation of $GABA_A$ receptors under basal conditions.

To further evaluate GABA's ability to regulate hippocampal neurogenesis in the absence of BDNF, we examined the long-term effects of pentobarbital, a $GABA_A$ receptor agonist. Following protocols published previously (Tozuka et al., 2005), wild type and BDNF mutant mice were administered a single dose of BrdU followed by 7 consecutive days of pentobarbital

or saline treatment. Animals were sacrificed 28 days after the initial BrdU administration and expression of calbindin in BrdU⁺ cells was examined (Figure 7B). Consistent with the finding of Tozuka et al. (2005), GABA_A receptor stimulation induced a 32% increase in the number of new calbindin⁺ granule cells in wild type animals ($P = 0.02$; $n = 4$). In contrast, pentobarbital treatment did not elicit any significant changes in the BDNF mutant hippocampus (Fig. 7B). These results suggest that BDNF is required for facilitating the effects of GABA on adult hippocampal neurogenesis.

Transcriptional analysis of the hippocampal neurogenic zone reveals alterations in BDNF mutant mice

We sought to ascertain molecular changes in the neurogenic microenvironment of the BDNF^{2L/2LCK-cre} mutants that might contribute to the observed deficits in adult hippocampal neurogenesis. For this, we selectively isolated cells residing in the SGZ and the inner segment of the dentate gyrus of wild type and mutant animals (10 to 12 weeks of age; $n = 4$) using laser capture microdissection. Figure 8 shows representative images of dentate gyrus before and after cells were captured by this method. RNA extracted from captured cells was amplified, labeled and hybridized to oligonucleotide arrays representing 34,000 mouse genes (Affymetrix). Others showed that RNA amplification does not result in a misrepresentation of the transcript composition of the original sample (Dafforn et al., 2004; Stoyanova et al., 2004). Only genes with a two-fold or higher change and a false discovery rate (FDR) p value < 0.05 were considered for further analysis. Using these criteria, 113 out of 34,000 genes exhibited altered expression in the BDNF mutant tissue, of which 46 were upregulated and 67 downregulated. Genes with modified expression in the BDNF mutants are listed in Supplement 1. A subset of those were validated using quantitative real time RT-PCR (qRT-PCR) (Table 1).

A marked decrease (15-fold) in levels of BDNF mRNA in the BDNF mutant tissue was confirmed (Supplement 1 and Table 1). Differential expression of genes associated with early developmental stages of new cells in the adult dentate gyrus was also observed. They included dramatic 3.4-fold and 5.3-fold increases in expression of DCX and calretinin, respectively, consistent with the results of the immunolabeling studies described earlier. Elevated content of these transcripts was confirmed by qRT-PCR (Table 1). Because expression of both of these molecules marks transient stages of differentiation of neural precursors in the adult dentate gyrus (Kempermann et al., 2004), these results further attest to developmental arrest of new neurons in the BDNF mutant dentate gyrus. Other notable changes included a near 4-fold increase in the levels of the GABA_A receptor subunit $\alpha 3$ (GABRA3) and a 3.5-fold reduction in EphA4 transcript content. Both of these were confirmed by qRT-PCR (Table 1). Overrepresentation of $\alpha 3$ subunits might lead to aberrant GABA_A receptor subunit composition and the observed suppression of GABA_AR-mediated responses in the mutants. EphA4, for its part, is a receptor tyrosine kinase that binds ephrin-A and B ligands to promote neural crest cell migration during early development (Robinson et al., 1997; Adams et al., 1999) and neuroblast migration in the adult subventricular zone (Conover et al., 2000). Therefore, its depletion in BDNF mutants might contribute to the observed migration deficits of new granule neurons. Finally, bone morphogenic protein receptor, type II (Bmpr2) transcript levels were vastly depleted (5.3-fold) in the mutant neurogenic zone (Table 1 and Supplement 1). Bmp signaling was demonstrated previously to be pivotal for migration of enteric neural crest cells (Goldstein et al., 2005). Furthermore, interactions of Bmpr2 with LIM kinase 1 were reported to induce dendritogenesis in cortical neurons (Lee-Hoeflich et al., 2004), an interesting finding in light of deficits in dendritic differentiation that we observed in BDNF mutants.

The genechip data were further analyzed using the Ingenuity Pathway analysis (IPA) software to elucidate how the identified changes in gene expression might combine to impact specific

signaling pathways and biological functions. IPA facilitates generation of functional biological networks using a genome-wide knowledge base. Figure 9 shows the top biological network associated with changes in gene expression elicited by lack of hippocampal BDNF. The neurotrophin/Trk, chemokine, phospholipid degradation and ERK/MAPK signaling pathways were identified as the most affected canonical pathways. The most significantly influenced functions were associated with nervous system development and with regulation of behavior. Within the nervous system development category, significant interactions were identified for genes implicated in neuronal migration (BDNF↓, DCX↑, MAP3I2↓, Slit2↑, EphA4↓, and NRXN1↓), neurogenesis (CBLN1↑, CRIM1↑, DCX↑, ID4↓, Sema 5A↓, Slit2↑, and BMPR2↓), proliferation of neuroblasts (BDNF↓ and ID4↓), neurite development (BDNF↓, Amigo1↓, DCX↑, EphA4↓, Slit2↑ and PTK2B↓) and synaptic transmission (BDNF↓, CBLN1↑, DLG2↓, KCNMA1↓, NRXN1↓, PTK2B↓, GABRA3↑). Five gene expression changes (BDNF↓, CART↑, PMCH↑, CRHBP↓ and EphA4↓) in the BDNF mutants were associated with modulation of behavior.

Together, these data provide further evidence that hippocampal BDNF depletion triggers developmental arrest of new neurons in the adult dentate gyrus. Furthermore, they provide molecular clues to the mechanisms underlying alterations in neurogenesis observed in the BDNF mutants.

Discussion

BDNF has been suspected to play an influential role in adult hippocampal neurogenesis. Evidentiary are reports indicating altered cell proliferation and diminished enhancement of neurogenesis by antidepressant treatment or dietary restriction in the hippocampi of BDNF^{+/−} mice (Lee et al., 2002; Sairanen et al., 2005). However, the specific effects of BDNF in the differentiation of adult-born granule neurons *in vivo* remained largely unknown. Here, we show that BDNF is required for later phases of neuronal maturation but not for survival or fate specification of new hippocampal cells. Indeed, new granule neurons in BDNF conditional mutant mice exhibited developmental arrest, reduced dendritic differentiation and deficient migration. An accumulation of immature neurons in the hippocampi of adult BDNF mutants was also evident. Furthermore, we demonstrated that the enhancing effects of GABA_A receptor activation on neurogenesis are attenuated in the absence of BDNF, suggestive of a mechanism that might underlie the developmental deficits that we observed in the mutants. Because adult hippocampal neurogenesis mediates cognition (Shors et al., 2001; Saxe et al., 2006; Saxe et al., 2007) and antidepressant efficacy (Santarelli et al., 2003), these findings illustrate a mechanism that might lead to deficits in learning and antidepressant efficacy reported previously in BDNF mutant mice (Korte et al., 1995; Minichiello et al., 1999; Xu et al., 2000; Monteggia et al., 2004).

Facilitation of differentiation of new granule neurons by BDNF

In agreement with a recent report (Donovan et al., 2008), we showed that a small proportion of proliferative cells in the adult dentate gyrus (DG) contain TrkB receptors, suggesting that BDNF exerts a limited direct effect on cycling progenitors. The 43% increase in the number of proliferating cells exhibited by BDNF mutants might reflect the lack of BDNF signaling on cycling cells. However, it was reported that BDNF does not influence hippocampal proliferation or self-renewal in a neurosphere culture system (Bull and Bartlett, 2005). Alternatively, the increased in hippocampal proliferation in BDNF mutants could be due to a homeostatic response to the inability of granule neurons to mature. Consistent with this, pathological insults to the brain such as ischemia elicit increased proliferation in the adult DG and this response was proposed to mediate replacement of lost neurons and preservation of hippocampal function (Liu et al., 1998; Jin et al., 2001). A similar alteration was described in

BDNF^{+/-} mice and attributed to reduced survival of new cells (Sairanen et al., 2005). However, the BDNF mutants examined here did not exhibit changes in cell survival when examined using two independent experimental approaches. The discrepancies might arise from background strain differences. Alternatively, they might be due to differential temporal patterns of BDNF depletion. Whereas in BDNF^{2L/2LCK-cre} mutants BDNF is depleted in post mitotic neurons, in BDNF^{+/-} mice, BDNF levels are reduced at all developmental stages. Therefore, if BDNF is secreted by cycling progenitors and acts in an autocrine manner, defects that would be evident in BDNF^{+/-} mice are expected to be absent in BDNF^{2L/2LCK-cre} mutants.

Our observation that BDNF mutants and wild types had comparable proportions of new cells that differentiated into neurons or glia supports the idea that BDNF is not required during early differentiation stages in the adult DG. The increased expression of TrkB in new hippocampal neurons that parallels their developmental progression to maturity also supports this model (Donovan et al., 2008). In contrast, lack of BDNF had deleterious effects on the terminal differentiation of new neurons. As shown here, most new granule neurons in adult BDNF mutants failed to fully mature as indicated by their lack of calbindin at 8 weeks post BrdU administration. The findings that calretinin expression persisted in significantly more BrdU-labeled cells at 4 and 8 weeks post treatment and that there was an accumulation of calretinin⁺ and DCX⁺ cells in the mutant SGZ are consistent with new cells being developmentally arrested at these stages.

Albeit exhibiting impaired maturation of new neurons, BDNF mutants had comparable numbers of new calbindin⁺ granule cells in the SGZ compared to wild types at 8 weeks post BrdU treatment. This suggests that a homeostatic response was triggered that effectively expanded the pool of new neurons. Even though the putative homeostatic response in the mutants was effective in maintaining normal numbers of new calbindin⁺ cells at 8 weeks post BrdU treatment, deficits in dendritic differentiation that we observed in new mutant neurons suggest that they are likely to be functionally compromised. Therefore, defects in hippocampal neurogenesis triggered by lack of BDNF might ultimately result in abnormal hippocampal activity. Future investigations should focus on ascertaining whether their synaptic integration is normal. In that context, it is also important to consider the potentially detrimental effects of the excess in developmentally arrested cells present in the mutant dentate gyrus. Their incorporation into the hippocampal circuitry could be faulty and adversely affect hippocampal function. Indeed, Jakubs et al. (2006) showed that increased neurogenesis in the adult rodent hippocampus elicited by status epilepticus resulted in elevated numbers of new granule neurons with abnormal functional connectivity. New cells generated in this pathological environment had reduced excitability due to aberrant excitatory and inhibitory synaptic drive.

Mechanisms leading to deficient granule cell differentiation in the absence of hippocampal BDNF

It remains unclear whether the effects on adult hippocampal neurogenesis in BDNF mutants are cell autonomous. Based on previous reports, it is plausible that BDNF acts directly on developing new neurons. For example, BDNF facilitated neuronal differentiation of cultured primary neurospheres derived from adult murine hippocampus and of neural precursor cells microdissected from adult DG (Bull and Bartlett, 2005; Babu et al., 2007). Furthermore, developing newly-generated neurons in the adult hippocampus express TrkB receptors (Donovan et al., 2008 and our findings). However, these findings do not exclude the possibility that BDNF facilitates adult neurogenesis through indirect mechanisms as discussed below (Supplementary Fig. 1).

Our analysis indicated that GABA_A receptor stimulation did not induce differentiation of granule neurons in the BDNF mutants and that basal activity of their GABA_A receptors might be reduced. The lack of response to GABA_A receptor blockade and stimulation might be due

to a ceiling effect under basal conditions in BDNF^{2L/2Lck-cre} mice. Alternatively, the compromised maturation of new mutant granule neurons might be due to perturbed GABA signaling in developing neurons through cell autonomous mechanisms. GABA exerts a depolarizing effect on neuronal precursors in the adult SGZ pivotal for their differentiation (Ge et al., 2005; Overstreet Wadiche et al., 2005; Tozuka et al., 2005). Indeed, switching the effect of GABA on developing cells in the SGZ from depolarizing to hyperpolarizing by knock down of NKCC1, a Cl⁻ importer, had detrimental effects on their differentiation and synapse formation (Ge et al., 2005). Specifically, new neurons had reduced dendritic branching and reduced GABAergic and glutamatergic synapses. Interestingly, new granule neurons in BDNF mutants also exhibit reductions in dendritic branching as demonstrated here. Therefore, altered responses of neural precursors in BDNF mutants to GABA might underlie their deficient differentiation.

Unlike mature granule cells, neural precursors in the adult hippocampus lack expression of GABA_Aα3 subunits (Overstreet Wadiche et al., 2005). We observed a near 4-fold increase in levels of GABA_Aα3 transcripts in the BDNF mutant SGZ in the absence of accompanying elevations in transcription of other GABA_AR subunits. Because this region is enriched with immature neurons, it is possible that lack of BDNF triggers ectopic expression of GABA_Aα3 that might lead to stoichiometry changes, aberrant GABA_AR assembly and reduced responses to GABA. Indeed, homomeric channels containing only GABA_Aα3 subunits showed no response to GABA application (Sigel et al., 1990). Therefore, a direct effect of BDNF that influences hippocampal neurogenesis might entail regulating expression of GABA_A receptors on neural precursors. Alternatively, BDNF could regulate GABA transmission to indirectly influence adult neurogenesis. For example, BDNF induces glutamic acid decarboxylase (GAD) expression and facilitates K⁺-evoked GABA release (Yamada et al., 2002; Elmariah et al., 2004). Therefore, deficits in GABA release could contribute to the observed alterations and would entail an indirect mechanism. BDNF might also exert its effects on new granule neurons by modulating expression of other growth factors. For example, BDNF was reported to modulate hippocampal expression of vascular endothelial growth factor, which for its part, facilitates adult hippocampal neurogenesis (Thakker-Varia et al., 2007).

Functional Implications

Adult hippocampal neurogenesis facilitates antidepressant efficacy and certain forms of hippocampal-dependent learning (Shors et al., 2001; Santarelli et al., 2003; Saxe et al., 2006; Saxe et al., 2007). These processes are compromised in BDNF mutants and as we showed here, granule cell differentiation in the adult DG is hindered by lack of BDNF signaling (Korte et al., 1995; Minichiello et al., 1999; Monteggia et al., 2004). Therefore, we propose that BDNF might mediate specific aspects of hippocampal function through enhancement of local neurogenesis. In agreement with this, environmental enrichment and different forms of antidepressant treatment induce both hippocampal neurogenesis and BDNF expression (Nibuya et al., 1995; Kempermann et al., 1997; van Praag et al., 1999; Madsen et al., 2000; Malberg et al., 2000). Moreover, the beneficial effects of these manipulations on neurogenesis are attenuated by depleted levels of BDNF (Lee et al., 2002). In summary, we conclude that BDNF is required for the terminal differentiation of new granule neurons in the adult hippocampus.

Experimental Methods

Animals

Mice with postnatal depletion of central BDNF (BDNF^{2L/2Lck-cre}) were generated as described previously (Rios et al., 2001). Briefly, for the generation of mice with floxed BDNF alleles, loxP sites were inserted around the single coding exon of BDNF. Thus, cre-mediated

recombination of floxed BDNF results in a null BDNF allele. BDNF conditional mutants were generated by crossing mice carrying floxed BDNF alleles with transgenic mice in which expression of cre recombinase was driven by the α -calcium/calmodulin protein kinase II (CamKII). The mutant mice are depleted of BDNF stores everywhere in the brain except the cerebellum. Female and male BDNF mutants and littermate controls, averaging 8–12 weeks of age and in a hybrid (129/C57Bl6) background, were used in all of the experiments described. Every mutant had a sex and age-matched control. All of the procedures described here were approved by the Institutional Animal Care and Use Committee at Tufts University and were in compliance with the NIH guide for the care and use of laboratory animals. All animals were given free access to water and food and housed in a 12-hour light/ dark cycle.

Hippocampal volume measurements

Cresyl violet-stained coronal serial sections were used to determine the total volume of the granule cell layer, the hilar region and the CA3 cell body area as described previously (Heine et al., 2004). For this, serial sections representing the full rostro-caudal extent of the hippocampus, 180 μ m apart were analyzed. The hilar region (hilus) was defined by drawing straight lines from the tip of the two GCL blades towards the tip of the CA3 layer. Part of the stratum radiatum/lucidum of the CA3 (Str Rad/Luc) was then delineated by placing an extra line between the tip of the inner GCL blade and the CA3/CA2 border. Volumes for each region for the were measured using OpenLab software.

Retroviral gene delivery using stereotaxic surgery

A murine stem cell virus (MSCV) retroviral vector (a gift from Dr. Naomi Rosenberg at Tufts University) with the endogenous 3' LTR promoter driving expression of green fluorescence protein (GFP) and a pSV- ψ -E-MLV packaging vector were transfected into HEK293T cells. Secreted virus was harvested and delivered to rostral and caudal sites of the dentate gyrus of anesthetized mice using the coordinates A/P: -2.0 mm, M/L ± 1.5 mm, D/V 2.0 mm and A/P: -3.0 mm, M/L ± 3.0 mm, D/V 3.0 mm. One microliter of the viral preparation (1×10^7 infectious units per ml) was administered using a 10 μ l Hamilton syringe with a 33-gauge needle attached to a digital stereotaxic apparatus and an infusion pump at a rate of 0.15 μ l/minute. After virus infusion was completed, the needle remained in place for 10 minutes before slow withdrawal. Mice were sacrificed for analysis 3 or 4 weeks after surgery.

5-bromo-2-deoxyuridine (BrdU) pulse chasing and immunochemistry

A thymidine analog, BrdU (75 mg/kg) (Sigma, Saint Louis, MO) was injected IP, 3 times at 2 hourly intervals to mark proliferating cells in the hippocampi of BDNF mutant and wild type mice similar to protocols published previously (Malberg et al., 2000; Nakagawa et al., 2002). Animals were deeply anesthetized 24 hours or 28 days after the last BrdU treatment and transcardially perfused with 10 ml of phosphate buffered saline (PBS) followed by 35 ml of 4% paraformaldehyde (PFA) solution. Brains were removed, post-fixed in 4% PFA, cryoprotected in a 30% sucrose solution and frozen in mounting media (Tissue-Tek, Torrance, CA) until further use. Thirty micron-thick sections representing the whole rostro-caudal extent of the hippocampus were rinsed in 1X PBS and incubated in 20% MeOH/3% H₂O₂ for 15 minutes to quench endogenous peroxidase activity. After washing, sections were incubated in citrate buffer (0.1M sodium citrate and 0.1M citric acid, pH 6.0) for 10 minutes at 90°C, followed by washing in 1X PBS and incubation in 2.0N HCl for 30 minutes as described previously (Nakagawa et al., 2002). Sections were blocked with 5% normal goat serum in 1X PBS for 30 minutes at room temperature and then incubated with rat anti-BrdU (1:250; Accurate Chemical, Westbury, NY) for 3 days at 4°C. Sections were then incubated with a biotinylated secondary antibody (goat anti-rat IgG; 1:200; Jackson ImmunoResearch, Westgrove, PA) for one hour at room temperature and BrdU signal was further amplified with

an avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA), followed by incubation with DAB substrate and counterstaining with cresyl violet.

For BrdU double immunolabeling studies, slide mounted sections were denatured as described above and blocked with 5% normal goat or donkey serum/0.1% Triton-X 100 in 1X PBS. Sections were then incubated with rat anti-BrdU (1:250) and mouse anti-GFAP (1:250, Chemicon, Temecula, CA), mouse anti-NeuN (1:50, Chemicon), goat anti-DCX (1:250, Santa Cruz, Santa Cruz, CA), rabbit anti-calretinin (1:500, Swant, Bellinzona, Switzerland), mouse anti-calbindin (1:500, Chemicon) or rabbit anti-trkB (1:500, Chemicon) for 3 days at 4°C. Sections were then incubated with the appropriate combination of fluorescence-conjugated secondary antibodies (1:200, Jackson ImmunoResearch). For all other experiments, the following primary antibodies were used: rabbit anti-GFP (1:500, Abcam, Cambridge, MA) and mouse anti-GFP (1:500, Chemicon).

Quantitation of BrdU⁺ cells

The number of BrdU⁺ cells was determined using a modified unbiased stereological protocol as described previously (Gould et al., 1999; Malberg et al., 2000). Briefly, BrdU⁺ cells in the SGZ were counted in every sixth section throughout the rostro-caudal extent of the dentate gyrus using a 63X objective. This approach ensured that the same cell was not counted in two sections. Cells in the outermost focal plane were not counted. The total number of BrdU⁺ cells was multiplied by six to signify the estimated total amount of proliferative cells in the entire dentate gyrus.

TUNEL Analysis

Apoptotic cell death was measured using the protocol and reagents of the ApopTag Plus Fluorescein *In Situ* Apoptosis Detection Kit (Chemicon, Temecula, CA). Briefly, slide mounted sections were treated with terminal deoxynucleotidyl transferase (TdT) to add digoxigenin-nucleotide triphosphates to DNA ends. Labeled fragments were bound to fluorescein conjugated anti-dioxigenin antibodies and the reacted sections were counterstained with 4'-6' diamino-2-phenylindole (DAPI) in order to mark the nucleus. DNase treated sections were used as a positive control. Apoptotic cells were counted in sections 180 µm apart representing the whole rostro-caudal extent of the hippocampus.

Confocal Analysis

Confocal microscopy was used to identify cells that co-labeled for multiple markers in the x, y, and z planes of an optical field. Cells were examined with a Leica (Heidelberg, Germany) DXREII microscope equipped with HeNe (688 nm), Kr (568 nm) and Ar (488 nm) laser sources. Optical sections were captured throughout 30 micron-thick tissue sections at 1µm-step intervals and settings were adjusted such that signals were below saturation levels. For analysis of co-localization of BrdU and markers with cytoplasmic expression, cells were scored as positive if the BrdU nuclear signal was surrounded in all three planes by the cytoplasmic signal. Analysis of GFP co-localization with specific cellular markers involved identification of cells in which the GFP signal co-localized in all planes with the cellular marker. Leica imaging software was used to analyze z-series scans.

Migration studies

Migration patterns of new granule neurons were studied using hippocampal sections from wild type and BDNF^{2L/2LCK-cre} mice that were administered retrovirus encoding GFP 4 weeks earlier. Tissue sections were immunolabeled for GFP and NeuN as described earlier. GFP⁺/NeuN⁺ neurons were categorized by the location of their cell bodies within the granule cell layer, which was divided into three horizontal segments using the OpenLab software program

(Improvisation). The percentage of new neurons falling in the inner, middle and outer segments of the granule cell layer (outermost being the cells bordering the molecular layer) were quantified in sections representing the whole rostro-caudal extent of the hippocampus.

Dendritic Morphometric Analysis

Analysis of dendrite morphology was studied using hippocampal sections from wild type and BDNF^{2L/2LCk-cre} mice that were administered a retrovirus encoding GFP 20 days earlier. Coronal tissue sections (40µm) were prepared and processed for immunolabeling using rabbit anti-GFP (1:1000 Invitrogen) and a fluorescence-conjugated secondary antibody (1:200 Jackson ImmunoResearch) as described earlier. Images were acquired with a Leica DXREII confocal microscope, also as described. Three-dimensional reconstructions of the dendritic arbor of GFP⁺ neurons were made from Z-series stacks of confocal images. For completion of the dendritic analysis, we followed protocols published previously (Ge et al., 2005). Briefly, two-dimensional projection images were traced using NeuronJ, a NIH ImageJ plugin and the total dendritic length of individual GFP⁺ neurons (n=10 cells per animal, 3–4 animals per group) was analyzed. Total dendrite branching of the same GFP⁺ neurons was counted manually. Sholl analysis for dendritic complexity was carried out using a separate NIH ImageJ plugin, which counted the number of dendrites that intersected a series of concentric circles at 10 µm intervals from the cell body. For each of the above measures, statistical significance (P<0.05) was assessed using a Students T-test.

GABA_AR studies

Experimental procedures involving the administration of GABA_AR agonist were followed as previously described (Tozuka et al., 2005). Briefly, the effects of GABA on cell proliferation were determined by administration of a GABA_AR antagonist picrotoxin (5mg/kg in PBS, n=4) or saline alone (n=4) for three consecutive days. On the fourth day, mice were administered 3 doses of BrdU (75 mg/kg) at 2 hourly intervals. Animals were sacrificed 24 hours after the last dose of BrdU and processed for BrdU immunohistochemistry as described above. To determine the effects of GABA on cell differentiation, animals received three doses of BrdU on the first day, followed by seven consecutive days of pentobarbital (50mg/kg in 1XPBS) or saline treatment. Saline (n = 4) and agonist (n = 4) treated animals were sacrificed 4 weeks after BrdU administration and processed for BrdU/Calbindin double immunolabeling studies as described above.

Laser capture microscopy (LCM) and RNA extraction and amplification

Brains from wild type and BDNF^{2L/2LCk-cre} mutant mice (n = 4; 10 to 12 weeks of age) were extracted and immediately frozen. Ten micron-thick cryostat sections representing 3 independent coronal levels of the hippocampus were obtained and dehydrated in a series of ethanols, stained in cresyl violet, followed by a 1 minute incubation in histoclear. Sections were then dried in a desiccator and bilateral laser microdissection of cresyl violet-stained cells in the subgranular zone and first row of cells of the dentate gyrus performed using the Arcturus Pixcell Ile LCM system. Total RNA was extracted from captured cells using the Picopure RNA extraction kit (Arcturus, Sunnyvale, CA), DNAsed and amplified (two rounds) and labeled with biotinylated UTP's using the MessageAmp II RNA amplification kit (Ambion, Austin, TX) as per the manufacturer's specifications.

Genechip analysis and interpretation of data

Target cRNAs from each experimental animal (n = 4 for each group) were fragmented and independently hybridized to GeneChip Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA). Genechips were then washed and stained with Streptavidin R-phycoerythrin (Molecular Probes, Carlsbad, CA) and following washes, scanned using the GeneChip scanner.

Data analysis was conducted using the Bioconductor suite of programs. The 3'/5' RNA degradation plot was used to confirm the quality of the RNA samples hybridized to microarrays. Background correction, normalization and summarization of the raw probe intensities were carried out using the GCRMA protocol with default options (Wu et al., 2004) and the RankProd algorithm was used to calculate the list of differentially expressed genes (Hong et al., 2006). RankProd also incorporates a non-parametric permutation test to calculate the associated p-value and false discovery rate (FDR) for the significance of detection that allowed for a 0.05 cut-off for the list of differentially expressed genes at a significant FDR. A list of genes (>2-fold change and FDR value < 0.05) with altered expression in the BDNF mutants was uploaded into the Ingenuity Pathway Analysis (IPA) Software for further analysis (www.ingenuity.com). IPA provides a network for all known direct and indirect interactions between genes, protein and small molecules. The top subnetworks predicted by IPA were further analyzed for interpretation of the gene expression data.

Quantitative RT-PCR analysis

RNA extracted from laser-captured cells was treated with DNase, amplified as described earlier and tested for genomic DNA contamination in PCR reactions. Reverse transcription to generate cDNA was conducted with 2 µg of RNA and using 200 units of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and 150 ng of random primers (Invitrogen, Carlsbad, CA) in a 20 µl reaction. Real time PCR amplification was performed using a MX-4000 Stratagene cyler and SYBR green PCR master mix (Qiagen, Valencia, CA). For each primer set, the specificity of the product amplification was confirmed by dissociation curve analysis and agarose gel electrophoresis. Furthermore, curves were created using serial dilutions and the efficiencies for each primer set was calculated. The amplification efficiency for all the primers used in this study was >90 %. Fifty cycles of a two step protocol were used; 95°C for 10 min; 50 cycles with 95°C 30 s, 57°C or 60°C 30 s, and 72°C 30 s. The transferring receptor2 (Trfr2) gene was used as the normalizer. The following primer sequences were used for the analysis: Calretinin, 5'acctggagattgtgctctgc3' (Forward) and 5'acctggagattgtgctctgc3' (Reverse); GABAα3, 5'taacagcctcagccactttg3' (Forward) and 5'gagtgggcaggaagaatctg3' (Reverse); DCX, 5'tttcccaggcaatgcttaac3' (Forward) and 5'ccttgccaaacattccagct3' (Reverse); BDNF, 5'gaaagtcccgtatccaaag3' (Forward) and 5'ccagccaattctctttt3' (Reverse); EphA4, 5'cagctcccctgaattctctg3' (Forward) and 5'aaccacggcttctagtgtcg3' (Reverse); Bmpr2, 5'gtccacctgggtcatctcc3' (Forward) and 5'cctcctctgcaaggtacac3' (Reverse); Trfr2, 5'caggagcaaagtccccata3' (Forward) and 5'tgctcacataccgtagctca3' (Reverse). All samples were analyzed in triplicates and non-template controls were included to ascertain any level of contamination. Amplification products ranged from 100 to 124 bp. Data obtained were analyzed using the comparative C_t method. For each primer set a validation experiment was performed to demonstrate that the PCR efficiencies were approximately equal.

Statistical Analysis

Data were presented as mean ± the standard error of the mean (SEM). Statistical significance between groups was determined on a student's t-test for experiments comparing control and mutants. An analysis of variance (ANOVA) was used to assess statistical significance between multiple groups. Significance was scored when $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. TrkB receptor expression in cycling progenitor cells and immature neurons in the adult dentate gyrus

A–C, Representative confocal images showing TrkB expression (red) in BrdU⁺ precursors (green) (arrow) in adult wild type dentate gyrus twenty four hours after BrdU administration. **D–F**, Representative confocal images showing TrkB receptor expression (green) in BrdU (blue) and NeuN (red)-containing immature neurons (arrow) in wild type dentate gyrus 5 days following BrdU administration. Scale bars =10μm

Figure 2. Organization and cytoarchitecture of the $BDNF^{2L/2LCk-cre}$ mutant hippocampus is grossly normal

Volumetric measurements of the granule cell layer (A), hilus (B) and CA3 region (C) of wild type (WT) and $BDNF^{2L/2LCk-cre}$ conditional mutant (CM) mice. Representative coronal sections containing hippocampus from wild type (D and F) and BDNF conditional mutant (E and G) mice showing comparable levels and patterns of NeuN (D and E) and synaptophysin (F and G) immunolabeling.

Figure 3. Proliferation of hippocampal progenitor cells in wild type (WT) and BDNF conditional mutant (CM) mice

A, Representative images of dentate gyrus from animals that received BrdU 24 hours earlier show robust increase of BrdU⁺ cells (dark brown staining) in the BDNF mutant (bottom) compared to the wild type control (top). Sections were counterstained with cresyl violet. **B**, BDNF mutants (n = 9) exhibited a significant 43% increase in BrdU⁺ cells compared to wild type animals (n = 8); *, $P < 0.05$.

Figure 4. Survival of newly generated cells is normal in BDNF conditional mutant mice
A, The calculated proportion of surviving new cells is similar in wild type (36%) and BDNF mutant (41%) mice ($n = 12$). **B**, Cell death, quantified by the total amount of TUNEL⁺ cells, was similar between wild type ($n = 6$) and BDNF mutant ($n = 7$) mice ($P = n/s$).

Figure 5. BDNF regulates later phases of neuronal differentiation in the adult hippocampus

Wild types (WT) and BDNF conditional mutants (CM) ($n = 4$) had comparable proportions of new cells that expressed the neuronal marker NeuN (**A**) or the glial marker GFAP (**B**) in the adult DG. **C**, BrdU and calbindin double immunolabeling of hippocampal sections from wild types and BDNF conditional mutants that received BrdU 4 weeks earlier. A lower proportion of new cells in the DG of BDNF mutants expressed calbindin, a terminal differentiation marker ($n = 4$); *, $P = 0.008$. **D**, Percentage of the total number of BrdU+ cells that also contained calbindin 8 weeks after BrdU administration ($n = 4$); *, $P < 0.0001$. **E**, Representative images showing accumulation of immature calretinin+ neurons (red signal, arrows) in the SGZ of BDNF conditional mutants (bottom panel) compared to wild types (top panel). **F**, Quantification of number of calretinin-containing cells in the wild type and BDNF mutant SGZ ($n = 4$); *, $P = 0.02$. **G**, Percentage of the total number of BrdU+ cells that also contained calretinin 4 weeks after BrdU administration ($n = 4$); *, $P = 0.01$. **H**, Representative images

showing accumulation of immature DCX⁺ neurons (green signal) in the SGZ of BDNF conditional mutants compared to wild types.

Figure 6. Dendritic differentiation of adult-generated granule neurons is compromised in BDNF conditional mutants

Representative confocal images (A and C) and traces (B and D) of new granule neurons from wild type (A and B) and BDNF conditional mutant (C and D) mice that were marked *in vivo* with a GFP-encoding retroviral vector twenty days earlier; Scale bars: 25 μm. Newly generated neurons in BDNF conditional mutant mice (CM; n = 40 cells from 4 animals) displayed significantly decreased (E) dendrite branching and total length (F) when compared to wild type control cells (WT; n = 30 cells from 3 animals); * $P < 0.05$. Sholl Analysis for dendrite complexity (G) also indicated a significant decrease in dendritic complexity among BDNF

mutant cells (closed circles) when compared to wild type control cells (open circles) at a distance of 50 μm from the soma; *, $P = 0.02$; #, $P = 0.068$.

Figure 7. Newly generated neurons in BDNF conditional mutants exhibit deficient migration to outer segments of the dentate gyrus

A, To analyze granule cell migration, a retrovirus encoding GFP was delivered to the hippocampus of wild type (WT) and BDNF conditional mutant (CM) mice, and GFP⁺/NeuN⁺ cells analyzed 28 days later; Scale bar = 50μm. **B**, Quantification of the proportion of GFP⁺/NeuN⁺ cells in the inner (white bar), middle (grey bar) or outer (black bar) segments of the granule cell layer. Mutants had a significantly lower proportion of cells that reached the outermost segment (closest to molecular layer) of the granule cell layer when compared to wild type animals; n = 6; *, $P < 0.05$). A concomitant increase of new neurons was observed in the middle segment of the granule cell layer of BDNF mutants when compared to wild types; *, $P < 0.05$.

Figure 8. GABA_AR-mediated changes in proliferation and differentiation of neural precursors in the adult hippocampus are absent in BDNF mutants

A, Blockade of GABA_A receptors with picrotoxin (PT) induced a significant increase in proliferation in the DG of wild types compared to their saline (Sal) treated counterparts ($n = 4$); $F = 12$; *, $P = 0.01$ in Fishers PLSD test. Picrotoxin treatment had no effect on the BDNF mutants ($n = 4$). **B**, Quantification of new calbindin-containing cells in the adult DG of mice ($n = 4$) that received saline or pentobarbital treatment 4 weeks earlier. Whereas pentobarbital stimulation of GABA_A receptors significantly increased the number of mature new granule neurons in the wild types ($F = 10.8$), it had no effect on the BDNF mutants ($n = 4$); *, $P = 0.02$.

Figure 9. Laser capture microdissection (LCM) of cells in the hippocampal neurogenic region of adult mice

Representative images of hippocampal sections before (left panel) and after (right panel) LCM. Arrows indicate areas of dissected tissue.

Table 1

Validation of GeneChip data by quantitative RT-PCR. Unpaired *t*-tests were used to calculate *p* values.

Gene Name	Fold Difference	<i>p</i> -Value	Genechip Fold Difference
DCX	+ 2.5	0.01	+3.4
Calretinin	+4.9	0.02	+5.3
GABA α 3	+9.7	0.02	+3.9
EPHA4	-4.2	0.04	-3.5
BMPR2	-3.98	0.05	-5.4
BDNF	-129	0.002	-15.0