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Antiseizure Effects of TrkB Kinase Inhibition

Gumei Liu^{1,*}, Robert J Kotloski^{1,*}, and James O McNamara^{1,2,3}

¹Department of Neurobiology, University Medical Center, Durham, North Carolina, USA

²Department of Pharmacology and Cancer Biology, University Medical Center, Durham, North Carolina, USA

³Department of Neurology, Duke University Medical Center, Durham, North Carolina, USA

Summary

Objective—The principal molecular targets of conventional antiseizure drugs consist of ligand- and voltage-gated ion channels and proteins subserving synaptic function. Inhibition of the receptor tyrosine kinase TrkB limits epileptogenesis, but its effect on individual seizures is unknown. We sought to determine whether inhibition of TrkB kinase exerts an antiseizure effect.

Methods—We utilized the kindling model in combination with an inducible conditional knock-out of the *TrkB* gene (*Act-CreER TrkB^{flox/flox}* mice treated with tamoxifen), and also with a chemical-genetic approach in which mice carry a TrkB kinase with a phenylalanine to alanine substitution of residue 616 (*TrkB^{F616A}*), which allows inhibition of the kinase by a blood-brain barrier permeable small molecule, 1'-naphthylmethyl-4-amino-1-*tert*-butyl-3-(*p*-methylphenyl)pyrazolo[3,4-*d*]pyrimidine (1NMPP1).

Results—Following induction of kindling, reduction of TrkB protein levels in *Act-CreER TrkB^{flox/flox}* mice treated with tamoxifen was associated with reduced severity of behavioral seizures evoked by stimulation. Treatment with 1NMPP1 for two weeks following induction of kindling reversibly elevated both focal electrographic and generalized seizure thresholds in *TrkB^{F616A}*, but not wildtype (WT), mice. In contrast to kindled animals, treatment of naïve *TrkB^{F616A}* mice for two weeks had no detectable effect on electrographic seizure threshold (EST).

Significance—This study provides proof of concept of a novel molecular target for antiseizure drugs, namely the receptor tyrosine kinase TrkB.

Keywords

Epilepsy; Seizure; Kindling; TrkB; Treatment

Correspondence should be addressed to: Dr. James O. McNamara, Department of Neurobiology, Duke University Medical Center, Box 3676, Durham, NC 27710, USA. Telephone: (919) 684 0323, Fax: (919) 684 4431, jmc@neuro.duke.edu.

*These authors contributed equally to this work.

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Introduction

The epilepsies are one of the most common serious neurological disorders, afflicting approximately 1% of the population worldwide¹. Among these diverse disorders, temporal lobe epilepsy is particularly difficult because it is both common and commonly medically intractable. Despite introduction of more than ten antiseizure drugs in the past two decades, seizures persist in an estimated 30% of patients². The molecular targets through which antiseizure drugs control neuronal excitability consist of ligand- and voltage-gated ion channels, auxiliary proteins of ion channels, and diverse proteins subserving synaptic transmission³. Identification of additional molecular mechanisms controlling seizure threshold may reveal targets for development of a novel class of antiseizure drug.

The neurotrophin brain-derived neurotrophic factor (BDNF) and its receptor, tropomyosin-related kinase B (TrkB), are thought to promote epileptogenesis, the process by which a normal brain becomes epileptic (see 4, 5, 6, but also 7). Recently a chemical-genetic approach to inhibit TrkB kinase signaling in a model of epileptogenesis resulted in prevention of spontaneous seizures induced by status epilepticus⁸. The evidence implicating BDNF and TrkB in epileptogenesis notwithstanding, whether inhibiting TrkB signaling *de novo* in an adult animal elevates seizure threshold is unknown.

We therefore asked whether *de novo* inhibition of TrkB kinase elevated threshold for stimulation-evoked seizures in an adult mouse following kindling. We selected this model because FDA-approved drugs for treatment of partial seizures uniformly suppress seizures in the kindling model³. To inhibit TrkB signaling *de novo* in an adult mouse, we used an inducible conditional knock-out approach¹⁰ to reduce full-length TrkB protein. To selectively inhibit TrkB tyrosine kinase activity *de novo* in an adult mouse, we used a chemical-genetic approach¹¹. Our studies reveal that inhibition of TrkB kinase elevated the seizure threshold in kindled animals, thereby establishing a novel target for development of antiseizure drugs.

Material and Methods

Animals

TrkB^{flox/flox} mutant mice in a C57BL/6 background were crossed to a mouse carrying a transgene of chicken β -actin-*CreER* with a CMV enhancer¹⁰ (*Act-CreER*, Jackson Labs, strain 004453) to generate a mouse with inducible conditional reduction of TrkB. *TrkB^{F616A}* mice were in a C57BL/6 (Charles River) background. Animals were handled according to the NIH Guide for the Care and Use of the Laboratory Animals and the experiments were conducted under an approved protocol by the Duke University Animal Care and Use Committee.

Tamoxifen treatment

Act-CreER TrkB^{flox/flox} and control mice were treated with tamoxifen (7.5mg in sunflower oil at a concentration of 50mg/mL) by oral gavage for 5 days. Tamoxifen (Sigma) was prepared daily by dissolving in sunflower oil through continuous rotation in a 55°C oven for approximately 1–2 hours, filtered (0.2 μ m), and kept at 37°C. Vehicle-treated mice received

an equal volume of sunflower oil alone. Given the possibility of excretion of tamoxifen from one mouse and uptake by cage mates, tamoxifen-treated mice were not housed with vehicle-treated mice. No adverse effects from tamoxifen or vehicle treatment were noted.

1'-naphthylmethyl-4-amino-1-*tert*-butyl-3-(*p*-methylphenyl)pyrazolo[3,4-*d*]pyrimidine (1NMPP1) treatment

1NMPP1 was dissolved in DMSO and a 100mM stock solution was stored at -20°C until use. Mice were given 1NMPP1 through drinking water ($25\mu\text{M}$) and daily intraperitoneal (i.p.) injections ($16.6\mu\text{g/g}$). For oral treatment, an aliquot of the 100mM stock solution of 1NMPP1 was thawed every 2 days and diluted in solubilization solution (2.5% Tween-20 in 0.9% saline). For i.p. injection, 1NMPP1 solution was prepared fresh daily. Controls received the same volume of vehicle. No adverse effects from 1NMPP1 or vehicle treatment were noted.

Surgery and kindling

Under pentobarbital (60mg/kg) anesthesia, a bipolar electrode used for stimulation and recording was stereotactically implanted in the right hippocampus. After a post-operative recovery period of 1 week, the electrographic seizure threshold (EST) was determined by application of 1 sec train of 1 msec biphasic-rectangular pulses at 60Hz beginning at $60\mu\text{A}$; additional stimulations increasing by $20\mu\text{A}$ were administered at 1 min intervals until an electrographic seizure lasting 5 sec was detected. Subsequently, stimulations at the intensity of EST were administered following a previously described kindling protocol¹². The behavioral manifestations of seizures were classified according to a modification of the description of Racine¹³. Mice were stimulated until fully kindled, defined by the occurrence of 3 consecutive seizures of Class 4. The kindling procedures were performed by an individual blinded to genotype and treatment of the mice.

Regarding kindling development in the inducible conditional knock-out mice, as tamoxifen (TAM) was administered only after induction of kindling, no significant differences were expected or found between the two groups in kindling development, including EST (*Act-CreER TrkB^{flox/flox}*+TAM $104\pm 19\mu\text{A}$; controls $124\pm 25\mu\text{A}$, $p>0.05$), initial electrographic seizure duration (*Act-CreER TrkB^{flox/flox}*+TAM $24.3\pm 3.1\text{sec}$; controls $25.1\pm 2.8\text{sec}$, $p>0.05$), or stimulations to induce kindling (*Act-CreER TrkB^{flox/flox}*+TAM 18.8 ± 1.6 ; controls 15.6 ± 1.1 , $p>0.05$).

Similarly, because 1NMPP1 treatment was initiated only after induction of kindling, no significant differences were expected or found with respect to induction of kindling between 1NMPP1- and vehicle-treated *TrkB^{F616A}* mice. There was no significant difference in the number of stimulations required to induce kindling for *TrkB^{F616A}* mice (1NMPP1, 16.3 ± 3.9 ; vehicle, 16.8 ± 4.4 , $p>0.05$) or for 1NMPP1- and vehicle-treated WT mice (1NMPP1, 19.9 ± 5.2 ; vehicle, 18.8 ± 6.2 , $p>0.05$).

Brain homogenates and immunoblot

Approximately 30 minutes after the last evoked seizure, a lethal dose of pentobarbital (100mg/kg, i.p.) was administered and the animal was decapitated. The head was briefly

cooled in liquid nitrogen and the brain was dissected on ice. The right hemisphere, into which the electrode was implanted, was taken whole, frozen on powdered dry ice, and sectioned to confirm electrode placement. The left hippocampus was dissected and homogenized in RIPA buffer (150mM NaCl, 50mM Tris-HCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 2mM EDTA, pH 7.4) including 1.5mM sodium orthovanadate, 1mM phenylmethanesulfonylfluoride and proteinase inhibitor cocktail (Roche). Homogenates were centrifuged at 16,000g for 10 minutes at 4°C, and the supernatants were stored at -80°C. For western blotting, samples were resolved by SDS-PAGE and blotted with antibodies against p-Trk (pY515, Santa Cruz), TrkB (Cell Signaling), p-Akt (ser473, Cell Signaling), and β -actin (Sigma). HRP-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Molecular Probes) were used. Membranes were developed with ECL and when applicable, quantified using a standard curve of homogenates from WT mice and ImageJ software.

Statistical analysis

All results are presented as mean $\pm$ SEM and were analyzed by Mann-Whitney or Kruskal-Wallis tests unless otherwise stated. All p-values are for two-tailed tests. During the retest protocol for the *Act-CreER TrkB^{lox/flox}* mice, one outlier in the control group required >3sd from mean stimulations and was excluded from statistical comparison.

Results

Reduction of TrkB protein following kindling exerts antiseizure effects

Full-length TrkB protein was reduced *de novo* after kindling by administration of tamoxifen to *Act-CreER TrkB^{lox/flox}* mice (Figure 1A). Following induction of kindling, *Act-CreER TrkB^{lox/flox}* mice (n=10) were treated with tamoxifen (7.5 mg/day by oral gavage for 5 days). Controls consisted of vehicle-treated WT mice (n=5), tamoxifen-treated WT mice (n=5), and vehicle-treated *Act-CreER TrkB^{lox/flox}* mice (n=5). Seizures evoked by electrical stimulation following kindling (“retest stimulations”) were examined 16 days following the last treatment with tamoxifen or vehicle, as pilot experiments (not shown) revealed reductions of TrkB protein 7–10 days after tamoxifen. Retest stimulations identical to those used for induction of kindling for a given animal were administered twice daily until a Class 4 seizure was evoked (Figure 1A).

Seizures evoked by retest stimulations demonstrated a trend towards attenuation in *Act-CreER TrkB^{lox/flox}* mice treated with tamoxifen as compared to controls. While all controls and *Act-CreER TrkB^{lox/flox}* mice treated with tamoxifen exhibited an electrographic seizure with the first retest stimulation (Figure 2A), the initial retest stimulation evoked a behavioral seizure of Class 4 in 8 of 15 controls (3/5 WT+veh; 2/5 WT+TAM; 3/5 *Act-CreER TrkB^{lox/flox}*+TAM), but in only 1 of 10 *Act-CreER TrkB^{lox/flox}* mice treated with tamoxifen (Freeman-Halton test, p=0.08) (Figure 2B). No difference existed between the groups in the duration of the first electrographic seizure, as a ratio of the final seizure of the kindling protocol (*Act-CreER TrkB^{lox/flox}*+TAM 111 $\pm$ 9%; controls 100 $\pm$ 6%, p>0.05). Seizures evoked by subsequent stimulations also trended towards attenuation in the *Act-CreER TrkB^{lox/flox}* mice treated with tamoxifen as compared to controls, with more stimulations

required to induce a Class 4 seizure in the *Act-CreER TrkB^{flox/flox}* mice treated with tamoxifen (*Act-CreER TrkB^{flox/flox}*+TAM 5.1±1.0 stimulations; WT+veh 1.8±0.6; WT+TAM 3±1.0; *Act-CreER TrkB^{flox/flox}*+veh 2.4±0.8; Kruskal-Wallis, $p = 0.099$; *Act-CreER TrkB^{flox/flox}*+TAM (5.1±1.0) vs. all controls (2.4±0.5), Mann-Whitney, $p < 0.05$) (Figure 2C).

To determine whether tamoxifen treatment of *Act-CreER TrkB^{flox/flox}* mice reduced TrkB protein, treated and control animals were sacrificed following completion of the retest protocol and lysates from the left (contralateral to the electrode) hippocampus were assessed by western blot. Quantification of western blots revealed reductions of TrkB content in *Act-CreER TrkB^{flox/flox}* mice treated with tamoxifen, with average full-length TrkB levels equal to 55% (±6%) of the levels detected in WT mice (100±8%) (Figure 2D).

Long-term inhibition of TrkB kinase following kindling exerts antiseizure effects

The experiments above suggested that reduction of TrkB protein *de novo* following induction of kindling exerted an antiseizure effect, and raised two significant questions: 1) does the mechanism by which the reduced TrkB content impairs responses to stimulation involve TrkB kinase activity? 2) is the impaired response to retest stimulations reversible? To address these questions, we used a chemical-genetic approach.

Following induction of kindling, both WT and *TrkB^{F616A}* mice were treated with either 1NMPP1 (n=11 for *TrkB^{F616A}* mice; n=7 for WT) or vehicle (n=12 for *TrkB^{F616A}* mice; n=6 for WT) daily for two weeks, at which time retest stimulations identical to those used for induction of kindling were administered twice daily until a Class 4 seizure was evoked (Figure 1B).

Antiseizure effects of 1NMPP1 treatment of *TrkB^{F616A}* mice were evident in two ways: 1) an increase of the current required to evoke an electrographic seizure; 2) the number of stimulation-evoked electrographic seizures required to induce a Class 4 seizure. Regarding threshold, the initial retest stimulation evoked an electrographic seizure in all controls (25 of 25), including vehicle-treated WT, 1NMPP1-treated WT, and vehicle-treated *TrkB^{F616A}* mice. By contrast, the initial retest stimulation evoked an electrographic seizure in a minority (5 of 11) of 1NMPP1-treated *TrkB^{F616A}* mice (Freeman-Halton test, $p < 0.05$) (Figure 3A). Representative electrographic recordings from 1NMPP1- and vehicle-treated *TrkB^{F616A}* mice are shown in the supplemental figure. In sum, these data demonstrate that two weeks of 1NMPP1 treatment of *TrkB^{F616A}* mice elevated the EST in 6 of 11 *TrkB^{F616A}* mice but had no detectable effect in WT mice.

Antiseizure effects of 1NMPP1 treatment of *TrkB^{F616A}* mice were also evident in the failure of evoked electrographic seizures to induce behavioral seizures with tonic-clonic motor semiology (Class 4). That is, among mice in the three control groups (vehicle- and 1NMPP1-treated WT and vehicle-treated *TrkB^{F616A}* mice), a Class 4 behavioral seizure accompanied the first or second evoked electrographic seizure in 24 of 25 animals (Figure 3B). By contrast, among 1NMPP1-treated *TrkB^{F616A}* mice, a Class 4 behavioral seizure accompanied the first or second evoked electrographic seizure in 5 of 11 mice (Freeman-Halton test, $p < 0.05$) (Figure 3B). The number of stimulation-evoked electrographic seizures required to induce a Class 4 seizure was approximately two-fold more in the 1NMPP1-

treated *TrkB^{F616A}* mice (3.5 ± 0.9) compared to controls (WT+veh 1.5 ± 0.2 ; WT+1NMPP1 1.6 ± 0.2 ; *TrkB^{F616A}*+veh 1.6 ± 0.2 ; ANOVA, $p < 0.05$) (Figure 3C). Post-hoc analysis found a significant difference between the 1NMPP1-treated and vehicle-treated *TrkB^{F616A}* mice (Bonferroni's test, $p < 0.05$).

1NMPP1 inhibits TrkB kinase in *TrkB^{F616A}* mice

Meaningful interpretation of the antiseizure effects of 1NMPP1 requires demonstration of the efficacy and specificity of its inhibition of TrkB kinase activity. To examine its effects on TrkB kinase activity, the same mice used for testing antiseizure effects were sacrificed 30 minutes after the first evoked Class 4 seizure and biochemical assays performed. For biochemical assays, the hippocampus contralateral to stimulation was dissected and homogenized for immunoblotting. Immunoreactivity of pY515 was used as a surrogate measure of TrkB kinase activity¹⁴. These immunoblots revealed p-Trk immunoreactivity in hippocampal extracts of 1NMPP1-treated *TrkB^{F616A}* mice ($n=3$) was reduced to $45 \pm 10\%$ of the level of vehicle-treated *TrkB^{F616A}* mice ($n=3$) (t-test, $p < 0.01$) (Figure 3D&E). In contrast, no reduction was detected in 1NMPP1-treated ($n=5$) WT mice relative to vehicle-treated WT mice ($n=4$) (t-test, $p > 0.05$) (Figure 3D&E), demonstrating specificity of 1NMPP1 inhibition. No differences in TrkB protein content between vehicle- and 1NMPP1-treated groups were detected (Figure 3D). Importantly, there was no significant difference in behavioral seizure class (Chi-squared test, $p > 0.05$) or electrographic seizure durations (vehicle, 28 ± 3 seconds; 1NMPP1, 32 ± 4 seconds; t-test, $p > 0.05$) of the seizures evoked immediately prior to sacrifice between vehicle- and 1NMPP1-treated *TrkB^{F616A}* mice. Likewise, behavioral seizure class (Chi-squared test, $p > 0.05$) and electrographic seizure durations (vehicle, 25 ± 3 seconds; 1NMPP1, 27 ± 4 seconds; t-test, $p > 0.05$) were similar between the WT groups. In sum, these results demonstrate that 1NMPP1 treatment of *TrkB^{F616A}*, but not WT, mice partially reduces pY515 in hippocampal lysates, thereby providing evidence of partial inhibition of TrkB kinase activity.

Reversibility of antiseizure effects of TrkB kinase inhibition

Collectively these studies reveal that partial inhibition of TrkB kinase activity for two weeks exerted antiseizure effects in animals following kindling. We next asked whether the antiseizure effects were reversible. To address this question, *TrkB^{F616A}* mice underwent induction of kindling, subsequent treatment with vehicle or 1NMPP1 for two weeks, elimination of 1NMPP1 treatment for one week, and testing for antiseizure effects and TrkB kinase activity (Figure 1C). In contrast to results obtained in the presence of 1NMPP1 treatment, here the retest stimulation evoked an electrographic seizure in 6 of 8 1NMPP1- (75%) and 5 of 6 vehicle-treated (83.3%) *TrkB^{F616A}* mice. Moreover, the number of stimulations required to induce a Class 4 seizure was similar in vehicle- and 1NMPP1-treated *TrkB^{F616A}* mice (1.2 ± 0.2 [$n=6$] and 1.4 ± 0.2 [$n=8$], t-test, $p > 0.05$) (Figure 4A). To address whether TrkB kinase activity was similar in the vehicle- and 1NMPP1-treated *TrkB^{F616A}* mice, animals were sacrificed approximately 30 minutes after the last evoked seizure and subjected to biochemical analyses. In contrast to animals maintained on 1NMPP1 treatment until sacrifice, pY515 immunoreactivity in 1NMPP1-treated *TrkB^{F616A}* mice was similar to vehicle-treated controls (1NMPP1-treatment group was $112 \pm 9\%$ of controls, $n=4$ per group, t-test, $p > 0.05$) (Figure 4B&C). Importantly, behavioral seizure class

(Chi-squared test, $p > 0.05$) and electrographic seizure duration (vehicle, 29.3 ± 1.3 seconds; 1NMPP1, 29.6 ± 4 seconds; t-test, $p > 0.05$) of the evoked seizure prior to sacrifice was similar in the two groups. These findings demonstrate that inhibition of TrkB kinase activity by 1NMPP1 dissipates by one week after cessation of 1NMPP1-treatment of *TrkB^{F616A}* mice. Likewise the antiseizure effects evident in 1NMPP1-treated *TrkB^{F616A}* mice were no longer detectable when tested one week after cessation of 1NMPP1 treatment.

Acute inhibition of TrkB kinase activity does not exert antiseizure effects

Having demonstrated that inhibition of TrkB kinase for two weeks exerted antiseizure effects that dissipated by one week after terminating inhibition, we next asked whether brief inhibition of TrkB kinase activity is sufficient to exert antiseizure actions. To address this question, we determined whether a single treatment of *TrkB^{F616A}* mice with 1NMPP1 15 minutes prior to stimulation would be sufficient to inhibit a stimulation-evoked seizure (Figure 1D). In contrast to examination of long-term inhibition of TrkB kinase activity (Figure 1C) in which 1NMPP1 treatment was initiated immediately following kindling and continued for two weeks at which time responses to electrical stimulation were examined, here *TrkB^{F616A}* mice were left untreated for two weeks following kindling and treated with either vehicle or 1NMPP1 15 minutes prior to stimulation (Figure 1D). The 15 minute interval was selected because brain concentration of 1NMPP1 peaks between 15 and 20 minutes after an i.p. injection¹¹.

In contrast to two weeks of treatment with 1NMPP1, a single injection of 1NMPP1 in *TrkB^{F616A}* mice did not inhibit the stimulation-evoked seizure. No elevation of EST was detected, as evidenced by the initial retest stimulation evoking an electrographic seizure in each of the 1NMPP1- and vehicle-treated *TrkB^{F616A}* mice ($n = 6$ per group). Likewise no significant differences in the number of stimulation-evoked electrographic seizures required to induce a Class 4 seizure were found (vehicle-treated 1.50 ± 0.22 ; 1NMPP1-treated 1.17 ± 0.17 ; t-test, $p > 0.05$) (Figure 5A). To verify that a single i.p. injection of 1NMPP1 was sufficient to inhibit TrkB kinase activity, biochemical analyses were performed on hippocampal lysates isolated 30 minutes following the terminal seizure. A $45 \pm 11\%$ ($n = 4$ per group) reduction of pTrk immunoreactivity in the hippocampus was evident in 1NMPP1-treated compared to vehicle-treated *TrkB^{F616A}* mice (t-test, $p < 0.05$) (Figure 5B&C); this level of inhibition is similar to that observed after two weeks of 1NMPP1 treatment of *TrkB^{F616A}* mice ($45 \pm 10\%$ of vehicle-treated level) (Figure 3D&E). In summary, acute inhibition of TrkB kinase activity was not sufficient to exert an antiseizure effect.

Long-term inhibition of TrkB kinase activity does not exert antiseizure effects in naïve *TrkB^{F616A}* mice

A key remaining question is whether the antiseizure effect of TrkB kinase inhibition is specific to the hyperexcitable state. That is, might long-term inhibition of TrkB kinase activity elevate EST in naïve animals? To address this, naïve *TrkB^{F616A}* mice were implanted with a hippocampal electrode but not stimulated, then treated with 1NMPP1 or vehicle for 10 days (Figure 1E). At this point the naïve *TrkB^{F616A}* mice received a supplemental i.p. injection of 1NMPP1 or vehicle 15 minutes prior to assessment of EST. The initial EST was measured using an initial stimulus of 20 μ A. The current of subsequent

stimuli administered at 1 min intervals was increased in 25 μ A steps until an electrographic seizure of 5 seconds was evoked. No significant differences were detected in initial EST as evidenced by values of 128 $\pm$ 26 μ A and 103 $\pm$ 12 μ A for vehicle- (n=6) and 1NMPP1-treated (n=7) *TrkB^{F616A}* mice, respectively (t-test, $p>0.05$) (Figure 6A).

To further examine whether long-term inhibition of TrkB kinase activity exerts antiseizure effects in naïve animals, an additional experiment was performed in which each animal served as its own control. Naïve *TrkB^{F616A}* mice were implanted with hippocampal electrodes and the ESTs were assessed one week later. These mice were treated with either 1NMPP1 (n=4) or vehicle (n=5) for two weeks using the same protocol as in the initial experiments in kindled mice (Figure 1F). The EST was measured again after two weeks of treatment, thereby permitting comparison of the threshold in the same animal prior to and after two weeks of TrkB kinase inhibition. No significant effects of TrkB kinase inhibition were detected as evidenced by values of the ratios of EST after treatment compared to before treatment (1NMPP1-treated *TrkB^{F616A}* 1.13 $\pm$ 0.13; vehicle-treated *TrkB^{F616A}* 1.19 $\pm$ 0.76; t-test, $p>0.05$) (Figure 6B). In sum, long-term inhibition of TrkB kinase activity had no detectable effect on the EST of naïve animals.

Discussion

We hypothesized that inhibition of TrkB kinase activity would impair hyperexcitability following kindling. This hypothesis was tested by reducing TrkB protein using an inducible conditional knock-out of the *TrkB* gene¹⁰, and by reversibly inhibiting TrkB kinase activity using a chemical-genetic approach¹¹. Five principal findings emerged. 1) Reduction of TrkB protein following induction of kindling attenuates the seizure response to retest stimulations. 2) Treatment of *TrkB^{F616A}*, but not WT, mice with 1NMPP1 partially inhibited TrkB kinase activity in a reversible manner as evident in reduced pTrk immunoreactivity measured on western blots of hippocampal lysates. 3) Two weeks of 1NMPP1 treatment of *TrkB^{F616A}* mice elevated the focal EST in kindled, but not naïve, mice. 4) The antiseizure effect in kindled *TrkB^{F616A}* mice is reversible following termination of 1NMPP1 treatment. 5) In contrast to the antiseizure effects of two weeks of treatment with 1NMPP1, a single treatment sufficient to inhibit TrkB kinase activity for tens of minutes exerted no detectable antiseizure effect. We conclude that long-term inhibition of TrkB kinase activity, whether through reduction of full-length TrkB protein or through targeted inhibition of TrkB kinase activity, exerts antiseizure effects in the kindling model. These findings reveal a new molecular target for antiseizure drug development, the receptor tyrosine kinase TrkB.

The antiseizure effects of TrkB kinase inhibition exhibited novel features in comparison to conventional antiseizure medications. The time course of TrkB kinase inhibition required to exert antiseizure effects is unusual in this regard. Typically a single dose of a conventional antiseizure medication is sufficient to suppress seizures in the kindling and other models within tens of minutes following administration. By contrast, antiseizure effects were detected after two weeks of treatment with 1NMPP1, but not shortly after a single dose despite similar reductions of p-Trk immunoreactivity, a surrogate measure of TrkB kinase activity. Yet the antiseizure effects induced by two weeks of 1NMPP1 treatment were reversible as evident by their absence when tested one week following cessation of 1NMPP1

treatment, implying the mechanisms underlying persistence of the hyperexcitability remained intact despite inhibition of TrkB kinase activity for two weeks. The precise duration of TrkB kinase inhibition required to induce antiseizure effects is uncertain. The unusual time course of the antiseizure actions of TrkB kinase inhibition is consistent with a fundamental difference in the responsible molecular and cellular mechanisms compared to conventional antiseizure medications. The targets of antiseizure medications in current clinical use are mainly ligand- and voltage-gated ion channels, an auxiliary protein thereof, or some molecular component of GABA_A receptor containing synapses³; not surprisingly, the binding of a drug directly to such a target would be expected to immediately affect intrinsic neuronal excitability or synaptic function, thereby accounting for the time course of antiseizure effects of these agents. By contrast, inhibition of a receptor tyrosine kinase per se would not be expected to directly regulate activity of ion channels; instead regulation of neuronal excitability is likely to be a consequence of signaling events downstream from TrkB kinase. The fact that chronic, but not acute, inhibition of TrkB kinase activity exerts antiseizure effects in the kindling model is consistent with this proposal. Finally, we note that seizure threshold in a small portion of 1NMPP1-treated *TrkB^{F616A}* mice (non-responders) did not change after two weeks of treatment; whether there was a correlation between the extent of TrkB inhibition and elevation of seizure threshold is a subject of future study. Identifying the signaling pathway downstream from TrkB responsible for the antiseizure effects will provide a clue to the underlying molecular and cellular mechanisms.

The finding that TrkB kinase inhibition elevated the focal electrographic seizure threshold in kindled but not in naïve animals was unexpected. The inability of TrkB kinase inhibition to elevate seizure threshold in naïve animals was evident when studied with two different paradigms, one examining effects in different animals and a second examining effects within the same animal. By contrast, carbamazepine, an antiseizure medication commonly used to treat focal seizures, elevates the focal electrographic seizure threshold in both kindled and naïve animals¹⁵. Treatment with 1NMPP1 would be expected to inhibit TrkB kinase activity in both kindled and naïve animals; although inhibition of TrkB kinase by 1NMPP1 was verified only in kindled animals in the present study, the behavioral consequences of 1NMPP1 treatment of naïve *TrkB^{F616A}* mice reported by other authors attest to the efficacy of its inhibition of TrkB kinase. For example, 1NMPP1-treatment of *TrkB^{F616A}* mice can prevent recovery of visual cortical responses following monocular deprivation¹⁶, limit acquisition but not expression of conditioned incentive value¹⁷, and limit induction of tissue- and nerve injury-induced pain¹⁸. In each instance these effects of 1NMPP1 treatment were mediated by inhibition of TrkB kinase activity because they were evident in *TrkB^{F616A}*, but not WT, mice. Thus systemic treatment with 1NMPP1 almost certainly inhibited TrkB kinase activity in both naïve and kindled animals yet elevated the focal electrographic seizure threshold only in the kindled animals, as manifested by the lack of electrographic and behavioral seizure upon initial retest stimulation. This implies a difference in the mechanisms controlling the focal electrographic seizure threshold in naïve compared to kindled animals, the difference somehow enhancing sensitivity to the antiseizure effects of TrkB kinase inhibition.

Collectively, the present findings reveal a novel target for antiseizure drugs, the receptor tyrosine kinase TrkB. The elevation of seizure threshold in the kindled but not naïve animals raises the possibility that inhibition of TrkB signaling may be preferentially efficacious in a disease context. Importantly, a process predominantly active in the diseased state may allow for treatments with fewer side effects, which often limit current therapeutic approaches. It will be interesting to determine whether inhibition of TrkB kinase confers synergistic effects when used in combination with a conventional antiseizure drug. Future studies will seek to identify the signaling pathway activated by TrkB and the molecular effectors responsible for its antiseizure actions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A. Retest after 3-week tamoxifen treatment (*Act-CreER TrkB^{flox/flox}*) **B. Retest after 2-week 1NMPP1 treatment (*TrkB^{F616A}*)** **C. Retest at 1 week after a 2-week 1NMPP1 treatment** **D. Retest after acute 1NMPP1 treatment** **E. Testing threshold in naïve animals (protocol 1)** **F. Testing threshold in naïve animals (protocol 2)** **Figure 1. Treatment and retest protocols**

(A) Retest after 3-week tamoxifen treatment (*Act-CreER TrkB^{flox/flox}*) A bipolar electrode was implanted in the right hippocampus in adult *Act-CreER TrkB^{flox/flox}* or control mice and stimulations administered until 3 consecutive Class 4 seizures were evoked. At that point tamoxifen (7.5mg in sunflower oil) was given by oral gavage to *Act-CreER TrkB^{flox/flox}* mice, with controls receiving tamoxifen or vehicle, for 5 consecutive days. Retest stimulations were initiated 3 weeks after the final kindling stimulation. **(B) Retest after 2-week 1NMPP1 treatment (*TrkB^{F616A}*)** A bipolar electrode was implanted in the

right hippocampus in adult wildtype (WT) or *TrkB^{F616A}* mice and stimulations administered until 3 consecutive Class 4 seizures were evoked. At that point 1NMPP1 was added to the drinking water (25μM) and supplemented with a single daily dose intraperitoneally (i.p. 16.6μg/g); controls received vehicle in drinking water or i.p. Retest stimulations were initiated after 2 weeks of treatment with 1NMPP1 or vehicle. Treatments were continued until the animal was euthanized. Animals were euthanized for biochemical study 30 minutes following the last evoked Class 4 seizure. **(C) Retest at 1 week after a 2-week 1NMPP1 treatment** Treatments for kindled mice were terminated after 2 weeks. Drugs were allowed to clear for 1 week at which time retesting was conducted. **(D) Retest after acute 1NMPP1 treatment** Two weeks after animals were fully kindled, a single dose of 1NMPP1 (i.p. 16.6μg/g) or equal volume of vehicle was given to the mice 15 minutes prior to retest. Animals were sacrificed 30 minutes after the evoked Class 4 seizure during retest. **(E) Testing threshold in naïve animals (protocol 1)** Naïve *TrkB^{F616A}* mice were implanted with hippocampal electrodes and received either 1NMPP1 or vehicle through drinking water (25μM) for 10 days. At 15 minutes prior to threshold test, animals received an i.p. injection of 1NMPP1 or vehicle at 16.6μg/g. **(F) Testing threshold in naïve animals (protocol 2)** Naïve *TrkB^{F616A}* mice were implanted with hippocampal electrodes and allowed to recover from surgery for a week prior to the first threshold test. Immediately following the first threshold test, animals received either 1NMPP1 or vehicle for 2 weeks using the same treatment paradigm described for kindled animals. The electrographic seizure threshold (EST) was examined a second time 2 weeks later.

Figure 2. Treatment of *Act-CreER TrkB^{flox/flox}* mice with tamoxifen decreases hyperexcitability during the retest protocol

(**A**) Treatment of *Act-CreER TrkB^{flox/flox}* mice with tamoxifen does not increase the EST with the first stimulation of the retest protocol. (**B**) Treatment of *Act-CreER TrkB^{flox/flox}* mice with tamoxifen trends towards decreasing the percentage of animals exhibiting a Class 4 seizure with the first stimulation of the retest protocol, as compared to controls (Freeman-Halton, $p=0.08$). (**C**) Treatment of *Act-CreER TrkB^{flox/flox}* mice with tamoxifen trends towards increasing the number of evoked electrographic seizures needed to induce a Class 4 seizure, as compared to controls (Kruskal-Wallis, $p = 0.099$; *Act-CreER TrkB^{flox/flox}*+TAM (5.1 ± 1.0) vs. all controls (2.4 ± 0.5), Mann-Whitney, $p < 0.05$). Bars in scatter plot are mean $\pm$ SEM. (**D**) Reduction of TrkB protein in *Act-CreER TrkB^{flox/flox}* + tamoxifen mice. This representative western blot demonstrates approximately 50% reduction in full-length TrkB protein, as compared to WT mice, from hippocampal homogenate from *Act-CreER TrkB^{flox/flox}* mice following treatment with tamoxifen, as compared to WT mice.

Figure 3. Two-week 1NMPP1 treatment elevates electrographic and behavioral seizure thresholds and decreases p-Trk content in *TrkB^{F616A}* but not WT mice

(A) Increased seizure threshold in 1NMPP1-treated *TrkB^{F616A}* mice evident by reduced likelihood of an electrographic seizure compared to controls. In contrast to 100% of control mice (vehicle- or 1NMPP1-treated WT or vehicle-treated *TrkB^{F616A}*, $n = 6, 7$, and 12 respectively), only 45% of 1NMPP1-treated *TrkB^{F616A}* ($n = 11$) exhibited a focal electrographic seizure upon first stimulation (Freeman-Halton test, $*p < 0.05$). (B) Treatment of *TrkB^{F616A}* mice with 1NMPP1 decreases the percentage of animals exhibiting a Class 4 seizure with the first or second stimulation of the retest protocol, as compared to controls (Freeman-Halton test, $*p < 0.05$). (C) Number of evoked electrographic seizures required to induce a Class 4 seizure during retest. 1NMPP1-treated *TrkB^{F616A}* mice require significantly more stimulations than vehicle-treated *TrkB^{F616A}* mice (ANOVA, $p < 0.05$; *TrkB^{F616A}*+1NMPP1 3.5 ± 0.9 ; *TrkB^{F616A}*+veh 1.6 ± 0.2 ; Bonferroni's test, $*p < 0.05$). Bars in scatter plot are mean $\pm$ SEM. (D) Per protocol depicted in Figure 1B, WT and *TrkB^{F616A}* were euthanized thirty minutes after the first evoked Class 4 seizure and hippocampi dissected for immunoblotting. Representative immunoblots of pY515, full-length TrkB, and β -actin. (E) Quantification of pY515 content normalized to β -actin of three animals in each group. 1NMPP1-treated *TrkB^{F616A}* mice averaged a reduction to $45 \pm 10\%$ of the levels of p-Trk immunoreactivity seen in vehicle-treated *TrkB^{F616A}* mice (t-test, $*p < 0.01$).

Figure 4. Terminating 1NMPP1 treatment reverses inhibition of TrkB kinase activity and accompanying antiseizure effects

Per protocol depicted in Figure 1C, neither inhibition of TrkB kinase nor antiseizure effects were detectable 1 week following termination of 1NMPP1. **(A)** No difference in number of evoked electrographic seizures required to induce a Class 4 seizure was detected between mice that had earlier received vehicle or 1NMPP1 (t-test, $p > 0.05$). **(B)** Representative western blot reveals similar levels of hippocampal pY515 content following evoked seizure in *TrkB^{F616A}* mice when tested 1 week following termination of 1NMPP1 or vehicle. **(C)** Quantification of western blots including that shown in (B) reveals similar levels of hippocampal pY515 content in *TrkB^{F616A}* mice when tested 1 week following termination of 1NMPP1 or vehicle. ($n=4$ for each group, t-test, $p > 0.05$).

Figure 5. Acute inhibition of TrkB kinase activity does not exert antiseizure effects in kindling model

(A) Number of evoked electrographic seizures required to induce a Class 4 seizure 15 minutes following treatment of *TrkB^{F616A}* mice with vehicle or 1NMPP1. The average stimulation numbers are 1.50 ± 0.22 for control and 1.17 ± 0.17 for 1NMPP1-treated group (n=6 per group, t-test, $p > 0.05$). (B, C) Treatment of *TrkB^{F616A}* mice with 1NMPP1 15 minutes prior to an evoked seizure inhibited TrkB kinase activity in comparison to vehicle-treated controls as shown by representative western blot (B) and confirmed by quantitative analyses (t-test, $*p < 0.05$) (C).

Figure 6. Long term treatment with 1NMPP1 treatment exerts no detectable antiseizure effect in naïve *TrkB^{F616A}* mice

(A) Per protocol depicted in Figure 1D, *TrkB^{F616A}* mice were treated with 1NMPP1 or vehicle for 10 days prior to measurement of EST. Thresholds were $128 \pm 26 \mu\text{A}$ and $103 \pm 12 \mu\text{A}$ for vehicle- ($n=6$) and 1NMPP1-treated ($n=7$) *TrkB^{F616A}* mice respectively (t-test, $p>0.05$). (B) Per protocol depicted in Figure 1E, the initial EST was measured in absence of treatment in *TrkB^{F616A}* mice, after which 1NMPP1 or vehicle was administered for 2 weeks, at which time seizure threshold was measured a second time. The ratios of post-treatment

EST to pre-treatment EST were 1.13 ± 0.13 for 1NMPP1- and 1.19 ± 0.76 for vehicle-treated mice, respectively (t-test, $p > 0.05$).