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Reduction of TrkB Expression De Novo in the Adult Mouse Impairs Epileptogenesis in the Kindling Model

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Abstract

Elucidating the mechanisms of epileptogenesis in molecular terms can identify targets for therapies aimed at preventing epileptogenesis or limiting its progression. Genetic perturbations have implicated signaling by the neurotrophin, BDNF, and its receptor, TrkB, in limbic epileptogenesis. Whether this signaling is critical to epileptogenesis in the adult brain is unclear. We sought to determine whether reduced expression of TrkB de novo in the mature brain is sufficient to impair epileptogenesis in the kindling model. Treatment of adult *Act-CreER TrkB^{flox/flox}* mice with tamoxifen resulted in modest reductions of TrkB protein expression de novo in the adult that were detected in hippocampus but not other brain regions. Modest reduction of hippocampal TrkB content inhibited epileptogenesis induced by stimulation of hippocampus or amygdala. The data support the conclusion that reduction of TrkB expression in hippocampus de novo in the mature brain impairs epileptogenesis in the kindling model. These findings advance TrkB and its downstream signaling pathways as attractive targets for limiting the progression of epileptogenesis.

Keywords

epilepsy; temporal lobe epilepsy; neurotrophin; kindling; Cre-transgenic; transgenic

INTRODUCTION

Epilepsy is a severe and prevalent neurological disease for which current treatments are symptomatic and often ineffective. Epileptogenesis refers to the process by which a normal brain becomes epileptic. Understanding the cellular mechanisms of epileptogenesis in molecular terms may provide novel targets for development of drugs aimed at preventing epileptogenesis or limiting its progression. The proposition of Sir William Gowers (Gowers, 1881) that “seizures beget seizures” found experimental support in Goddard et al.’s discovery of the kindling model (Goddard et al., 1969). Goddard et al. demonstrated that repeated triggering of focal seizures led to lifelong increased sensitivity whereby an initially subconvulsive electrical stimulus triggered an intense tonic–clonic seizure. The power of advanced mouse genetic manipulations facilitates elucidation of the molecular mechanisms underlying epileptogenesis in the kindling model.

Neurotrophin signaling has been identified as a molecular mechanism by which fleeting changes in neuronal activity are converted to lasting changes in neuronal excitability, including long-term potentiation (LTP) of excitatory synapses between principal neurons (Kang et al., 1997; Minichiello et al., 1999; Messaoudi et al., 2002; Ying et al., 2002). Previous studies implicated the neurotrophin BDNF and its receptor, TrkB, in epileptogenesis. Expression of BDNF is increased following a seizure (Ernfors et al., 1991; Isackson et al., 1991; Springer et al., 1994), and enhanced activation of TrkB has been identified in multiple models of limbic epileptogenesis (Binder et al., 1999; He et al., 2002; Danzer et al., 2004; Xu et al., 2004). *BDNF* mRNA (Murray et al., 2000) and protein (Takahashi et al., 1999) content are increased in the hippocampus of temporal lobe epilepsy patients. Zhu and Roper (2001) demonstrated that application of BDNF to resected human hippocampal tissue increases excitatory and decreases inhibitory synaptic transmission. Striking impairments of epileptogenesis in the kindling model were identified in mice carrying only a single *BDNF* allele (Kokaia et al., 1995), while epileptogenesis was eliminated altogether in mice with a conditional deletion of *TrkB* (He et al., 2004). Whether the antiepileptogenic effects of the BDNF and TrkB mutant mice were due to impaired BDNF/TrkB signaling during development and/or in adulthood remained unclear, because the *BDNF* and *TrkB* alleles were eliminated during embryonic development in these experiments. Determining whether TrkB signaling in the adult brain is required for epileptogenesis is critical to elucidating the responsible cellular mechanisms and to evaluating the candidacy of TrkB as a target for antiepileptogenic therapies in adults.

We therefore sought a genetic method by which TrkB could be eliminated de novo from the brain of adult mice. Fusion of Cre recombinase to the ligand-binding domain of the estrogen receptor, which is mutated so that it selectively binds the exogenous ligand tamoxifen (CreER^{TM2}), permits inducing recombination by administering tamoxifen to adult transgenic mice (Metzger et al., 1995; Feil et al., 1996, 1997; Indra et al., 1999). Crossing a transgenic mouse expressing CreER^{TM2} driven by the *Actin* promoter (Hayashi and McMahon, 2002) with a mouse possessing “floxed” *TrkB* alleles resulted in mice from which TrkB could be eliminated de novo in the mature animal. Fortuitously, tamoxifen treatment of adult *Act-CreER TrkB^{flox/flox}* selectively reduced TrkB protein expression in the hippocampus; thus, not only was TrkB expression reduced de novo in the adult but the reduction was restricted anatomically to the hippocampus. We took advantage of this regional specificity by comparing and contrasting epileptogenesis induced by stimulation of the hippocampus and amygdala in the kindling model.

MATERIALS AND METHODS

Mice

TrkB^{flox/flox} mutant mice (Luikart et al., 2003) in a C57/B6 background (at least three backcrosses to pure C57/B6) were crossed with a mouse carrying a chicken β -*actin*-*CreER^{TM2}* (*Act-CreER*) transgene with a CMV enhancer (Hayashi and McMahon, 2002) (Jackson Labs strain 004453) to generate a mouse with inducible conditional elimination of TrkB. The first coding exon of the *TrkB* gene, which encodes the signal peptide and the first 40 amino acids of the N terminus of TrkB, is flanked by *loxP* sites in the *TrkB^{flox/flox}* mutant mice. A line of mice carrying the *Rosa26-lacZ* reporter transgene (Soriano, 1999) was crossed with the *Act-CreER* mice to identify cells in which Cre recombinase had been activated. The genotype of each animal was assessed twice using PCR of genomic DNA isolated from a tail cut before the experiment and another cutting from the tail taken after sacrifice. All procedures involving live animals conformed to institutional guidelines.

Cre probes: 5' CGCCGCATAACCAAGTGAAAC 3' and 5' AATTTACTGACCGTACACCA 3' (both internal to the *Cre* transgene).

TrkB probes: 5' ACACACACAGTATATTTTACC 3' and 5' CAAGAAGTCAGAGACCAGAGAGA 3' (which flank the 5' *loxP* site and a 200-bp spacer in the floxed *TrkB* allele).

Tamoxifen Treatment

Mice were treated with tamoxifen [7.5 mg in sunflower oil (50 mg/ml), Sigma] by oral gavage for 5 days. Tamoxifen was dissolved into sunflower oil by continuous rotation in a 55°C oven for ~1–2 h. The tamoxifen solution was filtered (0.2 µm) and kept at 37°C until used. Fresh tamoxifen solution was made daily. For the gavage procedure, mice were restrained by hand and placed in a supine position with the neck extended. A slightly curved feeding needle (22 gauge, Harvard Apparatus) marked for the proper length (the distance from the mouth to the costal margin) was gently inserted down the esophagus with minimal pressure, and the tamoxifen solution was delivered slowly through the feeding needle using a 1-cm³ syringe. Vehicle-treated mice received an equal volume of sunflower oil without tamoxifen. The mice were treated at ~2 months of age. Tamoxifen-treated mice were housed separately from vehicle-treated or untreated mice.

β-Galactosidase Staining

β-Galactosidase staining was used to determine the pattern of Cre activity following tamoxifen treatment in *Act-CreER* mice crossed with the *Rosa26-lacZ* reporter line. Nine days following tamoxifen treatment (14 days after the first dose of tamoxifen), a lethal dose of pentobarbital (100 mg/kg, i.p.) was administered, the mouse was decapitated, and the brain was removed and rapidly frozen on powdered dry ice. The fresh frozen brain was sectioned at 40 µm using a cryostat and mounted on Plus slides (Erie Scientific Company). Slide-mounted tissue sections were thawed into ice cold 4% paraformaldehyde in PBS for 15 min, washed three times in PBS for 5 min each, and then immersed in the X-gal staining solution [1 mg/ml X-Gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 (NP-40) in PBS] overnight in a 37°C incubator. Following staining, the slides were washed twice in PBS for 10 min, in dH₂O for 5 min, and mounted with 90% glycerol. Some slides were counterstained with neutral red to facilitate identification of anatomic structures.

Surgery and Kindling

Under pentobarbital (60 mg/kg, i.p.) anesthesia, a bipolar electrode used for stimulation and recording was implanted using stereotaxic guidance in the right amygdala using the following coordinates with bregma as the reference: 1.2 mm posterior, 2.9 mm lateral, 4.6 mm below dura. In other experiments, the bipolar electrode was implanted in the right hippocampus with the following coordinates: 2.9 mm posterior, 3.0 mm lateral, 3.0 mm below dura. After a postoperative recovery period of 1 week, the electrographic seizure threshold (EST) was determined by application of a 1 s train of 1 ms biphasic rectangular pulses at 60 Hz beginning at 20 µA using a Grass stimulator with constant current stimulus isolation units (PSIU6, Grass Technologies). Additional stimulations, increased in 10 µA steps, were administered at 1-min intervals until an electrographic seizure lasting at least 5 s was detected on the electroencephalogram (EEG) recorded from the site of stimulation, i.e., amygdala or hippocampus. Stimulations at the intensity of EST were subsequently administered following the standard kindling protocol that was described previously in detail (He et al., 2002). Video and EEGs were recorded using Harmonie software (Stellate Systems, Montreal, Quebec, Canada). The behavioral manifestations of seizures were classified according to a modification of the description of Racine (1972): (1) facial clonus; (2) head nodding; (3) unilateral forelimb clonus; (4) rearing with bilateral forelimb clonus; (5) rearing and falling (loss of postural control); (6) running or bouncing seizures; and (7) tonic hind limb extension. Unless specified otherwise, mice were stimulated until fully

kindled as defined by the occurrence of three consecutive seizures of Class 4 or greater. The kindling procedures were performed by an individual blinded to genotype of the animals.

Brain Homogenates and Immunoblot

Mice were treated with a lethal overdose of pentobarbital (100 mg/kg, i.p.) and decapitated. Following decapitation, the brain was removed and divided along the midline. The right hemisphere was frozen on powdered dry ice and subsequently sectioned to confirm electrode placement. The left hemisphere was dissected on ice into parietal cortex, amygdala/pyriform cortex, hippocampus, and cerebellum. The parietal cortex was dissected as the cortex overlying the hippocampus, from the rhinal fissure to the midline. The amygdala/pyriform cortex was dissected as the cortex lateral to the thalamus and inferior to the rhinal fissure. Each dissected region was homogenized in lysis buffer [20 mM Tris, pH 8.0, 137 mM NaCl, 1% Igepal, 10% glycerol, 1 mM phenylmethylsulfonylfluoride, and 1 Complete Mini protease inhibitor tablet (Roche, Mannheim, Germany)/10 ml]. The pellet was discarded following centrifugation at 0.3g for 10 min, and the protein concentration of the supernatant was determined using a BCA Protein Assay kit (Pierce, Rockford, IL). The homogenate was diluted to 2 mg/ml with lysis buffer, aliquoted, and stored at -80°C for further biochemical analysis.

For immunoblotting, the sample loading buffer [150 mM Tris-HCl (pH 6.8)/15% SDS/25% (v/v) glycerol/0.02% (w/v) bromophenol blue/12.5% (v/v) 2-mercaptoethanol] was added to the homogenate and boiled for 10 min before SDS-PAGE, transfer, and immunoblotting on protran nitrocellulose membrane (Whatman, Germany) as recommended by the manufacturer. The membrane was blocked with 3% nonfat dry milk or 3% bovine serum albumin in 1× TBS buffer containing 0.05% Tween-20, probed with mouse anti-TrkB antibody (1:500, BD Transduction Laboratories) and a mouse anti-β-actin antibody (1:10,000, Sigma) for 1 h at room temperature followed by three washes with blocking solutions and then with peroxidase-conjugated goat antimouse secondary antibodies (1:5,000, Jackson Immunoresearch) for another hour. The immunoblots were developed with enhanced chemiluminescence (ECL, Amersham Pharmacia, Buckinghamshire, United Kingdom).

To quantify TrkB protein expression, a standard curve was constructed by inclusion of a series of dilutions of homogenate with known protein content. The standard curve spanned the range of TrkB levels detected in the samples. The photographic film was scanned and band intensity was determined using volume quantification in ImageQuant (Molecular Dynamics). The value for each animal was calculated as the mean of three separate samples.

RESULTS

Characterization of Act-CreER Rosa26-lacZ Mice

To determine the anatomic distribution of the tamoxifen-induced Cre recombinase activity in adult mouse brain, β-galactosidase activity was examined in coronal sections of *Act-CreER* mice crossed to a *Rosa26-lacZ* reporter line. *Act-CreER Rosa26-lacZ* mice were treated with 7.5 mg tamoxifen daily for 5 days, sacrificed 5–7 days after the last dose, and β-galactosidase activity was assessed by X-gal staining. Tamoxifen treatment of mature (>2 months old) *Act-CreER Rosa26-lacZ* mice revealed particularly robust X-gal staining in the principal cell layers of hippocampus (right side of Fig. 1B); in addition, widespread X-gal staining was evident in neocortex, striatum, septum, ventral posterior thalamic nucleus, the periaqueductal gray, and the cerebellum (Fig. 1A). Importantly, no X-gal staining was detected in vehicle-treated *Act-CreER Rosa26-lacZ* mice (left side of Fig. 1B), demonstrating that activation of Cre recombinase activity required tamoxifen. Treatment of

Act-CreER Rosa26-lacZ mice with a single dose of tamoxifen (7.5 mg) underscored the X-gal staining in principal cell layers of hippocampus, especially robust in the dentate gyrus, with fewer cells stained in other regions (Fig. 1C). Analysis of Cre activity in tamoxifen-treated *Act-CreER Rosa26-lacZ* mice demonstrated recombination throughout the principal cells of the hippocampus (Fig. 1D); by comparison, sparser X-gal staining was evident within the amygdala/pyriform cortex, the most extensive recombination evident in the medial amygdaloid nucleus, posterodorsal part (Fig. 1E).

Characterization of *Act-CreER TrkB^{flx/flx}* Mice

Importantly, the efficacy of recombination must be examined directly in the *TrkB^{flx/flx}* in addition to the *Rosa26-lacZ* reporter line. To assess the anatomic distribution of the tamoxifen-induced elimination of TrkB in the brain of *Act-CreER TrkB^{flx/flx}* mice, content of TrkB protein was assessed by immunoblotting dissected brain regions. Like the *Act-CreER × Rosa26-lacZ* mice, *Act-CreER TrkB^{flx/flx}* mice were treated with 7.5 mg of tamoxifen daily for 5 days and sacrificed 14 days after the initial dose of tamoxifen. Hereafter, tamoxifen-treated *Act-CreER TrkB^{flx/flx}* mice will be referred to as *Act-CreER TrkB^{-/-}* mice. As predicted by the robust X-gal staining in the hippocampus of the reporter mouse (Fig. 1), clear reductions of full length TrkB were evident in hippocampus of the *Act-CreER TrkB^{-/-}* mice (Figs. 2A,C). Paralleling the sparser X-gal staining in other regions (Fig. 1), reductions of full length TrkB were not detected in neocortex, amygdala/pyriform cortex, or cerebellum of *Act-CreER TrkB^{-/-}* compared to controls (Figs. 2A,B). Despite clear reductions of full length TrkB (TrkB.FL), quantitative measures of truncated TrkB (TrkB.T) revealed no reduction in hippocampus of *Act-CreER TrkB^{-/-}* mice (Fig. 2C). The reduction of full length but not truncated TrkB in hippocampus implies that recombination occurred predominantly in neurons because full length TrkB expression is restricted to neurons whereas truncated TrkB is richly expressed in astrocytes (Frisen et al., 1993).

Variability in the extent of TrkB reduction among animals was evident despite identical treatments with tamoxifen (Figs. 2B, 4D, and 5D). For example, among three *Act-CreER TrkB^{-/-}* mice receiving the same regimen of tamoxifen (Fig. 2B), striking and more modest reductions of TrkB content were evident in animals CreER + TAM1 and CreER + TAM2, respectively, yet no reduction of TrkB content was evident in a third animal (CreER + TAM3) (Fig. 2B). Despite variability in reduction of TrkB content within hippocampus among *Act-CreER TrkB^{-/-}* mice, the absence of detectable reductions of TrkB content within amygdala/pyriform cortex was a consistent finding, even in animals with marked reduction of hippocampal TrkB content (e.g., CreER + TAM1, Fig. 2B).

Kindling Induced by Stimulation of Amygdala

A significant reduction of the rate of development of kindling was detected in *Act-CreER TrkB^{-/-}* mice as compared to controls. *Act-CreER TrkB^{flx/flx}* mice were treated with tamoxifen (referred to as *Act-CreER TrkB^{-/-}* mice) at 2 months of age to induce Cre recombinase activity before beginning the kindling protocol (Fig. 3, top). The three control groups (*Act-CreER TrkB^{flx/flx}* + vehicle, wild-type mice + tamoxifen, wild-type mice + vehicle) were pooled for analyses, because comparison of these groups by ANOVA revealed no significant differences ($P > 0.05$). Therefore, neither tamoxifen treatment in the absence of the transgenes nor the transgenes in the presence of vehicle affected initial electrographic seizure threshold, initial after discharge duration, or rate of kindling development. The number of stimulations required for development of kindling (three consecutive seizures of Class 4 or greater) in *Act-CreER TrkB^{-/-}* mice exceeded controls by 60% (*Act-CreER TrkB^{-/-}* 16.1 ± 2.1 stimulations vs. controls 10.1 ± 1.3 stimulations, $P < 0.05$) (Fig. 4C). *Act-CreER TrkB^{-/-}* mice did not differ from control mice with respect to current intensity required to induce the initial electrographic seizure (*Act-CreER TrkB^{-/-}* 210 ± 45 μ A vs.

controls $190 \pm 29 \mu\text{A}$, $P > 0.05$) (Fig. 4A). Likewise, no difference was detected in the duration of the first electrographic seizure (*Act-CreER TrkB*^{-/-} 11.2 ± 1.1 s vs. controls 12.8 ± 2.1 s, $P > 0.05$) (Fig. 4B).

The variable success in reducing TrkB with tamoxifen treatment in *Act-CreER TrkB*^{-/-} mice raised the question as to whether a relationship existed between the extent of TrkB reduction and the rate of kindling development. To address this question, TrkB protein content was quantified by immunoblot and the relationship between the % control TrkB content and the number of stimulations to induce kindling was examined (Fig. 4D). Linear regression analysis revealed that this relationship was statistically significant ($r^2 = 0.17$ and $P\text{-value} = 0.04$). The statistical significance notwithstanding, marked variation in the rate of kindling development was evident among animals exhibiting similar reductions (40–60%) of hippocampal TrkB content (Fig. 4D).

Kindling Induced by Stimulation of Hippocampus

The finding that reductions of TrkB protein content were detected in hippocampus but not amygdala/pyriform cortex following tamoxifen treatment of *Act-CreER TrkB*^{flx/flx} mice led us to examine development of kindling induced by direct stimulation of hippocampus in tamoxifen-treated *Act-CreER TrkB*^{flx/flx} mice. Here, we found a marked reduction in the rate of kindling development in *Act-CreER TrkB*^{-/-} mice as compared to controls. Similar to the amygdala stimulation experiments, comparison of the three control groups (*Act-CreER TrkB*^{flx/flx} + vehicle, wild-type mice + tamoxifen, wild-type mice + vehicle) revealed no significant differences (ANOVA, $P > 0.05$) and so these groups were pooled for subsequent analyses. The number of stimulations required to induce the first Class 4/5 seizure in the *Act-CreER TrkB*^{-/-} exceeded controls by 150% (*Act-CreER TrkB*^{-/-} 30.6 ± 5.0 vs. controls 12.1 ± 1.5 stimulations, $P < 0.01$) (Fig. 5C, third column). Similarly, the number of stimulations required to induce the fully kindled state in the *Act-CreER TrkB*^{-/-} exceeded controls by 145% (*Act-CreER TrkB*^{-/-} 36.5 ± 0.6 vs. controls 15.5 ± 1 stimulations, $P < 0.01$) (Fig. 5C, fourth column). Importantly, these measures underestimate the inhibitory effects of reduction of TrkB, because 2 of the 10 *Act-CreER TrkB*^{-/-} mice did not exhibit a seizure of Class 4 or greater despite 50 stimulations of the kindling protocol and were therefore arbitrarily assigned a value of 50. *Act-CreER TrkB*^{-/-} mice did not differ from control mice with respect to current intensity required to induce the initial electrographic seizure (*Act-CreER TrkB*^{-/-} $113 \pm 27 \mu\text{A}$ vs. controls $170 \pm 30 \mu\text{A}$, $P > 0.05$) (Fig. 5A). Likewise, no difference was detected in the duration of the first electrographic seizure (*Act-CreER TrkB*^{-/-} 18.8 ± 2.1 s vs. controls 20.4 ± 2.0 s, $P > 0.05$) (Fig. 5B).

The relationship between the reduction of TrkB protein content and the number of stimulations to induce kindling was also addressed in this experiment. Linear regression analysis of the relationship between percentage of control TrkB content and the number of stimulations to induce kindling (Fig. 5D) was highly significant ($r^2 = 0.65$ and $P\text{-value} < 0.01$). In contrast to this analysis of amygdala kindling, here there was quite consistent relationship between the extent of reduction of TrkB protein content and the impairment of kindling development.

DISCUSSION

We sought to determine whether reduced expression of TrkB de novo in the adult mammalian brain is sufficient to impair epileptogenesis in the kindling model. The principal findings are as follows: (1) Systemic treatment of adult *Act-CreER TrkB*^{flx/flx} mice with tamoxifen produced a modest reduction of TrkB protein content that was unexpectedly confined to the hippocampus; (2) Modest reductions of hippocampal TrkB content markedly

inhibited kindling induced by stimulation of hippocampus and also significantly inhibited kindling induced by stimulation of amygdala. These results demonstrate that reducing TrkB expression in the adult hippocampus is sufficient to impair epileptogenesis in the kindling model. These findings have important mechanistic and therapeutic implications.

Technical Considerations

The inducibility of the *Act-CreER* by treatment with tamoxifen provides the opportunity to examine the role of a single gene in a complex process of a living, adult mouse. Inducing recombination of a gene in neurons of the central nervous system de novo in the adult mouse has only recently been reported (Bradley et al., 2007; Erdmann et al., 2007; Guy et al., 2007), and in only one instance was the effect of recombination assessed at a behavioral level of analysis (Guy et al., 2007). In the present study, the *Act-CreER* mouse was used to probe the role of TrkB in epileptogenesis in the kindling model. The lack of effect of tamoxifen on epileptogenesis in control mice together with its efficacy in promoting recombination of the floxed *TrkB* alleles revealed a key role of TrkB in neurons of the adult hippocampus. The induced reductions of full length but not truncated TrkB provide evidence that the activity of the *CreER* was limited to neurons under these experimental conditions (Klein et al., 1990; Middlemas et al., 1991; Frisen et al., 1993; Fryer et al., 1996). The induction of recombination of the floxed *TrkB* alleles in hippocampus correlates with the locale of the greatest *Act-CreER* activity evident in studies of the reporter mouse (Fig. 1C). The cause of the restricted Cre expression is unknown, although it may be due to the promoter sequence of the transgene or the site in the mouse genome at which the transgene was inserted. Although reduction of TrkB content in a highly restricted distribution within regions outside hippocampus may escape detection, the immunoblot method was selected because it provided a method of reliably quantifying TrkB expression. These advantages notwithstanding, some unexpected technical limitations emerged. The extent of recombination varied among *Act-CreER TrkB^{flox/flox}* mice receiving identical doses of tamoxifen by oral gavage daily for 5 days; while tamoxifen treatment resulted in reductions of TrkB protein content of 40–70% in most animals, this treatment induced no detectable reduction in two animals stimulated in hippocampus and in three animals stimulated in amygdala (Figs. 4D and 5D). Some variability in the extent of tamoxifen-induced recombination was also noted in studies of *MECP2* recombination (Guy et al., 2007). Such failures seem most likely due to insufficient amounts of tamoxifen accessing hippocampal neurons, yet no hint of difficulties with administration or absorption of tamoxifen was evident in the experiments. That said, the variability encountered provided the opportunity to examine correlations between the extent of recombination and the behavioral phenotype among individuals animals.

Mechanistic Implications

Our findings raise the question as to the nature of the cellular consequences of TrkB activation within hippocampus that contribute to epileptogenesis in this model. Addressing this question must take into account three observations in the present study. The fact that reduction of TrkB content de novo in the adult is sufficient to produce marked antiepileptogenic effects excludes developmental consequences of absent TrkB as the critical mechanism (e.g., formation of inhibitory synapses); instead, this focuses consideration of cellular consequences of TrkB signaling in the mature brain. The fact that modest reductions of TrkB content within hippocampus markedly inhibited kindling induced by stimulation of hippocampus, and furthermore significantly inhibited kindling induced by stimulation of amygdala must be considered. Finally, the fact that these reductions of TrkB content within hippocampus resulted in a striking inhibition of kindling despite no change of initial excitability as assessed by current required to induce a focal seizure or the duration of the first evoked seizure must be considered. Stated differently, reductions of TrkB protein

predominantly in the hippocampus impaired enhanced propagation of electrographic seizure activity initiated by stimulation of two distinct sites (amygdala and hippocampus), regardless of whether TrkB content was significantly reduced at the site.

One plausible explanation is that induction of pathologic activity in the form of stimulation-evoked focal seizures results in long-term potentiation of excitatory synapses between principal neurons locally within the hippocampus of wild-type mice. Potentiation of these synapses would be expected to facilitate propagation of subsequent focal seizures, resulting in invasion of progressively expanding neural circuitry and behavioral seizures of greater intensity and duration. Consistent with this proposal is the finding that limbic seizures induce LTP of multiple excitatory synapses within entorhinal–hippocampal circuitry including the synapses made by the mossy fiber axons with CA3 pyramidal cells (Sutula and Steward, 1986; Goussakov et al., 2000). LTP of the mossy fiber synapse with CA3 pyramids may be particularly relevant to the present findings because seizures evoked by stimulation of hippocampus induce enhanced activation of TrkB localized to the mossy fiber pathway (Binder et al., 1999; He et al., 2002, 2004). Moreover, genetic elimination of TrkB or acutely inhibiting TrkB kinase activity impairs induction of LTP of the mossy fiber-CA3 pyramid synapse (Huang et al., 2008) and also of the Shaffer collateral-CA1 pyramid synapse [Xu et al., 2000; but see Huang et al. (2008)]. Thus, impairments of TrkB-dependent LTP of excitatory synapses locally within hippocampus may contribute to the inhibition of kindling induced by stimulation of amygdala or hippocampus. The trend toward more robust inhibition of kindling induced by stimulation of amygdala compared to hippocampus may reflect recruitment of extrahippocampal circuitry by seizures arising from amygdala.

Importantly, the hypothesized role of impaired LTP is compatible with the equivalent threshold for induction of focal seizure and with equivalent duration of the initial seizure evoked in tamoxifen-treated *Act-CreER TrkB^{flox/flox}* and control mice. That is, although inhibition of TrkB kinase activity or reduction of TrkB content impairs induction of LTP induced by high frequency stimulation of afferents of CA3 or CA1 pyramidal cells, the efficacy of basal synaptic transmission is not affected (Xu et al., 2000; Huang et al., 2008), and threshold and duration of the initially evoked seizure thus would be expected to be normal. The results of the present study differ from previous findings in *Synapsin-Cre TrkB^{flox/flox}* mice (He et al., 2004) in two ways, which are likely a consequence of the degree and/or timing of TrkB reduction. Conditional deletion of TrkB was previously shown to eliminate kindling induced by stimulation of amygdala (He et al., 2004) in contrast to the more modest inhibitory effects in the present study. Also conditional deletion of TrkB in the *Synapsin-Cre TrkB^{flox/flox}* mice was associated with increases of the focal seizure threshold. In contrast to the tamoxifen-treated *Act-CreER TrkB^{flox/flox}* mice of the present study, reduction of TrkB content locally within amygdala/pyriform cortex was identified in *Synapsin-Cre TrkB^{flox/flox}* mice, a factor likely to contribute to inhibition of kindling development. Developmental effects of loss of TrkB contributing to the difference in kindling phenotype also cannot be excluded. Finally, the reduction of TrkB dating back to embryonic development may contribute to the increased focal seizure threshold in the *Synapsin-Cre TrkB^{flox/flox}* mice.

In addition to potentiating excitatory synaptic transmission, TrkB activation may limit inhibitory synaptic transmission. That is, enhanced activation of TrkB has been shown to reduce the expression of the K^+-Cl^- transporter, KCC2, impairing Cl^- extrusion and thereby decreasing the ability of GABA_A receptors to mediate synaptic inhibition. Following rapid kindling in mice, expression of KCC2 is decreased in the hippocampus, and this reduction of KCC2 expression can be prevented by inhibiting TrkB signaling (Rivera et al., 2004).

Interestingly, decreased *KCC2* mRNA expression is found in the hippocampus and subiculum of patients with mesial TLE (Palma et al., 2006).

Therapeutic Implications

The present findings strongly support the candidacy of TrkB as a molecular target for antiepileptogenic therapy. The fact that reduction of TrkB content de novo in the adult brain inhibited epileptogenesis in the kindling model implies that therapies inhibiting TrkB and its downstream signaling pathways in adults may prevent development or progression of epilepsy. The fact that the reduction of TrkB content inhibited kindling induced by hippocampal stimulation is promising, because the hippocampus is thought to play an important role in medically refractory mesial temporal lobe epilepsy of humans (Falconer, 1974). The marked inhibition of hippocampal kindling caused by reductions of TrkB content approximating 40–70% suggests that modest reductions of TrkB content may prove sufficient to exert antiepileptogenic effects. The powerful antiepileptogenic effects of reductions of TrkB content localized to the hippocampus suggest that powerful antiepileptogenic effects may be achievable without inhibiting TrkB globally throughout the brain, thereby minimizing unwanted effects. Collectively, these findings raise the possibility that gene therapy targeting TrkB within a hippocampus from which seizures arise may limit progression of limbic epilepsy.

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FIGURE 1.

β-Galactosidase staining in a tamoxifen-treated *Act-CreER Rosa26-lacZ* mice. (A) β-Galactosidase activity throughout a coronal series of *Act-CreER Rosa26-lacZ* mice treated with tamoxifen for 5 days. For analysis of inducible Cre activity, *Act-CreER* mice were crossed to a *Rosa26-lacZ* reporter line, in which β-galactosidase expression reflects Cre activity, and then treated with tamoxifen. X-gal staining resulting from β-galactosidase activity is widespread in cortex, striatum, amygdala, hypothalamus, and hippocampus. (B) Control of Cre recombinase activity. Comparison of vehicle-treated (left) and tamoxifen-treated (for 5 days) (right) *Act-CreER Rosa26-lacZ* mouse. (C) Submaximal induction of Cre activity. An example of submaximal activation of Cre activity by a single dose of

tamoxifen (by mouth 7.5 mg once). Cre activity is predominately in the hippocampus, especially in the dentate gyrus, and in the pyriform cortex, with sporadic X-gal positive cells throughout the brain. Greater variability was seen in the degree of induced Cre activity with a single dose of tamoxifen. (D) Cre activity in the hippocampus. Cre activity of the tamoxifen-treated (5 days) *Act-CreER* mice in the *Rosa26-lacZ* reporter line revealed activation of Cre with varying anatomical distributions. Within the hippocampus the *Act-CreER* mouse has relatively uniform staining in all principle cell layers. (E) Cre activity in the amygdala-pyriform cortex. In the amygdala, the *Act-CreER* mouse has the most intense staining in the medial amygdaloid nucleus, posterodorsal part (MePD). Please note subtle color variations are due to slice thickness and differences in image acquisition. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

FIGURE 2.

Reduction of TrkB protein in Act-CreER TrkB^{-/-} mice. (A) TrkB immunoblot in multiple brain regions in wild type (WT) and *Act-CreER TrkB^{-/-}* (Act-CreER). Immunoblotting was done to examine TrkB protein levels in different brain regions following tamoxifen treatment in an *Act-CreER TrkB^{-/-}* mouse as compared to wild type control mice. Actin immunoblot is included as a loading control. (B) TrkB immunoblot in multiple tamoxifen-treated *Act-CreER TrkB^{flox/flox}* and control mice. Comparison of multiple tamoxifen-treated *Act-CreER TrkB^{flox/flox}* mice with control mice reveals a spectrum of efficiency in elimination of TrkB protein from the hippocampus (HPC), with no changes in TrkB in the amygdala-piriform cortex (APC). Mice presented in these experiments were used only to

examine TrkB levels and were not implanted and were not kindled, although the results are representative of mice used in kindling experiments. (C) Hippocampal TrkB protein quantification in *Act-CreER TrkB*^{-/-} and control mice. The concentration of full-length TrkB (TrkB.FL) in the hippocampus is decreased in *Act-CreER TrkB*^{-/-} mice, both amygdaloid ($n = 10$) and hippocampal kindled ($n = 10$), as compared to controls ($n = 15$ for APC kindling and 15 for HPC kindling). No difference is seen in TrkB.T protein in any of the groups. Mean + standard error of mean (SEM), * $P < 0.01$.

Amygdaloid Kindling **Hippocampal Kindling** **FIGURE 3.**

Kindling and tamoxifen protocols for *Act-CreER TrkB^{flx/flx}* mice. *Act-CreER TrkB^{flx/flx}* mice and wild type controls are allowed to mature to 2 months of age before treatment with tamoxifen or vehicle. Following treatment with tamoxifen (by feeding tube 7.5 mg $\times$ 5 days), mice were implanted with electrodes for kindling, either in the hippocampus or in the amygdala. Kindling was continued until the behavioral seizures progressed to three consecutive Class 4/5, at which point the mouse was considered fully kindled.

FIGURE 4.

Amygdaloid kindling in the *Act-CreER TrkB*^{-/-} mice. (A) Initial electrographic seizure threshold. *Act-CreER TrkB*^{-/-} mice did not differ from control mice in initial excitability as evidenced by an equivalent threshold to induce an electrographic seizure (*t*-test, $P > 0.05$). (B) Initial electrographic seizure duration. *Act-CreER TrkB*^{-/-} mice did not differ from control mice in initial excitability as evidenced by an equivalent duration of the first seizure (*t*-test, $P > 0.05$). (C) Kindling development. *Act-CreER TrkB*^{-/-} mice have delayed kindling development as compared with control mice, requiring significantly more stimulations to reach the fully kindled state (ANOVA, post hoc Bonferroni, $P < 0.05$). (D) Amygdaloid kindling and relative TrkB protein content in the hippocampus. Normal levels

of hippocampal TrkB are associated with more rapid kindling (control mice and three *Act-CreER TrkB*^{-/-} mice without a reduction in TrkB), while reduced levels of TrkB are associated with delayed kindling (seven *Act-CreER TrkB*^{-/-} mice with reduced TrkB) ($n = 10$ *Act-CreER TrkB*^{-/-} and 15 controls for A, B, C), mean + SEM, * $P < 0.05$.

FIGURE 5.

Hippocampal kindling in *Act-CreER TrkB*^{-/-} mice. (A) Initial electrographic seizure threshold. *Act-CreER TrkB*^{-/-} mice did not differ from control mice in initial excitability as evidenced by an equivalent threshold to induce an electrographic seizure (*t*-test, $P > 0.05$). (B) Initial electrographic seizure duration. *Act-CreER TrkB*^{-/-} mice did not differ from control mice in initial excitability as evidenced by an equivalent duration of the first seizure (*t*-test, $P > 0.05$). (C) Kindling development. *Act-CreER TrkB*^{-/-} mice have delayed kindling development as compared with control mice, requiring significantly more stimulations to reach the first Class 4/5 seizure and to reach the fully kindled state (ANOVA, post hoc Bonferroni, $P < 0.05$). (D) Hippocampal kindling and relative TrkB

protein content in the hippocampus. Normal levels of hippocampal TrkB are associated with more rapid kindling (control mice and two *Act-CreER TrkB*^{-/-} mice without a reduction in TrkB), while reduced levels of TrkB are associated with delayed kindling (eight *Act-CreER TrkB*^{-/-} mice with reduced TrkB) ($n = 10$ *Act-CreER TrkB*^{-/-} and 15 controls for A, B, C), mean + SEM, * $P < 0.05$.