Regression of Epileptogenesis by Inhibiting Tropomyosin Kinase B Signaling following a Seizure

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Objective: Temporal lobe epilepsy (TLE) is a devastating disease in which seizures persist in 35% of patients despite optimal use of antiseizure drugs. Clinical and preclinical evidence implicates seizures themselves as one factor promoting epilepsy progression. What is the molecular consequence of a seizure that promotes progression? Evidence from preclinical studies led us to hypothesize that activation of tropomyosin kinase B (TrkB)–phospholipase-C-gamma-1 (PLCγ1) signaling induced by a seizure promotes epileptogenesis.

Methods: To examine the effects of inhibiting TrkB signaling on epileptogenesis following an isolated seizure, we implemented a modified kindling model in which we induced a seizure through amygdala stimulation and then used either a chemical–genetic strategy or pharmacologic methods to disrupt signaling for 2 days following the seizure. The severity of a subsequent seizure was assessed by behavioral and electrographic measures.

Results: Transient inhibition of TrkB-PLCγ1 signaling initiated after an isolated seizure limited progression of epileptogenesis, evidenced by the reduced severity and duration of subsequent seizures. Unexpectedly, transient inhibition of TrkB-PLCγ1 signaling initiated following a seizure also reverted a subset of animals to an earlier state of epileptogenesis. Remarkably, inhibition of TrkB-PLCγ1 signaling in the absence of a recent seizure did not reduce severity of subsequent seizures.

Interpretation: These results suggest a novel strategy for limiting progression or potentially ameliorating severity of TLE whereby transient inhibition of TrkB-PLCγ1 signaling is initiated following a seizure.

despite the introduction of a panoply of new antiseizure drugs in the past quarter century, there has been no measurable improvement in the proportion of patients with newly diagnosed epilepsy rendered free of seizures.1 Approximately one-third of such patients experience recurrent seizures despite treatment by skilled clinicians with recently introduced therapeutics.1 These failures underscore the need to elucidate the mechanisms underlying the development and/or progression of epilepsy, a process termed epileptogenesis.2

Of the various forms of epilepsy, temporal lobe epilepsy (TLE) is both common and commonly debilitating.3,4 Notably, TLE is frequently progressive, with worsening of clinical course,5 comorbidities,6 and structural lesions.7,8 One factor that might contribute to this progression is the occurrence of seizures themselves, as first posited by Gowers, who proposed in 1881 that "seizures beget seizures."9 In support of this idea, longitudinal observations of a cohort of patients with newly diagnosed epilepsy revealed that individuals at low risk of recurrent seizures exhibited a progressive increase in risk with increasing number of seizures.5 Direct evidence that an isolated seizure could promote both development and progression of epilepsy emerged from preclinical observations.10 Repeatedly evoking brief, localized seizures induced a progressive increase in duration and severity of subsequent evoked seizures, a model termed kindling.10,11 Evoking many (eg, 70–80) such seizures culminated in recurrent seizures occurring without stimulation, often associated with fatality.12,13 Furthermore, the frequency and severity of seizures progressively increases long after the onset of epilepsy in diverse models including hypoxia–ischemia, status epilepticus...
transient inhibition of TrkB-PLCγ1 signaling following prolonged seizures prevents subsequent development of epilepsy.\textsuperscript{19,20} We therefore hypothesized that transiently inhibiting TrkB-PLCγ1 signaling by treatment for 2 days following an isolated seizure would inhibit progression of epilepsy. We used the kindling model to test our hypothesis because of the convenience afforded over timing of a seizure.

**Materials and Methods**

**Animals**
All animal procedures were approved by the institutional animal care and use committee at Duke University and conform to the US Public Health Service’s Policy on Humane Care and Use of Laboratory Animals, as well as the National Institutes of Health and Duke University institutional guidelines for the care and use of experimental animals. Animals were maintained on a 12-hour light/dark cycle with food and water available ad libitum. Wild-type (WT) adult 8- to 12-week-old C57BL/6 male mice were obtained from Charles River (Wilmington, MA). TrkB\textsuperscript{F616A} mice were originally obtained from Dr. David Ginty\textsuperscript{21} and backcrossed to the C57BL/6 line for at least 7 generations. This knockin mouse harbors a point mutation on the TrkB allele, substituting an alanine for phenylalanine within the adenosine triphosphate binding pocket of the TrkB kinase domain. This mutation renders TrkB protein uniquely susceptible to kinase inhibition by small molecule derivatives of the general kinase inhibitor PP1, including 1-(1,1-dimethylethyl)-3-(1-naphthalenylmethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (1NMPP1). Importantly, 1NMPP1 does not have any detectable effect in WT mice, and there are no differences in TrkB kinase activity in the TrkB\textsuperscript{F616A} compared to WT mice in the absence of this compound.\textsuperscript{19,21} Both male and female adult (8–12 weeks old) homozygous mice were used.

**Kindling**
Kindling experiments were performed as previously described.\textsuperscript{18,22} A bipolar stimulating and recording electrode was inserted into the right amygdala (1.0mm posterior, 2.9mm lateral to bregma; 4.6mm below dura). A skull screw was placed over the left frontal lobe as a ground electrode. After a postoperative recovery period of at least 5 days, animals were connected to a Grass Stimulator and monitored by both video and electroencephalogram (EEG). The current required to evoke an electrographic seizure with duration $\geq$5 seconds (electrographic seizure threshold [EST]) was determined by applying a 1-second train of 1-millisecond biphasic-rectangular pulses at 60Hz beginning at 20µA. Additional stimulations were given in 20µA increments at 1-minute intervals until an electrographic seizure was detected. Animals then received 2 stimulations per day at the EST, spaced at least 6 hours apart, with the behavioral seizure scores classified according to a modification of the Racine scale for mice: 0, normal activity; 1, arrest and rigid posture; 2, head nodding; 3, unilateral forelimb clonus; 4, rearing with bilateral forelimb clonus; 5, rearing and falling; 6, tonic–clonic seizures with violent running and/or jumping.\textsuperscript{23} The criterion for “kindled” was the occurrence of 3 consecutive seizures of class 4 or greater, with limb clonus/tonus lasting at least 12 seconds. Once “kindled,” subsequent evoked seizures were characterized by abrupt onset concomitantly of electrographic seizure activity in the stimulated amygdala and behavioral seizures culminating in class 4, 5, or 6. Electrographic seizure activity was typically followed by postictal depression of EEG activity in the stimulated amygdala during which animals exhibited immobility and occasional orofacial clonus; this sequence was followed by abrupt return of exploratory activity in the cage. For each seizure, 4 variables were quantified by offline analyses of video-EEG recordings by a blinded, trained observer: (1) the maximum behavioral seizure class, (2) the duration of electrographic seizure activity, (3) the duration of overt seizure activity defined as clonic and/or tonic behaviors of class 4 or greater, and (4) the total time elapsed between seizure onset and abrupt return of exploratory activity in the cage. For variables 2 through 4, data are presented normalized to the seizure evoked prior to treatment, thereby enabling each animal to serve as its own control.

Design of individual experiments is presented in panel A of each figure. All seizures following “kindling” (Fig 1A), whether designated seizure 1 or 2, were evoked with a stimulation intensity determined by assessing the EST a second time, administering stimulations in 20µA increments every 1 minute, beginning at 20µA, until an electrographic seizure (duration $\geq$5 seconds) was evoked. Notably, in experiments presented in Figures 1A–E, 2, 3, and 5, experimental design mandated that the behavioral pattern accompanying electrographic seizure 1 was class 4 or greater.

**Treatments**
Prior to each administration, a solution of 100mM 1NMPP1 was dissolved in a solubilization buffer (vehicle) containing 0.9% NaCl and 2.5% Tween-20 to a concentration of 1.67mg/ml and dosed at 16.6µg/g intraperitoneal (IP) every 12 hours for a total of 5 treatments. Vehicle injection served as a control. In addition to treatments by IP injection, either 1NMPP1 (25µM) or vehicle was included in drinking water for the 2 days of treatment (see Figs 2 and 4).

Peptides were prepared as previously described.\textsuperscript{20} The sequence of human TrkB amino acids 807 to 820 (LQNLAKASPVPYLDI) with the tyrosine at residue 817 phosphorylated (note that this corresponds to residue 816 in mouse TrkB protein) was conjugated at the N-terminus to the HIV transactivating protein transduction domain (tat: YGRKKRRQRRR) to facilitate membrane permeability (termed “pY816”). The HIV tat sequence conjugated to a scrambled peptide (LVApYQLKAPNDLS) served as a control (termed “Scr”). Peptides were synthesized and purified by Tufts Peptide Core Facility.
dissolved in sterile phosphate-buffered saline at 2mg/ml, stored at −80°C, thawed just prior to treatment administration, and given at a dose of 20mg/kg IP, for a total of 5 doses given 12 hours apart. The quality of each batch of peptide was assessed by reverse phase high-performance liquid chromatography, verifying that at least 95% elutes as a single peak.

FIGURE 1: Time and evoked seizure in kindled animals increased duration of subsequent electrographic and behavioral seizure, with a more robust effect of evoked seizure. (A) Schematic of experimental design for animals receiving evoked seizure 6 days after kindling. Control animals from experiments depicted in Figures 2B–E and 3B–E were pooled for representation. (B–E) Electrographic seizure duration, overt behavioral seizure duration, combined ictal/postictal duration, and seizure score for animals receiving control treatment after an evoked seizure (n = 28). (F) Schematic of experimental design for animals not receiving evoked seizure 6 days after kindling. Control animals from experiments depicted in Figure 4 were pooled for representation. (G–J) Electrographic seizure duration, overt behavioral seizure duration, combined ictal/postictal duration, and seizure score for animals not receiving control treatment after an evoked seizure (n = 24). Data were analyzed by 2-way analysis of variance with repeated measures and post hoc Bonferroni test. *p < 0.05, **p < 0.01, ***p < 0.001. EEG = electroencephalographic; Kind = kindled; Sz = seizure; Tx = treatment. [Color figure can be viewed at www.annalsofneurology.org]
Carbamazepine (Sigma, St Louis, MO) was dissolved in a solubilization buffer of 2% Tween-80 and 70% propylene glycol at a concentration of 2mg/ml and administered at a dose of 20mg/kg, a dose of carbamazepine sufficient to produce therapeutic blood levels.24 Treatments were given every 4 hours for 2 days. Solubilization buffer was used as a control.

**Statistical Analysis**

All data analyses were performed by individuals blinded to treatment group and experimental condition. Animals were randomized to treatment groups following completion of kindling and prior to any data analysis. Sample sizes were chosen based on power analysis. Unless otherwise stated, data are presented as mean ± standard error of the mean. Unless otherwise stated, comparisons between 2 groups were analyzed using 2-way analysis of variance with repeated measures and post hoc Bonferroni test. A \( p < 0.05 \) was considered significant.

**Results**

**Progression of Epileptogenesis**

To examine progression of epileptogenesis, we implemented a variation of the kindling model. To induce “kindling,” adult mice were subjected to repeated brief (1 second) low-intensity stimulations locally within the amygdala twice daily, resulting in evoked seizures of increasing duration and propagation. Animals were termed “kindled” following the third consecutive evoked seizure with score of class 4 or greater. Following a 6-day stimulus-free period, an additional seizure was evoked (seizure 1), and the electrographic and behavioral features were quantified (see Fig 1). Following an additional 8-day stimulation-free period, another seizure was evoked (seizure 2), and the electrographic and behavioral features were again quantified. The increased duration of electrographic and behavioral features of seizure 2 compared to seizure 1 provided a model of progression, enabling us to ask whether and when brief inhibition of TrkB signaling limited epileptogenesis. Notably, the increased duration of the seizure evoked 14 days after the final kindled seizure was due in part to time elapsed, as evident in animals in which no seizure was evoked during the 2-week interval.

**Chemical–Genetic Inhibition of TrkB Kinase after an Evoked Seizure Prevents Progression**

We previously demonstrated that TrkB kinase is activated following an evoked seizure in the kindling model.18 We therefore asked whether initiating inhibition of TrkB signaling immediately following an evoked seizure would prevent progression of seizure duration and severity. Data were analyzed by 2-way analysis of variance with repeated measures and post hoc Bonferroni test. A \( p < 0.05 \), \( ***p < 0.001 \). EEG = electroencephalographic; Sz = seizure; Tx = treatment. [Color figure can be viewed at www.annalsofneurology.org]
FIGURE 3: pY816 treatment after an evoked seizure reduces duration and severity of subsequent seizures evoked at either 6 or 14 days after treatment termination. (A) Schematic of experimental design for animals receiving subsequent seizure 6 days after treatment termination. (B–E) Electrographic seizure duration, overt behavioral seizure duration, combined ictal/postictal duration, and seizure score for animals receiving pY816 (n = 17) or Scr (n = 13) after an evoked seizure 6 days following treatment termination. (F) Schematic of experimental design for animals receiving subsequent seizure 14 days after treatment termination. (G–J) Electrographic seizure duration, overt behavioral seizure duration, combined ictal/postictal duration, and seizure score for animals receiving pY816 (n = 13) or Scr (n = 13) after an evoked seizure 14 days following treatment termination. Data were analyzed by 2-way analysis of variance with repeated measures and post hoc Bonferroni test. *p < 0.05, **p < 0.01, ***p < 0.001. EEG = electroencephalographic;Sz = seizure;Tx = treatment. [Color figure can be viewed at www.annalsofneurology.org]
progression seen in our kindling model. To accomplish this, we employed a chemical–genetic approach with TrkBFG16A mice, because this provides both molecular specificity and temporal control of inhibition of TrkB kinase activity.21 Initiating inhibition of TrkB kinase with 1NMPP1 immediately following seizure 1 in TrkBFG16A mice prevented the...

FIGURE 4: 1NMPP1 or pY816 treatment in the absence of a preceding evoked seizure has no effect on subsequent seizure severity. (A) Schematic of experimental design for 1NMPP1 experiments. (B–E) Electrographic seizure duration, overt behavioral seizure duration, combined ictal/postictal duration, and seizure score for animals receiving 1NMPP1 (n = 10) or vehicle (Veh; n = 10) in the absence of a preceding evoked seizure. (F) Schematic of experimental design for pY816 experiments. (G–J). Electrographic seizure duration, overt behavioral seizure duration, combined ictal/postictal duration, and seizure score for animals receiving pY816 (n = 16) or Scr (n = 14) in the absence of a preceding evoked seizure. Data were analyzed by 2-way analysis of variance with repeated measures and post hoc Bonferroni test. EEG = electroencephalographic; Sz = seizure; Tx = treatment. [Color figure can be viewed at www.annalsofneurology.org]
approximately 50% increase in duration of electrographic seizure evident in vehicle-treated animals (see Fig 2). Likewise, 1NMPP1 treatment of TrkB<sup>F616A</sup> mice eliminated the approximately 25% increase in duration of overt seizures and 75% increase in duration of combined ictal and postictal behavior of vehicle-treated animals. A similar trend was evident with respect to behavioral seizure class (p = 0.06) when comparing treatment with 1NMPP1 compared to vehicle.

Ascribing the effects of 1NMPP1 in the TrkB<sup>F616A</sup> mice to inhibition of TrkB kinase requires that 1NMPP1 treatment of WT mice be ineffective. The duration of both electrographic and behavioral features of seizure 2 exhibited striking increases in comparison to seizure 1 when WT animals (n = 9) were treated with 1NMPP1 immediately following seizure 1 (electrographic: 223%; overt behavioral seizure: 184%; combined ictal and postictal behavior: 168%). The ability of 1NMPP1 to limit progression in TrkB<sup>F616A</sup> mice contrasts sharply with its ineffectiveness in WT animals and implicates inhibition of TrkB kinase as the mechanism underlying the effect of 1NMPP1 in the TrkB<sup>F616A</sup> mice.

**pY816 Treatment after an Evoked Seizure Prevents Progression**

Our prior studies of SE-induced TLE implicated a causal role of a single signaling pathway downstream of TrkB, namely PLCγ1; this conclusion is based in part on the beneficial effects of pY816, a peptide that uncouples TrkB from PLCγ1. Importantly, an evoked seizure in the kindling model induced activation of PLCγ1 as assessed by a surrogate biomarker, the phosphorylation of PLCγ1 tyrosine 783. Moreover, treatment with pY816 immediately following repeated seizures inhibits TrkB-induced activation of PLCγ1. Together, these observations led us to ask whether treatment with pY816 following a seizure prevented progression in duration and severity of a subsequent seizure. Treatment of WT mice with pY816 immediately following seizure 1 eliminated the approximate 200% increase of duration of electrographic seizure, duration of overt seizure, and combined ictal and postictal behavior of seizure 2 evident in Scr-treated control animals (see Fig 3). pY816 treatment also induced significant reductions of the behavioral seizure class of seizure 2 in comparison to Scr controls.

**Inhibition of TrkB-PLCγ1 Signaling following an Evoked Seizure: Evidence of Persistent Regression of Epileptogenesis to an Earlier Stage**

Experiments described above demonstrate that transient inhibition of TrkB-PLCγ1 signaling following an evoked seizure limits progression of epileptogenesis, as evident in comparisons between treatment groups and controls for seizure 2 compared to seizure 1 (see Figs 2 and 3). Unexpectedly, this transient disruption also induced a regression of epileptogenesis evident in
comparisons of seizure 2 with either seizure 1 or the final kindled seizure. This effect was particularly notable in a cohort of animals treated identically to those described in the preceding paragraph with one exception, namely that the interval between seizures 1 and 2 was extended from 8 to 14 days. In comparison to seizure 1, treatment with pY816 produced a 50% reduction in electrographic seizure duration and ictal and postictal behavior for seizure 2, as well as a reduction in overt seizure duration by 75% (Table). Furthermore, a significant reduction in behavioral seizure class for seizure 2 compared to seizure 1 was noted for the pY816-treated group. These effects contrast sharply with the increases of seizure 2 compared to seizure 1 in the Scr-treated control groups. Comparison of the final kindled seizure with seizure 2 in pY816-treated animals further demonstrated a regression to an earlier state of epileptogenesis, as evident by a significant reduction in electrographic seizure duration, overt seizure duration, and seizure score (see Table). These results demonstrate that transient inhibition of TrkB-PLCγ1 signaling following a seizure induces persistent regression of epileptogenesis.

Similar effects were evident for treatment with 1NMPP1 or pY816 with an 8-day interval between seizure 1 and seizure 2, although these were less robust (see Figs 2 and 3, Table). Treatment of TrkBF616A mice with 1NMPP1 or WT mice with pY816 produced significant reductions in duration of overt behavioral seizure of seizure 2 by approximately 50% in comparison to seizure 1; these effects contrast sharply with the increases of seizure 2 compared to seizure 1 in the vehicle or Scr peptide control groups. Similar effects were evident with respect to behavioral seizure class.

**Ineffectiveness of pY816 and 1NMPP1 in the Absence of a Preceding Evoked Seizure**

The dramatic impact on epileptogenesis seen with transient inhibition of TrkB-PLCγ1 signaling following a seizure led us to ask whether such inhibition had similar effects in the absence of a preceding seizure; restated, we wondered whether the preceding seizure activity is necessary for regression of epileptogenesis induced by inhibition of TrkB-PLCγ1 signaling. To address this question, 6 days following the final seizure evoked to establish kindling (denoted here “seizure 1”), TrkBF616A mice were treated with 1NMPP1 for 2 days and a seizure (seizure 2) was evoked 6 days following cessation of 1NMPP1 (see Fig 4). No differences were detected between 1NMPP1- and vehicle-treated mice with respect to duration of electrographic or behavioral seizure or seizure class. The effects of pY816 in WT mice were investigated using a similar experimental design. No differences were detected between pY816 and Scr peptide-treated mice with respect to duration of electrographic or behavioral seizure or behavioral seizure class.

**Treatment with Carbamazepine after an Evoked Seizure Has No Effect on Subsequent Seizure Class or Duration**

The beneficial effects of inhibitors of TrkB-PLCγ1 signaling administered transiently following an evoked seizure raised the question of whether carbamazepine, a clinically effective antiseizure drug, might have similar benefits. To address this question, a similar experimental design was employed whereby carbamazepine was administered for 2 days following an evoked seizure (see Fig 5). Fifteen to 27 days after establishing “kindling,” seizure 1 was evoked and animals were treated with either carbamazepine (n = 7, 20mg/kg IP at 4-hour intervals for 2 days) or vehicle (n = 6). Fourteen days after the last dose, seizure 2 was evoked. No differences were detected between carbamazepine- and vehicle-treated animals with respect to duration of electrographic or behavioral seizures or seizure class. The increased variability in seizure duration and class notwithstanding, these results demonstrate that treatment with a clinically effective anticonvulsant immediately following an evoked seizure is not sufficient to ameliorate seizure-induced progression in duration and seizure class of subsequent seizures.

**Discussion**

We tested the hypothesis that transient inhibition of TrkB-PLCγ1 signaling following a seizure evoked in a kindled animal would limit progression of seizure-induced epileptogenesis. Four principal findings emerged: (1) either chemical-genetic inhibition of TrkB kinase or treatment with pY816 peptide initiated after an evoked seizure and continued for 2 days prevented the increased duration and severity of subsequent seizures; (2) unexpectedly, transient inhibition of TrkB-PLCγ1 signaling initiated following an evoked seizure also induced a regression to an earlier state of epileptogenesis, evident by reduced duration and severity of behavioral seizures; (3) treatment with inhibitors of TrkB-PLCγ1 signaling in the absence of a recent seizure did not reduce severity of subsequent evoked seizures; and (4) treatment with a clinically effective anticonvulsant (carbamazepine) transiently following an evoked seizure did not reduce severity of subsequent seizures. These results establish TrkB-PLCγ1 signaling as a molecular mechanism by which a seizure promotes progression of epileptogenesis.

TLE is a progressive disorder in some patients, as evidenced by increasing hippocampal and cortical atrophy monitored by magnetic resonance imaging (MRI). Clinical observations led Gowers to propose that seizures themselves could promote the worsening of epilepsy. Consistent with this notion, the risk of seizure recurrence increased with each subsequent seizure in a cohort of patients studied by Hauser and Lee. The increasing hippocampal atrophy detected by MRI in TLE patients correlated with increased...
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<thead>
<tr>
<th>Sz</th>
<th>EEG Sz Duration, s</th>
<th>Overt Sz Duration, s</th>
<th>Ictal &amp; Postictal Duration, s</th>
<th>Sz Score</th>
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<tr>
<td>pY816 after an evoked Sz &amp; 14-day interval between Sz 1 &amp; 2 (see Fig 3F–J)</td>
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<tr>
<td>Final kindled Sz</td>
<td>Scr: 39.9 ± 4.8; pY816: 47.2 ± 7.3*</td>
<td>Scr: 38.2 ± 4.2; pY816: 42.9 ± 6.0*</td>
<td>Scr: 80.2 ± 6.0; pY816: 82.3 ± 4.8</td>
<td>Scr: 4.8 ± 0.22; pY816: 4.8 ± 0.26b</td>
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<td>Sz 1</td>
<td>Scr: 43.6 ± 4.8; pY816: 41.9 ± 3.3*</td>
<td>Scr: 43.6 ± 3.8; pY816: 46.0 ± 3.6*</td>
<td>Scr: 77.4 ± 7.0; pY816: 77.6 ± 6.5</td>
<td>Scr: 4.6 ± 0.18; pY816: 5.0 ± 0.20b</td>
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<td>Sz 2</td>
<td>Scr: 48.9 ± 5.5; pY816: 25.2 ± 4.1</td>
<td>Scr: 47.5 ± 4.1; pY816: 13.6 ± 5.3</td>
<td>Scr: 113.0 ± 9.6; pY816: 61.7 ± 10.8</td>
<td>Scr: 5.23 ± 0.23; pY816: 3.1 ± 0.37</td>
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<td>1NMPP1/Veh after an evoked Sz &amp; 6-day interval between Sz 1 &amp; 2 (see Fig 2)</td>
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<tr>
<td>Final kindled Sz</td>
<td>Veh: 47.9 ± 5.3; 1NMPP1: 43.8 ± 4.5*</td>
<td>Veh: 47.2 ± 4.6; 1NMPP1: 44.1 ± 4.9*</td>
<td>Veh: 74.9 ± 4.1; 1NMPP1: 84.1 ± 7.6</td>
<td>Veh: 4.7 ± 0.16; 1NMPP1: 4.6 ± 0.18</td>
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<td>Sz 1</td>
<td>Veh: 42.6 ± 4.6; 1NMPP1: 43.8 ± 4.5*</td>
<td>Veh: 45.0 ± 4.8; 1NMPP1: 46.8 ± 4.1*</td>
<td>Veh: 85.8 ± 9.8; 1NMPP1: 91.0 ± 7.5</td>
<td>Veh: 4.5 ± 0.16; 1NMPP1: 4.9 ± 0.18a</td>
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<td>Sz 2</td>
<td>Veh: 56.6 ± 7.4; 1NMPP1: 29.2 ± 3.8</td>
<td>Veh: 49.1 ± 6.8; 1NMPP1: 26.2 ± 4.4</td>
<td>Veh: 139.8 ± 16.5; 1NMPP1: 65.3 ± 10</td>
<td>Veh: 4.9 ± 0.27; 1NMPP1: 4.0 ± 0.4</td>
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<td>pY816/Scr after an evoked Sz &amp; 6-day interval between Sz 1 &amp; 2 (see Fig 3A–E)</td>
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<tr>
<td>Final kindled Sz</td>
<td>Scr: 31.9 ± 2.8; pY816: 28.2 ± 2.8</td>
<td>Scr: 31.8 ± 3.4; pY816: 31.7 ± 3.4</td>
<td>Scr: 67.4 ± 7.3; pY816: 57.1 ± 3.4</td>
<td>Scr: 4.5 ± 0.21; pY816: 4.5 ± 0.17</td>
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<td>Sz 1</td>
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<td>Scr: 4.6 ± 0.21; pY816: 4.8 ± 0.18</td>
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<td>Scr: 115.8 ± 19.8; pY816: 48.9 ± 5.7</td>
<td>Scr: 5.4 ± 0.18; pY816: 3.7 ± 0.4</td>
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<td>1NMPP1/Veh without a preceding evoked Sz (see Fig 4A–E)</td>
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<td>Final kindled Sz</td>
<td>Veh: 33.9 ± 3.6; 1NMPP1: 37.5 ± 5.1</td>
<td>Veh: 40.8 ± 6.4; 1NMPP1: 40.8 ± 6.4</td>
<td>Veh: 81.9 ± 10.5; 1NMPP1: 82.0 ± 10.3</td>
<td>Veh: 4.5 ± 0.22; 1NMPP1: 4.8 ± 0.17</td>
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<td>Veh: 91.0 ± 9.9; 1NMPP1: 106.1 ± 12.4</td>
<td>Veh: 4.9 ± 0.23; 1NMPP1: 5.5 ± 0.17</td>
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<td>pY816/Scr without a preceding evoked Sz (see Fig 4F–J)</td>
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<td>Scr: 69.1 ± 4.1; pY816: 65.1 ± 3.4</td>
<td>Scr: 4.5 ± 0.17; pY816: 4.5 ± 0.14</td>
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<td>Sz 2</td>
<td>Scr: 52.5 ± 6.7; pY816: 39.6 ± 4.7</td>
<td>Scr: 56.9 ± 6.5; pY816: 48.6 ± 5.0</td>
<td>Scr: 111.4 ± 14.0; pY816: 85.9 ± 10.8</td>
<td>Scr: 5.1 ± 0.22; pY816: 5.0 ± 0.29</td>
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<td>CBZ after an evoked Sz (see Fig 5)</td>
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<td>Veh: 36.3 ± 6.5; CBZ: 38.0 ± 4.6</td>
<td>Veh: 35.5 ± 4.0; CBZ: 34.3 ± 3.3</td>
<td>Veh: 85.5 ± 10.1; CBZ: 94.4 ± 12.7</td>
<td>Veh: 4.3 ± 0.21; CBZ: 4.7 ± 0.36</td>
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<td>Sz 1</td>
<td>Veh: 44.8 ± 7.1; CBZ: 39.3 ± 5.9</td>
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<td>Veh: 76.9 ± 5.1; CBZ: 53.1 ± 6.4</td>
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<td>Veh: 58.0 ± 6.6; CBZ: 46.3 ± 8.9</td>
<td>Veh: 58.5 ± 5.9; CBZ: 57.4 ± 6.3</td>
<td>Veh: 122.0 ± 12.1; CBZ: 95.7 ± 11.7</td>
<td>Veh: 5.2 ± 0.3; CBZ: 5.4 ± 0.20</td>
</tr>
</tbody>
</table>

Statistical analyses were done by 2-way analysis of variance with repeated measures and post hoc Bonferroni test.

*\(p < 0.01\).

*\(p < 0.001\).

*\(p < 0.05\), significant differences in experimental (ie, 1NMPP1 or pY816) animals when compared to Sz 2.

CBZ = carbamazepine; EEG = electroencephalographic; Sz = seizure; Veh = vehicle.
seizure frequency.7,8,25 Discovery of the kindling model provided unambiguous preclinical evidence supportive of Gowers’ hypothesis.10 In this model, repeated administration of brief, low-intensity electrical stimuli to a limbic structure induces progressively enhanced sensitivity to subsequent stimuli evidenced by increased duration and propagation of electrographic and behavioral seizures.11,23 In contrast to the brief localized electrographic seizures without overt behavioral change evoked by initial stimuli, prolonged and widely propagated electrophysiological seizures accompanied by tonic–clonic seizures are evident following 10 to 15 stimulations.23 Spontaneous recurrent seizures (epilepsy), often associated with sudden unexpected death, emerge after evoking many (eg, 70–80) seizures.12,13 Importantly, the seizure itself, not the electrical stimulus, is the key variable causing epileptogenesis.26–28 Interestingly, spontaneously occurring subclinical seizures are thought to contribute to increased severity of subsequent seizures in other models of epilepsy. Animals in which spontaneous recurrent seizures arise after seizure-free latent periods of days to weeks following SE exhibit a progressive increase in frequency and severity of seizures long after onset of the initial seizure14,15,29; recurrent subconvulsive seizures are thought to promote progression of the epilepsy in these animals.15 A similar increase in seizure frequency and severity was observed in rats long after postnatal hypoxia–ischemia.30,31 Finally, inducing several brief seizures in an asymptomatic mouse carrying a mutation of SCN1A identified in humans with Dravet syndrome causes severe epilepsy and cognitive decline; remarkably, inducing similar brief seizures in WT mice has no overt deleterious consequences.16

Collectively, these findings imply that some molecular consequences of a seizure promote the worsening of epilepsy. Our findings implicate TrkB-PLCγ1 signaling as one such consequence. The effectiveness of 1NMPP1 in TrkBP6616A but not in WT animals provides unambiguous evidence implicating TrkB kinase in seizure-induced worsening. Among signaling pathways downstream of TrkB kinase, the efficacy of pY816 implicates PLCγ1 in particular.20 These findings raise the question of the cellular consequence of seizure-evoked TrkB-PLCγ1 signaling that promotes epileptogenesis. One plausible mechanism is the long-term potentiation (LTP) of excitatory synapses between principal neurons. Goddard proposed that repeatedly evoking electrographic seizures induces LTP of excitatory synapses at various “nodes” within a spatially distributed but synaptically connected network of limbic sites.32 A proposal supported by findings of Sutula and Steward.33,34 If causal, then disruption of LTP at any of these loci may impair propagation and severity of seizure activity. Immunohistochemical evidence of TrkB-PLCγ1 activation at hippocampal mossy fiber-CA3 and Shaffer collateral-CA1 synapses following kindling and SE suggests that these particular synapses may be two such “nodes.” TrkB inhibition prevents LTP of each of these synapses, further supporting the putative role of this mechanism.

Why is it that inhibition of TrkB-PLCγ1 signaling limits epileptogenesis only when introduced following a seizure? The suggestion that LTP is a mechanism by which enhanced TrkB-PLCγ1 signaling promotes epileptogenesis could provide an explanation. Inhibitors of TrkB-PLCγ1 signaling prevent high-frequency stimulation–induced LTP of excitatory synapses in in vitro preparations, a scenario akin to limiting seizure-promoted epileptogenesis.36–39 Importantly, inhibition of TrkB-PLCγ1 signaling has no effect on basal transmission of these synapses, paralleling its ineffectiveness when administered to an animal in the absence of a recent seizure. Similar parallels emerge with respect to protein synthesis inhibitors in that anisomycin has no effect on basal synaptic transmission yet inhibits stimulation-induced LTP of the Schaeffer collateral synapse with CA1 pyramidal cells.40–42

The requirement of a recently evoked seizure for the effectiveness of TrkB-PLCγ1 inhibitors exhibits striking parallels to previous studies of fear conditioning and an animal model of neuropathic pain. For example, recall of previously encoded classical fear conditioning introduces a period of lability during which perturbations such as an evoked seizure43 or transient inhibition of protein synthesis can eliminate the pathologic memory44; as in the present study, introducing these perturbations without prior recall of the encoded memory fails to eliminate the memory. Likewise, reversal of “neuropathic pain” can be accomplished by transient exposure to protein synthesis inhibitors administered following re-exposure to the pain stimulus; treatment with the protein synthesis inhibitors in the absence of a preceding pain stimulus are ineffective.45 Evidence of TrkB activation following the inciting stimuli in each of these models46,47 provides a rationale for determining whether transient inhibition of TrkB-PLCγ1 signaling will limit progression or reverse these plasticities.

Whereas limiting progression of epileptogenesis by TrkB-PLCγ1 inhibitors was consistent with our hypothesis, the regression to an earlier stage of epileptogenesis was unexpected. Two weeks after transient treatment following a seizure, both behavioral and EEG features of seizures were significantly reduced in comparison to the final kindled seizure in pY816- but not Scr-treated controls (see Fig 3F–J). Similar results were evident in both 1NMP1- and pY816-treated animals tested 8 days following treatment (see Figs 2 and 3A–E). Whether complete reversal of epileptogenesis can be achieved with additional trials of seizures followed by TrkB-PLCγ1 inhibitors remains to be determined. Likewise, whether transient inhibition of TrkB-PLCγ1 signaling following a spontaneous seizure (as distinct from evoked seizures in the kindling model) in
other models of TLE will limit progression or induce a remission is a subject of future study.

One possible confound in the interpretation of the current results, that the reduction in seizure class and duration is an anticonvulsant effect due to retained 1NMPP1 or peptide, is unlikely for several reasons. First, the chemical–genetic strategy for the experiments depicted in Figure 2 utilized the small molecule 1NMPP1, a compound with a half-life in the brain of approximately 30 minutes,49 which would be cleared well before seizure 2 evoked 6 days after treatment termination. Second, pY816 exhibits an anticonvulsant effect in the intra-amygdala kainate model of SE if administered 10 minutes to 24 hours before induction, but no effect if given 72 hours before induction,50 providing indirect evidence that the peptide is cleared from the brain within this time frame. Finally, the reduction in behavioral and EEG measures of seizures persisted when the latency between pY816 treatment and testing was increased from 6 days to 2 weeks (see Fig 3F–J).

The present findings suggest a novel strategy for managing patients with TLE. In contrast to continuous exposure to standard antiseizure drugs aimed at inhibiting a seizure, this strategy would consist of transient inhibition of TrkB-PLCγ1 signaling introduced following the occurrence of a seizure. Engaging the TrkB-PLCγ1 target selectively in a disease (eg, postseizure) context may limit progression and potentially ameliorate the severity of the epilepsy. The improved efficacy of automated seizure detection systems49,50 would facilitate notification of need to introduce treatment in a timely manner, thereby enhancing the feasibility of such an approach.

Acknowledgment

Funding was provided by the American Epilepsy Society Pre-doctoral Fellowship (K.K.), Wakeman Endowment (K.K., S.C.H.), NIH National Institute of Neurological Disorders and Stroke (NS056217, J.O.M.; F31NS078847, S.C.H.), NIH National Institute of General Medical Sciences (T32-GM007171, K.K., S.C.H.), and the CURE Taking Flight Award (Y.Z.H.).

We thank W. Qian for help with animal husbandry and all members of the McNamara laboratory for critical feedback.

Author Contributions

K.K., Y.Z.H., S.C.H., and J.O.M. conceived of the project. K.K. and K.K.S. performed experiments. K.K., K.K.S., and D.L.T. analyzed data. K.K., Y.Z.H., and J.O.M. wrote the manuscript with feedback from all authors.

Potential Conflicts of Interest

K.K. has a pending patent, “Compositions and Methods for the Treatment of Epilepsy,” which includes data presented in this article. Y.Z.H. owns equity in and J.O.M. is a founder of LVM Biosciences, a company that develops inhibitors of TrkB signaling for the treatment of epilepsy.

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