A Peptide Selectively Uncoupling BDNF Receptor TrkB from Phospholipase Cγ1 Prevents Epilepsy and Anxiety-like Disorder

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

Temporal lobe epilepsy is a common and devastating disorder that features recurrent seizures and is often associated with pathologic anxiety and hippocampal sclerosis. An episode of prolonged seizures (status epilepticus) is thought to promote development of human temporal lobe epilepsy years later. A chemical-genetic approach established proof of concept that transiently inhibiting the receptor tyrosine kinase, TrkB, following status epilepticus prevented epilepsy, anxiety-like behavior and hippocampal damage in a mouse model, providing rationale for developing a therapeutic targeting TrkB signaling. To circumvent the undesirable consequence that global inhibition of TrkB exacerbates neuronal degeneration following status epilepticus, we sought to identify both the TrkB-activated signaling pathway mediating these pathologies and a compound that uncouples TrkB from the responsible signaling effector. To accomplish these goals, we used genetically modified mice and a model of seizures and epilepsy induced by a chemoconvulsant. Genetic inhibition of TrkB-mediated phospholipase Cγ1 (PLCγ1) signaling suppressed seizures induced by a chemoconvulsant, leading to design of a peptide (pY816) that inhibited the interaction of TrkB with PLCγ1. We demonstrate that pY816 selectively inhibits TrkB-mediated activation of PLCγ1 both *in vitro* and *in vivo*. Treatment with pY816 prior to administration of a chemoconvulsant suppressed seizures in a dose- and time-dependent manner. Treatment with pY816 initiated after chemoconvulsant-evoked status epilepticus and continued for just three days

suppressed seizure-induction of epilepsy, anxiety-like behavior and hippocampal damage assessed months later. This study elucidates the signaling pathway by which TrkB activation produces diverse neuronal activity-driven pathologies and demonstrates therapeutic benefits of an inhibitor of this pathway in an animal model *in vivo*. A strategy of uncoupling a receptor tyrosine kinase from a signaling effector may prove useful in diverse diseases in which excessive receptor tyrosine kinase signaling contributes.

Dedication

I dedicate my dissertation work to my grandparents, mother, father and sister, who were encouraging me to chase my dreams.

I dedicate my dissertation to my wonderful wife, Wenjing Xu. We've been through both good and bad time together for a decade. You are my hero and soul mate.

I dedicate my dissertation to my daughter, Jocelyn W. Gu. You are a little angel and we are so proud to have you in our lives.

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List of Abbreviations

ACSF: artificial cerebrospinal fluid

AEDs: antiepileptic drugs

AMP: adenosine monophosphate

AP: anterior-posterior

ATP: adenosine triphosphate

BDNF: brain-derived neurotrophic factor

CaMK: Ca^{2+}/calmodulin-dependent protein kinases

CNO: clozapine-N-oxide

CNS: central nervous system

CREB: cyclic adenosine monophosphate-responsive element-binding protein

DAG: diacylglycerol

DBS: deep brain stimulation

DMSO: dimethyl sulfoxide

EEG: electroencephalography

EGFR: epidermal growth factor receptor

EP2: E prostanoid 2

ERK: extracellular signal-regulated kinase

FDA: US Food and Drug Administration

FGFR: fibroblast growth factor receptor

FJC: fluoro-Jade C

fMRI: functional magnetic resonance imaging

GABA: γ-aminobutyric acid

GAB1: growth factor receptor-bound protein 2-associated binder 1

GRB2: growth factor receptor-bound protein 2

HER2: human epidermal growth factor receptor 2

HIV-1 Tat: Human immunodeficiency virus type 1 trans-activating protein

HMGB1: high-mobility group protein B1

ILAE: International League Against Epilepsy

IL-1β: interleukin-1β

i.p.: intraperitoneal

IP3: inositol-1,4,5-trisphosphate

i.v.: intravenous

JAK: Janus kinase

KA: kainic acid

LTP: long term potentiation

NT4: neurotrophic factor 4

MAP: mitogen-activated protein

MEK: MAP kinase/ERK kinase

ML: medial-lateral

mTOR: mammalian target of rapamycin

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PIP2: phosphatidylinositol-4,5-bisphosphate

PKC: protein kinase C

PLCγ1: phospholipase Cγ1

PMSF: phenylmethylsulfonyl fluoride

PTB: phosphotyrosine-binding

PTSD: posttraumatic stress disorder

Ras: Rat sarcoma

REST: repressor element 1-silencing transcription factor

RIPA: Radio-Immune Precipitation Assay

RTK: receptor tyrosine kinase

SDS: sodium dodecyl sulfate

Shc: Src homology 2 domain containing

SH2: Src homology 2

SOS: son of sevenless

STAT3: signal transducer and activator of transcription-3

TLE: temporal lobe epilepsy

TrkB: tropomyosin related kinase B

DV: dorsal-ventral

VNS: vagus nerve stimulation

WT: wild type

1NMPP1: 1-(1,1-dimethylethyl)-3-(1-naphthalenylmethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine

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1. Introduction

1.1 Overview of Epilepsy

Epilepsy, a disorder of recurrent seizures, is a serious common neurological disorder, afflicting an estimated 1% of the population worldwide. The International League Against Epilepsy (ILAE) defines epilepsy as “a condition characterized by two or more recurrent epileptic seizures over a period longer than 24 hours, unprovoked by any immediate identified cause” (Commission on classification and terminology of the ILAE, 1989). Epilepsy syndromes fall into two broad categories: generalized and partial (or localization-related) syndromes. More than half of all cases of adult epilepsy are partial in nature, meaning that seizures originate in one or more localized foci (Browne and Holmes, 2001; Chang and Lowenstein, 2003). Among many partial epilepsies, temporal lobe epilepsy (TLE) is arguably the most common and devastating form (McNamara et al., 2006).

1.2 Temporal lobe epilepsy

Limbic epilepsy (synonyms include complex partial epilepsy, TLE, psychomotor epilepsy), refers to an electroclinical syndrome in which seizures emanate from the temporal lobe. The term TLE was included in the classification of the ILAE in 1989 under the group of “localization-related symptomatic epilepsies characterized by seizures with specific modes of precipitation”

(Commission on classification and terminology of the ILAE, 1989). TLE is the most prominent epilepsy syndrome in adults for two main reasons: (1) TLE constitute the single most common seizure type, accounting for approximately 40% of all cases in adults; (2) TLE is often quite resistant to available anticonvulsant drugs and an estimated 30% experience recurrent complex partial seizures despite optimal contemporary treatment (Arroyo et al., 2002). TLE is a devastating neurological disorder, because seizure attacks induce impairment of consciousness, thereby severely limiting performance of many normal functions (e.g., driving, maintaining employment, etc.). Therapy of TLE is symptomatic. There is no effective prevention or cure, apart from surgical intervention for a minority (Engel, 2001).

1.2.1 Clinical aspects of TLE

TLE is typically considered to be an acquired epilepsy syndrome that is triggered by a precipitating factor, such as head trauma, infection, tumor or more commonly an episode of prolonged seizures (also known as status epilepticus), although there are known familial forms of TLE (Engel et al., 1998). Patients with TLE typically experience unpredictable recurrent seizures and mood disorders including anxiety and depression. A pattern of hippocampal damage manifested by atrophy and neuron loss is commonly seen in humans with TLE.

1.2.1.1 Status epilepticus is a causal event of TLE

Status epilepticus refers to a state of continuous seizure activity: either continuous seizures lasting at least 5 minutes or 2 or more discrete seizures between which there is incomplete recovery of consciousness (Lowenstein and Alldredge, 1998). Retrospective studies of patients with medically refractory TLE revealed that the majority experienced an episode of continuous seizure activity (status epilepticus) years earlier (French et al., 1993). Longitudinal studies reveal that almost half of individuals experiencing *de novo* status epilepticus develop recurrent seizures (epilepsy) after a seizure-free latent period of variable duration (Annegers et al., 1987; Tsai et al., 2009). Together with the fact that induction of status epilepticus alone is sufficient to induce TLE in diverse mammalian species (Pitkanen, 2010), the occurrence of *de novo* status epilepticus is thought to contribute to development of TLE in both human and nonhuman mammals.

1.2.1.2 Psychiatric comorbid disorder of TLE: anxiety

Up to 50 or 60% of patients with chronic epilepsy have various comorbid mood disorders (Beyenburg et al., 2005), which worsen the patients' quality of life. People with epilepsy have higher risk of mood disorders relative to the general population, to other neurologic control groups, and to people with chronic non-neurologic disorders. In particular, increased psychopathology is more common in TLE than in generalized epilepsy (Torta and Keller, 1999). Among many psychiatric disorders associated with TLE, anxiety is the most prevalent

one, which can be seizure related or interictal. The symptoms of anxiety disorder include excessive worry and anxiety in association with the somatic symptoms of restlessness, poor concentration, sleep disturbance, fatigue, irritability, and muscle tension (Jackson and Turkington, 2005). Likewise, anxiety-like behavior which mimics many clinical aspects of human anxiety is observed in multiple animal models of TLE (Jialal et al., 2014; Liu et al., 2013).

1.2.1.3 Pathological abnormality of TLE-hippocampal sclerosis

Patients with TLE commonly have hippocampal sclerosis, first recognized in postmortem specimens over 180 years ago (Bouchet, 1826). It is found in approximately 50–75% of temporal lobe resections made for medically intractable TLE. Hippocampal sclerosis is featured with hippocampal atrophy, neuronal loss, gliosis, granule cell dispersion and mossy fiber sprouting, which are also evidenced in diverse animal models of TLE. Damage can be either unilateral or bilateral, and can also effect adjacent regions such as the parahippocampal gyrus and amygdala (Engel et al., 1998; Mathern et al., 1998). Whether this neuropathological abnormality is consequence or cause of the epilepsy, however, has been debated (Jefferys, 1999).

1.2.2 Animal model of TLE

Animal models of TLE which reflect the human condition are invaluable research tools. Because TLE commonly develops after status epilepticus, most

animal models involve use of this factor. Status epilepticus can be produced experimentally by chemical convulsants, hyperthermia or by in virtually continuous electrical stimulations. In addition to post-status epilepticus models, the kindling model is also commonly used to study TLE. Kindling entails repeated, low-intensity electrical stimulation of the amygdala, hippocampus, or other brain regions to induce a progressive and permanent seizure response (Buckmaster, 2004; McNamara et al., 2006).

1.2.2.1 Kainic acid (KA) amygdala infusion mouse model

Unilateral intra-amygdala KA microinfusion model of TLE was first described in rats and cats (Tanaka et al., 1988; Tanaka et al., 1985). This approach was recently applied in mice by Mouri et al., 2008. Briefly, status epilepticus is induced by infusion of KA into basolateral nucleus of right amygdala in mice. Status epilepticus is typically evident in electroencephalography (EEG) recordings 5-10 minutes after KA infusion. The behavioral seizures associated with electrographic seizures typically consist of immobility, followed by facial automatisms, unilateral or bilateral forelimb(s) clonus, and whole body clonus. Short periods of tonic-clonic seizures with loss of posture, running and jumping also occur during the seizures. Forty minutes after onset of status epilepticus, convulsive seizures are suppressed by administration of diazepam (10 mg/kg, i.p.) followed by lorazepam (6 mg/kg, i.p.) an hour later. Spontaneous recurrent seizures typically emerge 3 to 7 days after completion of

status epilepticus following a seizure free latent period. Both acute and chronic hippocampal damage was found following KA induced status epilepticus (Liu et al., 2013; Mouri et al., 2008). Importantly, the acute hippocampal neuronal death was correlated to the seizure severity of preceding status epilepticus (Engel et al., 2010). Epileptic mice also exhibited anxiety-like behavior when tested months after KA induced status epilepticus (Liu et al., 2013). KA amygdala infusion mouse model offers the advantage of directly activating limbic structures, unlike with systemic administration, ensuring that the epileptogenic focus is within the limbic system. Pathology consistent with hippocampal sclerosis, such as neuronal loss and mossy fiber sprouting, is also evident in injected hemisphere in this model (Mouri et al., 2008). This unilateral hippocampal pathology is also commonly found in patients with TLE (Engel and Pedley, 2008). Furthermore, the low mortality (5%-10%) and very early emergence of spontaneous recurrent seizures (3-5 days following status epilepticus) provide us a useful tool to study both status epilepticus induced by KA infusion and spontaneous recurrent seizures following status epilepticus.

1.2.3 Treatment of TLE

Initial management of patients with TLE is with antiepileptic drugs (AEDs), which control seizures in only half the patients (Devinsky, 2004). No prevention or disease modifying therapy is available. Patients with drug resistant seizures may be provided the option of resective surgery (Ojemann, 1997). Other

therapeutic approaches including vagus nerve stimulation (VNS), deep brain stimulation (DBS) and ketogenic diet have been applied clinically.

1.2.3.1 AEDs

Despite over 30 AEDs have been approved by U.S. Food and Drug Association (FDA), these medications fail to control seizures in 30-40% of patients and target symptoms rather than the underlying cause. The mechanisms of action of conventional AEDs for treatment of TLE fall into two major categories. One is to limit the sustained, repetitive firing of neurons, an effect mediated by promoting the inactivated state of voltage-activated Na^+ channels. A second mechanism appears to involve enhanced γ-aminobutyric acid (GABA)-mediated synaptic inhibition, an effect mediated either by a presynaptic or postsynaptic action. Importantly, no AED has been shown to prevent the development of epilepsy in patients prior to the first seizures. For some AEDs and recently identified targets of epileptogenesis, an anti-epileptogenic effect has actually been suggested in animal models. However, none of them has proved effective in humans (Loscher et al., 2013). Recent identification of novel anti-epileptic or anti-epileptogenic drug targets provides new avenues for treatment of TLE (discussed in section 1.2.4).

1.2.3.2 Surgical interventions

Despite control of seizures in approximately half of patients with epilepsy with medication, patients who are refractory to all forms of medical therapy are

candidates for resective surgery, which requires demonstration of a single site of origin of the seizures, the focus or epileptogenic zone, and location of that site in an area of the brain that can be resected with low risk of a new neurologic deficit. 70-80% of patients of properly selected patients with refractory TLE become seizure free after surgery. Unfortunately, most patients with medically refractory TLE do not have an exclusively unilateral interictal focus in the same temporal lobe (Devinsky, 2004).

1.2.3.3 Electric stimulation

In addition to AEDs and surgical removal of epileptic tissue, electric stimulation, including VNS and DBS have been applied in subgroups of patients who are refractory to available medications and not qualified for surgery. VNS is designed to control seizures by sending regular, mild pulses of electrical energy to the brain via the vagus nerve, which controls functions of the body that are not under voluntary control. 60-70% patients show reduction of seizure frequency following VNS, with less than 5% of patients become seizure free (Uthman, 2000). DBS involves implanting electrodes, which deliver electric pulses into specific areas of the brain, commonly in the anterior nucleus of the thalamus (Jobst, 2010).

1.2.3.4 Ketogenic diet

The ketogenic diet is a special high-fat, low-carbohydrate diet that helps to control seizures in some people with epilepsy (Baranano and Hartman, 2008).

The efficacy of ketogenic diet treatment in epilepsy is mainly known from observational studies and its underlying mechanism of ketogenic is largely unknown (Mosek et al., 2009). A recent study revealed that there was at least a 50% reduction in seizures in 32% of adult TLE patients treated with a ketogenic diet (Klein et al., 2014).

1.2.3.5 Other potential therapeutic approaches

In addition to FDA approved therapeutic approaches described above, recent works have yielded important conceptual advances in treatment of TLE. Among them on-demand control of seizures and restoration of inhibitory circuit in epileptic brain by transplantation of neuronal stem cells are most appealing alternatives. Using a computerized real-time, closed-loop response system, either optogenetic inhibition of excitatory principal cells, or activation of a sub-population of GABAergic cells of hippocampus stops spontaneous seizures immediately after detection of them (Krook-Magnuson et al., 2013). This finding establishes an important concept of on-demand control of spontaneous seizures. A chemical–genetic approach has also been applied to achieve localized suppression of neuronal excitability in a seizure focus, using combination of a modified muscarinic receptor and its selective, normally inactive and orally bioavailable agonist clozapine-N-oxide (CNO). Systemic administration of CNO suppresses focal seizures induced by chemoconvulsants. CNO also has a robust anti-seizure effect in a chronic model of focal neocortical epilepsy (Katzel et al.,

2014). On demand control of seizures provides a novel approach to treat intractable focal epilepsy while minimizing disruption of normal circuit function and limiting side effects involved in long-term treatment of AEDs.

Due to loss of GABA inhibition in human epileptic tissues and brains of epileptic animals, restoration of inhibitory circuits in brain provides an alternative approach for halting seizures in TLE patients. Transplantation of stem cells which are capable to differentiate into functional inhibitory neurons into epileptic brain provides such idea. Medial ganglionic eminence progenitors transplanted into mice hippocampus migrated and expressed genes and proteins characteristic for interneurons, differentiated into functional inhibitory neurons and received excitatory synaptic input. Transplantation of GABA-releasing cells suppressed the occurrence of electrographic seizures and restored behavioral deficits in spatial learning, hyperactivity and the aggressive response to handling in epileptic mice (Hunt et al., 2013). Successful derivation of inhibitory neurons from human pluripotent stem cells facilitates clinical translation of quality-controlled human cell sources that can engraft within human epileptic brain, induces inhibitory synaptic responses in host neurons and finally suppress seizure and abnormal behavior (Cunningham et al., 2014).

1.2.4 Molecular mechanisms of epileptogenesis

The current available medication listed above targets symptoms rather than the underlying cause. No preventive or disease modifying therapy for TLE is

available. To develop interventions for the prevention of TLE, recent studies have been focusing on elucidating the molecular mechanisms of epileptogenesis, how a normal brain becomes epileptic. Important potential master molecular regulators of epileptogenesis have been indentified in recent years, including diverse factors involving in inflammatory pathways, mammalian target of rapamycin (mTOR), adenosine kinase, repressor element 1-silencing transcription factor (REST), TrkB (will be elaborated in section 1.2) and many others. Albeit more than a dozen molecular targets of epileptogenesis have been identified, prevention of epileptogenesis is still an unmet medical challenge.

1.2.4.1. Inflammatory pathways

Critical role of inflammatory processes in epielsptogenesis has been well recognized as a result of supportive evidence in experimental models and in the clinical setting. Diverse regulators of inflammation including Inflammatory cytokines (e.g. interleukin-1β, [IL-1β]; High-mobility group protein B1, [HMGB1]), receptors of innate immunity (e.g. Toll-like receptor 4, [TLR4]), inflammation-regulating G protein coupled receptors (e.g. E prostanoid 2, [EP2]) and integrins (e.g. α4β1 integrin) are over-expressed or -activated in human and experimental epileptogenic tissue. Inhibition of IL-1β biosynthesis reduced acute seizures and drug resistant chronic epileptic activity in mice (Maroso et al., 2011). Administration of antagonists of TLR4 retarded seizure precipitation and decreased acute and chronic seizure recurrence in rodents (Maroso et al., 2010).

Inhibition of leukocyte-vascular interactions, either with blocking antibodies of α4 integrin or by genetically interfering with leukocyte mucin P-selectin glycoprotein ligand-1 function in mice, markedly reduced seizures. Treatment with blocking antibodies of α4 integrin 1 hour after onset of pilocarpine-induced status epilepticus significantly reduced convulsions and daily seizures measured from days 5-20 after induction of status epilepticus. Notably, mice were also received antibody treatment every other day for 20 days during monitoring (Fabene et al., 2008). Systemic administration of brain-permeable EP2 antagonists commencing hours after pilocarpine-induced status epilepticus reduced the formation of inflammatory cytokines and gliosis in hippocampus, maintained the integrity of the blood-brain barrier, and reduced delayed neurodegeneration in hippocampus. Intriguingly, behavioral seizure scoring and cortical EEG recording demonstrated that pharmacological inhibition of EP2 did not have an acute antiseizure effect in the same status epilepticus model, suggesting that the beneficial effects from EP2 antagonism might be caused through an anti-inflammatory mechanism (Jiang et al., 2013).

1.2.4.2 mTOR

mTOR is a serine/threonine protein kinase that is involved in diverse biological functions, including cell growth, survival and proliferation in the brain. The mTOR mediated signaling is strongly associated with epilepsy. Genetic mutations in many components of the mTOR pathway produce phenotypes that

include epilepsy. Pharmacological inhibition of mTOR by administration of mTOR inhibitor, rapamycin after the induction of status epilepticus decreased cell death, mossy fiber sprouting and aberrant dentate granule cell neurogenesis, though seizure frequency was not affected. Treatment with rapamycin prior to induction of status epilepticus increased latency to seizure emergence and reduced the frequency of spontaneous seizures. However initiation of treatment prior to induction of status epilepticus precludes rigorous study of preventive effect of rapamycin.

1.2.4.3 Adenosine kinase

Substantial evidence shown that adenosine is an endogenous anticonvulsant in brain. Adenosine exerts predominantly inhibitory functions by activation of A1 receptors in hippocampus. Presynaptic activation of A1 receptors causes a reduction in glutamate release probability by inhibiting calcium influx or interfering directly with the release process (Gundlfinger et al., 2007; Thompson et al., 1993; Wu and Saggau, 1994). Adenosine released during the seizure also significantly attenuated the depolarizing $GABA_AR$ responses (Ilie et al., 2012). Brain levels of adenosine are primarily regulated by the activity of adenosine kinase, which was found overexpressed in hippocampus of epileptic mouse. Injection of adenosine kinase inhibitor suppressed seizures and interictal spikes induced by unilateral KA hippocampus infusion (Gouder et al., 2004). Inhibitory action of adenosine can be used therapeutically to suppress seizures probably

through a novel mechanism by inhibition of DNA methylation, which is increased in epileptic brain. Transient augmentation of adenosine reduces pathological DNA methylation and prevents epileptogenesis in a mouse model of TLE (Williams-Karnesky et al., 2013).

1.2.4.4 REST and its effector enzymes

Epigenetic is the mechanism for regulating gene activity independent of DNA sequence mainly through DNA methylation and histone modification, thereby determines which genes are turned on or off. REST binds to a specific DNA sequence known as repressor element-1. In human genome, more than a thousand genes contain this site within their regulatory regions. REST enables many histone modifications to control fundamental transcription patterns like BDNF and potassium-chloride cotransporter 2 which are critical to circuit excitability. REST is strongly induced following status epilepticus. Administration of decoy oliognucleotides limiting this transcriptional repressor initiated after status epilepticus resulted in a 70% reduction in the number of spontaneous seizures during the ensuing 2 weeks (McClelland et al., 2011). However, whether suppression of seizures persists after withdrawal of oligonucleotide therapy has not been studied.

1.2.4.5 Other

In addition to well recognized molecular mechanisms of epileptogenesis listed above, recent identification of various potential drug targets including

miRNA-134, α2-adrenergic receptor and Janus kinase/signal transducer and activator of transcription-3 (JAK/STAT3) pathway provides potential therapies for TLE. Pharmacological depletion of a microRNA, miR-134, initiated after status epilepticus reduced the occurrence of spontaneous seizures when tested weeks later. Nevertheless, whether this treatment was preventive requires additional study because reductions of miR-134 persisted during recording (Jimenez-Mateos et al., 2012). Treatment with atipamezole, an α2-adrenergic receptor antagonist, after status epilepticus reduced the frequency of seizures but failed to prevent epilepsy or behavioral impairments (Pitkanen et al., 2004). The JAK/STAT3 pathway modulates gene transcription by transducing extracellular signals into the nucleus. Systemic administration of a JAK2 inhibitor, WP1066 immediately after the onset of pilocarpine-induced status epilepticus reduced frequency and severity of spontaneous seizures over a 2 weeks recording period. Importantly treatment with WP1066 did not affect the intensity or duration of status epilepticus or the associated neuronal death (Grabenstatter et al., 2014). However, initiation of treatment with WP1066 during the status epilepticus precludes meaningful interpretation of any preventive or disease modifying effect of this approach.

1.3 TrkB and its signaling

TrkB belongs to neurotrophin receptor tyrosine kinase (RTK) family. Binding of brain-derived neurotrophic factor (BDNF) or neurotrophin 4 (NT-4) to

TrkB results in receptor dimerization and activation of kinase. TrkB plays diverse roles in both human health and diseases, and its pivotal role in TLE has been well documented (McNamara and Scharfman, 2012).

1.3.1 Overview of RTK

RTKs are a family of cell surface receptors, many of which are important regulators of key cellular processes including proliferation, differentiation, cell migration and cell cycle control (Blume-Jensen and Hunter, 2001; Lemmon and Schlessinger, 2010). The diversity evident in the 58 distinct RTKs in humans notwithstanding, these receptors exhibit similar molecular structures with ligand-binding ectodomain, a single transmembrane domain, and a cytoplasmic component that includes the protein tyrosine kinase domain. These receptors convey signals by coupling ligand binding to the ectodomain to intracellular signaling cascades via recruiting adaptor proteins and enzymes to motifs in the cytoplasmic domain which contains distinct tyrosine residues that undergo phosphorylation upon receptor activation. Mutations of RTKs result in excessive activation of intracellular signaling pathways and cause a diversity of diseases including cancer, diabetes, and arteriosclerosis (Lemmon and Schlessinger, 2010). This led to development of a diversity of FDA-approved drugs, most of which are small molecules that target the adenosine triphosphate (ATP)-binding site of the kinase domain. While several tyrosine kinase inhibitors have successfully treated some human cancers, emergence of side effects due to lack

of target selectivity together with emergence of drug resistance has limited success with this strategy.

1.3.2 BDNF receptor TrkB and its signaling

Binding of cognate ligand, BDNF or NT-4 to TrkB results in receptor dimerization and activation of TrkB. TrkB is known to regulate neuronal structure and function and is important for synaptic plasticity (Bibel and Barde, 2000). TrkB conveys cell signaling through recruiting and activating adaptor proteins/enzymes to multiple tyrosine phosphorylated sites within its intracellular domain upon receptor activation. Enhanced activity of the TrkB tyrosine kinase results in phosphorylation of Y515 in the juxtamembrane region and Y816 on the carboxyl terminus of the receptor, thereby creating docking sites for Src homology 2 domain containing (Shc) and PLCγ1 respectively. Phosphorylation of Shc and PLCγ1 activates Shc/Rat sarcoma (Ras)/ mitogen-activated protein (MAP) kinase and PLCγ1 signaling respectively (Huang and Reichardt, 2003).

1.3.2.1 TrkB Y515-mediated signaling and function

Phosphorylation and recruitment of adaptors to Y515 leads to activation of the Ras/MAP kinase signaling cascade, which promotes neuronal differentiation and growth through extracellular signal-regulated kinase (ERK) and MAPK/ERK kinase (MEK), and to activation of the phosphatidylinositol 3-

kinase (PI3K) cascade, which promotes survival and growth of neurons and other cells through Ras or GRB-associated binder 1 (GAB1) (Reichardt, 2006).

TrkB mutated at the Shc binding site (*trkB*$^{SHC/SHC}$) supported survival and growth poorly relative to wild-type TrkB. TrkB-mediated neuronal survival was dependent on P13-kinase and to a lesser extent MEK activity, while growth depended upon both MEK and P13-kinase activities. These results indicate that the TrkB-Shc site is critical to both neuronal survival and axonal outgrowth (Atwal et al., 2000). *trkB*$^{SHC/SHC}$ mice also exhibited impairment of consolidation but not acquisition of fear conditioning to tone (Gruart et al., 2007).

1.3.2.2 TrkB Y816-mediated signaling and function

Phosphorylation of TrkB Y816 provides a docking site for an enzyme, PLCγ1, which is phosphorylated by the active receptor. Activated PLCγ1 hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP_2) to generate inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 promotes release of Ca^{2+} from internal stores, which results in the activation of enzymes such as Ca^{2+}/calmodulin-dependent protein kinases (CaMK). DAG stimulates DAG-regulated protein kinase C (PKC) isoforms. Point mutation of Y816F impairs phosphorylation of CaMKII, CaMKIV and cyclic adenosine monophosphate (AMP)-responsive element-binding protein (CREB) (Minichiello et al., 2002).

TrkB Y816-mediated PLCγ1 activation play a critical role in synaptic plasticity and learning. Both hippocampus mossy fiber-CA3 and CA3-CA1 LTP

were impaired in mice with TrkB mutated at the PLCγ1 binding site (*trkB*$^{PLC/PLC}$) (Gruart et al., 2007; He et al., 2010). *trkB*$^{PLC/PLC}$ mice also exhibit impairment of associative learning and acquisition of fear conditioning (Gruart et al., 2007; Musumeci et al., 2009). In contrast to TrkB mediated Shc signaling, which is critical to neuronal survival, TrkB mutated at the PLCγ1 binding site supported growth and survival well compared to wild type (Atwal et al., 2000).

1.4 TrkB and epilepsy

Extensive experimental evidence supports the assertion that the BDNF promotes limbic epileptogenesis by activation of its cognate receptor, TrkB. Expression of BDNF is dramatically increased following a seizure in multiple animal models (Ernfors et al., 1991; Isackson et al., 1991; Springer et al., 1994). BDNF mRNA (Murray et al., 2000) and protein content (Takahashi et al., 1999) are also increased in the hippocampus of humans with TLE. Enhanced activation of TrkB has been identified in multiple models of limbic epileptogenesis (Binder et al., 1999b; Danzer et al., 2004; He et al., 2002). Administration of BDNF and transgenic overexpression of BDNF enhance limbic epileptogenesis (Croll et al., 1999; Xu et al., 2000b). Striking impairments of epileptogenesis in the kindling model were identified in mice carrying only a single BDNF allele, while epileptogenesis was eliminated altogether in mice with a conditional deletion of TrkB in the CNS (He et al., 2004; Kokaia et al., 1995). Study of localization of TrkB activation following prolonged seizures using antibody

recognizing phosphor-tyrosine 816 of TrkB reveals enhancement of phosphor-TrkB immunoreactivity in mossy fiber axons and giant synaptic boutons and spines of apical dendrites of CA1 pyramidal cells of hippocampus (Helgager et al., 2013). The role of BDNF-TrkB signaling in epileptogenesis is still controversial, because localized viral-mediated delivery of both fibroblast growth factor-2 and BDNF into hippocampus, when epileptogenic damage was already in place, reduces spontaneous seizures and embanked neuronal damage (Paradiso et al., 2009). However, the effects of BDNF alone are not studied.

1.4.1 Inhibition of TrkB exerts an antiseizure effect

Emerging evidence suggests that activation of the BDNF receptor TrkB promotes development of epilepsy, but its effect on individual seizures is unknown. Utilizing the kindling model in combination with an inducible conditional knockout of the TrkB gene that treatment with tamoxifen down regulates TrkB expression in *Act-CreER TrkB*$^{flox/flox}$ mutant but not control mice and a chemical-genetic approach that genetically altered mice (*TrkB*^{F616A}) are unique in that a drug, 1-(1,1-dimethylethyl)-3-(1-naphthalenylmethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (1NMPP1), inhibits TrkB in their brains, Liu, et al. demonstrated that following induction of kindling, reduction of TrkB protein levels was associated with reduced severity of behavioral seizures evoked by stimulation. Treatment with 1NMPP1 for 2 weeks following induction of kindling reversibly elevated both focal electrographic and generalized seizure thresholds in *TrkB*^{F616A}, but not wild

type mice (Liu et al., 2014). These data together demonstrate an antiseizure effect of TrkB kinase inhibition.

1.4.2 Transient Inhibition of TrkB kinase after status epilepticus prevents development of TLE

Emerging evidence suggests that activation of the BDNF receptor TrkB promotes epileptogenesis caused by status epilepticus. A chemical-genetic approach established proof of concept that transiently inhibiting the receptor tyrosine kinase, TrkB, following status epilepticus prevented epilepsy and anxiety-like behavior in a mouse model of TLE (Liu et al., 2013). Only two weeks treatment of 1NMPP1 after completion of status epilepticus prevented epilepsy from developing in *TrkB*^{F616A} mice when tested many weeks later. This result demonstrates that transiently inhibition of TrkB kinase following the initial bout of prolonged seizures prevents the onset of epilepsy.

In sum, inhibition of TrkB exhibits both anticonvulsant and antiepileptogenic effects, establishing TrkB signaling as an attractive target for treatments of epilepsy in humans. However, the specific downstream signaling of TrkB, activation of which promotes these pathological consequences is still unclear.

2. Methods and Materials

2.1 Animals

Animals were handled according to the National Institutes of Health 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.

2.1.1 Mutant mice

Mutant mice on mixed background were backcrossed to C57BL/6 mice. The genotype of each animal was assessed using polymerase chain reaction (PCR) of genomic DNA isolated from tails.

2.1.1.1 *trkB*$^{PLC/PLC}$ mutant mice

trkB$^{WT/WT}$ and *trkB*$^{PLC/PLC}$ mutant mice on a C57BL/6-129 background were generated by cDNA knockin approach as described previously (Minichiello et al., 2002). In brief, PCR-based site-directed mutagenesis was used to induce a single point mutation (A to T position 2958) in TrkB cDNA that resulted in substituting phenylalanine for tyrosine 816 (Y816F), thereby disrupting the binding of PLCγ1. The mutant TrkB cDNA (TrkB^{PLC}) and control wild type TrkB cDNA (TrkB^{WT}) were knocked into the juxtamembrane exon of the mouse *trkB* gene. Adult male and female homozygous mutant *trkB* (*trkB*$^{PLC/PLC}$) and wild type knockin *trkB* (*trkB*$^{WT/WT}$) mice were used in this study.

2.1.1.2 *PLCγ1* mutant mice

PLCγ1 mutant mice were generated by targeted deletion of genomic sequences encoding the X domain and both Src Homology 2 (SH2) domains of *PLCγ1* as described previously (Ji et al., 1997). We crossed inbred strains of *PLCγ1* mutant mice on a 129/SvJ background to C57BL/6 for six generations. Note that the line generated with replacement vector TV-1 was used in these experiments. Homozygous disruption of *PLCγ1* (*−/−)* results in embryonic lethality at approximately embryonic day 9.0–9.5. Therefore, adult male and female heterozygotes *of PLCγ1* (*PLCγ1*$^{+/-}$) and wild type littermates (*PLCγ1*$^{+/+}$) were used in this study.

2.1.1.3 *TrkB*^{F616A} mutant mice

TrkB^{F616A} mice in a 129/J and C57BL/6 hybrid genetic background were provided by Dr. David Ginty (Chen et al., 2005). Briefly, *TrkB*^{F616A} knockin mice harbor a single point mutation, changing phenylalanine to alanine within the ATP binding pocket of kinase subdomain V, which renders it sensitive to inhibition of a small molecule derivative of the general kinase inhibitor protein phosphatase 1NMPP1. Adult male and female homozygous mice (*TrkB*^{F616A}) were used in this study.

2.1.2 Wild type mice

Wild type male C57BL/6 mice purchased from Charles River were used in some experiments.

2.2 Cell culture

Dissociated neuronal cultures were prepared as described previously (Huang et al., 2008). Briefly, cortical mixed neuron and glia cultures were prepared from pups of embryonic day 18 pregnant Sprague Dawley rats (Charles River). Cells were cultured in Neurobasal with B27 supplement for 10-12 days *in vitro* before use. Medium was replaced with artificial cerebrospinal fluid (ACSF) buffer *before* BDNF (10 ng/ml, Chemicon) stimulation. Fifteen minutes after addition of BDNF, cells were lysed in modified Radio-Immune Precipitation Assay (RIPA) buffer (20 mM Tris, pH 7.5, 137 mM sodium chloride, 1% NP40, 0.25% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and one complete Mini protease inhibitor cocktail tablet [Roche]/10 ml), briefly centrifuged, and the supernatant was used for western blotting.

2.3 Surgery and kainic acid microinfusion

Adult mice were anesthetized and placed in a stereotaxic frame. A guide cannula (Plastics One) was inserted above the right amygdala (coordinates from bregma: AP=-1.2 mm; ML= 3.0 mm). A bipolar electrode was placed into the left

dorsal hippocampus (coordinates from bregma: AP=-2.0 mm; ML=-1.6 mm; and DV=-1.5 mm below dura) (Figure 2A). After a 7-day postoperative recovery, animals were gently restrained and an infusion cannula (Plastics One) was inserted into the right amygdala through the guide cannula to a depth of 3.7 mm below the dura. Either KA (0.3 μg in 0.5 μl phosphate-buffered saline, PBS) or vehicle (0.5 μl of PBS) was infused into the right amygdala at the rate of 0.11 μl/min. Following completion of the infusion, the cannula was left in the right amygdala for two additional minutes. Subsequently, animals were housed individually for EEG telemetry and video monitoring with ad libitum access to food and water.

2.4 Status epilepticus and video-EEG monitoring

Continuous hippocampal EEG telemetry (Grass Instrument Co.) and time-locked video-monitoring were performed using Harmonie software (Stellate Systems). Monitoring started 15 minutes before amygdala KA infusion for recording baseline EEG and behavioral activity. Emergence of status epilepticus was typically evident in EEG recordings 0-10 minutes after completion of KA infusion. Behavioral seizures were classified according to a modification of Racine scale for mice (Borges et al., 2003; Racine, 1972): 0, normal activity; 1, arrest and rigid posture; 2, head nodding; 3, partial body clonus (unilateral forelimb clonus); 4, rearing with bilateral forelimbs clonus; 5, rearing and falling (loss of postural control); 6, tonic-clonic seizures with running and/or jumping.

For experiments in which pY816 or Scr was administered prior to infusion of KA, mice were monitored for 45 minutes following KA infusion. EEG patterns consistent with electrographic status epilepticus included any of the following: 1. discrete electrographic seizures; 2. waxing and waning epileptiform activity; 3. continuous, high amplitude, rapid spiking; 4. periodic epileptiform discharges on a relatively flat background as described (Treiman et al., 1990; Walton and Treiman, 1988). The duration of behavioral and electrographic status epilepticus was determined by analyses of video and EEG data by trained observers blinded to treatment of mice. A similar method was used in experiments examining effects of mutations (*trkB*$^{PLC/PLC}$ and *PLCγ1*$^{+/-}$) on status epilepticus induced by KA.

2.5 Video-EEG Monitoring for Detection of Spontaneous Recurrent Seizures

For experiments in which pY816 or Scr was administered following status epilepticus, diazepam (10 mg/kg, i.p.) was administered 40 minutes after the onset of status epilepticus followed by lorazepam (6 mg/kg, i.p.) one hour later to suppress status epilepticus and limit the mortality and morbidity. Animals underwent continuous video-EEG monitoring 24 hr per day, 7 days per week for the first two weeks after status epilepticus (day 1-14); at the end of the second week, monitoring was discontinued and animals were returned to home cages. After a two-week interval, monitoring was resumed for an additional two weeks

(day 29-42). Spontaneous recurrent seizures were identified by review of video-EEG files independently by each of two trained readers blinded to treatment of mice. The consistency of identifying spontaneous recurrent seizures between readers was ~90%; in instances in which readers disagreed, the events were excluded from this study. Spontaneous recurrent seizure was defined electrographically as high frequency (>5 Hz), high amplitude (>2 X baseline) rhythmic epileptiform activity with a minimal duration of 5 seconds. Behavioral correlates of these electrographic seizures ranged from Classes 1 through 6.

2.6 Immunoprecipitation

Animals were anesthetized with pentobarbital (200 mg/kg i.p.) and decapitated, and the head was quickly immersed in liquid nitrogen for 4 seconds to rapidly cool the brain. The hippocampi were rapidly dissected on ice and homogenized in RIPA buffer, incubated on ice for 15 minutes, and centrifuged at ~200,000 x g for 10 minutes at 4°C. The supernatant was collected and stored at -80°C until further analysis. TrkB was immunoprecipitated using TrkB antibody (1:1000, Millipore) and the lysate was incubated with 100 µL of protein A-Sepharose beads (Roche) overnight in 4°C. The beads/immune complexes were pelleted in a microcentrifuge and then resuspended in RIPA buffer and this procedure was repeated two additional times before adding 2 x sodium dodecyl sulfate (SDS) sample buffer and boiled. After SDS polyacrylamide gel

electrophoresis (PAGE), the gels were probed with antibodies to TrkB or PLCγ1 (1:1000, Cell signaling).

2.7 Western Blotting

Western blotting was conducted as previously described (He et al., 2010). Cell lysates and hippocampal homogenates were subjected to SDS-PAGE, transferred, and blots probed with antibodies to the following: p-PLCγ1 (pY783), PLCγ1, p-Akt (pS473), Akt, p-ERK (p44/p42) and ERK (1:1000, Cell signaling). The immunoreactivity of individual bands on western blots was measured by ImageJ software (National Institutes of Health) and normalized to immunoreactivity of PLCγ1, Akt or ERK respectively. Equivalent protein loading and transfer were monitored by β-actin (1:10000; Sigma) immunoreactivity. Shown are representative results of immunoblotting from at least three independent experiments.

2.8 Behavior tests

At 8 weeks after completion of status epilepticus, mice were subjected to open field test and light/dark box test to assess locomotor activity and anxiety-like behavior respectively.

2.8.1 Open field tests

A plexiglas arena (40 x 40 x 40 cm) was placed in a room with homogenous illumination (~ 300 lux). The arena was subdivided into sixteen 10 x 10 cm squares by lines marked on the floor. Each mouse was placed in the center of the open field and allowed to freely explore the apparatus for 5 minutes. A video camera mounted directly above the arena was used to monitor animal activity and movement. To assess locomotor activity, an investigator unaware of the treatment reviewed the video and recorded line crossing (frequency of crossing grid lines with all four paws) and the frequency of rearing (standing on hind legs or leaning against the walls of the arena).

2.8.2 Light/dark box test

The apparatus (20 x 40 x 40 cm) consisted of two acrylic compartments. The dark compartment (30 x 40 cm) and light compartment (40 x 40 x 40 cm, brightly illuminated at 600 lux) were separated by a divider with a 5.5 x 5.5 cm opening at floor level. Mice were placed into the dark side and allowed to move freely between the two chambers with the door open for 5 minutes. During behavior tests, a video camera was mounted directly above the arena to monitor animal activity and movement. An investigator unaware of the treatment reviewed the video and recorded: 1) line crossing (frequency of crossing grid lines with all four paws) and the frequency of rearing (standing on hind legs or leaning against the walls of the arena) to assess locomotor activity in open field

test; and 2) the latency to first entry into lighted compartment (entering the lighted side with all four paws) and the total time spent on the lighted side to assess anxiety-like behavior in light/dark box test.

2.9 Fluoro-Jade (FJC) staining

Degenerating neurons were visualized by FJC staining 24 hr after status epilepticus induced by infusion of KA into amygdala. Mice were sacrificed 24 hr after completion of status epilepticus and perfused with PBS containing heparin (1 U/ml) followed by 4% paraformaldehyde. Brains were removed, frozen by slow immersion in isopentane chilled in dry ice, cryoprotected, and sectioned. Serial 40 µm coronal sections were cut through the forebrain spanning the entire hippocampus. Sections were subjected to FJC (Millipore, MA USA) staining as previously described (Mouri et al., 2008). Stained sections were examined using a ZEISS AX10 microscopy system equipped with a 10x objective lens and fluorescein filter (excitation: 485 nm; emission: 525 nm). The number of FJC positive cells were counted in two adjacent sections from middle level (AP from Bregma: −1.82 mm) of hippocampus ipsilateral to KA infusion site by an observer blinded to genotype and treatment conditions with ImageJ software (Ferreira and Rasband, 2011) in a 260 X 260 µm field within hippocampal CA1 or CA3 a/b subfield.

2.10 Immunohistochemistry

Mice were deeply anesthetized and perfused with PBS containing heparin (1 U/ml) followed by buffered 4% paraformaldehyde. Brains were removed, frozen by slow immersion in isopentane chilled in dry ice, cryoprotected, and sectioned. Serial 40 µm coronal sections were cut through the forebrain spanning the entire hippocampus. Adjacent sections at middle level of hippocampus (AP from Bregma: −1.82 mm) were subjected to immunofluorescent staining. Neurons and astrocytes were visualized using antibodies against neuronal nuclei (mouse monoclonal to NeuN, 1:500; Millipore) and glial fibrillary acidic protein (rabbit polyclonal to GFAP, 1:500; Sigma) detected with Alexafluor 488 coupled anti-mouse and Alexafluor 594 coupled anti-rabbit secondary antibodies (Molecular Probes), respectively. Images were captured using a Leica TCS SL confocal microscopy system equipped with a 63x oil-immersion objective lens. NeuN-positive cell counting was performed by an investigator blinded to the treatment conditions with ImageJ software (Ferreira and Rasband, 2011) in a 260 X 260 µm field within the CA3b pyramidal cell layer. Mean counts were obtained from two adjacent sections ipsilateral to the infused site.

2.11 Quantitative analysis of EEG energy content

Quantitative analysis of EEG energy content was performed using the method described previously (Lehmkuhle et al., 2009). Experimental design

necessitated handling the animals (e.g. KA microinfusion, treatment with benzodiazepine or peptide) which resulted in EEG artifacts. EEG records were reviewed to detect these artifacts and these portions of recording were removed. To assure objectivity, detection and removal of such artifacts from power plots and corresponding EEG and subsequent analyses were performed by an investigator unaware of either treatment or genotype. We calculated running power in the gamma band (20-50 Hz) at a one-second resolution and smoothed the resulting time-series using a 5 minutes moving average filter. We averaged the values of the smoothed gamma power time-series during and following the period of status epilepticus and normalized it to the average of the baseline to give ratios representing gross activity.

2.12 Peptide and reagent

Human immunodeficiency virus type 1 (HIV-1) trans-activating (Tat) protein transduction domain (YGRKKRRQRRR) was conjugated to the N-terminus of a sequence of human TrkB (807-820) with tyrosine residue (817 of human and 816 of mouse and rat) phosphorylated (pY816, YGRKKRRQRRR-LQNLAKASPVpYLDI). A HIV-1 Tat protein transduction domain conjugated to a scrambled peptide (Scr, YGRKKRRQRRR-LVApYQLKIAPNDLS) served as control. Peptides were synthesized and purified by Tufts Peptide Core Facility. Peptides were dissolved in PBS at a concentration of 2 mg/ml and stored at -80°C until use. Aliquots of pY816 and Scr were thawed and injected

intravenously (i.v.) shortly thereafter. All reagents were purchased from Sigma unless specified otherwise.

2.13 Data analyses

Unless otherwise stated, All data are presented as the mean ± standard error of the mean (SEM). Unless otherwise stated, comparisons between two groups were analyzed using unpaired Student's t-test (parametric) or Mann-Whitney U test (nonparametric), while multi-group comparisons were analyzed using ANOVA followed by Tukey's (one-way ANOVA) or Bonferroni's (two-way ANOVA) *post-hoc* test (parametric) or Kruskal-Wallis test followed by Dunn's multiple comparisons test (nonparametric). A $p < 0.05$ was considered significant.

3. Results

This chapter has been adapted from a manuscript under review.

3.1 Inhibition of TrkB kinase exacerbates status epilepticus-induced neuronal degeneration

We recently demonstrated that transient inhibition of TrkB kinase commencing after status epilepticus can prevent epilepsy and anxiety-like disorder detected months later (Liu et al., 2013), thereby implicating TrkB kinase as a therapeutic target for prevention. That said, the pro-survival effects of TrkB signaling (Alcantara et al., 1997; Atwal et al., 2000) raised our concern that inhibition of TrkB kinase might exacerbate death of CNS neurons induced by status epilepticus (Henshall and Meldrum, 2012). To address this issue, we used FJC staining of hippocampal sections from mice euthanized 24 hr following status epilepticus to quantify neuronal degeneration (Mouri et al., 2008). These experiments utilized a chemical-genetic approach to selectively inhibit TrkB kinase by systemic administration of a blood-brain barrier permeable small molecule, 1NMPP1 (Chen et al., 2005) (Figure 1A). Substitution of alanine for phenylalanine at residue 616 within kinase subdomain V renders TrkB of mutant (*TrkB*^{F616A}) but not wild type (WT) mice sensitive to inhibition by 1NMPP1 (Chen et al., 2005); importantly TrkB of *TrkB*^{F616A} functions equally well in mutant compared to WT mice in the absence of 1NMPP1.

3.1.1 Status epilepticus induces neuronal degeneration

One well documented consequence of status epilepticus is the destruction of hippocampal neurons. We first set to confirm this finding by assessing neuronal degeneration 24 hours after completion of status epieltpicus induced by KA. Status epilepticus preferentially destroyed CA3 pyramidal cells in hippocampus ipsilateral to KA infusion site as evident by FJC staining in sections from both WT and *TrkB*^{F616A} animals treated with vehicle (Figures 1B, left panel and 1C). The pattern of cell death confirmed results of Mouri et al (2008). Sporadic cell degeneration was also observed in CA1 subfield of hippocampus ipsilateral to KA infusion site (Figures 1B and 1C).

3.1.2 Inhibition of TrkB kinase following status epilepticus exacerbates neuronal degeneration

By confirming neuron loss in hippocampus following status epilepticus, we set to ask whether inhibition of TrkB after status epilepticus exacerbates neuronal degeneration. Using doses demonstrated to inhibit status epilepticus-induced activation of TrkB (Liu et al., 2013), 1NMPP1 treatment of *TrkB*^{F616A} mice produced three- to ten-fold increases in the number of FJC positive cells in the CA3 and CA1 pyramidal cell layers of hippocampus in comparison to vehicle treated controls (Figures 1B and 1C). Importantly, treatment of WT animals with 1NMPP1 following induction of status epilepticus produced no significant differences from vehicle (Figure 1C). In sum, these results demonstrate

neuroprotective effects of TrkB kinase following status epilepticus and reveal a deleterious consequence of global inhibition of TrkB signaling in this context, namely exacerbation of neuronal degeneration.

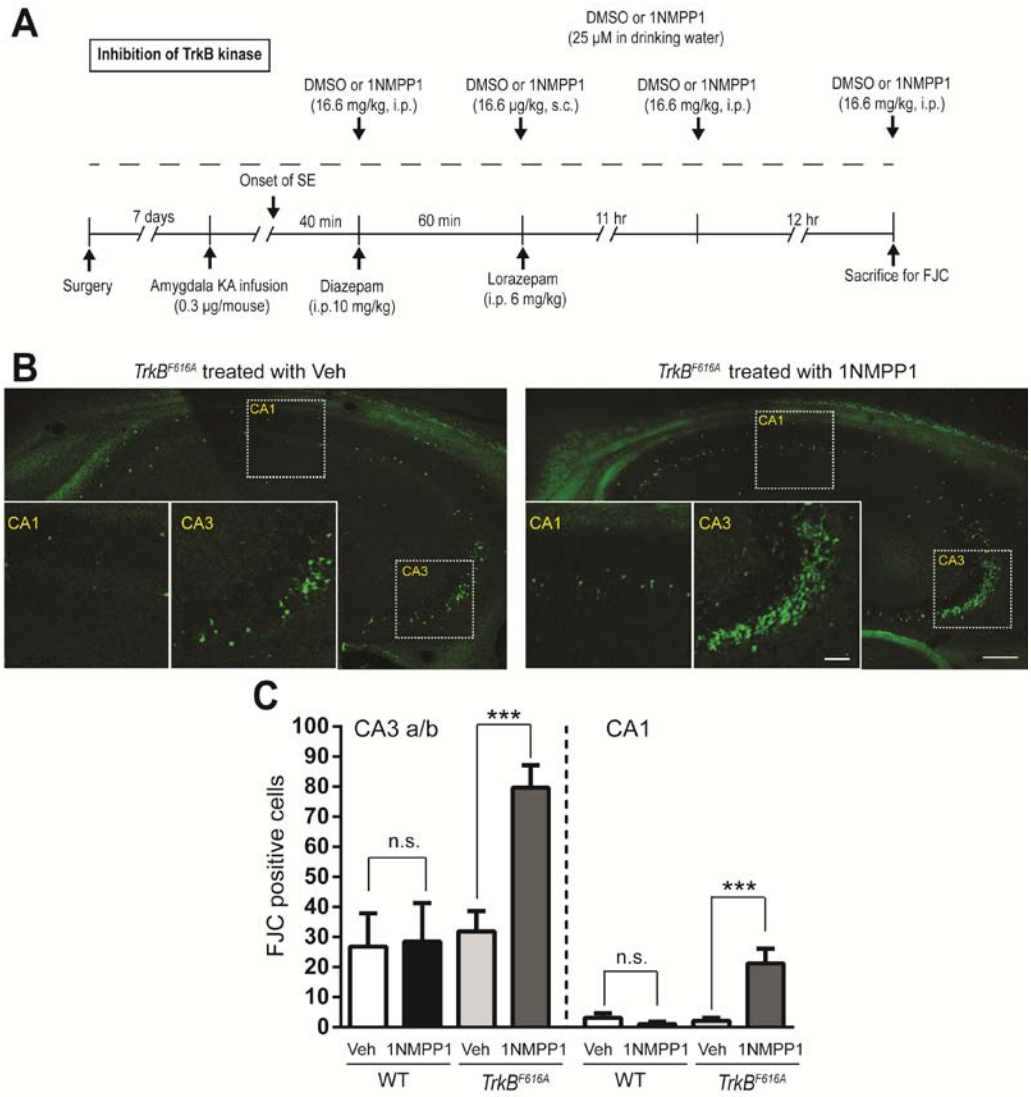

Figure 1. Inhibition of TrkB kinase exacerbates status epilepticus-induced neuronal degeneration

(A) Schematic of experimental design of assessment of neuronal degeneration 24 hr after status epilepticus induced by infusion of KA into amygdala. (B) Representative images of FJC staining in the hippocampus ipsilateral to KA infusion site in *TrkB*^{F616A} mice treated with either dimethyl sulfoxide (DMSO) (vehicle, Veh) or 1NMPP1; scale bar represents 200 μm. Insets: high-magnitude images of hippocampal CA1 and CA3 a/b subfields; scale bar represents 40 μm. (C) Counts of FJC positive cells within hippocampal subfield CA3 a/b or CA1 ipsilateral to infusion site of KA in WT or *TrkB*^{F616A} mice treated with either Veh (WT, n=4; *TrkB*^{F616A}, n=4) or 1NMPP1 (WT, n=6; *TrkB*^{F616A}, n=4). Data are presented as mean ± SEM and analyzed using Two-way ANOVA with *post hoc* Bonferroni's tests, ***p<0.001.

3.2 Antiseizure effect of limiting PLCγ1 signaling

The untoward consequences of global inhibition of TrkB kinase led us to seek the signaling pathway by which status epilepticus-induced activation of TrkB promotes epilepsy and anxiety-like disorder. That is, if the downstream pathways mediating the deleterious and beneficial effects of TrkB signaling induced by status epilepticus were distinct, then selective inhibition of the deleterious pathway could be an attractive strategy for drug development.

An antiseizure effect of TrkB kinase inhibition has been observed in seizures evoked by electrical stimulation (Liu et al., 2014) but the responsible signaling pathway downstream of TrkB is unknown. The pivotal role of TrkB mediated PLCγ1 signaling in kindling development (He et al., 2010; He et al.,

2014) led us to hypothesize that limiting TrkB-mediated activation of PLCγ1 in particular will suppress limbic seizures. To investigate the anticonvulsant effects of inhibition of TrkB-mediated PLCγ1 signaling, we first examined responses to prolonged seizures (status epilepticus) induced by KA amygdala infusion in *trkB*$^{PLC/PLC}$ mice, in which a phenylalanine is substituted for tyrosine at residue 816 TrkB, thereby disrupting TrkB-mediated PLCγ1 signaling. To further confirm PLCγ1 in particular is the dominant signaling pathway promoting the anticonvulsant effects in *trkB*$^{PLC/PLC}$ mice we subsequently tested the responses to KA infusion in mice heterozygous for PLCγ1 (PLCγ1$^{+/-}$).

3.2.1 Disruption of TrkB tyrosine 816 mediated signaling inhibits chemoconvulsant induced seizures

To test this hypothesis, our initial experiments utilized *trkB*$^{PLC/PLC}$ mice in which a phenylalanine is substituted for tyrosine at residue 816 of TrkB, thereby disrupting TrkB-mediated PLCγ1 signaling (Minichiello et al., 2002). Continuous seizures (status epilepticus) were induced by local infusion of KA into the right amygdala of adult mice and both behavior and EEG were monitored continuously for 45 minutes following KA infusion (Figures 2A and 2B, schematic diagram of experimental design). Quantification of EEG power (Figures 2C and 2D) and behavioral seizure score (Figures 2E and 2H) during 45 minutes following KA infusion revealed significant reductions in *trkB*$^{PLC/PLC}$ compared to *trkB*$^{WT/WT}$ mice. Visual inspection of duration of EEG seizures (Figure 2F) and convulsive motor

seizures (Figure 2G) during 45 minutes following KA infusion confirmed this observation. Infusion of KA induced prolonged electrographic and convulsive motor seizures in each WT control mouse (*trkB*$^{WT/WT}$, 20.0 ± 3.3 min and 21.4 ± 3.8 min, respectively) (Figures 2F and 2G). By contrast, the electrographic and convulsive motor seizures were reduced by 80-90% in *trkB*$^{PLC/PLC}$ mice (4.1 ± 1.5 min and 1.2 ± 0.8 min, respectively) (Figures 2F and 2G).

These findings demonstrate that genetically uncoupling TrkB from adaptor proteins and enzymes that bind the motif containing Y816 result in powerful anticonvulsant effects.

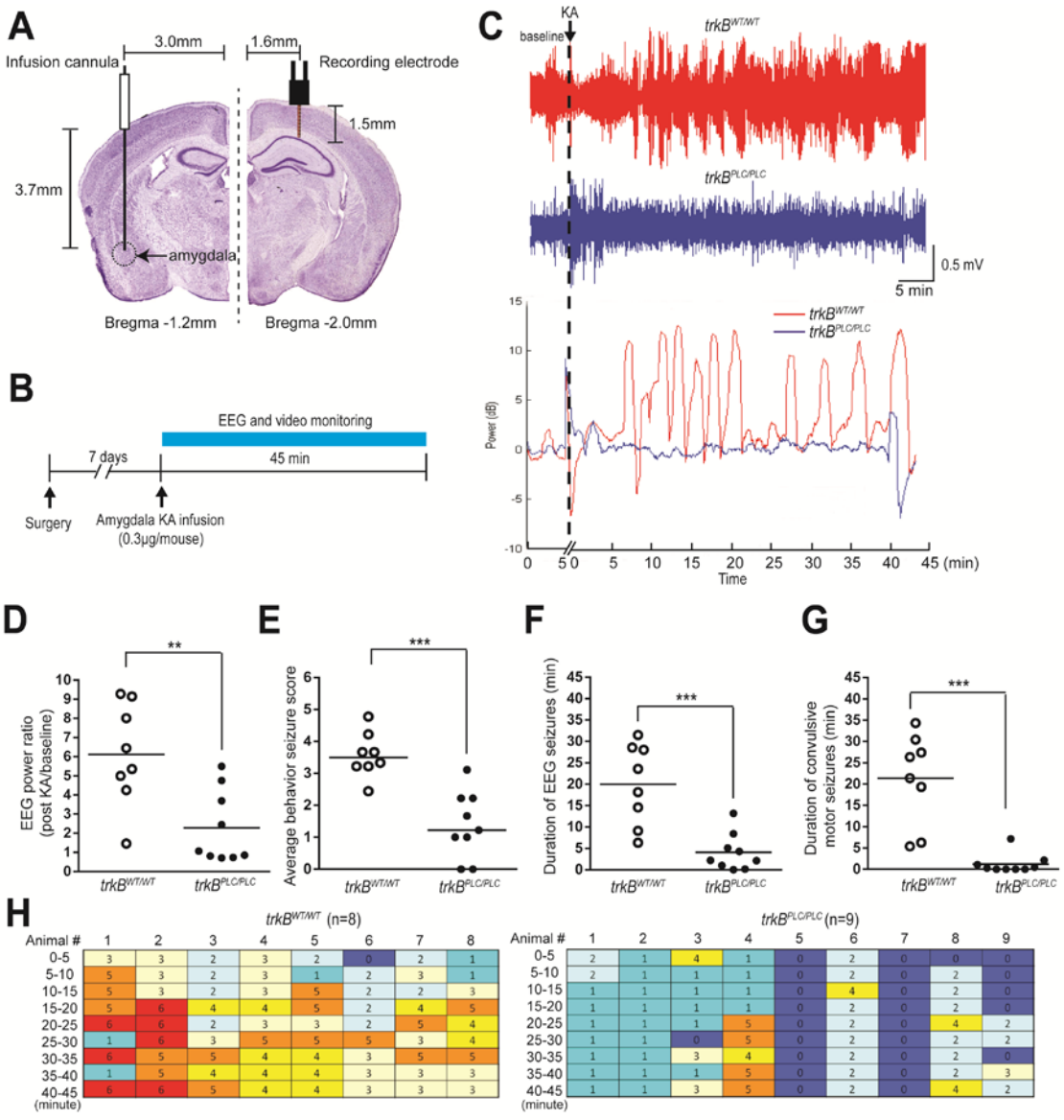

Figure 2 Inhibition of TrkB tyrosine 816 mediated signaling inhibits chemoconvulsant induced seizures

Schematics of sites of infusion cannula and recording electrode (A) and experimental design (B). (C) Representative EEG trace of *trkB*$^{WT/WT}$ (upper row) or *trkB*$^{PLC/PLC}$ (middle row) recording of 5 min prior to (baseline) and 45 min after KA infusion. The

representative plots of log_{10} power analyses of EEG adjusted to baseline power prior to KA infusion is presented immediately below, using a similar time scale. Average EEG power normalized to baseline (D) and average behavioral seizure scores (E), duration of EEG seizures (F) and convulsive motor seizures (G) were analyzed during 45 min following KA infusion in $trkB^{WT/WT}$ (n=8) and $trkB^{PLC/PLC}$ (n=9) mice. (H) Presents heat maps of maximum behavioral seizure score of each mouse during 5 min observation periods following infusion of KA. Data are presented from individual animals as well as mean and analyzed using Student's t-test (D, F and G) or median and analyzed using Mann-Whitney U test (E), n=8-9, **p<0.01 and ***p<0.001.

3.2.2 Impaired chemoconvulsant induced seizures in PLCγ1 heterozygous mice

Evidence that the pY816 motif of TrkB mediates the binding and activation of PLCγ1 suggested that the mechanism by which seizures were suppressed in $trkB^{PLC/PLC}$ mice was mediated by inhibiting PLCγ1 signaling. That said, multiple adaptor proteins and enzymes can bind a given motif of a RTK. To test whether limiting signaling thru PLCγ1 in particular is the mechanism of the anticonvulsant effects in the $trkB^{PLC/PLC}$ mice, we examined responses to KA infusion in mice heterozygous for PLCγ1 ($PLC\gamma1^{+/-}$) in comparison to wild type control. Notably, hippocampal expression of PLCγ1 protein is reduced by approximately 50% in $PLC\gamma1^{+/-}$ mice (He et al., 2014; Ji et al., 1997). Quantification of EEG power (Figure 3A) and behavioral seizure score (Figure 3B and 3E) during 45 minutes

following KA infusion revealed significant reductions in *PLCγ1*$^{+/-}$ compared to wild type control mice. This finding was confirmed by visual inspection of EEG seizures (Figure 3C) and convulsive motor seizures (Figure 3D) during 45 minutes after KA infusion. Infusion of KA induced prolonged electrographic and convulsive motor seizures in each WT control mouse (28.1 ± 2.8 min and 22.3 ± 4.3 min, respectively) (Figures 3C and 3D). By comparison, the electrographic and convulsive motor seizures evoked by KA were reduced by 65-70% in *PLCγ1*$^{+/-}$ mice (9.5 ± 2.2 min and 7.1 ± 1.9 min, respectively) (Figures 3C and 3D). These findings demonstrate that reducing the expression of PLCγ1 results in powerful anticonvulsant effects and support the conclusion that the dominant mechanism underlying the anticonvulsant effects of the *trkB*$^{PLC/PLC}$ mutation involves inhibition of PLCγ1 activation. Collectively, these findings provide a strong rationale for development of therapeutic agents that disrupt the interaction of TrkB with PLCγ1.

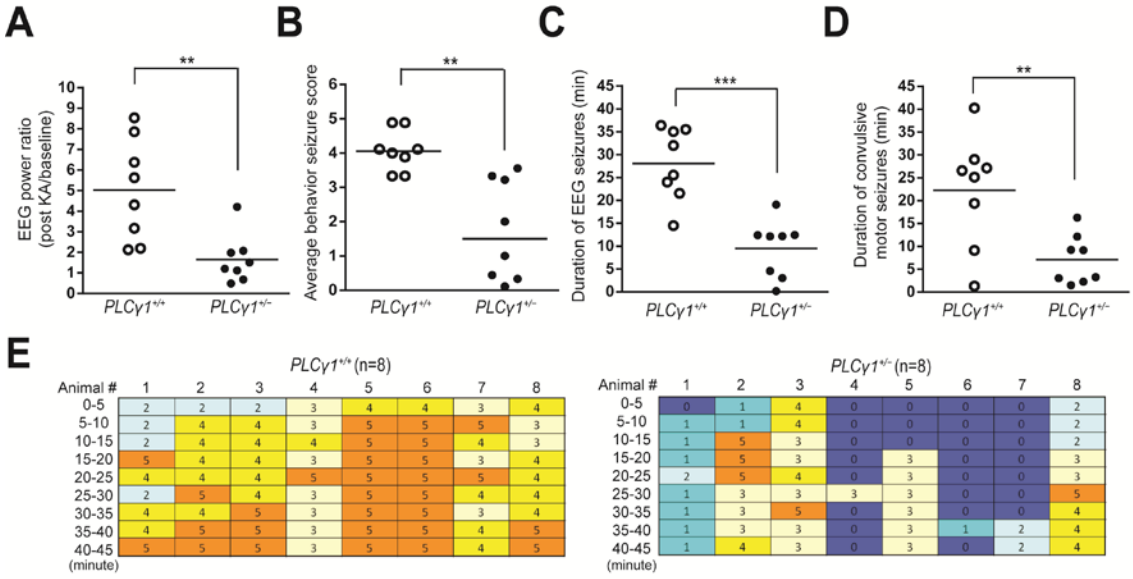

Figure 3 Inhibition of PLCγ1 inhibits chemoconvulsant induced seizures

Average EEG power normalized to baseline (A), average behavioral seizure scores (B), duration of EEG seizures (C) and convulsive motor seizures (D) were analyzed during 45 min following KA infusion in $PLC\gamma1^{+/+}$ (n=8) and $PLC\gamma1^{+/-}$ (n=8) mice. (E) Presents heat maps of maximum behavioral seizure score of each mouse during 5 min observation periods following infusion of KA. Data are presented from individual animals as well as mean and analyzed using Student's t-test (A, C and D) or median and analyzed using Mann Whitney U test (B), n=8, **p<0.01 and ***p<0.001.

3.3 pY816 peptide inhibits BDNF-mediated activation of PLCγ1

By demonstrating the signaling through which excessive activation of TrkB produces these pathological consequences, the next challenge will be how to achieve the pharmacological inhibition. Due to lack of selective PLCγ1

inhibitors, we adopted an alternative approach, namely to selectively uncouple PLCγ1 from activated TrkB. We designed a membrane-permeable peptide comprising human immunodeficiency virus (HIV)-1 trans-activating (Tat) protein transduction domain and a sequence of human TrkB which is required for binding of PLCγ1 to the motif of TrkB containing tyrosine 816 (pY816, YGRKKRRQRRR-LQNLAKASPVpYLDI) (Obermeier et al., 1993). A HIV-1 Tat conjugated to a randomly scrambled peptide (Scr, YGRKKRRQRRR-LYApYQLKIAPNDLS) served as a negative control for this study.

3.3.1 Design of pY816 peptide

The sequence of TrkB which is required for binding of PLCγ1 is determined based on the binding sequence of TrkA and PLCγ1 (Obermeier et al., 1993). The fact that PLCγ1 binding sequence of TrkB is heterogeneous compared to TrkA, TrkC and many other RTKs strengthen the likelihood that PLCγ1 binds to these RTKs with different affinities (Table 1, rows 4-12). And the fact that PLCγ1 binding sequence of TrkB is highly conserved among mouse, rat and human supports the idea that pY816 may work in human as well as mice (Table 1, rows 2-4).

Table 1 Alignment of PLCγ1 binding sequence of RTKs

RTKs	PLCγ1 SH2 binding sequence
Mouse TrkB	LQNLAKASPVYLDIL
Rat TrkB	LQNLAKASPVYLDIL

Human TrkB	LQNLAKASPVYLDIL
Human TrkA	LQALAQAPPVYLDVL
Human TrkC	LHALGKATPIYLDIL
Human EGFR (Y978)	ARDPQRYLVIQG
Human EGFR (Y1197)	TAENAEYLRVAP
Human ErbB2 (Y1248)	TAENPEYLGLDV
Human FGFR (Y766)	ALTSNQEYLDLSMPL
Human PDGFR1 (Y988)	RVDSDNAYIGVTYKN
Human PDGFR1 (Y1018)	RLSADSGYIIPLPDI

3.3.2 pY816 inhibits BDNF-mediated PLCγ1 activation *in vitro*

We first examined the concentration dependence and time course of pY816-mediated inhibition of TrkB-mediated PLCγ1 activation in primary cultured neurons *in vitro*. pY816 at 1, 5 or 10 μM was pre-incubated for 90 minutes prior to addition of BDNF. Cell lysates were then probed with p-PLCγ1 (pY783), p-Akt (pS473) and p-Erk (p44/p42) as surrogate measurement of activation of signaling pathways downstream of TrkB. pY816 at 10 μM significantly inhibited BDNF-induced PLCγ1 activation (Figures 4A and 4B). We next sought to test the time course by pre-incubating pY816 at 10 μM for 10, 30, 60, 90, or 120 minutes prior to addition of BDNF. pY816 treated 60-120 minutes prior to addition of BDNF produced marked inhibition of BDNF-mediated PLCγ1 activation (Figures 4C and 4D). Importantly, pY816-mediated inhibition was selective to PLCγ1 in that

BDNF-mediated increases of p-Akt and p-ERK were not affected. (Figures 4A and 4C, rows 3 and 5).

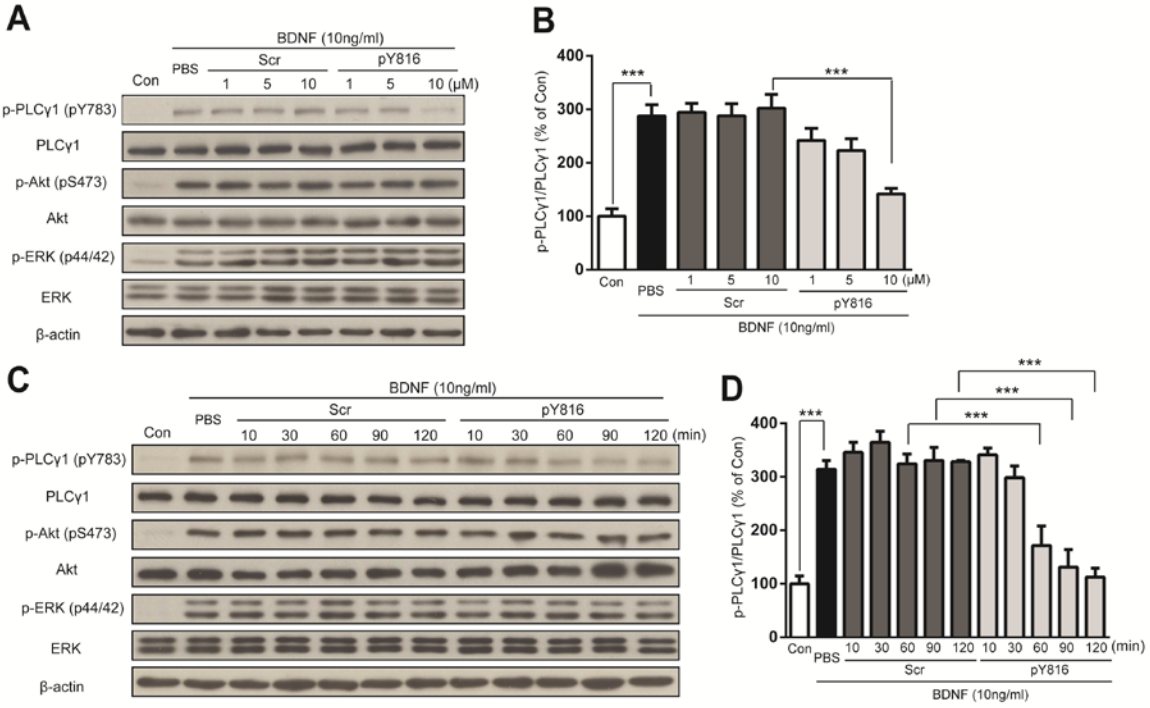

Figure 4 pY816 inhibits PLCγ1 activation *in vitro*

(A) and (B), either PBS or various concentrations of Scr or pY816 peptide (1, 5 or 10 μM) were preincubated with cultured neurons 90 min prior to addition of BDNF (100 ng/ml) (n=3). (C) and (D), either PBS or Scr or pY816 peptide (10 μM) were preincubated for various periods of time (10, 30, 60, 90 or 120 min) with cultured neurons prior to addition of BDNF (100 ng/ml) (n=3). (A) and (C) present representative western blots of p-PLCγ1 (pY783), PLCγ1, p-Akt (pS473), Akt, p-ERK (p44/p42), ERK and β-actin. (B) and (D) present quantification of ratios of immunoreactivity of p-PLCγ1

(pY783) to PLCγ1. Data are presented as mean ± SEM and analyzed using Two-way ANOVA with post hoc Bonferroni's test, n=3; ***p<0.001.

3.3.3 pY816 inhibits PLCγ1 activation *in vivo*

By demonstrating the effect of pY816 *in vitro*, the next question regarding the usefulness of pY816 is whether systemic administration of pY816 inhibits PLCγ1 *in vivo*. Intravenous (i.v.) injection of pY816 (10 mg/kg, 3 hours) reduced p-PLCγ1 (pY783) immunoreactivity by ~50% in comparison to Scr as revealed by western blotting of hippocampal lysates (Figures 5A and 5B). Importantly, the effects of pY816 were selective for PLCγ1 in that neither p-Akt nor p-ERK was affected (Figure 5A, rows 3 and 5).

3.3.4 pY816 disrupts TrkB-PLCγ1 binding *in vivo*

The effects of pY816 were likely mediated by uncoupling TrkB from PLCγ1 because systemic infusion of pY816 (10 mg/kg, 3 hours) inhibited the co-immunoprecipitation of TrkB and PLCγ1 in comparison to the Scr control peptide (Figure 5C). Collectively, these experiments demonstrate that pY816 selectively inhibits PLCγ1 activation in brain following systemic administration *in vivo.*

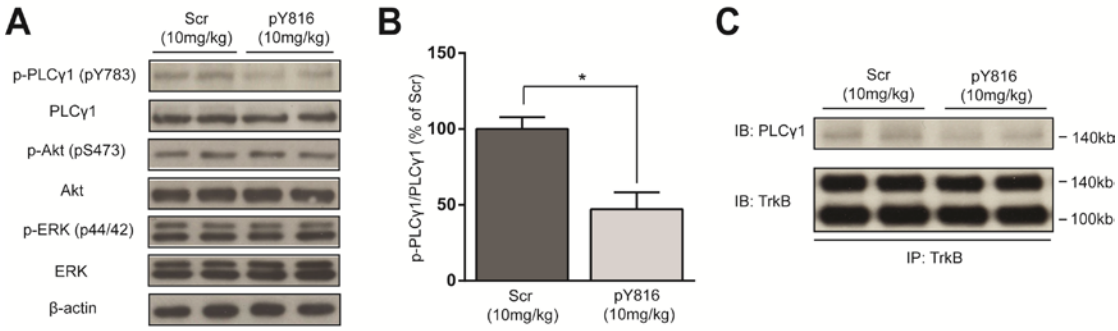

Figure 5 pY816 uncouples TrkB-PLCγ1 binding and inhibits PLCγ1 activation *in vivo*

(A) and (B) Scr or pY816 (10 mg/kg, i.v.) was injected into naïve mice (n=4) and animals were euthanized 3 hr later and hippocampal homogenates subjected to SDS-PAGE and western blotting. Compared to scrambled control peptide, pY816 peptide (10 mg/kg) reduces p-PLCγ1 (pY783) but not p-Akt or p-ERK immunoreactivity (A left panel); quantification of immunoreactivity of p-PLCγ1 (pY783) to PLCγ1 ratio is presented in (B). (C) Systemic infusion of pY816 peptide (10 mg/kg, i.v.) reduces co-immunoprecipitation of PLCγ1 with TrkB compared to Scr control in hippocampal homogenates isolated 3 hr after infusion. Data are presented as mean ± SEM and analyzed using Student's t-test, n=4; *$p<0.05$.

3.4 pY816 peptide inhibits chemoconvulsant-induced seizures

The genetic evidence that disrupting TrkB from PLCγ1 exerts antiseizure effects together with evidence that pY816 can inhibit PLCγ1 activation *in vitro* and *in vivo* led us to ask whether systemic administration of pY816 exerted

antiseizure effects in the KA model. Towards this end, either pY816 or Scr was administered (i.v.) prior to KA infusion and both EEG and behavioral seizures were monitored for 45 minutes following KA infusion (Figures 6A and 7A).

3.4.1 pY816 peptide inhibits chemoconvulsant-induced seizures in dose dependent manner

To optimize the effects, we first asked how varying doses of pY816 administration affected seizures evoked by KA. Both electrographic and behavioral seizures were analyzed by an investigator who was unaware of treatment. EEG seizures were quantified using power analyses and visual inspection. Behavioral seizures were quantified using modified Racine's scores and duration of convulsive motor seizures (Class 3-6). Infusion of KA into amygdala induced prolonged electrographic and convulsive motor seizures following i.v. infusion of Scr (Figures 6B-6E). By contrast, i.v. infusion of pY816 inhibited electrographic and convulsive motor seizures in a dose-dependent manner (Figures 6B-6E). The maximal inhibition of 90% was obtained with 10 mg/kg of pY816 (Figures 6B-6E).

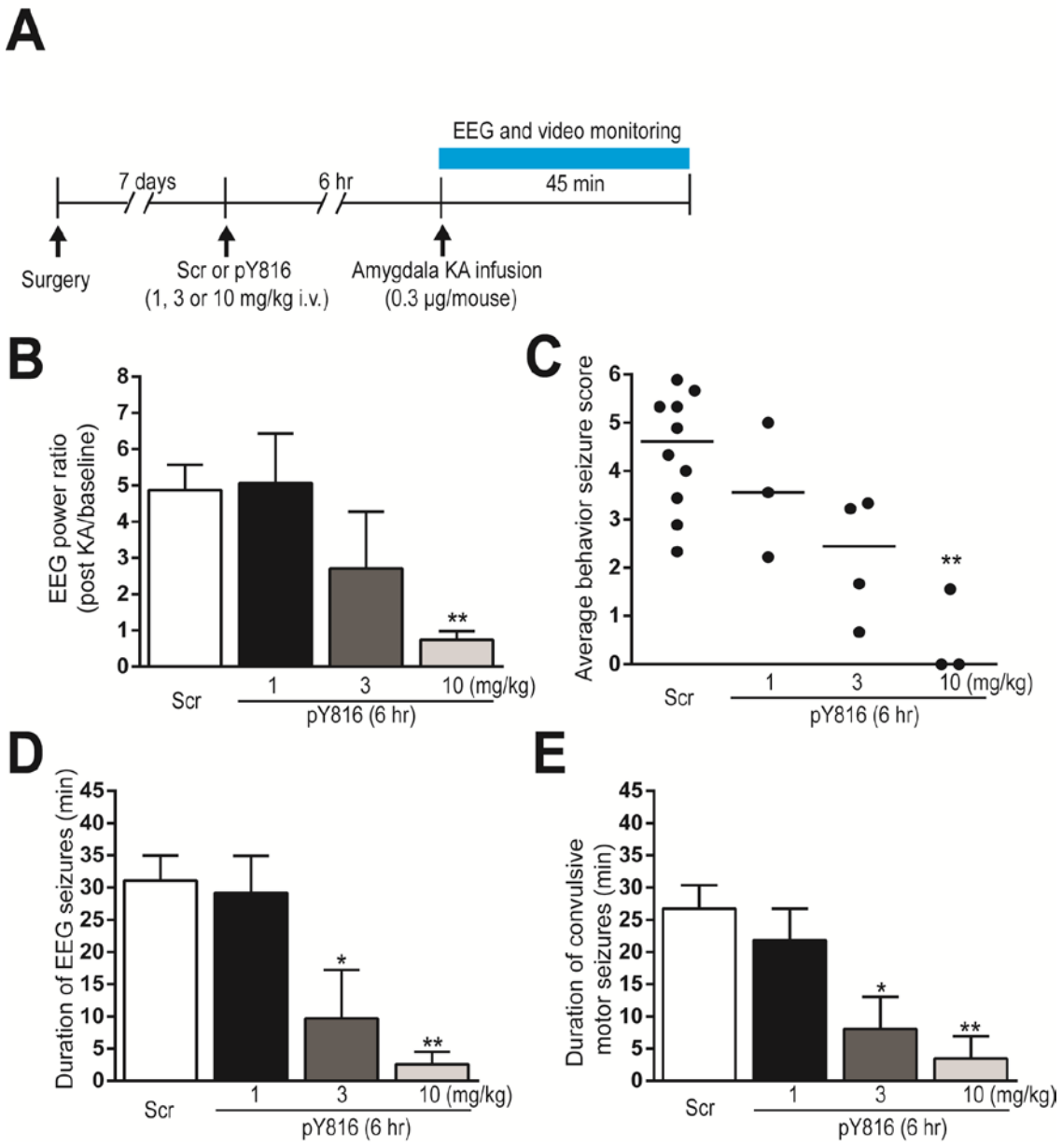

Figure 6 pY816 inhibits chemoconvulsant induced seizures in dose-dependent manner

(A) Schematic of experimental design of systemic administration of pY816 at various doses (n=3-4) prior to induction of seizures by infusion of KA into amygdala. Average EEG power normalized to baseline (B), average behavioral seizure scores (C), duration of

EEG seizures (D) and convulsive motor seizures (E) were analyzed during 45 min following KA infusion. Scr treated controls were included in experiments examining effects of doses (total of 10). Data are presented as mean ± SEM and analyzed using Two-way ANOVA with post hoc Bonferroni's test (B, D and E) or from individual animals as well as median and analyzed using Kruskal-Wallis test followed by Dunn's multiple comparisons test (C); *$p<0.05$ and **$p<0.01$.

3.4.2 pY816 peptide inhibits chemoconvulsant-induced seizures in time dependent manner

Using the dose (10 mg/kg) optimized above, pY816 was injected (i.v.) at various intervals (10, 30 minutes, 3, 6, 24 and 72 hours) prior to KA infusion (Figure 7A). By quantitatively measuring EEG and behavioral seizures, inhibitory effects were evident as early as 10 minutes following i.v. infusion and inhibition of 50-90% persisted for 24 hours, returning to control levels by 72 hours (Figures 7B-7E). Collectively, these results support the conclusion that pY816 inhibits the prolonged seizures induced by KA infusion in a dose- and time-dependent manner.

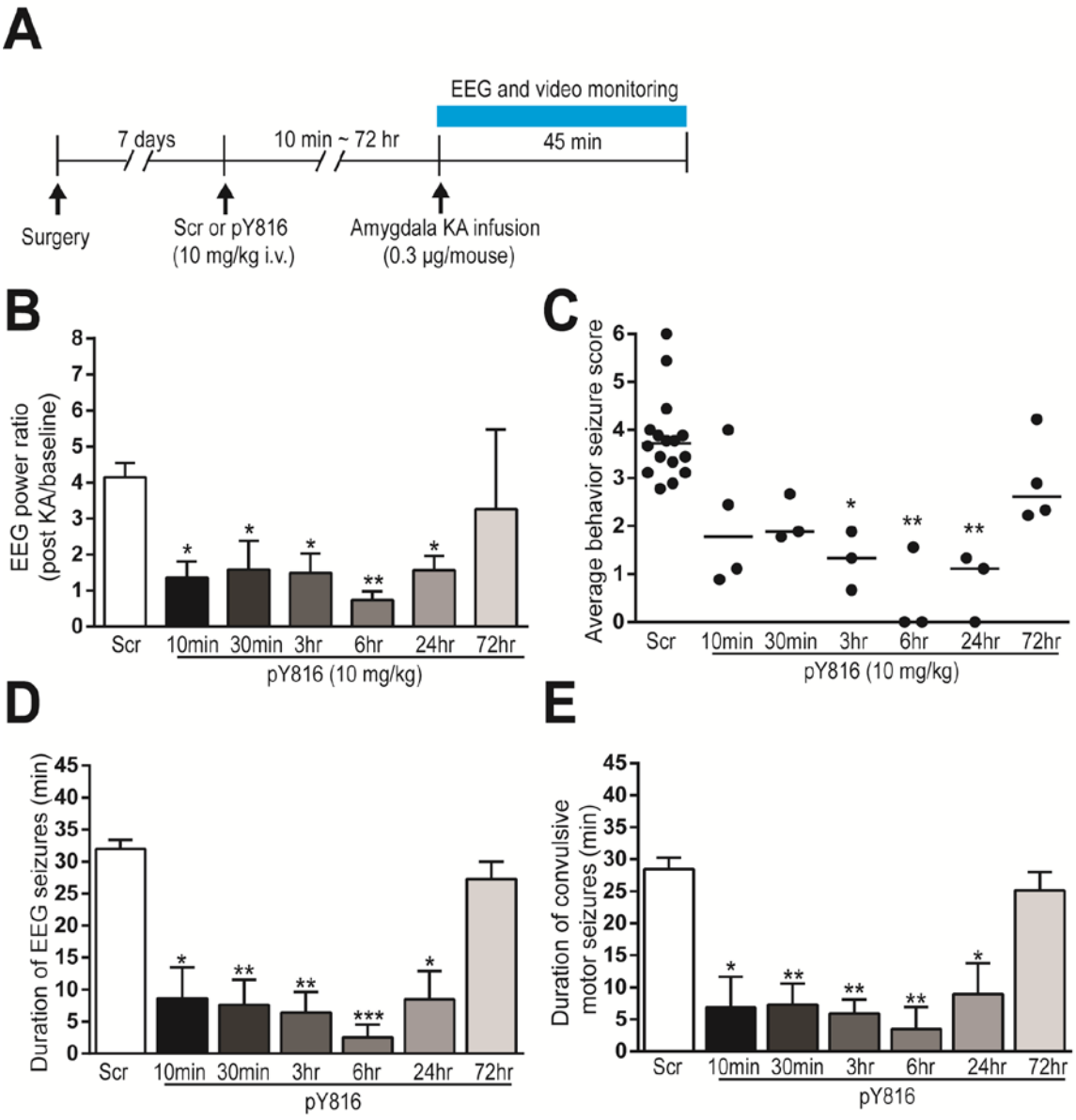

Figure 7 pY816 inhibits chemoconvulsant induced seizures in time-dependent manner

(A) Schematic of experimental design of systemic administration of pY816 at various intervals (n=3-4) prior to induction of seizures by infusion of KA into amygdala. Average EEG power normalized to baseline (B), average behavioral seizure scores (C), duration of

EEG seizures (D) and convulsive motor seizures (E) were analyzed during 45 min following KA infusion. Scr treated controls were included in experiments examining effects of doses (total of 18). Data are presented as mean ± SEM and analyzed using Two-way ANOVA with post hoc Bonferroni's test (B, D and E) or individual animals as well as median and analyzed using Kruskal-Wallis test followed by Dunn's multiple comparisons test (C); *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

3.5 Treatment with pY816 following status epilepticus spares neuroprotective effects of TrkB

Usefulness of a preventive agent in a clinical setting requires it to be effective when administered following the status epilepticus. In the experiments described above, pY816 was administered prior to induction of status epilepticus by microinfusion of KA. The key issue arising is whether administration of pY816 *following* chemoconvulsant-evoked status epilepticus prevents the resulting epilepsy and anxiety-like behavior.

Prior to addressing this issue, we asked whether the neuroprotective effects of TrkB signaling are inhibited by treatment with pY816 administered following status epilepticus. Status epilepticus was induced by microinfusion of KA into the amygdala and terminated after 40 minutes by treatment with diazepam (10 mg/kg, i.p.). At that point, animals received intravenous infusion of either Scr control peptide or pY816 (10 mg/kg) and were euthanized 24 hours later for histological studies (Figure 8A). As observed previously (Figures 1B and

1C), status epilepticus led to destruction of CA3 and CA1 pyramidal cells as detected by FJC staining in sections from animals treated with Scr control peptide (Figure 8B). In contrast to inhibition of TrkB kinase, similar numbers of FJC stained neurons were detected in hippocampal sections from animals treated with pY816 (Figure 8B), demonstrating that pY816 did not interfere with the neuroprotective effects of endogenous TrkB signaling.

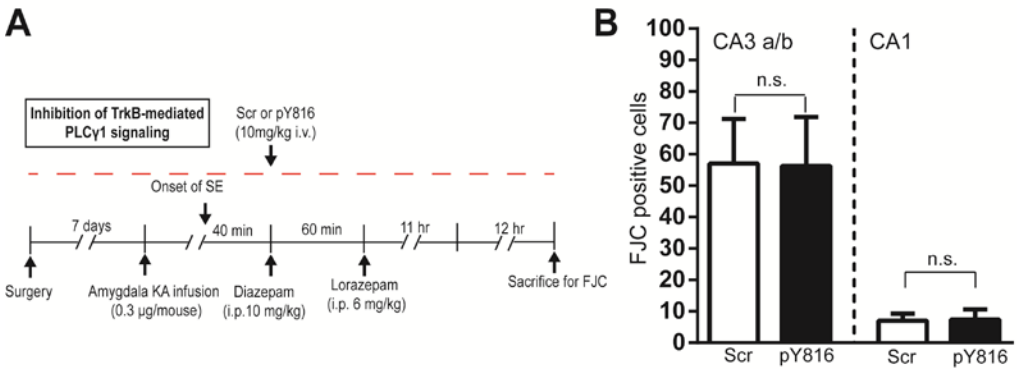

Figure 8 pY816 treatment following status epilepticus spares neuroprotective effects of TrkB kinase

(A) Schematic of experimental design of assessment of neuronal degeneration 24 hr after status epilepticus induced by infusion of KA into amygdala. (B) Counts of FJC positive cells within hippocampal subfield CA3 a/b or CA1 ipsilateral to infusion site of KA in mice treated with either Scr (n=7) or pY816 (n=8). Data are presented as mean ± SEM and analyzed using Two-way ANOVA, n.s. no significant difference.

3.6 Treatment with pY816 following status epilepticus prevents epilepsy and anxiety-like behavior

Transient inhibition of TrkB kinase initiated following status epilepticus prevents epilepsy and anxiety-like behavior induced by status epilepticus (Liu et al., 2013). The experiments above reveal that administration of pY816 prior to the chemoconvulsant inhibited the chemoconvulsant-evoked seizures. Here we asked whether administration of pY816 following chemoconvulsant-evoked status epilepticus prevented the resulting epilepsy and anxiety-like behavior.

3.6.1 Treatment with pY816 following status epilepticus inhibits PLCγ1 activation

To guide design of this experiment, we sought to understand the time course of PLCγ1 activation induced by prolonged seizures, the idea being that a critical period of enhanced activation following prolonged seizures may provide a therapeutic window during which inhibition of TrkB-PLCγ1 signaling may prevent TLE.

3.6.1.1 Activation of PLCγ1 is transiently increased following status epilepticus

To study the time course of PLCγ1 activation following status epilepticus, mice were euthanized immediately, 6, 24, 48 and 72 hours after the completion of status epilepticus. Hippocampus ipsilateral to the infusion site was

homogenized and subject to western blotting. By probing with p-PLCγ1 (pY783) antibody, we found that an episode of prolonged seizures induced a 2-3 fold increase of PLCγ1 activation that was maximal during the first 24 hr and returned to normal by 72 hours (Figures 9A and 9B).

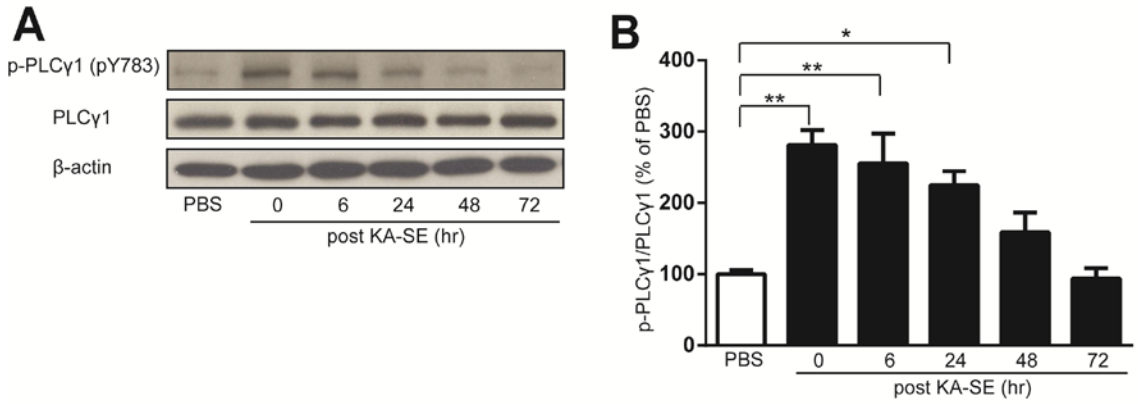

Figure 9 Transient enhancement of PLCγ1 activation following status epilepticus

(A) Representative western blot of hippocampal lysates of mice euthanized at various time points (0, 6, 24, 48 or 72 hr) after the completion of status epilepticus. (B) Quantification of immunoreactivity of p-PLCγ1 (pY783) to PLCγ1 ratio(n=3). Data are presented as mean ± SEM and analyzed using Two-way ANOVA with *post hoc* Bonferroni's test, n=3; *$p<0.05$ and **$p<0.01$.

3.6.1.2 pY816 peptide inhibits status epilepticus-induced PLCγ1 activation

By showing the time course of PLCγ1 activation following status epilepticus, we next asked whether pY816 treatment after the termination of status epilepticus can inhibit this transient enhancement of PLCγ1 activation. To

test this idea, various doses of pY816 (1, 10 and 20 mg/kg) were injected (i.v.) immediately after the termination of status epilepticus, and mice were euthanized 6 hours later. Compared to Scr controls (20 mg/kg), injection of pY816 immediately following the episode of status epilepticus inhibited PLCγ1 activation in a dose-dependent manner, inhibition approximating 75% with doses of 10 and 20 mg/kg (Figures 10A and 10B).

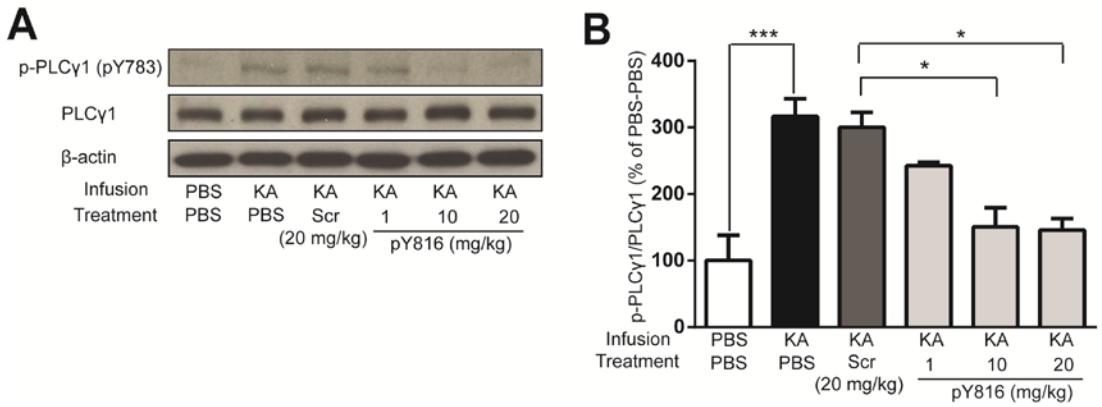

Figure 10 pY816 inhibits PLC1 activation following status epilepticus

(A) Representative western blots of hippocampal lysates of mice treated with PBS, Scr (20 mg/kg) or varying doses of pY816 (1, 10 and 20 mg/kg) immediately after completion of status epilepticus and euthanized 6 hr later. (B) Quantification of immunoreactivity of p-PLCγ1 (pY783) to PLCγ1 ratio is presented in bottom (n=3). Data are presented as mean ± SEM and analyzed using Two-way ANOVA with *post hoc* Bonferroni's test, n=3; *$p<0.05$ and ***$p<0.001$.

3.6.2 Severity of status epilepticus is equivalent prior to treatment of Scr and pY816

Prevention of development of TLE is an unmet clinical need. The above information was used to design an experiment addressing whether treatment with pY816 initiated following status epilepticus could prevent the resulting epilepsy and anxiety-like behavior. We asked whether administering pY816 (10 mg/kg, i.v.) immediately, 24 and 48 hours after diazepam treatment would prevent epilepsy and anxiety-like behavior.

To minimize its effects on KA-induced status epilepticus, we withheld treatment of peptides until diazepam was administered after 40 minutes of status epilepticus. Importantly, behavioral (Figures 11A and 11B) and electrographic (Figures 11C) seizures during status epilepticus prior to treatment with diazepam were similar in the Scr- and pY816-treated mice. Moreover, assessment of electrographic seizure number or duration in hippocampal EEG recordings during the 1 hour interval between diazepam and lorazepam or during the 1 hour after treatment with lorazepam by a blinded observer revealed no significant differences between Scr- and pY816-treated mice (Figures 11E and 11F, respectively). These results of visually inspected EEG were corroborated by quantitative measures of EEG power, which revealed no significant differences between Scr- and pY816-treated mice during the 1 hour intervals after treatment with diazepam or lorazepam (Figure 11D).

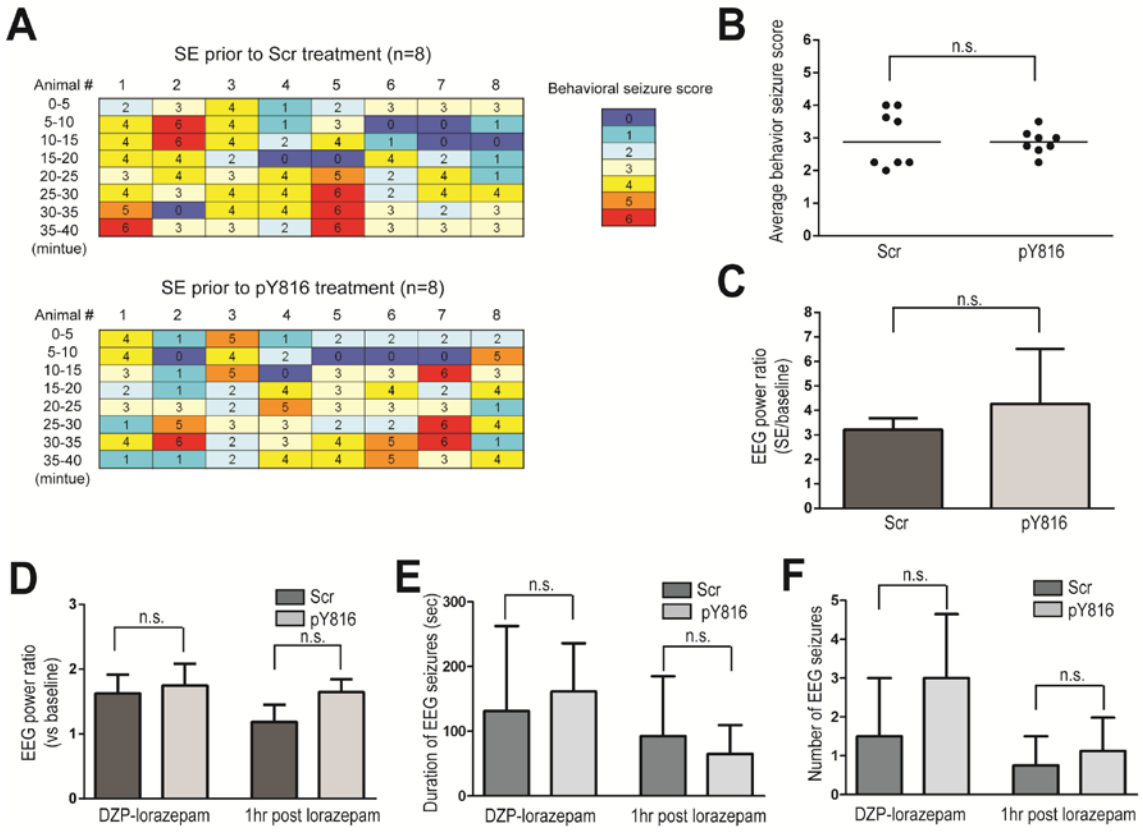

Figure 11 Behavioral and EEG measures reveal similarity of KA-evoked status epilepticus in animals treated with Scr or pY816 after status epilepticus

These data pertain to animals in experiment depicted in Figure 12A in which a total of three doses of either Scr or pY816 was administered following status epilepticus. (A) Heat map presents the maximum behavioral seizure exhibited in 5 min intervals in each mouse prior to treatment with Scr or pY816 peptide. Average behavioral seizure scores (B) and EEG signal energy analyses (C) of status epilepticus revealed no significant differences (n.s.) between animals subsequently treated with Scr or pY816 peptide. Additional measures of EEG power (D) and number (E) and duration (F) of electrographic seizures revealed similar activity during 1 hr between treatment with

diazepam and lorazepam and 1hr post lorazepam; note that in these experiments, either Scr or pY816 peptide was infused immediately following treatment with diazepam. Data are presented as mean ± SEM and analyzed using Student's t-test (C, D, E and F) or individual animals as well as median and analyzed using Mann-Whitney U test (B), n=8.

3.6.3 Treatment with pY816 following status epilepticus prevents development of spontaneous recurrent seizures

The transient enhancement of PLCγ1 activation following status epilepticus provides a therapeutic window during which inhibition of PLCγ1 by systemic administration of pY816 may prevent development of spontaneous seizures. To test this hypothesis, treatment with pY816 (10 mg/kg, i.v.) was initiated following termination of status epilepticus and repeated 24 and 48 hours thereafter. Spontaneous seizures were detected using continuous video-EEG recordings during days 1-14 and days 29-42 after status epilepticus (Figures 12A and 12B) and identified by review of video-EEG files by trained readers blinded to treatment of mice. Preventive effects of pY816 were evident during days 1-14 (Figures 12C and 12D). The latency to the onset of the first spontaneous seizure was delayed in pY816 treated animals compared to controls (Figure 12C, top panels). The initial spontaneous seizure was observed in 5 of 8 control animals within 3 days following prolonged seizures whereas only a single pY816 infused animal exhibited a seizure in this interval. A striking reduction (86%) in the number of spontaneous seizures was observed in pY816 compared to Scr

control animals during the two weeks immediately following status epilepticus (Scr: 18.1±1.7; pY816: 2.4 ± 0.6, $p < 0.001$, Student's t-test) (Figures 12C and 12D). Additional video-EEG recordings were conducted during a two week period (Days 29-42) initiated approximately four weeks after the last dose of pY816. Once again, a marked reduction (90%) in the number of spontaneous seizures was observed in pY816 compared to Scr control animals (Scr: 16.5 ± 3.5; pY816: 1.5 ± 0.4, $p < 0.001$, Student's t-test) (Figures 12C and 12D). Among the eight animals treated with pY816, two exhibited no seizures and the remaining six animals exhibited only 1-3 seizures. By contrast, control animals exhibited 7-36 spontaneous recurrent seizures during this same interval (Figures 12C and 12D).

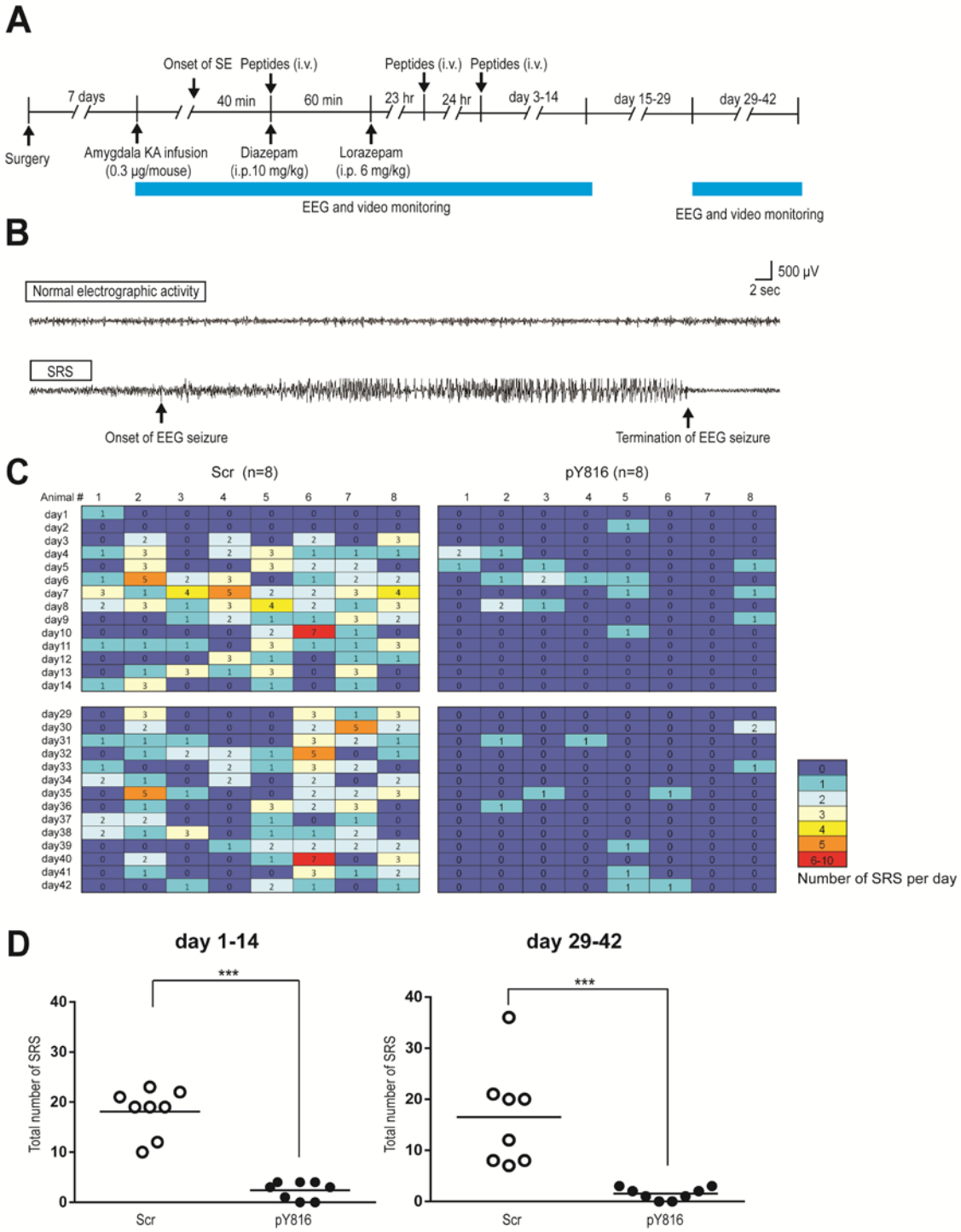

Figure 12 Treatment with pY816 after status epilepticus prevents development of spontaneous recurrent seizures

(A) Schematic of experimental design of 3 days treatment of pY816 initiated after completion of status epilepticus. (B) Representative EEG recording of an electrographic seizure (bottom) and normal activity (top). (C) Heatmap presents number of spontaneous recurrent seizures detected each day during days 1-14 and days 29-42 after status epilepticus; each animal was treated with either Scr or pY816 (10 mg/kg, i.v.) immediately, 24 hr and 48 hr after completion of status epilepticus. (D) Total number of SRSs during days 1-14 or days 29-42 for each animal treated with either Scr or pY816 (n=8). Data are presented from individual animals as well as mean and analyzed using Student's t-test, ***$p<0.001$.

3.6.4 Treatment of pY816 following status epilepticus prevents anxiety-like behavior

Patients with epilepsy commonly exhibit anxiety disorders and anxiety-like behavior has been documented in animal models of TLE (Beyenburg et al., 2005; Groticke et al., 2007). Anxiety-like behavior has been observed months following status epilepticus in the model studied here and was found to be prevented by transient inhibition of TrkB kinase commencing after status epilepticus (Liu et al., 2013). We therefore asked whether this behavioral abnormality induced by status epilepticus can also be prevented by treatment with pY816. To address this question, following completion of video-EEG recording during days 29-42, anxiety-like behavior was assessed using the light-dark emergence test (Bourin and Hascoet, 2003). This test is based on the

innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory nature of rodents to novel environment. Importantly, light-dark test has been validated in rodents using both anxiolytic and anxiogenic drugs (Kilfoil et al., 1989). In comparison to controls (n=7), mice undergoing status epilepticus followed by treatment with Scr exhibited a prolonged latency to enter the lighted compartment (Figure 13A) and spent less time in the lighted compartment (Figure 13B). By comparison to the Scr controls, mice given pY816 for 3 days following status epilepticus exhibited a significantly reduced latency to enter the lighted compartment (Figure 13A) and spent increased time in the lighted compartment (Figure 13B). Similarities in locomotor activity in an open field between two groups undergoing status epilepticus excluded differences in spontaneous activity as a confounding variable in the light-dark emergence results (data not shown). Collectively, these results demonstrate that treatment with pY816 for three days commencing after status epilepticus prevents status epilepticus-induced anxiety-like behavior, providing evidence of anti-anxiogenic effects of this peptide.

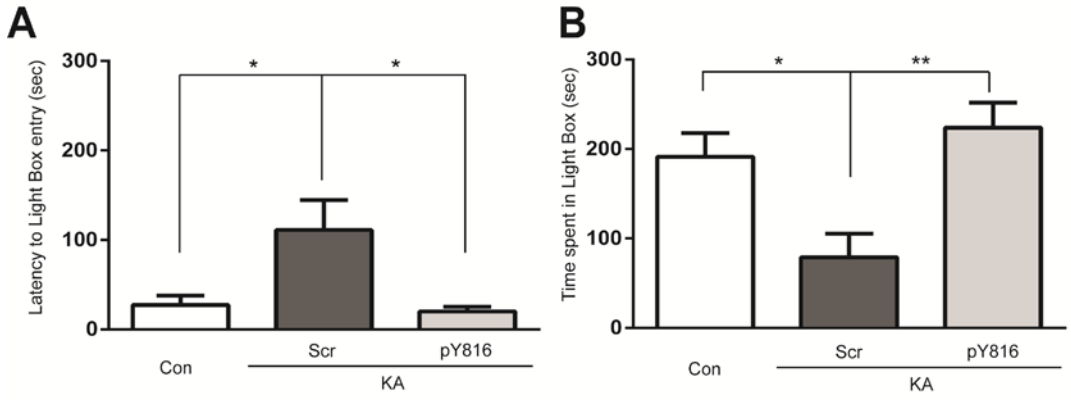

Figure 13 Treatment with pY816 after status epilepticus reverses anxiety-like behavior

Anxiety-like behavior assessed by latency to enter light-compartment (A) and by time spent in lighted compartment (B). Mice undergoing infusion of PBS into amygdala served as controls (Con, n=7). Data are presented as mean ± SEM and analyzed using Two-way ANOVA with *post hoc* Bonferroni's test. *$p<0.05$ and **$p<0.01$.

3.6.5 Treatment of pY816 following status epilepticus attenuates hippocampal sclerosis

Death of hippocampal neurons and reactive gliosis are well recognized neuropathological features of TLE in humans (Mathern et al., 1998) and similar features have been identified in the hippocampus ipsilateral to the KA-infused amygdala 2 weeks following status epilepticus (Mouri et al., 2008). Histological analyses of mice given Scr after status epilepticus and euthanized 10 weeks thereafter revealed ~50% reduction of neurons (NeuN immunoreactive cells) in hippocampus CA3b subfield compared to control animals undergoing PBS

infusion into amygdala (Figures 14A and 14B, rostral, $p<0.01$, middle, $p<0.001$), confirming results of Mouri et al. (2008). Compared to Scr treatment, pY816 significantly reduced the neuron loss by ~30% (Figures 14A and 14B, rostral, $p<0.05$, middle, $p<0.05$). Reactive gliosis evidenced by enlarged GFAP-immunoreactive cells with thickened processes in CA3b of hippocampus were observed following status epilepticus in Scr treated animals (Figure 14A, bottom row), confirming a previous report of Mouri et al. (2008). Importantly, these abnormalities were attenuated by pY816 treatment following status epilepticus (Figure 14A, bottom row).

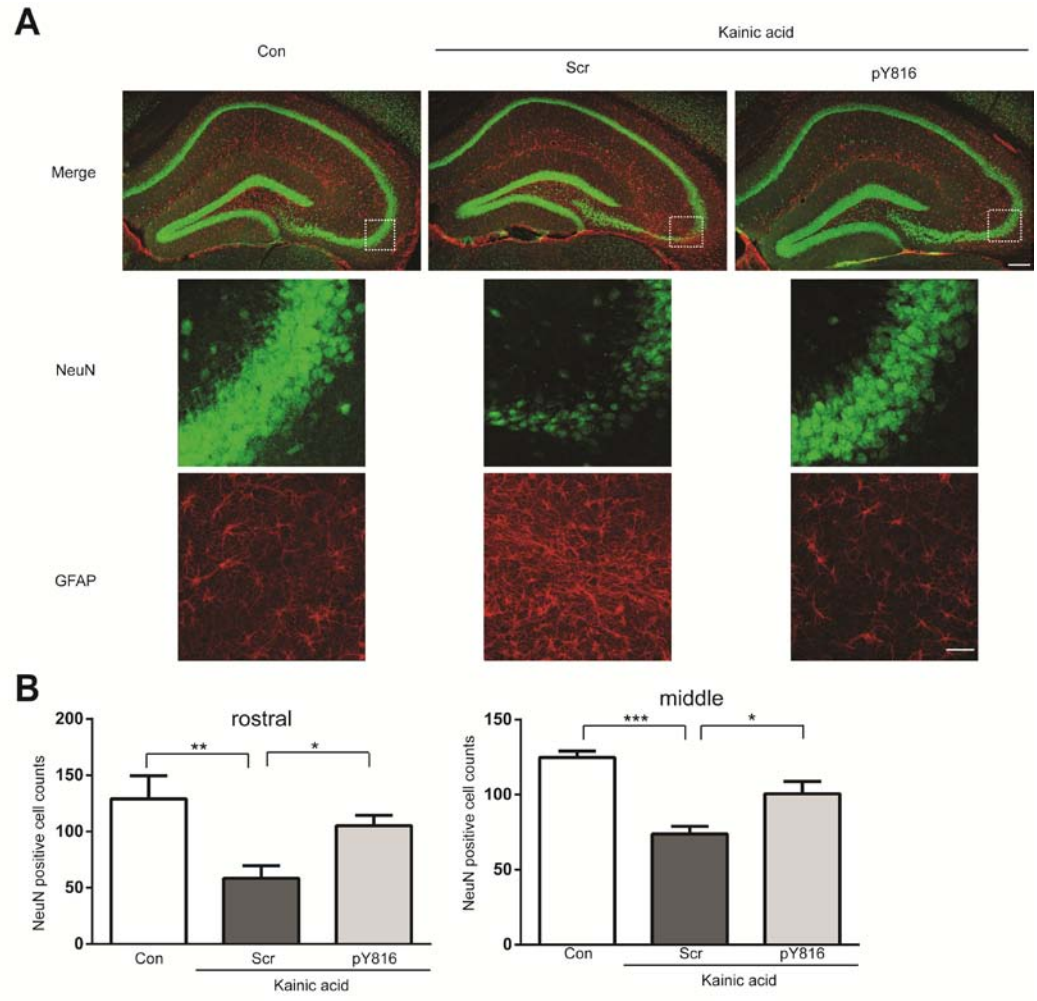

Figure 14 Treatment with pY816 after status epilepticus attenuates hippocampal damage

Representative Immunohistology study was performed on animals euthanized10 weeks after KA-status epilepticus in experiment depicted in Figure 4E. (A) Representative images of immunostaining of NeuN (green) and GFAP (red) in hippocampus ipsilateral to the infusion site in control (Con, PBS infusion), KA-Scr treated, and KA-pY816 treated mice, scale bar = 200 µm. Insets: Loss of NeuN positive cells together with enhanced GFAP immunoreactivity were evident in hippocampus CA3b subfield of KA-

Scr treated mice (middle column). This hippocampal damage was attenuated by pY816 (right column), scale bar = 30 μm. (B) Number of NeuN positive cells in ipsilateral hippocampus CA3b was reduced in mice undergoing KA-status epilepticus and treated with Scr thereafter compared to PBS controls ($p<0.001$). Treatment with pY816 inhibited loss of NeuN positive cells (*$p<0.05$). Data are presented as mean ± SEM and analyzed using Two-way ANOVA with Bonferroni *post hoc* tests, n=4-8.

4 Discussion

A chemical-genetic approach provided proof of concept evidence that inhibition of TrkB kinase suppresses both seizures (Liu et al., 2014) and seizure-induced development of TLE and anxiety-like behavior (Liu et al., 2013), establishing a strong rationale for developing a therapeutic. The objective of the present work was to seek an inhibitor of TrkB signaling for prevention of epilepsy caused by status epilepticus. We used genetically modified mice and a novel biologic together with biochemical and electrophysiological methods in studies of an animal model. Five principal findings emerged. 1) Global inhibition of TrkB signaling by targeting its kinase activity exacerbated neuronal death induced by status epilepticus, this untoward consequence leading us to seek a TrkB downstream signaling pathway promoting epilepsy and anxiety-like disorder. 2) Marked inhibition of status epilepticus was evident in mice with mutations that uncoupled TrkB from its signaling effector, PLCγ1, or of PLCγ1 itself. 3) pY816, a novel peptide designed to uncouple TrkB from PLCγ1, selectively inhibited TrkB-mediated activation of PLCγ1 both *in vitro* and *in vivo.* 4) Treatment with pY816 prior to administration of a chemoconvulsant inhibited chemoconvulsant-induced status epilepticus in a dose- and time-dependent manner *in vivo*. 5) Treatment with pY816 initiated after status epilepticus prevented anxiety-like disorder and inhibited epilepsy detected 1-2 months afterwards yet preserved the neuroprotective effects of TrkB signaling. In sum, these findings reveal PLCγ1 to

be the dominant signaling effector by which status epilepticus-induced TrkB activation promotes its epileptogenenic and anxiogenic consequences and identify a therapeutic with promise for advancement to the clinic.

To facilitate identification of a molecular target for a therapeutic, we sought to elucidate the signaling pathway through which the status epilepticus-induced activation of TrkB induced diverse pathologies. RTKs like TrkB convey signals by coupling binding of a ligand with their ectodomain to intracellular signaling cascades, a coupling accomplished by recruiting enzymes and/or adaptor proteins to motifs containing tyrosine residues that undergo autophosphorylation upon receptor activation. The robust inhibition of KA-evoked status epilepticus in the *trkB*$^{PLC/PLC}$ mutant mice demonstrate that genetically uncoupling TrkB from adaptor proteins and enzymes that bind the motif containing Y816 produces powerful anticonvulsant effects. That anticonvulsant effects similar to *trkB*$^{PLC/PLC}$ mice were observed in mice heterozygous for PLCγ1 implicates PLCγ1 as the dominant effector of the anticonvulsant effects of the *trkB*$^{PLC/PLC}$ mutation. This provides a strong rationale for development of therapeutic agents that disrupt the interaction of TrkB with PLCγ1. We focused on a motif within the carboxyl terminus of TrkB shown to be critical for its binding to PLCγ1 (Obermeier et al., 1993) and designed a peptide, pY816, to disrupt this interaction. The mechanism by which pY816 produces its desired consequences presumably involves its binding a sequence within the SH2 domains of PLCγ1, the net result being reduced binding of TrkB to PLCγ1; the ability of systemically

administered pY816 to reduce the co-immunoprecipitation of TrkB and PLCγ1 is consistent with this mechanism. One advantage of selectively uncoupling binding of TrkB with PLCγ1 is that other TrkB signaling pathways would be left intact (Lai et al., 2012; Minichiello et al., 2002), an expectation supported by pY816 inhibition of pY783 PLCγ1 immunoreactivity without affecting p-Erk or p-Akt immunoreactivity, either *in vitro* or *in vivo* (Figures 4 and 5). Notably, phosphorylation of Y515 of TrkB and the subsequent binding of the adaptor protein, Shc, results in neuroprotective effects in cultured sympathetic neurons (Atwal et al., 2000), raising the possibility that TrkB-mediated activation of the Shc signaling pathway may promote neuroprotection following status epilepticus *in vivo*. That said, the extent to which TrkB signaling through Shc or other pathways contributed to the neuroprotective effects evident in pY816-treated animals is uncertain.

A strategy of uncoupling a RTK from an effector protein similar to the present work was implemented in *in vitro* studies of the MET receptor tyrosine kinase (Bardelli et al., 1998). Identification of residues critical for the transforming potential of another RTK, a mutant MET receptor, led to design and electroporation of phosphopeptides that uncoupled signal transducers from MET; this resulted in inhibition of sprouting and migration of transformed cells in an *in vitro* assay (Bardelli et al., 1998). To the best of our knowledge, the strategy of treating a disease model *in vivo* by uncoupling a RTK from a signal transducer has not been successfully implemented. One reason may lie in limited

bioavailability of a peptide inhibitor. That said, fusion of the tat sequence to the phosphorylated TrkB peptide here facilitated its traversing cell membranes, accessing a target within the cytosol of neurons behind the blood-brain barrier. A second reason may lie in the many autophosphorylation sites within the cytoplasmic domain of an RTK such as the epidermal growth factor receptor (EGFR), each of which can recruit different SH2 and phosphotyrosine-binding domain (PTB)-containing proteins (Lemmon and Schlessinger, 2010), perhaps precluding activation of a single signaling pathway by a single small peptide. By contrast, the few identified autophosphorylation sites within the cytoplasmic domain of TrkB may reduce redundancy of signaling and render this strategy feasible.

The pY816 peptide may provide a therapeutic for prevention of TLE. Currently there is no preventive or disease modifying therapy available for TLE. Clinical observations together with studies of animal models support the conclusion that an episode of prolonged seizures contributes to the emergence of severe TLE years later (Annegers et al., 1987; French et al., 1993; Tsai et al., 2009). The seizure free latent period provides a therapeutic window for intervention aimed at preventing development of TLE (Loscher et al., 2013). In the model studied here, the transiently enhanced activation of PLCγ induced by status epilepticus occurred during the seizure free latent period (i.e. 3-5 days) following status epilepticus, leading us to examine the preventive effects of three days of treatment with pY816. That commencing treatment with pY816 following

40 minutes of status epilepticus was effective together with the short latency of access to emergency medical care of many patients with status epilepticus (Alldredge et al., 2001) enhances the feasibility of this approach to preventive therapy. That just 3 days of treatment was sufficient to exert these beneficial effects many weeks later could minimize potential unwanted consequences of long-term exposure to preventive therapy.

pY816 exhibits powerful antiepileptogenic and anti-anxiogenic effects when treatment is initiated immediately after completion of 40 minutes status epilepticus induced by KA amygdala infusion in mice. This finding provides a proof of concept that activation of TrkB-PLCγ1 signaling is required for the development of chronic recurrent seizures and anxiety-like behavior after status epilepticus, thus providing a strong rationale for developing this approach for clinical use. That said, it is important to further examine how long after the onset or completion of status epilepticus the treatment with pY816 can be initiated and retain its effectiveness. This information is critical to design of a clinical trial aimed at assessing the efficacy of pY816 for prevention of epilepsy following status epilepticus.

The antiepileptogenic and anti-anxiogenic effects of pY816 implicate TrkB-mediated activation of PLCγ1 as a critical molecular mechanism by which status epilepticus transforms the brain from normal to epileptic. What might be the cellular consequence of TrkB-mediated activation of PLCγ1 that promotes epileptogenesis? Modification of synaptic efficacy is a leading possibility

because immunohistochemical studies revealed status epilepticus-induced activation of TrkB to be localized to two distinct synapses within the hippocampus in the animal model studied here, the presynaptic boutons of mossy fiber synapses with CA3 pyramidal cells and spines of apical dendrites of CA1 pyramidal cells upon which Shaffer collateral axons of CA3 pyramidal cells synapse (Helgager et al., 2012). Long term potentiation (LTP) of excitatory synapses between principal neurons of the limbic system has long been advanced as an attractive mechanism underlying TLE (Scharfman, 1997; Sutula and Steward, 1987; Takei et al., 1997). Importantly, activation of TrkB is required for induction of presynaptic LTP of the mossy fiber-CA3 synapse (Pan et al., 2011) and for some forms of LTP of the Shaffer collateral-CA1 synapse (Kang et al., 1997; Minichiello et al., 2002; Xu et al., 2000a). Interestingly, status epilepticus appears to induce LTP of the excitatory mossy fiber-CA3 synapse, as evidenced by occlusion of LTP in hippocampal slices studied *ex vivo* following KA-induced status epilepticus (Goussakov et al., 2000). It seems plausible that status epilepticus-induced TrkB activation enhances the efficacy of either or both of these excitatory synapses, thereby facilitating the initiation and/or propagation of seizure activity through these circuits.

One puzzling facet of the present work is that treatment with pY816 prior to administration of the chemoconvulsant powerfully suppresses the chemoconvulsant induced status epilepticus yet treatment with pY816 after 40 minutes of status epilepticus does not affect the status epilepticus as measured

by quantitative assessments of behavior and EEG power. Reduction or loss of efficacy of an anti-epileptic agent as a function of the animal's experience of repeated seizures is a well recognized occurrence. Reduction or loss of anti-seizure effectiveness of benzodiazepines occurs following prolonged seizures, the mechanism thought to be due at least in part to internalization of synaptic $GABA_A$ receptors (Naylor et al., 2005). The mechanism by which 40 minutes of status epilepticus renders pY816 ineffective in inhibiting seizures remains to be elucidated.

The beneficial effects of pY816 in status epilepticus-induced abnormalities in the light-dark test implicate TrkB-mediated activation of PLCγ1 signaling in the pathogenesis of anxiety-like behavior. Anxiety disorders are observed in patients with epilepsy, most commonly those with TLE in particular (Beyenburg et al., 2005). Optogenetic activation of neuronal cell bodies within the basolateral nucleus of the amygdala induce anxiety-like behaviors in rodents, behaviors mediated in part by amygdala-induced activation of ventral hippocampal circuitry (Felix-Ortiz et al., 2013; Tye et al., 2011). It seems plausible that excessive activity within neurons of the basolateral nucleus of amygdala may produce lasting modifications of synapses within these circuits, the behavioral consequence of which is an anxiety disorder. Here the source of the abnormal activity could be the status epilepticus itself and/or the recurrent seizures observed for many weeks thereafter whereas clinically the source of such abnormal activity might be an emotionally traumatic experience (Helfer et

al., 1996; Wang et al., 2011). Demonstration of abnormal activity within the amygdala in functional magnetic resonance imaging (fMRI) studies of patients with anxiety disorders such as post traumatic stress disorder is consistent with this idea (Rauch et al., 2000). If this idea is correct, the effectiveness of just three days of treatment with pY816 initiated after status epilepticus advances this peptide as a potential candidate for prevention of anxiety disorders arising after traumatic experiences in humans.

5 Concluding Remarks

5.1 This study reveals a novel therapeutic strategy for disorders of excessive activation of RTKs

RTK signaling plays critical roles in cell function including proliferation, differentiation, survival, and migration (Blume-Jensen and Hunter, 2001; Lemmon and Schlessinger, 2010). High expression of or gain-of-function mutations in multiple RTKs including TrkB, KIT, EGFR, human epidermal growth factor receptor 2 (HER2) and fibroblast growth factor receptor (FGFR) family is thought to contribute a diversity of human cancers. In addition to human cancers, excessive activation of RTK signaling causes a variety of non-cancer disorders (Lemmon and Schlessinger, 2010). Aberrant activation of FGFRs causes craniosynostosis syndromes and a variety of severe skeletal dysplasias (Eswarakumar et al., 2005). Known mutations for craniosynostosis syndromes, such as Apert, Crouzon, Jackson-Weiss, and some cases of Pfeiffer syndrome are located within the gene encoding FGFR 2, whereas mutations for achondroplasia, hypochondroplasia, and thanatophoric dysplasia types I and II are located within the gene encoding FGFR 3 (Cohen, 1997). Gain-of-function mutation of Tie2 (angiopoietin receptor) has been linked to venous malformation, the most common developmental form of vascular dysmorphogenesis. Two unrelated families with venous malformation exhibited a single gain-of-function mutation in Tie2 in all affected individuals (Vikkula et al., 1996).

The critical role of excessive activation of RTK in human diseases led to development of drugs that inhibit RTK signaling, mainly small molecules targeting the cytoplasmic kinase domain or monoclonal antibodies targeting the ectodomain for cancer therapy. Each approach has been successful yet fraught with frequent emergence of drug resistance and side effects. Drug resistance to either small molecules or monoclonal antibodies often arises in cancer patients, due in part to selection driving emergence of drug resistant mutant receptors. Undesired side effects also result from global inhibition of an RTK by a small molecule or an antibody. These limitations encountered led us to consider an alternative strategy. In this study, we identified the immediate signaling effector mediating the untoward consequences of excessive RTK signaling and exploited this insight to develop a drug that uncoupled the RTK from its disease related signaling effector.

5.2 This study reveals a potential therapeutic strategy for disorders of excessive activation of TrkB

This study demonstrates that PLCγ1 is the dominant downstream signaling of TrkB, through which activation of TrkB promotes status epilepticus induced TLE. In addition to TLE induced by status epilepticus, elevated BDNF levels or TrkB activation have also been documented in a variety of neurological disorders, including catamenial epilepsy, posttraumatic epilepsy, neuropathic pain, autism spectrum disorders and posttraumatic stress disorder (PTSD),

suggesting that excessive activation of TrkB signaling plays a critical role in these pathologies.

5.2.1 Catamenial and posttraumatic epilepsy

Catamenial epilepsy refers to seizures that increase in frequency or severity at specific stages of the menstrual cycle. High levels of estrogen, the key regulator of menstrual cycle is thought to contribute to periovulatory seizures (Backstrom, 1976; Herzog et al., 1997). Estrogen is able to regulate BDNF levels in the brain (Scharfman and MacLusky, 2006; Solum and Handa, 2002). Sohrabji and colleagues showed that ovariectomy of adult female rats reduced BDNF levels in the brain, and estrogen treatment reversed the effect (Sohrabji et al., 1995). Treatment of female rats with estrogen led to epileptiform responses of CA3 pyramidal cells to mossy fiber activation, an effect blocked by K2521, a Trk antagonist (Scharfman et al., 2003). These findings suggest that TrkB signaling may contribute to catamenial epilepsy. Brain trauma can also cause development of epilepsy probably due to hyperexcitability resulted from disruption of synaptic connections. Using Schaffer collateral lesions in organotypic hippocampal slice cultures, Gills et al., demonstrated that blocking BDNF using TrkB-Fc reduced axonal sprouting by 50% and attenuates injury-induced hyperexcitability of hippocampal CA3 neurons (Gill et al., 2013). Axonal sprouting has been found in patients with post-traumatic epilepsy (McKinney et al., 1997). Therefore, these findings suggest that blocking the BDNF-TrkB signaling cascade shortly after

injury may be a potential therapeutic target for the treatment of post-traumatic epilepsy.

5.2.2 Neuropathic pain

Neuropathic pain characterized by continuous burning condition, such as hyperalgesia and allodynia is commonly associated with a primary lesion or dysfunction to the peripheral nervous system, or secondarily to injury of the CNS. Multiple lines of evidences suggest that BDNF and presumably TrkB are involved in the central sensitization and synaptic plasticity in the spinal cord. Spinal cord BDNF has been shown to contribute to the development and maintenance of neuropathic pain by activation of NMDA receptors (Geng et al., 2010). In the spinal nerve ligation model of neuropathic pain, BDNF expression is significantly increased in the spinal dorsal horn in spinal nerve ligation rats (Li et al., 2006). Systemic administration of a selective TrkB kinase inhibitor (1NMPP1 in *TrkB*^{F616A} mutant mouse) and intrathecal injection of BDNF antibody or K252a, an inhibitor of neurotrophin receptors kinase activity are able to prevent the development of tissue- or nerve injury-induced heat and the mechanical hypersensitivity in mice (Wang et al., 2009; Yajima et al., 2002), indicating that TrkB signaling is not only an important contributor to the induction of heat and mechanical hypersensitivity produced by tissue or nerve injury but also to the development and persistence of neuropathic pain. The mechanism underlying the role of BDNF-TrkB signaling pathway in neuropathic pain is still largely

unknown. One possible mechanism is through an increased expression of thermal transient receptor potential channels (Ciobanu et al., 2009).

5.2.3 Autism spectrum disorders

Autism is a developmental disorder characterized by marked deficits in communication and social interaction skills, abnormal behavior and language impairment. Converging lines of evidence support the involvement of BDNF in autism spectrum disorders. Higher BDNF level was found in archived samples of neonatal blood obtained from children with autistic spectrum disorders compared with normal controls (Nelson et al., 2001), suggesting that early BDNF-TrkB hyperactivity may play an etiological role in autism early in life. This finding is further supported by a pilot study demonstrating a higher concentration of serum BDNF in autistic and mental retardation patients compared with healthy controls (Miyazaki et al., 2004). Enhanced BDNF level was also found directly in the basal forebrain of autistic adults compared with normal adults (Perry et al., 2001). BDNF-TrkB hyperactivity may also explain the association of increased prevalence of seizures in autism, and similar behaviors observed in autism and fragile X syndrome (Halepoto et al., 2014; Tsai, 2005).

5.2.4 PTSD

Increased BDNF-TrkB signaling has also been implicated in PTSD, with significant increase in levels of peripheral BDNF of individuals with PTSD (Hauck

et al., 2010; Matsuoka et al., 2013). The levels of BDNF as well as TrkB along with epigenetic regulation of the BDNF gene in response to fear conditioning was also enhanced in rats subjected to single prolonged stress, an animal model of PTSD (Takei et al., 2011). Increased BDNF expression was observed in insular cortex during conditioned taste aversion reconsolidation (Wang et al., 2012) and intracranial injection of recombinant BDNF facilitated memory consolidation and reconsolidation in a passive avoidance learning paradigm (Samartgis et al., 2012). These findings suggest that activation of BDNF-TrkB signaling may contribute fear memory reconsolidation, a process in which consolidated memory can re-enter states of transient instability following reactivation. Memory reconsolidation plays an important role in the development of clinical symptoms, such as reexperiencing of trauma, in PTSD. Therefore, blocking reconsolidation by targeting BDNF-TrkB pathway offers the therapeutic possibility of weakening traumatic memories in PTSD (Debiec, 2012; Pitman, 2011). However, the role of BDNF-TrkB signaling in PTSD is still controversial. A decrease in signaling in the BDNF-TrkB pathway is widely accepted as a contributing factor in PTSD (Green et al., 2013). Systemic administration of TrkB receptor modulators have resulted in changes in phenotypes related to human PTSD, such as fear acquisition and extinction (Andero et al., 2011) and anxiety related phenotypes (Cazorla et al., 2010).

In sum, the fact that elevated BDNF level or TrkB activation has been evidenced in a variety of neurological disorders, together with the critical roles

that PLCγ1 plays in TrkB-mediated synaptic plasticity raises the possibility that the strategy of inhibiting TrkB-PLCγ1 signaling (e.g. using pY816) may be effective in many other types of epilepsies, neuropathic pain, autism and PTSD as well.

5.2.5 Down-regulation of BDNF-TrkB and neurological disorders

In addition to PTSD, multiple lines of evidence have implicated down-regulation of BDNF-TrkB signaling in the etiology, pathology, or therapeutic pharmacological responses of diverse neurodegenerative disorders, including Alzheimer's disease (Lee et al., 2005), Parkinson's disease (Howells et al., 2000; Scalzo et al., 2010) and Huntington's disease (Zuccato and Cattaneo, 2007), as well as a variety of common neuropsychiatric disorders, such as schizophrenia, bipolar disorder and major depressive disorders (Reinhart et al., 2015). Therefore, therapies aimed at inhibiting BDNF-TrkB signaling may have potential adverse impacts on both healthy and unhealthy brain. This study reveals an alternative strategy, namely blocking a specific disease related downstream pathway of TrkB for a short period of time, thereby providing the opportunity to achieve the therapeutic goal while minimizing possible adverse effects.

5.3 This study reveals a novel molecular mechanism of epileptogenesis

Important role of BDNF-TrkB signaling pathway in epileptogenesis has been well established yet the downstream signaling by which over-activation of TrkB promotes TLE is still unclear. In this study, treatment with pY816, which is able to uncouple TrkB-PLCγ1 binding and inhibit PLCγ1 *in vivo*, after completion of status epilepticus prevents development of spontaneous seizures, revealing a novel molecular mechanism of epileptogenesis. Importantly, to rigorously interpret the anti-epileptogenic effect of pY816, we 1) initiated the treatment with pY816 after completion of status epilepticus; 2) analyzed both EEG and behavioral seizures during status epilepticus and demonstrated that the brain insult (status epilepticus) was equivalent between treatment groups 3) resumed video-EEG monitoring approximately a month after termination of treatment and found frequency of seizures was significantly reduced in mice treated with pY816 compared to controls. In sum, proper experimental design enables us to claim (TrkB-mediated) PLCγ1 signaling as a novel molecular mechanism of epileptogenesis.

5.4 This study provides a novel compound for treatment of TLE

Transient treatment with pY816 prevented development of TLE and comorbid anxiety following status epilepticus, thereby rendering pY816 a

promising candidate for a clinical trial. The approval of 24 peptides for diverse clinical indications by the FDA in the recent past (2001-2013) (Kaspar and Reichert, 2013) attests to the feasibility of advancing peptides to the clinic. That said, we are unaware of any peptide approved by the FDA for a CNS indication. This may be due in part to limited bioavailability. To address this limitation, phosphorylated TrkB peptide was fused to an HIV Tat sequence which presumably facilitated its traversing cell membranes, accessing a target within the cytosol of neurons behind the blood-brain barrier (Kumar et al., 2006). HIV-Tat fused peptides have been successfully applied pre-clinically in multiple neurological disorders (Aarts et al., 2002; Liu et al., 2008; Tu et al., 2010; Yao et al., 2011). A HIV-Tat conjugated peptide (Tat-NR2B9c), which inhibited postsynaptic density-95 protein and exhibited neuroprotective effects in both rodent and primate models of stroke (Aarts et al., 2002; Cook et al., 2012), was able to sustain fewer ischemic infarcts compared placebo in human ruptured or unruptured intracranial aneurysm (Hill et al., 2012). Successful preclinical and clinical application of HIV-Tat fused peptide strengthens the feasibility of this strategy. The fact that the sequence of pY816 is identical in mouse and human implies that pY816 itself may prove useful in prevention of TLE and anxiety-like behaviors in humans.

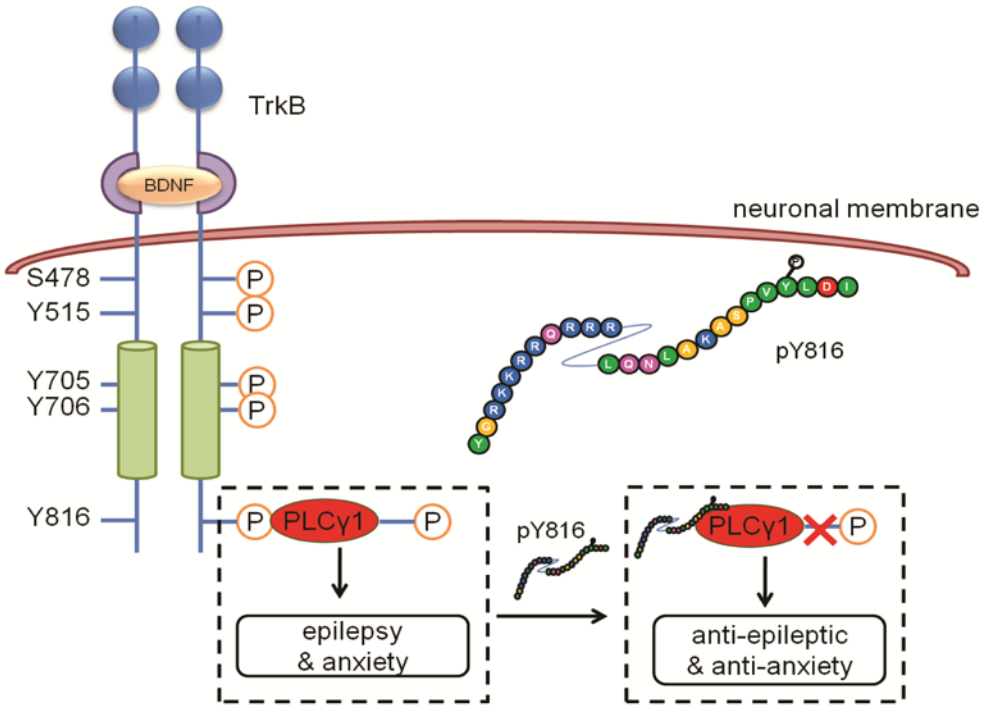

Figure 15 Schematic illustrating the main hypotheses

Schematic illustrating the main hypothesis. Among many autophosphorylation sites within the cytoplasmic domain of TrkB, phosphorylation of tyrosine residue 816 promotes epilepsy and anxiety-like behavior. pY816 peptide (YGRKKRRQRRR-LQNLAKASPVpYLDI) inhibits TrkB-mediated PLCγ1 activation and prevents both epilepsy and anxiety-like behavior caused by status epilepticus while sparing beneficial effects of TrkB signalling.

In sum, the present work establishes the therapeutic efficacy *in vivo* of a strategy in which a signal transducer (PLCγ1) is selectively uncoupled from a RTK (TrkB). Given the diversity of cancers and other diseases promoted by

excessive activation of RTK signaling, this strategy may be applicable to some RTKs in addition to TrkB (Figure 15).

6 Future Directions

6.1 Hypothesis

The experimental evidence described above supports the conclusions: 1) Treatment with pY816 prior to administration of chemoconvulsant exhibits powerful anticonvulsant effects; 2) Treatment with pY816 immediately after completion of status epilepticus, which lasts for only 3 days, prevents epileptogenesis. These results provide a strong rationale to develop pY816 as a therapeutic approach of TLE in human. That said, the difficulty of prevention clinical trial of TLE in human and the urgent need of drug which is able to control seizure and modify TLE progression in an already epileptic patients require initiation of treatment after diagnosis of first spontaneous seizures. The facts that enhancement of BDNF mRNA and protein levels were found in hippocampus removed from epileptic patients (Murray et al., 2000; Takahashi et al., 1999) and the idea that isolated seizure is sufficient to elicit TrkB activation (Binder et al., 1999a) led us hypothesize that treatment of pY816 after establishment of spontaneous seizures in a mouse model of TLE 1) suppresses seizures during treatment, exhibiting anticonvulsant effect; 2) halts seizures development after completion of treatment, exhibiting disease modifying effect. To exclude the possibility that inhibition of seizure after termination of treatment results from inhibition of preceding seizures, carbamazepine, a conventional AED, albeit

powerful anticonvulsant effect, failed to suppress seizure after drug withdraw (Ali et al., 2012) will be tested in the same mouse model of TLE.

6.2 Experimental design

To test this hypothesis, we will take advantage of mouse model of TLE in which status epilepticus is induced by intra-amygdala KA microinfusion. Mice will be subjected to continuous video-EEG recording for 6 weeks after induction of status epilepticus for monitoring of spontaneous seizures. After 2 weeks baseline recording of spontaneous seizures following status epilepticus (Liu et al., 2013; Mouri et al., 2008), either Scr or pY816 will be administered (20mg/kg, i.p. twice per day) for 2 weeks to assess the anticonvulsant effect of pY816. The mice will be recorded for additional 2 weeks after completion of treatment to assess the disease modifying effect of pY816. Using same experiment paradigm, a separate cohort of mice will be treated with carbamazepine. Spontaneous seizures will be monitored for 2 weeks before, during and after carbamazepine treatment.

6.3 Pitfalls and limitation

Given the powerful anticonvulsant effect of pY816, it is possible that suppression of seizure expression after withdrawal of pY816, if any, results from anticonvulsant effect of residue pY816, which is not eliminated from the brain. To address this concern, we set to monitor mice for additional 2 weeks to allow clearance of drug from the brain. Importantly, treatment with pY816 72 hours

(10mg/kg, i.v.) prior to infusion of KA failed to inhibit seizures (Figure 7) suggests that pY816 is cleared from brain within 3 days following a single injection (10mg/kg, i.v.). However, the pharmacokinetics of pY816 in brain following repeated injection (20mg/kg, i.p. twice per day for 2 weeks) need to be further addressed to tackle this question.

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Biography

I was born in Hohhot, China on November 27th, 1983. I received bachelor's degree in 2006 (Ocean University of China, CHINA) major in biology and minor in Japanese and master's degree in pharmacology (Chinese medicine) in 2009 (Beijing Normal University, CHINA). From 2007 to 2008, I visited Japan as an exchange research student and conducted research with Dr. Takuma Kazuhiro and Dr. Yukio Yoneda in Kanazawa University in Japan. Accomplishment of this study led to a first author publication on Journal of Neuroscience Research titled "Possible Protection by Notoginsenoside R1 against Glutamate Neurotoxicity Mediated by N-methyl-D-aspartate Receptors Composed of an NR1/NR2B Subunit Assembly". I was then admitted to Pharmacology PhD program at Duke University and joined Dr. James O. McNamara's lab in 2009. My research interest focuses on TrkB signaling and its role in epileptogenesis. Collaborated with Dr. Gumei Liu, we demonstrated that transient inhibition of TrkB kinase can prevent recurrent seizures in an animal model of temporal lobe epilepsy. Given the importance of this study, this finding was published in journal of Neuron. My thesis project focuses on designing a membrane-permeable small peptide that can interrupt a critical signaling pathway involving in epileptogenesis. A U.S. patent (U.S. #8507438) was approved and a manuscript was under review based on the findings of this study. I received 2013 Robert J. Fitzgerald Award from Department of Pharmacology and Cancer Biology, Duke University and 2014 Chinese Government Award for Outstanding

Self-financed Students Abroad. While enrolled at Duke, In addition to many to be submitted or under review manuscripts, I has published one first author paper, and coauthored another.

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