

Disease Modification of Epilepsy by Disruption of TrkB Signaling

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Dissertation submitted in partial fulfillment of  
the requirements for the degree of Doctor of Philosophy in the Department of  
Neurobiology in the Graduate School  
of Duke University

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ABSTRACT

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## Abstract

Epilepsy is the most common acquired neurological disorder and is characterized by spontaneous, recurrent seizures. Of the various forms of epilepsy, Temporal Lobe Epilepsy (TLE) has received intense clinical and research interest. Current therapeutic options for TLE are anti-convulsive and purely symptomatic. Improved treatments are needed that either (1) prevent epilepsy development or (2) ameliorate existing disease. TLE is commonly induced by a preceding episode of prolonged seizure activity (*status epilepticus*, or SE). Our lab previously demonstrated that disruption of TrkB-PLC $\gamma$ 1 signaling after SE prevented development of epilepsy, identifying a critical molecular signaling pathway in SE-induced TLE.

SE-induced TLE is analogous to associative memory formation in that both involve activity-determined plasticity. Memories can be “erased” by re-exposure to the inciting stimulus and inhibition of molecular mechanisms necessary for initial learning. Given the proposed parallels between epilepsy and memory as well as the central role of TrkB-PLC $\gamma$ 1 signaling in the development of TLE, I sought to test the effect of disrupting TrkB-PLC $\gamma$ 1 signaling following a seizure using the kindling model. I demonstrate that disruption of TrkB-PLC $\gamma$ 1 after an evoked seizure, but not TrkB-PLC $\gamma$ 1 inhibition in the absence of a preceding seizure, prevents progression in subsequent seizure severity and, in a subset of animals, results in partial remission.

In specimens from patients who underwent surgical resection for medically refractory TLE there is a striking increase in expression of the ligand for TrkB, BDNF. In a second study, I demonstrate that this increase, as well as an increase in TrkB-PLC $\gamma$ 1 signaling, is also seen in an animal model of SE-induced epilepsy exhibiting spontaneous seizures. I then demonstrate that administration of a peptide uncoupling TrkB from PLC $\gamma$ 1 induced a remission in seizure frequency that persists after treatment termination, while producing minimal overt unwanted consequences.

These studies elucidate a signaling pathway (TrkB-PLC $\gamma$ 1) underlying epilepsy progression and persistence, connect TLE to other disorder of pathologic plasticity like PTSD and neuropathic pain, and open the door to a novel therapeutic approach for treating patients with existing epilepsy.

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# **1. Introduction**

## ***1.1 Clinical Background on Temporal Lobe Epilepsy***

Epilepsy is the most common acquired chronic neurological disorder. This disease is characterized by spontaneous seizures, which are episodes of uncontrolled, synchronous neuronal firing within the central nervous system producing behavioral changes (Fisher et al 2014). Epileptic seizures are subdivided based on the origin of seizure onset, with focal seizures originating in a localized brain region and generalized seizures generated over a widespread brain area (Fisher et al. 2017). Over 10% of the population will experience a seizure in their lives, and 65 million individuals are diagnosed with epilepsy (Ngugi et al. 2010); in the United States alone the care of epileptic patients exceeds \$15 billion annually (Sander 2003). Current treatments are purely symptomatic, reducing seizure occurrence but not inducing a remission or inhibiting the progression of the disease (Pitkanen 2010). This therapeutic shortcoming in part contributes to the significantly increased mortality in patients with epilepsy (Thurman et al. 2017), highlighting the need for increased study of this disease.

Of the various forms of epilepsy, Temporal Lobe Epilepsy (TLE) has received intense clinical and research interest. TLE is the single most common form of focal epilepsy, affecting approximately 35% of epileptic patients (Tellez-Zenteno and Hernandez-Ronquillo 2012). For patients with TLE, frequent seizures hinder ability to gain employment, operate a motor vehicle, and participate fully in society. In addition,

TLE is commonly associated with psychiatric co-morbidities such as depression and anxiety disorder (Torta and Keller 1999), which patients report as having a greater impact on quality of life than seizure frequency alone (Choi et al. 2014, Boylan et al. 2004). Current therapy aims to augment inhibitory neurotransmission, suppress excessive excitatory neurotransmission, or reduce intrinsic neuronal excitability. These purely symptomatic anti-seizure medications are limited by their propensity to produce adverse effects—over 80% of patients on anti-seizure treatment will experience some form of these effects (Marson et al. 2005). Furthermore, despite the presence of over 20 FDA-approved drugs for the treatment of epilepsy, an estimated 30% of TLE patients remain refractory to medical therapy and exhibit seizures despite therapeutic blood levels of medication (Pitkanen et al. 2000). Improved treatments are needed that are disease modifying. A disease modifying treatment is one that either (1) prevents epileptogenesis, the process by which the brain is transformed from to epileptic or (2) ameliorates existing disease (i.e. is administered in a symptomatic patient and induces a reduction in seizure frequency).

Clinical observation of patients with TLE frequently identifies a preceding brain insult (e.g. infection, stroke, traumatic brain injury) in the months to year preceding onset of seizures. In particular, retrospective clinical studies have demonstrated a correlation between severe TLE and a previous episode of prolonged seizure activity, termed status epilepticus (SE), in otherwise healthy individuals (Cendes et al. 2003,

Falconer et al. 1964). In the laboratory, SE is sufficient to produce TLE in various animal models (Mouri et al 2008). Once established, TLE is frequently progressive with worsening of clinical course, co-morbidities, and structural lesions (Elwes et al. 1988, Kwan and Brodie 2000). This progression was first observed by the astute 19<sup>th</sup> century British neurologist Sir William Gowers, who noted that “seizures beget seizures” (Gowers 1901).

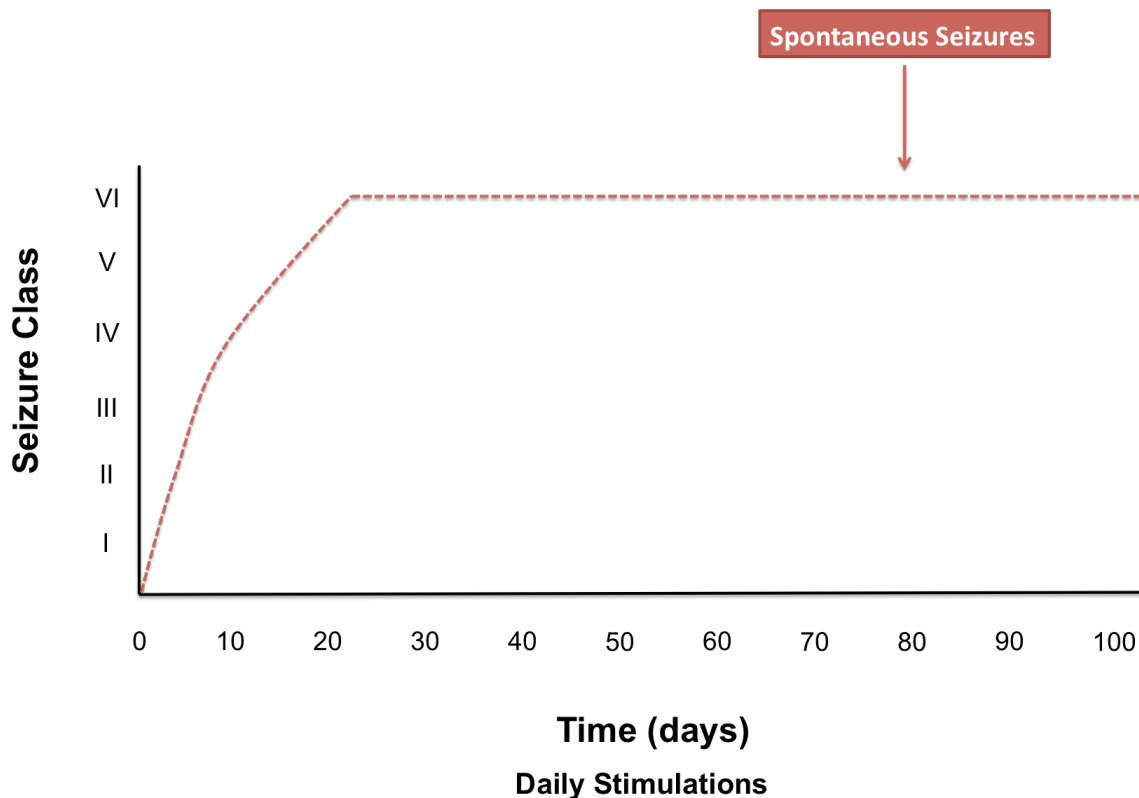
## ***1.2 Animal Models of TLE***

### **1.2.1 Kindling Model**

The kindling phenomenon, first described by Graham Goddard in the 1960s (Goddard 1967, Goddard et al. 1969), is an extension of Gowers’s observation regarding the progressive course of TLE and is a commonly used model of prolonged epileptogenesis. In this model, a stimulating electrode is implanted into various brain regions and repetitive stimulations are given to elicit focal electrographic seizures. The evoked electrographic seizures are initially brief, localized to the region of stimulation, and unaccompanied by detectable behavioral abnormalities. Repeated administration of this low intensity stimulus results in evoked seizures of increasing duration and more widespread propagation, the behavioral correlates of which are similar to temporal lobe and secondarily generalized tonic-clonic seizures of humans. This enhanced sensitivity to an initially subconvulsive stimulus persists for the life of the animal. This model has been extensively studied and characterized (McNamara 1999), which allows for

classification of behavioral seizure severity on a simple 0-6 scale (Borges et al., 2003; Racine, 1972): 0, normal activity; 1, arrest and rigid posture; 2, head nodding; 3, unilateral forelimb clonus; 4, rearing with bilateral forelimbs clonus; 5, rearing and falling; 6, tonic-clonic seizures with violent running and/or jumping.

The definition of an animal being “kindled” varies but one common definition is the occurrence of three consecutive class IV seizures with duration lasting greater than ten seconds. At this stage, the enhanced sensitivity to stimulation notwithstanding, animals do not exhibit spontaneous seizures. In contrast to the 10-15 stimulation evoked electrographic seizures required to induce “kindling”, evoking an additional 80 or so electrographic seizures causes the emergence of seizures occurring in the absence of a stimulation (i.e. spontaneous recurrent seizures or epilepsy—Figure 1). Persistent evoked seizures in these animals will ultimately lead to spontaneous recurrent seizures (SRS), hippocampal gliosis, and neuronal death (Cavazos et al. 1991, Lothman and Williamson 1993, Cavazos et al. 1994). A key advantage of this model is that seizure induction is controlled by the investigator.



**Figure 1: Pathologic activity induced epileptogenesis in the kindling model. In the kindling model, repeated evoked seizures lead to an increase in seizure class for subsequent evoked seizures. This persists for the life of the animal. However, spontaneous seizures in this model only appear after more than 80 stimulations.**

### **1.2.2 Intra-Amygdala Kainic Acid-Induced Status Epilepticus**

A shortcoming of the kindling model is the large number of stimulations required for the animal to exhibit SRS. By contrast, studies in animal models have demonstrated that a single episode of SE is sufficient to induce the occurrence of both SRS as well as histologic hallmarks of TLE like gliosis and neuronal death (Curia et al. 2008). Common methods of inducing SE include systemic administration of chemoconvulsants such as the cholinergic agonist pilocarpine or the kainite receptor

agonist kainic acid (Moriomoto et al. 2004). However, in contrast the unilateral histological damage seen in human TLE patients (Lewis et al. 2014), systemic administration of these agents frequently produces symmetric, bilateral damage (Sloviter et al. 2007). Furthermore, these models are complicated by mortality exceeding 30% (Helgager et al. 2013). To overcome these limitations, a common approach is focal injection of kainic acid (KA) into limbic structures such as hippocampus or amygdala. The intra-amygdala KA (IAK) model of SE was originally described in cats (Tanaka et al. 1985) and later adapted to rodents (Tanaka et al. 1988, Li et al. 2008, Mouri et al. 2008). In this model, KA is infused into the right amygdala with EEG recordings obtained from the left hippocampus. SE ensues 2-15 minutes after infusion, and is terminated by administration of the benzodiazepine diazepam 40 min later, with a dose of lorazepam given 60 min after diazepam (Mouri et al. 2008, Liu et al. 2013, Gu et al. 2015). SRS emerge beginning 3-5 days after SE. This model results in unilateral hippocampal damage (Mouri et al. 2008) with seizures observed first in the CA3 region of hippocampus ipsilateral to the site of infusion (Li et al. 2008); mortality is less than 5% (Liu et al. 2013). In stark contrast to the kindling model, SRS emerge on a much faster timescale. However, a commonality in both models is that epileptogenesis and emergence of SRS are induced by electrographic seizure activity.

### **1.3 Cellular and Molecular Mechanisms of Epileptogenesis**

Use of both models has allowed for study of the cellular and molecular mechanisms underlying epileptogenesis, which would help facilitate identification of targets for either preventative or remission-inducing therapy. This section will focus on mechanisms of epilepsy development; a subsequent section will discuss studies on epilepsy persistence and targets for remission.

A prominent feature in both resected specimens from patients with medically-refractory TLE (Klein et al. 2017) and animal models of SE-induced TLE (Li et al. 2008, Liu et al. 2013, Gu et al. 2015) is reactive astrogliosis, a situation where astrocytes increase in number, change their molecular expression, and alter their morphology to become enlarged while sending out multiple projections (Fawcett and Asher 1999). In clinical TLE, this is commonly found along the blood-brain barrier as well as in both neocortex and subcortical white matter and is thought to contribute to the MRI abnormalities in these patients (Garbelli et al. 2012). Studies in animal models have demonstrated that this pathology occurs in the days following induction of SE (Guo et al. 2017, Pernot et al. 2015) and persists when animals exhibit SRS (Liu et al. 2013, Gu et al. 2015, Sierra et al. 2015). The consequences of this cellular change on neural function is not completely understood, though changes in ionic gradients, metabolism of neurotransmitters, and expression of receptors are thought to play deleterious consequences (Sofroniew 2009). Understanding the cause and consequences of this process may lead to advances in

treatment options given the finding in genetically-modified mice that induction of progressive astrogliosis is sufficient to cause hyperexcitability and SRS (Robel et al. 2005).

In both SE and the kindling model, structural neuronal plasticity is thought to play a pivotal role in the disease process. One example found in both animal models (Okazaki et al. 1995, Danzer et al. 2010, Jinde et al. 2012, Singh et al. 2013), as well as in clinical TLE (Pitkanen et al. 2000) is mossy fiber sprouting. Here, the mossy fiber output from the dentate gyrus produces additional axon collaterals that frequently form synapses back on dentate granule cells, creating a recurrent excitatory loop. Other examples of structural changes occurring in TLE include the presence of ectopic granule cells (aberrantly located newborn neurons), increase in dendritic spines (Singh et al. 2013), and death of inhibitory interneurons (Sloviter 1987, De Lanerolle et al. 1989).

A central role for inflammation in epileptogenesis is well established and may serve as a target for prevention of SRS development. A broad array of inflammatory mediators, including cytokines, chemokines, and receptors are upregulated during the epileptogenic process. For example, expression of the cytokine HMGB1 is increased in both animal models and specimens from TLE patients (Maroso et al. 2010). Injection of a monoclonal antibody targeting this protein prevented the progression of seizure severity in the kindling model and reduced the frequency of seizures during treatment, though it had no effect on the number of SRS after treatment termination compared to control animals (Zhao et al. 2017).

Adenosine kinase represents another potential target for prevention of SRS development. Adenosine is an endogenous inhibitory neuromodulator whose levels are regulated via phosphorylation by adenosine kinase; inhibition of adenosine kinase increases levels of adenosine (Fedele et al. 2004). Adenosine kinase levels are known to be upregulated in both animal models of SE-induced TLE and human specimens (Li et al. 2008, Aronica et al. 2011). Brain specific upregulation of adenosine kinase results in hippocampal hyperexcitability and ultimately generation of spontaneous recurrent seizures (Li et al. 2008). Notably, implantation of adenosine kinase KO ES cell-derived neural progenitor grafts reduced SRS and astrogliosis in a model of SE-induced TLE (Li et al. 2008). In addition to its role in epileptogenesis, adenosine plays a critical role in survival from the epileptogenic insult, since upregulation of adenosine kinase results in uniform lethality after SE (Li et al. 2008) and knockout of the adenosine receptor results in lethal SE after experimental traumatic brain injury (Kochanek et al. 2006).

mTOR, a protein kinase with a variety of biologic functions, also plays a critical role in epileptogenesis. The role of mTOR signaling in genetic epilepsy syndromes has been long studied given the upregulation of this signaling cascade in the neurocutaneous disorder tuberous sclerosis (Curatolo and Moavero 2012). mTOR signaling plays a role in acquired epilepsies like TLE as well. Excessive activation of mTOR signaling complexes has been observed in hippocampus and temporal cortex of TLE patients refractory to medical therapy (Talos et al. 2018). Blockade of mTOR signaling by the FDA-approved

inhibitor rapamycin ameliorates histologic damage and cognitive co-morbidities characteristic of TLE, though seizure frequency was not reduced (Keng et al. 2013, Brewster et al. 2013).

#### ***1.4 BDNF-TrkB-PLC $\gamma$ 1 Signaling is a Key Molecular Mechanism of Epileptogenesis***

BDNF is a trophic factor released from neurons in an activity dependent manner (Hedrick et al. 2016, Harward et al. 2016) where it binds to the receptor tyrosine kinase TrkB, resulting in receptor dimerization and autophosphorylation of various serines and tyrosines along the intercellular domain. Phosphorylated residues serve as docking sites for adaptor proteins, subsequently activating a complex series of signaling cascades (McNamara et al. 2006). Two canonical pathways are activated by phosphorylation of TrkB Y515 and Y816. Phosphorylation of Y515 leads to recruitment and phosphorylation of adaptor proteins in the Ras/MAP kinase signaling cascade, resulting in neuronal differentiation, growth, and survival (McNamara et al. 2006). Phosphorylation of Y816 leads to docking and phosphorylation of the enzyme PLC $\gamma$ 1, which hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) to generate inositol- 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). IP<sub>3</sub> promotes the release of calcium from intracellular stores while DAG stimulates isoforms of protein kinase C, one consequence of which is gene transcription (Minichiello et al. 2002).

Several converging lines of evidence suggest a critical role for TrkB signaling in epileptogenesis. Biochemical and immunohistochemical studies have demonstrated

increases in BDNF, as well as levels of a surrogate marker of TrkB activation (phosphorylated TrkB) following SE (Isackson et al. 1991, Binder et al. 1999). Intraventricular infusion of TrkB “receptor bodies” (antibodies which sequester BDNF) impaired the development of TLE (Binder et al. 1999). In addition, knockout of TrkB in synapsin-expressing neurons eliminated the development of TLE in the kindling model (He et al. 2004).

These findings support a role of TrkB in epileptogenesis but do not directly address whether SE-induced TrkB activation is required for SE-induced TLE – answering this question requires the ability to selectively and transiently inhibit TrkB kinase after SE. To that end, a chemical-genetic strategy was utilized with a transgenic mouse (termed *TrkB<sup>F616A</sup> mouse*) containing a point mutation within the ATP binding pocket of TrkB, rendering this mouse uniquely susceptible to TrkB kinase inhibition by 1NMPP1, a blood-brain-barrier permeable small molecule derivative of the kinase inhibitor PP1 (Chen et al. 2005). Importantly, 1NMPP1 does not have any detectable effect in WT mice, and there are no detectable differences in TrkB kinase activity in the *TrkB<sup>F616A</sup>* compared to WT mice in the absence of this compound (Liu et al. 2013, Chen et al. 2005). This approach bypasses the off-target effects of available TrkB kinase inhibitors while permitting the temporal precision of pharmacological inhibition. Using this mouse in the IAK model, it was found that transient inhibition of TrkB kinase initiated following SE prevented development of TLE and co-morbid anxiety-like behavior (Liu

et al. 2013). The implications of this finding include: (1) it is indeed possible to intervene transiently following an insult and prevent epilepsy; and (2) TrkB is a potential therapeutic target to prevent epileptogenesis. To determine the downstream pathway by which TrkB mediates the development of epilepsy, genetic studies first demonstrated impairment of epileptogenesis in the kindling model in mice with point mutations of Y816 preventing the phosphorylation of this residue (TrkB<sup>Y816F</sup>), as well as in mice heterozygous for PLC $\gamma$ 1. This motivated the development of a membrane-permeable 3kDa peptide comprising HIV-1 Tat protein transduction domain and the 14aa sequence of TrkB required for binding of PLC $\gamma$ 1 (termed “pY816”). This peptide was shown to uncouple TrkB from PLC $\gamma$ 1 *in vivo* (Gu et al. 2015). Administration of pY816 immediately following SE prevented the development of SRS and psychiatric comorbidities (anxiety-like behavior) without exacerbating SE-induced neuronal death (Gu et al. 2015).

### ***1.5 Cellular & Circuit Mechanisms of Epileptogenesis: TrkB-Mediated Synaptic Plasticity at Excitatory Synapses***

The cellular and circuit consequences of enhanced TrkB activation that contribute to SE-induced TLE are beginning to be identified. One appealing hypothesis is that SE-induced TrkB activation promotes functional plasticity in hippocampal circuits.

Plasticity is defined as the CNS response to stimuli, and functional plasticity is focused on changes in the strength of synapses, the site of contact between individual neurons.

In the 1960s, studies in hippocampal slices demonstrated that high-frequency

stimulation of the perforant path (the primary input into hippocampus, forming synapses onto dentate granule cells) resulted in a robust increase in dentate granule cell responses to subsequent stimulation (Lomo 1966); this was subsequently termed “Long-Term Potentiation” or LTP (Douglas and Goddard 1975). Since its discovery, LTP has been hypothesized to be the cellular mechanism of learning and memory (Lynch 2004) as well as a cellular mechanism of epileptogenesis (Jarero-Basulto et al. 2018). Importantly, TrkB Y816 signaling plays a critical role in LTP at excitatory hippocampal synapses (Minichiello et al. 2002), leading to the hypothesis that TrkB-induced LTP of hippocampal excitatory synapses drives epileptogenesis following SE.

Evidence in support of this hypothesis includes prominent SE-induced TrkB activation in hippocampal mossy fiber giant boutons, an excitatory pre-synaptic terminal that synapses onto an excitatory post-synaptic neuron – CA3 pyramidal cells (Helgager et al. 2013). Because of the known role of TrkB-PLCg1 signaling in potentiating this excitatory synapse in slice preparations (Musumeci et al. 2009, Schildt et al. 2013, Kang and Schuman 1995, Stoop and Poo 1996), SE-induced activation of TrkB may serve to potentiate this synapse and thereby enhance excitatory transmission through the tri-synaptic hippocampal circuit. Such excessive excitatory transmission could result in epileptiform activity. Additional evidence includes the finding that prior induction of LTP at the perforant path input into the hippocampus accelerates kindling development (Sutula and Steward 1987). Furthermore, in a rat model of SE, LTP at the

MF-CA3 synapse is occluded (Goussakov et al. 2000) suggesting that potentiation has already occurred at this synapse as a consequence of prolonged seizure activity.

### **1.6 Cellular & Circuit Mechanisms of Epileptogenesis: TrkB-Mediated Modulation of Inhibitory Neurotransmission**

The effect of TrkB on development of SRS may also be through alterations in inhibitory neurotransmission. Immunohistochemistry studies following SE have shown an increase in BDNF levels at axon terminals of dentate granule cells synapsing onto inhibitory interneurons (Danzer and McNamara 2004). Furthermore, *in vitro* studies suggest that TrkB negatively modulates inhibitory transmission since BDNF heterozygote mice have reduced frequency of mIPSCs in dentate granule cells (Olofsdotter et al. 2000), a finding confirmed using the BDNF scavenger TrkB-Ig. Furthermore, BDNF reducing evoked IPSPs in the CA1 region in a dose dependent manner (Tanaka et al. 1997). These findings raise the possibility that TrkB activation in inhibitory neurons may reduce synaptic inhibition and promote SE-induced TLE.

One way TrkB-PLCg1 signaling may modulate inhibitory transmission is via downregulation of the chloride transporter KCC2 (River et al. 2004). Reduced expression of KCC2, seen under physiologic conditions in the immature brain, results in decreased extracellular chloride and loss of GABA-mediated inhibition (Ben-Ari et al. 1989). This change in neuronal intrinsic properties might lead to the reduction in synaptic inhibition and promote the generation of SRS. Importantly, studies in surgically-resected tissue from TLE patients found a 30% reduction in the number of cells expressing KCC2

mRNA (Huberfeld et al. 2007), suggesting that this is a plausible mechanism by which seizures originate.

## ***1.6 Mechanisms of Epilepsy Persistence***

A striking feature of epilepsy is that this disease is chronic, lasting for the lifetime of the patient. Insight into the mechanisms of epilepsy persistence may help identify therapeutic targets that can result in disease remission. Identified mechanisms of epilepsy persistence include the serine/threonine kinase mTOR, chronic inflammation, and aberrant granule cell integration. Inhibition of mTOR using rapamycin resulted in a reduction in seizure frequency when administered in epileptic animals following both SE-induced epileptogenesis (Zeng et al. 2009), trauma-induced epilepsy (Guo et al. 2013), and in the genetic epilepsy syndrome tuberous sclerosis (Zeng et al. 2008), though only in trauma-induced epilepsy was treatment transiently administered, hindering interpretation regarding whether the effect was curative or simply anti-convulsive. With regards to chronic neuro-inflammation, antagonism of the innate immune receptors IL-1R1 and TLR4 using a cocktail of inhibitors in the IAK also induced a partial reduction in seizure frequency (Iori et al. 2017). Finally, a pair of studies examined the effect of ablating newborn dentate granule cells following SE using selective expression of DTr in these cells, testing whether aberrant integration of these newly-generated neurons contributes to the expression of SRS. Ablation of peri-SE generated granule cells reduced

the frequency of SRS and prevented disease progression (Hosford et al. 2016, Hosford et al. 2017).

### **1.7 Parallels Between Epileptogenesis and Associative Learning**

Associative learning, one example of which is classical conditioning, involves associating a response with a previously neutral stimulus (Pavlov and Anrep 1928). In rodents this is commonly modeled with classical fear conditioning (CFC). In this model, a mouse is placed in a novel context and presented with an auditory tone. The final seconds of tone presentation coincides with a mild foot-shock. Subsequent exposure of the mouse to the context or presentation of the tone produces a robust freezing response by the mouse, which can be quantified and serves as a surrogate marker of defensive fear behavior (Morrison and Ressler 2014, Maren 2001). The circuitry involved in this process has overlap to those generating seizure activity in TLE, and includes both hippocampus and amygdala (Phillips and LeDoux 1992).

Like epileptogenesis, LTP is thought to play a critical role as the cellular mechanism of associative memory. Electrophysiological recordings of auditory pathways from MGN thalamus to the amygdala show increased synaptic efficacy in mice following CFC compared to naïve controls (McKernan and Shinnick-Gallagher 1997). Infusion of NMDAR antagonists, which are known to prevent induction of forms of amygdala LTP *in vitro* (Chapman et al. 2003) impairs acquisition of fear conditioning memory (Fanselow and Kim 1994). Using modern optogenetic techniques to specifically

activate auditory inputs from MGN thalamus into amygdala, it was recently demonstrated that depression (LTD) of neural pathways potentiated after CFC suppressed freezing behavior and this was rescued by subsequent restoration of LTP (Nabavi et al. 2014).

### ***1.8 Processes in Associative Learning: Consolidation & Re-Consolidation***

Formation of associative memories involves a two-step process. Short-term memory, which occurs on the scale of minutes, is mediated by an early phase of LTP (E-LTP) that is independent of protein synthesis and instead involves post-translational modification (e.g. phosphorylation) of existing proteins (Mansuy et al. 1992). Long term memory, whose cellular correlate is the late phase of LTP (L-LTP), occurs following a process termed “consolidation.” Conceptually, consolidation involves the stabilization of the memory via LTP of subsets of neurons distributed among various nodes throughout the brain (Rajasethupathy et al. 2015, Rothschild et al. 2017, Kitamura et al. 2017). Mechanistically, both L-LTP (Huang et al. 1994, Nguyen et al. 1994, Huang et al. 2000) and memory consolidation require protein synthesis (Schafe et al. 1999, Schafe and LeDoux 2000). More precise mechanisms of memory consolidation continue to be studied (Johansen et al. 2011) but a critical role for TrkB signaling has been demonstrated using both genetic and pharmacologic approaches (Musumeci et al. 2009, Ou and Gean 2006, Liu et al. 2004, Ou et al. 2010, Choi et al. 2012, Andero et al. 2007).

Recall of a memory can occur several months after consolidation has occurred (De-Monte et al. 2015). Importantly, following recall, memories are rendered “labile” and erasable (Misnin 1968) and an additional stage of protein synthesis is required for preservation of the memory (Nader et al. 2000, Alberini 2005). This process is termed “reconsolidation” (Alberini 2005), and when protein synthesis following the recall is inhibited, the memory erasure is termed “reconsolidation inhibition.” In contrast to extinction learning, in which repeated presentation of the tone or exposure to the context reduces subsequent freezing behavior, reconsolidation inhibition is not a form of new learning because of key behavioral differences in the two processes (Duvarci and Nader 2004). Instead, reconsolidation inhibition is thought to involve LTD of synapses previously potentiated following learning (Ryan et al. 2015). The molecular mechanisms of reconsolidation inhibition-- upstream of protein synthesis-- are just beginning to be elucidated. Roles for mTOR signaling (Blundell et al. 2008), PKA activity (Kida et al. 2002), and  $\beta$ -adrenergic signaling (Przybylski et al. 1999) have been identified. In contrast to consolidation, a specific role for TrkB signaling in reconsolidation has not been examined.

Of note, reconsolidation inhibition occurs in other animal models besides CFC. In a model of neuropathic pain involving intraplantar injection of capsaicin, subsequent mechanical hyperalgesia could be reversed by a combination of capsaicin re-injection and intrathecal anisomycin but not administration of either compound alone.

Furthermore, the LTP of spinal cord dorsal horn synapses seen after capsaicin injection was depotentiated after optogenetic activation of nerve fibers and anisomycin administration (Bonin and De Koninck 2014). These results suggest that reconsolidation inhibition is not specific to CFC and may extend to other models of plasticity, including pathologic forms.

## **2. Hypotheses**

### ***2.1 Disease Modification by Combining Seizure Activity with Inhibition of TrkB-PLC $\gamma$ 1 Signaling***

Inhibition of seizure-evoked TrkB-PLC $\gamma$ 1 signaling in a kindled animal reduces the progression in subsequent seizure severity and induces a reversion to earlier stage of epileptogenesis.

### ***2.2 Seizure Remission in The IAK Model of Epilepsy Using pY816 Peptide***

In the IAK model of TLE signaling, administration of pY816 peptide induces a remission in SRS while producing minimal undesired effects.

## **3. Methods and Materials**

### **3.1 Animals**

All described animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Duke University and conform to the National Institute of Health and Duke University institutional guidelines for the care and use of experimental animals. Animals were maintained on a 12-hour light/dark cycle with food and water available ad libitum. All experiments were conducted during the light portion of the cycle.

#### **3.1.1 Wild Type Mice**

Wild type (WT) adult (8-12 wk) C57/bl6 male mice were purchased from Charles River.

#### **3.1.2 *TrkB*<sup>F616A</sup> Mutant Mice**

*TrkB*<sup>F616A</sup> mice were originally obtained from Dr. David Ginty (Chen et al. 2005) and backcrossed to the C57/bl6 line for at least seven generations. This knockin mouse harbors a point mutation on the *TrkB* allele, substituting an alanine for phenylalanine within the ATP binding pocket of the *TrkB* kinase domain. This mutation renders *TrkB* protein uniquely susceptible to kinase inhibition by small molecule derivatives of protein phosphatase 1, including 1-(1,1-dimethylethyl)-3-(1-naphthalenylmethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (1NMPP1). Importantly, 1NMPP1 does not have any detectable effect in WT mice, and there are no differences in *TrkB* kinase activity in the

*TrkB<sup>F616A</sup>* compared to WT mice in the absence of this compound (Chen et al. 2005, Liu et al. 2013). Both male and female adult homozygous mice were used.

### **3.1.3 PLC $\gamma$ 1 Mutant Mice**

PLC $\gamma$ 1 heterozygous knockout mice were previously generated by targeted gene disruption of the PLC $\gamma$ 1 SH2 domains (Ji et al. 1997). Homozygous deletion of PLC $\gamma$ 1 results in embryonic lethality; therefore heterozygotes and WT littermate controls were used. All animals were backcrossed at least seven generations to C57/bl6.

## **3.2 Treatments and Reagents**

### **3.2.1 pY816 and Scr Peptides**

Peptides were prepared as previously described (Gu et al. 2015). The human sequence of TrkB amino acids 807-820 (LQNLAKASPVpYLDI) with the tyrosine at residue 817 phosphorylated (note that this corresponds to residue 816 in mouse and rat TrkB protein) was conjugated at the N-terminus to the HIV trans-activating protein transduction domain (tat; YGRKKRRQRRR) to allow membrane permeability. The HIV tat sequence conjugated to a scrambled peptide (LVApYQLKIAPNDLS) served as a control. Peptides were synthesized and purified by Tufts Peptide Core Facility, dissolved in sterile PBS at 2 mg/mL, stored at -80°C, and thawed just prior to treatment administration. Unless otherwise stated, treatments were given at a dose of 20 mg/kg IP.

For experiments using biotinylated-pY816 (bio-pY816) the N-terminus of the pY816 sequence was conjugated to biotin without the tat sequence present.

### **3.2.2 1NMPP1**

1NMPP1 was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mM and stored at -80°C until use. Prior to each administration, stock 1NMPP1 was dissolved in a solubilization buffer containing 0.9% NaCL and 2.5% Tween-20 to a concentration of 1.67 mg/mL and provide a dose of 16.6 ug/g IP. For oral treatments, 25 µM of 1NMPP1 was dissolved in solubilization buffer and provided as drinking water. Injection of DMSO and application of DMSO to solubilization buffer served as IP and oral treatment controls, respectively.

### **3.2.3 Carbamazepine**

For experiments involving IP injections, carbamazepine (Sigma) was dissolved in a solubilization buffer of 2% Tween-80 and 70% propylene glycol at a concentration of 2 mg/mL and administered at a dose of 20 mg/kg. Solubilization buffer was used as a control. For experiments involving chronic administration of carbamazepine the drug was incorporated in 1 g chocolate flavored pellets at a dose of 5 mg/pellet (Bio-Serv). Mice were given 5 fresh pellets each day, corresponding to a dose of 800 mg/kg/day for a 25 g mouse; animals routinely ate 4-5 pellets each day and any uneaten pellets were removed. This treatment strategy has been previously demonstrated to result in therapeutic blood levels of carbamazepine in rodents as well as a marked anticonvulsant effect in animal models of TLE (Ali et al. 2012, Iori et al. 2017).

### **3.3 Surgical Procedures**

Adult mice (8-12 wks of age) were anesthetized using isoflurane and placed on a stereotaxic frame.

#### **3.3.1 Surgical Procedure for Kainic Acid Microinfusion**

Once aligned in the stereotaxic frame, a 3.6 mm guide cannula (Plastics One) was inserted above the right amygdala (1.0 mm posterior, 2.9 mm lateral to bregma). A bipolar recording electrode was placed in the left dorsal hippocampus (2.0 mm posterior, 1.6 mm lateral to bregma; 1.7 mm below dura). A skull screw was placed over the left frontal lobe for mechanical stability and as a ground electrode. Dental cement was then used to create a skull cap. Animals were allowed to recover for 5-7 days before subsequent experimentation.

#### **3.3.2 Surgical Procedure for Kindling**

Once aligned in the stereotaxic frame, a bipolar stimulating and recording electrode was inserted above the right amygdala (1.0 mm posterior, 2.9 mm lateral to bregma; 4.6 mm below dura). A skull screw was placed over the left frontal lobe for mechanical stability and as a ground electrode. Dental cement was then used to create a skull cap. Animals were allowed to recover for 5-7 days before subsequent experimentation.

### **3.4 Induction of Status Epilepticus by Kainic Acid Microinfusion**

Induction of status epilepticus (SE) was performed by microinfusion of kainic acid (KA) as previously described (Mouri et al. 2008, Liu et al. 2013, Gu et al. 2015). After a postoperative recovery period, animals were gently restrained and a 4.6 mm infusion cannula (Plastics One) was inserted through the guide cannula to target the amygdala. KA (Sigma, 0.3  $\mu\text{g}$  in 0.5  $\mu\text{L}$  PBS) or vehicle (0.5  $\mu\text{L}$  PBS) was infused at a rate of 0.11  $\mu\text{L}/\text{min}$ . The infusion cannula remained in place for two minutes after infusion to reduce reflux.

Continuous hippocampal EEG telemetry and time-locked video monitoring were performed using the PolyView software (Grass Instruments). Monitoring started 10 min before infusion to obtain a baseline of EEG and behavioral activity. Status epilepticus (SE) was defined as the onset of high frequency, high amplitude EEG patterns consistent with electrographic seizure activity (Liu et al. 2013); SE generally began between 2-10 minutes after infusion. Behavioral seizures were classified according to a modification of the Racine scale for mice (Borges et al., 2003; Racine, 1972): 0, normal activity; 1, arrest and rigid posture; 2, head nodding; 3, unilateral forelimb clonus; 4, rearing with bilateral forelimbs clonus; 5, rearing and falling; 6, tonic-clonic seizures with violent running and/or jumping. Forty minutes after the onset of SE, diazepam (10 mg/kg IP) was administered. A dose of lorazepam (6 mg/kg IP) was administered one hour later.

The duration and number of electrographic seizures as well as the severity of behavioral seizures were determined by analyses of video and EEG data by blinded, trained observers. In addition, quantitative analysis of EEG energy content was performed using a method described previously (Lehmkuhle et al. 2009, Liu et al. 2013, Gu et al. 2015). A custom MATLAB (Mathworks Inc.) script calculated the running power in the 20-50 Hz band at a one-second resolution and smoothed the resulting time-series using a 5 min moving average filter. The values of the smoothed power time-series during SE were averaged and normalized to the average of the baseline to give ratios representing gross power increase during SE.

### ***3.5 Video-EEG Monitoring for Detection of Spontaneous Recurrent Seizures***

After termination of SE with diazepam and lorazepam, animals underwent continuous video-EEG monitoring using the Nicolet EEG system (Natus Medical) for varying periods of time. Recordings were analyzed for spontaneous recurrent seizures (SRS) by two blinded, trained observers. The consistency of identifying SRS between observers was verified to exceed 90%; in rare instances in which readers disagreed, such events were not scored as seizures. SRS were defined as high frequency (> 5Hz) high amplitude (> 2x baseline) rhythmic activity lasting at least 5 seconds. Animals were monitored for a 6-8 week period divided into three epochs. The first 2-4 weeks were a baseline period to observe for the occurrence of SRS. Only animals that exhibited at least one SRS were included in the final analysis. During the next two weeks, mice continued

to be monitored while also receiving treatment. pY816 and Scr peptides were administered at a dose of 20 mg/kg IP BID. Mice receiving carbamazepine received 20-25 mg/day in pellet form. In the final two weeks, treatment was terminated while animals continued to undergo continuous video-EEG monitoring. At the completion of this epoch animals were sacrificed for cannula and electrode localization.

### ***3.6 Kindling Model of Temporal Lobe Epilepsy***

Kindling experiments were performed as previously described (He et al. 2010, He et al. 2014, Liu et al. 2014). After a postoperative recovery period of at least 5 days, animals were connected to a Grass Stimulator (Astro-Med) and monitored by both video and EEG telemetry using the PolyView software. The electrographic seizure threshold (EST) was determined by applying a 1 s train of 1 msec biphasic-rectangular pulses at 60 Hz beginning at 20  $\mu$ A. Additional stimulations were given in 20  $\mu$ A increments at 1 min intervals until an electrographic seizure was detected. Animals then received two stimulations per day at the EST, with the behavioral seizure scores classified according to a modification of the Racine scale for mice (Borges et al., 2003; Racine, 1972): 0, normal activity; 1, arrest and rigid posture; 2, head nodding; 3, unilateral forelimb clonus; 4, rearing with bilateral forelimbs clonus; 5, rearing and falling; 6, tonic-clonic seizures with violent running and/or jumping. The criterion for “kindled” was the occurrence of three consecutive seizures of class 4 or greater, with limb clonus/tonus lasting greater than 12 s. Once kindled, any subsequent seizures were evoked by determining the EST using the

approach previously described (application of pulses in 20  $\mu$ A increments every 1 min, beginning at 20  $\mu$ A, until an electrographic seizure was detected); a reduction in the EST after kindling has previously been established (Racine 1975).

### **3.6.1 Effects of Chemical-Genetic Inhibition of TrkB Kinase Following a Kindled Seizure**

Six days after kindling, seizures were evoked at the new EST for one subset of animals. A separate cohort of animals was connected to the stimulation system for 10 minutes (the average duration to determine an animal's EST) but not stimulated. Animals were removed from the Grass Stimulator and immediately given a 16.6  $\mu$ g/g dose of 1NMPP1 or vehicle IP. Mice were then singly housed for two days with access to 25  $\mu$ M 1NMPP1 or vehicle in drinking water, while continuing to receive 16.6  $\mu$ g/g IP BID. Six days following termination of treatment, the EST was again determined. Upon completion of these experiments animals were sacrificed for histology.

### **3.6.2 Effects of pY816 Peptide Administered Following a Kindled Seizure**

Analogous to experiments described previously, seizures were evoked at the new EST for one subset of kindled animals while a separate cohort of animals were connected to the Grass Stimulator for 10 minutes but not stimulated. Mice were then given either pY816 or Scr peptide at a dose of 20 mg/kg IP. Treatments were continued for 2 days at 20 mg/kg BID, before allowing for either 6 (Section 4.4) or 14 days (Section

4.5) to elapse at which time the EST was again determined. Upon completion of these experiments animals were sacrificed for histology.

### **3.6.3 Effects of Carbamazepine Administered Following a Kindled Seizure**

Given the extremely short half-life of carbamazepine in the rodent (Loscher 2007) and variability in the number of stimulations to reach kindling, all animals had their post-kindling EST determined on the same experimental day (15-27 d after final kindled seizure; contrast with 6d latency for experiments in Section 3.6.1 and 3.6.2). After an evoked seizure, animals were treated with either carbamazepine IP (20 mg/kg) or vehicle, the dose of carbamazepine previously shown to exert anticonvulsant effects in the kindling model (Albertson et al. 1984) and produce therapeutic blood levels (Grabenstatter et al. 2007). Animals then received additional treatments Q4h for two days, before allowing for a 14-day treatment-free period. Subsequently, the EST was again determined. Upon completion of these experiments animals were sacrificed for histology.

### **3.6.4 Analysis of Kindling Experiments**

Video and EEG recordings were analyzed by a blinded, trained observer. In addition to the EST and seizure score, the electrographic seizure duration and behavioral seizure duration was determined.

### **3.7 Lysate Collection**

Lysate collection was performed as previously described (Gu et al. 2015, He et al. 2010). Animals were anesthetized with isofluroane and decapitated; the head was quickly immersed in liquid nitrogen and the hippocampi subsequently dissected on ice and homogenized in RIPA buffer. Homogenates were centrifuged at 200,000 g for 10 min at 4°C and the supernatant collected, protein content quantified using a Bradford assay, and samples stored at -80°C until further analysis.

### **3.8 Plasma Collection**

Falcon tubes were coated with 0.5M CaEDTA and then a mixture of 100 µL CaEDTA, 100 µL 20x Protease Inhibitor Cocktail (Roche), and 50 µL 100 mM sodium orthovanadate was added to each tube. Animals were anesthetized with isoflurane, decapitated, and blood collected in each tube. After collection, 700 µL of sterile PBS was added and the mixture was transferred to an eppendorf tube and centrifuged at 2000 g for 10 min at 4°C. The supernatant was collected, protein content quantified using a Bradford assay, and samples stored at -80°C until further analysis.

### **3.9 Perfusion for Histology**

Following deep isoflurane anesthesia, animals underwent transcatheter perfusion through the left ventricle with 10 mL of chilled 1 U/mL heparin (Sigma) in PBS at a rate of 5 mL/min, followed by 40 mL of 4% paraformaldehyde (Sigma) and 2 mM sodium orthovanadate at 5 mL/min. Brains were dissected and postfixed overnight at 4°C in 4%

paraformaldehyde, then cryoprotected in 30% sucrose. After cryoprotection, brains were frozen by slow immersion in chilled 2-methylbutane and stored at -80°C until cryosectioning. Sections were obtained at 40 µM thickness and stored in 30% sucrose at -80°C until further analysis.

### **3.10 BDNF ELISA**

BDNF sandwich ELISA was performed with the BDNF Emax ImmunoAssay System (Promega) using the manufacturer's guidelines and as previously described (Helgager et al. 2014, Szapacs et al. 2004). A 96-well plate was coated with a monoclonal mouse anti-BDNF antibody (1:1000) overnight at 4°C. Samples and standards were prepared and added to wells for two hours at room temperature, washed, and incubated with a polyclonal rabbit anti-BDNF antibody (1:500) for two hours at room temperature. This was followed by a wash and incubation with a peroxidase conjugated anti-IgY secondary antibody (1:200) for one hour at room temperature, followed by incubation with the provided detection reagent and absorbance reading with a SpectraMax Microplate Reader (Molecular Devices) at 450nm. BDNF concentrations for each sample were then normalized to a standard curve. Sensitivity of detection is reported to be 15 pg/mL and cross-reactivity is less than 3% (Promega).

### **3.11 Western blot**

Western blotting was performed as previously described (He et al. 2010, Helgager et al. 2014, Gu et al. 2015). Samples were diluted to 1 mg/mL using lysis buffer

and SDS-PAGE sample buffer containing beta-mercaptoethanol (Sigma) and then loaded onto SDS-Page gels and electrophoresed, transferred onto nitrocellulose membranes, blocked with 5% BSA, and probed with the following antibodies (1:1000 dilution, Cell Signaling unless otherwise specified): p-PLC $\gamma$ 1 (pY783), PLC $\gamma$ 1, p-TrkB (Y705/706, Santa Cruz), TrkB (1:2000), and beta-actin (1:10,000, Sigma). Immunoreactivity was measured using ImageJ (NIH).

### **3.12 Cresyl Violet Staining & Electrode/Cannula Localization**

For localization of cannula or electrode targeting, serial 40  $\mu$ m sections encompassing the cannula/electrode trajectory were obtained every 80  $\mu$ m. Slides were stained with cresyl violet as previously described (Tureyen et al. 2004). Sections were then imaged by a blinded, trained observer on an epifluorescent microscope and the cannula and/or electrode was localized using a standard mouse brain atlas (Paxinos 2013). Localization was determined by tracing the cannula/electrode track to the deepest point in the tissue.

### **3.13 pY816 ELISA**

In order to eventually detect the tat-pY816 *ex vivo*, a competitive ELISA strategy was developed. High-capacity streptavidin coated plates (Pierce) were incubated overnight at 4°C with a super-saturating amount of biotinylated-pY816 peptide and blocked with BSA. To generate a standard curve, antisera from a rabbit immunized against the amino acids 806-819 of phosphorylated mouse TrkB (LQNLAKASPVpYLDI)

was diluted 1:500K and incubated with 25  $\mu$ g of plasma and varying concentrations of tat-pY816. After incubating the antisera/tat-pY816/plasma mixture on the streptavidin plate coated with biotinylated-pY816 for 1 hour at room temperature, the plate was incubated with an HRP-conjugated goat anti-rabbit secondary antibody for 1 hour at room temperature and detected with SuperSignal ELISA Pico Chemiluminescent Substrate (ThermoFisher) using an Amersham Imager 600 (GE). Images were quantified using ImageJ (NIH).

### ***3.14 Open Field Testing***

Animals were brought to an antechamber ~3 hours before testing began in order to habituate. Mice were then singly transported into the behavioral room and placed in a 12 inch x 12 inch x 24 inch (L x W x H) chamber and given free exploration of the chamber for five min under ~300 lux illumination. Animals were video recorded during this period and movement was analyzed using a publically available MATLAB script (Patel et al. 2014) to determine extent of locomotion in inches.

### ***3.15 Classical Fear Conditioning***

Classical fear conditioning (CFC) experiments were performed as previously described (Musumeci et al. 2009). Mice were brought to an antechamber ~3 hours before testing began in order to habituate. Animals were then singly transported into the behavioral room and placed in the fear conditioning chamber (San Diego Instruments; 12 inch x 12 inch x 12 inch; steel beam floor; clear glass walls; acetic acid scent). After 120

s of habituation to the chamber, a tone of 2800 Hz frequency and 80 dB intensity was presented for 30 seconds, with the last two seconds coinciding with a footshock (0.5 mA). The animals were kept in the chamber for an additional 120 s before another tone-footshock pairing was administered, then kept in the chamber for an additional 30 s before being returned to their home cage.

To test for recall of context, animals were placed in the original fear conditioning chamber 24 hours after conditioning for four minutes (no tone or foot shock was presented during this period).

To test for recall of the cue (tone), animals were placed in a modified fear conditioning chamber (felt floor; signs on walls; vanilla scent). Animals were allowed to explore this chamber for two minutes, before the tone was presented for an additional two minutes.

Animal movement was determined by photobeam interruption of a 16 x 16 array spaced 0.75 inches apart. Freezing was defined as failure to break three new beams, a metric that has been previously described to have strong correlation with both manual freezing determination (Valentinuzzi et al. 1998) as well as video capture (Patel et al. 2014).

### ***3.16 Rotorod Testing***

Mice underwent rotorod testing as previously described (Laskowitz et al. 2007). For training and habituation, mice were placed on an automated rotating rod (Ugo

Basile) for two 60-second training trials at a constant speed (16 rpm). Testing trials involved four consecutive trials with the accelerating rotational speed of 4-40 rpm over five minutes, with an inter-trial interval of at least 15 minutes. Latency to fall was determined by break of a photobeam located below the rotorod.

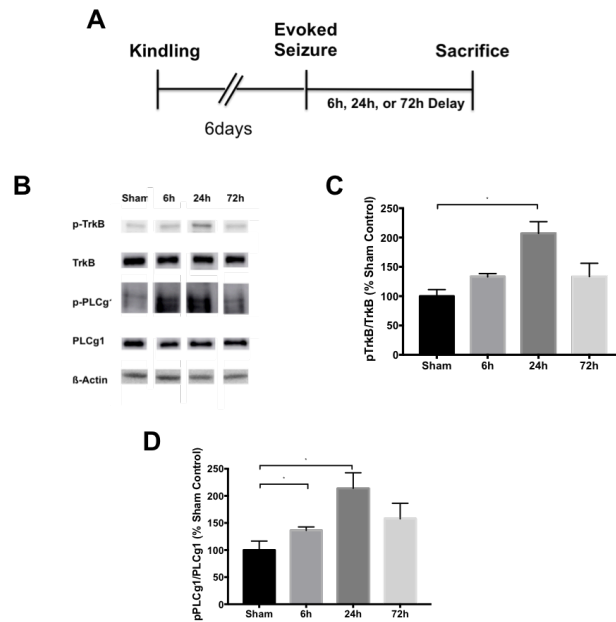
### ***3.17 Statistical Analysis***

All data analysis was performed by individuals blinded to treatment group or experimental condition. Data analysis was done in MATLAB (MathWorks) and Prism 7 software (GraphPad). Sample sizes were chosen based on power analysis. Unless otherwise stated, data are presented as mean  $\pm$  standard error of the mean (SEM), with individual data points depicted. Unless otherwise stated, comparisons between two groups were analyzed using an unpaired Student's t-test, while multi-group comparisons were analyzed using an ANOVA and post-hoc Bonferroni's test. A  $p < 0.05$  was considered significant.

## **4. Results: Disease Modification by Combining Seizure Activity with Inhibition of TrkB-PLC $\gamma$ 1 Signaling**

### ***4.1 Evoked Seizure in Kindled Mice Activates TrkB & PLC $\gamma$ 1***

To determine whether a seizure evoked in a kindled animal induced activation of TrkB-PLC $\gamma$ 1 signaling, we stimulated mice at the EST until kindled. Six days after kindling, a seizure was evoked and pooled bilateral hippocampal lysates were collected at varying timepoints (6h, n = 4; 24h, n = 4; 72h, n = 3), as depicted in Figure 2A. Kindled mice that did not receive an additional evoked seizure were used as controls (n = 4). Western blots were performed to detect p-TrkB and p-PLC $\gamma$ 1, surrogate markers for TrkB and PLC $\gamma$ 1 activation respectively; these were normalized to TrkB and PLC $\gamma$ 1 and presented as percent of sham control. Representative Western blots are shown in Figure 2B. An increase in the ratio of p-TrkB/TrkB was seen beginning six hours after an evoked seizure, peaked at 24 hours, and returned to baseline by 72 hours (Figure 2C). A similar pattern was seen for p-PLC $\gamma$ 1/PLC $\gamma$ 1 (Figure 2D). No differences were found in TrkB/Actin (sham: 100.0  $\pm$  11.2%, 6h: 95.9  $\pm$  5.9%, 24h: 108  $\pm$  5.8%, 72h: 100  $\pm$  10.0%) or PLC $\gamma$ 1/Actin (sham: 100.0  $\pm$  8.6%, 6h: 98.7  $\pm$  5.0%, 24h: 74  $\pm$  25.3%, 72h: 108.3  $\pm$  4.1%).



**Figure 2: Seizure Evoked in Kindled Mice Activates TrkB & PLC $\gamma$ 1.** (A) Schematic of experimental design for assessment of hippocampal lysates by western blot after an evoked seizure. (B) Representative western blots of p-TrkB (pY705/706), TrkB, p-PLC $\gamma$ 1 (Y783), PLC $\gamma$ 1, and Actin. (C-D) Quantification of p-TrkB/TrkB and p-PLC $\gamma$ 1/PLC $\gamma$ 1 following an evoked seizure, normalized to sham controls (n = 2-4). (D) Quantification of p-PLC $\gamma$ 1/PLC $\gamma$ 1, normalized to sham controls. Data are presented as mean  $\pm$  SEM and analyzed using two-way ANOVA with post hoc Bonferroni's test; \* p < 0.05

#### 4.2 Transient Inhibition of TrkB Kinase Only Following an Evoked Seizure Reduces Class and Duration of Subsequent Seizures

Given the above results, we tested the effect of TrkB kinase inhibition following an evoked seizure using a chemical-genetic approach. Initiating inhibition of TrkB kinase with 1NMPP1 immediately following an evoked seizure in *TrkB<sup>F616A</sup>* mice (left side of Figure 3A) significantly reduced the electrographic seizure duration (Figure 3B: 1NMPP1 Post Sz: 29.2  $\pm$  3.8 s, Vehicle Post Sz: 56.6  $\pm$  7.6 s; p < 0.01, two-way ANOVA

with repeated measures & post-hoc Bonferroni) and behavioral seizure duration (Figure 3C: 1NMPP1 Post Sz:  $63.5 \pm 10$  s, Vehicle Post Sz:  $139.8 \pm 16.5$  s;  $p < 0.01$ , two-way ANOVA with repeated measures & post-hoc Bonferroni) when compared to vehicle post-seizure controls. The severity of the subsequent seizure when compared to vehicle post-seizure controls, as evidenced by behavioral seizure class, was also reduced but did not reach statistical significance (Figure 3D). Furthermore, the administration of 1NMPP1 after an evoked seizure showed a strong trend toward a reduction in seizure threshold when compared to controls (Figure 3E).

Importantly, inhibition of TrkB kinase in the absence of a previous seizure (as depicted on the right side of Figure 3A) did not reduce the severity of subsequent seizures. Kindled *TrkB<sup>F616A</sup>* mice that received 1NMPP1 alone were indistinguishable from vehicle-treated mice with regards to subsequent increase in electrographic (Figure 3F) or behavioral (Figure 3G) seizure duration, increase in seizure class (Figure 3H), and reduction in EST (Figure 3I).

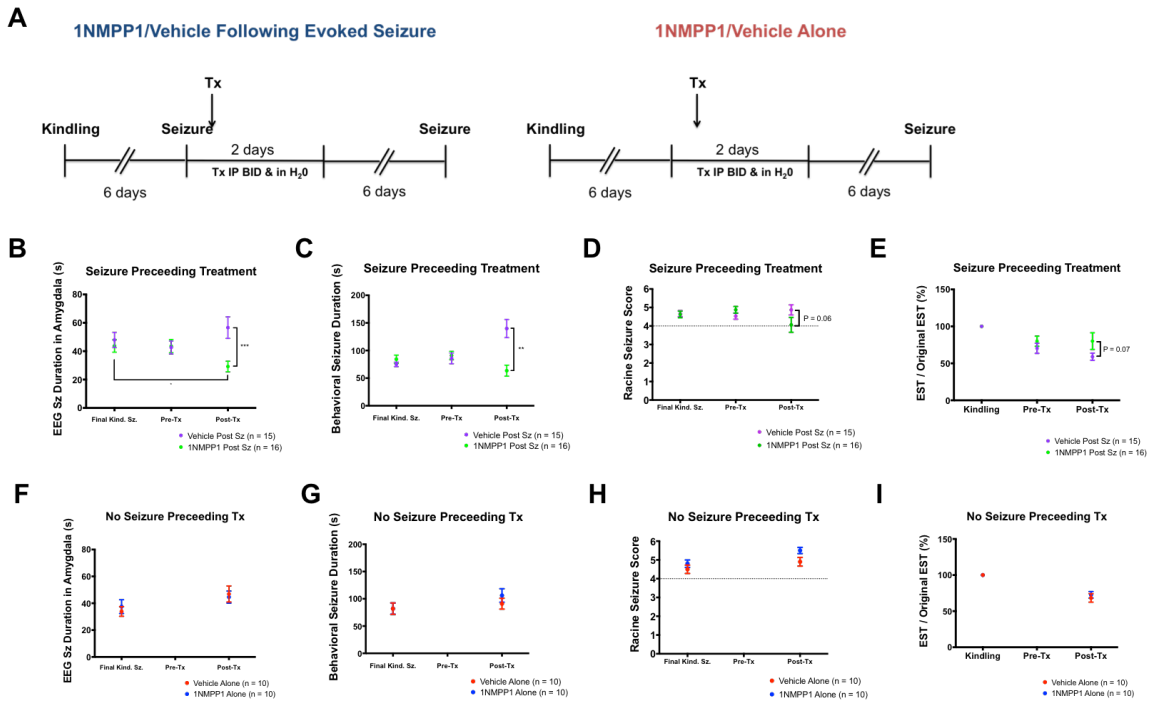
TrkB kinase inhibition following an evoked seizure not only prevented progression in seizure duration and class, but also exhibited a reversion to an earlier state of epileptogenesis in a subset of animals: 4 of 16 animals receiving 1NMPP1 post-seizure had a sub-convulsive seizure (less than Class IV) compared to one vehicle post-seizure control ( $p = 0.2$ , Fisher's exact test). All animals receiving 1NMPP1 or vehicle in the absence of a preceding evoked seizure had a convulsive seizure. Furthermore,

electrographic seizure duration post-treatment in animals receiving 1NMPP1 following an evoked-seizure was significantly reduced compared to the final kindled seizure (Post-Treatment:  $29.2 \pm 3.8$ , Final Kindling Sz:  $43.8 \pm 4.5$ s;  $p < 0.05$ , two-way ANOVA with repeated measures & post-hoc Bonferroni; Figure 3B).

In addition to comparing measurements as averages of individual animals, we analyzed the same data comparing the post-treatment seizure to the penultimate stimulation (final seizure for kindling in animals receiving 1NMPP1 or vehicle alone, in order to allow for each animal to serve as its own control. As depicted in Supplemental Figure 8, this analysis further illustrates that animals receiving 1NMPP1 following an evoked seizure showed a reduction in electrographic seizure duration, behavioral seizure duration, and average seizure score compared to vehicle post-seizure controls. A trend towards a reduction in EST was seen as well. In contrast, the average electrographic seizure duration, behavioral seizure duration, and seizure class exhibited similar increases and seizure threshold showed similar decreases for kindled animals treated with 1NMPP1 alone or vehicle alone.

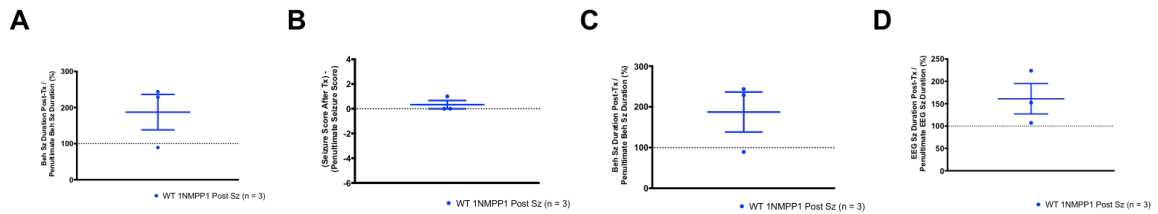
These findings demonstrate that elapsed time following kindling results in increase in seizure class and duration (Figure 3F-I). Induction of a seizure following kindling enhances the severity of subsequent evoked seizures, and inhibition of TrkB kinase, only if administered following an evoked seizure, prevented the progressive increase in seizure duration and class, reduced seizure class to subconvulsive levels in a

subset of animals, and reduced electrographic seizure duration post-treatment to less than that seen in the final kindled seizure (Figure 3B-E).



**Figure 3: Chemical-Genetic Inhibition of TrkB Kinase Only After an Evoked Seizure Reduces Severity of Subsequent Seizures. (A) Schematic of experimental design. (B-E) Electrographic seizure duration, behavioral seizure duration, seizure score, and EST in animals receiving 1NMPP1 (n = 16) or vehicle (n = 15) following an evoked seizure. (F-I) Electrographic seizure duration, behavioral seizure duration, seizure score, and EST for animals receiving 1NMPP1 (n = 10) or vehicle (n = 10) in the absence of a preceding evoked seizure. Data was analyzed by two-way ANOVA with repeated measures and post-hoc Bonferroni. \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001**

Meaningful interpretation of the above chemical-genetic approach requires that the effect of 1NMPP1 be mediated by TrkB kinase inhibition. 1NMPP1 treatment following an evoked seizure in WT kindled animals had no effect on subsequent seizure threshold or severity (Figure 4A-D)



**Figure 4: No Effect of 1NMPP1 After an Evoked Seizure in WT Animals. Change in (A) EST, (B) seizure score, (C) behavioral seizure duration, (D) electrographic seizure duration for post-treatment stimulation relative to pre-treatment stimulation.**

### ***4.3 pY816 Treatment Only After an Evoked Seizure Reduces Class and Duration of Subsequent Seizures***

Might inhibition of a single signaling pathway downstream of TrkB, when introduced following an evoked seizure, be sufficient to reduce the severity of subsequently evoked seizures? The pivotal role of TrkB-mediated PLC $\gamma$ 1 signaling in SE-induced TLE (Gu et al 2015) together with seizure-evoked activation of p-PLC $\gamma$ 1 (Figure 2), led us to test the effect of the pY816, a peptide that uncouples TrkB from PLC $\gamma$ 1 (Gu et al 2015). As shown on the left of Figure 5A, administration of pY816 peptide immediately following an evoked seizure in a kindled animal reduced the electrographic seizure duration (pY816 Post-Sz:  $27.4 \pm 8.5$ , Scr Post-Sz:  $64.5 \pm 9.8$ ;  $p < 0.001$ , two-way ANOVA with repeated measures & post-hoc Bonferroni) and behavioral seizure duration (pY816 Post-Sz:  $48.9 \pm 5.7$ , Scr Post-Sz:  $115.8 \pm 19.8$ ;  $p < 0.001$ , two-way ANOVA with repeated measures & post-hoc Bonferroni) compared to Scr post-seizure controls (Figure 5B-C). Furthermore, the seizure class for animals receiving pY816 following an evoked seizure was significantly reduced compared to Scr post-seizure

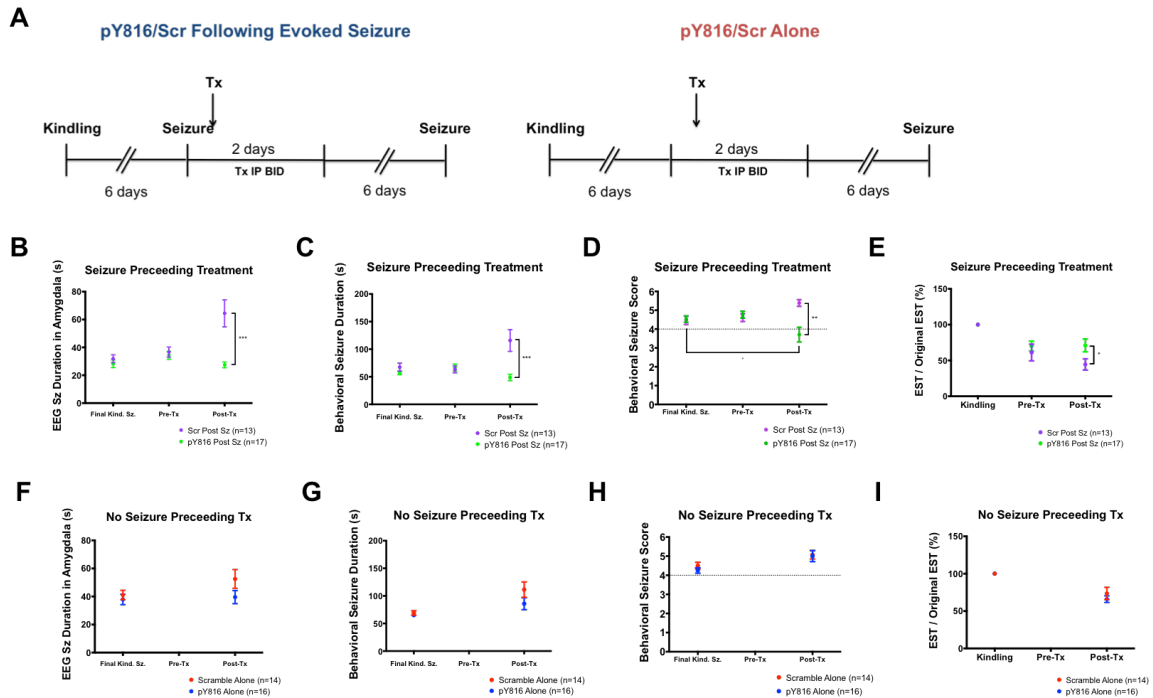
controls (Figure 5D: pY816 Post-Sz:  $3.7 \pm 0.4$ , Scr Post-Sz:  $5.4 \pm 0.18$ ;  $p < 0.05$ , two-way ANOVA with repeated measures & post-hoc Bonferroni). In addition, the administration of pY816, but not Scr, after an evoked seizure significantly increased threshold for subsequent seizures (Figure 5A: pY816:  $70.9 \pm 8.9\%$  of Kindling EST, Scr:  $44.5 \pm 7.8\%$ ;  $p < 0.05$  two-way ANOVA with repeated measures & post-hoc Bonferroni).

Animals that received pY816 alone (without preceding seizure activity, as depicted on the right side of Figure 5A) appeared indistinguishable from Scr-treated mice with regards to subsequent increase in seizure duration (Figure 5F-G) and seizure class (Figure 5H), as well as reduction in EST (Figure 5I).

Treatment with pY816 after an evoked seizure not only inhibited seizure progression but also, in a subset of animals, exhibited a reversion to an earlier state of epileptogenesis. Of the animals receiving pY816 following an evoked seizure, 5 of 17 had subconvulsive seizures (less than Class IV) while all Scr post-seizure controls had convulsive seizures ( $p < 0.05$ , Fisher's exact test). All animals receiving pY816 or Scr in the absence of a preceding evoked seizure had a convulsive seizure post-treatment. Seizure class post-treatment in animals receiving pY816 following an evoked-seizure was significantly reduced compared to the final kindled seizure (Post-Treatment:  $4.5 \pm 0.2$ , Final Kindling Sz:  $3.7 \pm 0.3$ ;  $p < 0.05$ , two-way ANOVA with repeated measures & post-hoc Bonferroni; Figure 5D).

Again, the same data was analyzed comparing the post-treatment seizure to the penultimate stimulation, shown in Supplemental Figure 9, allowing each animal to serve as its own control. Mice receiving pY816 following an evoked seizure showed a reduction in electrographic seizure duration, behavioral seizure duration, and average seizure score compared to vehicle post-seizure controls, as well as a reduction in EST. In contrast, the average electrographic seizure duration, behavioral seizure duration, and seizure class exhibited similar increases and seizure threshold showed similar decreases for kindled animals treated with pY816 alone or Scr alone.

These findings again demonstrate that elapsed time following kindling results in increase in seizure class and duration and induction of a seizure following kindling enhances the class and duration of subsequent evoked seizures. In addition, disruption of TrkB- PLC $\gamma$ 1 signaling, only following an evoked seizure, prevented the progressive increase in seizure class and duration and in a subset of animals reduced seizure class to subconvulsive levels.



**Figure 5: pY816 Treatment Only After an Evoked Seizure Reduces Severity of Subsequent Seizures. (A) Schematic of experimental design. (B-E) Electrographic seizure duration, behavioral seizure duration, seizure score, and EST for animals receiving pY816 (n = 17) or Scr (n = 13) after an evoked seizure. (F-I) Electrographic seizure duration, behavioral seizure duration, seizure score, and EST for animals receiving pY816 (n = 16) or Scr (n = 14) in the absence of a preceding evoked seizure. Data was analyzed by two-way ANOVA with repeated measures and post-hoc Bonferroni test. \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001**

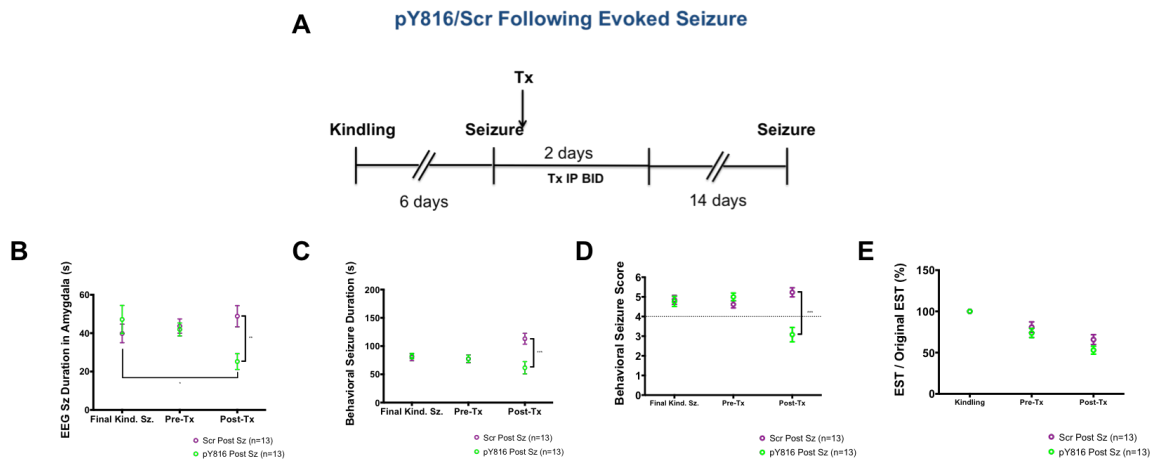
#### **4.4 Effect of pY816 Treatment After an Evoked Seizure Persists Up to Two Weeks After Treatment**

How long after treatment do the effects of pY816 after an evoked seizure persist?

To address this question, a separate kindled cohort were administered pY816 or Scr immediately following an evoked seizure (pY816: n = 13, Scr: n = 13) and allowed fourteen days for peptide clearance before being re-stimulated, as depicted in Figure 6A. Seizure duration (Figure 6B: electrographic duration—pY816 post sz: 25.2 ± 4.1s, Scr post

sz:  $48.9 \pm 5.5$ s;  $p < 0.01$ , two-way ANOVA with repeated measures & post-hoc Bonferroni; Figure 6C: behavioral seizure duration— pY816 post sz:  $61.7 \pm 10.8$ s, Scr post sz:  $113 \pm 9.6$ s;  $p < 0.001$ , two-way ANOVA with repeated measures & post-hoc Bonferroni) and seizure class (Figure 6D: pY816 post sz:  $3.0 \pm 0.4$ , Scr post sz:  $5.2 \pm 0.2$ ;  $p < 0.001$ , two-way ANOVA with repeated measures & post-hoc Bonferroni) were significantly reduced in animals that received pY816 following their evoked seizure compared to Scr post-seizure controls, even with a fourteen day clearance period. No effect was seen on seizure threshold (Figure 6E). Comparing the severity of the post-treatment stimulation to the penultimate stimulation confirmed that the reduction in seizure class and duration was seen even given a 14d clearance period following treatment (Supplemental Figure 10).

Again, treatment with pY816 after an evoked seizure not only inhibited seizure progression but also resulted in modification to a pre-kindled state in a subset of animals. Of the animals receiving pY816 following an evoked seizure, 8 of 13 had subconvulsive seizures (less than Class IV) while all Scr post-seizure controls had convulsive seizures ( $p < 0.05$ , Fisher's exact test). Furthermore, electrographic seizure duration post-treatment in animals receiving pY816 following an evoked-seizure was significantly reduced compared to the final kindled seizure (Post-Treatment:  $25.2 \pm 4.1$ , Final Kindling Sz:  $47.2 \pm 7.3$ s;  $p < 0.05$ , two-way ANOVA with repeated measures & post-hoc Bonferroni; Figure 6B).

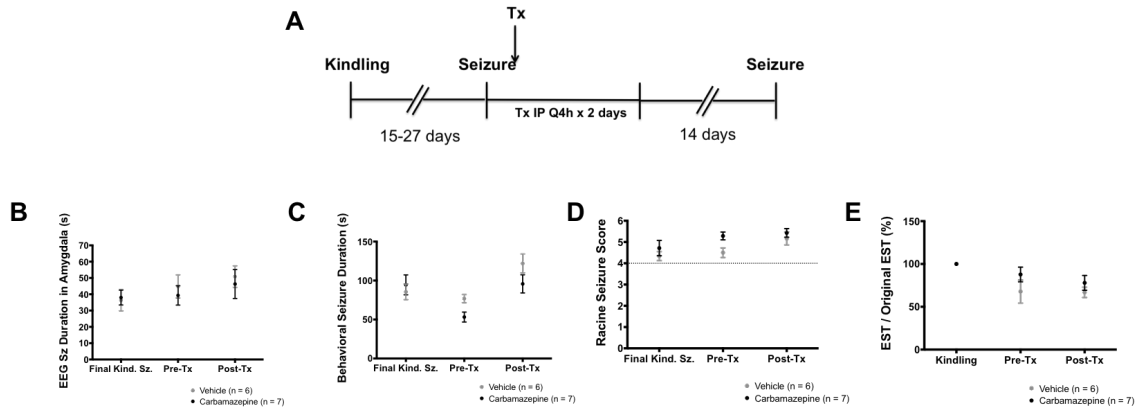


**Figure 6. Effect of pY816 Treatment After an Evoked Seizure Persists Up to Two Weeks After Treatment. (A) Experimental schematic. (B-E) Electrographic seizure duration, behavioral seizure duration, seizure score, and EST for animals receiving pY816 (n = 13) or Scr (n = 13) following an evoked seizure. Data was analyzed by two-way ANOVA with repeated measures and post-hoc Bonferroni test. \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001**

#### **4.5 Treatment with Carbamazepine After an Evoked Seizure Has No Effect on Subsequent Seizure Class or Duration**

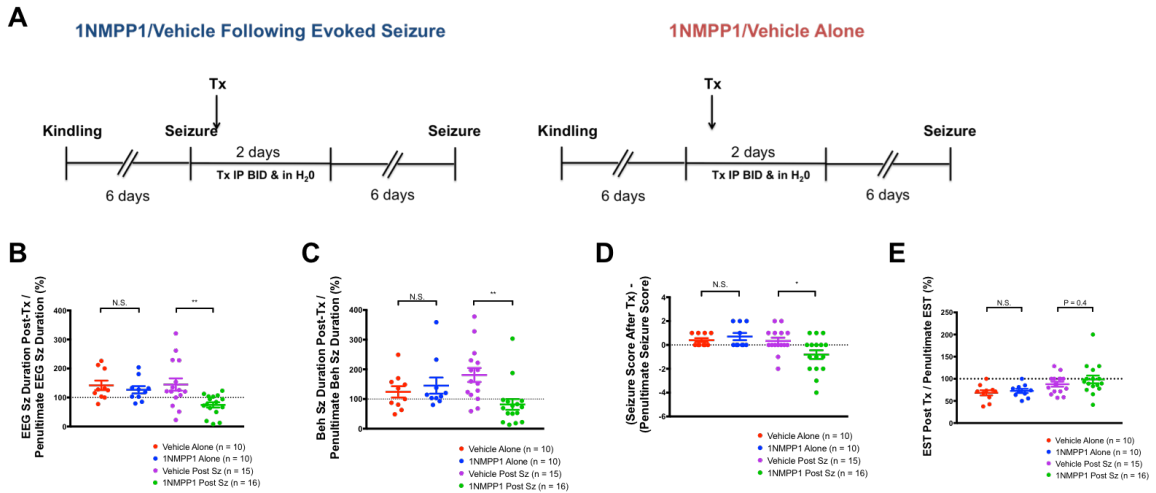
Finally, we sought to confirm that the inhibition of reconsolidation found following TrkB kinase inhibition or pY816 administration does not occur if treatment with the standard of care for TLE, carbamazepine (CBZ), was provided following an evoked seizure. A separate cohort of mice was stimulated until all animals were kindled. Fifteen to twenty-seven days after the last kindled seizure, animals were administered an evoked seizure followed by either CBZ or vehicle (Figure 7A; CBZ, n = 7, vehicle, n = 6) at 20mg/kg IP. Treatments continued every 4 hours for two days, mimicking the duration of 1NMPP1 or pY816 treatment. Fourteen days after the last dose, the EST was determined and seizure analyzed. As shown in Figure 7, CBZ treatment following an

evoked seizure had no effect on the increased duration (Figure 7B-C), increased seizure class (Figure 7D) or reduced threshold (Figure 7E) of subsequent seizures.

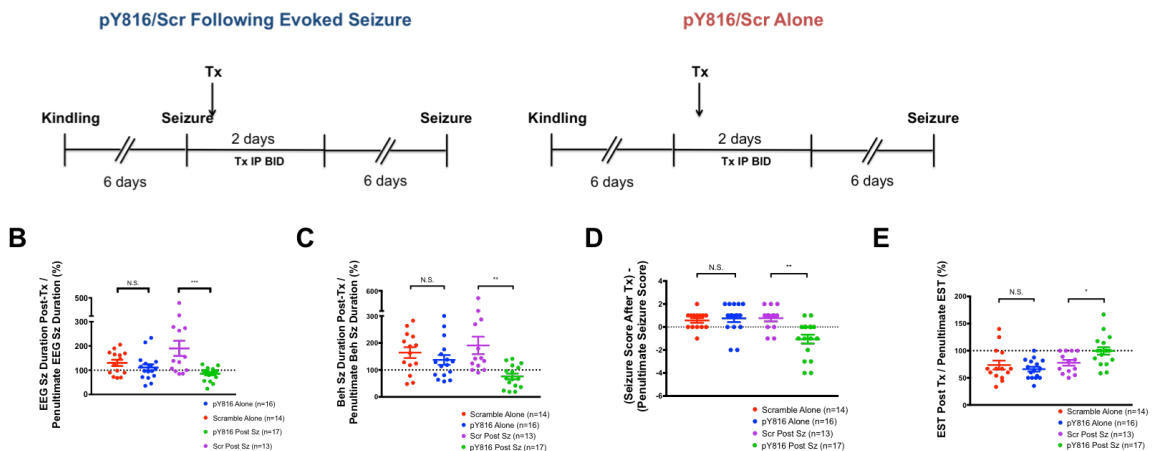


**Figure 7: Treatment with Carbamazepine After an Evoked Seizure Has No Effect on Subsequent Seizure Class or Duration. (A) Experimental design. (B-E) Electrographic seizure duration, behavioral seizure duration, seizure score, and EST for animals receiving CBZ or Veh following an evoked seizure. Data was analyzed by two-way ANOVA with repeated measures and post-hoc Bonferroni test. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$**

## 4.6 Supplemental Figures

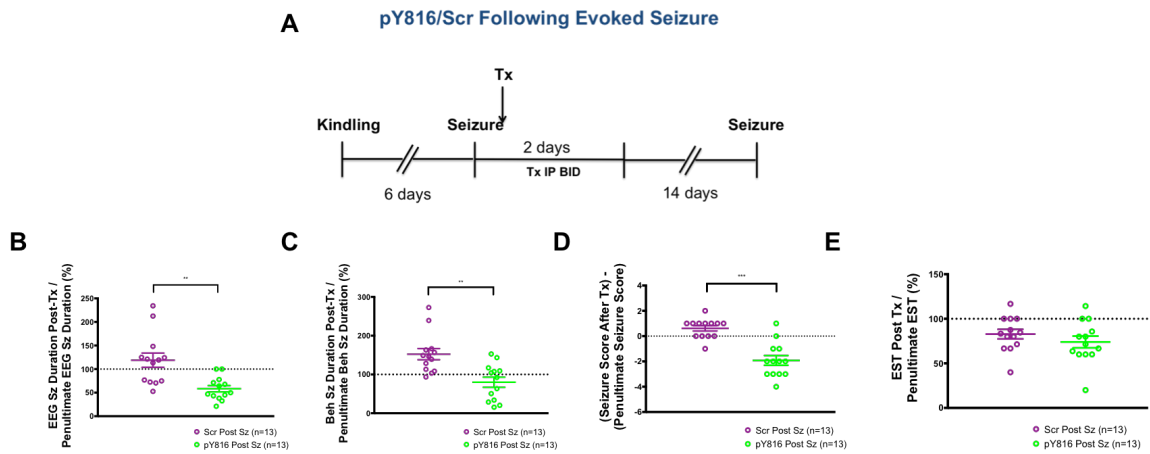


Supplemental Figure 8. Change in electrographic seizure duration, behavioral seizure duration, seizure score, and EST of post-treatment stimulation relative to penultimate stimulation for animals receiving either 1NMPP1 or vehicle in the presence or absence of a preceding evoked seizure. Data was analyzed by one-way ANOVA & post-hoc Bonferroni. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

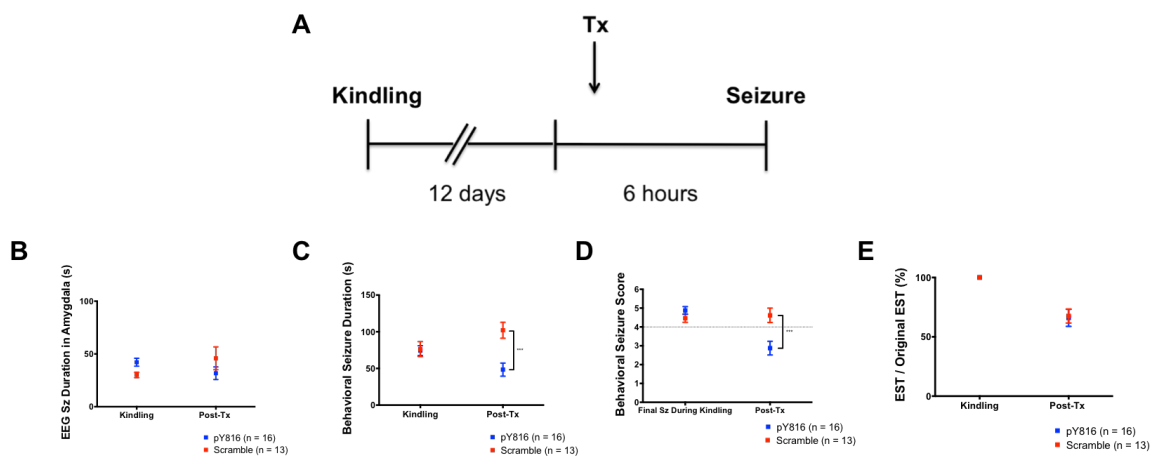


Supplemental Figure 9. Change in electrographic seizure duration, behavioral seizure duration, seizure score, and EST of post-treatment stimulation relative to penultimate stimulation for animals receiving pY816 or Scr in the presence or absence of a preceding evoked seizure, with post-treatment stimulation 6d after treatment

termination. Data was analyzed by one-ANOVA & post-hoc Bonferroni. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$



**Supplemental Figure 10. Change in electrographic seizure duration, behavioral seizure duration, seizure score, and EST of post-treatment stimulation relative to penultimate stimulation for animals receiving pY816 or Scr in the presence a preceding evoked seizure, with post-treatment stimulation 14d after treatment termination. Data was analyzed by one-ANOVA & post-hoc Bonferroni. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$**



**Supplemental Figure 11. Change in electrographic seizure duration, behavioral seizure duration, seizure score, and EST of post-treatment stimulation relative to**

penultimate stimulation for animals receiving pY816 or Scr six hours prior to evoked seizure. Data was analyzed by two-ANOVA with repeated measures & post-hoc Bonferroni. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

## **5. Discussion: Disease Modification by Combining Seizure Activity with Inhibition of TrkB-PLC $\gamma$ 1 Signaling**

We sought to test the effect of disruption of TrkB- PLC $\gamma$ 1 signaling following a seizure evoked in a kindled animal. Four principal findings emerged: 1) Evoked seizures in kindled animals activate TrkB & PLC $\gamma$ 1 signaling, with a peak at 24h and a return to baseline by 72h; 2) Chemical-genetic inhibition of TrkB kinase only after an evoked seizure reduces class and duration of subsequent seizures, and in a subset of animals reduces seizure class to subconvulsive levels; 3) treatment with pY816 only after an evoked seizure reduced class and duration of subsequent seizures, and in a subset of animals reduces seizure class to subconvulsive levels, with the effect persisting up to 2 weeks after treatment termination; 4) Treatment with the standard of care for TLE, carbamazepine, following an evoked seizure has no effect. We conclude that seizure-evoked TrkB-PLC $\gamma$ 1 signaling is necessary for progression of epileptogenesis in kindled animals and disrupting TrkB-PLC $\gamma$ 1 signaling after an evoked seizure produces a trend towards disease modification.

The experimental design deployed here uniquely enables study of seizure-evoked molecular consequences during epileptogenesis (the transformation of the brain from normal to epileptic) using the kindling model. In this model, repeated administration of initially subconvulsive electrical stimuli to a limbic structure induces progressive enhancement of sensitivity to subsequent stimuli evidenced by lowering of focal seizure threshold and enhanced duration and propagation of electrographic

seizures (Goddard 1969, Racine 1975). This enhancement is paralleled by behavioral seizures of increasing severity and duration, with tonic-clonic seizures evident following 10-15 stimulations (Racine 1975). While there is heterogeneity in the definition of a “kindled” state, we used the previously established criterion of three consecutive tonic-clonic seizures with duration of longer than 10s (He et al. 2010, He et al. 2014). At this stage, enhanced sensitivity to stimulation notwithstanding, animals do not exhibit spontaneous seizures. Repeatedly evoking seizures in these animals will ultimately lead to spontaneous seizures (i.e. seizures arising in the absence of stimulation) and other features characteristic of TLE, including hippocampal gliosis and neuronal death (Cavazos et al. 1991, Lothman and Williamson 1993, Cavazos et al. 1994). The critical causal variable in kindling epileptogenesis is the occurrence of an electrographic seizure because stimuli that fail to evoke ictal activity do not induce seizure progression (Racine 1972). Restated, consequences of the evoked electrographic seizure are what cause progression in this model. The standard method for testing potential therapeutics in kindling deploys treatment before each stimulation-evoked electrographic seizure (Baker-Haliski et al. 2015), with the primary readout being the number of stimulations to reach a kindled state. If the drug is present during the evoked electrographic seizure, its effects on epileptogenesis may be due to either attenuation of the electrographic seizure itself (McNamara et al. 1988) or disruption of the consequences of the electrographic seizure; critically with such a design the contribution of each cannot be disentangled.

Our experimental design sought to address the molecular consequences of an evoked electrographic seizure and therefore utilized interventions introduced following, not preceding, the evoked seizure. Our studies demonstrate progression of epileptogenesis as a consequence of both time (Figure 5, compare post-treatment stimulation to final kindled seizure for Scr-treated animals) and, more significantly, as a consequence of an additional evoked seizure (Figure 3, Figure 5, Figure 6; compare post-treatment stimulation to final kindled seizure for Scr-treated and Scr post-seizure animals). Intervention with either TrkB kinase inhibition or pY816 following an evoked seizure prevented subsequent seizure progression and returned severity to that seen during the final kindled seizure.

One possible confound in the interpretation of these results, that the reduction in seizure class and duration is due to retained 1NMPP1 or peptide, is unlikely for several reasons. First, the chemical-genetic strategy for the experiments depicted in Figure 3 utilized the small molecule 1NMPP1, a compound with a half-life in the brain of approximately 30 minutes (Wang et al. 2003), and would be cleared well before the post-treatment stimulation six days after treatment termination. Second, pY816 exhibits a robust anti-convulsant effect in the intra-amygdala kainate model of status epilepticus if administered 10 minutes to 24 hours before induction, but no effect if given 72 hours before induction (Gu et al. 2015), providing indirect evidence that the peptide is cleared from the brain within this time frame. Furthermore, administration of pY816 six hours

prior to stimulation in a kindled animal significantly reduces seizure duration and behavioral seizure score (Supplemental Figure 11), while administration six days prior to stimulation has no effect (Figure 5F-I). Finally, the reduction in seizure class and duration persisted when the latency between pY816 treatment termination and post-treatment stimulation was increased from six days to two weeks (Figure 6).

Our approach to the study of kindling is similar to previous studies in classical fear conditioning (CFC) and neuropathic pain, two other models where a fleeting experience causes persistent plasticity in the adult CNS (McKernan and Shinnick-Gallagher 1997, Woolf and Ji 2001). In CFC, a mouse is placed in a novel context and presented with an auditory tone; the final seconds of tone presentation coincide with a mild foot-shock. Subsequent exposure of the mouse to the context or presentation of the tone produces a freezing response by the mouse, which serves as a surrogate measure for memory recall (Morrison and Ressler 2014). Long-term memory recall is prevented by administration of the protein synthesis inhibitor anisomycin in the hours following foot-shock (Schafe et al. 1999, Schafe and LeDoux 2000), and this period of protein synthesis-dependent memory formation is termed “consolidation” (Johansen et al. 2011). Notably, re-exposure to the tone results in a period of “reconsolidation” where additional protein synthesis is required for subsequent memory retrieval (Nader et al. 2000). In a model of neuropathic pain, a period of reconsolidation also exists since the behavioral phenotype in this model (mechanical hyperalgesia) can also be reversed by a

combination of neuronal activity and anisomycin (Bonin and De Koninck 2014). Importantly, in the absence of re-exposure or neuronal activity, anisomycin alone has no effect in either model (Nader et al. 2000, Bonin and De Koninck 2014). Our findings in kindling share a requirement for neuronal activity since inhibition of TrkB kinase or pY816 administration in the absence of an evoked seizure had no effect on subsequent seizures. Notably, in contrast to the complete reversal of freezing behavior or mechanical hyperalgesia, disruption of TrkB-PLC $\gamma$ 1 signaling after an evoked seizure only produced sub-convulsive seizures in a subset of animals. This partial reduction is similar to studies in CFC that showed administration of the mTOR inhibitor rapamycin during the period of reconsolidation only slightly reduced subsequent freezing behavior (Blundell et al. 2008). Future experiments will test whether, like CFC, anisomycin administration after an evoked seizure returns an animal to a pre-kindled (i.e. naïve unstimulated) state. An additional outstanding question is whether multiple seizure-treatment pairings produces a more robust effect and complete reverses the enhanced sensitivity to low intensity electrical stimulations. These findings underscore the requirement for perturbing molecular signaling cascades after induction of neuronal activity, and inform future studies on memory reconsolidation by raising the possibility that TrkB-PLC $\gamma$ 1 signaling may be an upstream mechanism driving protein synthesis during this process.

What are the cellular and circuit consequences by which disruption of seizure-evoked TrkB-PLC $\gamma$ 1 prevents subsequent seizure progression? In answering this question, it is important to note that one consistent finding from these experiments is that effects of TrkB kinase inhibition or pY816 following an evoked seizure are more robust for seizure class and duration than for seizure threshold. One plausible explanation stems from the hypothesis that repeated evoking of electrographic seizures induces LTP of excitatory synapses between excitatory neurons at various “nodes” throughout the limbic system (Sutula and Steward 1987, Represa et al. 1989, Danzer et al. 2010), which mediates the increase in seizure class and duration; in contrast, reduction in EST is likely caused by changes in excitability in the immediate vicinity of the stimulating electrode. Any elevation of seizure threshold requires alterations specifically in the “driver” of ictal activity-- the stimulated amygdala-- while disruption at any of the synapses having undergone LTP would lead to a reduction in seizure class and duration. With regard to seizure threshold, electrophysiological studies of slices from amygdala-kindled rats suggests that the mechanisms of such changes in local excitability involve both enhanced glutamatergic transmission as well as reduction of both spontaneous and evoked inhibitory transmission (Rainnie et al. 1992). With regard to which synapses having undergone LTP are subsequently disrupted after inhibition of seizure-evoked TrkB-PLC $\gamma$ 1, insight from other models of epileptogenesis may prove informative. Previous studies have demonstrated SE-induced TrkB activation at the

presynaptic MF-CA3 and postsynaptic Schaffer collateral-CA1 synapses (Helgager et al. 2013). TrkB activation has been shown at the MF-CA3 synapse after partial kindling as well (Binder et al 1999). The seizure-induced activation of TrkB at these synapses suggests that these may be the sites for the effects of TrkB-PLC $\gamma$ 1 disruption following an evoked seizure in this study. The CA3-CA1 synapse is a particularly appealing locale because electrophysiological studies suggest that reconsolidation of LTP occurs at this synapse. Researchers induced LTP in the Schaeffer collateral pathway from CA3 to CA1 and then waited 6-8 hours before either applying anisomycin or combining anisomycin with tetanic stimulation (Foncesca et al. 2006). Only in the latter case was functional plasticity reversed.

These findings inform strategies for targeting TrkB signaling in the treatment of epilepsy, suggesting that the optimum timing for inhibiting TrkB-PLC $\gamma$ 1 in the treatment of epilepsy is following an individual's seizure. There is evidence that a patient with TLE symptomatically controlled by anti-convulsive medication can exhibit a "breakthrough" seizure, after which their disease progresses to becoming medically-refractory (Ettinger and Adiga 2008). A clinical trial design for disruption of TrkB-PLC $\gamma$ 1 might involve administration of treatment after such a seizure, with the hope of preventing subsequent progression of disease to a medically-refractory state. An important caveat of these findings is that, at this stage in kindling, mice do not exhibit spontaneous seizures, the defining characteristic of epilepsy. However, the kindling

model features both pathologic activity (in the form of evoked seizures) as well as an increase in the activation of TrkB- PLCg1. Increased expression of the ligand for TrkB, BDNF, in resected hippocampi from medically refractory TLE patients (Murray et al. 2000, Mathern et al. 1997, Takahashi et al. 1999) suggest that disruption of this signaling pathway may have therapeutic benefits in patients already exhibiting seizures. This hypothesis remains to be tested in animal models.

## **6. Results: Seizure Remission in The IAK Model of Epilepsy by Peptide that Uncouples BDNF Receptor TrkB from Effector PLC $\gamma$ 1**

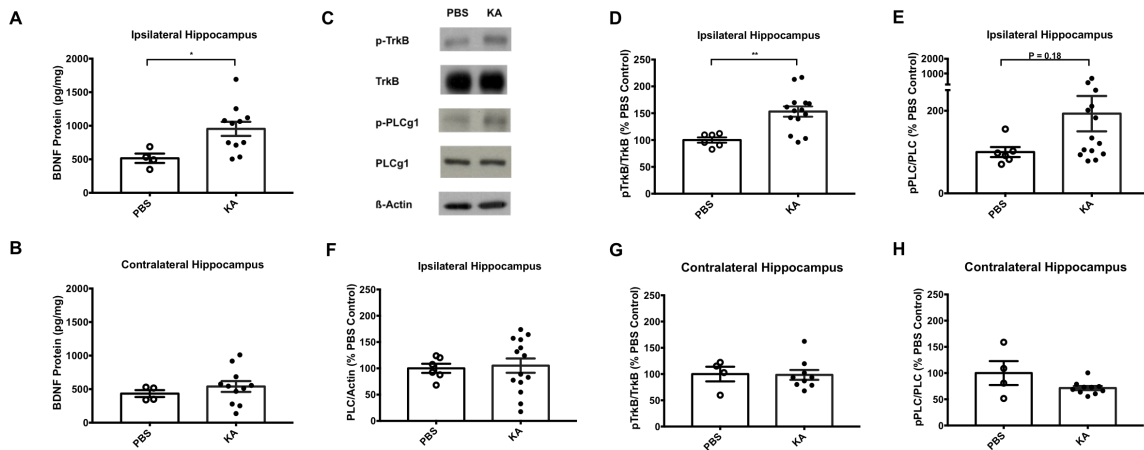
Our previous work (Chapter 4-5) demonstrated that fleeting treatment with pY816 prevented progression of epileptogenesis and partially reversed the enhanced excitability in the kindling model. Importantly, these beneficial effects of pY816 were detected only in the presence of enhanced TrkB-PLC $\gamma$ 1 signaling. Interestingly, the presence of enhanced expression of BDNF mRNA and protein in hippocampal neurons of patients with severe TLE suggests persistent increases of BDNF/TrkB/PLC signaling. Together these findings led us to ask: might epileptic animals in the IAK model exhibit increased BDNF like human TLE as well as persistent activation of TrkB signaling? And, if so, might fleeting treatment with pY816 reverse the enhanced excitability evident as SRS? We addressed these questions with the following experiments.

### ***6.1 Upregulation of BDNF Protein and TrkB-PLC $\gamma$ 1 Signaling in the IAK Mouse Model of TLE***

To determine whether the striking increase in BDNF mRNA (Murray et al. 2000, Mathern et al. 1997) and BDNF protein (Takahashi et al. 1999) in hippocampi of surgically resected specimens from patients with medically-refractory TLE was also seen in our animal model, we sacrificed mice 25-28 days after either KA or PBS infusion into the amygdala and collected hippocampal lysates ipsilateral and contralateral to infused amygdala. We analyzed BDNF protein levels in a subset of lysates (KA n = 11, PBS n = 4)

by ELISA. A significant increase in BDNF protein in KA-infused animals was found in hippocampus ipsilateral (KA:  $953.6 \pm 105.2$  pg/mg, PBS:  $515 \pm 69.7$  pg/mg;  $p < 0.05$ , Student's t-test) but not contralateral (KA:  $539.5 \pm 80.3$  pg/mg, PBS:  $433.7 \pm 51.4$  pg/mg;  $p = 0.46$ , Student's t-test) to the site of infusion (Figure 12A-B).

Using Western blot, we analyzed p-TrkB and p-PLC $\gamma$ 1 (surrogate markers for TrkB and PLC $\gamma$ 1 activation). Values were normalized to total TrkB and PLC $\gamma$ 1 and then presented as a percentage of the average PBS-infused control. Representative Western blots are shown in Figure 11C. A significant increase in p-TrkB/TrkB (KA:  $153.1 \pm 9.6\%$ , PBS:  $100 \pm 5.0\%$ ;  $p < 0.01$ , Student's t-test; Figure 12D) ipsilateral to the site of infusion was seen. A two-fold increase in p-PLC $\gamma$ 1/PLC $\gamma$ 1 ipsilateral to infusion was also found (KA:  $193 \pm 42.8\%$ , PBS:  $100 \pm 12\%$ ;  $p = 0.18$ , Student's t-test; Figure 12E) but did not reach statistical significance. This was likely due to the variability in PLC $\gamma$ 1 protein levels in KA-infused animals (standard deviation for PLC $\gamma$ 1/Actin in KA group:  $50.8\%$  vs PBS group:  $21.2\%$ , Figure 12F). Contralateral changes in p-TrkB/TrkB (KA:  $98.4 \pm 9.4\%$ , PBS:  $100 \pm 13.9\%$ ;  $p = 0.9$ , Student's t-test) and p-PLC $\gamma$ 1/PLC $\gamma$ 1 (KA:  $71.4 \pm 3.9\%$ , PBS:  $100 \pm 22.8\%$ ;  $p = 0.08$ , Student's t-test) were examined in a subset of lysates (KA  $n = 9$ , PBS  $n = 4$ ), and no significant differences were found between the two groups (Figure 12G-H).



**Figure 12: Upregulation of BDNF protein, p-TrkB, and p-PLCγ1 Ipsilateral to Infusion Site in IAK Model. (A) BDNF ELISA for ipsilateral hippocampus. (B) BDNF ELISA for contralateral hippocampus. (C) Representative Western blot for ipsilateral hippocampus. (D) p-TrkB/TrkB for ipsilateral hippocampus. (E) pPLG1/PLCγ1 for ipsilateral hippocampus. (F) PLCγ1/Actin for ipsilateral hippocampus. (G) p-TrkB/TrkB for contralateral hippocampus. (H) p-PLCγ1/PLCγ1 for contralateral hippocampus. All data was analyzed using unpaired Student's T-test. \*  $p < 0.05$ , \*\*  $p < 0.01$**

## **6.2 Remission of SRS Following Two Week pY816 Treatment in Epileptic Animals**

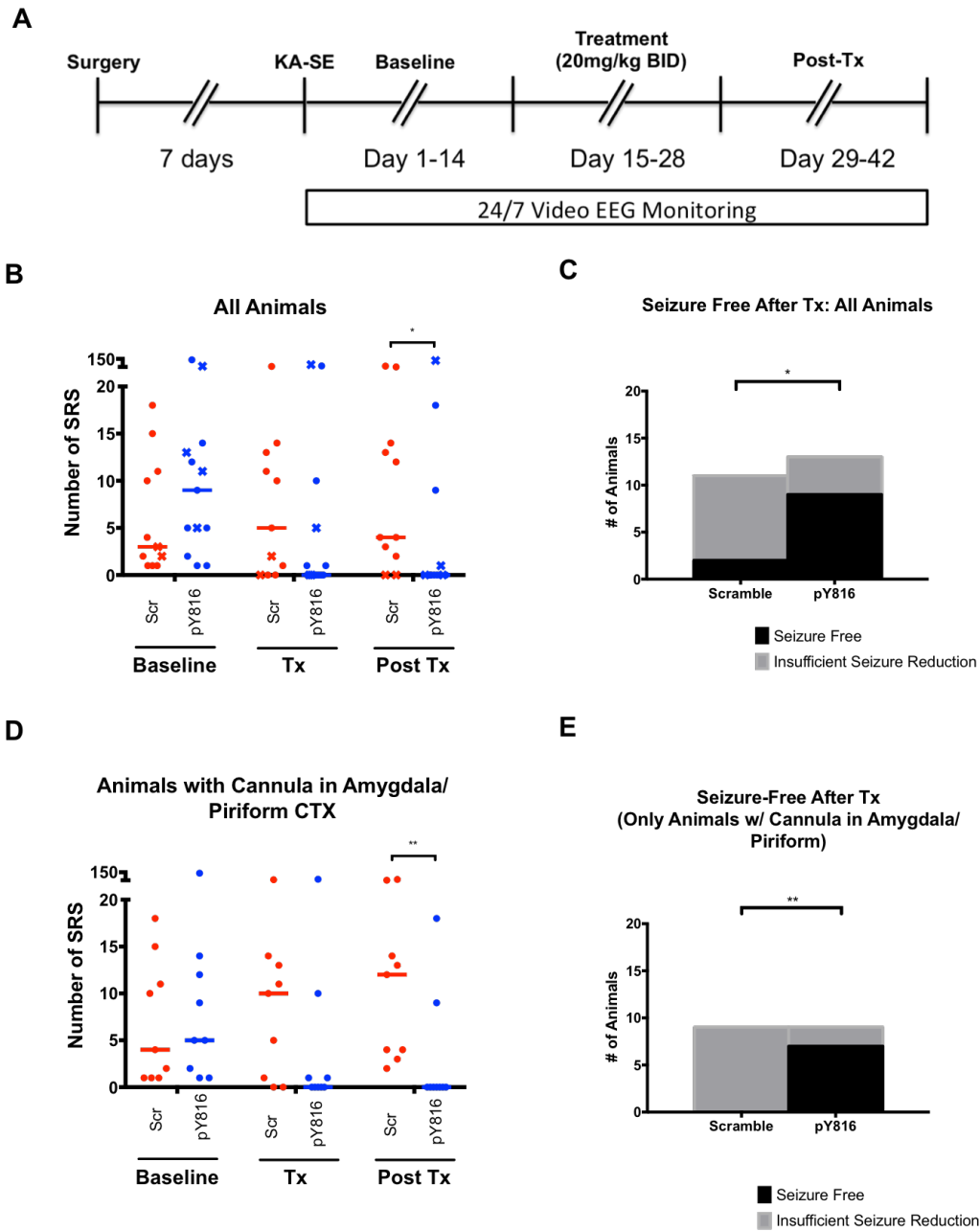
Based on these biochemical findings, we next asked what effect administration of pY816 would have on mice exhibiting SRS in the IAK model. Over the course of five independent experiments, mice were infused with KA and monitored using continuous 24/7 video-EEG telemetry for two weeks for the occurrence of baseline SRS, as depicted in Figure 13A. Mice exhibiting at least one SRS subsequently received either pY816 ( $n = 13$ ) or Scr ( $n = 11$ ), administered IP at 20 mg/kg BID for two weeks. Treatment was then terminated while animals continued to be monitored for the final two weeks of experimentation.

As depicted in Figure 13B, we found a trend toward a reduction in the frequency of SRS in the pY816 group during the two-week treatment phase (median frequency: pY816: 0 SRS, Scr: 5 SRS;  $p = 0.24$ , Mann-Whitney test). Remarkably, this reduction persisted after treatment termination (median frequency: pY816: 0 SRS, Scr: 4 SRS;  $p < 0.05$ , Mann-Whitney test). Almost 70% (9/13) of pY816 treated animals were seizure-free after treatment, while fewer than 20% (2/11) of Scr treated animals showed a remission ( $p < 0.05$ , Fisher's exact test; Figure 13C).

Previous studies of the IAK model by multiple investigators including ourselves reveal persistence of SRS after SE (Mouri et al. 2008, Iori et al. 2017, Liu et al. 2013, Gu et al. 2015). Thus we were puzzled to find remission of SRS in two Scr treated animals. Histological analyses by blinded investigators localized the injection site to the caudate in these two animals, in contrast to localization in amygdala/pyriform cortex for the other control animals. Importantly, kindled seizures evoked by stimulation of amygdala, but not caudate, induce striking increases of hippocampal BDNF levels, suggesting that seizures arising from amygdala but not caudate activate distinct circuits and only the latter induces activation of BDNF/TrkB/PLC $\gamma$ 1 signaling. This potential confound was recognized in analyses performed after the third cohort was studied. Rather than relying solely on post-hoc analysis, two additional cohorts were studied prospectively with inclusion criteria for a subgroup analysis requiring verified site of injection in limbic structures (amygdala or pyriform cortex).

Analyzing animals with verified site of injection in limbic structures showed no remission of SRS in any Scr treated animal. Once again there was a significant reduction in the number of SRS following treatment with pY816 (median frequency: pY816: 0 SRS, Scr: 12 SRS;  $p < 0.01$ , Mann-Whitney test; Figure 13D). Moreover, seven of nine pY816 treated animals were seizure-free after treatment, while all Scr treated animals had at least one SRS ( $P < 0.01$ , Fisher's exact test; Figure 13E).

These findings demonstrate that fleeting treatment of epileptic animals with pY816 induces a remission of SRS in the majority of animals.



**Figure 13: Remission of SRS Following 2wk pY816 Treatment in Epileptic Animals. (A) Experimental schematic. (B-C) Results for all animals exhibiting SRS at baseline, depicted as total SRS frequency (B) as well as proportion of animals seizure free after treatment (C). Animals with cannula not localized to amygdala/piriform cortex are depicted with as an 'x'. (D-E) Results for animals with cannula localized to amygdala/piriform cortex, depicted as total SRS frequency (D) as well as proportion**

of animals seizure free after treatment (E). Data was analyzed with Mann-Whitney test (B, D) or Fisher's Exact test (C, E). \*  $p < 0.05$ , \*\*  $p < 0.01$

### ***6.3 Transient Reduction in SRS by Carbamazepine is Insufficient to Induce Remission***

Given that pY816 treatment reduced the frequency of seizures during the two-week treatment period, the possibility exists that transient inhibition of SRS in the IAK model is sufficient to induce a remission. We therefore infused additional animals with KA and monitored them for four weeks to observe the development of SRS. Six animals had at least one SRS during this baseline period and were administered carbamazepine pellets, a commonly used anticonvulsant for treatment of TLE, for two week at a dose of 800mg/kg/day. Carbamazepine treatment effectively suppressed SRS in all but one animal, which had a single seizure while on treatment. Termination of treatment resulted in recurrence of SRS in all animals, at a frequency similar to that seen in the baseline period (Figure 14). This result suggests that the transient inhibition of SRS is insufficient to induce a remission.

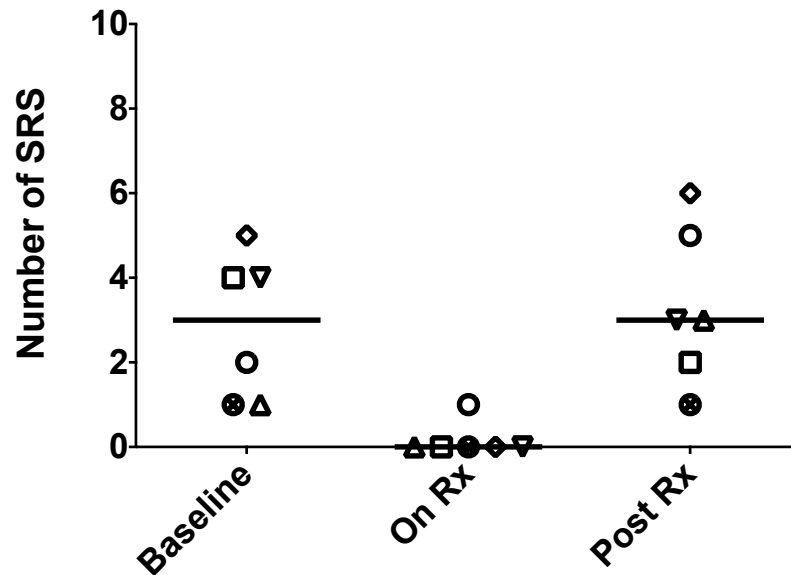


Figure 14: Transient Reduction in SRS by Carbamazepine is Insufficient to Induce Remission. Mice that exhibited SRS during the baseline period had a reduction in SRS frequency on treatment but not after treatment termination.

#### **6.4 Prolonged Treatment With pY816 Does Not Produce Overt Unwanted Consequences**

The promising finding that two-week treatment of an epileptic animal with pY816 induced a remission of SRS in 75% of treated animals coupled with the identity of TrkB sequence in both mouse and human raises the possibility of using pY816 peptide as a therapeutic biologic for the treatment of patients with TLE. One possible limiting factor in advancing pY816 to the clinic is the presence of overt toxicity from treatment administration as a result of chronic inhibition of TrkB-PLC $\gamma$ 1 signaling. To begin to address these questions, a separate cohort of animals underwent two week, twice-daily IP treatment with 20mg/kg pY816 (n = 6) or Scr (n = 6). Daily body weight was recorded. In addition, animals were monitored in the open field test on the eighth day of

treatment. No difference in body weight or locomotion was seen between the two groups, providing evidence against overt toxicity from two-week pY816 treatment (Figure 15A-B). In addition, rotarod testing was done on the tenth day of treatment to more rigorously assess motor function. No difference was seen between the two treatment groups for average latency to fall (pY816:  $258.2 \pm 7.0$ s, Scr:  $233.9 \pm 17.0$ s;  $p = 0.2$ , Student's t-test).

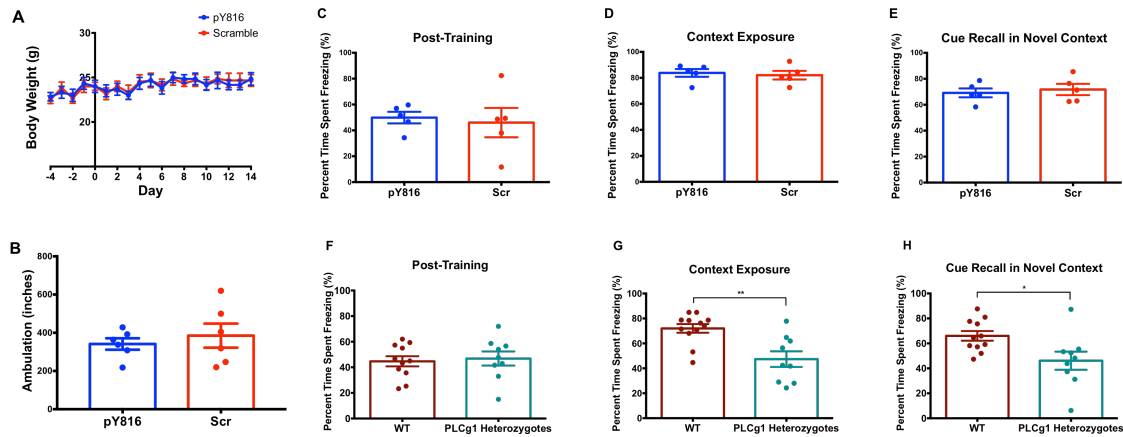
Previous studies have demonstrated a critical role for TrkB signaling in LTP in both the hippocampus (Kang and Schuman 1995, Schildt et al. 2013, Stoop and Poo 1996) and amygdala (Meis et al. 2011, Musumeci et al. 2009). As LTP may be the cellular mechanism underlying learning and memory (Lynch 2004), one potential undesired consequence of chronic inhibition of TrkB-PLC $\gamma$ 1 signaling is amnesia.

To address possible impairments in learning and memory from two-week treatment with pY816, a separate cohort of animals received two week, twice-daily IP treatment with 20mg/kg pY816 (n = 5) or Scr (n = 5). Three hours after the last treatment, mice underwent the CFC protocol as previously described (Musumeci et al. 2009). Briefly, mice were placed in Context A and administered two tone-shock pairings spaced two minutes apart, with 30s of monitoring after the final tone-shock pairing. One day later the mouse was re-exposed to Context A for four minutes. The mouse was placed in Context B two days after training for four minutes, with tone presentation in the final two minutes. Both groups of animals exhibited minimal freezing behavior

before foot shock (pY816: 13.8%  $\pm$  1.8%, Scr: 13.9%  $\pm$  1.5%;  $p = 0.9$ , Student's t-test).

Furthermore, both groups demonstrated increased freezing behavior following foot shock (Figure 15C; pY816: 49.9%  $\pm$  4.5%, Scr: 46.0%  $\pm$  11.4%;  $p = 0.8$ , Student's t-test), as well as upon exposure to the shock context (Figure 14D; pY816: 83.8%  $\pm$  3.0%, Scr: 82.1%  $\pm$  3.3%;  $p = 0.7$ , Student's t-test) and upon exposure to the cue in a novel context (Figure 15E; pY816: 69.2%  $\pm$  3.4%, Scr: 71.7%  $\pm$  43.3%;  $p = 0.7$ , Student's t-test), with no difference in freezing behavior between pY816 and Scr treated animals. As a positive control, the identical experiment was carried out in animals heterozygous for PLC $\gamma$ 1 ( $n = 9$ ) and WT littermate controls ( $n = 11$ ). PLC $\gamma$ 1 heterozygotes displayed equivalent increased freezing behavior following foot shock (Figure 15F), demonstrating acquisition of the memory. However, in contrast to animals treated with pY816, PLC $\gamma$ 1 heterozygotes displayed impaired freezing behavior upon both exposure to the shock context (Figure 15G; PLC $\gamma$ 1 heterozygotes: 47.4  $\pm$  6.2%, WT: 72.0  $\pm$  3.5%;  $p < 0.01$ , Student's t-test) and presentation of the cue (Figure 15H; PLC $\gamma$ 1 heterozygotes: 46.1  $\pm$  7.2%, WT: 66.0  $\pm$  3.9%;  $p < 0.05$ , Student's t-test) when compared to littermate controls.

These results suggest that two-week treatment with pY816 produces no overt unwanted consequences.



**Figure 15: Prolonged Treatment With pY816 Does Not Produce Overt or On-Target Toxicity. (A) Body weight for pY816 or Scr treated animals. (B) Ambulation in open field for pY816 or Scr treated animals. (C) Freezing immediately following CFC training for pY816 or Scr treated animals. (D) Freezing upon re-exposure to CFC context 24h after training for pY816 or Scr treated animals. (E) Freezing upon presentation of tone cue 48h after training for pY816 or Scr treated animals. (F) Freezing immediately following CFC training for PLC $\gamma$ 1 Heterozygote & WT Mice. (G) Freezing upon re-exposure to CFC context 24h after training for PLC $\gamma$ 1 Heterozygote & WT Mice. (H) Freezing upon re-exposure to tone cue 48h after training for PLC $\gamma$ 1 Heterozygote & WT Mice. Body weight analyzed using RMANOVA. All other data analyzed using unpaired Student's t-test, \*  $p < 0.05$ , \*\*  $p < 0.01$**

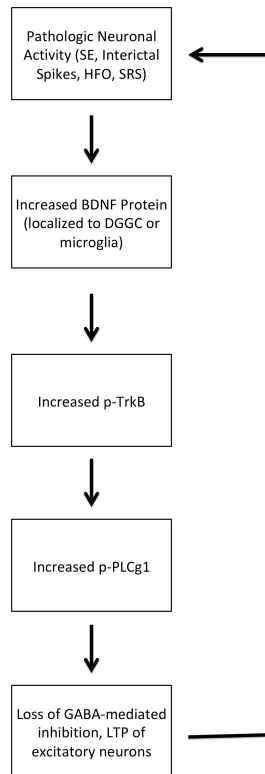
## **7. Discussion: Seizure Remission in The IAK Model of Epilepsy by Peptide that Uncouples BDNF Receptor TrkB from PLC $\gamma$ 1**

We sought to test the pY816 peptide in an IAK model of epilepsy after spontaneous seizures occurred. Four principal findings emerged: 1) In alignment with the clinical phenotype, there is an upregulation of BDNF protein and TrkB-PLC $\gamma$ 1 signaling in the hippocampus ipsilateral to the infused amygdala in the IAK model; 2) Two-week treatment with pY816 induced a remission in SRS following treatment termination; 3) Transient reduction of SRS using the standard of care for TLE, carbamazepine, was unable to induce a remission in SRS; 4) Two-week treatment with pY816 produced no signs of overt toxicity and did not impair hippocampal- or amygdala –dependent memory.

Increase in BDNF protein and TrkB- PLC $\gamma$ 1 activation aligns the IAK model with the clinical phenotype of TLE and provides a rationale for studying disruption of this signaling cascade. An increase in BDNF protein has been observed in hippocampi of surgically resected specimens from patients with medically-refractory TLE (Takahashi et al. 1999). The cellular source of this increase is likely the dentate granule cells, given the increase in BDNF mRNA in these cells in epileptic human tissue (Murray et al. 2000, Mathern et al. 1997). Our lab has previously demonstrated an increase in TrkB-PLC $\gamma$ 1 signaling immediately following SE (Liu et al. 2013, Gu et al. 2015), presumably as a consequence of activity-driven release of BDNF (Harward et al. 2016, Hedrick et al.

2016) and subsequent activation of TrkB. Increases in p-TrkB localized to the boutons of mossy fiber synapses with CA3 pyramidal cells and spines of apical dendrites of CA1 pyramidal cells in two different animal models immediately following SE (Helgager et al. 2013). However, we are the first to examine BDNF levels and TrkB-PLC $\gamma$ 1 signaling chronically (weeks to months) following SE, after the emergence of seizures.

What accounts for the increased BDNF and activation of TrkB- PLC $\gamma$ 1 seen in the more chronic stages of the disease? One hypothesis is that the emergence of pathologic activity following the latent period of epileptogenesis drives the release of BDNF and subsequent activation of TrkB-PLC $\gamma$ 1. TrkB is known to regulate the expression of BDNF through CaM KIV and CREB (Sheih and Ghosh 1999, Vaynman et al. 2004), which are known to be downstream of PLC $\gamma$ 1 (Minichiello et al. 2002, Musumeci et al. 2009). Since BDNF protein itself is increased in these hippocampi, a positive feedback loop may result in the persistent upregulation of this signaling cascade, with pathologic activity driving BDNF release and TrkB-PLC $\gamma$ 1 activation, which subsequently increased BDNF expression (Figure 16). This feedback loop may play a role in other diseases like addiction, where it was recently demonstrated that each episode of cocaine administration activates TrkB and potentiates synapses in the nucleus accumbens, and administration of a brain-penetrant noncompetitive TrkB antagonist tat-peptide reduces motivation for cocaine self-administration (Verhij et al. 2016).



**Figure 16: Model for BDNF-TrkB-PLC $\gamma$ 1 mediated persistence of SRS in TLE. Pathologic activity drives the release of BDNF and downstream activation of TrkB-PLC $\gamma$ 1. This persistently upregulated signaling cascade drives the cellular changes underlying persistence of epilepsy.**

What is the nature of activity that drives the release of the upregulated BDNF and persistent TrkB-PLC $\gamma$ 1 activation in TLE? One possible explanation is that ictal activity is the source. The fact that carbamazepine treatment suppresses seizures but does not induce a remission suggests that seizure activity *per se* does not drive the hypothesized feed-forward loop. It may be that other forms of pathologic activity may persist during carbamazepine treatment and drive BDNF release. For example, interictal spikes are not suppressed following carbamazepine treatment in the kindling model (Gigli and Gotman 1991). Another potential form of activity is high frequency

oscillations (Jones et al. 2015). Surgical resection of brain regions generating this activity achieves better seizure control than removal of the seizure focus alone (Jacobs et al. 2010), suggesting that this form of activity may play a critical role in epilepsy persistence. Future experiments will record from hippocampus ipsilateral to the site of infusion and attempt to identify the nature of activity that drives the elevation of BDNF-TrkB-PLC $\gamma$ 1 signaling in epileptic animals.

What is the cellular source of the increased BDNF and locale of increased p-TrkB and p-PLC $\gamma$ 1 required for epilepsy persistence, and what are the subsequent molecular consequences of persistent activation of this signaling cascade? BDNF mRNA is increased in the dentate granule cells of patients with TLE (Murray et al. 2000, Mathern et al. 1997). Furthermore, in the IAK model, seizures are thought to originate in the ipsilateral CA3 region (Li et al. 2008). Therefore, one hypothesis is that the increased BDNF is localized to the mossy fibers, with the persistently elevated TrkB-PLC $\gamma$ 1 signaling located at the presynaptic MF-CA3 terminals as seen immediately following SE (Helgager et al. 2013). An alternative hypothesis is based on the extensive gliosis seen in human patients with TLE (Wolf et al. 1997) and in the IAK model (Jiminez-Pacheco et al. 2016). Since BDNF released from microglia can result in enhanced plasticity in neocortex (Parkhurst et al. 2013), microglia may be the source of increased BDNF. With regards to molecular consequences, one potential molecular target is the potassium-chloride co-transporter KCC2. This protein maintains the chloride gradient required for

GABA-mediated inhibitory transmission (Kaila et al. 2014), and reduced expression results in a shift to GABA-mediated depolarization (Ben-Ari et al. 1989). Such a change would lead to reduced inhibitory transmission and might promote the generation of SRS. In support of this hypothesis, surgically resected specimens from patients with TLE show a 30% reduction in the number of cells expressing KCC2 mRNA, and these cells demonstrate depolarizing GABA potentials (Huberfeld et al. 2007). In animal models, genetic knockout of KCC2 leads to seizure development and lethality within the first three weeks of life (Woo et al. 2002). In adult mice, inhibition of KCC2 with an antagonist caused epileptiform activity both *in vitro* and when infused into dorsal hippocampus *in vivo* (Sivakumaran et al. 2015). Importantly, BDNF-TrkB-PLC $\gamma$ 1 dependent regulation of KCC2 has been demonstrated. Exposure of rat hippocampal slice cultures to BDNF produces a TrkB-mediated reduction in both KCC2 mRNA and protein (Rivera et al. 2002), and KCC2 mRNA and protein was increased in the hippocampus of a kindled animal (Rivera et al. 2002). With regard to signaling cascades downstream of TrkB mediating KCC2 expression, studies in genetically modified mice demonstrate that in the absence of TrkB-PLC $\gamma$  signaling, KCC2 expression is increased (Rivera et al. 2004). This suggests that the mechanism by which pY816 induces a remission might involve upregulation of KCC2 to pre-epileptic levels, restoring inhibitory transmission.

Uncoupling TrkB-PLC $\gamma$ 1 signaling using pY816 has advantages over other therapeutic strategies identified in preclinical studies to induce SRS remission in TLE. It has been demonstrated that rapamycin, an FDA-approved inhibitor of mTOR signaling, reduces SRS frequency when administered in epileptic rats (Zeng et al. 2009). However, the treatment paradigm continued for the duration of the study. This not only limits the interpretation of the result, since the effect of treatment termination was not examined, but also limits the clinical applicability since the frequency of adverse effects following chronic rapamycin treatment is greater than 30%, leading to a 5% discontinuation rate (Yee et al. 2006). Others have tested the effect of eslicarbazepine, a voltage-gated calcium and sodium channel blocker, in both rat and mouse models of SE-induced TLE, and found that transient, six-week treatment beginning nine days following SE did reduce frequency of SRS. However, EEG recording was not initiated until two months after treatment termination, so whether animals exhibited SRS before beginning treatment is unknown. Furthermore, eslicarbazepine at therapeutic doses resulted in overt motor impairment in naïve mice (Doeser et al. 2015), limiting the clinical application of this compound. Other studies have targeted neuro-inflammatory responses during the epileptogenic process as disease-modifying targets. Antagonism of the innate immune receptors IL-1R1 and TLR4 using a cocktail of inhibitors in another mouse model of SE-induced TLE did induce a reduction in seizure frequency, but importantly did not induce a remission. Furthermore, treatment was initiated after two observed seizures

(Iori et al. 2017), and whether this effect occurs if treatment begins in the more chronic stages of disease was not explored. In the IAK model, implantation of a silk polymer releasing adenosine 9 weeks after SE, in animals confirmed to be exhibiting SRS, did reduce seizure frequency (Williams-Karnesky et al. 2013), but did not induce a remission. However, the mechanism of action is thought to be via inhibition of adenosine kinase, limiting clinical application because of hepatic toxicity from systemic administration (Boison 2013). Transplantation of interneuron progenitors into epileptic mice (Casalia et al. 2017) and selectively ablation newborn dentate granule cells born around the period of SE (Hosford et al. 2016, Hosford et al. 2017) have both been shown to significantly reduced seizure frequency. However, the translatability of such a strategy is currently not feasible.

Our current study may have profound implications for patients suffering from TLE. Seizures in this disease hinder quality of life and ability to obtain employment, and an estimated 25-35% of patients are refractory to current medical therapy (Kwon and Brodie 2000, Juul Jensen 1986). The results of this study, combined with the identity of TrkB pY816 sequence in mouse and human raises the possibility of pursuing pY816 peptide as a therapeutic biologic. A challenge for clinical trials for epilepsy prevention is the large patient size needed (Franco et al. 2016) to achieve adequate power, since a small percentage of patients with identifiable brain insults ultimately go on to develop epilepsy. This is not an issue in a trial designed for remission, where the subject pool

already has SRS. A clinical trial design to test pY816 might involve enrollment of patients undergoing evaluation for temporal lobectomy. After being withdrawn from anti-seizure medications for foci localization, patients could receive transient treatment with pY816. Primary outcome would be lack of seizures after treatment termination; if seizures were to recur patients could continue on to surgical resection. The identification of a single signaling pathway whose disruption is sufficient to induce a remission represents a pivotal step forward in the treatment of epilepsy.

## **8. Conclusions and Future Directions**

### ***8.1 Disease Modification by Combining Seizure Activity with Inhibition of TrkB-PLC $\gamma$ 1 Signaling***

It has previously been established that SE-induced TLE is a disorder of pathologic-activity determined plasticity, where a fleeting experience causes persistent, aberrant circuit activity in the adult CNS (Goddard et al. 1969, Lynch and Seubert 1989, Sutula and Steward 1987) that persists for the life of the animal. Specifically, TLE involves LTP of excitatory synapses between excitatory neurons (Goussakoff et al. 2000, Remigio et al. 2017, O'Leary et al. 2016). LTP is an attractive cellular mechanism of learning and memory (Lynch 2004), and memories have long been known to be “erasable”, with a critical role of neuronal activity in this process (Misanin et al. 1968). More recently, studies have demonstrated that re-exposure to a conditioned stimulus combined with inhibition of molecular pathways necessary for learning renders associative memories reversible (Nader et al. 2000, Blundell et al. 2008). This idea was subsequently extended to another disease of pathologic activity determined plasticity (Ji and Woolf 2001), neuropathic pain. Here, phenotypes of the induced plasticity—specifically hyperalgesia and LTP of dorsal horn synapses—were reversed by a combination of activity and inhibition of protein synthesis (Bonin and De Koninck 2014).

Given the proposed parallels between epileptogenesis and memory formation (Goddard and Douglas 1975) as well as the central role of TrkB-PLC $\gamma$ 1 signaling in the development of epilepsy (Liu et al. 2013, Gu et al. 2015), I sought to test how the

combination of seizure activity and disruption TrkB-PLC $\gamma$ 1 signaling affected seizure progression in a model of TLE. I utilized the kindling model of TLE, where repetitive evoked seizures lead to subsequent seizure progression and eventually spontaneous recurrent seizures (Goddard 1967), in combination with chemical-genetic and pharmacologic perturbation of TrkB-PLC $\gamma$ 1 signaling. Three measures of seizure severity-- seizure class, behavioral seizure duration, and electrographic seizure duration-- served as surrogate markers for pathologic plasticity in this model. Four principal findings emerge from this study. 1) Evoked seizures in kindled animals activate TrkB & PLC $\gamma$ 1 signaling, with a peak at 24h and a return to baseline by 72h. 2) Chemical-genetic inhibition of TrkB kinase only after an evoked seizure reduces severity of subsequent seizures, and in a subset induced reversion to an earlier state of epileptogenesis. 3) pY816 treatment only after an evoked seizure reduced severity of subsequent seizures, and in a subset induced reversion to an earlier state of epileptogenesis, with the effect persisting up to 2 weeks after treatment termination. 4) Treatment with the standard of care for TLE, carbamazepine, following an evoked seizure has no effect.

What is the node where the disrupted plasticity is seen after inhibition of TrkB-PLC $\gamma$ 1 following an evoked seizure? Stated another way, where is the lability induced and reconsolidation disrupted in kindling following inhibition of this signaling cascade? Future directions will combine electrophysiology and imaging using *in vitro*

preparations to begin to answer this question. It has been shown that 2-photon glutamate uncaging over single spines results in enlargement in spine volume; this structural plasticity correlates with function (Harward et al. 2016, Hedrick et al. 2016). Using this *in vitro* approach we can test the cellular locales where reversal of plasticity can occur. Experiments will involve inducing structural plasticity by uncaging glutamate over single spines in various brain regions, followed by a latent period. Then, an additional uncaging pulse, in combination with chemical-genetic and pharmacological inhibition of TrkB-PLC $\gamma$ 1 signaling, will allow for examination for changes in spine volume. Electrophysiological readouts can complement interpretation of changes in synaptic structure. Once locales where plasticity reversal can occur have been identified, slices from kindled animals can be obtained to study (1) occlusion of structural plasticity at these sites and (2) whether spine size can be reduced by a combination of glutamate uncaging and TrkB-PLC $\gamma$ 1 inhibition.

What is the pattern of activity needed to induce lability? Is ictal activity required, or is subconvulsive stimulation sufficient? A disadvantage to the kindling protocol used is a situation where testing the biologic system by necessity perturbed the system (i.e. determination of the EST necessarily induces a seizure). Future experiments will test whether a subconvulsive stimulation in a kindled animal is sufficient to induce lability, and whether different patterns of activity are more efficacious in allowing for reconsolidation inhibition.

What are the molecular mechanisms by which lability is induced and reconsolidation prevented? In both CFC and neuropathic pain, activation of NMDAR is required for subsequent reconsolidation inhibition (Bonin and DeKoninck 2014, Mamou et al. 2006); in CFC this is specifically NR2B-containing NMDARs (Milton et al. 2013, Holehonnur et al. 2016). In addition, protein synthesis is known to be essential for reconsolidation after exposure in both CFC and neuropathic pain (Bonin and DeKoninck 2014, Nader et al. 2000, Blundell et al. 2008, Glover et al. 2014). However, the steps between NMDAR-activation and protein synthesis, as well as the consequences of protein synthesis, are unknown. This study raises the possibility that the sequence of molecular events involves NMDAR activation, TrkB-PLC $\gamma$ 1 signaling, and eventual protein synthesis. Subsequent experiments will test whether, like CFC and neuropathic pain, reconsolidation inhibition occurs if protein synthesis is inhibited after an evoked seizure. In addition, it is not known whether lability in the kindling model requires NMDAR activation. In order to address this question, it is first essential to determine whether ictal activity is required or whether a subconvulsive stimulation is sufficient to induce lability, since NMDAR antagonists are anti-convulsive in this model (Sato et al. 1988, Morimoto et al. 1991, Gilbert 1988). If subconvulsive stimulations are sufficient to induce lability, the role of NMDAR activation can be directly tested; if ictal activity is required, low-doses of NMDAR antagonists may provide insight since these doses

prevent structural plasticity during kindling progression without suppressing seizures *per se* (Sutula et al. 1996).

How might a seizure allow for subsequent reversion to an earlier stage of epileptogenesis? One possible consequence of NMDAR activation and downstream TrkB-PLC $\gamma$ 1 signaling is alteration of the DNA methylation landscape. DNA is hypomethylated following status epilepticus, allowing for subsequent gene transcription. In contrast, during the chronic stages of epilepsy the DNA becomes hypermethylated, suppressing gene transcription (Machnes et al. 2013, Williams-Karnesky et al. 2013). A plausible, testable hypothesis is that kindling also induces hypermethylation which is reversed by an evoked seizure, allowing for subsequent gene transcription which is required for re-consolidation.

My findings provide a more specific molecular mechanism for reconsolidation beyond protein synthesis. This allows for identification of the molecules whose synthesis is required for this process. Future studies can analyze the synaptic proteome of either hippocampal or amygdala lysates following an evoked seizure in the presence or absence of pY816. To start, excitatory synaptosomal fractions from these lysates will be analyzed using a traditional LS-MS/MS approach. Following this discovery proteomics experiment, candidate proteins will be chosen; proteins of interest are those regulated by an evoked seizure and normalized by pY816 treatment. From these candidates a peptide library will be generated for use in a subsequent Multiple Reaction Monitoring (MRM)

proteomics strategy (Wolf-Yadlin et al. 2007). Potential molecular targets will then be validated using traditional antibody-based approaches like Western blot, allowing for the identification of the molecular repertoire required for reconsolidation.

## **8.2 Seizure Remission in The IAK Model of Epilepsy by Peptide that Uncouples BDNF Receptor TrkB from PLC $\gamma$ 1**

Previous studies have demonstrated that TrkB-PLC $\gamma$ 1 signaling is a molecular mechanism by which SE leads to the subsequent development of TLE (Liu et al. 2012, He et al. 2010, He et al. 2014). Administration of the pY816 peptide following SE was shown to prevent development of SRS and psychiatric co-morbidities without exacerbating SE-induced neuronal death (Gu et al. 2015). While these results raise the possibility of pursuing TrkB-PLC $\gamma$ 1 inhibition as a therapeutic strategy to *prevent* the development of TLE, a related challenge is ameliorating *existing* disease. I therefore tested the pY816 peptide in an IAK model of epilepsy long after seizures emerged. Four principal findings emerged. 1) In alignment with the clinical phenotype, there is an upregulation of BDNF protein and TrkB-PLC $\gamma$ 1 signaling in the hippocampus ipsilateral to the infused amygdala in the IAK model. 2) Two-week treatment with pY816 induced a remission in SRS following treatment termination. 3) Transient reduction in SRS using the standard of care for TLE, carbamazepine, was unable to induce a remission in SRS. 4) Two-week treatment with pY816 produced no signs of overt toxicity and did not impair hippocampal- or amygdala –dependent memory.

What is the nature of activity that drives the release of the upregulated BDNF and persistent TrkB-PLC $\gamma$ 1 activation in TLE? Based on the results from the kindling model (Chapter 4), one possible explanation is that ictal activity is the source. The fact that carbamazepine treatment suppresses seizures in both human patients and our model but does not induce a remission suggests that seizure activity *per se* does not drive the hypothesized feed-forward loop required for disease persistence. Importantly, it is not known what effect carbamazepine treatment has on BDNF protein and p-TrkB and p-PLC $\gamma$ 1; this will need to be determined in future experiments. I hypothesize that despite inhibiting SRS, carbamazepine-treatment does not normalize TrkB-PLC $\gamma$ 1 signaling, which explains the recurrence of SRS after treatment termination. It may be that other forms of pathologic activity may persist during carbamazepine treatment and drive BDNF release. For example, interictal spikes are not suppressed following carbamazepine treatment in the kindling model (Gigli and Gotman 1991). Another potential form of activity is high frequency oscillations (Jones et al. 2015). Surgical resection of brain regions generating this activity achieves better seizure control than removal of the seizure focus alone (Jacobs et al. 2010), suggesting that this form of activity may play a critical role in epilepsy persistence. Future experiments will record from hippocampus ipsilateral to the site of infusion and attempt to identify the nature of activity that drives the elevation of BDNF-TrkB-PLC $\gamma$ 1 signaling.

What is the cellular source of the increased BDNF and locale of increased p-TrkB and p-PLC $\gamma$ 1 required for epilepsy persistence? Use of an antibody specific to TrkB Y816 combined with confocal microscopy can shed light on this question. An experiment comparing TrkB Y816 immunoreactivity between epileptic mice and PBS-infused controls during the period where SRS is observed (4 weeks after SE) can identify the cellular locales of increased p-TrkB in epileptic animals; simultaneously examination of BDNF immunohistochemistry using an antibody rigorously assessed for specificity (Dieni et al. 2012) will add to the interpretation by identifying potential sites of BDNF release. Subsequent experiments analyzing p-TrkB and BDNF immunoreactivity in epileptic animals after pY816- and Scr-treatment animals can help identify changes following uncoupling of TrkB-PLC $\gamma$ 1 that may produce a remission in SRS.

What are the molecular mechanisms by which upregulated TrkB-PLC $\gamma$ 1 signaling drives epilepsy persistence? One hypothesis is that changes in either the hippocampal excitatory or inhibitory synaptic proteome, mediated by TrkB-PLC $\gamma$ 1 signaling, may mediate the permanence of TLE. Future studies will pursue this question using a proteomics approach analogous to those used previously (Wolf-Yadlin et al. 2007, Uezo et al. 2016). Excitatory and inhibitory synaptosomal fractions from epileptic animals treated with either pY816 or Scr, along with control animals infused with PBS will be analyzed using a traditional LS-MS/MS approach. Following this discovery proteomics experiment, candidate proteins will be chosen; proteins of interest are those

regulated by SRS and normalized by pY816 treatment. From these candidates a peptide library will be generated for use in a subsequent Multiple Reaction Monitoring (MRM) proteomics strategy (Wolf-Yadlin et al. 2007). Potential molecular targets will then be validated using traditional antibody-based approaches like Western blot.

## References

- Aktekin, B., Dogan, E.A., Oguz, Y. and Senol, Y., 2006. Withdrawal of antiepileptic drugs in adult patients free of seizures for 4 years: a prospective study. *Epilepsy & Behavior*, 8(3), pp.616-619.
- Alberini, C.M., 2005. Mechanisms of memory stabilization: are consolidation and reconsolidation similar or distinct processes?. *Trends in neurosciences*, 28(1), pp.51-56.
- Albertson, T.E., Joy, R.M. and Stark, L.G., 1984. A pharmacological study in the kindling model of epilepsy. *Neuropharmacology*, 23(10), pp.1117-1123.
- Alfonsa, H., Merricks, E.M., Codadu, N.K., Cunningham, M.O., Deisseroth, K., Racca, C. and Trevelyan, A.J., 2015. The contribution of raised intraneuronal chloride to epileptic network activity. *Journal of Neuroscience*, 35(20), pp.7715-7726.
- Ali, A., Dua, Y., Constance, J.E., Franklin, M.R. and Dudek, F.E., 2012. A once-per-day, drug-in-food protocol for prolonged administration of antiepileptic drugs in animal models. *Epilepsia*, 53(1), pp.199-206.
- Andero, R., Heldt, S.A., Ye, K., Liu, X., Armario, A. and Ressler, K.J., 2011. Effect of 7, 8-dihydroxyflavone, a small-molecule TrkB agonist, on emotional learning. *American Journal of Psychiatry*, 168(2), pp.163-172.
- Aronica, E., Zurolo, E., Iyer, A., de Groot, M., Anink, J., Carbonell, C., van Vliet, E.A., Baayen, J.C., Boison, D. and Gorter, J.A., 2011. Upregulation of adenosine kinase in astrocytes in experimental and human temporal lobe epilepsy. *Epilepsia*, 52(9), pp.1645-1655.
- Barbarosie, M., Louvel, J., Kurcewicz, I. and Avoli, M., 2000. CA3-released entorhinal seizures disclose dentate gyrus epileptogenicity and unmask a temporoammonic pathway. *Journal of Neurophysiology*, 83(3), pp.1115-1124.
- Barker-Haliski, M.L., Friedman, D., French, J.A. and White, H.S., 2015. Disease modification in epilepsy: from animal models to clinical applications. *Drugs*, 75(7), pp.749-767.
- Ben-Ari YE, Cherubini EN, Corradetti RE, Gaiarsa JL. Giant synaptic potentials in immature rat CA3 hippocampal neurones. *The Journal of physiology*. 1989 Sep 1;416(1):303-25.

Berkovic, S.F., Andermann, F., Olivier, A., Ethier, R., Melanson, D., Robitaille, Y., Kuzniecky, R., Peters, T. and Feindel, W., 1991. Hippocampal sclerosis in temporal lobe epilepsy demonstrated by magnetic resonance imaging. *Annals of neurology*, 29(2), pp.175-182.

Bertram, E., 2007. The relevance of kindling for human epilepsy. *Epilepsia*, 48(s2), pp.65-74.

Binder, D.K., Routbort, M.J. and McNamara, J.O., 1999. Immunohistochemical evidence of seizure-induced activation of trk receptors in the mossy fiber pathway of adult rat hippocampus. *Journal of Neuroscience*, 19(11), pp.4616-4626.

Blundell, J., Kouser, M. and Powell, C.M., 2008. Systemic inhibition of mammalian target of rapamycin inhibits fear memory reconsolidation. *Neurobiology of learning and memory*, 90(1), pp.28-35.

Boison, D., 2013. Adenosine kinase: exploitation for therapeutic gain. *Pharmacological Reviews*, 65(3), pp.906-943.

Bonin, R.P. and De Koninck, Y., 2014. A spinal analog of memory reconsolidation enables reversal of hyperalgesia. *Nature neuroscience*, 17(8), p.1043.

Botterill, J.J., Brymer, K.J., Caruncho, H.J. and Kalynchuk, L.E., 2015. Aberrant hippocampal neurogenesis after limbic kindling: relationship to BDNF and hippocampal-dependent memory. *Epilepsy & Behavior*, 47, pp.83-92.

Boylan, L.S., Flint, L.A., Labovitz, D.L., Jackson, S.C., Starner, K. and Devinsky, O., 2004. Depression but not seizure frequency predicts quality of life in treatment-resistant epilepsy. *Neurology*, 62(2), pp.258-261.

Brewster, A.L., Lugo, J.N., Patil, V.V., Lee, W.L., Qian, Y., Vanegas, F. and Anderson, A.E., 2013. Rapamycin reverses status epilepticus-induced memory deficits and dendritic damage. *PLoS One*, 8(3), p.e57808.

Buchin, A., Chizhov, A., Huberfeld, G., Miles, R. and Gutkin, B.S., 2016. Reduced efficacy of the KCC2 cotransporter promotes epileptic oscillations in a subiculum network model. *Journal of Neuroscience*, 36(46), pp.11619-11633.

Buckmaster, P.S. and Lew, F.H., 2011. Rapamycin suppresses mossy fiber sprouting but not seizure frequency in a mouse model of temporal lobe epilepsy. *Journal of Neuroscience*, 31(6), pp.2337-2347.

Cain, D.P., 1989. Long-term potentiation and kindling: how similar are the mechanisms?. *Trends in neurosciences*, 12(1), pp.6-10.

Casalia, M.L., Howard, M.A. and Baraban, S.C., 2017. Persistent seizure control in epileptic mice transplanted with gamma-aminobutyric acid progenitors. *Annals of neurology*, 82(4), pp.530-542.

Cavazos JE, Das I, Sutula TP. Neuronal loss induced in limbic pathways by kindling: evidence for induction of hippocampal sclerosis by repeated brief seizures. *Journal of Neuroscience*. 1994 May 1;14(5):3106-21.

Cavazos, J.E., Golarai, G. and Sutula, T.P., 1991. Mossy fiber synaptic reorganization induced by kindling: time course of development, progression, and permanence. *Journal of Neuroscience*, 11(9), pp.2795-2803.

Cazorla, M., Jouvenceau, A., Rose, C., Guilloux, J.P., Pilon, C., Dranovsky, A. and Prémont, J., 2010. Cyclotraxin-B, the first highly potent and selective TrkB inhibitor, has anxiolytic properties in mice. *PLoS One*, 5(3), p.e9777.

Cendes, F., Andermann, F., Dubeau, F., Gloor, P., Evans, A., Jones-Gotman, M., Olivier, A., Andermann, E., Robitaille, Y., Lopes-Cendes, I. and Peters, T., 1993. Early childhood prolonged febrile convulsions, atrophy and sclerosis of mesial structures, and temporal lobe epilepsy An MRI volumetric study. *Neurology*, 43(6), pp.1083-1083.

Chapman, P.F., Ramsay, M.F., Krezel, W. and Knevet, S.G., 2003. Synaptic plasticity in the amygdala. *Annals of the New York Academy of Sciences*, 985(1), pp.114-124.

Chen, M., Arumugam, T.V., Leanage, G., Tieng, Q.M., Yadav, A., Ullmann, J.F., She, D.T., Truong, V., Ruitenber, M.J. and Reutens, D.C., 2017. Disease-modifying effect of intravenous immunoglobulin in an experimental model of epilepsy. *Scientific reports*, 7, p.40528.

Chen, X., Ye, H., Kuruvilla, R., Ramanan, N., Scangos, K.W., Zhang, C., Johnson, N.M., England, P.M., Shokat, K.M. and Ginty, D.D., 2005. A chemical-genetic approach to studying neurotrophin signaling. *Neuron*, 46(1), pp.13-21.

Choi, D.C., Gourley, S.L. and Ressler, K.J., 2012. Prelimbic BDNF and TrkB signaling regulates consolidation of both appetitive and aversive emotional learning. *Translational psychiatry*, 2(12), p.e205.

Choi, H., Hamberger, M.J., Munger Clary, H., Loeb, R., Onchiri, F.M., Baker, G., Hauser, W.A. and Wong, J.B., 2014. Seizure frequency and patient-centered outcome assessment in epilepsy. *Epilepsia*, 55(8), pp.1205-1212.

Coyle, J.T., Molliver, M.E. and Kuhar, M.J., 1978. In situ injection of kainic acid: a new method for selectively lesioning neuronal cell bodies while sparing axons of passage. *Journal of Comparative Neurology*, 180(2), pp.301-323.

Curatolo, P. and Moavero, R., 2012. mTOR inhibitors in tuberous sclerosis complex. *Current neuropharmacology*, 10(4), pp.404-415.

Curia, G., Longo, D., Biagini, G., Jones, R.S. and Avoli, M., 2008. The pilocarpine model of temporal lobe epilepsy. *Journal of neuroscience methods*, 172(2), pp.143-157.

Danzer, S.C., He, X., Loepke, A.W. and McNamara, J.O., 2010. Structural plasticity of dentate granule cell mossy fibers during the development of limbic epilepsy. *Hippocampus*, 20(1), pp.113-124.

De Lanerolle, N.C., Kim, J.H., Robbins, R.J. and Spencer, D.D., 1989. Hippocampal interneuron loss and plasticity in human temporal lobe epilepsy. *Brain research*, 495(2), pp.387-395.

Dieni, S., Matsumoto, T., Dekkers, M., Rauskolb, S., Ionescu, M.S., Deogracias, R., Gundelfinger, E.D., Kojima, M., Nestel, S., Frotscher, M. and Barde, Y.A., 2012. BDNF and its pro-peptide are stored in presynaptic dense core vesicles in brain neurons. *J Cell Biol*, pp.jcb-201201038.

Do-Monte, F.H., Quinones-Laracuente, K. and Quirk, G.J., 2015. A temporal shift in the circuits mediating retrieval of fear memory. *Nature*, 519(7544), p.460.

Doeser, A., Dickhof, G., Reitze, M., Uebachs, M., Schaub, C., Pires, N.M., Bonifácio, M.J., Soares-da-Silva, P. and Beck, H., 2014. Targeting pharmacoresistant epilepsy and epileptogenesis with a dual-purpose antiepileptic drug. *Brain*, 138(2), pp.371-387.

Douglas, R.M. and Goddard, G.V., 1975. Long-term potentiation of the perforant path-granule cell synapse in the rat hippocampus. *Brain research*, 86(2), pp.205-215.

Dragunow, M. and Goddard, G.V., 1984. Adenosine modulation of amygdala kindling. *Experimental neurology*, 84(3), pp.654-665.

Duvarci, S. and Nader, K., 2004. Characterization of fear memory reconsolidation. *Journal of Neuroscience*, 24(42), pp.9269-9275.

Elwes, R.D., Johnson, A.L. and Reynolds, E.H., 1988. The course of untreated epilepsy. *Bmj*, 297(6654), pp.948-950.

Engel Jr, J., Pedley, T.A. and Vaughn, B., 1998. Epilepsy: A Comprehensive Textbook, Volume One. *Journal of Clinical Neurophysiology*, 15(3), pp.277-278.

Ettinger, A.B. and Adiga, R.K., 2008. Breakthrough seizures—approach to prevention and diagnosis. *US Neurology*, 4(1), pp.40-2.

FALCONER, M.A., SERAFETINIDES, E.A. and CORSELLIS, J.N., 1964. Etiology and pathogenesis of temporal lobe epilepsy. *Archives of neurology*, 10(3), pp.233-248.

Fanselow, M.S. and Kim, J.J., 1994. Acquisition of contextual Pavlovian fear conditioning is blocked by application of an NMDA receptor antagonist D, L-2-amino-5-phosphonovaleric acid to the basolateral amygdala. *Behavioral neuroscience*, 108(1), p.210.

Fawcett, J.W. and Asher, R.A., 1999. The glial scar and central nervous system repair. *Brain research bulletin*, 49(6), pp.377-391.

Fedele, D.E., Koch, P., Scheurer, L., Simpson, E.M., Möhler, H., Brüstle, O. and Boison, D., 2004. Engineering embryonic stem cell derived glia for adenosine delivery. *Neuroscience letters*, 370(2-3), pp.160-165.

Fisher, R.S., Acevedo, C., Arzimanoglou, A., Bogacz, A., Cross, J.H., Elger, C.E., Engel, J., Forsgren, L., French, J.A., Glynn, M. and Hesdorffer, D.C., 2014. ILAE official report: a practical clinical definition of epilepsy. *Epilepsia*, 55(4), pp.475-482.

Fonseca, R., Nägerl, U.V. and Bonhoeffer, T., 2006. Neuronal activity determines the protein synthesis dependence of long-term potentiation. *Nature neuroscience*, 9(4), p.478.

Franco, V., French, J.A. and Perucca, E., 2016. Challenges in the clinical development of new antiepileptic drugs. *Pharmacological research*, 103, pp.95-104.

Garbelli, R., Milesi, G., Medici, V., Villani, F., Didato, G., Deleo, F., D'Incerti, L., Morbin, M., Mazzoleni, G., Giovagnoli, A.R. and Parente, A., 2012. Blurring in patients with temporal lobe epilepsy: clinical, high-field imaging and ultrastructural study. *Brain*, 135(8), pp.2337-2349.

Gigli, G.L. and Gotman, J., 1991. Effects of seizures and carbamazepine on interictal spiking in amygdala kindled cats. *Epilepsy research*, 8(3), pp.204-212.

Gilbert, M.E., 1988. The NMDA-receptor antagonist, MK-801, suppresses limbic kindling and kindled seizures. *Brain research*, 463(1), pp.90-99

Glover, E.M., Ressler, K.J. and Davis, M., 2010. Differing effects of systemically administered rapamycin on consolidation and reconsolidation of context vs. cued fear memories. *Learning & Memory*, 17(11), pp.577-581.

Glover, E.M., Ressler, K.J. and Davis, M., 2010. Differing effects of systemically administered rapamycin on consolidation and reconsolidation of context vs. cued fear memories. *Learning & Memory*, 17(11), pp.577-581.

Goddard, G.V. and Douglas, R.M., 1975. Does the engram of kindling model the engram of normal long term memory?. *Canadian Journal of Neurological Sciences*, 2(4), pp.385-394.

Goddard, G.V., 1967. Development of epileptic seizures through brain stimulation at low intensity. *Nature*, 214(5092), p.1020.

Goddard, G.V., 1967. Development of epileptic seizures through brain stimulation at low intensity. *Nature*, 214(5092), p.1020.

Goddard, G.V., McIntyre, D.C. and Leech, C.K., 1969. A permanent change in brain function resulting from daily electrical stimulation. *Experimental neurology*, 25(3), pp.295-330.

Goldring, S. and Gregorie, E.M., 1984. Surgical management of epilepsy using epidural recordings to localize the seizure focus: review of 100 cases. *Journal of neurosurgery*, 60(3), pp.457-466.

Gowers, W.R., 1901. *Epilepsy and other chronic convulsive diseases: their causes, symptoms, and treatment*. Churchill.

Grabenstatter, H.L., Clark, S. and Dudek, F.E., 2007. Anticonvulsant effects of carbamazepine on spontaneous seizures in rats with kainate-induced epilepsy: comparison of intraperitoneal injections with drug-in-food protocols. *Epilepsia*, 48(12), pp.2287-2295.

Gu, B., Huang, Y.Z., He, X.P., Joshi, R.B., Jang, W. and McNamara, J.O., 2015. A peptide uncoupling BDNF receptor TrkB from phospholipase C $\gamma$ 1 prevents epilepsy induced by status epilepticus. *Neuron*, 88(3), pp.484-491.

Guo, D., Zeng, L., Brody, D.L. and Wong, M., 2013. Rapamycin attenuates the development of posttraumatic epilepsy in a mouse model of traumatic brain injury. *PLoS one*, 8(5), p.e64078.

Guo, D., Zou, J. and Wong, M., 2017. Rapamycin attenuates acute seizure-induced astrocyte injury in mice in vivo. *Scientific reports*, 7(1), p.2867.

Harward, S.C., Hedrick, N.G., Hall, C.E., Parra-Bueno, P., Milner, T.A., Pan, E., Laviv, T., Hempstead, B.L., Yasuda, R. and McNamara, J.O., 2016. Autocrine BDNF–TrkB signalling within a single dendritic spine. *Nature*, 538(7623), p.99.

He, X.P., Minichiello, L., Klein, R. and McNamara, J.O., 2002. Immunohistochemical evidence of seizure-induced activation of trkB receptors in the mossy fiber pathway of adult mouse hippocampus. *Journal of Neuroscience*, 22(17), pp.7502-7508.

He, X.P., Pan, E., Sciarretta, C., Minichiello, L. and McNamara, J.O., 2010. Disruption of TrkB-mediated phospholipase C $\gamma$  signaling inhibits limbic epileptogenesis. *Journal of Neuroscience*, 30(18), pp.6188-6196.

Hebb, D.O., 1961. Distinctive features of learning in the higher animal. *Brain mechanisms and learning*, pp.37-46.

Hedrick, N.G., Harward, S.C., Hall, C.E., Murakoshi, H., McNamara, J.O. and Yasuda, R., 2016. Rho GTPase complementation underlies BDNF-dependent homo- and heterosynaptic plasticity. *Nature*, 538(7623), p.104.

Hiyoshi, T. and Wada, J.A., 1992. Lasting Nature of Both Transfer and Interference in Amygdaloid Kindling in Cats: Observation Upon Stimulation with 11-Month Rest Following Primary Site Kindling. *Epilepsia*, 33(2), pp.222-227.

Holehonnur, R., Phensy, A.J., Kim, L.J., Milivojevic, M., Vuong, D., Daison, D.K., Alex, S., Tiner, M., Jones, L.E., Kroener, S. and Ploski, J.E., 2016. Increasing the GluN2A/GluN2B ratio in neurons of the mouse basal and lateral amygdala inhibits the modification of an existing fear memory trace. *Journal of Neuroscience*, 36(36), pp.9490-9504.

Hosford, B.E., Liska, J.P. and Danzer, S.C., 2016. Ablation of newly generated hippocampal granule cells has disease-modifying effects in epilepsy. *Journal of Neuroscience*, 36(43), pp.11013-11023.

Hosford, B.E., Rowley, S., Liska, J.P. and Danzer, S.C., 2017. Ablation of peri-insult generated granule cells after epilepsy onset halts disease progression. *Scientific reports*, 7(1), p.18015.

Hosford, D.A., Simonato, M., Cao, Z.H.E.N., Garcia-Cairasco, N., Silver, J.M., Butler, L., Shin, C.H.E.O.L.S.U. and McNamara, J.O., 1995. Differences in the anatomic

distribution of immediate-early gene expression in amygdala and angular bundle kindling development. *Journal of Neuroscience*, 15(3), pp.2513-2523.

Huang, Y.Y., Li, X.C. and Kandel, E.R., 1994. cAMP contributes to mossy fiber LTP by initiating both a covalently mediated early phase and macromolecular synthesis-dependent late phase. *Cell*, 79(1), pp.69-79.

Huang, Y.Y., Martin, K.C. and Kandel, E.R., 2000. Both protein kinase A and mitogen-activated protein kinase are required in the amygdala for the macromolecular synthesis-dependent late phase of long-term potentiation. *Journal of Neuroscience*, 20(17), pp.6317-6325.

Huberfeld, G., Wittner, L., Clemenceau, S., Baulac, M., Kaila, K., Miles, R. and Rivera, C., 2007. Perturbed chloride homeostasis and GABAergic signaling in human temporal lobe epilepsy. *Journal of Neuroscience*, 27(37), pp.9866-9873.

Iori, V., Iyer, A.M., Ravizza, T., Beltrame, L., Paracchini, L., Marchini, S., Cerovic, M., Hill, C., Ferrari, M., Zucchetti, M. and Molteni, M., 2017. Blockade of the IL-1R1/TLR4 pathway mediates disease-modification therapeutic effects in a model of acquired epilepsy. *Neurobiology of disease*, 99, pp.12-23.

Iori, V., Iyer, A.M., Ravizza, T., Beltrame, L., Paracchini, L., Marchini, S., Cerovic, M., Hill, C., Ferrari, M., Zucchetti, M. and Molteni, M., 2017. Blockade of the IL-1R1/TLR4 pathway mediates disease-modification therapeutic effects in a model of acquired epilepsy. *Neurobiology of disease*, 99, pp.12-23.

Isackson, Paul J., et al. "BDNF mRNA expression is increased in adult rat forebrain after limbic seizures: temporal patterns of induction distinct from NGF." *Neuron* 6.6 (1991): 937-948

Jacobs, J., Zijlmans, M., Zelmann, R., Chatillon, C.É., Hall, J., Olivier, A., Dubeau, F. and Gotman, J., 2010. High-frequency electroencephalographic oscillations correlate with outcome of epilepsy surgery. *Annals of neurology*, 67(2), pp.209-220.

Jarero-Basulto, J.J., Gasca-Martínez, Y., Rivera-Cervantes, M.C., Ureña-Guerrero, M.E., Feria-Velasco, A.I. and Beas-Zarate, C., 2018. Interactions Between Epilepsy and Plasticity. *Pharmaceuticals*, 11(1), p.17.

Ji, Q.S., Winnier, G.E., Niswender, K.D., Horstman, D., Wisdom, R., Magnuson, M.A. and Carpenter, G., 1997. Essential role of the tyrosine kinase substrate phospholipase C- $\gamma$ 1 in mammalian growth and development. *Proceedings of the National Academy of Sciences*, 94(7), pp.2999-3003.

Jimenez-Pacheco, A., Diaz-Hernandez, M., Arribas-Blázquez, M., Sanz-Rodriguez, A., Olivos-Oré, L.A., Artalejo, A.R., Alves, M., Letavic, M., Miras-Portugal, M.T., Conroy, R.M. and Delanty, N., 2016. Transient P2X7 receptor antagonism produces lasting reductions in spontaneous seizures and gliosis in experimental temporal lobe epilepsy. *Journal of Neuroscience*, 36(22), pp.5920-5932.

Jinde, S., Zsiros, V., Jiang, Z., Nakao, K., Pickel, J., Kohno, K., Belforte, J.E. and Nakazawa, K., 2012. Hilar mossy cell degeneration causes transient dentate granule cell hyperexcitability and impaired pattern separation. *Neuron*, 76(6), pp.1189-1200.

Johansen, J.P., Cain, C.K., Ostroff, L.E. and LeDoux, J.E., 2011. Molecular mechanisms of fear learning and memory. *Cell*, 147(3), pp.509-524.

Jones, R.T., Barth, A.M., Ormiston, L.D. and Mody, I., 2015. Evolution of temporal and spectral dynamics of pathologic high-frequency oscillations (pHFOs) during epileptogenesis. *Epilepsia*, 56(12), pp.1879-1889.

Juul-Jensen P. Epidemiology of intractable epilepsy. In: Schmidt D, Morselli P, eds. Intractable epilepsy. New York: Raven Press, 1986:5–11.

Kaila, K., Price, T.J., Payne, J.A., Puskarjov, M. and Voipio, J., 2014. Cation-chloride cotransporters in neuronal development, plasticity and disease. *Nature Reviews Neuroscience*, 15(10), p.637.

Kang, H. and Schuman, E.M., 1995. Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. *Science*, 267(5204), pp.1658-1662.

Kida, S., Josselyn, S.A., de Ortiz, S.P., Kogan, J.H., Chevere, I., Masushige, S. and Silva, A.J., 2002. CREB required for the stability of new and reactivated fear memories. *Nature neuroscience*, 5(4), p.348.

Kononenko, N.L., Claßen, G.A., Kuijpers, M., Puchkov, D., Maritzen, T., Tempes, A., Malik, A.R., Skalecka, A., Bera, S., Jaworski, J. and Haucke, V., 2017. Retrograde transport of TrkB-containing autophagosomes via the adaptor AP-2 mediates neuronal complexity and prevents neurodegeneration. *Nature communications*, 8, p.14819.

Kwan, P. and Brodie, M.J., 2000. Early identification of refractory epilepsy. *New England Journal of Medicine*, 342(5), pp.314-319.

Kwan, P. and Brodie, M.J., 2000. Early identification of refractory epilepsy. *New England Journal of Medicine*, 342(5), pp.314-319.

Labiner, D.M., Butler, L.S., Cao, Z., Hosford, D.A., Shin, C.H.E.O.L.S.U. and McNamara, J.O., 1993. Induction of c-fos mRNA by kindled seizures: complex relationship with neuronal burst firing. *Journal of Neuroscience*, 13(2), pp.744-751.

Laskowitz, D.T., McKenna, S.E., Song, P., Wang, H., Durham, L., Yeung, N., Christensen, D. and Vitek, M.P., 2007. COG1410, a Novel Apolipoprotein E-Based Peptide, Improves Functional Recovery in a Murine Model of Traumatic Brain Injury. *Journal of neurotrauma*, 24(7), pp.1093-1107.

Lee, J.L., Everitt, B.J. and Thomas, K.L., 2004. Independent cellular processes for hippocampal memory consolidation and reconsolidation. *Science*, 304(5672), pp.839-843.

Li, T., Ren, G., Lusardi, T., Wilz, A., Lan, J.Q., Iwasato, T., Itohara, S., Simon, R.P. and Boison, D., 2008. Adenosine kinase is a target for the prediction and prevention of epileptogenesis in mice. *The Journal of clinical investigation*, 118(2), pp.571-582.

Liu, G., Gu, B., He, X.P., Joshi, R.B., Wackerle, H.D., Rodriguiz, R.M., Wetsel, W.C. and McNamara, J.O., 2013. Transient inhibition of TrkB kinase after status epilepticus prevents development of temporal lobe epilepsy. *Neuron*, 79(1), pp.31-38.

Liu, I.Y., Lyons, W.E., Mamounas, L.A. and Thompson, R.F., 2004. Brain-derived neurotrophic factor plays a critical role in contextual fear conditioning. *Journal of Neuroscience*, 24(36), pp.7958-7963.

Löscher, W., 2007. The pharmacokinetics of antiepileptic drugs in rats: consequences for maintaining effective drug levels during prolonged drug administration in rat models of epilepsy. *Epilepsia*, 48(7), pp.1245-1258.

Lossius, M.I., Hessen, E., Mowinckel, P., Stavem, K., Erikssen, J., Gulbrandsen, P. and Gjerstad, L., 2008. Consequences of antiepileptic drug withdrawal: A randomized, double-blind study (Akershus Study). *Epilepsia*, 49(3), pp.455-463.

Lothman, E.W. and Williamson, J.M., 1993. Rapid kindling with recurrent hippocampal seizures. *Epilepsy research*, 14(3), pp.209-220.

Lynch, G. and Seubert, P., 1989. Links between Long-Term Potentiation and Neuropathology An Hypothesis Involving Calcium-Activated Proteases. *Annals of the New York Academy of Sciences*, 568(1), pp.171-180.

Lynch, M. A. "Long-term potentiation and memory." *Physiological reviews* 84.1 (2004): 87-136.

Mahan, A.L. and Ressler, K.J., 2012. Fear conditioning, synaptic plasticity and the amygdala: implications for posttraumatic stress disorder. *Trends in neurosciences*, 35(1), pp.24-35.

Mamou, C.B., Gamache, K. and Nader, K., 2006. NMDA receptors are critical for unleashing consolidated auditory fear memories. *Nature neuroscience*, 9(10), p.1237.

Mansuy, I.M., Mayford, M., Jacob, B., Kandel, E.R. and Bach, M.E., 1998. Restricted and regulated overexpression reveals calcineurin as a key component in the transition from short-term to long-term memory. *Cell*, 92(1), pp.39-49.

Marson, A., Jacoby, A., Johnson, A., Kim, L., Gamble, C., Chadwick, D. and Medical Research Council MESS Study Group, 2005. Immediate versus deferred antiepileptic drug treatment for early epilepsy and single seizures: a randomised controlled trial. *The Lancet*, 365(9476), pp.2007-2013.

Mathern, G.W., Babb, T.L., Micevych, P.E., Blanco, C.E. and Pretorius, J.K., 1997. Granule cell mRNA levels for BDNF, NGF, and NT-3 correlate with neuron losses or supragranular mossy fiber sprouting in the chronically damaged and epileptic human hippocampus. *Molecular and chemical neuropathology*, 30(1-2), pp.53-76.

McIntyre, D.C. and Gilby, K.L., 2008. Mapping seizure pathways in the temporal lobe. *Epilepsia*, 49(s3), pp.23-30.

McKernan, M.G. and Shinnick-Gallagher, P., 1997. Fear conditioning induces a lasting potentiation of synaptic currents in vitro. *Nature*, 390(6660), p.607.

McNamara, J.O., 1999. Emerging insights into the genesis of epilepsy. *Nature*, 399(Supplementary), p.A15.

McNamara, J.O., Huang, Y.Z. and Leonard, A.S., 2006. Molecular signaling mechanisms underlying epileptogenesis. *Science Signaling*, 2006(356), pp.re12-re12.

McNamara, J.O., Russell, R.D., Rigsbee, L.C. and Bonhaus, D.W., 1988. Anticonvulsant and antiepileptogenic actions of MK-801 in the kindling and electroshock models. *Neuropharmacology*, 27(6), pp.563-568.

Meis, S., T. Endres, and V. Lessmann. "Postsynaptic BDNF signalling regulates long-term potentiation at thalamo-amygdala afferents." *The Journal of physiology* 590.1 (2012): 193-208.

Milton, A.L., Merlo, E., Ratano, P., Gregory, B.L., Dumbreck, J.K. and Everitt, B.J., 2013. Double dissociation of the requirement for GluN2B- and GluN2A-containing

NMDA receptors in the destabilization and restabilization of a reconsolidating memory. *Journal of Neuroscience*, 33(3), pp.1109-1115.

Minichiello, L., Calella, A.M., Medina, D.L., Bonhoeffer, T., Klein, R. and Korte, M., 2002. Mechanism of TrkB-mediated hippocampal long-term potentiation. *Neuron*, 36(1), pp.121-137.

Misanin, J.R., Miller, R.R. and Lewis, D.J., 1968. Retrograde amnesia produced by electroconvulsive shock after reactivation of a consolidated memory trace. *Science*, 160(3827), pp.554-555.

Morimoto, K., Fahnstock, M. and Racine, R.J., 2004. Kindling and status epilepticus models of epilepsy: rewiring the brain. *Progress in neurobiology*, 73(1), pp.1-60.

Morimoto, K., Katayama, K., Inoue, K. and Sato, K., 1991. Effects of competitive and noncompetitive NMDA receptor antagonists on kindling and LTP. *Pharmacology Biochemistry and Behavior*, 40(4), pp.893-899.

Moser, V.C., 1989. Screening approaches to neurotoxicity: a functional observational battery. *Journal of the American College of Toxicology*, 8(1), pp.85-93.

Mouri, G., Jimenez-Mateos, E., Engel, T., Dunleavy, M., Hatazaki, S., Paucard, A., Matsushima, S., Taki, W. and Henshall, D.C., 2008. Unilateral hippocampal CA3-predominant damage and short latency epileptogenesis after intra-amygdala microinjection of kainic acid in mice. *Brain research*, 1213, pp.140-151.

Murray, K.D., Isackson, P.J., Eskin, T.A., King, M.A., Montesinos, S.P., Abraham, L.A. and Roper, S.N., 2000. Altered mRNA expression for brain-derived neurotrophic factor and type II calcium/Calmodulin-dependent protein kinase in the hippocampus of patients with intractable temporal lobe epilepsy. *Journal of Comparative Neurology*, 418(4), pp.411-422.

Musumeci, G., Sciarretta, C., Rodríguez-Moreno, A., Al Banchaabouchi, M., Negrete-Díaz, V., Costanzi, M., Berno, V., Egorov, A.V., und Halbach, O.V.B., Cestari, V. and Delgado-García, J.M., 2009. TrkB modulates fear learning and amygdalar synaptic plasticity by specific docking sites. *Journal of Neuroscience*, 29(32), pp.10131-10143.

Nabavi, S., Fox, R., Proulx, C.D., Lin, J.Y., Tsien, R.Y. and Malinow, R., 2014. Engineering a memory with LTD and LTP. *Nature*, 511(7509), p.348.

Nader, K., Schafe, G.E. and Le Doux, J.E., 2000. Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. *Nature*, 406(6797), p.722.

Ngugi, A.K., Bottomley, C., Kleinschmidt, I., Sander, J.W. and Newton, C.R., 2010. Estimation of the burden of active and life-time epilepsy: a meta-analytic approach. *Epilepsia*, 51(5), pp.883-890.

Nguyen, P.V., Abel, T. and Kandel, E.R., 1994. Requirement of a critical period of transcription for induction of a late phase of LTP. *Science*, 265(5175), pp.1104-1107.

O'Leary, H., Bernard, P.B., Castano, A.M. and Benke, T.A., 2016. Enhanced long term potentiation and decreased AMPA receptor desensitization in the acute period following a single kainate induced early life seizure. *Neurobiology of disease*, 87, pp.134-144.

Ohkawa, N., Saitoh, Y., Suzuki, A., Tsujimura, S., Murayama, E., Kosugi, S., Nishizono, H., Matsuo, M., Takahashi, Y., Nagase, M. and Sugimura, Y.K., 2015. Artificial association of pre-stored information to generate a qualitatively new memory. *Cell reports*, 11(2), pp.261-269.

Okazaki, M.M., Evenson, D.A. and Victor Nadler, J., 1995. Hippocampal mossy fiber sprouting and synapse formation after status epilepticus in rats: visualization after retrograde transport of biocytin. *Journal of Comparative Neurology*, 352(4), pp.515-534.

Olofsson, K., Lindvall, O. and Asztely, F. "Increased synaptic inhibition in dentate gyrus of mice with reduced levels of endogenous brain-derived neurotrophic factor." *Neuroscience* 101.3 (2000): 531-539.

Ou, L.C. and Gean, P.W., 2006. Regulation of amygdala-dependent learning by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol-3-kinase. *Neuropsychopharmacology*, 31(2), p.287.

Ou, L.C., Yeh, S.H. and Gean, P.W., 2010. Late expression of brain-derived neurotrophic factor in the amygdala is required for persistence of fear memory. *Neurobiology of learning and memory*, 93(3), pp.372-382.

Panja, D., Kenney, J.W., D'Andrea, L., Zalfa, F., Vedeler, A., Wibrand, K., Fukunaga, R., Bagni, C., Proud, C.G. and Bramham, C.R., 2014. Two-stage translational control of dentate gyrus LTP consolidation is mediated by sustained BDNF-TrkB signaling to MNK. *Cell reports*, 9(4), pp.1430-1445.

Patel, T.P., Gullotti, D.M., Hernandez, P., O'Brien, W.T., Capehart, B.P., Morrison III, B., Bass, C., Eberwine, J.E., Abel, T. and Meaney, D.F., 2014. An open-source toolbox for automated phenotyping of mice in behavioral tasks. *Frontiers in behavioral neuroscience*, 8, p.349.

Pavlov, I.P. and Anrep, G.V., 1928. *Conditioned reflexes: An investigation of the physiological activity of the cerebral cortex*. London: Oxford University Press.

Phillips, R.G. and LeDoux, J.E., 1992. Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behavioral neuroscience*, 106(2), p.274.

Pinel, J.P. and Rovner, L.I., 1978. Electrode placement and kindling-induced experimental epilepsy. *Experimental neurology*, 58(2), pp.335-346.

Pitkänen, A. and Sutula, T.P., 2002. Is epilepsy a progressive disorder? Prospects for new therapeutic approaches in temporal-lobe epilepsy. *The Lancet Neurology*, 1(3), pp.173-181.

Pitkänen, A., 2010. Therapeutic approaches to epileptogenesis—hope on the horizon. *Epilepsia*, 51(s3), pp.2-17.

Pitkänen, A., Nissinen, J., Lukasiuk, K., Jutila, L., Paljärvi, L., Salmenperä, T., Karkola, K., Vapalahti, M. and Ylinen, A., 2000. Association between the density of mossy fiber sprouting and seizure frequency in experimental and human temporal lobe epilepsy. *Epilepsia*, 41(s6).

Przybylski, J., Rouillet, P. and Sara, S.J., 1999. Attenuation of emotional and nonemotional memories after their reactivation: Role of  $\beta$  adrenergic receptors. *Journal of Neuroscience*, 19(15), pp.6623-6628.

Racine, R.J., 1972. Modification of seizure activity by electrical stimulation: II. Motor seizure. *Electroencephalography and clinical neurophysiology*, 32(3), pp.281-294.

Rainnie, D.G., Asprodini, E.K. and Shinnick-Gallagher, P.A.T.R.I.C.I.A., 1992. Kindling-induced long-lasting changes in synaptic transmission in the basolateral amygdala. *Journal of Neurophysiology*, 67(2), pp.443-454.

Rajasethupathy, P., Sankaran, S., Marshel, J.H., Kim, C.K., Ferenczi, E., Lee, S.Y., Berndt, A., Ramakrishnan, C., Jaffe, A., Lo, M. and Liston, C., 2015. Projections from neocortex mediate top-down control of memory retrieval. *Nature*, 526(7575), p.653.

Ramirez, S., Liu, X., Lin, P.A., Suh, J., Pignatelli, M., Redondo, R.L., Ryan, T.J. and Tonegawa, S., 2013. Creating a false memory in the hippocampus. *Science*, 341(6144), pp.387-391.

Remigio, G.J., Loewen, J.L., Heuston, S., Helgeson, C., White, H.S., Wilcox, K.S. and West, P.J., 2017. Corneal kindled C57BL/6 mice exhibit saturated dentate gyrus long-

term potentiation and associated memory deficits in the absence of overt neuron loss. *Neurobiology of disease*, 105, pp.221-234.

Represa, A., La Salle, G.L.G. and Ben-Ari, Y., 1989. Hippocampal plasticity in the kindling model of epilepsy in rats. *Neuroscience letters*, 99(3), pp.345-350.

Rivera, C., Li, H., Thomas-Crusells, J., Lahtinen, H., Viitanen, T., Nanobashvili, A., Kokaia, Z., Airaksinen, M.S., Voipio, J., Kaila, K. and Saarma, M., 2002. BDNF-induced TrkB activation down-regulates the K<sup>+</sup>-Cl<sup>-</sup> cotransporter KCC2 and impairs neuronal Cl<sup>-</sup> extrusion. *J Cell Biol*, 159(5), pp.747-752.

Robel, S., Buckingham, S.C., Boni, J.L., Campbell, S.L., Danbolt, N.C., Riedemann, T., Sutor, B. and Sontheimer, H., 2015. Reactive astrogliosis causes the development of spontaneous seizures. *Journal of Neuroscience*, 35(8), pp.3330-3345.

Rothschild, G., Eban, E. and Frank, L.M., 2017. A cortical-hippocampal-cortical loop of information processing during memory consolidation. *Nature neuroscience*, 20(2), p.251.

Ryan, T.J., Roy, D.S., Pignatelli, M., Arons, A. and Tonegawa, S., 2015. Engram cells retain memory under retrograde amnesia. *Science*, 348(6238), pp.1007-1013.

Sander, J.W., 2003. The epidemiology of epilepsy revisited. *Current opinion in neurology*, 16(2), pp.165-170.

Sato, K., Morimoto, K. and Okamoto, M., 1988. Anticonvulsant action of a non-competitive antagonist of NMDA receptors (MK-801) in the kindling model of epilepsy. *Brain research*, 463(1), pp.12-20.

Saucier, D.M. and Corcoran, M.E., 1992. Characteristics of dorsal and ventral striatal kindling in rats. *Epilepsy research*, 11(2), pp.131-139.

Schafe, G.E. and LeDoux, J.E., 2000. Memory consolidation of auditory pavlovian fear conditioning requires protein synthesis and protein kinase A in the amygdala. *Journal of Neuroscience*, 20(18), pp.RC96-RC96.

Schafe, G.E., Atkins, C.M., Swank, M.W., Bauer, E.P., Sweatt, J.D. and LeDoux, J.E., 2000. Activation of ERK/MAP kinase in the amygdala is required for memory consolidation of pavlovian fear conditioning. *Journal of Neuroscience*, 20(21), pp.8177-8187.

Schildt, S., Endres, T., Lessmann, V. and Edelman, E., 2013. Acute and chronic interference with BDNF/TrkB-signaling impair LTP selectively at mossy fiber synapses in the CA3 region of mouse hippocampus. *Neuropharmacology*, 71, pp.247-254.

Shieh, P.B. and Ghosh, A., 1999. Molecular mechanisms underlying activity-dependent regulation of BDNF expression. *Journal of neurobiology*, 41(1), pp.127-134.

Sierra, A., Martín-Suárez, S., Valcárcel-Martín, R., Pascual-Brazo, J., Aelvoet, S.A., Abiega, O., Deudero, J.J., Brewster, A.L., Bernales, I., Anderson, A.E. and Baekelandt, V., 2015. Neuronal hyperactivity accelerates depletion of neural stem cells and impairs hippocampal neurogenesis. *Cell stem cell*, 16(5), pp.488-503.

Singh, S.P., He, X., McNamara, J.O. and Danzer, S.C., 2013. Morphological changes among hippocampal dentate granule cells exposed to early kindling-epileptogenesis. *Hippocampus*, 23(12), pp.1309-1320.

Sivakumaran, S., Cardarelli, R.A., Maguire, J., Kelley, M.R., Silayeva, L., Morrow, D.H., Mukherjee, J., Moore, Y.E., Mather, R.J., Duggan, M.E. and Brandon, N.J., 2015. Selective inhibition of KCC2 leads to hyperexcitability and epileptiform discharges in hippocampal slices and in vivo. *Journal of Neuroscience*, 35(21), pp.8291-8296.

Sloviter, R.S., 1987. Decreased hippocampal inhibition and a selective loss of interneurons in experimental epilepsy. *Science*, 235(4784), pp.73-76.

Sloviter, R.S., Zappone, C.A., Bumanglag, A.V., Norwood, B.A. and Kudrimoti, H., 2007. On the relevance of prolonged convulsive status epilepticus in animals to the etiology and neurobiology of human temporal lobe epilepsy. *Epilepsia*, 48(s8), pp.6-10.

Sofroniew, M.V., 2009. Molecular dissection of reactive astrogliosis and glial scar formation. *Trends in neurosciences*, 32(12), pp.638-647.

Specchio, L.M., Tramacere, L., La Neve, A. and Beghi, E., 2002. Discontinuing antiepileptic drugs in patients who are seizure free on monotherapy. *Journal of Neurology, Neurosurgery & Psychiatry*, 72(1), pp.22-25.

Stoop, R. and Poo, M.M., 1996. Synaptic modulation by neurotrophic factors: differential and synergistic effects of brain-derived neurotrophic factor and ciliary neurotrophic factor. *Journal of Neuroscience*, 16(10), pp.3256-3264.

Sutula, T. and Steward, O., 1987. Facilitation of kindling by prior induction of long-term potentiation in the perforant path. *Brain research*, 420(1), pp.109-117.

Sutula, T., Koch, J., Golarai, G., Watanabe, Y. and McNamara, J.O., 1996. NMDA receptor dependence of kindling and mossy fiber sprouting: evidence that the NMDA receptor regulates patterning of hippocampal circuits in the adult brain. *Journal of Neuroscience*, 16(22), pp.7398-7406.

Szapacs, M.E., Mathews, T.A., Tessarollo, L., Lyons, W.E., Mamounas, L.A. and Andrews, A.M., 2004. Exploring the relationship between serotonin and brain-derived neurotrophic factor: analysis of BDNF protein and extraneuronal 5-HT in mice with reduced serotonin transporter or BDNF expression. *Journal of neuroscience methods*, 140(1-2), pp.81-92.

Takahashi, M., Hayashi, S., Kakita, A., Wakabayashi, K., Fukuda, M., Kameyama, S., Tanaka, R., Takahashi, H. and Nawa, H., 1999. Patients with temporal lobe epilepsy show an increase in brain-derived neurotrophic factor protein and its correlation with neuropeptide Y. *Brain research*, 818(2), pp.579-582.

Tanaka, S., Kondo, S., Tanaka, T. and Yonemasu, Y., 1988. Long-term observation of rats after unilateral intra-amygdaloid injection of kainic acid. *Brain research*, 463(1), pp.163-167.

Tanaka, T., Kaijima, M., Yonemasu, Y. and Cepeda, C., 1985. Spontaneous secondarily generalized seizures induced by a single microinjection of kainic acid into unilateral amygdala in cats. *Electroencephalography and clinical neurophysiology*, 61(5), pp.422-429.

Téllez-Zenteno, J.F. and Hernández-Ronquillo, L., 2012. A review of the epidemiology of temporal lobe epilepsy. *Epilepsy research and treatment*, 2012.

Thurman, D.J., Logroscino, G., Beghi, E., Hauser, W.A., Hesdorffer, D.C., Newton, C.R., Scorza, F.A., Sander, J.W., Tomson, T. and Epidemiology Commission of the International League Against Epilepsy, 2017. The burden of premature mortality of epilepsy in high-income countries: A systematic review from the Mortality Task Force of the International League Against Epilepsy. *Epilepsia*, 58(1), pp.17-26.

Torta, R. and Keller, R., 1999. Behavioral, psychotic, and anxiety disorders in epilepsy: etiology, clinical features, and therapeutic implications. *Epilepsia*, 40(s10).

Treit, D. and Fundytus, M., 1988. Thigmotaxis as a test for anxiolytic activity in rats. *Pharmacology Biochemistry and Behavior*, 31(4), pp.959-962.

Türeyen, K., Vemuganti, R., Sailor, K.A. and Dempsey, R.J., 2004. Infarct volume quantification in mouse focal cerebral ischemia: a comparison of triphenyltetrazolium

chloride and cresyl violet staining techniques. *Journal of neuroscience methods*, 139(2), pp.203-207.

Vaynman, S., Ying, Z. and Gomez-Pinilla, F., 2004. Hippocampal BDNF mediates the efficacy of exercise on synaptic plasticity and cognition. *European Journal of Neuroscience*, 20(10), pp.2580-2590.

Vetencourt, J.F.M., Sale, A., Viegi, A., Baroncelli, L., De Pasquale, R., O'leary, O.F., Castrén, E. and Maffei, L., 2008. The antidepressant fluoxetine restores plasticity in the adult visual cortex. *Science*, 320(5874), pp.385-388.

Wang, H., Shimizu, E., Tang, Y.P., Cho, M., Kyin, M., Zuo, W., Robinson, D.A., Alaimo, P.J., Zhang, C., Morimoto, H. and Zhuo, M., 2003. Inducible protein knockout reveals temporal requirement of CaMKII reactivation for memory consolidation in the brain. *Proceedings of the National Academy of Sciences*, 100(7), pp.4287-4292.

Wang, Y., Xu, C., Xu, Z., Ji, C., Liang, J., Wang, Y., Chen, B., Wu, X., Gao, F., Wang, S. and Guo, Y., 2017. Depolarized GABAergic Signaling in Subicular Microcircuits Mediates Generalized Seizure in Temporal Lobe Epilepsy. *Neuron*, 95(1), pp.92-105.

Williams-Karnesky, R.L., Sandau, U.S., Lusardi, T.A., Lytle, N.K., Farrell, J.M., Pritchard, E.M., Kaplan, D.L. and Boison, D., 2013. Epigenetic changes induced by adenosine augmentation therapy prevent epileptogenesis. *The Journal of clinical investigation*, 123(8), pp.3552-3563.

Wolf-Yadlin, A., Hautaniemi, S., Lauffenburger, D.A. and White, F.M., 2007. Multiple reaction monitoring for robust quantitative proteomic analysis of cellular signaling networks. *Proceedings of the National Academy of Sciences*, 104(14), pp.5860-5865.

Wolf, H.K., Aliashkevich, A.F., Blümcke, I., Wiestler, O.D. and Zentner, J., 1997. Neuronal loss and gliosis of the amygdaloid nucleus in temporal lobe epilepsy. *Acta neuropathologica*, 93(6), pp.606-610.

Woo, N.S., Lu, J., England, R., McClellan, R., Dufour, S., Mount, D.B., Deutch, A.Y., Lovinger, D.M. and Delpire, E., 2002. Hyperexcitability and epilepsy associated with disruption of the mouse neuronal-specific K-Cl cotransporter gene. *Hippocampus*, 12(2), pp.258-268.

Woolf, C.J. and Salter, M.W., 2000. Neuronal plasticity: increasing the gain in pain. *science*, 288(5472), pp.1765-1768.

Yee, K.W., Zeng, Z., Konopleva, M., Verstovsek, S., Ravandi, F., Ferrajoli, A., Thomas, D., Wierda, W., Apostolidou, E., Albitar, M. and O'Brien, S., 2006. Phase I/II study of the mammalian target of rapamycin inhibitor everolimus (RAD001) in patients with relapsed or refractory hematologic malignancies. *Clinical Cancer Research*, 12(17), pp.5165-5173.

Zeng, L.H., Xu, L., Gutmann, D.H. and Wong, M., 2008. Rapamycin prevents epilepsy in a mouse model of tuberous sclerosis complex. *Annals of neurology*, 63(4), pp.444-453.

Zhu, K., Yuan, B., Hu, M., Feng, G.F., Liu, Y. and Liu, J.X., 2017. Reduced abnormal integration of adult-generated granule cells does not attenuate spontaneous recurrent seizures in mice. *Epilepsy research*, 133, pp.58-66.

## Biography

Kamesh Krishnamurthy was born in Walnut Creek CA on August 11, 1987. He received a Bachelors degree in 2010 from the University of Pittsburgh, in Political Science (American Politics) and Bioengineering (Neural Engineering) with minors in Neuroscience and Chemistry. He then enrolled in the Duke Medical Scientist Training Program in 2010. During his preclinical years he worked with Dr. David Berkoff on the use of technology to improve patient education; this led to a publication in the International Journal of Sports Physical Therapy titled “Corrected Error Video versus a Physical Therapist Instructed Home Exercise Program: Accuracy of Performing Therapeutic Shoulder Exercises” (Berkoff DJ, Krishnamurthy K et al. 2016). He then received my first grant (Wakeman Fellowship for Neurobiology MD-PhD students) and joined the Neurobiology PhD program in 2012. He first rotated in the lab of Dr. Daniel Laskowitz, where I worked on a book chapter titled “Cellular and molecular mechanisms of secondary neuronal injury following traumatic brain injury” (Krishnamurthy K and Laskowitz DT 2016). His thesis project has focused on the mechanisms of epilepsy progression and persistence, and has been funded by an American Epilepsy Society Pre-Doctoral Fellowship. He will receive both an MD and PhD in 2019.