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Expansion of the dentate mossy fiber-CA3 projection in the BDNF-enriched mouse hippocampus

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Abstract

Structural changes that alter hippocampal functional circuitry are implicated in learning impairments, mood disorders and epilepsy. Reorganization of mossy fiber (MF) axons from dentate granule cells is one such form of plasticity. Increased neurotrophin signaling is proposed to underlie MF plasticity, and there is evidence to support a mechanistic role for brain-derived neurotrophic factor (BDNF) in this process. Transgenic mice overexpressing BDNF in forebrain under the α -calcium/calmodulin-dependent protein kinase II promoter (TgBDNF mice) exhibit spatial learning deficits at 2–3 months of age, followed by the emergence of spontaneous seizures at ~6 months. These behavioral changes suggest that chronic increases in BDNF progressively disrupt hippocampal functional organization. To determine if the dentate MF pathway is structurally altered in this strain, the present study employed Timm staining and design-based stereology to compare MF distribution and projection volumes in transgenic and wild-type mice at 2–3 months, and at 6–7 months. Mice in the latter age group were assessed for seizure vulnerability with a low dose of pilocarpine given 2 hrs before euthanasia. At 2–3 months, TgBDNF mice showed moderate expansion of CA3-projecting MFs (~20%), with increased volumes measured in the suprapyramidal (SP-MF) and intra/infrapyramidal (IIP-MF) compartments. At 6–7 months, a subset of transgenic mice exhibited increased seizure susceptibility, along with an increase in IIP-MF volume (~30%). No evidence of MF sprouting was seen in the inner molecular layer. Additional stereological analyses demonstrated significant increases in molecular layer (ML) volume in TgBDNF mice at both ages, as well as an increase in granule cell number by 8 months of age. Collectively, these results indicate that sustained increases in endogenous BDNF modify dentate structural organization over time, and may thereby contribute to the development of pro-epileptic circuitry.

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Conflict of interest

The authors declare no conflict of interest, financial or otherwise.

Author Contributions

All authors have approved the final manuscript reporting this work. KG and CI conceived and planned the experiments. KG and CI carried out pilocarpine experiments. CP performed histology, in situ hybridization and quantitative densitometry. BM performed ELISA and Western blotting experiments. PC and CI performed Timms staining, and CI carried out all stereological assessments. KG, CI, CP, and BM participated in analysis and interpretation of data, including statistics. CI and KG wrote the manuscript.

Keywords

dentate granule cells; brain-derived neurotrophic factor; TrkB; hippocampus; seizure; epilepsy

Introduction

Morphological changes in the hippocampus occur in response to a variety of conditions that elicit altered neuronal activity within its circuitry, including long-term potentiation, specific types of learning, exercise and recurrent seizures (Ramirez-Amaya et al., 2001; Sutula, 2002; Galimberti et al., 2006; Gomes da Silva et al., 2012; McGonigal et al., 2012). Reorganization of mossy fiber (MF) axons arising from dentate gyrus granule cells is one such form of structural plasticity. Changes in synaptic connectivity with MF axon sprouting can result in expanded innervation of the CA3 pyramidal field, and extensive growth of collaterals in the dentate hilus and supragranular inner molecular layer (IML; Blaabjerg and Zimmer, 2007). Levels of neurotrophin expression in hippocampus are also dynamically regulated by neuronal activity, and modulating these levels can alter MF distribution (Patterson et al., 1992; Gall, 1993; Elmer et al., 1998; Gomes da Silva et al., 2012; Schjetnan and Escobar, 2012). When infused into hippocampus, nerve growth factor (NGF) and neurotrophin-3 (NT3) promote enhanced growth of MF axons into CA3 stratum oriens (Van de Zee et al., 1995; Adams et al., 1997; Xu et al., 2002), while the effects of the most abundant endogenous neurotrophin in hippocampus, brain-derived neurotrophic factor (BDNF), are somewhat inconsistent; some studies report that BDNF infusion into hippocampus has no effect on MF organization, while others have demonstrated significant expansion of MF-CA3 projections following BDNF administration in vivo (Vaidya et al., 1999; Xu et al., 2004; Schjetnan and Escobar, 2008).

Seizure activity increases BDNF expression throughout the hippocampus and promotes reactive sprouting of MFs, suggesting that BDNF-TrkB signaling stimulates this morphological response and thereby contributes to mechanisms of epileptogenesis (Gall, 1993; Binder et al., 2001; Danzer et al., 2010; Helgager et al., 2012). Application of K252a, a pan-inhibitor of the Trk family of neurotrophin receptors, blocks BDNF-stimulated MF growth both in vitro and in vivo (Koyama et al., 2004; Schjetnan and Escobar, 2008). In transgenic mice with reduced BDNF expression, or decreased signaling through its receptor TrkB (Liu et al., 2013), epileptogenesis is suppressed, as is seizure-associated reorganization of MFs (Kokaia et al., 1995; Vaidya et al., 1999; Lahtinen et al., 2002; He et al., 2004, 2010; Kotloski et al., 2010). Conversely, mice overexpressing TrkB receptors show greater susceptibility to seizure development, as do rats that receive chronic hippocampal infusions of BDNF (Scharfman et al. 2002a; Heinrich et al., 2011). Similarly, mice that constitutively overexpress c-myc-tagged BDNF under the β -actin promoter (actin-BDNF mice) develop spontaneous seizures and display more severe seizures than wild-type (WT) mice after kainate treatment (Croll et al., 1999). However actin-BDNF mice do not exhibit structural changes in MFs, although granule cell dendritic morphology appears more complex (Qiao et al., 2001; Tolwani et al., 2002).

While MF reorganization and susceptibility to spontaneous seizures are not invariably correlated (Buckmaster and Lew, 2011; Lee et al., 2012), the wealth of evidence implicating BDNF/TrkB signaling in epileptogenesis and MF plasticity prompted us to examine mice that overexpress BDNF in forebrain under the α -calcium/calmodulin-dependent protein kinase II (CAMKII α) promoter (Huang et al., 1999; hereafter termed TgBDNF mice]. Spontaneous seizures begin to emerge in a subpopulation of these mice at ~5–6 months of age, however prior to this, at 2–3 months, cognitive disruptions are detectable, including mild impairments in spatial learning (Cunha et al., 2009; Weidner et al., 2011). This progression of behavioral changes suggests that chronic increases in BDNF disrupt hippocampal functional organization over time to eventually promote epileptogenesis, and that structural changes stimulated by BDNF may contribute to this. To determine if changes in MF organization emerge under the influence of excess BDNF in this strain, the present study employed Timm's silver sulfide staining and design-based stereology to compare the distribution and volume of MF projections to CA3 in TgBDNF mice and their wild-type (WT) littermates. Genotype effects on the MF system was first quantified in young adults (2–3 months old), before spontaneous seizures develop, and before epileptiform responses in CA3 can be elicited in hippocampal slice cultures prepared from these mice (Weidner et al., 2011). Examination of MFs at this age was performed to avoid the confounding influence of seizure activity on MF organization. These results were then compared to MF measurements obtained from mice at older, "seizure-prone" ages (6–7 months old), following assessment of susceptibility to pilocarpine-induced seizures at this age. Additional morphological comparisons of hippocampus across age and genotype included stereological estimations of total dentate gyrus-granule cell layer (DG-GCL) and molecular layer (ML) volumes, as well as total granule neuron number.

Experimental Procedures

Animals

Transgenic TgBDNF mice were obtained from Jackson Laboratories (strain #006579; Bar Harbor, ME). The strain is maintained on a C57Bl6 background, and in addition to the endogenous BDNF gene, carries a transgene encoding rat BDNF under control of 8.5 kb of the CAMKII α promoter (Huang et al., 1999). Expression of the transgene occurs throughout forebrain. Mice were housed and bred under standard 12hr/12hr light/dark cycle conditions, with water and food ad libitum. All animal procedures were carried out according to experimental protocols approved by the Florida Atlantic University Institutional Animal Care and Use Committee in accordance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and all efforts were made to reduce animal discomfort and suffering. To obtain litters composed of transgenic and wild-type (WT) offspring, TgBDNF males were mated with WT C57Bl/6J females (Jackson Labs). Genomic DNA was isolated from tail samples of offspring, and genotyping was carried out by PCR detection of the transgene (94°C-30 sec, 55°C-30 sec, 72°C-45 sec; 30 cycles) using the following primers: 5'-CAAATGTTGCTTGCTGCTGGTG-3' and 5'-GTCAGTCGAGTGCACAGTTT-3'. Brain tissue was collected from young adult mice at 2–3 months of age, and from older mice aged 6–7 months. An effort was made to reduce the number of animals used, and toward this end experiments included both males and females as prior behavioral/seizure data reported

for this strain were obtained from female mice (Papaleo et al., 2011) and from male mice (Cunha et al., 2008; Weidner et al., 2011). Spontaneous seizures were never observed during handling, cage changing or transport of 2–3 month-old transgenic (Tg) mice, but were occasionally observed in older breeders housed in our vivarium facilities.

In situ Hybridization and Quantitative Densitometry

Untreated, young adult males (n=4 per genotype) were euthanized with sodium pentobarbital (150 mg/kg; i.p.) at 8–12 wks of age. Following decapitation, brains were rapidly dissected, frozen in chilled isopentane (–50°C), and stored at –80°C. Fresh frozen sections were cut in a cryostat (–20°C) at 30µm and tissue was collected on charged glass slides (Superfrost Plus, Fisher Scientific) in 4 sets of serial sections. Alternate series were used for in situ hybridization and Timm's staining of sections at this age. Sections through the entire hippocampus (1 in 4) were hybridized with ³⁵S-labeled BDNF cRNA according to described methods (Guthrie and Gall, 2003). The 540-base BDNF antisense transcript contains 384 bases complementary to the coding region of mature BDNF (GenBank sequence NM_012513). Control labeling of selected brain sections was carried using a sense transcript generated from the same template. Hybridization was conducted at 60°C for 16–18 hrs with the cRNA at a final concentration of 1×10^7 cpm/ml. Post-hybridization treatment included incubation with RNase A. All hybridizations included sections from transgenic and WT littermates, and sections from matched pairs were exposed on the same sheet of autoradiography film. Labeling specificity was verified by hybridization of sense BDNF RNA. Sections were processed for film (Kodak Biomax MR) followed by emulsion (Kodak NTB) autoradiography, with exposure times of 3 days and 4–5 weeks, respectively. Film autoradiograms were digitized and labeling densities calibrated relative to radiolabeled standards (American Radiolabeled Corp., St. Louis, MO), using NIH Image 6.2 analysis software (Wayne Rasband, NIH). Density was sampled from a minimum of 8 sections per animal (n= 4 per genotype) at matched rostrocaudal levels, and separate measurements were collected from the dentate gyrus granule cell layer, and the CA3 and CA1 pyramidal cell layers, in both hemispheres. Densities were sampled within the boundaries of the labeled cell body layer and background density sampled in the corpus callosum was subtracted from these measures for each section. Section means were used to calculate mean labeling density per neuronal population (dpm/mg protein) for each animal. Group mean values $\pm$ standard error of the mean (SEM) were calculated from these values, and significance of genotype was evaluated using Student's paired t-test comparisons, with significance defined as $p < 0.05$. Sections processed for emulsion autoradiography were counterstained with neutral red, and photomicrographs were collected using an Olympus AX70 microscope equipped with Magnafire digital camera. No specific labeling patterns were observed when sections were incubated with radiolabeled sense RNA sequence. All figures were assembled using Adobe Photoshop CS3, with adjustments made for size, brightness and contrast.

BDNF Enzyme-Linked Immunosorbent Assay (ELISA)

Additional mice at 2–3 months of age (n=4 WT, 5 Tg) were euthanized as above, decapitated, and hippocampi were rapidly dissected from the brains on ice. Tissue samples collected from left and right hemispheres were frozen separately on dry ice and stored at –80°C. Tissue was homogenized on ice in Cell Lysis buffer (#9803, Cell Signaling

Technology, Danvers, MA) to which 1 mg/ml complete protease inhibitors and 1 mg/ml complete phosphatase inhibitors (Roche Applied Biosystems) were added. Lysates were centrifuged at 14,000 rpm for 20 min at 4°C and supernatants were collected and assayed for protein content by Qubit assay (Invitrogen, Carlsbad, CA). Aliquots were stored at -80°C, prior to performing ELISA assays or Western blotting. ELISA measurement of total BDNF protein content was performed according to the manufacturer's instructions for the BDNF Emax immunoassay system (Promega, Madison, WI, USA). Duplicate samples (100 µg protein/well) incubated overnight at 4°C, and a standard curve was generated for each assay using serial dilutions of recombinant BDNF peptide provided in the Emax kit. Following final chromagen color development, absorbance was measured at 450nm using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA), and BDNF sample concentrations were calculated using SoftMax Pro software. T-test comparisons of group mean values (+/- SEM) were used to determine significance of genotype ($p < 0.05$).

Western Blotting

Lysates prepared as above were diluted in Laemmli buffer and heat-denatured (95°C) for 5 min, prior to separation by 12% SDS-PAGE (BioRad TGX gels). For detection of mature BDNF (mBDNF), proteins (75µg) were transferred to nitrocellulose membranes (0.45µm) and blocked in 5% milk in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8.2) for 1 hr at RT. Blots then incubated for 2 nights at 4°C in the same blocker containing rabbit anti-BDNF (1:250; #sc-546, Santa Cruz Biotech, Santa Cruz, CA) and mouse anti-alpha tubulin (1:1000; #T9026, Sigma, St. Louis, MO). Control lanes included 5 ng of human recombinant mBDNF peptide (PeproTech, Rocky Hill, NJ). After rinsing in TBS containing 0.1% Tween 20 (TBST), membranes incubated in a cocktail of Dylight infrared dye-labeled IgGs (Thermo Scientific) specific for rabbit (dye 680) and mouse (dye 800), both diluted 1:5000 in TBST, for 1 hr. Following rinsing, blot images were examined and digitized using the Li-Cor Odyssey Fc imaging system (Li-Cor Biosciences Lincoln, NE).

Assessment of Seizure Susceptibility

A cohort of older animals was briefly assessed for spontaneous seizures ($n=5$ WT, 5 Tg) at 6–7 months of age, when seizures can be elicited in a subpopulation of TgBDNF mice of both sexes by handling and transport (Papaleo et al., 2011; Weidner et al., 2011). As there are individual differences in susceptibility to spontaneous seizures at this age, we also challenged the mice with a low dose of pilocarpine to assess vulnerability to chemical seizure induction. Mice were first observed for spontaneous seizures during a 2 hr period prior to pilocarpine treatment. During this time mice were transported in cages, weighed, handled repeatedly, and moved from the home cage to a new cage containing novel objects to explore. Following this period of observation, mice were injected with scopolamine methyl bromide (1 mg/kg, i.p.; Sigma-Aldrich), followed 15 min later by pilocarpine hydrochloride (200 mg/kg, pH 7.5, i.p.; Tocris Bioscience), and were placed individually in test cages. The pilocarpine dose used was at the low end of the range typically used to reliably induce seizures in normal C57BL/6J mice (~300–400 mg/kg, Muller et al., 2009; Schauwecker, 2012). Seizure behaviors were scored according to the modified Racine scale of Winawer et al. (2011), which defines seizure stages specifically in pilocarpine-treated mice. Latency to first stage 2 seizure (mild, non-continuous tremor), as well as onset and

duration of subsequent seizure behaviors, was recorded. Mice were observed for 2 hrs following pilocarpine treatment, with the exception of one transgenic mouse that progressed within the first hour to sustained stage 5 seizures (clonus, rearing, jumping and falling). This animal showed signs of acute respiratory distress and was immediately euthanized by sodium pentobarbital overdose (150 mg/kg, i.p.). All other mice were euthanized as described at 2 hrs. Brains were rapidly dissected, snap frozen in chilled isopentane (-45°C), and were stored at -80°C prior to cryosectioning.

Timm's Silver Sulfide Staining

Brain sections from naive 2–3 month-old mice ($n=9$ WT, $n=7$ TG), and 7 month-old mice ($n=3$ WT, 2 Tg) were processed according to a modified Timm's method for fresh frozen sections to test for detectable changes in MF projections across genotype and age. Additional tissue was pooled from 6–7 month old mice ($n=5$ WT, 5 Tg) that were treated with pilocarpine 2 hrs prior to euthanasia as described above. For 2–3 month old mice, a total of 7 transgenic mice and 9 controls were used for MF volume analyses. For 6–7 month old mice, a total of 7 transgenic mice and 8 WT mice were used. Slide-mounted, serial coronal sections ($30\mu\text{m}$) through the hippocampus were stored at -20°C prior to Timm staining. Sections were thawed and air-dried at RT, immersed in phosphate-buffered (pH 7.4) 0.5% sodium sulphide solution for 2 min, rinsed twice in phosphate buffer, and were then fixed in 96% ethanol and rehydrated. Subsequently, sections from paired littermates were stained according to described methods by immersion in citrate-buffered hydroquinone-silver lactate developer containing gum arabic as a protective colloid (Danscher, 1981; Slomianka and Geneser, 1997). Incubation in developer solution was carried out for 45 min in the dark at 40°C . Sections were then thoroughly rinsed in water, and counterstained with cresyl violet or Giemsa. This modified Timm's staining procedure produces dense labeling of MF axons, and has been used successfully in prior studies by our laboratory and others for quantifying the size of MF terminals in frozen sections through the rodent hippocampus (Isgor et al., 2004a; Bhatti et al., 2007; Kadam and Dudek, 2007). An alternate series of sections for each animal was postfixed in 4% paraformaldehyde for 1 hr, followed by Nissl staining for basic qualitative assessment of hippocampal laminar organization.

Stereological Estimates of MF, ML and DG-GCL Volumes

Parameters of unbiased stereological techniques used to obtain volume estimates for MF projections (total and individual compartments as specified below), ML and DG-GCL are summarized in Table 1. MF volumes were estimated following processing of fresh frozen tissue sections for Timm staining as described above. Additional untreated WT and TgBDNF mice were euthanized with sodium pentobarbital overdose at 2–3 months ($n=4$ per genotype) or at 8 months of age ($n=3$ TgBDNF, 4 WT mice) via intracardial perfusion with phosphate buffered saline followed by 4% paraformaldehyde. Brains were removed, postfixed overnight, and cryoprotected with 30% sucrose in 0.1 M phosphate buffer (PB, pH 7.4) for 48 hr at 4°C . Frozen coronal serial sections were cut at $30\mu\text{m}$ thickness and were collected starting from the midline crossing of the anterior commissure, and ending at the section where the DG of the hippocampus was no longer invisible. Sections were collected in PB and mounted on slides. All quantitative analyses were conducted on the entire

hippocampus. On the day of staining, slides were dried in an oven at 70°C for 1 hr. Following the protocol of Iniguez et al. (1985), slides were incubated in Giemsa staining solution diluted 1:10 in buffer (67mmol KH₂PO₄) at 60°C for 10 min, rinsed in buffer for 1 min and differentiated in 3 changes of 99% ethanol, cleared in Xylene and coverslipped with Permount.

MF volume measurements were made in both the SP-MF, consisting of axons in CA3 stratum lucidum (apical dendritic compartment), and the IIP-MF, consisting of axons in CA3 stratum oriens (basal dendritic compartment). All volumes were estimated using a systematic random sampling scheme outlined in Table 1 using the Cavalieri principle (Gundersen and Jensen, 1987). In this procedure, the cross-sectional areas of each MF compartment, the ML or DG-GCL were estimated by an automated point-counting technique using a grid of test points superimposed upon the structure of interest using StereoInvestigator software (MicroBrightField, Inc., Colchester, VT). Volumes of the SP-MF, IIP-MF, ML and DG-GCL were estimated from the total number of points that fell within the respective terminal fields or regions, at the specified sampling interval and nominal section thickness measured, as described in Bhatti et al. (2007) and Isgor et al. (2004a–b). Rostral and caudal extents of the ML and the DG-GCL were determined following the convention of Paxinos and Franklin (2003). Counts were performed under brightfield illumination on a Zeiss Axiophot microscope interfaced with a CCD color video camera, with sections displayed on a high-resolution monitor at a final magnification of 250X. Total MF terminal field volume was calculated by adding SP-MF and IIP-MF volumes for each animal. Group mean volume estimates per genotype and age groups (+/- SEM) were calculated for the SP-MF, IIP-MF, total MF, ML and DG-GCL. Statistical comparisons for each volume estimate were made by a two-way ANOVA with Genotype (WT, Tg) and Age (2–3 months, 6–8 months) as between-subjects variables, followed by post-hoc t-test comparisons.

Stereological Estimate of Total Granule Neuron Number

Total numbers of granule cells were estimated using the Optical Fractionator principle (West et al., 1991) with StereoInvestigator software (MicroBrightField Inc., Colchester, VT) on a Zeiss Axioplan microscope using a 100X oil-immersion lens. Details of the stereological procedures are summarized in Table 2. Total granule cell number (N) is calculated using the formula $N = \Sigma Q^- \times (t/h) \times (1/asf) \times 1/ssf$, where Q^- = total number of cells counted, t = section thickness, h = height of optical disector, asf = area of sampling fraction = $a(\text{frame})/a(x,y \text{ step})$ and ssf = section sampling fraction. Coefficients of error (CE) of the granule cell estimates were calculated according to Gundersen et al. (1999) and ranged between 4 to 6%. Using the same systematic random sampling scheme and the sections selected for volume measurements, granule neurons were counted using an optical disector 10µm deep, centered within the z-axis of the histological preparation to avoid knife errors and other biases. Each section was surveyed at equal sample distances (x,y step) by a motorized stage attached to the microscope that was under computer control. Neurons were counted by the unit counting method (i.e., neuronal nuclei), when they first came into focus, and for each x,y step, counts were derived from a known fraction of the total area by using an unbiased counting frame 10µm × 10µm in dimension. Estimates were derived by

multiplying the sum of the neurons that were counted by the reciprocal of the fraction of the layer that was sampled (derived from the section sampling interval; x,y step size and section thickness). Using these sampling parameters estimates were calculated in 90–150 dissectors per animal. Rarely occurring small, elongated or irregularly shaped nuclei containing granular-appearing chromatin were considered glial cells and excluded from analyses. Mean estimated granule neuron numbers ($\pm$ SEM) were calculated for each genotype (WT, TgBDNF) and age group (2–3 months, 8 months), and compared by two-way ANOVA followed by post-hoc t-test comparisons.

Results

Hippocampal BDNF Expression

Increased forebrain expression of BDNF mRNA under the CAMKII α promoter has been documented in the TgBDNF mice (Huang et al., 1999; Xie et al., 2010). We used in situ cRNA hybridization to quantify this increase within specific neuronal subfields in hippocampus. As shown in Figure 1, levels of BDNF cRNA hybridization density in the dentate granule cell (GCL) were elevated approximately 2-fold. In CA3 and CA1 pyramidal cell layers, density measured 1.6-fold and 2.5-fold higher, respectively, compared to levels measured in WT littermates. Quantitation of total BDNF content (mBDNF and pro-BDNF) by ELISA confirmed that BDNF protein levels were increased more than 2-fold in the transgenic hippocampus (68.7 pg/mg protein $\pm$ 8.7 SEM for TgBDNF mice versus 27.6 pg/mg protein $\pm$ 1.1 SEM for WT; $p < 0.005$, unpaired t-test). Part of this increase was reflected as a change in the abundance of mBDNF as detected by immunoblotting. Hippocampal lysates from TgBDNF mice showed increased band intensity for mBDNF at ~14 kDa in comparison to lysates from WT mice (Fig. 1F).

Seizure Susceptibility

Responses to transport/handling, pilocarpine administration, and corresponding anatomical measures are summarized in Table 3. Of the five older TgBDNF mice tested, only one exhibited a single episode of spontaneous seizure (stage 4; rearing and bilateral forelimb clonus) during handling and weighing, lasting 30 seconds. Responses to chemical seizure induction varied across the treated transgenic animals, with two of five reaching stage 4–5 seizures. Following pilocarpine administration, one transgenic mouse rapidly progressed to sustained stage 5 seizures, and was euthanized at 25 min after injection due to signs of respiratory distress. A second transgenic mouse progressed to stage 4 (tonic/clonic seizures, rearing) within 20 min of injection, whereas the remaining three, including the animal that had previously exhibited a spontaneous seizure, did not progress beyond stage 3 (continuous tremor, shaking, tail extension) during the 2 hr post-treatment observation period. None of the WT mice displayed spontaneous seizures, or progressed beyond stage 3, in response to the 200 mg/kg dose of pilocarpine.

Mossy Fiber Terminal Field Volumes

Table 3 summarizes MF volume estimates specifically for the pilocarpine-treated mice, and Figures 2A–H show representative hippocampal sections from mice at 2 and 6 months of age, illustrating robust Timm staining in both the SP-MF and IIP-MF compartments. Two-

way ANOVAs showed a significant main effect of age in the SP-MF volume [$F=30.10$, $p<0.0001$] and significant main effects of age [$F=33.31$, $p<0.0001$] and genotype [$F=12.01$, $p=0.003$] in the IIP-MF volume. When the entire MF system volume (SP-MF + IIP-MF) was analyzed with a two-way ANOVA, significant main effects of age [$F=21.94$, $p=0.002$] and genotype [$F=7.07$, $p=0.016$] were detectable. These significant effects were followed by appropriate post-hoc comparisons as noted below.

At 2–3 months of age, mean estimated terminal field volumes in the SP-MF compartment were $0.841 \pm 0.035 \text{ mm}^3$ for WT ($n=9$) and $1.030 \pm 0.058 \text{ mm}^3$ for transgenic mice ($n=7$, Fig. 2L). The IIP-MF compartment mean volumes were $0.318 \pm 0.022 \text{ mm}^3$ for WT mice and $0.406 \pm 0.025 \text{ mm}^3$ for TgBDNF mice (Fig. 2M). Post-hoc comparisons (t-tests) showed a significant ~20% increase in the SP-MF volume, and a ~25% expansion of the IIP-MF volume in transgenic mice compared to WT controls [$p=0.039$, Figs. 2L–M]. When total MF volume (SP-MF + IIP-MF) was analyzed, post-hoc comparisons showed a moderate (~20%) overall increase in MF volume in TgBDNF mice compared to age-matched WT mice [$p=0.041$, data not shown].

At 6–7 months of age, mean estimated SP-MF volumes were $1.190 \pm 0.026 \text{ mm}^3$ for WT ($n=8$) and $1.252 \pm 0.085 \text{ mm}^3$ for TgBDNF mice ($n=7$; Fig. 2L). In the IIP-MF compartment, mean estimated volumes were $0.462 \pm 0.033 \text{ mm}^3$ for WT mice (Fig. 3c, e, g) and $0.617 \pm 0.056 \text{ mm}^3$ for transgenic mice (Fig. 2M). Post-hoc comparisons demonstrated a significant ~30% increase in the volume of the IIP-MF compartment in the older transgenic mice compared to WT controls [$p=0.01$]. However the same analysis did not reveal a significant increase for SP-MF volume in this age group. When MF volume was analysed in its entirety (SP-MF + IIP-MF), no significant change in overall terminal field size was detected between genotypes at older ages, due to the SP-MF bundle making a larger contribution to overall volume relative to the IIP-MF bundle. However, maturation-dependent enlargements were observed in the SP-MF (~40%) and IIP-MF compartments (~45%), resulting in a 40% increase in total MF (SP-MF + IIP-MF) volume in 6–7 month old WT mice compared to their younger counterparts [$p=0.01$]. There was no observable increase in Timm-stained terminals in the dentate inner molecular layer at any age in the TgBDNF mice. However, among the older TgBDNF mice, the three mice that displayed either a spontaneous seizure event or a more severe seizure response (stages 4–5) to pilocarpine exhibited the largest increases in MF volumes, mostly attributable to expansion of the IIP-MF compartment (see Table 3, transgenic mice #3–5).

Dentate Gyrus-Granule Cell Layer Volume

Qualitative assessment of Nissl-stained hippocampal sections from all WT and TgBDNF mice showed overall normal organization of the dentate, however in some sections from the older transgenic mice, portions of the granule cell layer appeared mildly enlarged compared to age-matched control tissue, with some cells appearing dispersed outward toward the deep molecular layer (Figs. 2I–K). Quantitative estimations of granule cell layer volume showed that at 2–3 months of age, mean estimated DG-GCL volumes were $0.910 \pm 0.043 \text{ mm}^3$ for WT ($n=4$) and $1.057 \pm 0.060 \text{ mm}^3$ for TgBDNF mice ($n=4$, Fig. 3A). At 8 months of age, mean estimated DG-GCL volumes were $0.975 \pm 0.100 \text{ mm}^3$ for WT ($n=4$) and $1.130 \pm$

0.107 mm³ for TgBDNF mice (n=3). A two-way ANOVA showed no significant main effects of age, genotype, or interaction between age and genotype with this sample size.

Total Number of Granule Neurons

At 2–3 months of age, the mean estimated total number of granule neurons were [(0.621 $\pm$ 0.011) $\times$ 10⁶] for WT (n=4) and [(0.658 $\pm$ 0.032) $\times$ 10⁶] for TgBDNF mice (n=4, Fig. 3B). These results are comparable to those reported previously for several strains of laboratory mice at 9 weeks of age (Abussad et al., 1999). At 8 months of age, mean estimated total number of granule neurons were [(0.617 $\pm$ 0.011) $\times$ 10⁶] for WT (n=4) and [(0.739 $\pm$ 0.038) $\times$ 10⁶] for TgBDNF mice (n=3, Fig. 3C). A two-way ANOVA showed a significant main effect of genotype [F =11.31, p =0.006] and interaction between age and genotype [F =4.66, p =0.05]. Furthermore, post-hoc comparisons showed that 8 month-old TgBDNF mice had significantly higher numbers of granule neurons compared to age-matched WT mice [$\sim$ 20%, p =0.04].

Molecular Layer Volume

At 2–3 months of age, mean estimated ML volumes were 2.237 $\pm$ 0.114 mm³ for WT (n=4) and 2.723 $\pm$ 0.212 mm³ for TgBDNF mice (n=4, Fig. 3C). At 8 months of age, mean estimated ML volumes were 2.792 $\pm$ 0.181 mm³ for WT (n=4) and 3.268 $\pm$ 0.155 mm³ for TgBDNF mice (n=3, Fig. 3D). A two-way ANOVA showed significant main effects of genotype [F =7.54, p =0.019] and age [F =9.87, p =0.009]. Post-hoc comparisons showed that TgBDNF mice had larger ML volumes in both age groups compared to WT controls [$\sim$ 20%, p s = 0.04]. Post-hoc comparisons also showed an age-related enlargement in ML volume in WT mice between 2–3 month-old and 8 month-old age groups [$\sim$ 25%, p =0.041].

Discussion

Hippocampal BDNF expression

In the TgBDNF strain, transgene expression parallels the postnatal spatiotemporal pattern of neuronal CAMKII α expression in forebrain, becoming detectable during the first postnatal week and increasing to adult levels by 3–4 weeks (Bayer et al., 1999; Huang et al., 1999). As shown here, by 8–12 weeks of age, levels of hippocampal BDNF mRNA measure significantly higher than those in WT mice by quantitative in situ hybridization. This is accompanied by an elevation in total BDNF protein as measured by ELISA. An increase in the abundance of mBDNF contributes to this, as reflected by increased band intensity on immunoblots, a result consistent with that reported for cortical samples from the TgBDNF mice (Xie et al., 2010; Papaleo et al., 2011).

Mossy fiber organization

BDNF is normally expressed by most hippocampal neurons, including granule cells, and its localization to dense core vesicles in mossy fiber terminals indicates an anterograde influence on target neurons in CA3, in addition to potential autocrine/paracrine effects within the granule cell population (Gall, 1993; Dieni et al., 2012). This factor promotes granule cell axon growth and targeting in vitro, stimulates IML MF sprouting in

hippocampal slices, expands MF innervation of CA3 stratum oriens when infused in vivo, and regulates MF synaptic plasticity (Patel and McNamara, 1995; Koyama et al., 2004; Schjetnan and Escobar, 2008, 2012; Tamura et al., 2009; Schild et al., 2013). As shown here by Timms staining, TgBDNF mice show moderate but significant enlargement of both the SP-MF and IIP-MF projections at 2–3 months of age, relative to WT mice. This expansion results in a ~20% increase in the total volume of the MF system. By 6 months of age, the IIP-MF compartment undergoes further enlargement beyond that associated with ongoing maturation. This temporal pattern parallels the emergence of distinct hippocampal-associated behavioral traits in this strain. Young adults (2–3.5 months old) exhibit deficits in spatial working memory across a variety of tasks, but prior to 5 months, do not show spontaneous seizures (Cunha et al., 2009; Papaleo et al., 2011). By 6 months, ~55–60% of mice display spontaneous seizures, a proportion that increases to ~85% by 16–20 months (Papaleo et al., 2011; Weidner et al., 2011).

The most notable increases measured in IIP-MF volume occurred in those transgenic mice that showed increased seizure susceptibility when tested at 6 months. At this age, prior seizure events, acting in combination with increased BDNF, may have exacerbated structural changes in MF projections. Weidner et al. (2011) showed that at 17–22 months of age, a subset of TgBDNF mice (4 out of 7) displayed robust NPY-immunoreactivity (IR) in MFs, a pattern of expression typically indicative of seizures. Sprouting of MFs in the IML is also frequently, though not always (Buckmaster and Lew, 2012), associated with recurring seizures, however we did not observe Timm staining in the IML of TgBDNF mice at any age, including the animal that exhibited a spontaneous seizure. Long-term video recording of TgBDNF mice is currently underway to monitor the number and frequency of spontaneous seizures to determine if these measures correlate with MF changes.

While we are unable to separate the individual contributions of increased BDNF and any undetected prior seizure events to the MF changes seen in older TgBDNF mice, recognition of this caveat is what prompted us examine the MF pathway in younger animals, before evidence of hippocampal hyperexcitability is detected. Increases in MF volume were measurable in these mice at 2–3 months, demonstrating that elevated endogenous BDNF per se can alter MF organization. At this age, TgBDNF mice show mild impairments in spatial learning and short-term memory, including delayed task acquisition on the Morris water maze (MWM) and paired-trial T-maze (Cunha et al., 2009; Papaleo et al., 2011). While these cognitive disruptions can be attributed to the direct effects of increased BDNF on synaptic function (Minichiello, 2009; Schild et al., 2013), the observed morphological changes in MFs may contribute as well. The MF-CA3 pathway plays a central role in the encoding of spatial information, and disruptions in its normal structural organization have been associated with learning deficits in a variety of rodent models (Cremer et al., 2000; Mineur et al., 2002; Kesner et al., 2007; Holahan et al. 2010).

The asymmetry seen in the altered MF pathway of older TgBDNF mice is of interest as evidence indicates that the SP and IIP systems are functionally distinct and respond differentially to pathological insults and environmental manipulations, including those that affect hippocampal BDNF levels (Scharfman et al., 2002b; Crusio and Schwegler, 2005; Romer et al., 2011). In adult rodents, IIP-MFs expand to a greater extent than SP-MFs in

response to neurogenic stimuli, including environmental enrichment and chemical seizure induction (Romer et al., 2011). The enrichment-related increase in neurogenesis is accompanied by an increase in the relative proportion of the IIP-MF volume to the total volume of the MF system (Romer et al., 2011). With seizures (kainate-induced), levels of infrapyramidal granule cell neurogenesis measure higher than those in the suprapyramidal blade, and IIP-MF size appears to be co-regulated with this, with post-seizure born granule cells projecting most, though not all, of their axons within the IIP-MF compartment (Choi et al., 2007; Romer et al., 2011). Furthermore, pilocarpine-induced seizures in rats produce more MF sprouting in the IML of the infrapyramidal versus suprapyramidal blade, and in vitro slice recordings demonstrate larger evoked responses in CA3 with stimulation of the infrapyramidal versus suprapyramidal ML (Scharfman et al., 2002b). Infrapyramidal granule cells also exhibit larger population spikes with perforant path stimulation compared to suprapyramidal cells, indicating that infrapyramidal cells tend to be more excitable following seizure development, and may contribute to activating the seizure-prone hippocampus more than suprapyramidal cells (Scharfman et al., 2002b). While we did not find significant genotype-related changes in SP-MF volume in older transgenic mice with Timms staining, this does not rule out the possibility of plasticity within the SP-MF compartment. MF sprouting alters functional connectivity through the growth of individual MF terminals and collaterals and the establishment of new synapses, and Timms staining does not provide anatomical evidence of such sprouting. Higher resolution analyses, including electron microscopic examination of MF synapse distribution and number in both MF compartments, will be needed to determine if changes in MF axon distribution in TgBDNF mice are accompanied by increases in the number, density or size of MF boutons in CA3 stratum oriens and stratum lucidum. Such changes have been shown by electron and confocal microscopy in kindled and pilocarpine-treated rodents, along with the emergence of more complex spines on CA3 target neurons (Represa and Ben-Ari, 1992; Frotscher et al., 2006; McAuliffe et al., 2011).

The present finding of MF expansion contrasts with a prior study of mice that overexpress BDNF under the β -actin promoter (Qiao et al., 2001). Strain differences in the MF response could be due to differing levels of BDNF, or the cellular context of BDNF expression. Expression of transgenes under the β -actin promoter is widespread, occurs in most cell types including glia, and is constitutive (Okabe et al., 1997). Under the CAMKII α promoter, BDNF transgene expression localizes primarily to glutamatergic forebrain neurons, and like the native CAMKII α gene, responds to changes in neural activity (Huang et al., 1999). BDNF expression is normally upregulated by neural stimulation via calcium response elements (CREs) in the native BDNF gene promoter (Tao et al. 1998, 2002). The CAMKII α promoter sequence reportedly lacks CREs, and increased neural activity reduces CAMKII α mRNA expression (but increases enzyme activity), while reduced activity increases its expression (Neve and Bear, 1989; Murray et al., 1995; Wang et al., 2008). The latter response has been successfully exploited in TgBDNF mice to maintain high levels of cortical BDNF during dark rearing to counteract the effects of sensory deprivation on visual cortex (Gianfranceschi et al., 2003). BDNF transgene expression may be similarly sustained in hippocampus during phases of relatively low physiological activity.

Molecular and granule cell layer volumes

In addition to the changes measured in the SP-MF and IIP-MF compartments, gross morphological analyses indicated a marked expansion of the ML in 2–3 month-old TgBDNF mice compared to normal littermates. This occurred in absence of a statistically significant change in either total granule neuron number or GCL volume. Although adult granule cell neurogenesis is ongoing at these ages, the monthly contribution of new cells to the total size of the rodent (rat) granule cell population is reportedly only about 6% and hence may not result in significant increases in total cell counts (Cameron and McKay, 2001). Since in young adult transgenic mice the total granule neuron number did not differ from that of age-matched controls, the increase in ML volume may result from alterations in the granule cell dendritic field, reflecting potential changes in apical dendrite morphology and/or expanded input to these dendrites, including projections from entorhinal cortex. Increases in granule cell dendritic complexity have been reported in mice that over-express BDNF under the β -actin promoter (Tolwani et al., 2002). Dentate granule cells are proposed to gate or attenuate the entry of entorhinal-derived activity into the hippocampus, thereby reducing the likelihood that aberrant activity will propagate through the hippocampus (Heinemann et al., 1992; Acsady and Kali, 2007; Hsu, 2007; Krueppel et al., 2011). An increase in excitatory cortical input to granule cells, fostered by sustained elevations in forebrain BDNF, may eventually overcome this gating function, leaving mice of this strain seizure susceptible.

Changes in granule cell number

At later, seizure prone ages (8 months), TgBDNF mice show a significant increase in total granule neuron number compared to controls, and a tendency for dispersion of some cells into the deep molecular layer. Similar increases in granule cell number have been seen in seizure-prone mice with disrupted insulin-like growth factor-1 signaling caused by knockout of the enzyme protein L-isoaspartate (D-aspartate) *O*-methyltransferase (*Pcm1*^{−/−} mice; Farrar et al., 2005). These knockout mice also exhibit dispersion of granule cells, as do other rodent epilepsy models (Liang et al., 2013). Dispersion is also observed in humans with temporal lobe epilepsy, and there is evidence this aberrant positioning of granule cells may involve seizure-related impairments in reelin signaling within the dentate (Houser, 1990; Haas and Frotscher, 2010).

The increase in granule cell number in TgBDNF mice raises the possibility that cells generated in the adult dentate subgranular zone (SGZ) contribute to the expansion of the IIP-MF projection seen in the older, seizure-prone TgBDNF mice. As noted above, studies employing environmental enrichment and seizure induction have shown selective, albeit not exclusive, targeting of MF axons from new granule neurons to the infrapyramidal compartment (Choi et al., 2007; Romer et al., 2011). It is well known that seizures stimulate adult hippocampal neurogenesis (reviewed by Parent (2007) and Koiaia (2011)). By 8 months of age some of the TgBDNF mice are likely to have experienced spontaneous seizures, in combination with over-expression of BDNF, with both factors potentially contributing to changes in granule cell number. This does not rule out effects on the established granule cell population, since both mature and immature granule cells express TrkB receptors.

As with seizure activity, environmental enrichment increases hippocampal BDNF expression and stimulates production of adult-born granule cells in comparison to mice housed in control conditions, an effect that is blocked by reducing endogenous BDNF (Rossi et al., 2006; Choi et al., 2007; Kuzumaki et al., 2011). Type 1 stem cells in the SGZ, and immature doublecortin-positive granule cells express full-length TrkB receptors (Donovan et al., 2008). The latter express TrkB as they progress through early phases of maturation, and later establish synaptic contacts with target CA3 neurons over the 2–8 weeks it takes them to functionally integrate (Donovan et al., 2008). This process is crucial to normal hippocampal function and to specific types of learning in particular (Faulkner et al., 2008; Deng et al., 2010). Conditional depletion of BDNF in vivo reduces spatial learning abilities, and as with TrkB depletion, impairs the development and maturation of adult-born granule cells (Bergami et al., 2008; Chan et al., 2008; Waterhouse et al., 2012; Vigers et al., 2012). Further study will be needed to determine if BDNF over-expression in hippocampus affects SGZ stem cell proliferation rates, the maturation and long-term survival of new granule cells, or the trajectories of their axons.

Conclusions

Our results demonstrate expansion of MF projections to CA3 in transgenic mice that over-express BDNF in hippocampus, and develop spontaneous seizures with increasing age. Before reaching older, seizure-prone ages, young adult transgenic mice show mild enlargement of MF terminal fields in both the SP-MF and IIP-MF compartments, resulting in a ~20% increase in MF system volume overall, relative to age-matched WT controls. When maturation-related changes in MF volume are accounted for, a subset of older transgenic mice (6–7 months old, showing seizure susceptibility) exhibit significant enlargement of the infrapyramidal projection. While the changes seen with Timms staining are suggestive of MF sprouting, the volumetric data do not show this, and additional anatomical analyses will be needed to assess alterations in the growth of MF terminals, bouton size and distribution, and possible postsynaptic changes in CA3 neurons.

TgBDNF mice also exhibit increased ML volume at both “pre-seizure” (i.e., 2–3 month-old) and “post-seizure” (i.e., 8 month-old) ages when compared to WT mice, an effect that is accompanied in older mice by a marked increase in the total number of granule neurons. Taken together with previous behavioral studies of these mice across their lifespan (Cunha et al., 2009; Papaleo et al., 2011; Weidner et al., 2011), the results indicate that chronic increases in endogenous BDNF modify hippocampal structural organization, adding to the body of evidence implicating BDNF in cumulative anatomical and functional changes in limbic circuitry that, over time, may promote epileptogenesis (Heinrich et al., 2011; McNamara and Scharfman, 2012; Liu et al., 2013).

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Abbreviations

BDNF	brain-derived neurotrophic factor
DG	dentate gyrus
GCL	granule cell layer
IIP	intra/infrapyramidal
IML	inner molecular layer
mBDNF	mature BDNF
ML	molecular layer
MF	mossy fiber
PB	phosphate buffer
proBDNF	prepro-brain-derived neurotrophic factor
SP	suprapyramidal
TBS	Tris-buffered saline
TgBDNF	transgenic CAMKIIa-BDNF mouse strain
WT	wild-type

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Research Highlights

BDNF expression is chronically increased in the hippocampus of TgBDNF mice

Mossy fiber volume in CA3 is enlarged by 2–3 months of age in TgBDNF mice

At seizure-prone ages (6–7 months) mossy fiber volume increases further

By 8 months, granule cell number is also significantly increased in TgBDNF mice

Figure 1.

(A–B) Pseudocolored autoradiograms of forebrain sections illustrating differences in BDNF mRNA levels across genotypes. (A) Expression pattern seen in a wild-type (WT) mouse. (B) Expression in a transgenic mouse. Levels are elevated in hippocampal CA1 and CA3 pyramidal cells, as well as in dentate gyrus (DG) granule cells, and in cortex (Cor), basolateral amygdala (Bla), and striatum (St). (C–D) Dark-field photomicrographs showing localization of ^{35} S-BDNF cRNA labeling in sections from a WT (C) and a Tg mouse (D), processed for emulsion autoradiography. Silver grain density in the Tg hippocampus is

higher in both the pyramidal and granule cell layers (Gcl). (E) Bar graph comparing BDNF cRNA hybridization density within neuronal populations in the hippocampus of WT and Tg mice. Bars indicate group mean values $\pm$ SEM. * = $p < 0.01$, Student's paired t-test. (F) Representative immunoblot showing that hippocampal lysates from TgBDNF mice (lanes 3 and 5) contain more mature BDNF (mBDNF, ~14 kDa) than those from WT mice (lanes 4 and 6). Lane 2 contains 5 ng of recombinant mBDNF peptide. GC, dentate granule cells. Bar in D = 750 μ m in A–B, and 360 μ m in C–D.

Figure 2.

(A–B) Representative coronal sections of dorsal hippocampus from 2 month-old WT (A) and TgBDNF mice (B) showing Timm-stained MF terminal fields. Short arrowheads indicate the infra/intrapyrarnidal mossy fiber (IIP-MF) compartment, and long arrowheads indicate the suprapyramidal mossy fiber (SP-MF) compartment. Scale bar in B=200 μ m. (C–D) Sections through dorsal hippocampus from 6 month-old WT (C) and TgBDNF mice (D) showing Timm-stained MFs. Arrowheads indicate the IIP-MF compartment, and in panel D, show sprouting of IIP-MFs into more distal aspects of stratum oriens (dSO). Bars=200 μ m.

(E–F) Sections through the ventral hippocampus of a 6 month-old WT (E) and age-matched TgBDNF mouse (F). Arrowheads indicate the IIP-MF terminal fields. Bars=100 μ m. (G–H) Higher magnification images of the IIP-MFs shown in E and F (bars=50 μ m). Arrowheads in G indicate a typical patchy pattern of terminal staining seen in WT mice, and in H, the more continuous, dense staining across CA3 seen in this transgenic animal. (I–J) Nissl-stained sections through the dorsal hippocampus of a 6 month-old WT (I) and age-matched transgenic mouse (J). The latter animal exhibited a stage 4 spontaneous seizure during observation. Bars=100 μ m. (K) Higher magnification images of the boxed areas in I and J. Portions of the granule cell layer appear mildly enlarged in this transgenic mouse compared to that of the WT. Bars=85 μ m. (L–M) Bar graphs comparing mean SP-MF (L) and IIP-MF (M) terminal field volumes ($\pm$ SEM) across age. Moderate expansion of both compartments is detected in young adult TgBDNF mice compared to WT controls (L). In the older transgenic mice, a robust enlargement of the IIP-MF compartment is seen (M). Measures from WT mice indicate maturation/age-dependent increases in MF projections as well. * p <0.05. spDG, suprapyramidal blade of dentate gyrus; ipDG, infrapyramidal blade of dentate gyrus.

Figure 3.

(A–C) Bar graphs comparing dentate gyrus granule cell layer volume, molecular layer volume, and total granule neuron number estimates plotted as group means $\pm$ SEMs for each genotype and age group. * $p < 0.05$.

Table 1

Stereological Parameters of the Volume Estimation Procedures

	Section sampling interval	Range of sections used	x, y point-to-point distance (μm)	Range of point counts obtained
SP-MF	Every 4 th	12–15	100	342–420
IIP-MF	Every 4 th	12–15	50	253–303
DG-GCL	Every 6 th	10–12	200	365–455
ML	Every 6 th	10–12	120	329–485

SP-MF: suprapyramidal mossy fibers, IIP-MF: intra/infrapyramidal mossy fibers, DG-GCL: dentate gyrus-granule cell layer, ML: dentate gyrus molecular layer

Table 2

Stereological Parameters for the Optical Fractionator

Experimental groups	Range of sections analyzed	$a(x,y \text{ step}) (\mu\text{m})$	Range of total cells counted (Q^-)
2–3 month-old WT	10–14	110×110	303–480
2–3 month-old TgBDNF	10–12	110×110	323–444
8 month-old WT	11–13	110×110	317–414
8 month-old TgBDNF	10–14	110×110	297–411

Sections were probed with disector samples with $a(\text{frame}) = 10 \times 10 \mu\text{m}^2$ and a height (h) of $10 \mu\text{m}$ at an average of 120 sampling sites.

Table 3

Behavioral & Structural Measures of Pilocarpine-Treated Mice

Animal ID	Spontaneous Seizure	Peak Response to Pilocarpine	Latency to Peak Response	SP-MF Vol (mm ³)	IIP-MF Vol (mm ³)	Total MF Vol (mm ³)
WT1	none	Stage 3	25 min	1.223	0.509	1.732
WT2	none	Stage 3	35 min	1.190	0.475	1.665
WT3	none	Stage 3	30 min	1.208	0.516	1.724
WT4	none	Stage 3	27 min	1.212	0.497	1.709
WT5	none	Stage 3	20 min	1.200	0.521	1.721
TG1	none	Stage 3	20 min	0.967	0.434	1.401
TG2	none	Stage 3	15 min	1.113	0.476	1.589
TG3	yes stage 4	Stage 3	25 min	1.386	0.684	2.070
TG4	none	Stage 4	20 min	1.437	0.776	2.213
TG5	none	Stage 5 (euthanized)	25 min	1.472	0.721	2.193