

TrkB and Epileptogenesis

by

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Date: August 27, 2007

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

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Abstract

Discovering the cellular and molecular mechanisms underlying the pathophysiology underlying the development of epilepsy is key to the creation of improved treatments. The neurotrophins and their receptors, in particular BDNF and TrkB, are likely candidates to be involved in the process by which a normal brain becomes epileptic (epileptogenesis). The work presented in the dissertation has investigated the hypothesis that TrkB is a central factor in epileptogenesis in multiple animal models of epilepsy.

Conditional deletion of TrkB in the *Syn-Cre TrkB^{-/-}* mouse prevented nearly all epileptogenesis in the kindling model, despite the ability to have a tonic-clonic seizure. Reduction of TrkB *de novo* in mature *Act-CreER TrkB^{-/-}* mice also delayed epileptogenesis in the kindling model. Additionally, *Syn-Cre TrkB^{+/-}* and *Act-CreER TrkB^{-/-}* mice had impaired persistence of the hyperexcitable state following kindling. It remained unclear from these findings whether reduction of TrkB during and/or following induction of kindling was responsible for the impaired persistence. The inducible *Act-CreER TrkB^{flox/flox}* mice were used to reduce TrkB only after the fully kindled state had been reached and demonstrated that loss of TrkB after completion of kindling impairs persistence of the hyperexcitable state.

Status epilepticus is a medical emergency defined by prolonged continuous seizure activity. Conditional deletion of TrkB in the *Syn-Cre TrkB^{-/-}* mice prevents

sustained seizure activity evident in wild type mice following pilocarpine injection. Furthermore, the *Syn-Cre TrkB^{-/-}* mice may also retain greater sensitivity to diazepam following status epilepticus than control mice. Together with biochemical evidence of TrkB activation during status epilepticus, these findings suggest that TrkB activation is required for persistence of status epilepticus.

In conclusion, the findings in this dissertation demonstrate TrkB to be a molecular mechanism critical for: 1) epileptogenesis in the kindling model; 2) persistence of hyperexcitability in the kindling model; 3) persistence of limbic status epilepticus in a chemoconvulsant model. These discoveries provide the basis for developing novel therapeutic approaches to three distinct and devastating aspects of the limbic epilepsy in humans. These aspects are: 1) preventing progression of limbic epilepsy to a medically refractory state; 2) reversal of medically refractory limbic epilepsy; 3) medically refractory status epilepticus.

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1. Introduction

Understanding epileptogenesis at a cellular and molecular level may provide novel targets for pharmacologic interventions to treat or prevent epilepsy. Kindling is a commonly studied animal model of epileptogenesis in which repeated pathological activity in the form of evoked focal seizures produces a permanent hyperexcitable state representative of epilepsy (D. C. McIntyre, 2006; T. P. Sutula and J. Ockuly, 2006). However, the mechanisms underlying the pathological plasticity in the kindling model are still being elucidated. Neurotrophin signaling is a promising potential molecular mechanism to convert the fleeting activation of repeated focal seizures into long-lasting changes seen in the epileptic brain (J. O. McNamara et al., 2006).

I hypothesize that activation of the neurotrophin receptor TrkB is required for limbic epileptogenesis in the mature brain and that continued TrkB signaling is required for the persistence of the hyperexcitable state following kindling. I have tested this hypothesis using conditional deletion of TrkB (*Syn-Cre TrkB^{lox/lox}*) to circumvent the neonatal lethality caused by complete elimination of TrkB (R. Klein et al., 1993) and studied epileptogenesis, persistence of the kindled state, and status epilepticus in these mice. I have used an inducible conditional knock-out of TrkB (*Act-CreER TrkB^{lox/lox}*) to examine the role of TrkB in the mature brain for epileptogenesis and the role of TrkB in the persistence of the undiminished hyperexcitable state following kindling

development. The initial steps in generating more advanced transgenic mice to further control the elimination of TrkB (BAC *Syn-CreER TrkB^{flox/flox}*) and thereby more precisely define the role of TrkB in epilepsy were taken.

1.1 Epilepsy

Epilepsy is a prevalent and severe chronic neurological disorder defined by the repeated occurrence of unprovoked seizures. Epilepsy occurs in approximately 1% of the population (J. W. Sander and S. D. Shorvon, 1996) or approximately 65 million people worldwide. About 5-10% of the population will experience a seizure at some point in their lives and a new diagnosis of epilepsy can occur at any point in a person's life, although the incidence of new cases is highest in the young and in the elderly (W. H. Theodore et al., 2006). Therefore, with the population's increasing life expectancy, the number of cases of epilepsy is expected to rise in the near future. While a few types of epilepsy spontaneously resolve, many individuals with epilepsy are burdened with a life-long struggle to control their disease. Epilepsy not only has direct medical consequences for the individual, but also a multitude of social costs and limitations. For instance, many states have driving restrictions for individuals who have had a seizure, severely limiting mobility and employment opportunities. In fact, when measured in terms of disability-adjusted life years (DALYs), epilepsy has a global disease burden equal to lung cancer in men and breast cancer in women (C. J. L. Murray and A. D. Lopez, 1994). Not only is epilepsy a brutal disease, but the available treatments can be

nearly as difficult to tolerate because of severe side effects. Many of the current pharmacological therapies for epilepsy act to treat seizures symptomatically by generally dampening neuronal excitability and, unsurprisingly, this therapeutic approach causes complications in the course of normal daily life. For these reasons, research into the basic mechanisms of epilepsy is particularly important since identification of molecular actors required for the pathophysiology of epilepsy would provide enticing targets for treatments that may be more effective and may act with less effects on normal brain functioning.

1.1.1 Clinical Presentation

The *sine qua non* of epilepsy is repeated unprovoked seizures. A seizure is a fleeting change in behavior due to the abnormal, synchronous firing of populations of CNS neurons. Seizures often present with physical and/or psychic manifestations, such as loss of consciousness and tonic-clonic muscle contractions. The clinical categorization of seizures is based on the scheme of the International League Against Epilepsy (1989), which divides seizures broadly into partial and generalized seizures. Partial seizures are isolated to a focal location in the brain, in contrast to generalized seizures which involve both hemispheres of the brain. Partial seizures are further divided depending on whether consciousness is interrupted. Simple partial seizures do not cause loss of consciousness, while complex partial seizures cause the sufferer to become unconscious. Partial seizures can secondarily generalize, in which the initially focal electrographic

activity spreads to involve both hemispheres. Generalized seizures are classified as absence, myotonic, clonic, tonic, tonic-clonic, and atonic seizures.

Partial epilepsy is the most common type of epilepsy (approximately 50% of epilepsy cases). Partial epilepsy often originates in the temporal lobe, although a precise focus is not always identified. The natural history of partial epilepsy often includes a previous insult (e.g. complicated febrile seizures lasting longer than 15 minutes and recurring within 24 hours, CNS infection, or head trauma) that occurred several years earlier. Partial epilepsy is important not just because of its prevalence, but also because of the difficulty in effective treatment. Even with appropriate medical management, 30 – 40% of patients will continue to have uncontrolled seizures (P. Kwan et al., 2001).

The pathologic features seen in the epileptic brain can be either causative of the disease or resultant from the disease. In some instances, such as vascular malformation, neoplasm, or trauma, the pathology is clearly causative. In other cases, such as hippocampal sclerosis, the relationship to the disease is less clear. Hippocampal sclerosis, the atrophy of the hippocampus with neuronal loss and gliosis, was identified in patients with epilepsy in 1825 (C. Bouchet and M. Cazauviel, 1825), and its significance has been debated since. Neuronal loss in hippocampal sclerosis usually is approximately 50% of cells in all pyramidal cell fields and in the dentate gyrus (J. H. Kim, 2001). Hippocampal sclerosis is a common finding in temporal lobe epilepsy (present in 84.6% of cases) and is less common in non-temporal lobe epilepsy (present in

38.5% of cases) (J. H. Margerison and J. A. Corsellis, 1966). Sclerosis and neuronal loss can also be found in many other brain structures, in particular the amygdala (about 25% of cases; (J. H. Margerison and J. A. Corsellis, 1966)). The amygdala is often considered to be part of the epileptic circuit in the brain and, in support of this assertion, one series of surgical cases found amygdala-only resections to be curative in 8 of 70 patients (R. Jooma et al., 1995).

The diagnosis of epilepsy is typically made from a history of repeated unprovoked seizures and may be confirmed by long-term video electroencephalogram (EEG) monitoring. EEG, brain MRI, and PET may be used to localize the lesion. The causes of symptomatic epilepsy are numerous and include genetic mutations, perinatal complications, stroke, head injury, infections, and many others. Additionally, idiopathic epilepsy does not have a defined cause although it is thought that the majority of cases are due to genetic conditions.

Another important type of seizure is status epilepticus, in which the seizure activity is continuous for an extended period of time (at least 5 to 30 minutes, but status epilepticus can last days). The causes of status epilepticus are numerous and include uncontrolled seizures in an individual with epilepsy (34% of status epilepticus cases, (R. J. DeLorenzo et al., 1996)), as well as from encephalitis, brain tumor, alcohol withdrawal, and other metabolic imbalances. Status epilepticus can either be convulsive or non-convulsive, which can cause difficulties with diagnosis. Status epilepticus is a relatively

common condition, with nearly 200,000 cases a year in the US, and has a high cost in terms of life (42,000 deaths per year in the US) and also in fiscal terms (\$3.8 - 7 billion a year) (R. J. DeLorenzo et al., 1996). Importantly, while status epilepticus is associated with epilepsy in some cases and may have important mechanistic similarities with seizures in epilepsy, status epilepticus is not synonymous with epilepsy since it does not necessarily involve repeated seizures and it is often precipitated by other medical conditions.

1.1.2 Treatment of Epilepsy

Current pharmacological treatments for epilepsy focus on symptomatic relief, not modification of the underlying pathophysiology, and therefore may be properly classed as anti-seizure or anti-convulsant drugs. Pharmacological treatments for epilepsy center on manipulating the net excitability of neurons by limiting firing by slowing the recovery of Na⁺ channels from desensitization, blocking Ca²⁺ channels, by potentiating inhibitory GABAergic synaptic transmission, or by blocking excitatory glutamatergic synaptic transmission. In the most common type of epilepsy, complex partial epilepsy, optimal seizure control is only achieved in approximately one-third of patients (P. Kwan et al., 2001). Surgical resection may be employed in a few select cases (<1%, (J. Engel, 2003)) and may be curative.

1.2 Research models

Studies of epilepsy can be arranged along a spectrum ranging from clinical studies in human patients to basic science studies at the molecular scale. While clinical trials on human patients with epilepsy are obviously the final step in the introduction of a new treatment, many earlier steps are necessary to identify a therapeutic target, to determine its potential, and to design a method to manipulate that target. These steps need to be done in a laboratory setting with well-defined models of epilepsy that accurately represent the disease. Finally, studies using animal models not only promote the development of novel treatments for human patients, but human patients also guide the work on animal models. For instance, a genetic deficit isolated from a family with epilepsy can be recreated in a mouse model to further our knowledge of that family's disease as well as epilepsy in general.

1.2.1 Animal models

Animal models of epilepsy allow for the study of epilepsy in ways that would be prohibitively expensive and often unethical in human subjects. The range of animal models in part reflects the diversity seen in different types of epilepsy. Additionally, while some animal models of epilepsy seek to exactly mimic the human condition, often models intentionally recreate epilepsy in a manner such that particular aspects of the disease are highlighted. This enlarged representation of specific facets of epilepsy allows for the pathological processes underlying those facets of the disease to be

examined in detail. Wada commented that the features of a useful model of epilepsy include 1) precise experimental control over the site of seizure induction; 2) an ability to create an epileptic site without producing gross pathology; 3) good control over the initiation of individual seizures and their progressive development; 4) easy induction of a seizure by an identified stimulus; 5) eventual development of spontaneous seizures; and 6) persistence of the epileptic state for long periods (J. A. Wada, 1976). For example, the kindling model of epilepsy allows for precise control by the experimenter over the timing and focus of seizures and therefore permits each stage of epileptogenesis during the progression to a permanent epileptic state with spontaneous seizures to be examined in a precise manner.

1.2.1.1 Kindling model

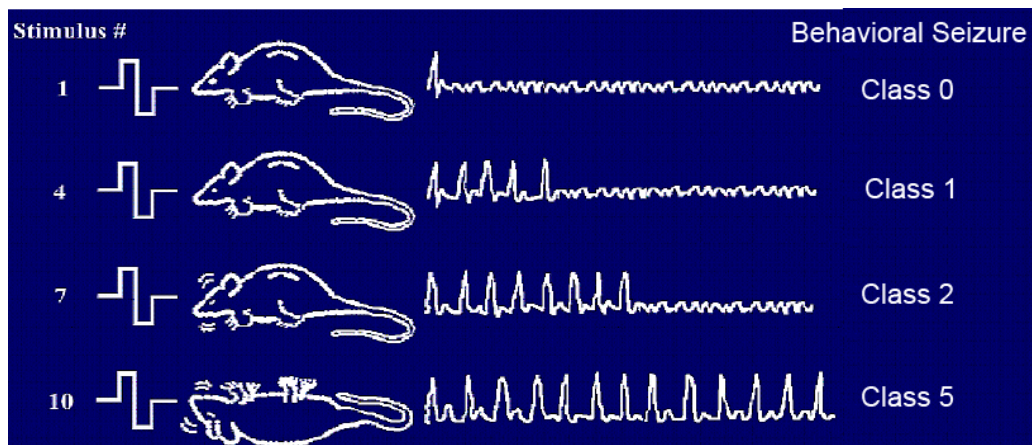


Figure 1: The kindling model of epilepsy.

The kindling model of epilepsy is produced by repeatedly evoking electrographic seizures. In this example, the same intensity stimulation that produces a short electrographic seizure and no behavioral seizure on the first stimulation

produces a longer electrographic seizure and behavioral freezing (Class 1) on the 4th stimulation. The 7th stimulation produces a longer electrographic seizure and head nodding (Class 2) and the 10th stimulation, still at the same intensity as the 1st stimulation, produces a lengthy electrographic seizure with rearing and loss of balance (Class 5).

Kindling is an animal model of epileptogenesis in which repeated evoked seizures gradually develop from focal electrographic seizures into secondarily generalized tonic-clonic seizures (Figure 1). The kindling phenomenon was discovered serendipitously by Graham Goddard (G. V. Goddard, 1967), when he was attempting to disrupt learning with repeated electrical stimulation of the hippocampus. Instead he found that the repeated stimulations evoked seizures that increased in intensity and that this induced hyperexcitability persisted for the life of the animal (G. V. Goddard et al., 1969). Systematic study of the kindling phenomenon revealed that repeated stimulations first reduce the threshold to evoke an electrographic seizure at the site of stimulation, after which the stimulations evoke a reproducible pattern of lengthening electrographic seizures accompanied by spread to other brain structures and behavioral seizures that also increase in severity. Racine (R. J. Racine, 1972a) developed a classification scheme for the behavioral seizures that is employed in nearly all kindling studies: Class 1) facial clonus; 2) head nodding; 3) unilateral forelimb clonus; 4) rearing with bilateral forelimb clonus; 5) rearing and falling with loss of postural control.

The kindling model is typically performed through placement of a bipolar electrode into a limbic structure, commonly the amygdala or the hippocampus, of a

rodent. The key prerequisite of each kindling stimulation is that repetitive, synchronized firing of neurons, i.e. an “afterdischarge”, is elicited (R. J. Racine, 1972b). The afterdischarge is evoked by an electrical stimulation consisting of 1ms biphasic pulses applied at a frequency of 50-100 Hz, often at the lowest intensity that evokes an afterdischarge at the site of stimulation. The stimuli are given at an interval long enough so that the recovery from the preceding stimulation does not affect the current stimulation, often twice a day with at least a 4 hour inter-stimulus interval (R. J. Racine et al., 1973). Often the stimulations are repeated until the animal has several (3 to 5) consecutive seizures of Class 4 or greater. The hyperexcitable state resulting from these repeated stimulations is permanent (J. A. Wada et al., 1974). This is the end of the most clearly epileptogenic process in the kindling model since continued stimulations will plateau in regards to the behavioral seizure produced, but continued stimulations will eventually (>80-90 stimulations) generate an animal with spontaneous seizures (J. P. Pinel and L. I. Rovner, 1978; U. Sayin et al., 2003).

The known acute effects following repeated evoked seizures are predominantly functional changes at existing synapses. These alterations occur as early as the first evoked seizure, when both the amount of current carried by NMDA receptors (I. Mody and U. Heinemann, 1987) and the amount of GABA_A receptor mediated inhibition increases in dentate granule cells (M. W. Oliver and J. J. Miller, 1985). The increase in NMDA-dependent current may reduce the normal gating function of the dentate

granule cells and allows easier propagation of activity to the CA3 pyramidal cells. The increase in inhibition is likely an inadequate response to limit the increasing excitability and the loss of the increased inhibition is coincident with the development of spontaneous seizures (U. Sayin et al., 2003). The increased inhibition seen early in kindling highlights the need to distinguish pathological changes which underlie the disease from protective changes made to limit the pathology.

The putative mechanisms underlying the long-term plasticity seen in the kindling model can be divided into two interrelated categories, permanent functional changes and morphological changes. The mechanisms by which the acute changes effect the long-term increase in excitability are not fully known, although influx of Ca^{2+} through the NMDA receptors (A. E. West et al., 2001) and increased signaling by neurotrophins (e.g. BDNF) through their receptors (e.g. TrkB) are two likely mechanisms for mediating the permanent alternations seen in the kindling model. For example, messenger RNA for the neurotrophin BDNF is increased in the hippocampus after a single epileptiform discharge (P. J. Isackson et al., 1991) and limbic seizures increase BDNF protein (Q. Yan et al., 1997; J. S. Rudge et al., 1998) and cause phosphorylation of its receptor, TrkB (D. K. Binder et al., 1999a; X. P. He et al., 2002).

Morphological changes are evident later in the kindling progresses. One of the most striking changes is the emergence of new synapses by the mossy fiber axons (T. Sutula et al., 1988). The mossy fibers, named by Ramon y Cajal (S. Ramon y Cajal, 1911)

because of the numerous varicosities along their length, are the axons of the dentate granule cells which synapse on the CA3 pyramidal cells and interneurons in stratum lucidum (SLINs). After as few as 3 Class 5 kindled seizures these axons can be seen forming aberrant synapses in the inner molecular layer of the dentate gyrus and also into the infrapyramidal layer of CA3 (T. Sutula et al., 1988). The number of synapses in these abnormal locations increases with additional evoked seizures and the synapses are permanent (J. E. Cavazos et al., 1991). The synapses formed by these abnormal axons are predominately excitatory (P. S. Buckmaster et al., 2002) and may contribute to the hyperexcitability of the kindled state.

Kindling also induces both cell loss and neurogenesis. Increased cell death as measured by TUNEL staining can be found in the hilus of the dentate gyrus after a single seizure in the kindling protocol (J. Bengzon et al., 1997). Repeated stimulations (>70 Class 5 seizures) lead to sufficient neuronal loss to be detected by stereological counting in the dentate gyrus, CA1, and CA3 (J. E. Cavazos et al., 1994; R. Kotloski et al., 2002), a pattern similar to hippocampal sclerosis in human patients. Furthermore, specific populations of interneurons undergo cell death coincident with the decrease in inhibition and the emergence of spontaneous seizures (U. Sayin et al., 2003). In addition to cell death, studies have also shown increased cell proliferation in the dentate gyrus as measured by BrdU staining (J. Bengzon et al., 1997) and these newborn cells are functionally integrated into the hippocampus (H. E. Scharfman et al., 2002). These

changes in the cellular composition of the hippocampus are a clear way in which the transitory seizures are translated into permanent alterations.

Glial cells also proliferate and hypertrophy during kindling (A. Hansen et al., 1990; M. Khurgel and G. O. Ivy, 1996) and undergo microglial activation (J. Szyndler et al., 2002). While glia responses to kindling have not been studied extensively, it is possible that they contribute to the hyperexcitable state through their management of the extracellular environment or perhaps even by direct release of glutamate onto neurons (Fellin et al., 2004).

1.2.1.2 Status epilepticus models

Another approach to studying epilepsy involves status epilepticus. Animal models of status epilepticus are often used to study epileptogenesis since many animals will develop late-onset spontaneous seizures weeks after a bout of status epilepticus. While status epilepticus may be induced in many ways (such as pilocarpine, kainic acid, or electrical stimulation), in all cases the continuous seizure activity becomes self-sustaining and persists even after the inciting stimulus is gone. This model of epileptogenesis can be thought of as mimicking the human condition in which prolonged febrile convulsions are thought to lead to the development of spontaneous seizures (epilepsy) later in life (K. B. Nelson and J. H. Ellenberg, 1978). Additionally, these models can be used to study status epilepticus itself, which is in dire need of improved understanding and improved treatments.

The pilocarpine model of epilepsy utilizes the cholinergic agonist pilocarpine to induce seizures and was first developed by Turski and colleagues (W. A. Turski et al., 1983; W. A. Turski et al., 1984). Cholinergic activation likely acts to trigger increased glutamate release and, importantly, to allow the increased glutamate release to be maintained (E. A. Cavalheiro et al., 2006). Rats subjected to status epilepticus without use of anti-seizure treatments, in which status epilepticus usually lasts from 6 to 12 hours, uniformly develop spontaneous seizures (J. P. Leite et al., 1990; E. A. Cavalheiro et al., 1991). The development of spontaneous seizures after status epilepticus displays a progression of increasing intensity seizures, similar to the progression seen in the kindling model. The pathologies seen in the pilocarpine model are numerous and include cell death (W. A. Turski et al., 1983; W. A. Turski et al., 1984)(L. Covolan and L. E. Mello, 2000), increased cell proliferation (L. Covolan et al., 2000), aberrant cell migration (L. E. Mello et al., 1992), abnormal axons (J. V. Nadler et al., 1980; L. E. Mello et al., 1993) and dendrites (I. Spigelman et al., 1998), functional changes at synapses (M. H. Abegg et al., 2004), and changes to intrinsic excitability of neurons (dendritic channelopathies) (C. Bernard et al., 2004). *Bdnf* mRNA has been shown to increase in expression in the dentate gyrus following status epilepticus (G. Mudo et al., 1996) and phosphorylation of TrkB increases (D. K. Binder et al., 1999a; X. P. He et al., 2002).

Some drugs, such as benzodiazepines and barbiturates, can potentially attenuate status epilepticus and prevent the development of spontaneous seizures through

reduction of the initial insult (T. Lemos and E. A. Cavalheiro, 1995). However, other pharmacologic interventions, such as the non-competitive NMDA receptor antagonist MK-801, can prevent the development of spontaneous seizures without overtly affecting status epilepticus (A. C. Rice and R. J. DeLorenzo, 1998). MK-801 has even been shown to prevent emergence of late-onset spontaneous seizures when given 1.5 hours after status epilepticus has begun (A. Prasad et al., 2002), implying that a window of opportunity for prevention of epileptogenesis exists even after a considerable period of continuous seizure activity has occurred. Indeed, it seems possible that epileptogenesis may still be prevented even after the status epilepticus has occurred.

1.2.2 Validity of animal models

The kindling model supposes a progressive nature to epilepsy. In 1881 William Gowers proposed that “seizures beget seizures” based on the worsening of the condition of his patients who suffered repeated seizures in the face of limited treatments (W. R. Gowers, 1881). While the progressive nature of epilepsy has been debated, evidence suggests that at least some forms of epilepsy, such as temporal lobe epilepsy, are progressive. For instance, temporal lobe epilepsy is more likely to develop late-onset intractability than epilepsy with a neocortical focus (A. T. Berg et al., 2006). This observation has important implications for the treatment of epilepsy, so that rapid and complete control of the seizures is a priority and the mechanisms by which the

individual seizures contribute to the disease process (epileptogenesis) are also a treatment target.

The progressive nature of epilepsy can be seen by increasing pathology with increased seizures. Studies have demonstrated a correlation between the duration of temporal lobe epilepsy and a decrease in the size of the hippocampus measured by MRI (W. H. Theodore et al., 1999). Further analysis of the patients examined revealed a correlation with not only duration of epilepsy, but in particular with frequent seizures (T. Salmenpera et al., 1998). In fact some studies have found that the strongest correlation is not with the duration of epilepsy, but with the frequency of refractory seizures (R. Kalviainen et al., 1998). Evidence exists that epilepsy becomes more difficult to treat with duration as well. In a survey of 2200 patients, a longer duration of epilepsy was associated with a reduced probability of being seizure-free for a year (F. Semah et al., 1998). Berg and colleagues found a similar increase in intractable epilepsies with time (A. T. Berg et al., 2006). However, all these studies of epilepsy progression are potentially confounded by the possibility that treatment alters the natural history of the disease.

Despite evidence for progression in epilepsy, little direct evidence exists for kindling in humans. Given that kindling occurs in every animal tested, including a wide range of mammals including primates (J. A. Wada et al., 1978), it is likely that the same pathological plasticity would occur in humans subjected to repeated electrical

stimulations. Although, kindling is more difficult in some primates (rhesus monkeys) (J. A. Wada et al., 1978), suggesting that kindling development in humans may be significantly slower than is seen in rodent models. A case report of complex partial seizures developing following repeated thalamic stimulation with depth electrodes for uncontrolled chronic pain (M. Smramka et al., 1977) suggests that the kindling phenomenon is possible in humans.

However, the question is not whether kindling can occur in humans, but whether the kindling process underlies the progression of epilepsy. In a clinical finding supporting Gower's dictum that "seizures beget seizures", Elwes and colleagues (R. D. Elwes et al., 1988) found that the interval between seizures decreased with repeated seizures in a retrospective study of 183 patients who presented for initial treatment. This phenomenon is consistent with the observation in kindling that repeated seizures lower the threshold for future seizures (R. J. Racine, 1972b). While humans with epilepsy often do not show the progressive changes seen in the early stages of the kindling model, it may be that these individuals are at a stage comparable to the later stages of kindling, in which the repeated stimulations reliably evoke a tonic-clonic seizure with little obvious progression from one stimulation to the next (D. C. McIntyre, 2006). In the kindling model it is only after numerous stimulations, at the stage when the behavioral seizure progression is less obvious, that spontaneous seizures occur. An analogous condition in humans may be that of anomalous pathological activity

originating from a focal pathology, such as a cortical malformation, tumor, or a site of trauma, “kindles” the brain.

Correlates between the later stages of kindling and epilepsy can be seen in some clinical cases. The earliest progressive stages of the pathophysiology may be clinically silent if no behavioral seizures are produced. However, as the kindling model demonstrates that repeated seizures cause more severe seizures and eventually results in seizures that are independent of the inciting stimulation. Evidence consistent with such a hypothesis is that patients undergoing surgical treatment for epilepsy, who have failed pharmacological management of their epilepsy and therefore have experienced many seizures, are more likely to have their epilepsy successfully treated by surgery if both the causal lesion (e.g. a tumor) and the hippocampus and amygdala are removed, even if the tumor does not directly involve these structures (L. M. Li et al., 1999). Often microscopic analysis of the tissue after resection reveals that the hippocampus and amygdala do in fact have pathological changes (H. K. Wolf et al., 1993).

1.2.3 Identification and validation of treatment targets

The most common animal model for the identification of new anti-seizure drugs is the maximal electroshock (MES) model, in which a strong current is applied through the brain on the animal to evoke a stereotyped tonic-clonic seizure. This model was first used by Merritt and Putnam (T. J. Putnam and H. H. Merritt, 1937) to identify phenytoin as an anti-convulsant. The success of phenytoin as a treatment for epilepsy in part

contributed to MES becoming the preferred model for testing for efficacy in treating generalized tonic-clonic seizures. Given that currently available treatments for epilepsy were identified by their ability to inhibit seizures acutely in a model without the long-term aspects of epilepsy, it is not surprising that these treatments are anti-convulsants without having anti-epileptogenic activity.

The kindling model has been used for initial drug testing and has led to the identification of the drug levetiracetam as a treatment for epilepsy, which had not been identified in previous models. Levetiracetam was demonstrated to both inhibit evoked seizures in fully kindled rats (W. Löscher and D. Honack, 1993), as well as specifically delaying kindling development (W. Löscher et al., 1998). Additionally, phenobarbital (J. M. Silver et al., 1991), diazepam (M. Schmutz et al., 1985), and valproic acid (J. M. Silver et al., 1991) impair kindling development.

Pilocarpine-induced status epilepticus, while a commonly used model to study epilepsy, has not been used in the discovery of new drug treatments, although the understanding of known treatments has been advanced by study in this model. Additionally proposals to identify drugs with anti-epileptogenic potential by looking for an effect on the development of late-onset spontaneous seizures following status epilepticus have been put forward (W. Löscher, 2006). Optimal treatment of status epilepticus, both acutely and with regard to long-term sequelae, is studied using the pilocarpine model (A. C. Rice and R. J. DeLorenzo, 1998; A. Prasad et al., 2002).

1.3 Neurotrophins and neurotrophin receptors

The first neurotrophin identified was nerve growth factor (NGF) by Hamburger, Levi-Montalcini and colleagues as they were searching for the soluble factor released from targets of innervation that allowed the survival of the neurons (R. Levi-Montalcini, 1987). Brain-derived neurotrophin factor (BDNF) was isolated as a neurotrophin isolated from 3kg of pig brain that was not blocked by anti-NGF antiserum (Y. A. Barde et al., 1982). Based on the similarities between NGF and BDNF, neurotrophin-3 (NT-3) (K. R. Jones and L. F. Reichardt, 1990) and neurotrophin-4 (NT-4) (N. Y. Ip et al., 1992) were identified based on sequence homology. Of the receptors for the neurotrophins, the low affinity neurotrophin receptor p75 was identified first (D. Johnson et al., 1986) and the tropomyosin-related kinases (Trk) receptors were initially identified as an oncogenic fusion protein with tropomyosin from colon carcinoma in humans (D. Martin-Zanca et al., 1986) and were later identified as high-affinity receptors for the neurotrophins (D. R. Kaplan et al., 1991). Eventually four neurotrophins (NGF, BDNF, NT-3, and NT-4) were identified along with three high-affinity Trk receptors, which selectively bind the neurotrophins (NGF binds TrkA, BDNF and NT-4 bind TrkB, and NT-3 binds TrkC plus TrkB and TrkC at lower affinity) (Figure 2 A) (M. Barbacid, 1995). The p75 receptors bind all the neurotrophins and bind with higher affinity to the pro-neurotrophins (R. A. Segal, 2003). Neurotrophins and both the high and low affinity receptors have been identified in all vertebrate species examined (F. Hallbook, 1999).

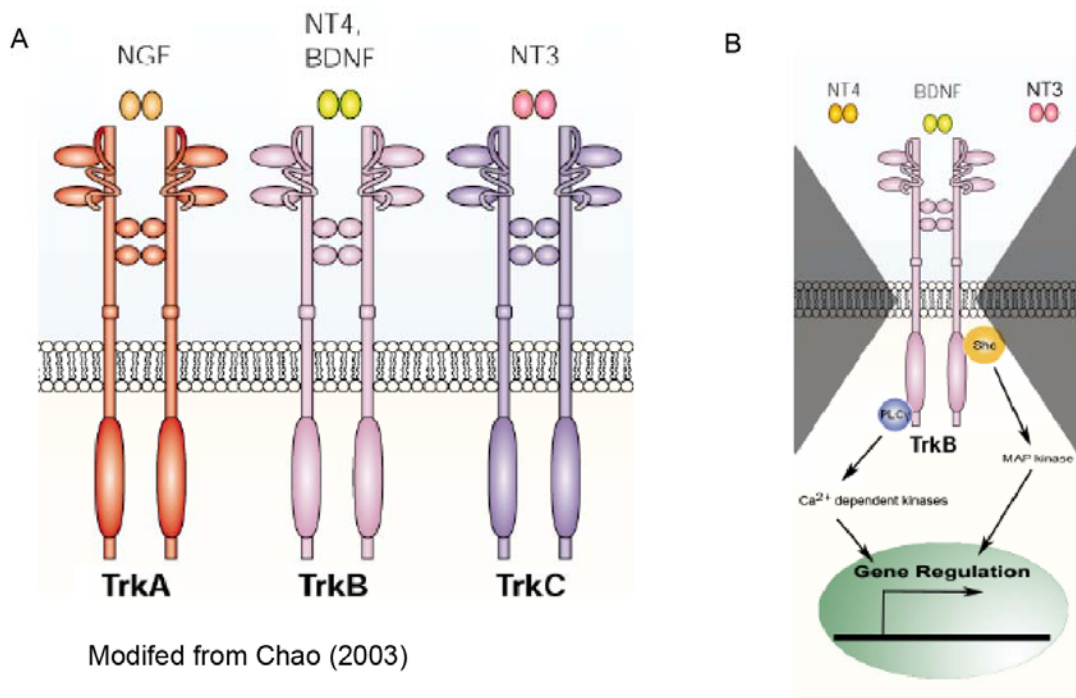


Figure 2: The neurotrophins and their receptors.

A) The neurotrophins and their high-affinity receptors. The neurotrophin NGF binds TrkA, BDNF and NT-4 bind TrkB, and NT-3 binds TrkC with high affinity and both TrkB and TrkC at lower affinity. B) Ligands and downstream signaling of the TrkB receptor. BDNF, NT-4 and NT-3 can all activate TrkB. TrkB activation leads to binding of Shc and PLC γ . BDNF, NT-4 and NT-3 can all activate TrkB. TrkB activation leads to binding of Shc and PLC γ to phosphorylated tyrosine residues which lead to further downstream signaling, including regulation of gene expression.

The neurotrophin signaling cascade is initiated by the neurotrophin binding to its receptor, causing dimerization of receptor, and autophosphorylation of docking sites for adapter proteins (E. J. Huang and L. F. Reichardt, 2001) (Figure 2 B). The adapter proteins dock to the phosphorylated receptor and initiate signaling cascades within the cell. For example, activation of TrkB is known to activate Shc, PLC- γ , and PI-3 kinase

cascades (E. J. Huang and L. F. Reichardt, 2001). Neurotrophin signaling is known to signal both at the cell membrane and also from internalized receptor in signaling endosomes (C. L. Howe and W. C. Mobley, 2005), although the signaling endosomes may be only applicable to select cases.

During development neurotrophins play a critical roles in neuronal survival and in activity-dependent plasticities. Knock-out mutations of the various neurotrophins and their receptors result in loss of specific cell populations and neonatal lethality (M. Barbacid, 1995), with the exception of the viable NT-4 null mice (X. Liu et al., 1995). In an example of activity dependent plasticity, the neurotrophin BDNF and its receptor TrkB are key players in the organization of ocular dominance columns in response to activity in the retinas (R. J. Cabelli et al., 1995; R. A. Galuske et al., 1996; R. J. Cabelli et al., 1997). While the contribution of neurotrophins and their receptors to survival of neurons can be deduced for knock-out mice despite neonatal lethality, the role of neurotrophins in the mature nervous system has had to be studied using heterozygotes, pharmacological blockades (receptor bodies and kinase inhibitors), over-expression or exogenous application of neurotrophins, and recently conditional knock-out mice.

In addition to a role in development, the neurotrophin BDNF and its receptor TrkB are present in the mature brain. Furthermore, the expression of the neurotrophins in the mature brain is activity dependent (C. M. Gall, 1993), implying that the neurotrophins are actively signaling in the mature brain. The actions of BDNF/TrkB in

the mature nervous system can be broadly categorized into functional changes at existing synapses and morphological changes to cells, although these categories are obviously interrelated.

The effects of BDNF/TrkB on synaptic transmission are generally considered to promote excitatory synaptic transmission. BDNF has been shown to facilitate glutamatergic synaptic transmission (V. Lessmann et al., 1994; H. Kang and E. M. Schuman, 1995; E. S. Levine et al., 1995; N. Takei et al., 1997) and to increase NMDA receptor currents through phosphorylation of the NMDA receptor (P.-C. Suen et al., 1997). BDNF has also been shown to illicit rapid depolarization of cells, on the same time scale as traditional neurotransmitters (K. W. Kafitz et al., 1999). BDNF/TrkB signaling has also been shown to attenuate inhibitory synaptic transmission in dissociated neuronal cultures (Q. Cheng and H. H. Yeh, 2003; R. A. Wardle and M. M. Poo, 2003), as well as in CA1 pyramidal cells in acute hippocampal slices (T. Tanaka et al., 1997). In the hippocampus BDNF and TrkB act to increase both excitatory and inhibitory synaptic transmission, although the effect on excitatory synaptic transmission is greater (M. M. Bolton et al., 2000). Consistent with effects on both excitatory and inhibitory signaling, BDNF/TrkB signaling has also been shown to play a key role in homeoplasticity, the changes of synaptic strengths to maintain a constant level of activity, in the cortex (L. C. Rutherford et al., 1997; L. C. Rutherford et al., 1998). Increases in the presynaptic

machinery effected by BDNF/TrkB signaling have been described (L. D. Pozzo-Miller et al., 1999).

BDNF/TrkB signaling is also known to affect the morphology of neurons. The effects of BDNF/TrkB on the structure of neurons is generally consistent with TrkB acting to promote excitatory synaptic transmission. For example, TrkB has been shown to increase spine number and dendritic branching in CA1 pyramidal cells (W. J. Tyler and L. Pozzo-Miller, 2003), dentate granule cells (S. C. Danzer et al., 2002), cortical pyramidal neurons (H. W. Horch and L. C. Katz, 2002), and cerebellar Purkinje cells (A. Shimada et al., 1998).

The effects of BDNF and TrkB are also seen in long-term potentiation (LTP). Exogenous BDNF is known to enhance LTP, while blocking BDNF/TrkB signaling impairs LTP (A. Figurov et al., 1996; H. Kang et al., 1997; G. Chen et al., 1999). The impaired LTP seen in mice with reduced BDNF signaling is restored by exogenous application of BDNF (M. Korte et al., 1995; M. Korte et al., 1996; S. L. Patterson et al., 1996). The action of BDNF/TrkB signaling on LTP appears to be through the phospholipase γ (PLC γ) pathway but not the Shc pathway (L. Minichiello et al., 2002).

1.4 BDNF/TrkB and Epilepsy

BDNF and TrkB are promising candidates for mediating the development of epilepsy. The hypothesis that neurotrophins and their receptors may be involved in epilepsy was launched by the discovery that seizures induce a large increase in *Ngf*

mRNA in the dentate gyrus (C. M. Gall and P. J. Isackson, 1989). Later it was found that following seizures BDNF is increased (P. J. Isackson et al., 1991; H. Nawa et al., 1995; Q. Yan et al., 1997; J. S. Rudge et al., 1998) and TrkB is activated (D. K. Binder et al., 1999a; X. P. He et al., 2002). Additionally BDNF and TrkB are expressed in brain structures, such as the hippocampus, known to be relevant for the development of epilepsy (M. Hofer et al., 1990). *Bdnf* mRNA (K. D. Murray et al., 2000) and protein (M. Takahashi et al., 1999) have also been found to be increased in the hippocampus of the TLE patients. Furthermore, many of the previously described actions of BDNF/TrkB in the mature nervous system are consistent with causing an increase in net excitability. Together these pieces of evidence build a strong case that BDNF and TrkB may be responsible for the pathological plasticity which consolidates the effects of individual seizures into a permanent hyperexcitable state.

Following these observational studies, manipulations of the BDNF/TrkB signaling pathway were shown to have effects consistent with being pro-epileptogenic. Zhu and Roper (W. J. Zhu and S. N. Roper, 2001) demonstrated that exogenously BDNF applied to resected hippocampal tissue from human patients can act to increase excitatory and decrease inhibitory synaptic transmission, likely through TrkB. Mice missing one allele of *Bdnf* have delayed kindling (M. Kokaia et al., 1995), while transgenic over-expression of BDNF resulted in spontaneous seizures and a reduced threshold for kainic acid-induced status epilepticus (S. D. Croll et al., 1999). Bolus

injection of BDNF in the hippocampus with each kindling stimulation accelerated the rate of kindling (B. Xu et al., 2004). Scavenging the ligands for TrkB with an intraventricular infusion of TrkB receptor body (a fusion protein of the ligand binding domain of TrkB with the Fc portion of immunoglobulin) also delayed kindling (D. K. Binder et al., 1999b). Importantly, receptors bodies designed to inhibit TrkA or TrkC signaling did not affect kindling development. Furthermore, the degree of inhibition of kindling by the TrkB receptor body was correlated with the penetration of the receptor body into the hippocampus, perhaps indicating the anatomical location at which TrkB activation is playing a role in the kindling process. While the TrkB receptor body will block both BDNF and NT-4 signaling, BDNF is the more likely candidate since the viable NT-4 knock-out mice kindle normally (X. P. He et al., 2006). Of note, the NT-3 heterozygous mouse has delayed kindling development (E. Elmer et al., 1997), although the TrkC receptor body, which should scavenge NT-3 did not have an effect (D. K. Binder et al., 1999b). Further research into which pathways originating from activated TrkB may be responsible for its pro-epileptogenic actions found no difference in kindling rates in mice with a point mutation of which eliminated the Shc binding site on TrkB (X. P. He et al., 2002). This finding highlights the likely shared mechanisms between kindling and LTP since the Shc point mutation mice also did not display a deficit in induction of LTP in hippocampal slices (L. Minichiello et al., 2002) and

encourages the examination of the PLC γ point mutation mice in the kindling paradigm, which showed a deficiency in the induction of LTP (L. Minichiello et al., 2002).

1.5 Transgenic mice

1.5.1 Cre/loxP transgenics

The use of site-specific recombinases (SSRs) has allowed for DNA rearrangements to be precisely made in transgenic mice at stages later than the embryonic stem cell. The use of SSRs eventually allowed for conditional elimination of genes in transgenic mice (H. Gu et al., 1994). By controlling expression of the SSR, this approach provided a tool to examine the effect of loss of a gene from a particular cell population at a particular time. Such an approach is required to study the gene in an adult mouse when elimination of the gene during development results in neonatal lethality, for example TrkB.

The *Cre/loxP* system uses the Cre recombinase from the P1 bacteriophage and its DNA recognition site, *loxP* (H. Gu et al., 1994). The Cre (cyclization recombinase) recombinase is especially suited for use in transgenic animals since no co-factors are required for it to act. The 34 base-pair *loxP* (locus of crossing over [X] of P1) sites consists of a pair of 13 base-pair inverted repeat sequences with a 8 base-pair spacer sequence in the center that is asymmetric and gives the *loxP* its directionality. When Cre recombinase encounters two *loxP* sites it promotes reciprocal strand exchange between

the two sites. Therefore the relative directions of the *loxP* sites determine the result of the recombination. Two *loxP* sites oriented in opposite directions will invert the intervening stretch of DNA following recombination, while two *loxP* sites with the same orientation will excise the intervening DNA. The Cre/*loxP* system has been used in many transgenic mice (C. Gaveriaux-Ruff and B. L. Kieffer, 2007), in which a gene or portion of a gene is flanked by *loxP* sites (“floxed”) and expression of the Cre recombinase is controlled by a promoter active in the desired cell populations at the desired time in the development of the transgenic mouse, such that the floxed gene will be eliminated conditionally following expression of the recombinase.

In order to add a level of inducibility to the *Cre/loxP* system of conditional knockouts, Metzger and colleagues (D. Metzger et al., 1995) designed a system in which the intracellular location of the Cre protein can be controlled. Since Cre must be in the nucleus to bind to act, confining Cre to the cytosol renders it effectively inactive while translocation to the nucleus allows Cre to excise the floxed sequence. Steroid receptors natively have this property of ligand-induced translocation of the previously purely cytosolic protein to the nucleus. In normal physiology in the absence of its ligand the steroid receptor is bound to HSP-90 in the cytosol and in this state the nuclear localization signal peptide is not exposed. Binding of the ligand to the steroid receptor displaces the HSP-90 and induces a conformational change that exposes the nuclear localization signal. The exposed nuclear localization signal causes translocation of the

receptor to the nucleus. To use this process to control the subcellular localization of Cre, a Cre recombinase-estrogen receptor fusion protein (Cre-ER^{TM2}) was made. Furthermore the ligand binding domain of the estrogen receptor was mutated at two sites so that it was no longer responsive to endogenous estrogen, but only to the selective estrogen receptor modulator (SERM) tamoxifen (ER^{TM2}) (R. Feil et al., 1996; R. Feil et al., 1997; A. K. Indra et al., 1999). As with an unmodified Cre, this CreER^{TM2} fusion protein is then put under the transcriptional control of a selected promoter. The final result, when combined with a floxed DNA sequence, is that in the absence of tamoxifen the CreER^{TM2} protein is confined to the cytosol and the floxed sequence remains intact. When tamoxifen is given to the transgenic animal, it binds to the mutated estrogen receptor portion of the fusion protein and exposes the nuclear localization signal. The CreER^{TM2} moves to the nucleus where it binds to the *loxP* sites and eliminates the floxed sequence from the genome.

In cases where the conditional recombination would be more informative if it could be induced at will, the CreER^{TM2} provides that ability. The inducibility of the system allows for a greater range of choices for the promoter chosen to drive expression of the recombinase. For example, a widely expressed chicken β -*actin* promoter with a CMV enhancer has been used (S. Hayashi and A. P. McMahon, 2002) in an effort to generate a global knock-out, but under temporal control. Additionally, this inducible knock-out system provides for greater flexibility in experiments, for instance

recombination can be induced after the disease model has already been accomplished. Such an experiment could closely mimic the situation of treating a patient who already has the disease. In an example of the potential of this system, Guy and colleagues (J. Guy et al., 2007) used Cre-mediated excision of a floxed *STOP* cassette to allow expression of *Mecp2* in a mouse model of Rett's syndrome (a *Mecp2* knock-out mouse) and showed reversal of the deficits in the mature mouse.

1.5.2 BAC transgenics

The wealth of information regarding the mouse genome along with an increasing catalogue of expression patterns of genes is a phenomenal advance for the development of transgenic mice. While traditional plasmid-derived transgenic mice are incredibly useful, they have two drawbacks. First, the size restrictions imposed by the plasmid require that most often only a portion of the gene promoter is used, if the promoter sequence has even been identified. Other regulatory elements are rarely known and usually too distant to be included in the plasmid construct. Secondly, the small size of the DNA construct inserted into the genome means that the particular insertion site will influence the expression of the transgene. The local genomic regulatory elements present near the site of insertion will affect the expression of the transgene, and therefore each insertion site may produce a different expression pattern.

The use of bacterial artificial chromosomes (BACs) to generate transgenic mice addresses both of these issues (N. Heintz, 2001). Libraries of large (100-300 kb) spans of

the mouse genome are readily available. Manipulation of the BACs in *E. coli* allows for relatively simple modification of the BAC using homologous recombination so that the chosen DNA construct can be inserted into the start site of an endogenous gene. The large size of the BAC often encompasses the important regulatory elements of the endogenous gene to facilitate expression of the transgene with fidelity to the expression pattern of the endogenous gene. Furthermore, the large size of the BAC buffers it from the effects of regulatory elements at the insertion site. Overall, this approach to the generation of transgenic mice, along with the catalogue of data concerning expression patterns of genes allows the rapid generation of cell-type specific transgenic mice.

2. *Synapsin1-Cre TrkB^{flox/flox}* mice

2.1 Introduction

Understanding the mechanisms of limbic epileptogenesis in cellular and molecular terms may lead to effective pharmacologic prevention of this disease. The astute British neurologist, William Gowers, noted worsening of the epileptic condition in some patients and proposed that seizures themselves may beget seizures (W. R. Gowers, 1881). Discovery of the kindling model by Goddard et al. (G. V. Goddard et al., 1969) validated Gowers' idea; in this model, repeated induction of brief, focal seizures by application of initially subconvulsive electrical stimuli eventually results in prolonged, intense focal and tonic-clonic seizures. Once established, this enhanced sensitivity to electrical stimulation persists for the life of the animal.

It remains unclear how pathological levels of neuronal activity in the form of focal seizures might be transduced into the cellular and molecular mechanisms underlying epileptogenesis. The idea emerged that extracellular signaling molecules might be pivotal in the transduction process. Converging lines of evidence support the suggestion that the neurotrophin, BDNF, may promote limbic epileptogenesis by activation of its cognate receptor, TrkB. Thus BDNF promotes dendritic outgrowth of cortical neurons and also promotes long-term potentiation of excitatory synaptic transmission (A. K. McAllister et al., 1996; S. L. Patterson et al., 1996; M. Korte et al.,

1998; L. Minichiello et al., 1998; D. Muller et al., 2000; B. Xu et al., 2000; L. Minichiello et al., 2002). These structural and synaptic plasticities have been implicated in epileptogenesis (J. V. Nadler et al., 1980; T. Sutula and O. Steward, 1987; G. Golarai and T. P. Sutula, 1996; I. Spigelman et al., 1998). Limbic seizures induce 4-10 fold increases in BDNF expression (Isackson et al., 1991). Interestingly, increased activation of TrkB has been identified in the mossy fiber pathway of hippocampus (Binder et al., 1999a; He et al., 2002), with activation occurring at the same site and time as the increased BDNF expression. Moreover, intraventricular infusion of TrkB receptor bodies that scavenge ligands and presumably limit activation of TrkB partially inhibited kindling development (D. K. Binder et al., 1999b). Although mice lacking both *BDNF* alleles in the germline die in the neonatal period, elimination of one *BDNF* allele also partially inhibited kindling development (M. Kokaia et al., 1995).

Taken together, the above evidence led us to hypothesize that BDNF mediates activation of its receptor, TrkB, in the hippocampus during limbic epileptogenesis and that BDNF-mediated activation of TrkB is required for epileptogenesis in the kindling model. Crossing mice with floxed alleles of *BDNF* or *TrkB* to mice in which Cre recombinase is driven by a *Synapsin1* promoter (*Syn-Cre*) resulted in mice in which *BDNF* or *TrkB* alleles were selectively eliminated from a subset of central nervous system neurons, and the mutant mice are viable in adulthood. We tested these hypotheses by

quantifying epileptogenesis and hippocampal TrkB activation in wild type and *Syn-Cre* conditional *BDNF* *-/-* and *TrkB* *-/-* mutant mice.

2.2 Methods

2.2.1 Mice

BDNF and *TrkB* mutant mice in a 129/C57/ICR background were generated with *Cre/loxP* technology (H. Gu et al., 1994) as described previously (Y. Zhu et al., 2001; L. M. Monteggia et al., 2004). For the *BDNF* mutant mice, exon 5, the exon encoding the entire BDNF protein, was flanked with 2 *loxP* sites (“floxed”). For the *TrkB* mutant mice, exon 1 of the *TrkB* gene, which encodes the signal peptide and the first 40 amino acids of the N terminus of TrkB, was floxed. Crossing either a *BDNF* or a *TrkB* floxed mouse to a transgenic mouse carrying Cre driven by a *Synapsin1* promoter (*Syn-Cre*) generated progeny in which expression of the floxed gene was selectively eliminated in a subset of CNS neurons (Figure 3). To further assess the pattern of expression in areas relevant to the present study, *Syn-Cre* mice were crossed to *Rosa-26* reporter mice (P. Soriano, 1999). Patterns of expression of NeuN (red) and β -galactosidase (green) were assessed in immunohistochemical experiments using primary antibodies from Chemicon. *In situ* hybridization using a probe from exon 5 of the *BDNF* gene was performed to confirm that the pattern of *BDNF* recombination was the same as that seen in *Rosa-26* reporter mice. An antisense probe was produced by *in vitro* transcription using T7 RNA

polymerase with a 350 bp linearized clone from the coding exon 5 of the *BDNF* gene. Similarly, *in situ* hybridization using a 420 bp antisense probe spanning the tyrosine kinase domain of the TrkB protein was performed. All other steps were performed as previously described by Luikart (B. W. Luikart et al., 2005).

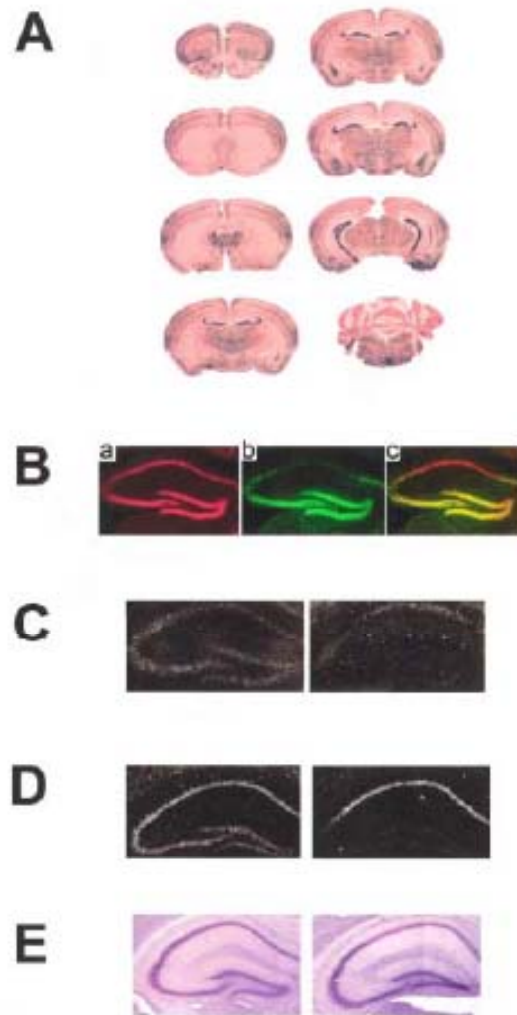


Figure 3: Characterization of Syn-Cre Reporter and BDNF and TrkB mutant mice.

A) β -galactosidase staining in coronal sections of reporter mice. For analysis of *Syn-Cre* expression, *Syn-Cre* mice were crossed to a *Rosa-26* reporter line in which β -galactosidase expression reflects Cre expression. Note that β -galactosidase activity (blue) is widespread in neocortex, pyriform cortex, amygdala, thalamus, hypothalamus, hippocampus and brainstem. B) Double-labeling immunoanalysis of *Syn-Cre* in hippocampus. Overlapping (right, yellow) immunoreactivities of NeuN (left, red) and β -galactosidase (center, green) indicate that Cre-mediated recombination specifically occurs in dentate granule cells and CA3 pyramidal neurons. C) Selective elimination of BDNF mRNA from hippocampus of *BDNF* *-/-*. *In situ* hybridization using a probe for *BDNF* exon 5 in *+/+* mice (left) revealed abundant BDNF transcript in all hippocampal subregions including CA1-3, dentate hilus and dentate gyrus. However, *BDNF* expression was ablated in the dentate gyrus and CA3 but persisted in CA1 region reflecting less effective recombination in CA1 pyramidal cells in *-/-* mice (right). D) Selective elimination of TrkB mRNA from hippocampus. *In situ* hybridization using an antisense *TrkB* probe in *+/+* mice (left) revealed abundant TrkB transcript in all hippocampal subregions including CA1-3, dentate hilus and dentate gyrus. However, as in (C), *TrkB* expression was ablated in the dentate gyrus and CA3 but maintained in CA1 region in *-/-* mice (right). E) Nissl-stained sections of *TrkB* *+/+* (left) and *-/-* (right) mice reveal no overt abnormalities within the hippocampus.

In the present study, the genotype of each animal was assessed twice using PCR of genomic DNA isolated from tail (before experiment) and liver (after sacrifice). As specified in the following sentences, symbols in parentheses following genotype specify presence (+) or absence (-) of the *BDNF* or *TrkB* allele. Seven *Syn-Cre* / *BDNF*^{wt/wt} (*+/+*), 16 *Syn-Cre* / *BDNF*^{wt/flox} (*+/+*), 16 *Syn-Cre* / *BDNF*^{flox/flox} (*+/+*), 16 *Syn-Cre*⁺ / *BDNF*^{wt/flox} (*+/-*) and 16 *Syn-Cre*⁺ / *BDNF*^{flox/flox} (*-/-*) were included in kindling experiments. Because both *Syn-Cre* / *BDNF*^{wt/flox} (*+/+*) and *Syn-Cre* / *BDNF*^{flox/flox} (*+/+*) carried 2 *BDNF* alleles and exhibited no significant differences with respect to kindling development in comparison to *Syn-Cre* / *BDNF*^{wt/wt} (*+/+*) mice (data not shown), the data from these three genotypes were pooled and used as wild type controls. Similarly, the rate of kindling development did

not differ among 6 *Syn-Cre⁻/TrkB^{wt/wt}* (+/+), 5 *Syn-Cre⁺/TrkB^{wt/wt}* (+/+), and 5 *Syn-Cre⁻/TrkB^{flox/flox}* (+/+) and thus these mice were pooled and used as wild type controls for 16 *Syn-Cre⁺/TrkB^{wt/flox}* (+/-) and 4 *Syn-Cre⁺/TrkB^{flox/flox}* (-/-).

The mice used for kindling experiments were 2 to 4 months old. The body weight was similar among three groups of *BDNF* mice (28.75±0.96 g for +/+, 28.80±1.58 g for +/-, 29.00±1.57 g for -/-, $p>0.05$). The *TrkB* -/- mice exhibited a 25% reduction of body weight (29±1 g for +/+, 32±1 g for +/-, and 22±2 g for -/-, $p<0.003$).

2.2.2 Surgery and kindling

Under pentobarbital (60mg/kg) or isofluorane (2%) anesthesia, a bipolar electrode used for stimulation and recording was stereotactically implanted in the right amygdala using the following coordinates with bregma as the reference: 1.2 mm posterior, 2.9 mm lateral, 4.6 mm below dura. After a post-operative recovery period of 2 weeks, the electrographic seizure threshold (EST) was determined by application of a 1 sec train of 1 msec biphasic rectangular pulses at 60 Hz beginning at 60 μ A. Additional stimulations increasing by 20 μ A were administered at one minute intervals until an electrographic seizure lasting at least 5 seconds was detected on the electroencephalogram (EEG) recorded from the amygdala. Stimulations at the intensity of EST were subsequently administered following the standard amygdaloid kindling protocol that was described previously in detail (X. P. He et al., 2002). EEG and behavioral seizures were observed and recorded. The behavioral manifestations of

seizures were classified according to a modification of the description of Racine (R. J. Racine, 1972a): 1, facial clonus; 2, head nodding; 3, unilateral forelimb clonus; 4, rearing with bilateral forelimb clonus; 5, rearing and falling (loss of postural control); 6, running or bouncing seizures; and 7, tonic hind limb extension. Unless specified otherwise, mice were stimulated until fully kindled as defined by the occurrence of 3 consecutive seizures of Class 4 or greater. The maintenance of the kindled state was tested two weeks after the final stimulation of the kindling protocol with a single stimulation to induce a seizure of Class 4 or greater. The surgery and kindling procedures were performed by an individual blinded to genotype of the animals. Unstimulated control animals of each genotype underwent surgical implantation of an electrode in amygdala and were handled identically but were not stimulated.

To test for seizure-induced increase of p-Trk immunoreactivity, an additional stimulation of the same intensity used in the kindling procedure was administered after a 2 week stimulation free period following the 3rd consecutive seizure of Class 4 or greater to the *BDNF* mutant mice and their controls. Twenty-four hours after the last stimulation, both experimental and control animals were perfused transcardially under deep pentobarbital anesthesia with ice-cold 4% paraformaldehyde in 0.1M phosphate buffered saline (1x PBS) containing 1 mM sodium orthovanadate (PBSV) for 5 min at a flow rate of 7.5 ml/min. The brains were removed, post-fixed in the same solution overnight at 4°C and cryoprotected in 20% sucrose in 1x PBSV until they sank. Coronal

sections of 40 μm were cut in a cryostat at -20°C , mounted in PBSV on slides, air-dried, frozen and stored at -80°C until use. Subsets of the sections were stained with methyl green pyronine-Y (MGPY) for analysis of electrode placement, which was performed without knowledge of genotype or experimental treatment. Only animals with correct electrode placement in the amygdala were included in the statistical analysis. All kindling data are presented as mean \pm SEM and analyzed by one-way ANOVA with post hoc Bonferroni's test.

2.2.3 Electroshock-induced seizures

One or two drops of local anesthetic (lidocaine, 1%) were placed in each conjunctiva. Approximately 30-60 seconds later, the animal was picked up gently and a cup electrode was placed over each cornea and current was administered (Wahlquist Instrument Co., Salt Lake City, UT). To induce maximal seizures, a stimulus of 200 mA at 60 Hz for a duration of 200 msec was administered. Seizure onset occurred virtually instantaneously with the onset of current flow. The duration of tonic hindlimb flexion, tonic hindlimb extension, and time to recovery were recorded. To induce minimal seizures, an initial stimulus of 5 mA at 60 Hz for a duration of 200 msec was administered, and additional stimuli of 2 mA increments were administered at 30 minute intervals until a seizure occurred. Seizure onset was immediate and the initial phase observed in all animals consisted of flexion of the head with head nodding and face clonus and clonus of all four extremities; the duration was similar among all

animals. Later stages of the seizures varied in pattern and duration. The investigator evaluating seizure pattern and duration was blinded to genotype.

2.2.4 BDNF and p-Trk immunohistochemistry

An affinity-purified rabbit anti-BDNF polyclonal antibody (0.3 µg/ml, a gift of Amgen) recognizing recombinant human BDNF was used for BDNF immunohistochemistry. The specificity of this rabbit polyclonal anti-BDNF antibody for BDNF was established by sequential affinity purification and verified by elimination of immunoreactivity in *BDNF* null mice (Q. Yan et al., 1997). Antibody pY490, a peptide affinity-purified polyclonal Trk antibody raised in rabbits, (1:10, New England Biolabs, Beverly, MA) directed against a synthetic phospho-Y 490 peptide corresponding to residues 485 to 493 (IENPQY*FSD) of human TrkA was used to detect the phosphorylated form of Trk receptor following the protocol described previously (D. K. Binder et al., 1999b; X. P. He et al., 2002). The signals of both BDNF and phospho-Trk (p-Trk) immunoreactivities were amplified by ABC method and visualized by diaminobenzidine (DAB) development and were done in nearby sections from the same brains. For BDNF immunohistochemistry, an antigen retrieval method was used to enhance BDNF detection. Sections were incubated in 20 mM Tris-HCl pH 9.0 and 150 mM NaCl at 85°C for 30 minutes. For p-Trk immunohistochemistry, two ABC incubations and biotinyl tyramide (1:100 in PBSV with 5% normal goat serum (NGS), applied between 2 ABC reactions (Bio-RAD, Richmond, CA)) were used to increase p-

Trk immunoreactivity. To facilitate quantitative comparisons, sections isolated from stimulated and unstimulated animals of different genotypes were processed in batches within the same Coplin jars using the identical solutions and durations of incubations, washes, etc. p-Trk immunoreactivity was quantified by a densitometric method as reported previously (X. P. He et al., 2002). Briefly, images of the p-Trk immunoreactivity were captured and measured with a computer assisted image analyzer. The gray values from distinct subregions were compared with that in corpus callosum, which had less variation and higher gray value reflecting less immunoreactivity, thereby permitting each animal to serve as its own control. The results are presented as relative p-Trk immunoreactivity, which reflects the percentage of reduction in gray value, higher reduction reflecting more p-Trk immunoreactivity. Data are mean \pm SEM and analyzed by one-way ANOVA with post hoc Bonferroni's test.

To examine the specificity of antibodies in immunohistochemical detection, peptide competition experiments were performed. The immunogen used to raise the pY490 antibody, the phosphorylated form of peptide 490 (p-490, 300 nM, New England Biolabs, Beverly, MA), or a negative control peptide, the unphosphorylated form of peptide 490, were used to confirm the specificity of the p-Trk immunoreactivity. The peptides used for competition were preincubated at room temperature with the primary antibody solution for 1 hour. After slides were quenched, blocked and permeabilized, the primary antibody solution co-incubated with the designated peptide was applied to

the sections. The remaining steps followed the standard protocol of immunohistochemistry.

2.2.5 Kainic acid treatment

Like kindling, kainic acid induced status epilepticus is an animal model of limbic epilepsy (T. Tanaka et al., 1982; E. Tremblay and Y. Ben-Ari, 1984). Although the early stages of epileptogenesis are less easily quantitated in this model than with the kindling model, kainic acid induced status epilepticus is much less labor intensive than the kindling model, and it evokes increased expression of BDNF (M. M. Dugich-Djordjevic et al., 1992b; Q. Yan et al., 1997; J. S. Rudge et al., 1998; R. Katoh-Semba et al., 1999; I. A. Scarisbrick et al., 1999; T. Saarelainen et al., 2001; A. K. Shetty et al., 2003) and increased p-Trk immunoreactivity (D. K. Binder et al., 1999b); Danzer et al., 2004). Because of the greater ease of inducing limbic seizures with kainic acid, this model was used instead of kindling in some experiments. *BDNF* *+/+* and *-/-* mice were injected with kainic acid (15 mg/ kg, i.p.) dissolved in saline or with saline alone. The animals were observed continuously for behavioral seizures after injection. Additional injections of kainic acid were administered to mice that did not exhibit a behavioral seizure of class 4 or greater during one hour following the first injection. Pentobarbital (50mg/kg, i.p.) was given to terminate status epilepticus after 4 hours of continuous seizure activity. Both kainic acid and saline treated mice were decapitated 24 hours after the first injection under deep pentobarbital anesthesia.

2.2.6 Hippocampal homogenate preparation

Following decapitation, the mouse head was quickly dipped into liquid nitrogen for 4 seconds to rapidly cool the brain. Two hippocampi from each animal were dissected on ice and homogenized in lysis buffer [20 mM Tris, pH 8.0, 137 mM NaCl, 1% Igepal, 10% glycerol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride, and 1 Complete Mini protease inhibitor tablet (Roche, Mannheim, Germany)/10 ml]. In some experiments, the hippocampi were isolated, sectioned with a McIlwain Tissue Chopper (400 μ m) in a plane perpendicular to the longitudinal axis of the hippocampus, and dissected into dentate gyrus, CA3, and CA1 prior to homogenization as previously described (J. E. Kraus et al., 1994). The pellet was discarded following centrifugation at 16,000 g for 10 min and the supernatant was aliquoted and stored at -80°C for further biochemical analysis.

2.2.7 Immunoprecipitation and western blot

For immunoprecipitation experiments, 500 μ g of hippocampal homogenate from each animal was incubated with 15 μ l of pY490 primary antibody for 4 hours at 4°C . Immunoprecipitated proteins were collected from pellets by centrifugation at 1000 g for 5 minutes after an overnight incubation with 30 μ l protein A/G beads (Pierce, Rockford, IL). Pellets were washed 3 times in lysis buffer, once in dH₂O, resuspended in sample loading buffer and boiled for 5 minutes before SDS-PAGE, transfer, and immunoblotting on polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA) as

recommended by the manufacturer. The membrane was blocked with 3% non-fat dry milk in 1X PBS buffer containing 1 mM sodium orthovanadate and 0.05% Tween-20, probed with mouse anti-TrkB antibody (1:1000, BD Transduction Laboratories) for 1 hr at room temperature followed by peroxidase conjugated goat anti-mouse secondary antibodies (1:1000, Jackson ImmunoResearch) for another hour. The immunoblots were developed with enhanced chemiluminescence (ECL, Amersham Pharmacia, Buckinghamshire, United Kingdom).

To quantitate TrkB protein expression in some experiments, PVDF membrane was developed by ECL plus and exposed to ImageQuant phospho-imager (Molecular Dynamics, Sunnyvale, CA). Band intensity was determined using volume quantification. A standard curve was generated by loading different amounts of hippocampal homogenate. Measurements from all samples fell within the linear range of the standard curve and were then analyzed by one-way ANOVA.

2.2.8 NT-3, NT-4 and BDNF ELISA

A specific sandwich E_{max}[®] ImmunoAssay system (Promega, Madison, WI), with a protocol of direct acid treatment for protein samples which can improve sensitivity (A. J. Okragly and M. Haak-Frendscho, 1997), was used to determine NT-3, NT-4 and BDNF protein levels according to manufacturer's suggestion. Briefly, the supernatant of hippocampal homogenate was diluted with 4 volumes of Dulbecco's PBS (DPBS), acid-treated by the addition of 1 µl 1N HCl per 50 µl of sample, to adjust the pH to less than

3.0, incubated for 15 minutes, and then neutralized with 1N NaOH to a pH approximating of 7.6. The acid treated protein samples were added to the 96-well plate pre-coated with the primary antibody (anti-human NT-3 polyclonal Ab, 1:500; anti-human NT-4 polyclonal Ab, 1:250; anti-BDNF monoclonal Ab, 1:1000, respectively) in carbonate coating buffer, pH 9.7, overnight at 4°C and blocked by 1x block and sample buffer for 1 hour. After an incubation of 6 hours at room temperature with shaking, a secondary antibody (anti-NT-3 monoclonal Ab, 1:4000; anti-NT-4 monoclonal Ab, 1:5000; anti-human BDNF polyclonal Ab, 1:500, respectively) was added to thoroughly washed wells and incubated overnight at 4°C. Horseradish peroxidase (HRP) conjugated detection antibody (anti-mouse IgG for NT-3 and NT-4, 1:100; anti-IgY for BDNF 1:200) was added to wells and incubated for 2.5 hours at room temperature. Color development was achieved by reaction with a tetramethylbenzidine (TMB)-peroxidase solution and terminated after 10 min with 1N HCl. Optical absorbance was read at 450 nm with a microplate reader and the immunoreactivity determined by comparison to a standard curve. All ELISA results are presented as mean \pm SEM and analyzed by one-way ANOVA with post hoc Bonferroni's test.

2.3 Results

2.3.1 Neuron-Specific *BDNF* Conditional Knockout Mice

To selectively eliminate *BDNF* expression from CNS neurons, mice in which exon 5 of the *BDNF* gene was floxed were crossed to *Syn-Cre* mice. As a first step in analysis of expression of the *Syn-Cre* transgene, *Syn-Cre* mice were crossed to a *Rosa-26* reporter line in which recombination results in expression of β -galactosidase.

Immunohistochemical analysis of β -galactosidase activity revealed widespread expression in pyriform cortex, nuclei within amygdala, thalamus, hypothalamus, and brainstem (Figure 3A). Expression within neocortex was largely restricted to the lower boundary of layer IV (Figure 3A). Because limbic epileptogenesis is associated with increased activation of TrkB in the mossy fiber pathway of hippocampus (X. P. He et al., 2002), the effects of the recombination were studied in detail in the hippocampus. High levels of Cre activity were evident in the dentate granule and CA3 pyramidal cells with lower levels of activity in the CA1 pyramidal cells as reflected by drastically reduced β -galactosidase activity (Figure 3A). More detailed analysis revealed robust expression of β -galactosidase in the dentate granule cells and neurons of the dentate hilus as well as CA3 pyramidal cells (Figure 3B, center), as reflected in overlapping (yellow, Figure 3B, right) immunoreactivity of β -galactosidase (green, Figure 3B, center) and the neuronal marker NeuN (red, Figure 3B, left). The presence of effective recombination of the *BDNF* exon 5 per se was confirmed by *in situ* hybridization as evident in the absence of

silver grains overlying the dentate granule and CA3 pyramidal cells in *BDNF* *-/-* mice (Figure 1C, right) in comparison to wild type controls (Figure 1C, left). Note the persistence of silver grains overlying the CA1 pyramidal cells in both the wild type and *BDNF* mutant mice (Figure 1C, left and right respectively), reflecting less effective recombination in CA1 pyramidal cells. Whether the absence of Cre recombinase in some CNS neurons is due to the exclusion of critical regulatory elements from the *Synapsin1* promoter within the transgene and/or the site in the genome at which the transgene inserted is uncertain.

Measuring BDNF protein expression in hippocampal homogenates with an ELISA assay revealed that the majority (79%) of BDNF was eliminated in the null mutant mice (*BDNF* *-/-* and *+/+* were 100 ± 18 and 512 ± 33 ng/g, respectively, $p < 0.05$) (Figure 2A left). Immunohistochemical experiments were subsequently performed to assess the anatomic distribution of the residual BDNF in the hippocampus of *BDNF* *-/-* mutants. To enhance the sensitivity of detecting BDNF expression, BDNF immunohistochemistry was performed on additional animals of all three genotypes one day following a Class 4 or 5 kindled seizure. Confirming previous reports (Q. Yan et al., 1997; S. C. Danzer et al., 2004), the most prominent BDNF immunoreactivity in unstimulated *+/+* mice was evident in the mossy fiber pathway of the hippocampus (arrows, Figure 2Ba); marked increases in immunoreactivity in the mossy fiber pathway and the pyramidal cell layers were evident following a kindled seizure (Figure 2Bd).

This immunoreactivity was markedly reduced in the unstimulated +/- mice as compared to unstimulated +/+ mice (Figure 2Bb), but increases were evident following a kindled seizure in the +/- mice (Figure 2Be). The immunoreactivity was eliminated in the unstimulated -/- mice and no increase was seen even after a kindled seizure (Figures 2Bc and f, respectively); the residual immunoreactivity evident in the -/- mice (Figure 2Bc and f) was similar to that seen in the absence of primary antibody (not shown) and was likely due to nonspecific binding of secondary antibody. Taken together, the findings from *in situ* hybridization, measures of BDNF protein, and immunohistochemistry demonstrate the efficacy of the Cre recombinase in virtually eliminating BDNF expression in the dentate granule and CA3 pyramidal cells but reveal some residual *Bdnf* mRNA expression in CA1 pyramidal cells. The ELISA results reveal some residual BDNF protein in the -/- mutants, but this protein escaped detection in the immunohistochemical experiments even after induced seizures.

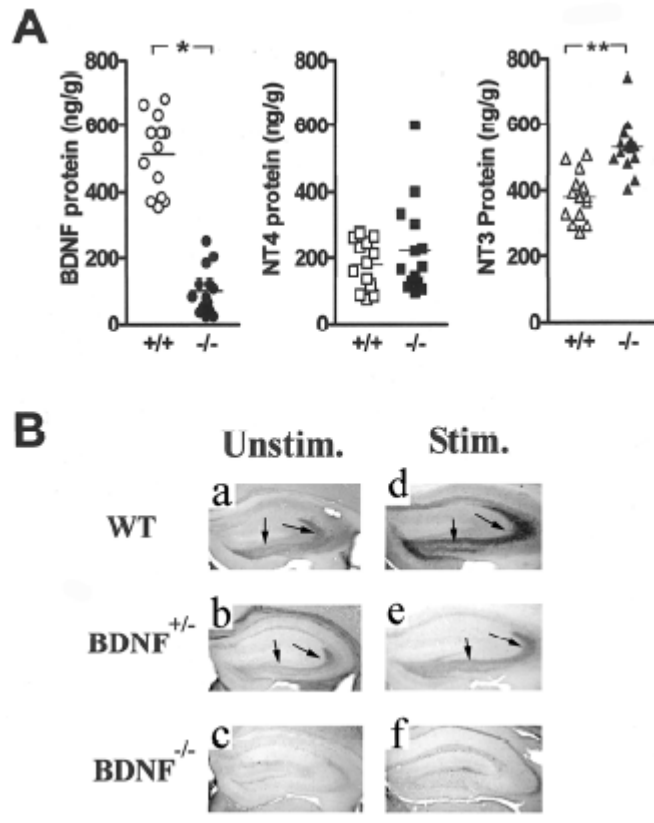


Figure 4: Prominent Reduction of BDNF Expression in *BDNF*^{-/-} Mice.

A) Neurotrophin expression in hippocampus of *BDNF*^{+/+} and *BDNF*^{-/-} mice. The content of BDNF (left), NT4 (center) and NT3 (right) were measured from hippocampal homogenates of *BDNF*^{+/+} and *BDNF*^{-/-} by sandwich ELISA method. Each symbol represents result from an individual mouse. The data are mean \pm SEM. Single asterisks (*) refer to $p < 0.05$; double asterisk (**) refers to $p < 0.01$ by one-way ANOVA with post hoc Bonferroni's test. B) Elimination of BDNF immunoreactivity in *BDNF* mutant mice. BDNF immunohistochemistry was performed by specific BDNF antibody in the coronal sections from *BDNF*^{+/+} (a, d) and *BDNF*^{+/-} (b, e) and *BDNF*^{-/-} (c, f) mice in the absence of a stimulation (a, b, c) or sacrificed 24 hours after an evoked Class 4 or 5 kindled seizure (d, e, f). Arrows denote immunoreactivity in dentate hilus and stratum lucidum. Note the immunoreactivity was eliminated in the unstimulated *BDNF*^{-/-} mouse even after a kindled seizure; the residual immunoreactivity evident in the *BDNF*^{-/-} mice was similar to that seen in the absence of primary antibody (not shown) and most likely due to nonspecific binding of secondary antibody. Scale bar, 650 μ m.

2.3.2 Partial inhibition of kindling development in BDNF null mutant mice

To our surprise, *BDNF* *-/-* mice exhibited only a modest impairment of limbic epileptogenesis as evident in the increased number of stimulations required to elicit behavioral seizures in comparison to *+/+* controls with intermediate effects evident in *+/-* mice (Figures 3A, C). These differences were most apparent in the number of stimulations required to evoke a limbic seizure termed Class 2 (Figure 3C; 12.1 ± 2.5 and 5.9 ± 0.5 for *-/-* and *+/+* respectively, $p < 0.01$). Once the first Class 2 seizure had been evoked, similar numbers of stimulations were sufficient to induce progression through subsequent classes of kindled seizures for all three genotypes. The number of stimulations required to induce the 3rd consecutive Class 4 or 5 seizure was 14.8 ± 0.9 , 16.9 ± 1.6 , and 22.5 ± 3.8 for *+/+*, *+/-*, and *-/-* mice respectively (Figures 3A, C, $p < 0.05$). Despite the increased number of stimulations required to induce behavioral seizures in the *-/-* mice, an increase of electrographic seizure duration approximated 300-400% in all three genotypes during the development of kindling (Figure. 3B). No significant differences in current required to induce the initial electrographic seizure were evident ($155 \pm 14 \mu\text{A}$, $172 \pm 30 \mu\text{A}$, and $230 \pm 56 \mu\text{A}$ for *+/+*, *+/-*, and *-/-* mice respectively).

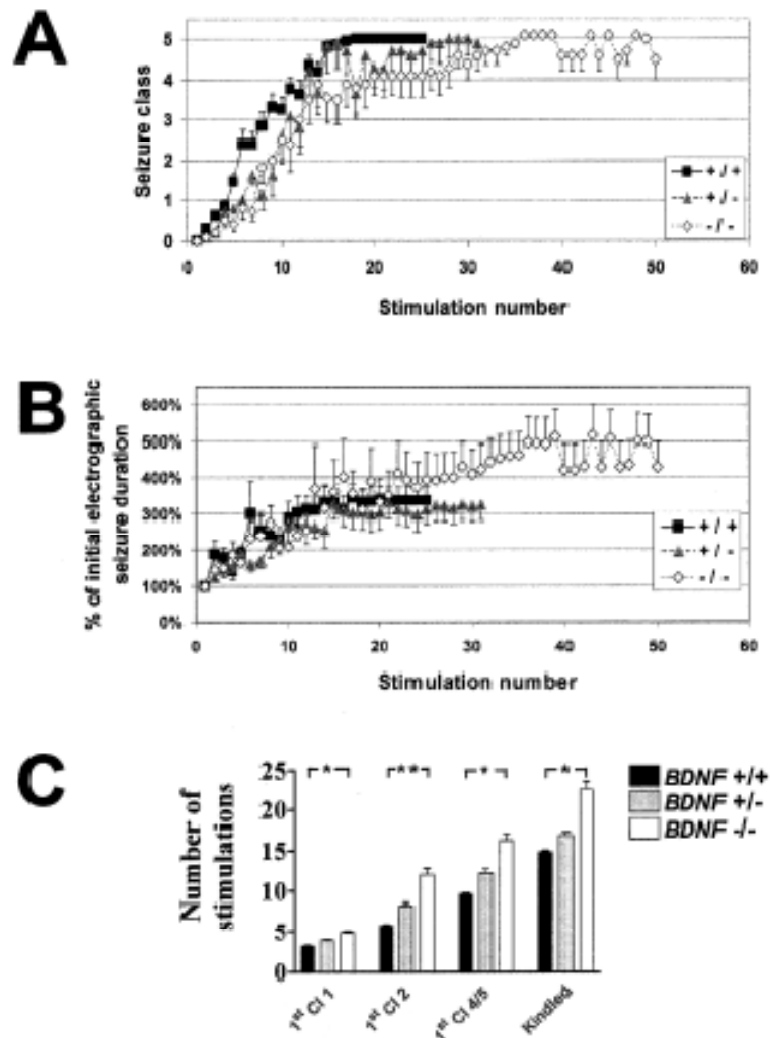


Figure 5: Partial Inhibition of Kindling Development in *BDNF*^{-/-} Mice.

Kindling development is presented as behavioral seizure class (A) and electrographic seizure duration (B) (y-axis). Stimulation number (x-axis) refers to the number of stimulations that evoked an electrographic seizure with duration of at least 5 sec. C) Number of stimulation to reach different seizure classes in +/+ (n= 21), +/- (n= 10) and -/- (n= 10) mice. Fully kindled stage is defined by the occurrence of 3 consecutive seizures of class 4 or greater. All data are presented as mean \pm SEM. *: $p < 0.05$, **: $p < 0.01$, one-way ANOVA with post hoc Bonferroni's test.

2.3.3 Seizure-induced increased phospho-Trk (p-Trk) immunoreactivity in *BDNF* mutant mice

Our previous work established the presence of increased phosphorylation of the BDNF receptor, TrkB, in the mossy fiber pathway of hippocampus during limbic epileptogenesis (D. K. Binder et al., 1999b; X. P. He et al., 2002). The concordance in the temporal and spatial patterns of increased BDNF expression and TrkB phosphorylation during limbic epileptogenesis (P. J. Isackson et al., 1991; C. M. Gall, 1993; D. K. Binder et al., 1999b) led us to hypothesize that the release and subsequent binding of BDNF to TrkB triggered the increased phosphorylation of TrkB detected in our immunohistochemical and biochemical studies (D. K. Binder et al., 1999b; X. P. He et al., 2002). Confirming our previous results (D. K. Binder et al., 1999b; X. P. He et al., 2002), increased p-Trk immunoreactivity was evident in the mossy fiber pathway of stimulated +/+ mice in comparison to unstimulated control +/+ mice (see arrows in Figure 4d compared to 4a). The increased immunoreactivity was evident in the dentate hilus and stratum lucidum of CA3a, b, and c bilaterally (only one hippocampus shown) in all brain sections examined; no overt changes of p-Trk immunoreactivity were noted in CA1, in stratum radiatum of CA3, or in the molecular layer of the dentate gyrus. Surprisingly, a seizure evoked in *BDNF* -/- mice evoked increased p-Trk immunoreactivity in the mossy fiber pathway similar to that of +/+ mice (compare Figure 4f with 4c); likewise seizures evoked in +/- mice resulted in increased p-Trk immunoreactivity in the mossy fiber pathway (compare Figure 4e with 4b).

Quantitative analyses confirmed the findings and demonstrated significant increases of p-Trk immunoreactivity of similar magnitude following evoked kindled seizures in +/+, +/-, and -/- mice in the hilar border and dentate hilus (Figure 5A). However, the seizure-induced increases of p-Trk immunoreactivity in stratum lucidum in -/- mice were slightly less than in +/+ and +/- mice (Figure 5B) and were not significantly different from unstimulated -/- animals.

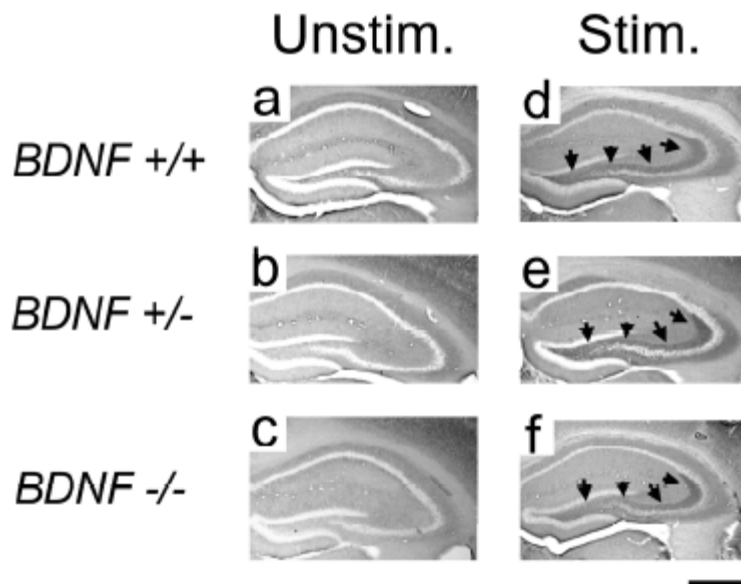


Figure 6: Seizure-Induced p-Trk Immunoreactivity in *BDNF* Mutant Mice.

A-C) p-Trk immunoreactivity in unstimulated +/+, +/-, and -/- mice. Note the absence of detectable immunoreactivity in dentate hilus and CA3 stratum lucidum of hippocampus. D-F) p-Trk immunoreactivity in *BDNF* +/+, +/-, and -/- mice 24 hr after a Class 4 or 5 seizure evoked by stimulation of right amygdala. *Arrows* denote immunoreactivity in dentate hilus and stratum lucidum in stimulated +/+, +/-, and -/- mice. Scale bar, 650 μ m.

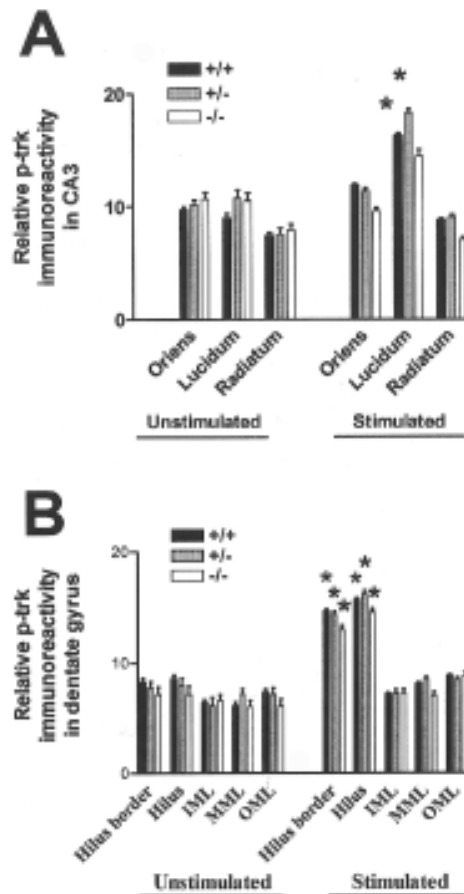


Figure 7: Quantitative Analyses of p-Trk Immunoreactivity in Hippocampal Subregions of Stimulated and Unstimulated *BDNF*^{+/+}, *BDNF*^{+/-}, and *BDNF*^{-/-} Mice.

P-Trk immunoreactivity was analyzed in dentate gyrus (A) and CA3 (B) regions of *BDNF*^{+/+} and *BDNF*^{-/-} mice sacrificed 24 hours after kindled seizures. Relative p-Trk immunoreactivity refers to the mean \pm SEM of the percentage reduction in gray value in the given subregions compared with corpus callosum (see Materials and Methods); high values reflect more intense immunoreactivity. * $p < 0.05$, compared with unstimulated mice (one-way ANOVA with post hoc Bonferroni's test).

Our previous studies established that TrkB in particular was the Trk receptor undergoing phosphorylation during epileptogenesis in *+/+* mice (D. K. Binder et al., 1999b; X. P. He et al., 2002). Because the p-Trk (pY490) antibody (Ab) used for

immunohistochemistry can detect the phosphorylated tyrosine residue in TrkA, TrkB, or TrkC (R. A. Segal et al., 1996), we wondered whether TrkB itself exhibited increased phosphorylation in the hippocampus of *BDNF* *-/-* mice during epileptogenesis. To test this idea, an alternative model of limbic epileptogenesis was used, namely kainate status epilepticus, because it is far less labor intensive than kindling yet increased phosphorylation of Trk is evident in this model as well (D. K. Binder et al., 1999b). Biochemical methods were used because these methods permit directly determining whether TrkB itself undergoes increased phosphorylation, whereas the antibody available for the immunohistochemical method does not permit distinguishing among individual Trks. *BDNF* *+/+* and *-/-* mice were sacrificed 24 hours after kainic acid induced seizures. The phosphorylated Trk receptors were immunoprecipitated with the pY490 antibody; the presence of TrkB in the immunoprecipitated phosphorylated Trk receptors was assessed by probing immunoblots of the immunoprecipitates with an antibody specific to TrkB. A representative experiment (Figure 6A) reveals increased p-TrkB immunoreactivity following seizures induced in both *+/+* and *-/-* mice, a result confirmed in two additional experiments (not shown). Importantly, no differences in TrkB content per se were detected in immunoblots of hippocampal homogenates in the absence of prior immunoprecipitation as reflected in a representative experiment (Figure 6B), thereby demonstrating that the increases of p-TrkB at 24 hours after seizures represented post-translational modifications of similar numbers of TrkB molecules.

Together, the immunohistochemical and biochemical results establish that TrkB in hippocampus undergoes phosphorylation following seizures in both *BDNF* $+/+$ and $-/-$ mice.

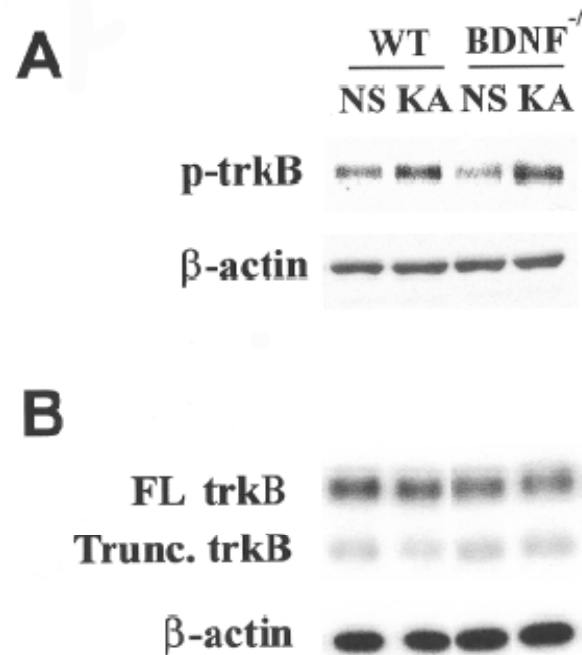


Figure 8: Increased Phosphorylation of Hippocampal TrkB Receptor during Epileptogenesis in *BDNF*^{-/-} Mice.

A) A representative western blot analysis of p-TrkB. The hippocampal homogenate from $+/+$ and $-/-$ mice sacrificed 24 hrs after saline or kainic acid treatment were immunoprecipitated with pY490 antibody followed by immunoblotting with anti-TrkB antibody. Similar results were obtained in two additional experiments. **B)** A representative western blot analysis of hippocampal TrkB expression 24 hrs following seizures in *BDNF* $+/+$ and $-/-$ mice. Similar results were obtained in at least five additional experiments. Top: Hippocampal homogenate was probed by TrkB antibody; bottom: the same blot was stripped and reprobed with anti-β-actin antibody (Chemicon) for loading control.

2.3.4 Neurotrophin expression in hippocampus of wild type and *BDNF* mutant mice

The fact that seizures can evoke activation of TrkB despite striking reductions of BDNF raised the possibility of compensatory responses of other neurotrophins that might activate TrkB in the *BDNF* *-/-* mice. To address this question, neurotrophin-4 (NT4) and neurotrophin-3 (NT3) content was measured in hippocampal homogenates of *BDNF* *+/+* and *-/-* mice. No significant difference was detected in the constitutive expression of NT4 protein between *+/+* and *-/-* mice (Figure 2A middle). In contrast to NT4, a significant (39%) increase of constitutive expression of NT3 protein in hippocampus was detected in *-/-* compared to *+/+* mice (529 ± 21 and 379 ± 19 ng/g for *-/-* and *+/+*, respectively, $p < 0.01$) (Figure 2A left). Together these findings demonstrate a compensatory response evident in increased expression of hippocampal NT3 protein content in *BDNF* *-/-* mutants compared to *+/+* mice, raising the possibility that compensatory increases in NT3 expression may contribute to TrkB activation and limbic epileptogenesis in the *BDNF* *-/-* mice.

2.3.5 Neuron specific TrkB conditional knockout mice

The tight association between enhanced phosphorylation of TrkB and limbic epileptogenesis in the *BDNF* *-/-* mice provided additional circumstantial evidence that TrkB is required for limbic epileptogenesis. We reasoned that any compensatory responses of ligands, such as the increased NT-3 expression in the *BDNF* *-/-* mice, may

be less efficient or absent when targeting the neurotrophin receptor itself. We therefore crossed the *Syn-Cre* mice to mice in which the *TrkB* allele was floxed, permitting selective elimination of *TrkB* from CNS neurons in an anatomic pattern similar to that of *BDNF* elimination. The presence of effective recombination of the *TrkB* exon 1 in a pattern similar to the *BDNF* allele was confirmed by *in situ* hybridization as evident in the absence of silver grains overlying the dentate granule and CA3 pyramidal cells in *TrkB* *-/-* mice (Figure 1D, right) in comparison to wild type controls (Figure 1D, left). Note the persistence of silver grains overlying the CA1 pyramidal cells in both the wild type and *TrkB* *-/-* mutant mice (Figure 1D, left and right respectively), reflecting less effective recombination in CA1 pyramidal cells. Nissl staining revealed a cellular pattern in *TrkB* *-/-* hippocampus (Figure 1E left) indistinguishable from wild type mice (Figure 1E right). The results of *in situ* hybridization were further assessed by measures of TrkB protein by western blotting of microdissected hippocampal subregions dentate, CA3, and CA1 (Figure 7). Western blotting was performed instead of immunohistochemistry because available anti-TrkB antibodies did not selectively label TrkB in immunohistochemistry experiments in our hands. No full-length TrkB protein was detectable in either dentate or CA3 of *TrkB* *-/-* in comparison to wild type controls, whereas a partial reduction of TrkB protein was evident in CA1. Residual expression of truncated TrkB was detected in all three subregions, presumably reflecting the expression of the truncated but not the full-length isoform in glia (J. Frisen et al., 1993),

which would not be expected to express the Cre recombinase driven by the neuron specific promoter, *Synapsin1*.

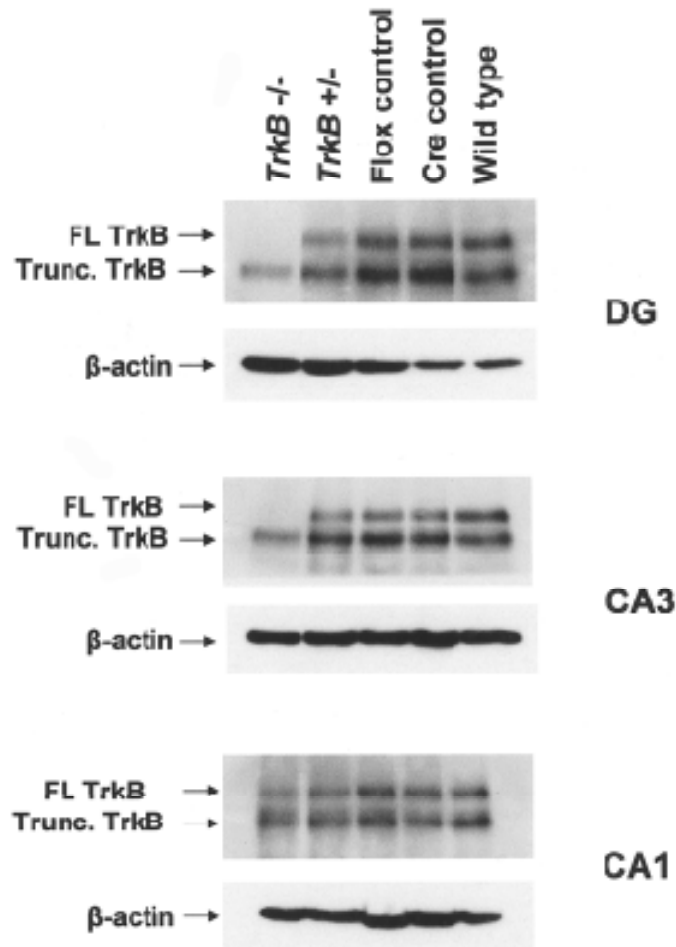


Figure 9: Efficacy of Excision of Floxed *TrkB* Alleles.

Western blot using anti-TrkB antibody (BD Transduction Laboratories, Franklin Lakes, NJ) was performed on homogenates of microdissected hippocampus of wild type, Cre control (*Syn-Cre*⁺ / *TrkB*^{wt/wt}), flox control (*Syn-Cre*⁻ / *TrkB*^{flox/flox}), *TrkB* ^{+/-} (*Syn-Cre*⁺ / *BDNF*^{wt/flox}) and *TrkB* ^{-/-} (*Syn-Cre*⁺ / *BDNF*^{flox/flox}). Note the absence of detectable full-length TrkB in DG and CA3 in *TrkB* ^{-/-} mice yet abundant full-length TrkB in CA1. By contrast, note relative preservation of truncated TrkB in DG and CA3 regardless of genotype. The blot was probed for β-actin as a control for loading and transfer.

2.3.6 Prevention of kindling development in *TrkB* mutant mice

In sharp contrast to the *BDNF* *-/-* mice, it was not possible to induce limbic epileptogenesis in *TrkB* *-/-* mice as evident in the absence of evoked behavioral seizures even after 48-50 stimulations (Figure 8A). Measures of evoked electrographic seizure duration also revealed inhibition of epileptogenesis. That is, in contrast to the 200% increase of electrographic seizure duration evident by the tenth stimulation of the *TrkB* *+/+* and *+/-* mice, only a 100% increase of electrographic seizure duration was evident by the 21st stimulation of the *TrkB* *-/-* mice and no further increase was observed even after 48-50 stimulations (Figure 8B). Importantly, the development of kindling in the *+/+* mice in this experiment was similar to that in the *+/+* mice in the *BDNF* experiment as evident in progressive increase of behavioral seizure intensity and electrographic seizure duration induced by similar numbers of repeated stimulations (Figures 3 and 8). Although no behavioral evidence of kindling development was detected in *TrkB* *-/-* mice, only modest inhibition of kindling development was evident in the *TrkB* *+/-* compared to *+/+* mice; the number of stimulations required to induce the 3rd consecutive Class 4 or 5 seizure was 9.5 ± 1.2 and 14.5 ± 1.9 for *+/+* and *+/-* mice respectively (Figures 8A, 8C, $p < 0.05$).

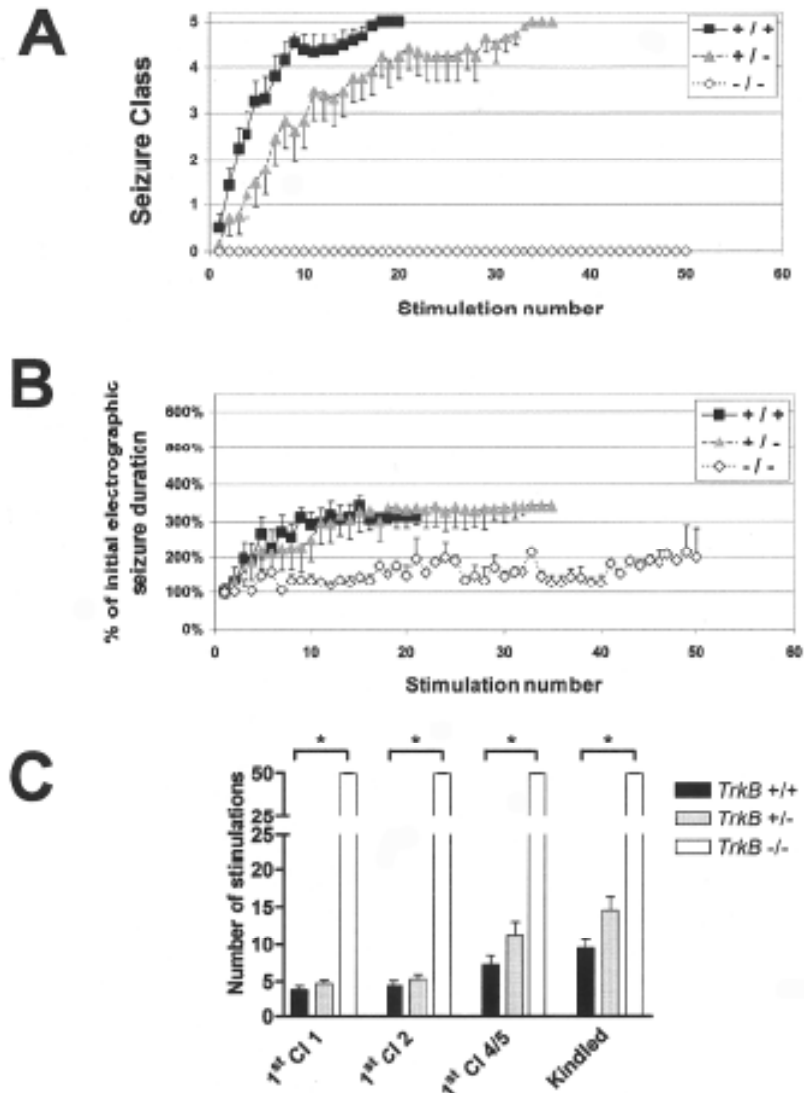


Figure 10: Striking Inhibition of Kindling Development in *TrkB*^{-/-} Mice.

Kindling development is presented as behavioral seizure class (A) and electrographic seizure duration (B) (y-axis). Stimulation number (x-axis) refers to the number of stimulations that evoked an electrographic seizure with duration of at least 5 sec. C) Number of stimulations required to reach different seizure classes in *+/+* (n= 16), *+/-* (n= 16) and *-/-* (n= 4) mice. Fully kindled stage is defined by the occurrence of 3 consecutive seizures of class 4 or greater. All data are presented as mean \pm SEM. *: p<0.05, **: p<0.01, one-way ANOVA with post hoc Bonferroni's test.

Significant increases were identified in the current required to trigger the initial electrographic seizure in the *TrkB* *-/-* mice (610 ± 18 , 675 ± 16 , and 960 ± 22 μA , in *+/+*, *+/-*, and *-/-* mice respectively, $p < 0.05$). Importantly, differences in current intensity required to evoke the initial electrographic seizure do not correlate with differences in epileptogenesis as measured in the kindling paradigm (R. J. Racine, 1972a). In the present study, subsets of both *+/+* and *+/-* mice exhibited elevated current intensities similar to those of the *-/-* mice, yet epileptogenesis proceeded in a pattern both quantitatively and qualitatively indistinguishable from the subset of *+/+* and *+/-* mice with lower current intensities. Finally, the duration of the initially evoked electrographic seizure was not significantly different among the three genotypes (12 ± 2 , 10 ± 1 , 9 ± 2 sec in *+/+*, *+/-*, and *-/-* mice respectively, $p = 0.372$) (Figure 8B). Together these findings indicate that elevated current thresholds evident in the *TrkB* *-/-* mice are not sufficient to explain the impairment of epileptogenesis.

2.3.7 Electroconvulsive Shock Seizures

Despite the absence of overt histological abnormality or defective motor function, the possibility persisted that the absence of *TrkB* impacted neuronal structure and/or function such that the *TrkB* *-/-* mice were incapable of exhibiting the behavioral endpoint of kindling, namely a tonic-clonic seizure. To address this possibility, electroconvulsive shocks were administered by transcorneal stimulation, and the occurrence and duration of seizures were recorded by an investigator blinded to

genotype. A maximal stimulus evoked typical tonic-clonic seizures in mice of all three genotypes. The duration of initial tonic flexion was 3.0 ± 0.1 , 3.0 ± 0.1 , and 2.9 ± 0.2 sec in *TrkB* +/+, +/-, and -/- mice respectively ($p > 0.05$). The duration of tonic hindlimb extension was 12.2 ± 1.7 , 11.2 ± 0.7 , and 11.0 ± 1.3 sec in *TrkB* +/+, +/-, and -/- respectively ($p > 0.05$). Thus the *TrkB* -/- mice are fully capable of expressing a behavioral endpoint of kindling, a tonic-clonic seizure.

Additional experiments determined the threshold of current required to evoke a seizure and also determined whether a predominantly clonic seizure could be evoked in both *TrkB* +/+ and -/- mice. A minimal electroshock seizure was evoked in all +/+ and -/- mice; the stereotypical pattern of the initial phase, consisting of flexion of the head with clonic movements of the head and face and all four extremities, was observed in all +/+ and -/- mice. The current required to evoke the seizure was similar in +/+ (9.7 ± 0.7 mA) and -/- (10.3 ± 0.7 mA) mice; the duration was slightly but not significantly longer in the +/+ (45 ± 14 sec) compared to the -/- (30 ± 7 sec) mice. Thus the *TrkB* -/- mice are capable of expressing a predominantly clonic seizure with features similar to Class 1 and 2 kindled seizures.

2.4 Discussion

We hypothesized that the neurotrophin BDNF mediates activation of its receptor, TrkB, in the hippocampus during epileptogenesis and that BDNF-mediated activation of TrkB is required for epileptogenesis in the kindling model. We tested these hypotheses

by quantifying epileptogenesis and hippocampal TrkB activation of wild type, *BDNF* *-/-*, and *TrkB* *-/-* mice. Four principal findings emerged. 1. Despite marked reductions of BDNF expression in multiple populations of CNS neurons, only a modest impairment of epileptogenesis was observed in the *BDNF* *-/-* mice. 2. Although BDNF was virtually eliminated from the dentate granule and CA3 pyramidal cells, significant increases of Trk activation were detected in some but not all parts of the mossy fiber pathway of *BDNF* *-/-* mice. 3. Measures of other neurotrophins revealed an increase in NT3 but not NT4 protein in the hippocampus of *BDNF* *-/-* mice. 4. In sharp contrast to *BDNF* *-/-* mice, no behavioral evidence of epileptogenesis was detectable in *TrkB* *-/-* mice, yet *TrkB* *-/-* mice exhibited electroshock-induced tonic-clonic and clonic seizures similar to wild type controls. 5. Maintenance of the hyperexcitable state following kindling is impaired in the *TrkB* *+/-* mice. These findings demonstrate that TrkB can be phosphorylated and epileptogenesis can proceed in *BDNF* *-/-* mice, albeit both phosphorylation and epileptogenesis are impaired in comparison to wild type mice. By contrast, elimination of the TrkB receptor itself selectively prevents the plastic response of epileptogenesis in the kindling model while preserving the ability to express both electrographic and behavioral seizures indistinguishable from wild type controls.

Contrary to our initial hypothesis, epileptogenesis was readily inducible in the *BDNF* *-/-* mice, with a modest inhibition evident only in the early stages. Also contrary to our initial hypothesis, increased activation of TrkB was detected during

epileptogenesis in the *BDNF* *-/-* mice although the immunohistochemical analyses revealed more modest activation compared to wild type mice. What mediated the increased TrkB activation in the mossy fiber pathway during epileptogenesis in the *BDNF* *-/-* mice? The increased expression of NT-3 protein in the *BDNF* *-/-* mice is an interesting possibility, both because of NT3's ability to activate TrkB (R. Klein et al., 1991; D. Soppet et al., 1991; I. Farinas et al., 1998) and the localization of NT-3 to the dentate granule cells (P. Ernfors et al., 1990; P. C. Maisonpierre et al., 1990), whose mossy fiber axons coincide with the spatial distribution of the activated TrkB. Indeed the increased expression of NT-3 may also contribute to epileptogenesis in the *BDNF* *-/-* mice because the partial inhibition of epileptogenesis reported in NT-3 heterozygotes (E. Elmér et al., 1997) implies that endogenous NT-3 can promote epileptogenesis in the kindling model. Additional possibilities include residual BDNF that may have escaped detection with the immunohistochemistry or even transactivation of the TrkB receptor through a G-protein coupled receptor as mediated by activation of an A_{2A} adenosine receptor (F. S. Lee and M. V. Chao, 2001).

The present findings demonstrate that TrkB is required for limbic epileptogenesis in the kindling model. In contrast to the modest inhibition of epileptogenesis evident in the *TrkB* *+/-* mice, no behavioral seizure of any type was observed after even 50 electrographic seizures evoked by focal stimulation of the amygdala in *TrkB* *-/-* mice. Importantly, electrical stimulation of the amygdala successfully evoked electrographic

seizures in *TrkB* *-/-* as well as *TrkB* *+/+* and *+/-* mice; this is important because the focal electrographic seizure is the absolute prerequisite for epileptogenesis in the kindling model (R. J. Racine, 1972a) . Whereas the duration of the initial evoked electrographic seizure was similar among *TrkB* *+/+*, *+/-*, and *-/-* mice, the progressive increase in duration of the electrographic seizure with successive stimulations was delayed and the magnitude attenuated in *TrkB* *-/-* compared to *TrkB* *+/+* and *+/-* mice. Interestingly, the duration of the electrographic seizure reached 200% of control by the 21st stimulation in the *TrkB* *-/-* mice and did not increase further even after 48-50 stimulations; whether the modest increases observed reflect residual TrkB locally in the amygdala or a TrkB independent process is uncertain. Just as focal electrographic seizures of similar duration were initially induced in *TrkB* *-/-* mice compared to *TrkB* *+/+* and *+/-* mice, tonic-clonic seizures of equivalent duration and intensity were evoked by a maximal electroshock stimulus of *TrkB* *+/+*, *+/-* and *-/-* mice. Moreover, a minimal electroshock stimulus of similar intensity triggered clonic seizures of head, face, and limbs resembling Class 1 and 2 kindled seizures in both *TrkB* *+/+* and *-/-* mice. These results demonstrate that the structural and functional connections required to exhibit behavioral seizures are present in the *TrkB* *-/-* mice. While fully capable of exhibiting both focal electrographic and electroshock-induced clonic and tonic-clonic seizures, the plasticity required for transforming a normal brain to an epileptic brain in the kindling model was selectively eliminated in the *TrkB* *-/-* mice. In contrast to the present findings,

intraventricular infusion of a TrkB ligand-scavenging protein in adult rats only partially inhibited kindling development (D. K. Binder et al., 1999b). We suspect that the limited spatial distribution of the TrkB ligand-scavenging protein underlies the partial effects previously observed.

A major focus of epilepsy research in the past decade has centered on elucidating the mechanisms of epileptogenesis in cellular and molecular terms, with the goal being to develop small molecules for preventing epilepsy in high risk individuals. Availability of animal models has been critical to this effort. Kindling is an animal model that has been widely studied in part because it permits quantifying epileptogenesis *in vivo*. Genetic and pharmacological perturbations of diverse intercellular signaling pathways using neurotrophins as well as glutamate, GABA, norepinephrine, adenosine, opiates, and other transmitters led to identification of a subset of ligand-receptor interactions that can regulate epileptogenesis in the kindling model (McNamara et al., 1987; Löscher, 2002). Among this subset, perturbations thought to limit activation of NMDA or promote activation of GABA_A receptors exert the most powerful inhibitory effects on epileptogenesis; yet in each instance, the inhibition is only partial such that behavioral and electrophysiological evidence of epileptogenesis is evident in the presence of the intervention (W. S. Schwark and M. Haluska, 1987; J. O. McNamara et al., 1988; K. H. Holmes et al., 1990; J. M. Silver et al., 1991; W. Löscher et al., 1998).

The present work demonstrates that a conditional deletion of the neurotrophin receptor TrkB from subsets of CNS neurons actually prevents behavioral evidence of epileptogenesis in the kindling model. This study is the first to demonstrate that TrkB, or any other signaling pathway, plays an essential, not merely regulatory, role in epileptogenesis in the kindling model. This requirement focuses the search for mechanisms of epileptogenesis in this model on structural and functional consequence(s) of TrkB activation. The enhanced activation of TrkB in the mossy fiber pathway in particular provides one anatomic locale for the search. Potential structural consequences of enhanced TrkB activation include formation of basilar dendrites and sprouting of mossy fiber axons of the dentate granule cells; these morphological plasticities have been identified in animal models of epilepsy (J. V. Nadler et al., 1980; T. Sutula and O. Steward, 1987; G. Golarai and T. P. Sutula, 1996; I. Spigelman et al., 1998) and are thought to underlie aberrant recurrent excitatory synaptic circuits promoting hyperexcitability. Because biolistic transfection of a dentate granule cell in explant culture with BDNF, but not NGF, is sufficient to increase axonal branching and basilar dendrite number (S. C. Danzer et al., 2002), these morphological plasticities may be one mechanism by which enhanced TrkB activation in the mossy fiber pathway could promote epileptogenesis. A potential functional consequence of enhanced TrkB activation that might promote epileptogenesis is long term potentiation (LTP) of excitatory synaptic transmission. Collectively, previous studies support the proposal

that induction of LTP is necessary but not sufficient for epileptogenesis in the kindling model (T. Sutula and O. Steward, 1987; G. Golarai and T. P. Sutula, 1996). Analyses of LTP using both pharmacological and genetic perturbations implicate activation of TrkB by BDNF in development of long lasting LTP at the Shaffer collateral-CA1 synapse (Patterson et al., 1996; Korte et al., 1998; Minichiello et al., 1999;1999b; Minichiello et al., 1999a; Muller et al., 2000; Xu et al., 2000b; He et al., 2002)2002a); whether enhanced activation of TrkB would promote formation of LTP in the mossy fiber-CA3 synapse or other synapses in the mossy fiber pathway is presently unclear.

The present findings raise the questions of precisely where, when, and how TrkB activation is required for epileptogenesis in the kindling model. Determining whether TrkB activation is required for epileptogenesis in additional models of limbic epilepsy must also be addressed. Development of transgenic mice expressing Cre recombinase in desired subsets of CNS neurons under temporal control will provide valuable tools to address these questions.

3. Reduction of TrkB in β -actin CreER *TrkB*^{flox/flox} mice delays kindling development

3.1 Introduction

Understanding the cellular and molecular mechanisms underlying epileptogenesis is a prerequisite for a rational approach to identify molecular targets for

prevention of epilepsy. Epilepsy is a severe and prevalent neurological disease for which current treatments are often ineffective and accompanied by severe side effects. Neurotrophin signaling is a known mechanism for the conversion of neuronal activity into lasting changes in brain functions, such as in the formation of ocular dominance columns (R. J. Cabelli et al., 1995; R. A. Galuske et al., 1996; R. J. Cabelli et al., 1997). Many studies have documented an important role for the neurotrophin BDNF and its receptor TrkB in epileptogenesis in the kindling model. Kindling is delayed in mice with only a single BDNF allele (M. Kokaia et al., 1995), infusion of BDNF into the hippocampus with each kindling stimulation increases epileptogenesis (B. Xu et al., 2004). Binder and colleagues (D. K. Binder et al., 1999b) demonstrated that TrkB receptor bodies, which scavenge BDNF and NT-4, delayed kindling when delivered into the ventricles of rats while the receptor bodies for other Trks did not have an effect. However, a limitation of this study was that the infusion pump could only deliver the TrkB receptor body for 11 days, so it was not possible to determine if kindling was only delayed or prevented. My colleagues and I (X. P. He et al., 2004) built upon this finding by demonstrating that mice with a conditional deletion of TrkB in a subset of CNS neurons completely prevented the development of behavioral seizures in the kindling model, despite the generation of up to 50 electrographic seizures. However, the conditional knock-out mice had TrkB eliminated from early in development and

therefore developmental deficits stemming from loss of TrkB could have contributed to the phenotype.

3.1.1 Rationale for using an inducible recombinase

The use of an inducible conditional knock-out of TrkB will allow for developmental effects from loss of TrkB to be avoided and for elimination of TrkB at different points within the kindling model. Fusion of the Cre recombinase to the ligand-binding domain of the estrogen receptor, which is mutated to bind only tamoxifen (CreER^{TM2}), allows for induction of recombination by administering tamoxifen to the transgenic mouse (D. Metzger et al., 1995; R. Feil et al., 1996; R. Feil et al., 1997; A. K. Indra et al., 1999). Furthermore, while it was hypothesized that the *Actin* promoter would produce a more widespread reduction than the *Synapsin1* promoter used in the *Syn-Cre* mouse, the apparently restricted pattern of elimination of TrkB in the tamoxifen-treated β -*actin-CreER TrkB^{lox/lox}* mouse implicates hippocampal TrkB in particular as playing a role in kindling development. The less severe phenotype seen with amygdaloid kindling in the *Act-CreER TrkB^{-/-}* mice, as compared to the phenotype seen with the *Syn-Cre TrkB^{-/-}* mice (Figure 2.8), lead us to examine hippocampal kindling development, in which TrkB is reduced at the site of stimulation. Overall, these studies will permit more precise conclusions regarding the role of TrkB in the mature brain and also suggest but do not prove an anatomical locale in which TrkB may be promoting epileptogenesis in the kindling model.

3.2 Methods

3.2.1 Mice

TrkB^{lox/lox} mutant mice in a C57/B6 background (at least 3 backcrosses to pure C57/B6) were crossed to a mouse carrying a transgene of chicken β -actin-*CreER^{TM2}* with a CMV enhancer (S. Hayashi and A. P. McMahon, 2002) (*Act-CreER*, Jackson Labs, strain 004453) to generate a mouse with inducible conditional elimination of TrkB. The *TrkB^{lox/lox}* mutant mice have exon 1 of the *TrkB* gene, which encodes the signal peptide and the first 40 amino acids of the N terminus of TrkB, flanked by *loxP* sites, *i.e.* floxed. A line of mice carrying the *Rosa26-lacZ* reporter transgene (P. Soriano, 1999) was also used to identify cells that had undergone recombinase activity (Jackson Labs, strain 002955). The genotype of each animal was assessed twice using PCR of genomic DNA isolated from a tail cut before the experiment and another cutting from the tail taken after sacrifice.

3.2.2 Tamoxifen treatment

Mice were treated with tamoxifen (7.5mg in sunflower oil (50mg/mL), Sigma) by oral gavage. Tamoxifen was put into solution by continuous rotation in a 55°C oven for approximately 1-2 hours. The tamoxifen solution was filtered (0.2 μ m) and kept at 37°C until used. Fresh tamoxifen solution was made each day. For the gavage procedure mice were restrained by hand and placed in a supine position with the neck extended.

A slightly bent feeding needle (22 gauge, Harvard Apparatus) marked for the proper length (the distance from the mouth to the bottom of the rib cage) was gently inserted with minimal pressure and the tamoxifen solution was delivered slowly through the feeding needle using a 1cc syringe. Vehicle-treated mice received an equal volume of sunflower oil alone. The mice were treated at approximately 2 months of age. Because of the possibility of excretion of tamoxifen from one mouse followed by uptake by cage mates, tamoxifen-treated mice were not housed with vehicle-treated or untreated mice.

3.2.3 β -galactosidase staining

β -galactosidase staining was used to determine the pattern of Cre activity in mice crossed to the *Rosa26-lacZ* reporter line. Mice were given a lethal dose of pentobarbital (100mg/kg), rapidly decapitated, and the brain was removed and placed on powdered dry ice. The fresh frozen brain was sectioned at 40 μ m using a cryostat and mounted on Plus slides (Erie Scientific Company). Slide-mounted tissue sections were thawed into ice cold 4% paraformaldehyde in PBS for 15 min, washed 3 times in PBS for 5 min each, and then completely immersed in the X-gal staining solution [1 mg/ml X-Gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 (NP-40) in PBS] overnight in a 37°C incubator. Following staining, the slides were washed twice in PBS for 10 min, in H₂O for 5 min, and mounted with 90% glycerol. Some slides were counterstained with neutral red to facilitate identification of anatomic structures.

3.2.4 *In situ* hybridization

The riboprobes for the TrkB *in situ* hybridization were prepared using a PCR amplicon of sequence in the TrkB kinase domain (~450bps) and transcribed into digoxigenin-labeled RNA using a MAXIscript® kit (Ambion) and digoxigenin-labeled UTP (Roche). For the *in situ* experiment fresh frozen brain sections (16 µm thick) on Plus slides were thawed into ice-cold 4% PFA in PBS for 20 minutes. After fixing the sections all steps were done under RNase-free conditions until after the hybridization. After fixing, the sections were dehydrated in a series of ethanol baths and allowed to dry before being rehydrated and then acetylated with acetic anhydride (Sigma) for 10 minutes. Following acetylation the sections were again dehydrated in an ethanol series, dried, warmed in the hybridization oven (65°C) and covered with the pre-heated (80°C for 5 min) hybridization solution [50% formamide, 5x SSC, 5x Denhardt's solution, 250 µg/ml yeast RNA, 0.5 mg/ml salmon testes DNA, and 400 ng/ml RNA probe]. A coverslip was placed over the sections with hybridization solution, the slides were placed in a humidified chamber (50% formamide in DEPC-treated water), and incubated at 65°C overnight. The following day the sections were washed in a series of formamide/SSC solutions, blocked with rabbit IgG (Chemicon) and 1% blocking reagent (Roche). The HRP-coupled anti-digoxigenin antibody (1:200, DakoCytomation) was applied for 1 hour at room temperature. The HRP was used to enzymatically deposit cyanine-3 fluorophore (Cy3) on the tissue through the use of a TSA-Cy3 amplification

system (PerkinElmer). The slides were dehydrated and mounted with Krystalon (Harleco). The fluorescent signal was visualized using a Leica confocal microscope, with excitation at 532 nm. Images are maximum projections of an image stack that spanned the section.

3.2.5 Surgery and kindling

Under pentobarbital (60mg/kg) anesthesia, a bipolar electrode used for stimulation and recording was stereotactically implanted in the right amygdala using the following coordinates with bregma as the reference: 1.2 mm posterior, 2.9 mm lateral, 4.6 mm below dura, or in the right hippocampus with the following coordinates: 2.9 mm posterior, 3.0 mm lateral, 3.0 mm below dura. After a post-operative recovery period of 1 week, the electrographic seizure threshold (EST) was determined by application of a 1 sec train of 1 msec biphasic rectangular pulses at 60 Hz beginning at 20 μ A using a Grass stimulator with constant current stimulus isolation units (PSIU6, Grass Technologies). Additional stimulations, increased in 10 μ A steps, were administered at 1 minute intervals until an electrographic seizure lasting at least 5 seconds was detected on the electroencephalogram (EEG) recorded from the site of stimulation, i.e. amygdala or hippocampus. Stimulations at the intensity of EST were subsequently administered following the standard kindling protocol that was described previously in detail (section 2.2.2) (X. P. He et al., 2002). Video and EEGs were recorded using Harmonie software (Stellate Systems, Montreal, Quebec, Canada). The behavioral manifestations of seizures

were classified according to a modification of the description of Racine (R. J. Racine, 1972a): 1) facial clonus; 2) head nodding; 3) unilateral forelimb clonus; 4) rearing with bilateral forelimb clonus; 5) rearing and falling (loss of postural control); 6) running or bouncing seizures; and 7) tonic hind limb extension. Unless specified otherwise, mice were stimulated until fully kindled as defined by the occurrence of 3 consecutive seizures of Class 4 or greater. The kindling procedures were performed by an individual blinded to genotype of the animals.

3.2.6 Brain homogenates and immunoblot

Mice were treated with a lethal overdose of pentobarbital (100mg/kg, i.p.) and rapidly decapitated. Following decapitation, the mouse brain was placed on ice and divided along the midline. The right hemisphere, in which the electrode was placed, was taken whole and frozen on powdered dry ice. This hemisphere was sectioned to confirm electrode placement. The left hemisphere was dissected on ice into striatum, parietal cortex, amygdala-piriform cortex, hippocampus, and cerebellum. Each dissected region was homogenized in lysis buffer [20 mM Tris, pH 8.0, 137 mM NaCl, 1% Igepal, 10% glycerol, 1 mM phenylmethylsulfonylfluoride, and 1 Complete Mini protease inhibitor tablet (Roche, Mannaheim, Germany)/10 ml]. The pellet was discarded following centrifugation at 0.3 g for 10 min and the protein concentration of the supernatant was determined using a BCA Protein Assay kit (Pierce, Rockford, IL).

The homogenate was diluted to 2 mg/mL with lysis buffer, aliquoted, and stored at –80°C for further biochemical analysis.

For immunoblotting, the sample loading buffer [150 mM Tris·HCl (pH 6.8)/15% SDS/25% (vol/vol) glycerol/0.02% (wt/vol) bromophenol blue/12.5% (vol/vol) 2-mercaptoethanol] was added to the homogenate and boiled for 10 minutes before SDS-PAGE, transfer, and immunoblotting on protran nitrocellulose membrane (Whatman, Germany) as recommended by the manufacturer. The membrane was blocked with 3% non-fat dry milk or 3% bovine serum albumin in 1X TBS buffer containing 0.05% Tween-20, probed with mouse anti-TrkB antibody (1:500, BD Transduction Laboratories) and a mouse anti- β -actin antibody (1:10,000, Sigma) for 1 hr at room temperature followed by 3 washes with blocking solutions and then with peroxidase-conjugated goat anti-mouse secondary antibodies (1:5000, Jackson Immunoresearch) for another hour. The immunoblots were developed with enhanced chemiluminescence (ECL, Amersham Pharmacia, Buckinghamshire, United Kingdom).

To quantify TrkB protein expression in some experiments, a standard curve was constructed by inclusion of a series of dilutions of homogenate with known protein content. The standard curve spanned the range of TrkB levels detected in the samples. Band intensity was determined using volume quantification in ImageQuant (Molecular Dynamics).

3.3 Results

3.3.1 Characterization of mice

The initial characterization of the *Act-CreER* mice involved determining the brain regions in which tamoxifen treatment induced recombinase activity, in addition to determining whether there was any recombinase activity in the absence of tamoxifen. Using a *Rosa26-lacZ* reporter line is a preferable method to determine the pattern of recombinase activity since it is far easier to localize the presence of expression of β -galactosidase, which can be visualized using an X-gal reaction which enhances the signal through the enzymatic activity of β -galactosidase, rather than to attempt to visualize the loss of TrkB expression.

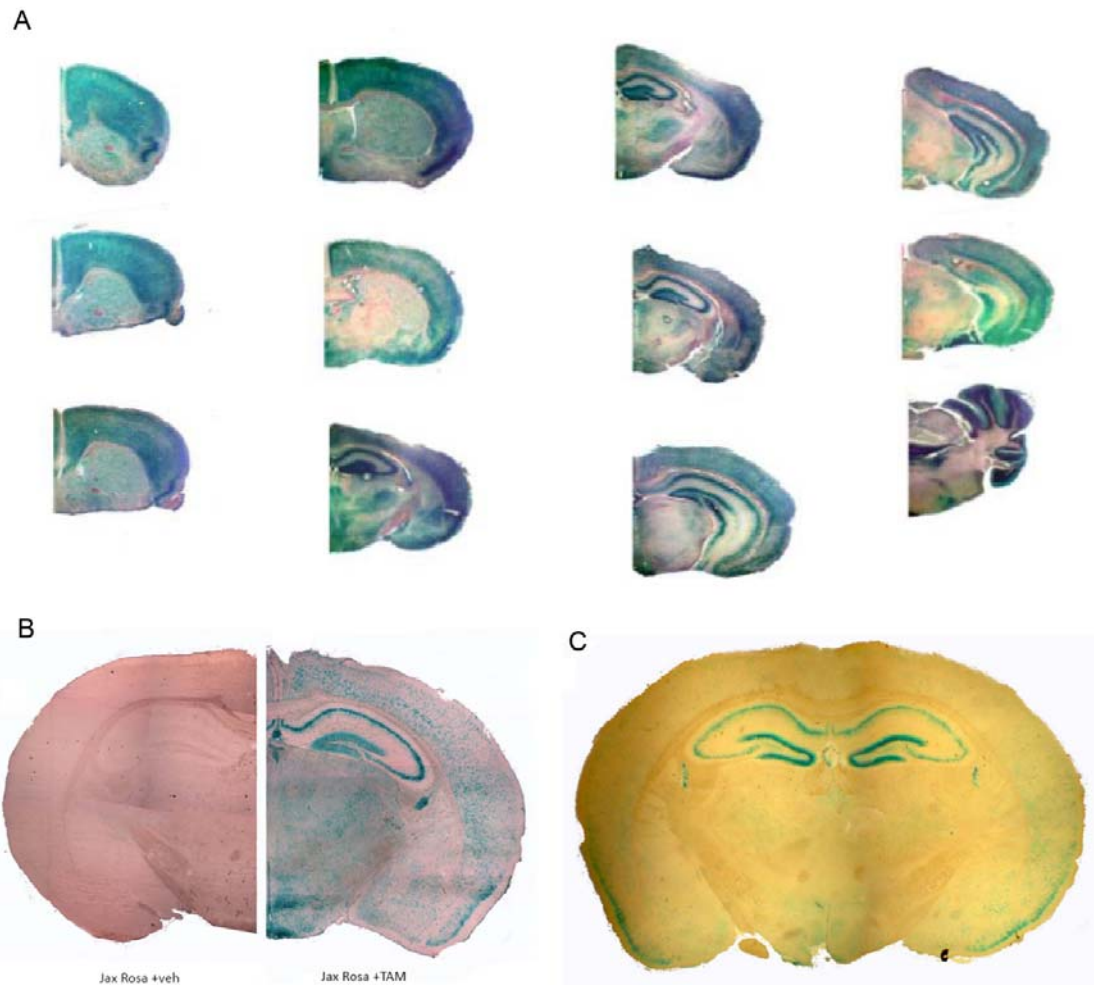


Figure 11: β -galactosidase staining in a tamoxifen-treated *Act-CreER Rosa26-lacZ* mice.

A) β -galactosidase activity throughout a coronal series of tamoxifen-treated *Act-CreER Rosa26-lacZ* mice. For analysis of inducible Cre activity, *Act-CreER* mice were crossed to a *Rosa26-lacZ* reporter line, in which β -galactosidase expression reflects Cre activity, and then treated with tamoxifen. X-gal staining (blue) resulting from β -galactosidase activity is widespread in cortex, striatum, amygdala, hypothalamus, and hippocampus. B) Control of Cre recombinase activity. Comparison of vehicle treated (left) and tamoxifen-treated (right) *Act-CreER Rosa26-lacZ* mouse. C) Sub-maximal induction of Cre activity. An example of sub-maximal activation of Cre activity by a single dose of tamoxifen (PO 7.5 mg). Cre activity is predominately in the hippocampus, especially in the dentate gyrus, and in the

piriform cortex, with sporadic X-gal positive cells throughout the brain. Greater variability was seen in the degree of induced Cre activity with a single dose of tamoxifen.

Induction of recombinase activity by tamoxifen in mature (>2 months old) *Act-CreER Rosa26-lacZ* mice revealed widespread activation of Cre in the brain (Figure 11 A). *Act-CreER Rosa26-lacZ* mice were treated with 7.5 mg tamoxifen daily for 5 days, sacrificed 5-7 days after the last dose, and β -galactosidase activity was assessed by X-gal staining. Staining was present in all layers of neocortex, striatum, septum, throughout the hippocampus, ventral posterior thalamic nucleus, the periaqueductal gray, and the cerebellar Purkinje cells (Figure 11 A). Comparison of tamoxifen-treated *Act-CreER Rosa26-lacZ* mice with vehicle-treated *Act-CreER Rosa26-lacZ* mice uncovered no evidence for Cre activity in the absence of tamoxifen (no “leakiness”) (Figure 11 B). Submaximal activation of Cre with a single 7.5 mg dose of tamoxifen demonstrated a similar pattern of activity, albeit with a smaller percentage of cells induced in each area (Figure 11 C). The exception is the hippocampus and the piriform cortex, which displayed significant recombinase activity even with a single dose of tamoxifen.

A comparison of Cre activity in tamoxifen-treated *Act-CreER Rosa26-lacZ* mice and *Syn-CreER Rosa-lacZ* mice revealed grossly similar levels of recombination, but with different anatomical distributions (Figure 12 A & B). Within the hippocampus the pattern of Cre activity was as previously described for the *Syn-Cre Rosa26-lacZ* mouse (Figure 3), with more activity in the dentate granule cells and the CA3 pyramidal cells as

compared to the CA1 pyramidal cells (Figure 12 A right). Activation of Cre activity in the hippocampus of the tamoxifen-treated *Act-CreER Rosa26-lacZ* mouse showed uniform activity throughout the principal cell layers (Figure 12 A center). Within the amygdala and piriform cortex the clearest discrepancy between the two lines is that the *Act-CreER Rosa26-lacZ* mouse has more recombination in the medial nuclei of the amygdala (medial amygdaloid nucleus, posterolateral part) (Figure 12 B center), while the *Syn-Cre Rosa26-lacZ* mouse has more recombination in the lateral nuclei (basolateral amygdaloid nucleus, anterior part, and lateral amygdaloid nucleus, dorsolateral part) (Figure 12 B right).

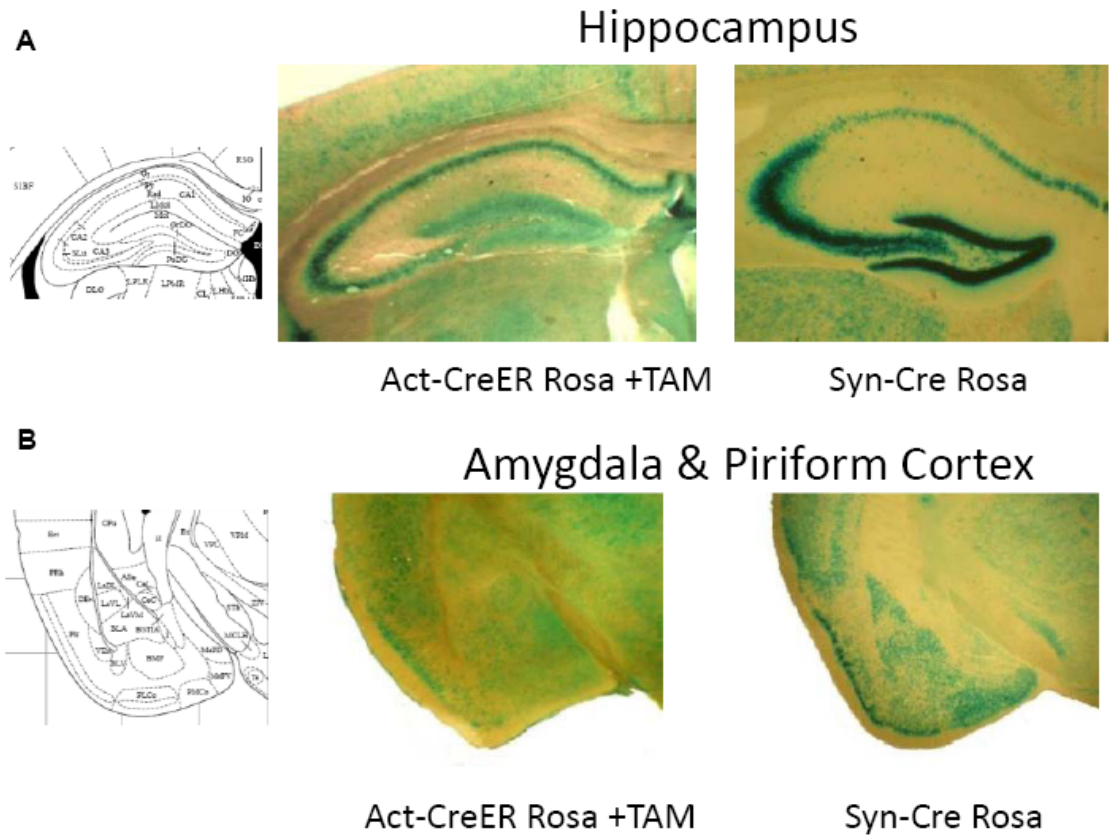


Figure 12: Comparison of Cre activity in *Syn-Cre* and *Act-CreER* mice.

A) Cre activity in the hippocampus. Comparison of Cre activity in the *Syn-Cre* and the tamoxifen-treated *actin-CreER* mice in the *Rosa26-lacZ* reporter line revealed similar levels of Cre activity, but with varying anatomical distributions. Within the hippocampus the *Syn-Cre* mouse has more intense staining in the dentate gyrus and CA3 than in CA1, while the *Act-CreER* mouse has relatively uniform staining in all principle cell layers. **B) Cre activity in the amygdala-piriform cortex.** In the amygdala the *Syn-Cre* mouse has the most intense staining in the basolateral amygdaloid nucleus, anterior part (BLA) and the lateral amygdaloid nucleus, dorsolateral part (LaDL), while the *Act-CreER* mouse has the most intense staining in the medial amygdaloid nucleus, posterodorsal part (MePD).

Importantly, the efficacy of recombination of the floxed gene, *TrkB^{lox/flox}*, must be examined in addition to the *Rosa26-lacZ* reporter. Immunoblot of dissected brain regions and *in situ* hybridization were used to examine the extent of elimination of TrkB following tamoxifen treatment of the *Act-CreER TrkB^{lox/flox}* mice (hereafter *Act-CreER TrkB^{-/-}* to signify the β -actin-*CreER TrkB^{lox/flox}* mice after induction of Cre activity by tamoxifen). Following treatment with 5 daily doses of 7.5 mg of tamoxifen and 9 days for elimination of existing *TrkB* mRNA and protein, the brains of *Act-CreER TrkB^{-/-}* were dissected into neocortex, hippocampus, amygdala-piriform cortex, and cerebellum. Surprisingly, immunoblot for TrkB revealed a less wide-spread reduction in TrkB protein levels than expected from the *Act-CreER Rosa26-lacZ* mice (Figure 13 A). Full length TrkB protein was reduced in the hippocampus of *Act-CreER TrkB^{-/-}* mice to the same degree as in *Syn-Cre TrkB^{-/-}* mice, approximately two-fold reduced from the wild type mouse. Truncated TrkB in the hippocampus was reduced as well, but only in the *Act-CreER TrkB^{-/-}* mouse. No clear differences between the *Act-CreER TrkB^{-/-}* mouse and the wild type mouse were seen in either full-length or truncated TrkB in neocortex, amygdala-piriform cortex, or cerebellum. The *Syn-Cre TrkB^{-/-}* mouse had a significant reduction in TrkB in the amygdala-piriform cortex, and smaller reductions in full-length TrkB in the cortex and in the cerebellum. The increase in truncated TrkB in the *Syn-Cre TrkB^{-/-}* mouse in the hippocampus, neocortex, and amygdala-piriform cortex as

compared to the wild-type mouse was not a consistent finding. The actin loading controls show equivalent protein loading for all lanes (Figure 13 A bottom).

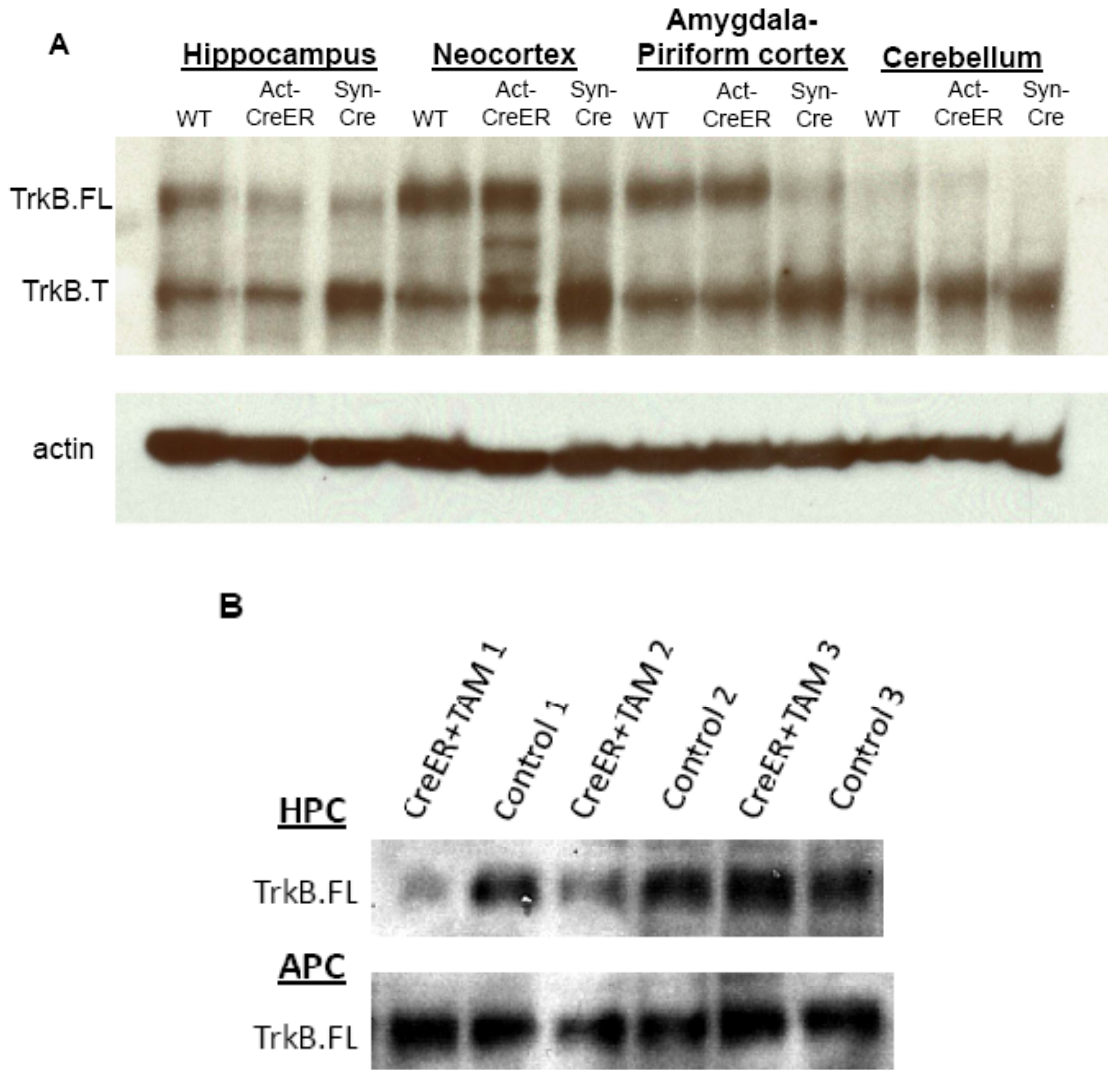


Figure 13: Reduction of TrkB protein in *Act-CreER TrkB*^{-/-} mice.

A) TrkB immunoblot in multiple brain regions in wild type (WT), *Act-CreER TrkB*^{-/-} (*Act-CreER*), and *Syn-Cre TrkB*^{-/-} (*Syn-Cre*) mice. Immunoblotting was done to examine TrkB protein levels in different brain regions following tamoxifen treatment in an *Act-CreER TrkB*^{-/-} mouse. A wild type mouse and a *Syn-Cre TrkB*^{-/-}

mouse are included for comparison. Actin immunoblot is included as a loading control. B) TrkB immunoblot in multiple tamoxifen-treated *Act-CreER TrkB^{lox/lox}* and control mice. Comparison of multiple tamoxifen-treated *Act-CreER TrkB^{lox/lox}* mice with control mice reveals a spectrum of efficiency in elimination of TrkB protein from the hippocampus (HPC), with no changes in TrkB in the amygdala-piriform cortex (APC).

In addition to the dissociation between the Cre recombinase activity seen in the *Rosa26-lacZ* reporter mouse (Figure 11 A) and in the *TrkB^{lox/lox}* mouse (Figure 13 A), the degree of reduction of TrkB varied in *Act-CreER TrkB^{-/-}* mice despite identical treatment with tamoxifen. The reduction in hippocampal TrkB varied from a greater than two-fold reduction to no detectable reduction (Figure 13 B top). No clear reduction in TrkB levels in the amygdala-piriform cortex were seen in the *Act-CreER TrkB^{-/-}* mice, despite recombination in the hippocampus in the same mouse (Figure 13 B bottom). Overall, our experience with the efficacy of reduction of TrkB following tamoxifen treatment in the *Act-CreER TrkB^{lox/lox}* mice revealed that tamoxifen treatment using this protocol results in 80% of mice (24 of 30) having hippocampal TrkB levels reduced to <75% of control mice.

To determine if the dissociation between the *Rosa26-lacZ* reporter mouse and the *TrkB^{lox/lox}* mouse was present at the level of *TrkB* mRNA, an *in situ* hybridization was performed. A non-quantitative *in situ* hybridization with a digoxigen-labeled riboprobe that specifically recognizes full-length *TrkB* was done using wild type, *Act-CreER TrkB^{-/-}*, and *Syn-Cre TrkB^{-/-}* mice (Figure 14). The wild type mouse showed strong TrkB expression throughout the principal cell layers of the hippocampus and through the

amygdala and piriform cortex (Figure 14 A & B left). A significant reduction in *TrkB* mRNA in the hippocampus was seen in the *Act-CreER TrkB^{-/-}* mouse and in *Syn-Cre TrkB^{-/-}* mouse, as compared to a wild type mouse (Figure 14 A). In the *Syn-Cre TrkB^{-/-}* mouse, *TrkB* mRNA in the CA1 pyramidal cells clearly remained, although reduced, but *TrkB* mRNA was nearly absent from CA3 pyramidal cells and only slightly less so in dentate granule cells (Figure 14 A right). In the *Act-CreER TrkB^{-/-}* mouse hippocampal *TrkB* was greatly reduced, with the greatest amount of *TrkB* mRNA remaining in the CA1 pyramidal cells, within the dentate gyrus including the hilus, and neurons outside the pyramidal cell layer (stratum oriens and stratum lucidum in CA3, stratum radiatum in CA1) (Figure 14 A center). *TrkB* mRNA was absent from the amygdala and greatly reduced in the piriform cortex of the *Syn-Cre TrkB^{-/-}* mouse (Figure 14 B right). *TrkB* mRNA levels were slightly reduced in the amygdala but unchanged in the piriform cortex in the *Act-CreER TrkB^{-/-}* mouse as compared to a wild type mouse (Figure 14 B center).

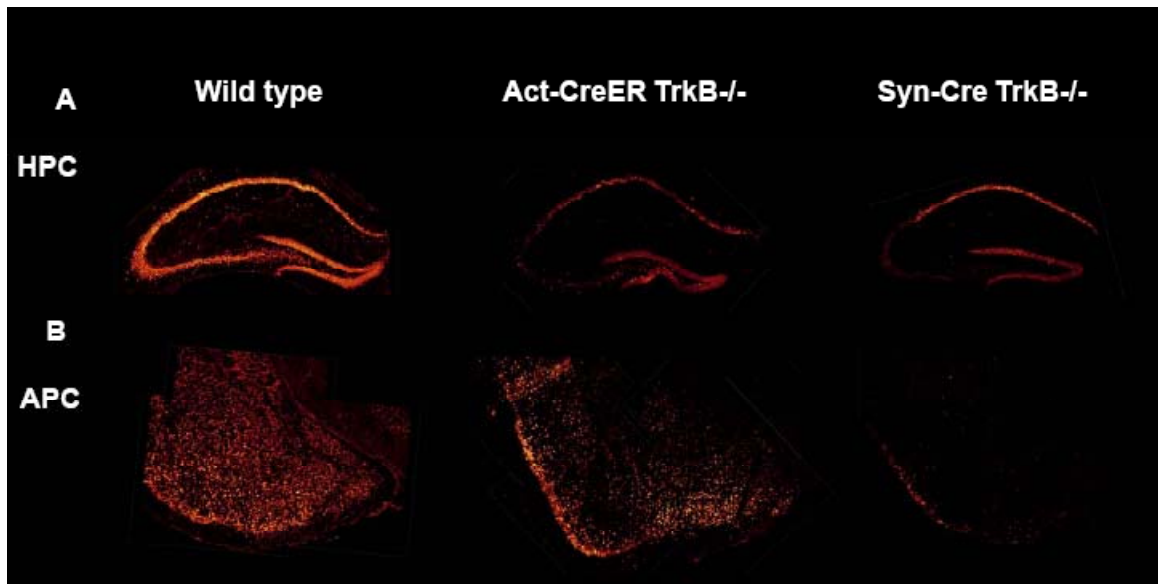


Figure 14: *TrkB* *in situ* hybridization in wild type, *Act-CreER TrkB*^{-/-}, and *Syn-Cre TrkB*^{-/-} mice.

A) *TrkB* *in situ* hybridization in hippocampus. *In situ* hybridization reveals a dramatic reduction in *TrkB* mRNA in the hippocampus of the *Act-CreER TrkB*^{-/-} mouse, with the only some sparing of *TrkB* mRNA in the CA1 pyramidal cells and a few sporadic cells throughout. *Syn-Cre TrkB*^{-/-} mice have a *TrkB* mRNA remaining only in the CA1 pyramidal cells. The wild type mouse shows *TrkB* mRNA throughout the hippocampus. B) *TrkB* *in situ* hybridization in amygdala-piriform cortex (APC). *In situ* hybridization in the amygdala and piriform cortex reveals a decrease in *TrkB* mRNA in the amygdala but not the piriform cortex in the *Act-CreER TrkB*^{-/-} mouse that was undetected at the protein level. The *Syn-Cre TrkB*^{-/-} mouse has a dramatic reduction in *TrkB* mRNA throughout the amygdala with only some sparing in the piriform cortex. The wild type mouse shows *TrkB* mRNA throughout the amygdala and piriform cortex.

3.3.2 Kindling

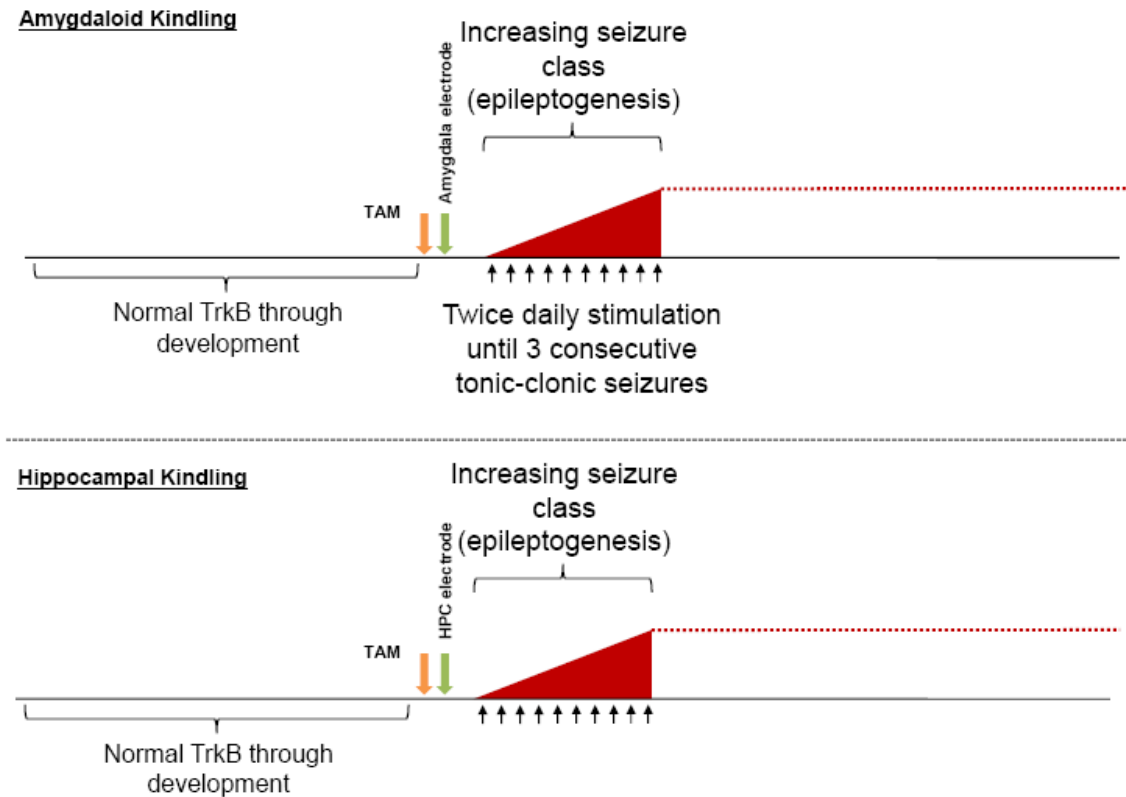


Figure 15: Kindling and tamoxifen protocols for *Act-CreER TrkB^{flox/flox}* mice.

Act-CreER TrkB^{flox/flox} mice and wild type controls are allowed to mature to 2 months of age before treatment with tamoxifen or vehicle. Following treatment with tamoxifen (PO 7.5mg x5d), mice were implanted with electrodes for kindling, either in the hippocampus or in the amygdala. Kindling was continued until the behavioral seizures progressed to 3 consecutive Class 4 or greater seizures, at which point the mouse was considered fully kindled.

3.3.2.1 Amygdaloid kindling

Overall, the development of kindling was significantly delayed in tamoxifen treated *Act-CreER TrkB^{-/-}* mice stimulated in the amygdala as compared to control mice

(*Act-CreER TrkB^{fllox/fllox}* + vehicle, wild-type mice + tamoxifen, wild-type mice + vehicle) (Figure 4.7 B). Comparison of the various control groups by ANOVA did not reveal any significant differences ($p > 0.05$) and therefore these groups were analyzed as a single control group. *Act-CreER TrkB^{fllox/fllox}* mice were treated with tamoxifen at 2 months of age to induce Cre recombinase activity before beginning the kindling protocol (Figure 15 top). *Act-CreER TrkB^{-/-}* mice did not differ from control mice in their excitability at the first stimulation, as evidenced by similar electrographic seizure threshold (*Act-CreER TrkB^{-/-}* $210 \pm 45 \mu\text{A}$ vs. controls $190 \pm 29 \mu\text{A}$, $p > 0.05$) (Figure 16 A left) or in the duration of the first electrographic seizure (*Act-CreER TrkB^{-/-}* 11.2 ± 1.1 sec vs. controls 12.8 ± 2.1 sec, $p > 0.05$) (Figure 16 A right). Upon repeated stimulations *Act-CreER TrkB^{-/-}* mice were found to be deficient in the development of kindling, requiring significantly more stimulations to reach the fully kindled state (3 consecutive seizures of Class 4 or greater) than control mice (*Act-CreER TrkB^{-/-}* 16.1 ± 2.1 stimulations vs. controls 10.1 ± 1.3 stimulations, $p < 0.05$) (Figure 16 B). Given the difference in the phenotype in the *Act-CreER TrkB^{-/-}* mice as compared to the *Syn-Cre TrkB^{-/-}* mice, the possible effects of loss of TrkB at the site of stimulation were considered. Since the *Act-CreER TrkB^{-/-}* mice display a loss of TrkB in the hippocampus, hippocampal kindling in the *Act-CreER TrkB^{-/-}* mice was explored.

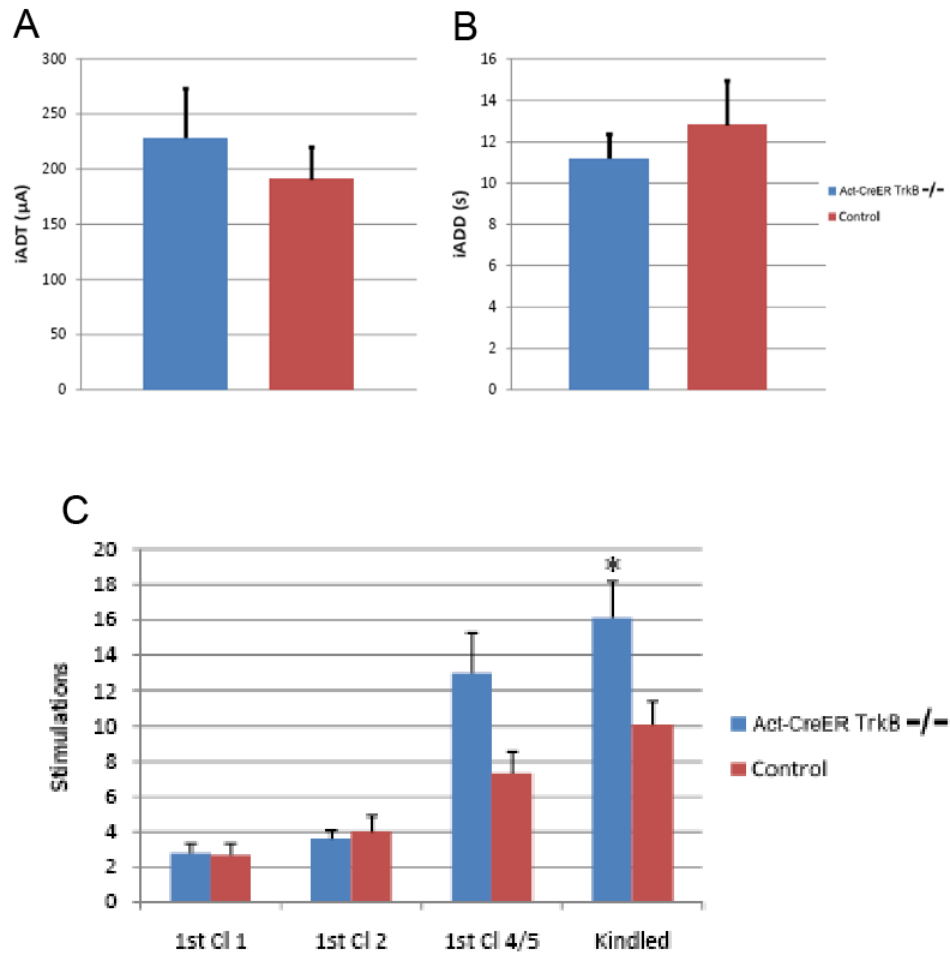


Figure 16: Amygdaloid kindling in the *Act-CreER TrkB*^{-/-} mice.

A) Initial electrographic seizure threshold. *Act-CreER TrkB*^{-/-} mice did not differ from control mice in initial excitability as evidenced by an equivalent threshold to induce an electrographic seizure ($p > 0.05$). B) Initial electrographic seizure duration. *Act-CreER TrkB*^{-/-} mice did not differ from control mice in initial excitability as evidenced by an equivalent duration of the first seizure ($p > 0.05$). C) Kindling development. *Act-CreER TrkB*^{-/-} mice have delayed kindling development as compared with control mice, requiring significantly more stimulations to reach the fully kindled state ($p < 0.05$). Mean + SEM.

3.3.2.2 Hippocampal kindling

Overall, the rate of development of kindling was significantly delayed in *Act-CreER TrkB^{-/-}* mice stimulated in the hippocampus as compared to control mice (*Act-CreER TrkB^{lox/lox}* + vehicle, wild-type mice + tamoxifen, wild-type mice + vehicle) (Figure 17 B). Comparison of the various control groups did not reveal any significant differences in the measured variables (ANOVA, $p > 0.05$) and therefore these groups were combined into a single control group for the purpose of statistical analysis. *Act-CreER TrkB^{lox/lox}* mice were treated with tamoxifen at 2 months of age to induce Cre recombinase activity before beginning the kindling protocol (Figure 15 bottom). The *Act-CreER TrkB^{-/-}* mice did not display a significant difference in their initial excitability as compared to controls, either in the electrographic seizure threshold (*Act-CreER TrkB^{-/-}* $113 \pm 27 \mu\text{A}$ vs. controls $170 \pm 30 \mu\text{A}$, $p > 0.05$) (Figure 17 A left) or in the duration of the first electrographic seizure (*Act-CreER TrkB^{-/-}* $18.8 \pm 2.1 \text{ sec}$ vs. controls $20.4 \pm 2.0 \text{ sec}$, $p > 0.05$) (Figure 17 A right). Upon repeated stimulations significantly more stimulations were required for *Act-CreER TrkB^{-/-}* mice to achieve their first Class 4/5 seizure (*Act-CreER TrkB^{-/-}* 30.6 ± 5.0 stimulations vs. controls 12.1 ± 1.5 stimulations, $p < 0.01$) and to reach the fully kindled state (3 consecutive seizures of Class 4 or greater) than control mice (*Act-CreER TrkB^{-/-}* 36.5 ± 4.2 stimulations vs. controls 15.5 ± 1.3 stimulations, $p < 0.01$) (Figure 17 B). Additionally 2 *Act-CreER TrkB^{-/-}* mice did not have any Class 4 or

greater seizures within the 50 stimulations of the kindling protocol, although they did develop Class 1-2 behavioral seizures.

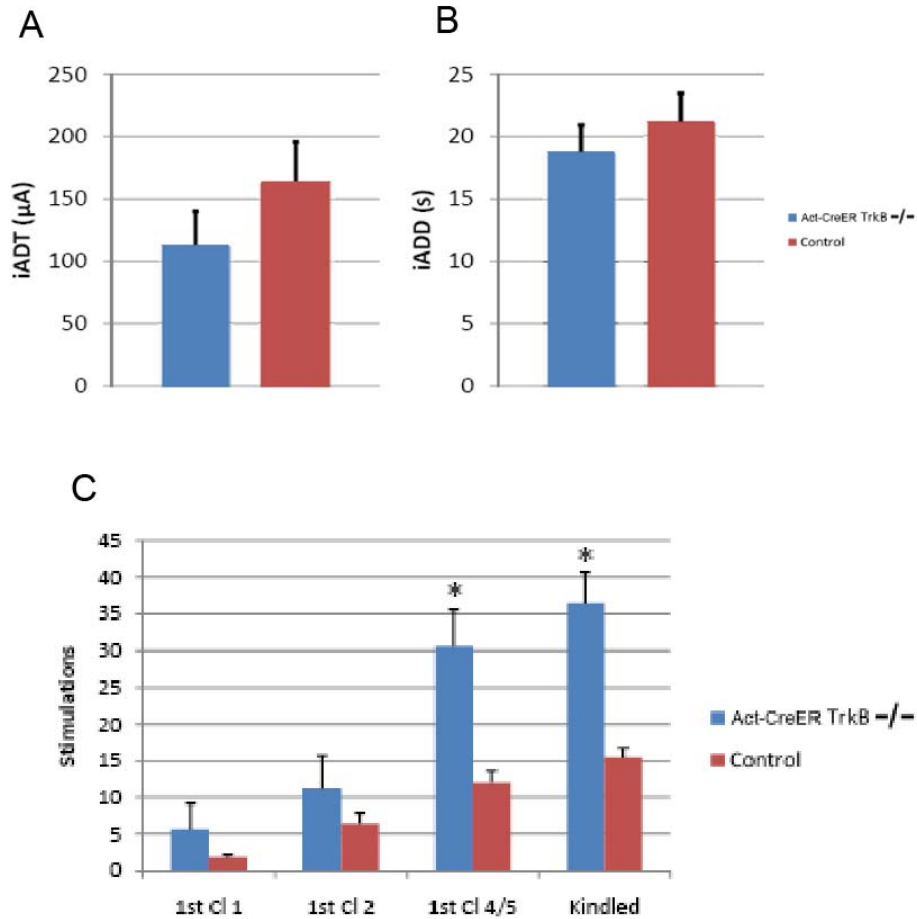


Figure 17: Hippocampal kindling in *Act-CreER TrkB*^{-/-} mice.

A) Initial electrographic seizure threshold. *Act-CreER TrkB*^{-/-} mice did not differ from control mice in initial excitability as evidenced by an equivalent threshold to induce an electrographic seizure ($p > 0.05$). B) Initial electrographic seizure duration. *Act-CreER TrkB*^{-/-} mice did not differ from control mice in initial excitability as evidenced by an equivalent duration of the first seizure ($p > 0.05$). C) Kindling development. *Act-CreER TrkB*^{-/-} mice have delayed kindling development as compared with control mice, requiring significantly more stimulations to reach the 1st Cl 4/5 seizure ($p < 0.01$) and to reach the fully kindled state ($p < 0.01$). Mean + SEM.

3.3.3 Evidence for elimination of TrkB

3.3.3.1 Reduction of TrkB and delay in amygdaloid kindling

Experience with the *Act-CreER TrkB^{-/-}* mice revealed variability in the extent of TrkB reduction detected in tamoxifen-treated mice. We therefore quantified TrkB protein content in the hippocampus in both control mice and in the *Act-CreER TrkB^{-/-}* mice and sought to determine if a relationship exists between the extent of TrkB reduction and the rate of kindling development. Analysis of the TrkB protein levels in the *Act-CreER TrkB^{-/-}* mice from the amygdaloid kindling experiment revealed 7 of the 10 mice had reduced levels of TrkB protein in the hippocampus (<75% of wild type levels). The threshold of < 75% of the average protein content in control mice was chosen as it encompassed the variability seen within control mice and divided the clearly different groups within the tamoxifen-treated *Act-CreER TrkB^{lox/lox}* mice (see Figure 18 A inset as an example). Analysis of the kindling rates for *Act-CreER TrkB^{-/-}* mice with reduced TrkB (“successes”) showed a greater delay in reaching the fully kindled state (19.3 ± 4.8 stimulations) than *Act-CreER TrkB^{-/-}* mice with normal levels of TrkB in the hippocampus (“failures”) (8.7 ± 2.7 stimulations) or control mice (10.1 ± 1.3 stimulations) (Figure 18 B).

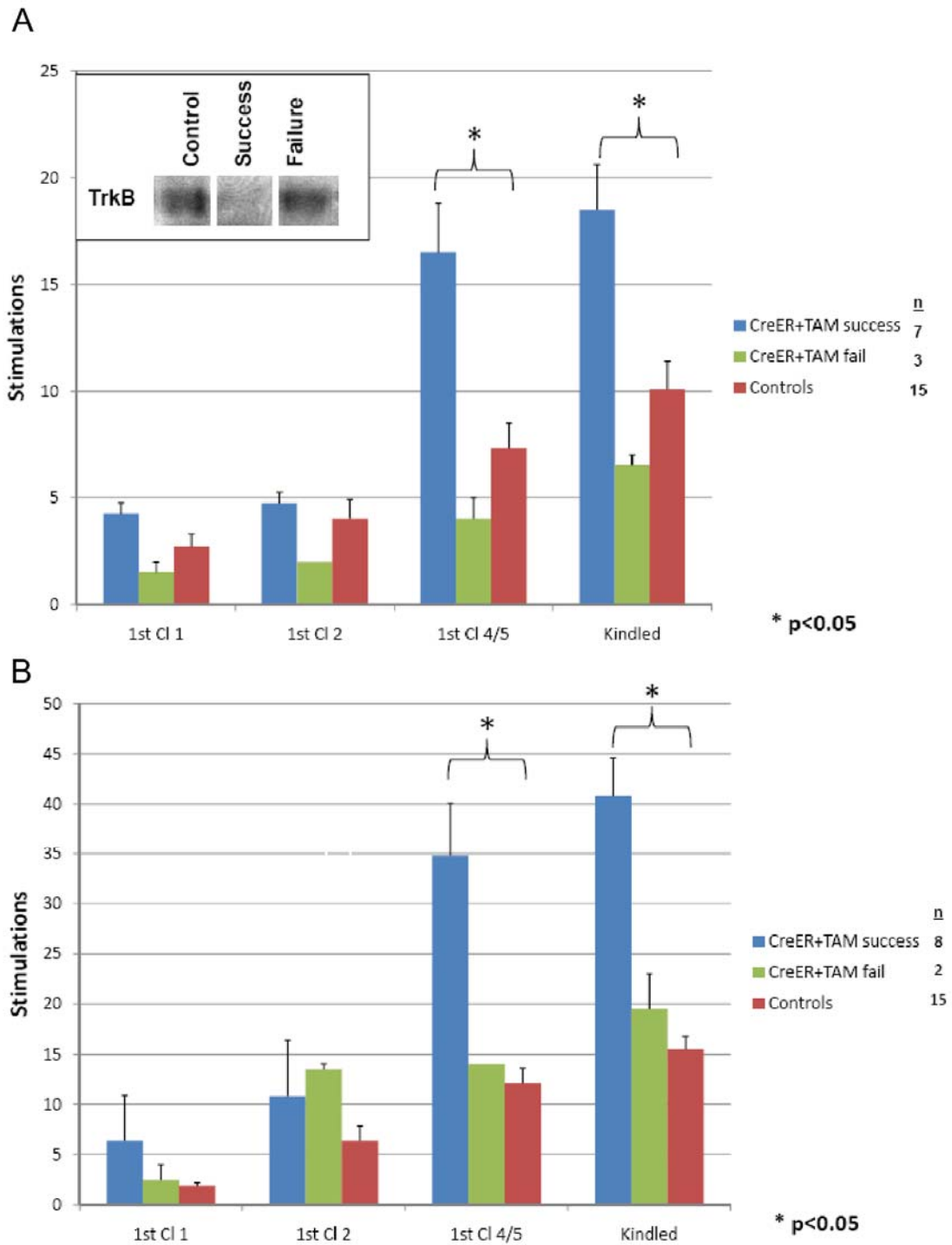


Figure 18: TrkB elimination and delay in kindling development.

A) Amygdaloid kindling in *Act-CreER TrkB*^{-/-} mice with a reduction in TrkB protein. The subset (n = 7) of *Act-CreER TrkB*^{flx/flx} mice with a reduction in TrkB (<75% wild type levels) following tamoxifen treatment showed a delay in amygdaloid kindling, while tamoxifen-treated *Act-CreER TrkB*^{flx/flx} mice without a reduction in TrkB (n = 3) did not show a delay in kindling, as compared to controls (n=15). Inset: A representative immunoblot showing TrkB protein in a control mouse, a successfully tamoxifen-treated *Act-CreER TrkB*^{flx/flx} mouse, and an *Act-CreER TrkB*^{flx/flx} mouse with failed tamoxifen treatment. B) Hippocampal kindling in *Act-CreER TrkB*^{-/-} mice with a reduction in TrkB. The subset (n = 8) of *Act-CreER TrkB*^{flx/flx} mice with a reduction in TrkB (<75% wild type levels) following tamoxifen treatment showed a delay in hippocampal kindling, while tamoxifen-treated *Act-CreER TrkB*^{flx/flx} mice without a reduction in TrkB (n = 2) did not show a delay in kindling, as compared to controls (n=15). Mean + SEM, * p<0.05 ANOVA with post-hoc Bonferroni's test.

3.3.3.2 Reduction of TrkB and delay in hippocampal kindling

Analysis of TrkB levels in the *Act-CreER TrkB*^{-/-} mice revealed 8 of the 10 mice had levels of TrkB protein in the hippocampus below the range seen in control mice (<75% of control levels). Analysis of the kindling rates for only *Act-CreER TrkB*^{-/-} mice with reduced TrkB ("successes") showed a greater delay in kindling rate, with 40.8 ± 3.8 stimulations required to become fully kindled, while *Act-CreER TrkB*^{-/-} mice without a reduction in TrkB ("failures") required 19.5 ± 3.5 stimulations and controls required 15.5 ± 1.3 stimulations (Figure 18 A).

3.4 Discussion

3.4.1 Summary and Conclusions

The essential finding of this study is that reduction of TrkB *de novo* in the mature mouse is sufficient to delay kindling development. By using an inducible conditional

knock-out of TrkB (*Act-CreER TrkB^{lox/lox}* mice), kindling development was examined following the elimination of TrkB selectively in the mature mouse. The principal findings of these studies are: 1) Reduction of TrkB in the mature mouse is sufficient to delay kindling from the amygdala as well as kindling from the hippocampus. 2) The delay in kindling is associated with the reduction in TrkB within the group of tamoxifen-treated *Act-CreER TrkB^{lox/lox}* mice (recombination “successes” vs. “failures”). 3) The delay in kindling caused by reduced TrkB is most consistent with an anti-epileptogenic effect, since the initial excitability was not affected although TrkB was already reduced. These results further support the role of TrkB in the kindling model and identify TrkB as a direct actor in epileptogenesis in the mature brain.

3.4.2 Loss of TrkB in the mature animal and epileptogenesis

Elimination of TrkB selectively in the adult mouse is sufficient to delay kindling. This result provides further support that TrkB plays a direct role in epileptogenesis in the kindling model, in agreement with previous modifications of BDNF/TrkB signaling in the mature animal (D. K. Binder et al., 1999b; B. Xu et al., 2004). Furthermore, the normal initial excitability in the *Act-CreER TrkB^{-/-}* mice implies that the effect of loss of TrkB is to inhibit plasticity (anti-epileptogenic) rather than to reduce the basal excitability of neurons (anti-convulsant). Finally, the association with reduction of TrkB protein in the hippocampus suggests that it is a relevant brain structure. All of these

restrictions on the mechanism by which loss of TrkB delays kindling will help to isolate the cellular and molecular events underlying the phenotype.

The mechanisms by which loss of TrkB could delay kindling development are likely either through limiting excitatory synaptic transmission or potentiating inhibitory synaptic transmission. BDNF/TrkB signaling is known to potentiate excitatory transmission as been demonstrated *in vitro* (M. M. Bolton et al., 2000) and *in vivo* (S. D. Croll et al., 1999). BDNF/TrkB signaling and rapid kindling have also been shown to diminish the effect of inhibitory synaptic transmission by decreasing the expression of the K⁺-Cl⁻ transporter KCC2, impairing Cl⁻ extrusion and thereby decreasing the ability of GABA receptors to hyperpolarize the cell. Following rapid kindling in mice, expression of the KCC2 is decreased in the hippocampus and does not return to basal levels within 24 hours (C. Rivera et al., 2002), implying that the stimulation has produced a long-lasting change in KCC2 expression. Furthermore, this change in KCC2 expression has been shown to be BDNF/TrkB-dependent and blocking TrkB signaling prevents or even reverses the decrease in KCC2 seen after seizure activity (C. Rivera et al., 2004). Maintained or augmented inhibition would clearly impair the development of hyperexcitability. Furthermore, decreased KCC2 mRNA expression is found in the hippocampus and subiculum of human temporal lobe patients (E. Palma et al., 2006).

Anatomically, the connections between the dentate granule cells and the CA3 pyramidal cells in the hippocampus provide a locale where increases of excitatory

synaptic transmission and decreases of inhibitory synaptic transmission colocalize. The mossy fiber (MF) terminals of dentate granule cells synapse onto glutamatergic CA3 pyramidal cells and also onto GABAergic interneurons in stratum lucidum (SLINs), which in turn inhibit the CA3 pyramidal cells. Because of differential expression of Ca²⁺ channels at the presynaptic sites of the MF-CA3 synapse and of the MF-SLIN synapse (K. A. Pelkey et al., 2006), the two synapses can undergo independent regulation. As might be expected, following high-frequency stimulation (HFS), the MF-CA3 synapse is potentiated while the MF-SLIN synapse is unchanged or impaired (G. Maccaferri et al., 1998). As TrkB is known to be activated in the mossy fiber pathway during kindling (D. K. Binder et al., 1999a; X. P. He et al., 2002) and the *Syn-Cre TrkB*^{-/-} mice are deficit in LTP at the MF-CA3 synapse (Huang et al., in review), this synapse is at the crossroads of many different pieces of circumstantial evidence regarding the role of TrkB signaling in epileptogenesis. The development of transgenic mice with elimination of TrkB selectively in the pre- and post-synaptic cells of this synapse (dentate granule cells and CA3 pyramidal cells) will precisely define the role of TrkB at this synapse during epileptogenesis.

3.4.3 Differences between the *Act-CreER TrkB*^{-/-} and *Syn-Cre TrkB*^{-/-} mice

The inducible nature of the recombination in the *Act-CreER TrkB*^{flx/flx} mice was expected to address possible developmental impacts of loss of TrkB in the *Syn-Cre TrkB*^{-/-} mice. While the *Act-CreER TrkB*^{-/-} mice demonstrate that loss of TrkB in maturity is

sufficient to delay kindling, the phenotype in the kindling model in the *Act-CreER TrkB*^{-/-} mice differs from the phenotype seen in the *Syn-Cre TrkB*^{-/-} mice in important ways. *Act-CreER TrkB*^{-/-} mice showed evidence of epileptogenesis, albeit delayed, with both amygdaloid and hippocampal kindling, rather than the complete absence of behavioral seizures seen with amygdaloid kindling in the *Syn-Cre TrkB*^{-/-} mice (Figure 10) (X. P. He et al., 2004). Furthermore, the *Syn-Cre TrkB*^{-/-} mice showed a significant increase the threshold to evoke an electrographic seizure while *Act-CreER TrkB*^{-/-} had normal initial excitability in both the amygdala (Figure 16 A) and in the hippocampus (Figure 17 A).

A variety of causes could explain the differences between the two experiments. Firstly, the different pattern of TrkB elimination from the brain could be critical. The *Syn-Cre TrkB*^{-/-} and *Act-CreER TrkB*^{-/-} mice have a similar reduction of hippocampal TrkB, but the loss of TrkB in the *Syn-Cre TrkB*^{-/-} mice is mostly in the dentate gyrus and the CA3 pyramidal cells, where the elimination of TrkB is nearly complete, while the remaining hippocampal TrkB in the *Act-CreER TrkB*^{-/-} mice is distributed throughout the hippocampus (Figure 14 A). The *Syn-Cre TrkB*^{-/-} mice have a reduction of TrkB in the amygdala while the *Act-CreER TrkB*^{-/-} do not. The increased electrographic seizure threshold in the *Syn-Cre TrkB*^{-/-} mice is likely due to local effects of loss of TrkB in the amygdala. Furthermore, cortical TrkB may also play a role, since the behavioral seizures used to score kindling likely involve cortical neurons. As evidence for kindling induced plasticity in the cortex, amygdaloid kindling has been demonstrated to cause functional

rearrangements in motor cortex (G. C. Teskey et al., 2002). In sum, the additive effects of loss of TrkB in each of these structures may be sufficient to explain the differences in kindling in the *Syn-Cre TrkB^{-/-}* and *Act-CreER TrkB^{-/-}* mice.

Secondly, the loss of TrkB in the *Syn-Cre TrkB^{-/-}* mice could have caused a developmental defect that, combined with the effect of loss of TrkB in the mature mouse, resulted in the complete lack of behavioral seizures in the kindling model. Attempts to recapitulate the developmental time course of TrkB elimination from the *Syn-Cre TrkB^{-/-}* mice in the *Act-CreER TrkB^{flox/flox}* mice were unsuccessful since abundant recombinase activity could not be induced except through oral gavage, which is not possible until the mouse is nearly mature.

3.4.4 Hippocampal TrkB and Kindling

The association of reduced hippocampal TrkB levels with delayed kindling within the *Act-CreER TrkB^{-/-}* mice (“successes” vs. “failures”), further strengthens the causative role of loss of TrkB and suggests that hippocampal TrkB may be essential. The suggestion that hippocampal TrkB may play a key role in epileptogenesis in the kindling model was suggested by previous work by Binder and colleagues (D. K. Binder et al., 1999b), in which delay in kindling development was correlated with penetration of the TrkB receptor bodies into the hippocampus but not into the striatum, and by Xu and colleagues (B. Xu et al., 2004), in which bolus BDNF infusions into the hippocampus increased the rate of kindling from the perforant path. Additionally, many of the

putative mechanisms underlying the actions of TrkB in epileptogenesis are described within the hippocampus (C. R. Bramham and E. Messaoudi, 2005).

Furthermore, extensive loss of TrkB from the site of stimulation is not required to delay kindling, since kindling is delayed in *Act-CreER TrkB^{-/-}* mice when the stimulating electrode is in the amygdala, which has no detectable decrease in TrkB protein levels by immunoblot (Figure 13 A), even when recombination had caused a loss of TrkB from the hippocampus (Figure 13 B). This result addresses an important unanswered question regarding kindling in the *Syn-Cre TrkB^{-/-}* mouse. In the *Syn-Cre TrkB^{-/-}* mice it is unclear if loss of TrkB from the amygdala is completely responsible for the lack of epileptogenesis in the kindling model. Since no behavioral seizures occur in the *Syn-Cre TrkB^{-/-}* mice, electrographic seizure activity is presumably not propagating into the motor cortex. Electrographic seizure activity in the hippocampus or septum is associated with “wet dog shakes” (M. Lerner-Natoli et al., 1984), which are also not seen in the *Syn-Cre TrkB^{-/-}* mice. While the amygdaloid kindling in the *Act-CreER TrkB^{-/-}* mice cannot reveal if seizure activity propagates out of the amygdala in the *Syn-Cre TrkB^{-/-}*, the experiments do reveal that apparent wild type levels of TrkB in the amygdala are not sufficient for normal amygdaloid kindling. Of note, as the stimulations in this experiment were delivered at a supra-threshold level, the effects of reduced TrkB on the initial plasticity of the kindling model, the reduction of the

electrographic seizure threshold at the site of stimulation, were not examined. This presents another aspect of epileptogenesis that could potentially involve TrkB.

Given the wide-spread recombination seen following tamoxifen treatment in the *Act-CreER Rosa26-lacZ* mouse, *a priori* there is no reason to exclude a reduction in TrkB in nearly any brain area in the *Act-CreER TrkB^{-/-}* mouse. However, in an immunoblot of homogenized brain regions, the reduction of TrkB is seen only in the hippocampus (Figure 13 A). However, a comparison of *TrkB* mRNA in the amygdala of the *Act-CreER TrkB^{-/-}* mouse (Figure 14 B center panel), where a slight reduction is seen in comparison to wild type mice, with TrkB protein in the immunoblot (Figure 13 A), where no reduction is seen, reveals a disassociation. Since the absence of evidence for reduced TrkB from an immunoblot is not sufficient to rule out loss of TrkB from important cell populations, experiments that directly target hippocampal TrkB are required to implicate the hippocampus as the location at which TrkB signaling is required.

3.4.4 Future directions

While this study has strengthened the hypothesis that TrkB is required in the mature brain for epileptogenesis, many questions remain. Discovering whether TrkB mediates or only modulates epileptogenesis will advance our understanding of the pathophysiology of epileptogenesis and determine the potential for therapeutics aimed at inhibiting TrkB. The *Act-CreER TrkB^{-/-}* may have insufficiently reduced TrkB signaling to replicate the phenotype of the *Syn-Cre TrkB^{-/-}* mice. However, this question

can likely be addressed with a chemical-genetic approach to inhibition of TrkB kinase activity (X. Chen et al., 2005). Studies by Huang and colleagues (Huang et al., in review) has shown that inhibition of TrkB kinase activity in this mouse impairs MF-CA3 LTP to a greater degree than the *Syn-Cre TrkB^{-/-}* mouse, and this pharmacological approach will inhibit TrkB throughout the brain.

Uncovering the brain structures in which TrkB acts during epileptogenesis, and the relations of those structures to the epileptic focus, is necessary to further our knowledge of the commonalities and differences within epilepsy and, if necessary, will guide the tailoring of clinical treatments to each individual pathological profile of epilepsy. Experiments using viral RNAi techniques to reduce TrkB selectively in well-defined structures can elucidate the locations at which TrkB acts to promote epileptogenesis as well as being an important first step towards a clinical therapy.

4. Reduction of TrkB Impairs Persistence of the Kindled State

4.1 Introduction

The defining symptom of epilepsy is repeated unprovoked seizures which are the result of a hyperexcitable brain. The pathology underlying the persistence of the hyperexcitable state is poorly understood. The study of the development of epilepsy can identify the origins of the pathology which underlies the chronic disease state.

Inhibition of signaling from the neurotrophin BDNF and its receptor TrkB have been shown to delay epileptogenesis in the kindling model (M. Kokaia et al., 1995; D. K. Binder et al., 1999b; X. P. He et al., 2004), although the time course for the required TrkB signaling is not known. Inducible reduction of TrkB by use of the CreER^{TM2}/loxP system allows for testing for a requirement of TrkB in the earliest stages of maintenance of the hyperexcitable state.

4.1.1 Rationale for studying persistence of hyperexcitability in the kindling model

Previous work exploring the role of TrkB in the development of kindling has led to the hypothesis that TrkB participates in the plasticity by which individual fleeting seizures permanently changing the excitability of the brain (epileptogenesis). The molecular and cellular events responsible for the pathological plasticity during epileptogenesis are the direct predecessors of the functional and structural changes that

underlie the persistence of the epileptic condition. By reducing the level of TrkB only after the hyperexcitable state has been achieved, the effects of blocking TrkB signaling on the persistence of the hyperexcitable state can be studied without the confounding variable of abnormal development of the kindled state.

4.2 Methods

4.2.1 Mice

4.2.1.1 *Syn-Cre TrkB^{+/-lox}* mice

TrkB^{+/-lox} mutant mice with exon 1 of a single allele of the *TrkB* gene, which encodes the signal peptide and the first 40 amino acids of the N terminus of TrkB flanked by *loxP* sites (i.e. floxed) were crossed to a transgenic mouse carrying Cre driven by a *Synapsin1* promoter (*Syn-Cre*) generated progeny in which expression of the floxed gene was selectively eliminated in a subset of CNS neurons. The *Syn-Cre TrkB^{+/-lox}* mice were on a mixed background of 129/C57/ICR. The pattern of recombinase activity in *Syn-Cre* mice, visualized with the *Rosa-26* reporter mice (P. Soriano, 1999) (Figure 2.1 A), by TrkB *in situ* hybridization (Figure 2.1 D and 4.4 A & B right), as well as with immunoblots probed for TrkB (Figure 2.7 and 4.3 A), has been previously characterized (X. P. He et al., 2004). The genotype of each animal was assessed twice using PCR of genomic DNA isolated from tail cut before the experiment and another cutting from the tail taken after sacrifice.

4.2.1.2 *Act-CreER TrkB^{lox/lox}* mice

TrkB^{lox/lox} mutant mice in a C57/B6 background (at least 3 backcrosses to pure C57/B6) were crossed to a mouse carrying a transgene of chicken β -actin-*CreER* with a CMV enhancer (S. Hayashi and A. P. McMahon, 2002) (*Act-CreER*, Jackson Labs, strain 004453) to generate a mouse with inducible conditional elimination of TrkB. The pattern of recombination in a *Rosa26-lacZ* reporter line (Figure 11) and tamoxifen-induced elimination of the floxed *TrkB* gene at a mRNA level (Figure 14 A & B middle panels) and at a protein level (Figure 13 A&B) have been described earlier in section 3.3.1. The genotype of each animal was assessed twice using PCR of genomic DNA isolated from a tail cut before the experiment and another cutting from the tail taken after sacrifice. A diagram of the several experimental protocols for the *Act-CreER TrkB^{lox/lox}* mice is shown in Figure 20.

4.2.2 Tamoxifen treatment

Tamoxifen treatment of the *Act-CreER* mice to induce Cre activity was described in detail in section 3.2.2. Briefly, mice were treated with tamoxifen (7.5mg in sunflower oil (50mg/mL), Sigma) by oral gavage for 5 days. Vehicle-treated mice received an equal volume of sunflower oil alone. The mice were treated at approximately 2 months of age or after completion of kindling in one set of experiments.

4.2.3 Surgery and kindling

The surgery and kindling protocols were discussed in detail previously in sections 2.2.2 and 3.2.5. In brief, a bipolar electrode used for stimulation and recording was stereotactically implanted in the right amygdala using the following coordinates with bregma as the reference: 1.2 mm posterior, 2.9 mm lateral, 4.6 mm below dura, or in the right hippocampus with the following coordinates: 2.9 mm posterior, 3.0 mm lateral, 3.0 mm below dura. After a post-operative recovery period of 1 week, the electrographic seizure threshold (EST) was determined and stimulations at the EST were subsequently administered following the standard kindling protocol that was described previously in detail (X. P. He et al., 2002). Video and EEGs were recorded using Harmonie software (Stellate Systems, Montreal, Quebec, Canada). The behavioral manifestations of seizures were classified according to a modification of the description of Racine (R. J. Racine, 1972a): 1) facial clonus; 2) head nodding; 3) unilateral forelimb clonus; 4) rearing with bilateral forelimb clonus; 5) rearing and falling (loss of postural control); 6) running or bouncing seizures; and 7) tonic hind limb extension. Unless specified otherwise, mice were stimulated until fully kindled, as defined by the occurrence of 3 consecutive seizures of Class 4 or greater. Testing for maintenance of the kindled state was done following a 1 week or a 3 week stimulation-free period, using the same stimulation parameters as for kindling. Testing for maintenance was continued until a Class 4 or greater seizure was evoked and, if multiple stimulations were needed,

they were delivered twice daily as in kindling protocol. The kindling procedures were performed by an individual blinded to genotype of the animals.

4.2.4 Brain homogenates and immunoblot

The methods used for preparing brain homogenates and for performing immunoblots were presented in detail in section 2.2.6 and 3.2.6. Succinctly, the right hemisphere, into which the electrode was implanted, was taken whole and frozen on powdered dry ice. This hemisphere was sectioned to confirm electrode placement. The left hemisphere was dissected on ice into striatum, parietal cortex, amygdala-piriform cortex, hippocampus, and cerebellum. Each region was homogenized, aliquoted, and stored at -80°C.

For immunoblotting, homogenates were run on a SDS-PAGE gel and transferred to a protran nitrocellulose membrane. Membranes were blocked, probed with a mouse anti-TrkB antibody (1:500, BD Transduction Laboratories) and a mouse anti- β -actin antibody (1:10,000, Sigma) for 1 hr at room temperature, washed, and probed with a peroxidase-conjugated goat anti-mouse secondary antibodies (1:5000, Jackson ImmunoResearch) for another hour. The immunoblots were developed with enhanced chemiluminescence (ECL, Amersham Pharmacia, Buckinghamshire, United Kingdom) and exposed to film.

To quantify TrkB protein expression in some experiments, a standard curve of a series of dilutions of homogenate was included. The standard curve spanned the range

of TrkB levels detected in the samples and assured that the signal intensities were within the linear range. Band intensity was determined using volume quantification in ImageQuant (Molecular Dynamics).

4.3 Results

4.3.1 *Syn-Cre TrkB +/-* mice have reduced persistence of hyperexcitability following kindling

Mice with a single *TrkB* allele were found to be deficient in persistence of the hyperexcitable state following kindling. In the study of the *Syn-Cre TrkB^{-/-}* mice (Chapter 2 and (X. P. He et al., 2004)), *Syn-Cre TrkB^{+/-}* mice were found to have a minimal delay in kindling (14.5 ± 1.9 stimulations to reach the fully kindled state vs 9.5 ± 1.2 for control mice) (Figure 19 A), but unlike the *Syn-Cre TrkB^{-/-}* mice, the *Syn-Cre TrkB^{+/-}* mice reached the fully kindled state (3 consecutive Class 4 or greater seizures). The persistence of the hyperexcitable state was tested two weeks after the completion of kindling by giving the animal a single stimulus, the expectation being that the stimulus would evoke a seizure of Class 4 or greater. For the *Syn-Cre TrkB^{+/+}* (control) mice the duration of the electrographic seizure evoked was $106\% (\pm 8\%)$ of the previous seizure, which was the final seizure of the kindling protocol; by contrast, for *Syn-Cre TrkB^{+/-}* mice the duration was $79\% (\pm 6\%)$ of the previous seizure ($p < 0.05$) (Figure 19 B). In all cases the final stimulation of the kindling protocol and the stimulation to test persistence of the kindled state evoked a Class 4 or greater seizure.

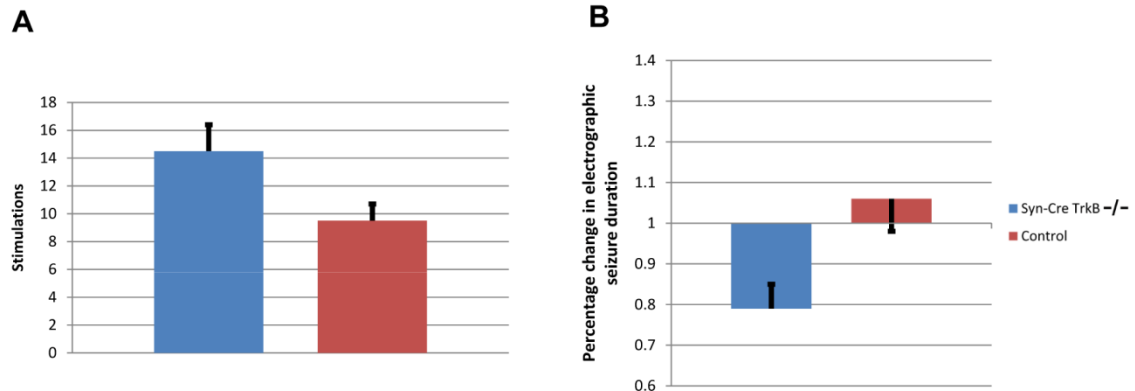


Figure 19: Syn-Cre TrkB^{+/-} mice have reduced excitability following a 2 week rest period.

A) Stimulations to reach the fully kindled state. *Syn-Cre TrkB^{+/-}* mice have delayed kindling development as compared with *Syn-Cre TrkB^{+/+}* (control) mice, requiring more stimulations to reach the fully kindled state. **B) Change in electrographic seizure duration over the rest period.** Following the completion of the kindling protocol, animals are rested for two weeks without stimulation. Persistence of the hyperexcitable state was assessed by administration of an additional stimulation at the same intensity as the final stimulation of the kindling protocol. Duration of the evoked electrographic seizure as a ratio of the duration of the electrographic seizure at the end of the kindling protocol is plotted for wild type controls (TrkB ^{+/+}) and TrkB ^{+/-} mice . Gray bars mark mean value \pm SEM.

4.3.2 Persistence of the hyperexcitable state following amygdaloid kindling in the *Act-CreER TrkB^{-/-}* mice

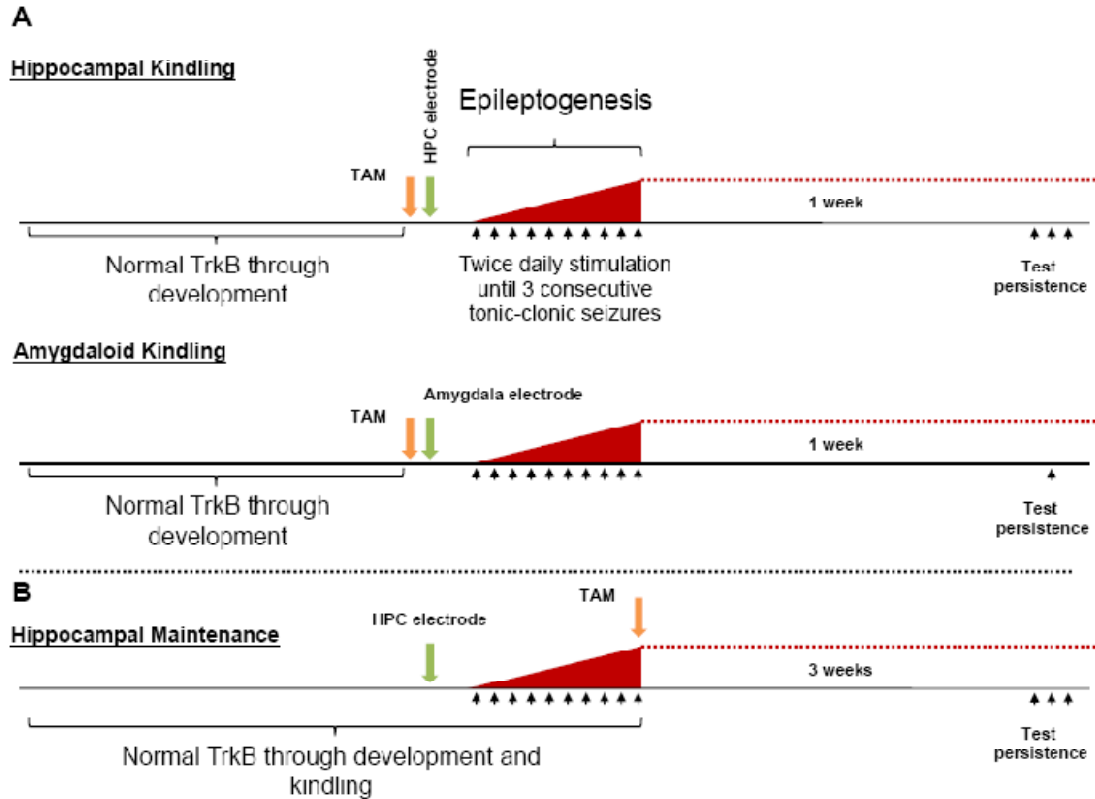


Figure 20: Kindling and tamoxifen protocols for *Act-CreER TrkB^{flox/flox}* mice.

A) Amygdaloid and hippocampal kindling (TAM-kindling). To test the role of TrkB in the development of kindling *Act-CreER TrkB^{flox/flox}* mice and wild type controls are allowed to mature to 2 months of age before treatment with tamoxifen or vehicle. Following treatment with tamoxifen (PO 7.5mg x5d), mice were implanted with electrodes for kindling, either in the hippocampus or in the amygdala. Kindling was continued until the behavioral seizures progressed to 3 consecutive Class 4 or greater seizures, at which point mice were not stimulated, “rested”, for 1 week before maintenance of the hyperexcitable state was tested. After 1 week stimulations resumed and continued until 1 Class 4 or greater seizure was evoked. B) Hippocampal kindling (kindling-TAM). To test the role of TrkB specifically in maintenance, *Act-CreER TrkB^{flox/flox}* mice and wild type control mice were implanted with hippocampal electrodes, kindled to 3 consecutive Class 4 or greater seizures. The mice were then treated with either tamoxifen (PO 7.5mg x5d) or vehicle, allowed to rest for a total of 3 weeks from the last kindled seizure, and then maintenance was

tested. Maintenance stimulations continued until a Class 4 or greater seizure was evoked.

Maintenance of the hyperexcitable state following amygdaloid kindling in the tamoxifen-treated *Act-CreER TrkB^{lox/lox}* mice (hereafter, *Act-CreER TrkB^{-/-}* mice) was not impaired. As previously described in section 3.3.2.1, amygdaloid kindled *Act-CreER TrkB^{-/-}* mice were delayed in kindling development in comparison to control mice (*Act-CreER TrkB^{-/-}* 16.1 ± 2.1 stimulations; controls 10.1 ± 1.3 stimulations, p<0.05) (Figure 21 A). Following completion of kindling, mice were rested for 1 week without stimulation before maintenance of the hyperexcitable state was tested. All amygdaloid kindled mice had a Class 4 or greater on the first or second stimulation. During the maintenance testing no difference was found in the duration of the first electrographic seizure, as a ratio of the final seizure of the kindling protocol, between the *Act-CreER TrkB^{-/-}* mice (100 ± 16%) as compared to control mice (107 ± 5%, p>0.05) (Figure 21 B). While the entire group of *Act-CreER TrkB^{-/-}* mice failed to demonstrate a deficit in persistence of the hyperexcitable state, which was expected given the phenotype in the Syn-Cre *TrkB^{+/-}* mice, previous experience with the variable efficacy of tamoxifen-induced reduction in TrkB suggested that the extent of reduction in TrkB should be examined in the *Act-CreER TrkB^{-/-}* mice.

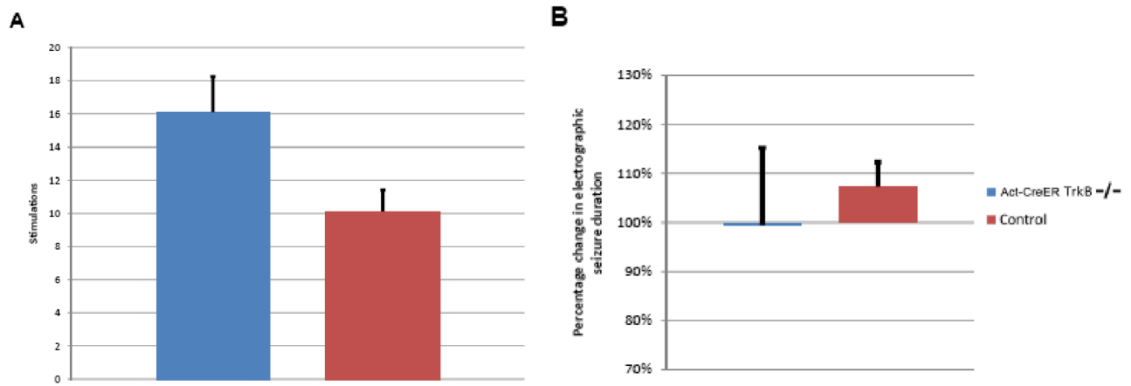


Figure 21: Persistence of the hyperexcitable state following amygdaloid kindling.

A) Stimulations to become fully kindled. *Act-CreER TrkB^{-/-}* mice (n=10) required significantly more stimulations to reach the fully kindled state than control mice (n=15, p<0.05). **B) Change in electrographic seizure duration across the rest period.** *Act-CreER TrkB^{-/-}* mice have no impairment in maintenance of the kindled state, as evidenced by no reduction in electrographic seizure duration across the rest period, as compared to control mice. Mean ± SEM.

Analysis of the maintenance of the kindled state for the group of successfully treated *Act-CreER TrkB^{lox/lox}* mice (hippocampal TrkB <75% of control mice, n=7) showed a non-significant deficit in maintenance of the hyperexcitable state (*Act-CreER TrkB^{-/-}* 80.6 ± 23.9%; controls 107.3 ± 5.0%; p=0.099) (Figure 22 A), although the size of the effect was comparable to the result from the *Syn-Cre TrkB^{+/-}* mice.

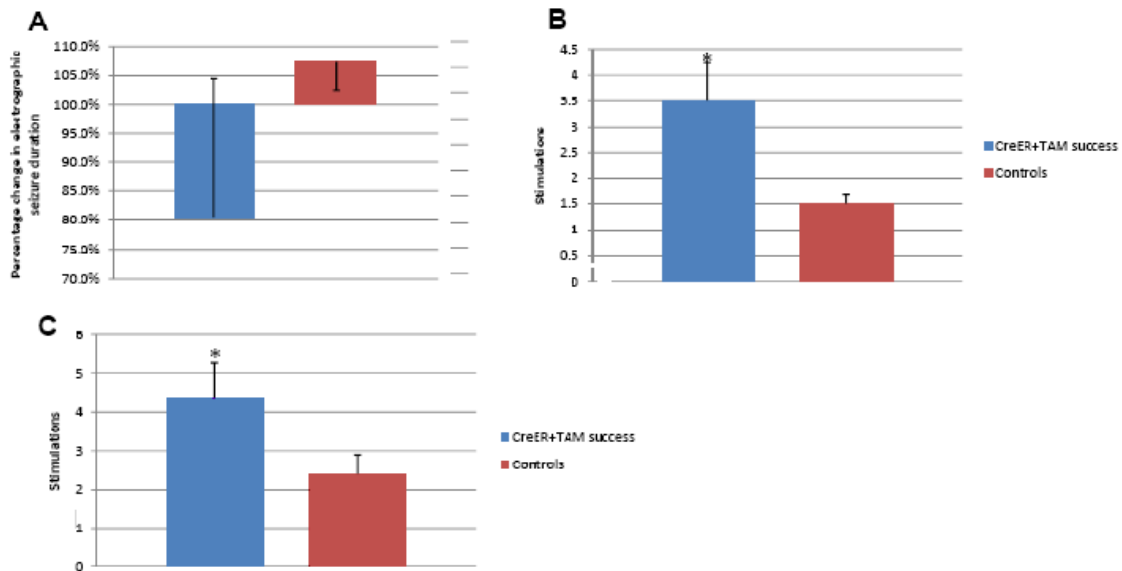


Figure 22: Loss of TrkB and deficit in persistence of the hyperexcitable state.

A) Change in electrographic seizure duration over the rest period. Following amygdaloid kindling, *Act-CreER TrkB*^{-/-} mice with a reduction in hippocampal TrkB (<75% wild type levels) (n=7) had a non-significant trends towards a shorter duration seizure, as a ratio of the final kindled seizure, than control mice (n=15, p=0.099). **B)** Stimulations for the complete maintenance testing. Following hippocampal kindling, *Act-CreER TrkB*^{-/-} mice with a reduction in hippocampal TrkB (<75% wild type levels) (n=8) required more stimulations to evoke a Class 4 or greater seizure following the rest period than control mice (n=15, p<0.05). **C)** Stimulations for the complete maintenance testing. Following hippocampal kindling, *Act-CreER TrkB*^{-/-} (after kindling) mice with a reduction in hippocampal TrkB (<75% wild type levels) (n=9) required more stimulations to evoke a Class 4/5 seizure following the rest period than control mice (n=15, p<0.05). Mean ± SEM.

4.3.3 Persistence of the hyperexcitable state following hippocampal kindling in *Act-CreER TrkB*^{-/-} mice (TAM-kindling)

Maintenance of the hyperexcitable state following hippocampal kindling was deficient in the *Act-CreER TrkB*^{-/-} mice. As was seen in the *Syn-Cre TrkB*^{+/-} mice and the amygdaloid kindled *Act-CreER TrkB*^{-/-} mice, the *Act-CreER TrkB*^{-/-} mice had delayed kindling development as compared to control mice (section 3.3.2.2 and Figure 23 A).

Following completion of kindling, with the exception of the 2 *Act-CreER TrkB*^{-/-} mice that did not fully kindle, mice were rested for 1 week before persistence of the hyperexcitable kindled state was tested. Unlike amygdaloid kindled animals, following hippocampal kindling, stimulations given to test persistence of the hyperexcitable state after a stimulus-free interval do not uniformly evoke a Class 4 or greater seizure (A. Nanobashvili et al., 2000; M. Kokaia et al., 2001). Testing for persistence of the kindled state 1 week after hippocampal kindling in control mice in this experiment revealed that 87% had a Class 4 or greater seizure within two stimulations (vs 100% of amygdaloid kindled control mice) and the duration of the electrographic seizure was 93% of the last seizure of the kindling protocol (vs. 107% for amygdaloid kindled control mice).

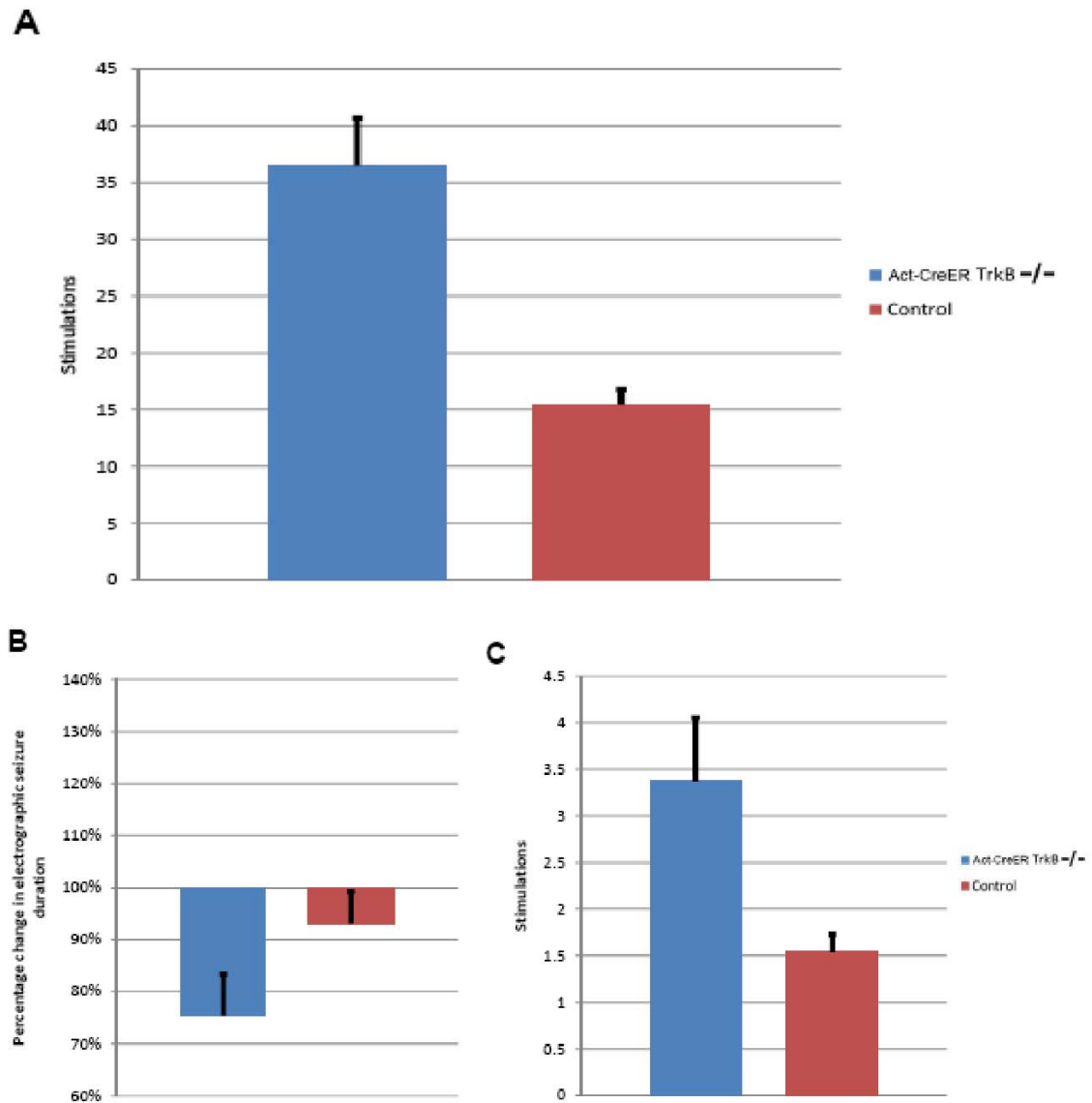


Figure 23: Persistence of the hyperexcitable state following hippocampal kindling in the *Act-CreER TrkB*^{-/-} mice.

A) Stimulations to reach the kindled state. *Actin-CreER TrkB*^{-/-} mice (n=10) required significantly more stimulations to reach the fully kindled state than control mice (n=15, p<0.01). **B)** Change in electrographic seizure duration across rest period. *Act-CreER TrkB*^{-/-} mice have a reduced ratio of electrographic seizure duration of the 1st maintenance seizure as compared to the duration of the last kindled seizure, as compared to control mice (p<0.05). **C)** Stimulations required for complete

maintenance testing. *Act-CreER TrkB*^{-/-} mice require significantly more stimulations to have a Class 4 or greater seizure during the maintenance testing, as compared to control mice (p<0.05). Mean ± SEM.

Reduction of TrkB in the *Act-CreER TrkB*^{-/-} mice impaired persistence of the hyperexcitable state following hippocampal kindling. Only 3 of 8 *Act-CreER TrkB*^{-/-} mice had a Class 4 or greater seizure with the first or second stimulation (Figure 25 A left), indicating that they had maintained hyperexcitability over the course of a week without stimulations, while a significantly greater proportion of control mice had a Class 4 or greater seizure with the first or second stimulation, 13 of 15 (p<0.05, Fisher Exact test) (Figure 5. A right). There is a non-significant trend for the duration of first seizure of the maintenance protocol, as a ratio of the final stimulation of kindling, to be less in the *Act-CreER TrkB*^{-/-} mice than in control mice (*Act-CreER TrkB*^{-/-} 75.4 ± 8.0% vs. controls 93.0 ± 6.2%; p=0.051) (Figure 23 B). Furthermore, during the complete course of the maintenance testing protocol more stimulations were required to evoke a Class 4 or greater seizure in the *Act-CreER TrkB*^{-/-} mice than in control mice (*Act-CreER TrkB*^{-/-} 3.4 ± 0.7 stimulations vs. controls 1.5 ± 0.2 stimulations, p<0.05) (Figure 23 C). Again, given the variability in the efficacy of the tamoxifen-induced recombination, the extent of reduction of TrkB was examined in the *Act-CreER TrkB*^{-/-} mice.

Persistence of the kindled state was deficient in the subset of *Act-CreER TrkB*^{-/-} mice with a successful reduction of hippocampal TrkB protein (< 75% of control TrkB). *Act-CreER TrkB*^{-/-} mice with reduced hippocampal TrkB (n=8) required 3.5 ± 0.8

stimulations to induce a Class 4 or greater seizure following the stimulation-free period, while control mice required 1.5 ± 0.2 stimulations ($p < 0.05$) (Figure 22 B). Of note, *Act-CreER TrkB^{-/-}* mice with normal hippocampal TrkB levels ($n=2$) were not different from *Act-CreER TrkB^{-/-}* mice with reduced hippocampal TrkB in the number of stimulations to evoke a Class 4 or greater seizure (*Act-CreER TrkB^{-/-}* “failures” 3 ± 1 stimulations; *Act-CreER TrkB^{-/-}* “successes” 3.5 ± 0.8 stimulations; control mice 1.5 ± 0.2 stimulations), although this is a small sample to draw inferences from.

4.3.4 Persistence of hyperexcitability following hippocampal kindling in the *Act-CreER TrkB^{-/-}* (post kindling) mice

The previous findings of impaired persistence of the kindled state with the Syn-Cre TrkB^{+/-} mice and the hippocampal kindled *Act-CreER TrkB^{-/-}* mice suggested the possibility that TrkB is required for complete persistence of the hyperexcitable state. However, both the Syn-Cre TrkB^{+/-} mice and the *Act-CreER TrkB^{-/-}* mice had reduced TrkB during kindling development as well as during the stimulation-free period, allowing the possibility of a subtle effect of loss of TrkB during the development of kindling to impair persistence. However, the inducible nature of the recombination in the *Act-CreER TrkB^{lox/lox}* mice allows for reduction of TrkB de novo following kindling.

To address the role of TrkB specifically in the persistence of the hyperexcitable state following kindling, *Act-CreER TrkB^{lox/lox}* mice were treated with tamoxifen only after the completion of kindling (hereafter *Act-CreER TrkB^{-/-}* (post-kindling)), along with control mice also treated with tamoxifen or vehicle alone following completion of

kindling (*Act-CreER TrkB^{lox/lox}* mice + vehicle, wild-type mice + tamoxifen, and wild-type mice + vehicle). Comparison of the different control groups by ANOVA revealed no differences ($p>0.05$) and therefore they were combined as a single control group for analysis. As expected, there were no differences between the two groups in any of the measures of kindling development, including electrographic seizure threshold (*Act-CreER TrkB^{-/-}* (post-kindling) $104 \pm 19 \mu\text{A}$ vs. controls $124 \pm 25 \mu\text{A}$, $p>0.05$) (Figure 24 A), initial electrographic seizure duration (*Act-CreER TrkB^{-/-}* (post-kindling) 24.3 ± 3.1 sec vs. controls 25.1 ± 2.8 sec, $p>0.05$) (Figure 24 B), or stimulations to become fully kindled (*Act-CreER TrkB^{-/-}* (post-kindling) 18.8 ± 1.6 stimulations vs. controls 15.6 ± 1.1 stimulations, $p>0.05$) (Figure 24 C).

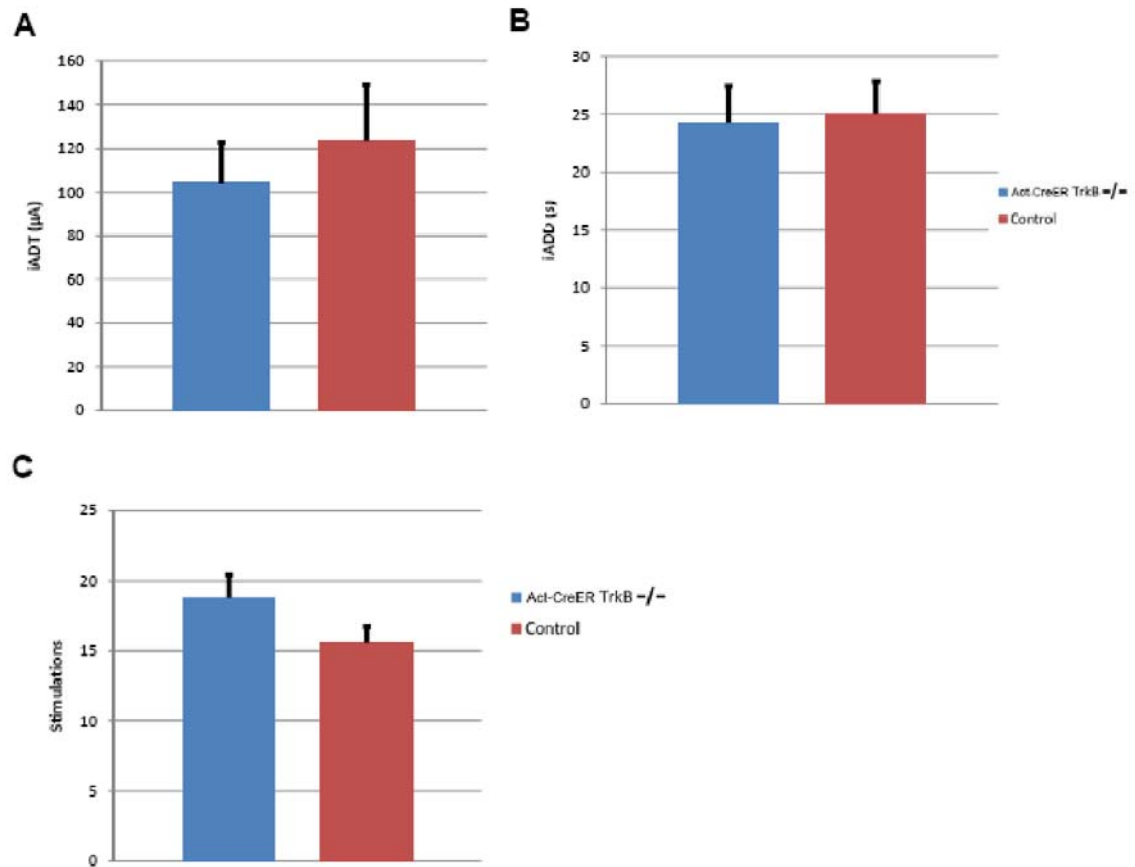


Figure 24: Hippocampal kindling in *Act-CreER TrkB^{-/-}* (post-kindling) mice.

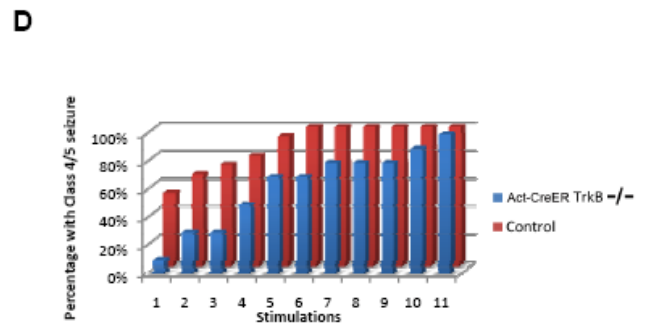
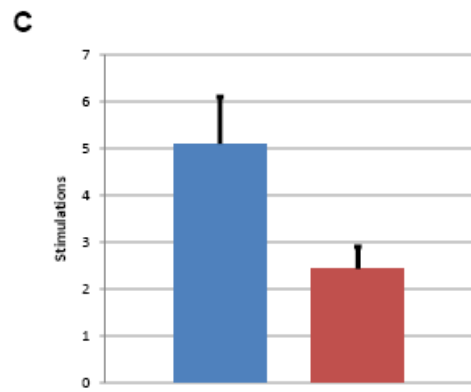
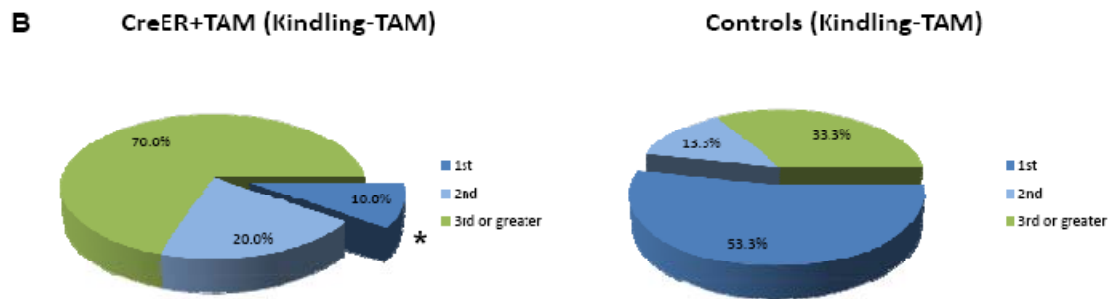
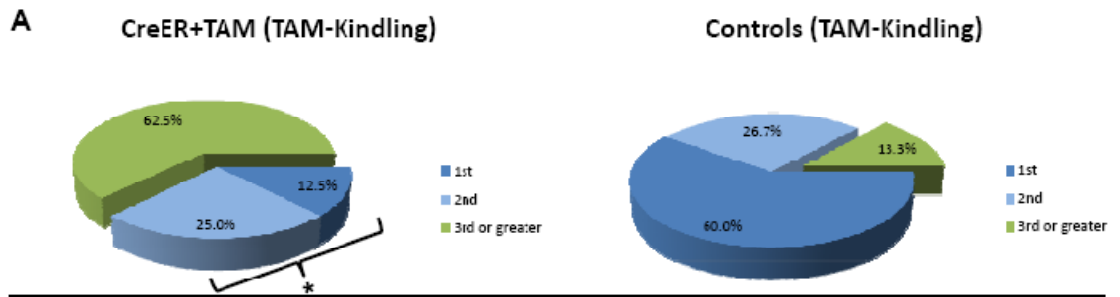
A) Initial afterdischarge threshold. *Act-CreER TrkB^{-/-}* (post-kindling) mice (n=10) did not differ from control mice (n=15) in initial threshold to induce an electrographic seizure ($p>0.05$). **B) Initial electrographic seizure duration.** *Act-CreER TrkB^{-/-}* (post-kindling) mice did not differ from control mice in the duration of the initial electrographic seizure ($p>0.05$). **C) Stimulations to complete kindling.** *Act-CreER TrkB^{-/-}* (post-kindling) mice do not differ in the number of stimulations to reach the fully kindled state as compared with control mice ($p>0.05$). Mean + SEM.

Reduction of TrkB only after the hyperexcitable state was established through hippocampal kindling impaired maintenance of the hyperexcitable state. Following kindling, the mice were treated with tamoxifen (7.5 mg per day for 5 days) or vehicle (150 μL sunflower oil), rested for a total of 3 weeks (16 days after the last treatment with

tamoxifen), and then maintenance of the hyperexcitable state was tested. The interval of 16 days was chosen as previous experiments had demonstrated that a reduction in TrkB protein could be seen 9 days after the 5 day tamoxifen treatment regimen. Additionally the previous study (section 4.3.3) in which the *Act-CreER TrkB^{lox/lox}* mice were treated with tamoxifen before kindling from the hippocampus demonstrated that a rest period of 7 days with a reduction in TrkB was sufficient to cause a detectable deficit in persistence of the hyperexcitable state. Three weeks provide for 5 days of tamoxifen treatment, 9 days for the TrkB protein levels to decrease, and 7 days for the reduced TrkB levels to have its effect on persistence of the kindled state.

With the initial maintenance test stimulation, only 1 of 10 *Act-CreER TrkB^{-/-}* (post-kindling) mice had a Class 4 or greater seizure (Figure 25 B left), indicating that the hyperexcitable state persisted, while the initial maintenance stimulation evoked a Class 4 or greater seizure in a significantly greater proportion of control mice, 8 of 15 ($p < 0.05$, Fisher Exact test) (Figure 25 B right). For the complete course of the maintenance testing protocol the *Act-CreER TrkB^{-/-}* (post-kindling) mice required more stimulations to induce a Class 4 or greater seizure than control mice (*Act-CreER TrkB^{-/-}* (post-kindling) 5.1 ± 1.0 stimulations vs. controls 2.4 ± 0.5 stimulations, $p < 0.05$, one outlier in control group (stims = 12) was $> 3sd$ from mean was excluded from statistical comparisons) (Figure 25 C). Comparison of the percentage of mice having a Class 4 or greater seizure through the course of the maintenance testing in the *Act-CreER TrkB^{-/-}* (post-kindling)

and control mice demonstrates the impaired persistence of the hyperexcitable state in the *Act-CreER TrkB^{-/-}* (post-kindling) mice (Figure 25 D). During the maintenance testing no difference existed between the two groups in the duration of the first electrographic seizure, as a ratio of the final seizure of the kindling protocol (*Act-CreER TrkB^{-/-}* (post-kindling) $111 \pm 9\%$ vs. controls $100 \pm 6\%$, $p > 0.05$) (Figure 25 E).



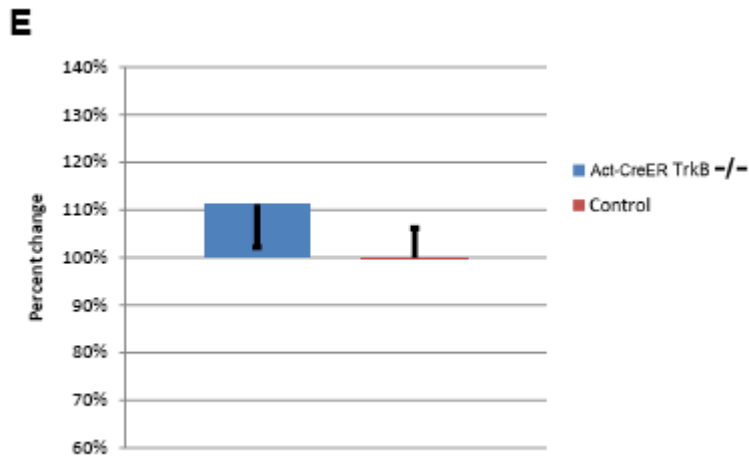


Figure 25: Comparison of persistence of the hyperexcitable state in hippocampal kindled Act-CreER TrkB^{-/-} mice treated with tamoxifen before or after kindling.

A) Persistence of hyperexcitability for Act-CreER TrkB^{-/-} mice. Following the rest period, a significantly smaller proportion of hippocampal kindled Act-CreER TrkB^{-/-} mice (n=10) had a Class 4/5 seizure with the 1st or 2nd stimulation as compared to control mice (n=15, * p<0.05) **B) Persistence of hyperexcitability for Act-CreER TrkB^{-/-} (post-kindling) mice.** Following the rest period, a significantly smaller proportion of hippocampal kindled Act-CreER TrkB^{-/-} (post-kindling) mice (n=10) had a Class 4/5 seizure with the 1st stimulation as compared to control mice (n=15, * p<0.05) **C) Total stimulations required to evoke Class 4 or greater seizure during the maintenance testing.** Act-CreER TrkB^{-/-} (post-kindling) mice require significantly more stimulations to have a Class 4 or greater seizure during the maintenance testing, as compared to control mice (p<0.05). **D) Percentage of Act-CreER TrkB^{-/-} (post-kindling) mice and control mice having a Class 4/5 seizure at each maintenance stimulation.** **E) Change in electrographic seizure duration across the rest period.** Act-CreER TrkB^{-/-} (post-kindling) mice demonstrate no reduction in electrographic seizure duration across the rest period (ratio of duration of 1st seizure of maintenance testing to the duration of the last seizure of the kindling protocol), as compared to control mice (p>0.05). Bar graphs are mean ± SEM.

Analysis of the TrkB levels in the Act-CreER TrkB^{-/-} (post-kindling) mice revealed 9 of 10 mice had reduced levels of TrkB protein in the hippocampus (<75% of wild type levels) as compared to control mice. Comparison of only Act-CreER TrkB^{-/-} (post-

kindling) mice with reduced TrkB with control mice showed a significant deficit in persistence of the kindled state (4.4 ± 0.9 stimulations for a Class 4 or greater seizure vs. 2.4 ± 0.5 stimulations for controls, $p < 0.05$) (Figure 22 C).

4.4 Discussion

4.4.1 Summary and Conclusions

This study supports the hypothesis that TrkB is required for the persistence of the kindled state. In addition to the study of mice deficient in TrkB during kindling development, the inducibility of recombination in the *Act-CreER TrkB^{flox/flox}* mice permitted the examination of the effects of reduced TrkB specifically on persistence of the hyperexcitable state, using mice that were kindled in the presence of normal levels of TrkB. The principal findings of these studies are: 1) Reduction in TrkB impairs persistence of the hyperexcitable state following kindling; 2) Reduction of TrkB selectively in the mature mouse impairs persistence of the hyperexcitable state; 3) Reduction in TrkB selectively after the establishment of the kindled state impairs persistence of the kindled state. In identifying one of the molecular actors underlying the continued hyperexcitable state, these studies advance TrkB inhibition as a therapeutic target for impairing the persistence of the epileptic state and furthermore may provide insight into the cellular and molecular underpinnings of the kindled state.

4.4.2 Possible mechanisms underlying loss of TrkB causing impaired persistence of the hyperexcitable state

The mechanisms by which reduced levels of TrkB lead to impaired persistence of the undiminished kindled state likely involve deficits in the development or preservation of alterations in morphology and gene expression. While post-translational modifications to existing proteins may maintain short-term plasticity, changes in gene expression and neuronal structure are likely required for the long-term changes in the brain. One mechanism for consolidation of short-term alterations into long lasting changes is Ca^{2+} influx through the NMDA receptor (A. E. West et al., 2001).

Interestingly, blocking the NMDA receptor with the non-competitive antagonist MK-801 has been found to cause a phenotype similar to reduction of TrkB with regards to kindling. MK-801 delays kindling (J. O. McNamara et al., 1988) and partially inhibits seizures in fully kindled animals (J. O. McNamara et al., 1988{Williamson, 1989 #165}). Treatment of kindled rats with long-term MK-801 (28 days) results in impaired persistence of the kindled state, even when the anti-convulsant effect of MK-801 is avoided by stopping treatment 1 week before testing for persistence of the kindled state (M. Yoshida et al., 1997). This result lends support to the hypothesis that an opportunity for treatment exists even after the kindling process has finished, at least for early time points after kindling.

Another possibility that cannot be excluded is that reduction of TrkB acts as an anti-convulsant. An anti-convulsant effect would have been expected to cause a

reduced initial excitability in the *Act-CreER TrkB*^{-/-} mouse which was not seen (Figure 16 A & B, 17 A & B). However, the initial evoked seizure often does not include a behavioral component, while the seizure evoked by the maintenance test stimulation is a behavioral Class 4 or greater seizure in control mice. Therefore the anti-convulsant effect of reduced TrkB may be limited to the behavioral seizure in the kindled mouse. Such a selective anti-convulsant effect has been described for the NMDA receptor antagonist MK-801 (J. M. Williamson and E. W. Lothman, 1989), for which the proposed mechanism was that MK-801 acts to prevent seizure spread and generalization rather than inhibiting focal seizures. Of note, despite a reduction in the behavioral seizure class evoked by the first maintenance stimulation in the *Act-CreER TrkB*^{-/-} (post-kindling) mice as compared to control mice (Figure 25 B), there was no difference in the duration of the focal electrographic seizure (Figure 25 E). This may imply that the effect of loss of TrkB on persistence of the hyperexcitable state is different, depending on whether TrkB was reduced during kindling or only after kindling.

Comparison of the pattern of activation of TrkB and known cellular and molecular alterations following kindling can help to identify the mechanism by which TrkB affects persistence of the hyperexcitable state. The increase in TrkB activation following a kindled seizure begins at 12 hours after the seizure and returns to baseline by 1 week (D. K. Binder et al., 1999a), which defines the window of time in which reduced TrkB signaling could prevent a plastic change following the last kindled

seizure; reversal of some alteration due to a reduction in TrkB signaling below basal levels could occur at any time point, although this mechanism might be expected to lead to a reduction in initial excitability in the *Act-CreER TrkB^{-/-}* mice, which was not seen (Figure 17 A). For instance, while structural changes such as sprouting of mossy fiber axons are present within 30 minutes of the end of kindling (T. Sutula et al., 1988) and therefore not a likely target. However, if the synapses of these sprouted axons are not formed by the time the tamoxifen treatment starts 24 hours after the last kindled seizure, the role of BDNF/TrkB signaling in the formation of excitatory synapses (A. Martinez et al., 1998; B. Alsina et al., 2001; B. W. Luikart et al., 2005) may be relevant, and since reduced TrkB levels could prevent the formation of these synapses. One available mechanism involves the role of TrkB in the maintenance of synapses in the adult mouse, which has been demonstrated for excitatory synapses in the visual cortex (S. Chakravarthy et al., 2006) and at the neuromuscular junction, where continued TrkB signaling is required to maintain post-synaptic receptor clustering, and thereby the maintenance of the synapse itself (M. Gonzalez et al., 1999). However, this mechanism would require that the pathological synapses were selectively vulnerable to elimination by reduced TrkB signaling. By these means reduced TrkB could result in the loss of excitatory synapses between glutamatergic neurons, which may be responsible for persistence of the kindled state. Another structural change that could preserve the short-term plasticity is the addition of new neurons, however this mechanism is unlikely

since increased neurogenesis is not seen following only 3 Class 4 or greater seizures (J. M. Parent et al., 1998).

Long-lasting changes to the brain may also occur as changes in the structure of the synapse itself, leading to changes in synaptic efficacy. BDNF and TrkB have been shown to increase excitatory synaptic transmission within the hippocampus (H. Kang and E. M. Schuman, 1995; K. W. Kafitz et al., 1999)) and in particular BDNF/TrkB may have a role specifically in late LTP (L-LTP) (H. Kang and E. M. Schuman, 1996; H. Kang et al., 1997; M. Korte et al., 1998; E. Messaoudi et al., 2002). Indeed, kindling and LTP likely share mechanisms at a cellular and molecular level, though they are not synonymous (R. J. Racine et al., 1983; T. Sutula and O. Steward, 1986, 1987). It may be that this late phase of LTP, which is dependent on transcription and translation, is most analogous to the maintenance stage of kindling, when the earlier short-term plastic changes need to be consolidated into permanent changes. For L-LTP, and perhaps for kindling as well, following the initial stimulation short-term potentiation of the synapse occurs, lasting a few hours. Following this initial stage, two stages of L-LTP occur, one requiring protein synthesis and lasting days (LTP2) and a second requiring gene transcription and protein synthesis and lasting tens of days (LTP3) (W. C. Abraham, 2003; C. R. Raymond, 2007). If the post-kindling period is similar to LTP2/3, then a loss of TrkB could be impairing this consolidation step. The similar inhibitory effect of MK-801 in both kindling and LTP support this comparison as well. Finally, as evidence that

long-term alternations are made permanent over an extended time course and BDNF signaling is important to this process, reduction of hippocampal BDNF 12 hours after a training trial impairs retention at 7 days but not at 2 days (P. Bekinschtein et al., 2007).

4.4.3 Future Directions

Further elucidation of the requirements for TrkB signaling in the persistence of the hyperexcitable state following kindling will improve our understanding of the cellular and molecular basis of persistence of the hyperexcitable state and clarify the potential for TrkB as a therapeutic target to reverse the epileptic state. A key question is whether reduction of TrkB reverses the hyperexcitable state or whether the effect of reduced TrkB has an anti-convulsant effect. Differentiation of these possibilities would require the ability to transiently inhibit TrkB signaling, so that the effects of reduced TrkB signaling on persistence of the underlying hyperexcitable state can be separated from any effects of reduced TrkB signaling on the evoked seizure used to evaluate the underlying hyperexcitability.

The discovery of the role of TrkB in the persistence of the kindled state also raises the possibility that the effect of loss of TrkB on kindling development is also essentially a defect in maintenance. For kindling to progress the changes induced by a stimulation need to be maintained and then added to by the next stimulation. If loss of TrkB impairs the maintenance between one stimulation and the next in the kindling model, it would

lead to a delay in kindling or even prevention of kindling if none of the stimulation-induced changes persisted until the next stimulation.

These studies have only examined the effects of a reduction of TrkB starting immediately after kindling. Like LTP, the plasticity of epileptogenesis likely has multiple stages during and following kindling, as the alterations caused by the repeated seizures are passed through various molecular actors until they are consolidated into a permanent increase in excitability in the brain. Determining requirements for TrkB in the formation of the hyperexcitable state could be accomplished by inducing recombination in the *Act-CreER TrkB^{lox/flox}* mice at later time points following kindling. Additionally, kindling or other models such as pilocarpine status epilepticus could be used to generate a mouse with spontaneous recurrent seizures, and TrkB could then be reduced in a mouse that is already clearly epileptic. The time point at which TrkB signaling is no longer needed, if such a time exists, could then be fixed. The time course of the deficit in persistence of the hyperexcitable state following reduction of TrkB could also be examined. Following induction of recombination in the *Act-CreER TrkB^{lox/flox}*, the electrographic seizure threshold and evoked seizure could be monitored to determine if the hyperexcitable state continues to decay over time or whether it simply plateaus at a level of reduced hyperexcitability without returning to the basal level. TrkB mutant mice utilizing a chemical-genetic approach (X. Chen et al., 2005), which permits the efficient and specific pharmacological inhibition of TrkB kinase activity, could determine

if the reduction of TrkB needs to continue indefinitely or if a limited period of reduced TrkB signaling is sufficient to treat the pathological state, which has very important clinical implications. Finally, studies using a virally-mediated RNAi approach can define the anatomical requirements for TrkB signaling and are an important first step towards a clinically useful intervention.

The implications of a possible role for TrkB in the persistence of the kindled state, independent of the effects of reduced TrkB on kindling development, are profound. If reduction of TrkB signaling can either prevent or reverse the pathology underlying the epileptic state, a treatment to reduce TrkB signaling could be broadly applicable to many individuals with epilepsy. Such a treatment may likely be applicable to other conditions caused by pathological plasticity resulting in aberrant activity, such as addiction and chronic pain.

5. Conditional Deletion of TrkB Impairs Sustained Seizure Activity in the Pilocarpine Model

5.1 Introduction

Status epilepticus, a condition characterized by prolonged persistence seizure activity, is a serious medical condition. Status epilepticus affects 126,000 to 195,000 individuals each year in the United States (R. J. DeLorenzo et al., 1996) and mortality within the first month after status epilepticus is near 25%, which is comparable to the mortality rate for gun shot wounds and myocardial infarction (A. R. Towne et al., 1994). Furthermore, those that survive the initial insult of status epilepticus suffer cognitive impairment (10-35% (A. Krumholz, 1999)), later development of epilepsy (41% (D. C. Hesdorffer et al., 1998)), and recurrent status epilepticus (13% (R. J. DeLorenzo et al., 1996)).

Current treatments for status epilepticus are often ineffective and may not prevent long-term sequelae. Approximately 40 %- 80% of patients in status epilepticus will fail to respond to the initial treatment (refractory status epilepticus (D. M. Treiman et al., 1998; D. M. Treiman, 1999)). A longer duration of status epilepticus is associated with a worse clinical outcome (A. R. Towne et al., 1994; D. H. Lowenstein and B. K. Alldredge, 1998; S. Shorvon, 2001). Therefore further study into the cellular and molecular mechanisms of acute pathophysiology of status epilepticus and potential mechanisms leading to late consequences is critical.

The pilocarpine model of epilepsy is a well-established model in which injection with the cholinergic agonist pilocarpine leads to a bout of status epilepticus, with the late onset of spontaneous seizures (W. A. Turski et al., 1983; W. A. Turski et al., 1984). The pilocarpine model of epilepsy is often used to most closely mimic the human condition of an instance of status epilepticus, such as complex febrile seizures (K. B. Nelson and J. H. Ellenberg, 1978), leading to later development of epilepsy. In the pilocarpine model it is known that the length of status epilepticus is a key determinant of whether late onset recurrent spontaneous seizures emerge (T. Lemos and E. A. Cavalheiro, 1995). Given the striking deficit in epileptogenesis seen with kindling mice with a conditional deletion of TrkB (X. P. He et al., 2004), we attempted to examine epileptogenesis in the pilocarpine model using these mice.

5.1.1 Rationale for studying the pilocarpine model of status epilepticus

Previously my colleagues and I demonstrated that conditional elimination of TrkB in the *Syn-Cre TrkB^{-/-}* mice is sufficient to prevent epileptogenesis in the kindling model (X. P. He et al., 2004). However, the *Syn-Cre TrkB^{-/-}* mice did not reach a point in the kindling model when spontaneous seizures could be expected (>80 Class 4/5 seizures (U. Sayin et al., 2003), so the effect of elimination of TrkB at the stage most analogous to the human condition of epilepsy could not be examined. To further define the role of TrkB in epileptogenesis the *Syn-Cre TrkB^{-/-}* mice were examined using the pilocarpine model of epilepsy. Since in the kindling model the deficit in the *Syn-Cre TrkB^{-/-}* mice

was not in the initial evoked seizure (section 2.3.6) and the *Syn-Cre TrkB^{-/-}* had normal tonic-clonic seizures with maximal electroshock (section 2.3.7), it was hypothesized that the *Syn-Cre TrkB^{-/-}* mice would have equivalent status epilepticus following pilocarpine treatment. This hypothesis is supported by the findings of Lähteinen and colleagues (S. Lähteinen et al., 2002), that inhibition of TrkB signaling by over-expression of truncated TrkB receptors decreases the probability but does not eliminate late onset spontaneous seizures without affecting kainic acid-induced status epilepticus. Given the lack of epileptogenesis in the kindling model, it was anticipated that the *Syn-Cre TrkB^{-/-}* mice might display a complete elimination of late onset spontaneous recurrent seizures.

To our surprise, we were not able to induce equivalent status epilepticus in the *Syn-Cre TrkB^{-/-}* and WT mice; however, the exceptional differences in status epilepticus in the *Syn-Cre TrkB^{-/-}* mice compared to control mice elucidates possible mechanisms fundamental to sustaining seizure activity during status epilepticus and the role of TrkB acutely in status epilepticus.

5.2 Methods

5.2.1 Mice

TrkB^{lox/lox} mutant mice were generated with *Cre/loxP* technology (H. Gu et al., 1994) as described previously (Y. Zhu et al., 2001). The *TrkB^{lox/lox}* mutant mice have exon 1 of the *TrkB* gene, which encodes the signal peptide and the first 40 amino acids of the

N terminus of TrkB flanked by *loxP* sites (i.e. floxed). Crossing a *TrkB^{lox/lox}* mouse to a transgenic mouse carrying Cre driven by a *Synapsin1* promoter (*Syn-Cre*) generated progeny in which expression of the floxed gene was selectively eliminated in a subset of CNS neurons. The *Syn-Cre TrkB^{lox/lox}* mice were on a mixed background of 129/C57/ICR.

The pattern of recombinase activity in *Syn-Cre* mice, visualized with the *Rosa-26* reporter mice (P. Soriano, 1999) (Figure 2.1 A), by *in situ* hybridization for *TrkB* mRNA (Figure 2.1 D and 4.4 A & B right), as well as with immunoblots of probed for TrkB protein (Figure 2.7 and 4.3 A), has been previously characterized (He et al., 2004). In the present study, the genotype of each animal was assessed twice using PCR of genomic DNA isolated from tail cut before the experiment and another cutting from the tail taken after sacrifice.

5.2.2 Surgery

Under pentobarbital (60mg/kg) anesthesia, two bipolar electrodes (160 μ m diameter steel wire with insulation (California Fine Wire Company) for recording were stereotactically implanted, one in the left parietal cortex using the following coordinates with bregma as the reference: 1.1 mm posterior, 2.9 mm lateral, 1.2 mm below dura, and a second bipolar electrode was implanted in the right hippocampus using the following coordinates with bregma as the reference: 2.0 mm posterior, 0.8 mm lateral, 1.5 mm below dura. Mice were allowed a post-operative recovery period of 5-7 days before pilocarpine-induced status epilepticus.

5.2.3 Pilocarpine-induced status epilepticus and video EEG monitoring

Mice were placed in the recording chamber and allowed to acclimate while connected with wired leads for recording EEG from the electrodes in the cortex and the hippocampus. Harmonie software (Stellate Systems, Montreal, Quebec, Canada) was used to record (sampling frequency of 200 Hz) both the electrical potentials from the cortex and hippocampus, which were passed through Grass amplifiers (high-pass filter 5Hz, low-pass filter 10kHz), as well as a video recording of the mice throughout the experiment. *Syn-Cre⁺ TrkB^{lox/lox}* (hereafter *Syn-Cre TrkB^{-/-}*) mice, *Syn-Cre⁺ TrkB^{+/+}* mice, *Syn-Cre⁻ TrkB^{lox/lox}* mice, and wild-type mice were injected with N-methyl-scopolamine in normal saline (1mg/kg, i.p.) and 15 minutes later with pilocarpine (475 mg/kg, i.p.) in normal saline. The animals were observed continuously for behavioral seizures after injection. Additional injections of pilocarpine (10% of the original dose) were administered to mice that did not exhibit a behavioral seizure of Class 4 or greater during the first hour following pilocarpine injection. After the initiation of continuous seizure activity, status epilepticus was allowed to continue for 3 hours. Diazepam (10 mg/kg, i.p.) was given to terminate status epilepticus at 3 hours after the beginning of continuous seizure activity, with additional doses (50% of the original dose) given if status epilepticus had not terminated within 30 minutes. The recordings continued through the end of status epilepticus. Following status epilepticus, mice were maintained on a heated pad and supplemented with soft food and hydration gel. Video

EEG was also recorded for 10 minutes at 24 hours and 48 hours after status epilepticus. As an additional control, *Syn-Cre TrkB^{-/-}* and wild-type mice were treated with normal saline instead of pilocarpine, but otherwise treated identically through the recording protocol. At 48 hours after status epilepticus mice were given a lethal dose of pentobarbital (100 mg/kg, i.p.) and transcardially perfused using a peristaltic pump with ice cold PBS with heparin (50 U/mL) until the blood was completely flushed from the animal. The mouse was then perfused with 2% paraformaldehyde in PBS at a rate of 4 mL/minute for 10 minutes. The brain was then removed, post-fixed in 2% paraformaldehyde for 1 hour at 4°C, cryoprotected in 30% sucrose in PBS until the brain sunk, frozen in a 2-methyl-butane and dry ice bath at -25°C, and stored at -80°C.

5.2.4 Analysis of video EEG data

Video EEG recordings were scored for (1) latency from pilocarpine to the first seizure, (2) latency from pilocarpine to the beginning of status epilepticus, (3) continuation of status epilepticus for the full 3 hours, and (4) latency from treatment with diazepam to termination of status epilepticus. The first seizure and the beginning of status epilepticus were marked by coincident behavioral seizure activity and electrographic seizure activity both in cortex and in hippocampus. The termination of status epilepticus was marked by the absence of behavioral seizure activity and the absence of electrographic seizure activity in both electrodes. Video EEG recordings were scored by two individuals, one of whom was blinded to genotype and treatment.

Digital electrographic recordings from the cortical and hippocampal electrodes were exported from the Harmonie software (Stellate Systems, Montreal, Quebec, Canada) as text and imported into MATLAB (The Math Works) for further analysis. Spectral analysis of the EEG recording was done using the built-in SPECTROGRAM command in MATLAB (Hamming window 1000, overlap 400).

5.3 Results

5.3.1 *Syn-Cre TrkB*^{-/-} mice have a normal latency to develop seizures and status epilepticus following pilocarpine injection

Administration of pilocarpine (475mg/kg) with no more than a single boost uniformly induced status epilepticus in the *Syn-Cre TrkB*^{-/-} mice and control mice (*Syn-Cre TrkB*^{+/+}, *TrkB*^{flox/flox}, and *TrkB*^{+/+} mice) implanted with cortical and hippocampal electrodes. No differences were seen among the various control groups (*Syn-Cre TrkB*^{+/+}, *TrkB*^{flox/flox}, and *TrkB*^{+/+} mice) in any of the measurements (ANOVA, $p > 0.05$) and therefore these animals were combined as a single control group for purposes of analysis. No differences in the baseline recordings before pilocarpine injection were noticed between the *Syn-Cre TrkB*^{-/-} mice and the control group (Figure 27 A). Following injection with pilocarpine seizures were identified both behaviorally and electrographically. No difference in latency from the pilocarpine injection to the first seizure was evident in the *Syn-Cre TrkB*^{-/-} mice (13.2 ± 2.1 min) compared to control mice (17.2 ± 3.8 min, $p > 0.05$) (Figure 26 A). The latency from the pilocarpine injection to the initiation of status

epilepticus following pilocarpine injection was also measured. Again, no significant difference in latency from the pilocarpine injection to the beginning of status epilepticus was seen in the *Syn-Cre TrkB*^{-/-} mice (28.3 ± 6.6 minutes), as compared to control mice (40.4 ± 5.8 minutes, $p > 0.05$) (Figure 26 B). Additionally, the same proportion of *Syn-Cre TrkB*^{-/-} mice required a boost of pilocarpine to initiate status epilepticus as control mice (1 of 6 *Syn-Cre TrkB*^{-/-} mice vs. 2 of 12 control mice). In sum, neither the trend towards shorter latency to the initial seizure or nor to status epilepticus was significantly different in the *Syn-Cre TrkB*^{-/-} mice as compared to controls.

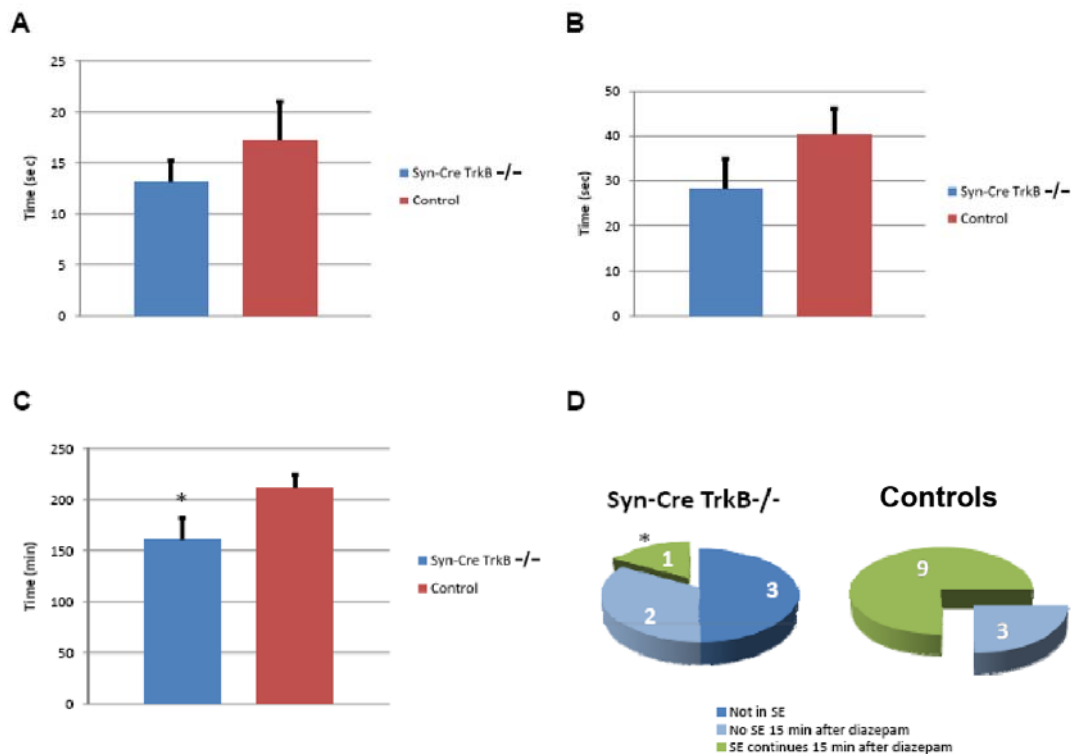


Figure 24: Pilocarpine-induced status epilepticus in *Syn-Cre TrkB*^{-/-} mice.

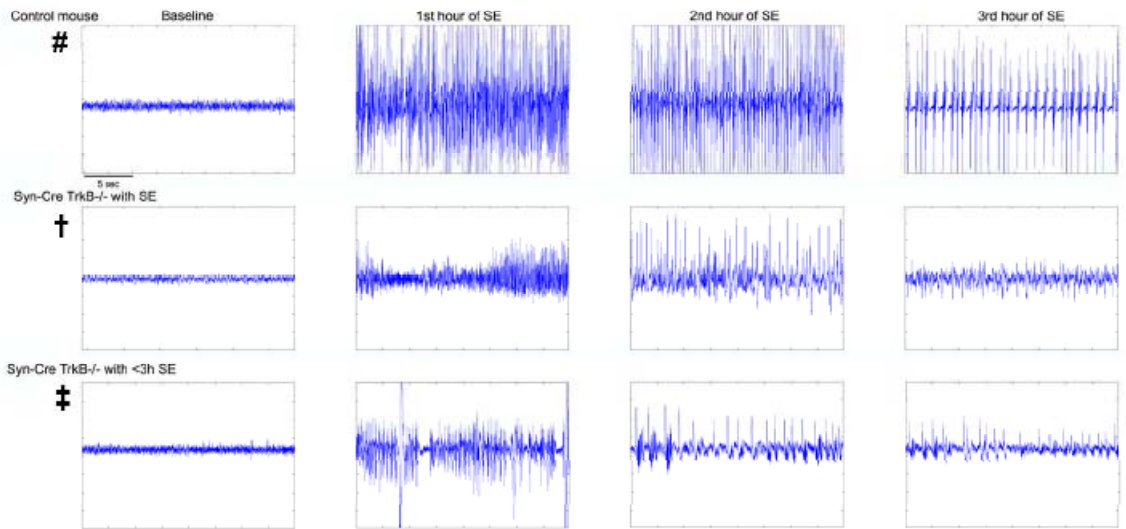
A) Latency from pilocarpine to 1st seizure. No difference in latency from injection with pilocarpine until the first seizure was seen in *Syn-Cre TrkB*^{-/-} mice as compared to control mice ($p>0.05$). B) Latency from pilocarpine to status epilepticus. No difference in latency from injection with pilocarpine until initiation of status epilepticus was seen in *Syn-Cre TrkB*^{-/-} mice as compared to control mice ($p>0.05$). C) Duration of status epilepticus. A significantly shorter total duration of status epilepticus was found in *Syn-Cre TrkB*^{-/-} mice as compared to control mice ($p<0.05$). D) Status following diazepam treatment. The proportion of *Syn-Cre TrkB*^{-/-} mice that continued to have status epilepticus 10 min after diazepam is significantly less than the proportion in control mice still in SE (Fisher Exact, $p<0.05$). Note that 3 *Syn-Cre TrkB*^{-/-} mice spontaneously terminated status at 101, 108, and 160 minutes, while no control mice terminated status until after injection with diazepam. Bar graphs are mean + SEM.

5.3.2 Status epilepticus differs in *Syn-Cre TrkB*^{-/-} compared to wild type mice

Once status epilepticus had begun in control mice, continuous behavioral and electrographic seizure activity persisted in all cases ($n=12$) until the benzodiazepine diazepam was given after 3 hours of status epilepticus. Electrographic activity recorded from a control mouse shows a normal baseline and then continued seizure activity through the 3 hours of status epilepticus, with only a small reduction in frequency over that time (Figure 27 A top row). Additionally, behavior consistent with continued seizure activity (i.e. uninterrupted whole-body clonus without obvious deliberate movements), was maintained throughout the 3 hour period for all control mice. This result is consistent with our laboratory's extensive previous experience with pilocarpine-induced status epilepticus; that is, once status epilepticus begins it will continue for at least 3 hours unless the animal dies.

In this respect the status epilepticus in the *Syn-Cre TrkB^{-/-}* mice is unique in that status epilepticus remitted spontaneously within 3 hours in half of the mice (3 of 6 mice). Electrographic activity recorded from the hippocampus of a *Syn-Cre TrkB^{-/-}* mouse in which status epilepticus terminated spontaneously reveals a normal baseline (Figure 27 A bottom row 1st panel), clear continuous seizure activity in the first hour of status (Figure 27 A bottom row 2nd panel), albeit with a lower amplitude than control mice, but the frequency is visibly decreased in the second hour of status epilepticus (Figure 27 A bottom row 3rd panel). At the start of the third hour of status epilepticus the seizure activity remained but began to lose its rhythmicity (Figure 27 A bottom row 4th panel) and by the end of the third hour the EEG recording had returned to baseline (Figure 27 B bottom panels), and seizure activity was no longer present either electrographically or behaviorally. These mice continued to have some isolated seizures, but they also had periods of several minutes without electrographic seizure activity and they appeared to move around deliberately within the recording chamber, as opposed to being propelled only through seizure-induced myoclonus.

A



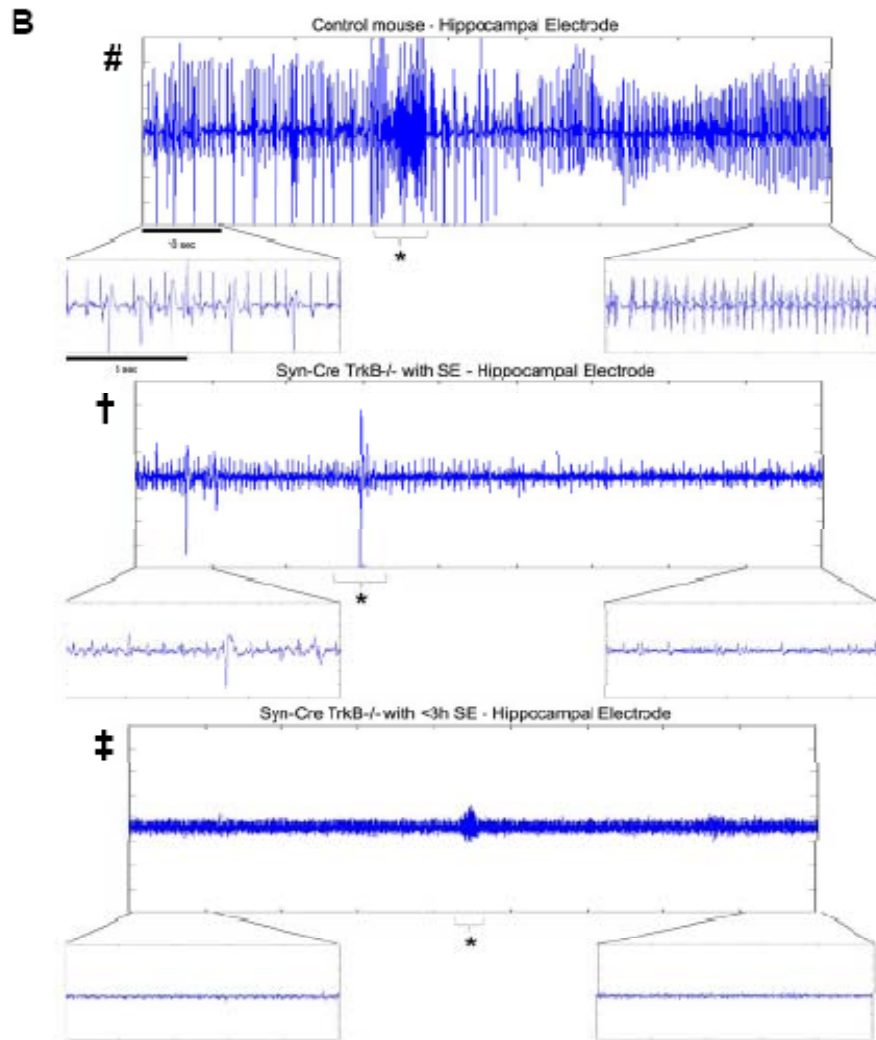


Figure 25: Electrographic recordings of status epilepticus and diazepam treatment.

A) Electrographic recordings from the hippocampus at baseline and during SE in a representative control mouse, a *Syn-Cre TrkB^{-/-}* mouse with 3 hours of SE, and a *Syn-Cre TrkB^{-/-}* mouse with spontaneous resolution of SE. Note the glyphs #, †, and ‡ are used to denote these representative mice throughout. B) Electrographic recordings from the hippocampus at diazepam treatment (artifact marked by *) from a representative control mouse, a *Syn-Cre TrkB^{-/-}* mouse with 3 hours of SE, and a *Syn-Cre TrkB^{-/-}* mouse with spontaneous resolution of SE (<3h SE).

5.3.3 Latency to terminate status epilepticus following diazepam injection

Three hours after the initiation of status epilepticus, all mice were injected with the benzodiazepine diazepam (10mg/kg, i.p.) to terminate status epilepticus. Analysis of the video and EEG recordings was used to determine the end of status epilepticus. The end of status epilepticus was marked when behavioral seizure activity and continuous electrographic seizure activity had ceased in both cortex and hippocampus. Most often the termination of seizure activity occurred in both cortex and hippocampus at the same time, although persistent low frequency activity but with an amplitude above baseline would sometimes continue in either cortex or hippocampus (perhaps similar to periodic lateralized epileptiform discharges (PLEDs) described in human EEGs). Additionally, as previously mentioned 3 of 6 *Syn-Cre TrkB^{-/-}* mice were no longer in status epilepticus at 3 hours, although they continued to have isolated seizures until they were treated with diazepam as well.

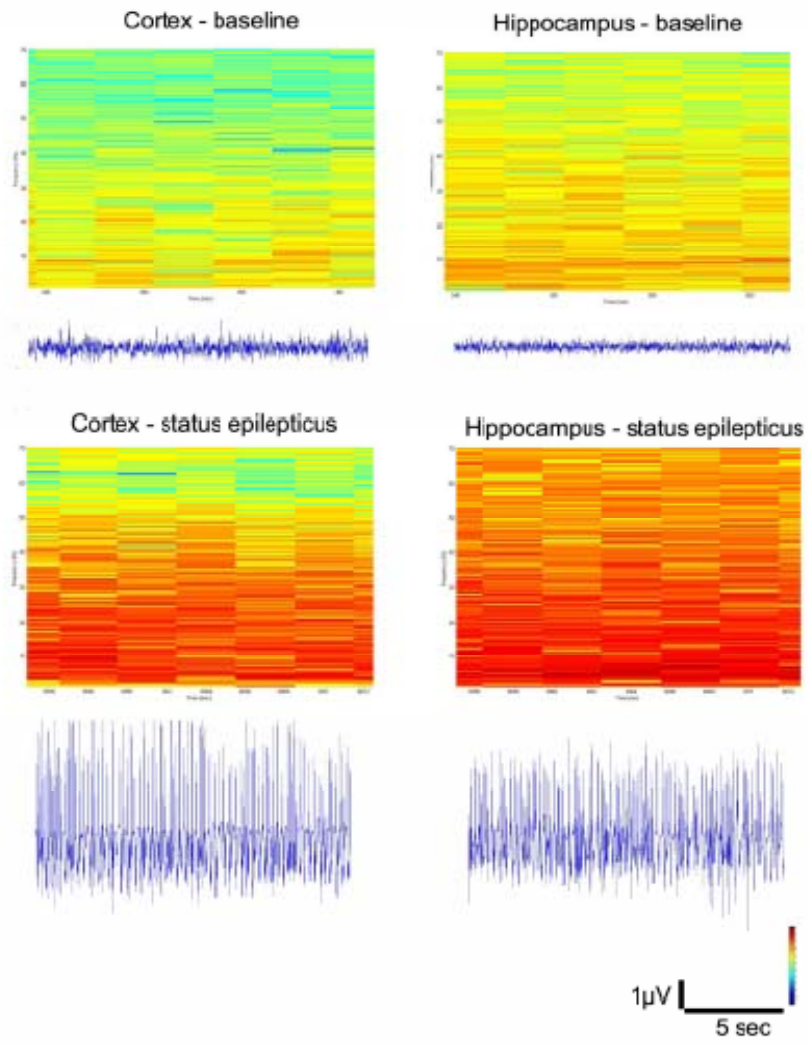
5.3.4 Spectral analysis of status epilepticus

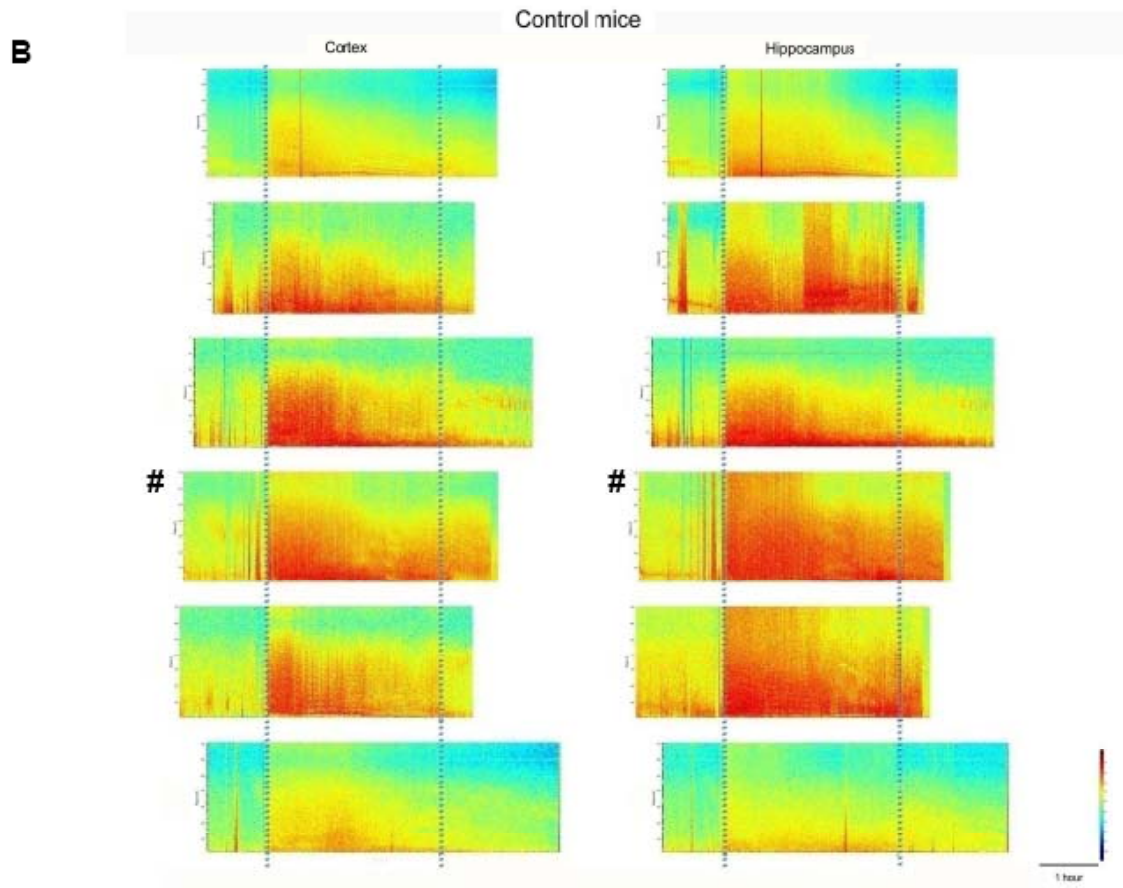
Spectrograms of the cortical and the hippocampal EEG activity for each animal confirmed the observations from visual analyses of the video EEG recordings and, additionally, revealed apparent differences in the spectral composition of the electrographic seizures during status epilepticus (Figure 28 B & C). A benefit of the spectrograms is that they condense many hours of EEGs recordings into a single image,

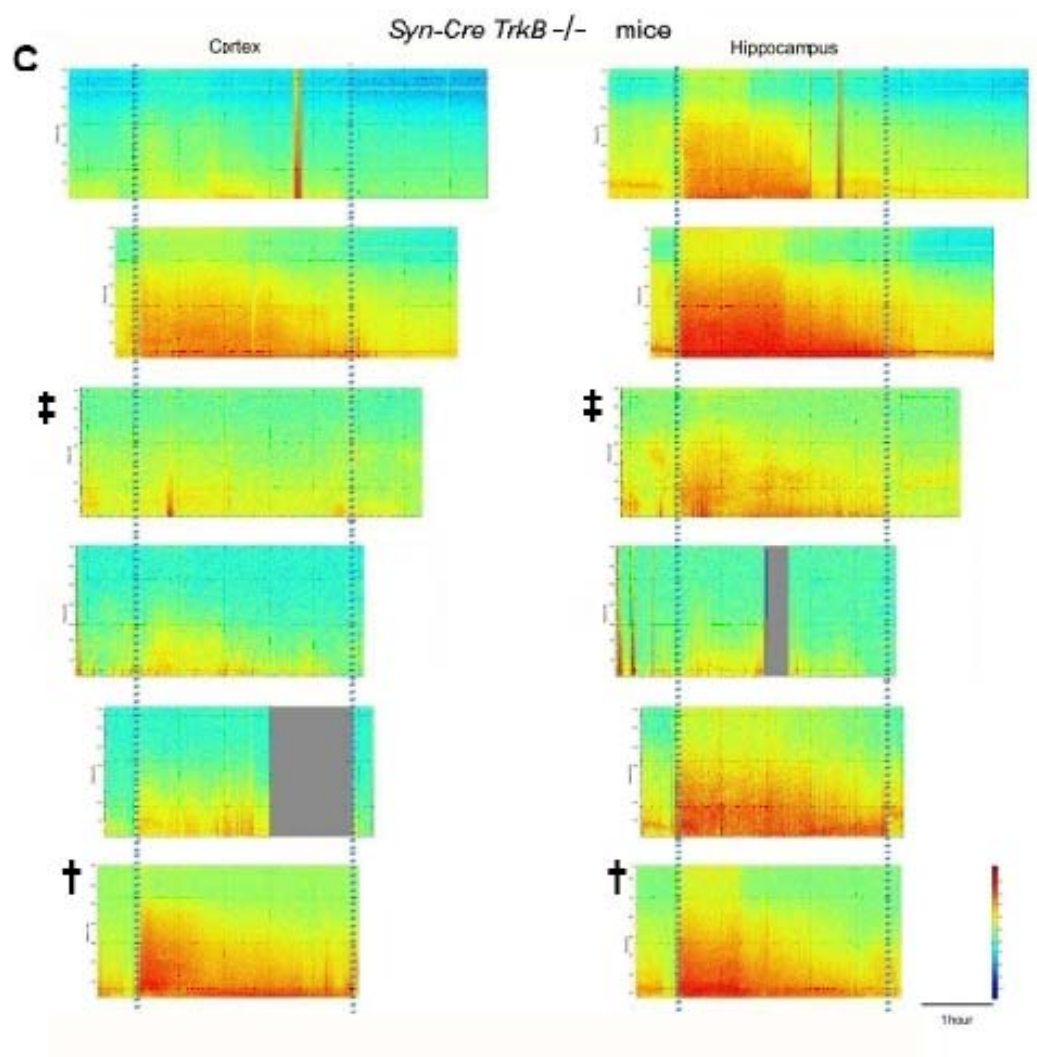
simplifying comparisons within and between groups. No clear differences in baseline (before pilocarpine injection) recordings were evident between the *Syn-Cre TrkB^{-/-}* mice and control mice in either un-processed EEG traces (Figure 27 A 1st column) or by spectral analysis (Figure 28 D left vs right side). Cortical activity was unsynchronized and low amplitude in both groups and hippocampal activity consisted of theta rhythm (6-12 Hz) (Figure 28 A 2nd row). The spectrograms present the amplitudes of activity across the frequency spectrum. For example, for a control mouse in the baseline condition the spectrogram for cortical activity shows low amplitude activity (green and yellow) across the spectrum (Figure 28 A 1st row, left), corresponding to the EEG recording. The spectrogram for the hippocampal recording shows a more intense (red) band at the frequency of theta rhythm (Figure 28 A 1st row, right). During status epilepticus high amplitude and high frequency activity is present in both the cortex and the hippocampus (Figure 28 A 4th row) and the spectrograms show corresponding high-intensity (red) bands across the frequency spectrum (Figure 28 A 3rd row). Despite no noticeable differences in the behavioral seizures during the first thirty minutes of status epilepticus (before spontaneous termination of status epilepticus in some of the *Syn-Cre TrkB^{-/-}*), the spectrograms revealed less high amplitude activity (> 40 Hz) in the *Syn-Cre TrkB^{-/-}* mice as compared to controls but with similar intensity at lower frequencies (compare area between the dashed lines in Figures 3.3 B & C). Only 2 of the 6 *Syn-Cre TrkB^{-/-}* mice displayed high amplitude high frequency activity during the first hour of

status epilepticus, as compared to 9 of 12 control mice. Spectral analysis of the EEG recordings at the time surrounding diazepam treatment corroborate the conclusions from visual analysis of the EEG traces (Figure 27 B); high-amplitude high-frequency activity is either absent in *Syn-Cre TrkB^{-/-}* mice or ends soon after diazepam treatment, while this activity often continues tens of minutes beyond treatment with diazepam in control mice (Figure 28 E).

A







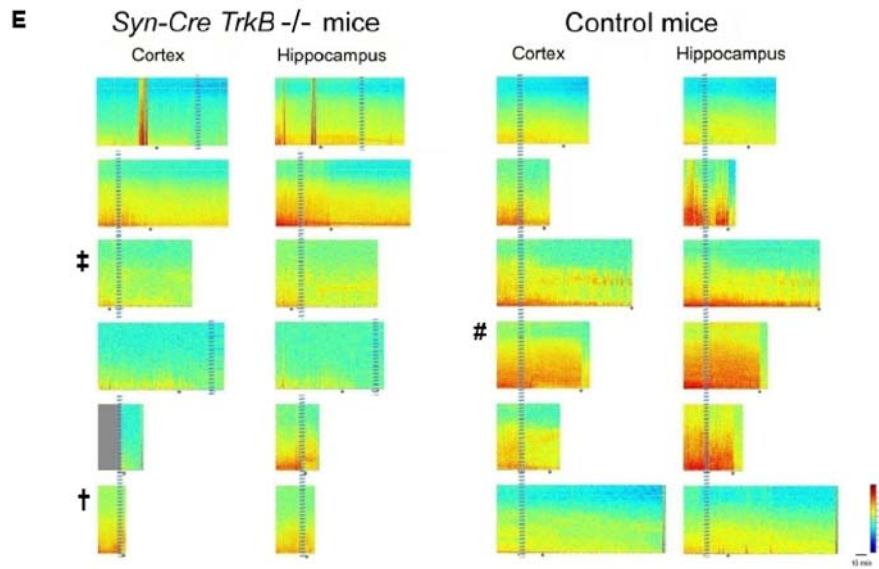
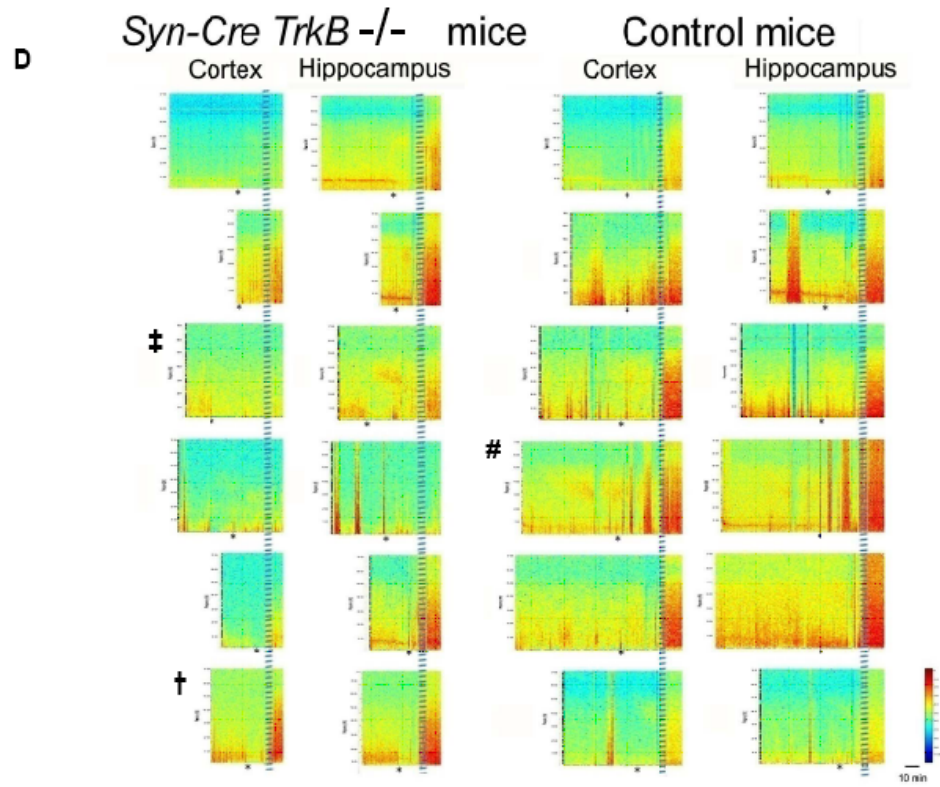


Figure 26: Spectral analysis of EEG recordings.

Spectrograms of EEG recordings present time along the abscissa and frequency along the ordinate. The amplitude of activity at each frequency is presented as a color map (blue to red). A) Comparison of electrographic recordings and spectrograms for a control mouse. Cortical and hippocampal recordings are presented at baseline and during SE, with the corresponding spectrograms. Note theta rhythm in baseline hippocampus at 6 – 12 Hz, with corresponding band in spectrogram. B) Spectrograms of cortical and hippocampal recordings from control mice. The first dashed line marks the initiation of SE and the second dashed line marks treatment with diazepam 3 hours later. C) Spectrograms of cortical and hippocampal recordings from *Syn-Cre TrkB*^{-/-} mice. As with control mice, the dashed lines mark the initiation of SE and treatment with diazepam 3 hours later. Gray boxes are instances without recordings, due to technical problems. D) Spectrograms of baseline recordings, showing pilocarpine treatment (*), and start of SE (dashed line) for *Syn-Cre TrkB*^{-/-} mice and controls. E) Spectrograms covering treatment with diazepam (dashed line) and the end of SE (*) for *Syn-Cre TrkB*^{-/-} mice and controls.

5.4 Discussion

5.4.1 Summary and Conclusions

The initial hypothesis leading to this study was that conditional deletion of TrkB in the *Syn-Cre TrkB*^{-/-} mice would inhibit or even prevent the occurrence of spontaneous recurrent seizures in the pilocarpine status epilepticus model, just as this mouse had failed to display evidence of epileptogenesis in the kindling model (X. P. He et al., 2004). However significant differences in status epilepticus were found which prohibit meaningful comparison of the development of late-onset spontaneous recurrent seizures in *Syn-Cre TrkB*^{-/-} mice to control mice, the difference in sustained seizure activity is a fascinating indication that TrkB may have an important acute role in status epilepticus. Two principal findings emerged from this work: 1) induction of behavioral and electrographic status epilepticus by pilocarpine is equivalent (equivalent sensitivity to

pilocarpine and equivalent latency to develop status) in *Syn-Cre TrkB^{-/-}* and wild type mice; 2) pilocarpine-induced status epilepticus remits spontaneously in some *Syn-Cre TrkB^{-/-}* mice but not control mice. These findings demonstrate that expression of the neurotrophin receptor TrkB is required to sustain pilocarpine status epilepticus.

5.4.2 Implications for sustaining seizure activity during status epilepticus

The *Syn-Cre TrkB^{-/-}* mice are more susceptible than control mice to endogenous mechanisms to terminate status epilepticus. The spontaneous resolution of status epilepticus within 3 hours was unique to the *Syn-Cre TrkB^{-/-}* mice within this experiment and is unique in the laboratory's experience with pilocarpine-induced status epilepticus. Importantly, the phenotype in the *Syn-Cre TrkB^{-/-}* mice is not due to pilocarpine having a reduced effect in these mice. Although a non-significant difference, the latency from pilocarpine injection to the first seizure and to the beginning of status epilepticus is less in the *Syn-Cre TrkB^{-/-}* mice than in control mice (Figure 26 A & B). Additionally, the same proportion of *Syn-Cre TrkB^{-/-}* mice required a boost of pilocarpine to initiate status epilepticus as control mice (1 of 6 *Syn-Cre TrkB^{-/-}* mice vs. 2 of 12 control mice).

The role of BDNF/TrkB signaling in status epilepticus has been previously examined in animal models. Increases in BDNF and phosphorylation of TrkB have been reported as early as 20 minutes after kainic acid injection in the sensory and motor cortex (R. Aloyz et al., 1999). This time course contrasts with the peak of BDNF/TrkB signaling seen in the hippocampus, which peaks at 24-48 hours after status epilepticus (M. M.

Dugich-Djordjevic et al., 1992a; G. Mudo et al., 1996). Given the deficit in sustaining the seizure activity is seen within 3 hours in the *Syn-Cre TrkB*^{-/-} mice, this implies that if the phenotype is due to a direct result of reduced TrkB during status epilepticus, cortical TrkB may be critical.

Whether the inability to sustain continuous seizure activity is due to failure of excitatory synaptic transmission, increased inhibition, or a deficit in synchronization of the activity is unclear from examination of status epilepticus alone. BDNF/TrkB signaling clearly leads to an increase in net excitability under many conditions and previous work has demonstrated that TrkB can modulate both excitatory and inhibitory synaptic transmission. In cultured hippocampal neurons TrkB colocalizes with both presynaptic and postsynaptic markers of both excitatory and inhibitory synapses (C. C. Swanwick et al., 2004), implying that BDNF/TrkB signaling could directly act at both excitatory and inhibitory synapses.

Exogenous BDNF has been shown to enhance spontaneous and evoked excitatory synaptic transmission in cultured hippocampal neurons (V. Lessmann et al., 1994; E. S. Levine et al., 1995), with both presynaptic and postsynaptic contributions (J. Alder et al., 2005), and in acute hippocampal slices from mature animals (H. Kang and E. M. Schuman, 1995). The mechanisms elucidated for BDNF and TrkB in LTP may be particularly informative for understanding the role of BDNF/TrkB signaling in sustained seizure activity. In support of this comparison, treatment of hippocampal slice cultures

with bicuculline, an in vitro model of continuous seizure activity, has been shown to occlude LTP at the CA3-CA1 synapse (M. H. Abegg et al., 2004). One of the roles of BDNF may be to maintain an adequate number of docked synaptic vesicles to prevent synaptic fatigue following repeated stimulation (A. Figurov et al., 1996; L. Minichiello et al., 1999; B. Xu et al., 2000), which may be relevant for the continued excitatory synaptic transmission during status epilepticus. Therefore lack of TrkB in the *Syn-Cre TrkB^{-/-}* mice could result in fewer docked vesicles at excitatory synaptic terminals and with a smaller pool of vesicles these synapses could fatigue during the continuous seizure activity of status epilepticus. The result would be a failure of excitatory synaptic transmission. These studies suggest that reduced TrkB signaling may impair continued excitatory synaptic transmission during status epilepticus, thereby leading to termination of status epilepticus in the *Syn-Cre TrkB^{-/-}* mice. Furthermore, chronic inhibition of BDNF/TrkB signaling in dissociated hippocampal cultures results in a reduced frequency of mEPSCs (M. M. Bolton et al., 2000), suggesting the *Syn-Cre TrkB^{-/-}* mice may have a basal deficit in excitatory synaptic transmission even before pilocarpine treatment.

Alterations in inhibitory synaptic transmission also clearly occur during status epilepticus (R. L. Macdonald and J. Kapur, 1999). Reductions in inhibition may underlie the persistence of seizure activity during status epilepticus through internalization of inhibitory GABA_A receptors. GABA_A receptors accumulate intracellularly in cultured

hippocampal cells when they undergo epileptiform bursting secondary to exposure to magnesium-free media (H. P. Goodkin et al., 2005). Furthermore, Hewitt and Bains (S. A. Hewitt and J. S. Bains, 2006) found evidence for TrkB activation found evidence for TrkB activation leading to the silencing of GABA synapses in hypothalamic neuroendocrine cells, likely through internalization of the receptors, given that the process is dynamin-dependent. Together these results suggest that TrkB may decrease GABA signaling by causing the internalization of GABA_A receptors. Therefore, reduced TrkB signaling in the *Syn-Cre TrkB^{-/-}* mice may allow for undiminished inhibitory synaptic transmission during status epilepticus and allow spontaneous termination. Furthermore, Bolton and colleagues (M. M. Bolton et al., 2000) found that chronic inhibition of BDNF/TrkB signaling in dissociated hippocampal cultures resulted in an increased number of GABAergic terminals, suggesting that the *Syn-Cre TrkB^{-/-}* mice may have enhanced inhibitory synaptic transmission before pilocarpine treatment that may enhance their ability to terminate status epilepticus.

A role for BDNF/TrkB in the formation of networks during development has been described (F. Aguado et al., 2003; M. A. Carmona et al., 2006), but whether these mechanisms are active in the *Syn-Cre TrkB^{-/-}* mouse or in the mature brain is not known. Theoretically changes in the network organization could disrupt the synchronicity characteristic of seizure activity. Regardless of the mechanism, if elimination of TrkB

allows endogenous mechanisms to terminate status epilepticus, a pharmacological treatment that blocks TrkB signaling would be useful for treating status epilepticus.

In an important experiment which is the converse of this work, over-expression of TrkB in transgenic mice lead to a greater proportion of animals developing SE with a low dose of kainic acid and having more severe SE and more prevalent cell death, although no differences in the emergence of spontaneous seizures were seen (S. Lahtinen et al., 2003). Previously Lahtinen and colleagues had found that reduced TrkB signaling in a mouse over-expressing truncated TrkB did not affect kainic acid-induced status epilepticus (S. Lahtinen et al., 2002). The more severe phenotype in the *Syn-Cre TrkB^{-/-}* mice than in the mice over-expressing truncated TrkB may be due to a greater reduction in TrkB signaling by removing the receptor rather than relying on a dominant-negative mechanism of the truncated receptor, for instance these transgenic mice did not have a deficit in LTP (T. Saarelainen et al., 2000), or it may be the result of differences between the pilocarpine and kainic acid models.

5.4.3 Implications for treatment of status epilepticus

Status epilepticus is a common and serious medical condition, with 120,000 to 200,000 cases yearly in the United States (R. J. DeLorenzo et al., 1996) and having a mortality of approximately 25% (A. R. Towne et al., 1994). A common complication of status epilepticus is the failure to respond to two different anticonvulsants , often a benzodiazepine and fosphenytoin, and development of refractory status epilepticus.

Previous studies found that 38% of patients with convulsive status epilepticus (D. M. Treiman et al., 1998) and 82% of patients with non-convulsive status epilepticus (D. M. Treiman, 1999) developed refractory status epilepticus. Furthermore, with continued status epilepticus the treatment becomes more difficult (S. Shorvon, 2001), the pathology becomes greater (D. H. Lowenstein and B. K. Alldredge, 1998), and the outcome for the patient becomes worse (A. R. Towne et al., 1994). These facts stress the importance of improving the treatment of status epilepticus, especially if the responsiveness to benzodiazepines could be improved.

Conditional deletion of TrkB in the *Syn-Cre TrkB^{-/-}* mice may have prevented the decrease in responsiveness to benzodiazepines normally associated with status epilepticus. Two of the three *Syn-Cre TrkB^{-/-}* mice with 3 hours of status epilepticus responded to diazepam more rapidly than 9 of 12 control mice. The proposed mechanism of reduced TrkB signaling preventing endocytosis of GABA_A receptors (H. P. Goodkin et al., 2005; S. A. Hewitt and J. S. Bains, 2006) and allowing endogenous inhibitory synaptic transmission to terminate status epilepticus is consistent with a greater responsiveness to benzodiazepines, since the same GABA_A receptors on the cell surface that could be activated by GABA during the course of status epilepticus to cause remission of the continuous seizure activity would also be available for the exogenous diazepam to bind. Furthermore, BDNF activation of TrkB has been shown to cause

increased expression of the $\alpha 4$ subunit of the GABA_A receptor, which is insensitive to benzodiazepines (D. S. Roberts et al., 2006).

The increased sensitivity to diazepam in the *Syn-Cre TrkB*^{-/-} mice may be similar to the phenotype seen in rats pre-treated with the non-competitive NMDA receptor antagonist MK-801 before kainic acid status epilepticus (A. C. Rice and R. J. DeLorenzo, 1998). The MK-801 pre-treated rats have continuous seizure activity for 4 hours after kainic acid injection, but status epilepticus terminates within minutes following treatment with diazepam. In comparison, control rats need two diazepam treatments, after 1 hour and after 3 hours of status epilepticus, to terminate seizure activity within 4 hours. It should be noted that in the mice described in this study and in the work of Rice and DeLorenzo the animals showing an increased sensitivity to benzodiazepine treatment were different from control animals before the start of pilocarpine treatment, therefore the status epilepticus they experienced may have been qualitatively different, even if it continued for 3 or 4 hours.

5.4.4 Implications for studying the development of spontaneous seizures

The shorter duration of status epilepticus in the *Syn-Cre TrkB*^{-/-} mice (Figure 26 C) precludes drawing meaningful interpretations from studying the development of spontaneous recurrent seizures in these mice. The duration of pilocarpine-induced status epilepticus has a direct effect on the propensity to develop late-onset seizures (T. Lemos and E. A. Cavalheiro, 1995). Therefore, if the *Syn-Cre TrkB*^{-/-} had a reduced

propensity to develop late onset spontaneous recurrent seizures, the effect could not be attributed unambiguously to lack of TrkB during the period of epileptogenesis.

Furthermore, the spectral analysis of the EEG recordings from status epilepticus (Figure 28 B & C), the spontaneous remission of status epilepticus in 3 of 6 *Syn-Cre TrkB^{-/-}* mice, and the rapid response to diazepam of 2 of 3 *Syn-Cre TrkB^{-/-}* mice all suggest that status epilepticus in the *Syn-Cre TrkB^{-/-}* is qualitatively different in intensity from control mice. In contrast to the study of the *Syn-Cre TrkB^{-/-}* mice in the kindling model (X. P. He et al., 2004), in which the initial kindled seizure and MES seizures were equivalent to control mice (sections 2.3.6 and 2.3.7), pilocarpine-induced status epilepticus was not equivalent in the *Syn-Cre TrkB^{-/-}* and control mice, suggesting that status epilepticus is more complex than just a prolonged seizure.

While these experiments did not advance our understanding of the role of TrkB in epileptogenesis in the pilocarpine-induced status epilepticus model, a contribution towards the improvement of the treatment of status epilepticus would be a life-saving accomplishment in itself.

5.4.5 Future directions

Further study into the role of TrkB in status epilepticus could advance our understanding of the pathophysiology of status epilepticus while possibly identifying a new therapeutic target. The chief concern is the time at which the reduction in TrkB acts to impair the sustained seizure activity. To address this concern the time course of TrkB

activation during pilocarpine status epilepticus and localization of the activated TrkB. If the activated TrkB could be localized to specific axons or dendrites, it would provide a framework for developing more informed hypotheses regarding the cellular and molecular mechanisms of status epilepticus. If a reduction in TrkB after the initiation of status epilepticus is sufficient, then a therapy inhibiting TrkB signaling could be used clinically as an adjuvant (e.g. given 1 hour after initiation of status epilepticus upon admission to the emergency department) to current treatments for status epilepticus. The results in a subset of *Syn-Cre TrkB^{-/-}* mice suggest that inhibiting TrkB signaling could potentiate the effect of diazepam. If the reduction in TrkB was required before the beginning of status epilepticus, a therapy to inhibit TrkB may still be useful as a chronic treatment for individuals prone to development status epilepticus, allowing a better response to exogenous benzodiazepine treatments or perhaps spontaneous remission of status epilepticus by endogenous mechanisms, as was seen in 3 of 6 *Syn-Cre TrkB^{-/-}* mice. However chronic treatment to inhibit TrkB would require the unlikely finding of minimal side effects.

Many of these questions could be addressed using a chemical-genetic approach to efficiently and specifically inhibit TrkB kinase activity (X. Chen et al., 2005). This approach would allow for rapid inhibition of TrkB signaling and the effects of reduced TrkB signaling before or after the initiation of status epilepticus could be tested. Pairing of TrkB inhibition with a time course of benzodiazepine treatment would establish if

reduced TrkB signaling improves the efficacy of benzodiazepines in treating status epilepticus. The discovery of a role for TrkB acutely in status epilepticus supports the more detailed examination of in vivo and in vitro models of status epilepticus to uncover the cellular and molecular mechanisms by which TrkB affects the sustained seizure activity.

6. BAC *Synapsin1-CreER* mice

6.1 Introduction

The importance of transgenic mice to progress in the biomedical sciences cannot be overstated. The bi-directional flow of information from human conditions being replicated in mouse models and discoveries from transgenic mouse models leading to improvements in treatment for human disease is a boon for translational research. Genetically altered mice have allowed for the dissection of important cellular and molecular processes at the level of individual gene products. Current techniques in transgenic mouse technology permit the genetic manipulations to be restricted to certain cell types while inducible systems provide the ability to temporally control the transgene.

The studies described previously in this dissertation have successfully used conditional and inducible conditional mutations to implicate TrkB as an important actor in the development and persistence of kindling and in the sustained seizure activity of status epilepticus. However, TrkB likely plays different roles in different cell populations within the brain. Understanding which of these actions are involved in promoting epileptogenesis, in limiting epileptogenesis, or are uninvolved requires cell type specific elimination of TrkB. Given the number of different cell types that are involved, even within a single structure such as the hippocampus, we sought to design a

system for the rapid generation of transgenic mice with inducible, cell-type specific elimination of TrkB.

6.1.1 Rationale for using BAC transgenics

To develop a transgenic mouse with an inducible cell-type specific elimination of TrkB, we combined the advantages of the inducible CreERTM2/loxP system with bacterial artificial chromosome (BAC) transgenics. Previous work has demonstrated that BAC transgenics faithfully represent the expression pattern of the endogenous gene (N. Heintz, 2001). Therefore, given knowledge of the expression pattern of the endogenous gene, BAC transgenic mice can be designed to express a transgene in any population of cells for which a cell-type specific promoter can be identified. Given the success with the *Synapsin1* promoter in the *Syn-Cre TrkB*^{-/-} mice, a BAC containing the *Synapsin1* gene was chosen for modification with the CreER^{TM2} recombinase. The methods used to prepare the DNA construct and for evaluating the resultant transgenic lines were optimized to facilitate the creation of future transgenic lines using other BACs. In sum, a functional BAC transgenic mouse with inducible recombination was created and valuable knowledge was gained to improve the making of future BAC transgenic mice.

6.2 Methods

6.2.1 Mice

Rosa26-lacZ reporter mice on a C57/B6 background (P. Soriano, 1999) were used to identify the pattern of Cre recombinase activity in the newly generated mice. The *Rosa26-lacZ* mice have the *lacZ* gene downstream of a transcriptional and translational *STOP* cassette. The *STOP* cassette is flanked by *loxP* sites so that following Cre recombinase activity the *STOP* cassette is excised and expression of the *lacZ* gene produces β -galactosidase. The β -galactosidase is visualized by an X-gal staining reaction, in which β -galactosidase activity results in the deposition of a blue precipitate.

TrkB^{lox/lox} mutant mice (Y. Zhu et al., 2001), with the first exon of the *TrkB* gene flanked by *loxP* sites, on a mixed background of 129/C57/ICR were used to conditionally eliminate the *TrkB* gene from cells following Cre recombinase activity. These mice have been previously described (section 2.2.1) (X. P. He et al., 2004).

Additionally, mice with an inducible form of the Cre recombinase (CreER^{TM2}) were generated in the course of this study.

6.2.2 BAC selection and recombineering

The bacterial artificial chromosome (BAC) containing the *Synapsin1* gene from a female C57/B6 mouse was selected using a search on NCBI (www.ncbi.nlm.nih.gov). BAC RP23-141-N-10 was selected since it provided ~100 kb of sequence 5' of the gene,

the full 60 kb of exon-intron sequence of the *Synapsin1* gene, and ~25 kb of sequence 3' of the gene (Figure 29). The amount of flanking sequence should allow for the BAC to include enough regulatory elements to control expression with fidelity to expression of the endogenous gene. The BAC was acquired from the Children's Hospital of Oakland Research Institute BACPAC Resources Center (bacpac.chori.org). The BAC was contained within a pBACe3.6 plasmid in *E. coli*. Verification of the proper BAC was done by PCR amplification and sequencing of the end sequences and a diagnostic rare-cutting restriction enzyme digest, which was run on a pulsed field gel and confirmed to match the predicted pattern (Figure 30 A).

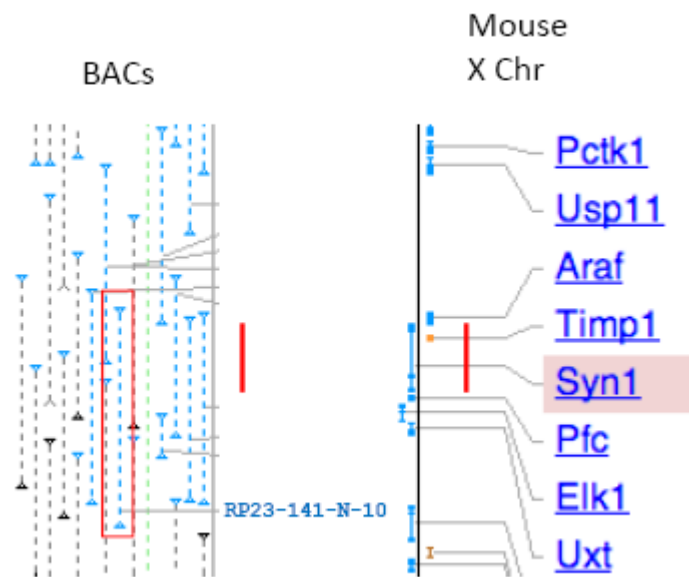


Figure 27: Selection of BAC.

A bacterial artificial chromosome (BAC) (dashed blue line boxed in red) was selected from the NCBI website to contain the entire *Synapsin1* gene (red bar) with

both 5' and 3' regulatory elements. Available BACs are shown on the left and the genes of the mouse X chromosome are shown on the right.

A BAC-modifying vector was constructed containing the Cre-estrogen receptor fusion construct (*Cre-ER^{TM2}*) with a neomycin resistance cassette with FRT sites, flanked by two ~500 bp regions amplified from the BAC (the A & B boxes) (Figure 30 left). The amplicons for the A and B boxes had restriction enzymes cut sites added by PCR to facilitate insertion into the BAC-modifying vector. These A and B box regions provide the sites for homologous recombination between the BAC-modifying vector and the BAC and were designed to replace the ATG-start site of the *Synapsin1* gene with the ATG-start site of the *CreERTM2* construct. Additionally, the first 30 bp of the *Synapsin1* gene were eliminated to knock-out the *Synapsin1* gene in the BAC. The correct configuration of the BAC-modifying vector containing the A and B boxes was confirmed by PCR amplification and sequencing.

A digestion fragment of the BAC-modifying vector containing the A box, the CreERTM2 cDNA, NeoR cassette flanked by FRT sites, and the B box were electroporated into a strain of *E. coli* designed for recombineering (EL-250) which already contained the BAC. These *E. coli* have a temperature-inducible recombinase genes, so that recombination will occur when the bacteria are grown at 37°C, but no further recombination will occur when the bacteria are kept at 30°C. The recombineering *E. coli* containing both the BAC and the BAC-modifying vector were

grown at 37°C to allow recombination between the A and B boxes and the homologous regions in the BAC, thereby replacing the first 30 bp of the *Synapsin1* gene with the *CreER* and NeoR cassette. The recombineering *E. coli* was then returned to 30°C and plated at low density to isolate individual bacterium. Antibiotic selection was used to grow only bacteria carrying the BAC (ChlorR) with the CreER^{TM2} fragment (NeoR) incorporated. The individual colonies were screened by PCR for correct insertion of the CreERTM2 fragment into the *Synapsin1* gene in the BAC. Proper recombination was also tested by restriction enzyme digest followed by pulsed-field electrophoresis of the products of the digest (Figure 30 B). In this case the CreER^{TM2}-NeoR construct added an additional cut site for the restriction enzyme, so that fragment 1 in lane A of the pulsed field gel is cleaved in to fragments 2 and 3 in lane B (Figure 30 right). An arabinose-induced Flp recombinase in the EL-250 *E. coli* was used to eliminate the NeoR cassette from the modified BAC through addition of arabinose to the media. The final modified BAC was confirmed through PCR screening, restriction enzyme digest with pulsed-gel electrophoresis (Figure 30 C), and sequencing of the insertion site. Elimination of the NeoR cassette causes a 1.4 kb downward shift in band 3 in lane B to band 4 in lane C (Figure 30 right).

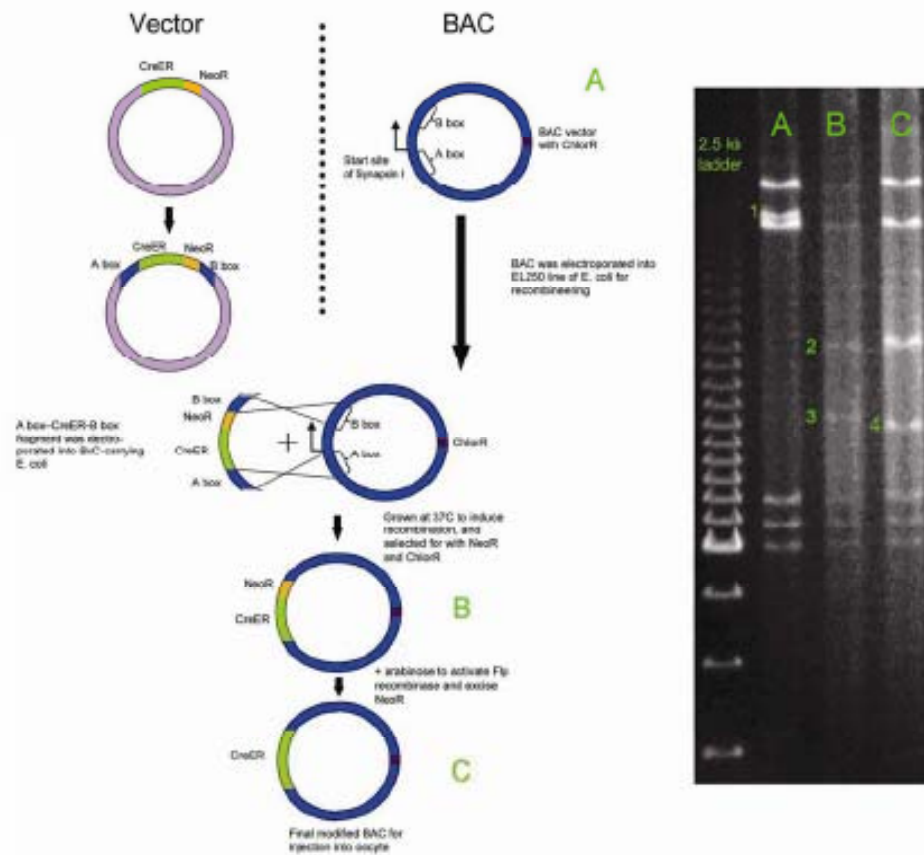


Figure 28: Recombineering the BAC to insert *CreER^{TM2}*.

(left panel) A flowchart demonstrates the addition of the homologous sequences flanking the start site of the gene of interest (A and B boxes) to the plasmid carrying *CreERTM* and a neomycin-resistance selectable marker. The *CreERTM* and *NeoR* flanked by the A and B boxes replace the first 30 base pairs of the gene of interest in the BAC when the temperature-sensitive recombination is induced. Treatment with arabinose activates a Fli recombinase with excises the *NeoR* from the modified BAC. (right panel) A pulsed field gel of BAC DNA digested with *SwaI* demonstrates the modification of the BAC. Lane A is the unmodified BAC. The *CreERTM* modification adds a cut site, which cleaves fragment 1 into fragments 2 and 3 (lane B). Elimination of the *NeoR* marker reduces the size of fragment 3 by 1.4kb, resulting in fragment 4 (lane C).

6.2.3 Injection into oocyte and implantation

(Work done by Bernd Gloss and Jimmy Gross)

Blastocyst Injection:

a. Superovulation: To obtain the maximum number of embryos from the minimum number of females, female mice serving as embryo donors were superovulated using gonadotropins (lyophilized powders from National Hormone & Peptide Program, UCLA Medical Center) and prepared in PBS + sterile saline. Females 4-6 weeks of age are injected once intraperitoneally with 5 IU (0.1cc) of pregnant mare's serum. They were injected similarly 46hr later with 5 IU (0.1cc) of human chorionic gonadotropin and placed with fertile males for mating.

b. Embryo isolation: Superovulated females were mated with wild type males produce the fertilized eggs necessary for micro-injections. Blastocysts were taken from 3.5 days post-conception females following sacrifice by cervical dislocation. Embryos were collected in FHM or M16 mediums.

c. Transfer of blastocysts to pseudopregnant females: Female mice of 28-34 grams which had been plugged by a vasectomized male, 0.5 or 2.5 days earlier are anesthetized with Avertin (125-250mg/kg). Prior to incision, the area surrounding the area to be incised was swabbed with a full strength solution of betadine, rinsed with ethanol, swabbed again with betadine and finally rinsed a second time with ethanol. A <1cm longitudinal incision through the skin was made at the dorsal midline below the last rib.

A small incision was made lateral to the spine over the ovary through the skin opening. The ovary, oviduct and uterus were lifted from the cavity via the fat pad. A 28 gauge hypodermic needle was used to pierce the proximal uterus, into which 5-8 blastocysts were transferred with a thin Pasteur pipette. The organs were returned to the cavity and the transfer repeated for the other horn of the uterus. The wound was closed with 1-2 stitches and the skin stapled with 1-2 staples. (1-2 drops of 2.5% bupivacaine is applied to each incision prior to body wall and skin closure) After recovery, mice were returned to the shelves and followed up with post-operative observation.

6.2.4 *In situ* hybridization for Cre

(Work done by A. Soren Leonard)

Mice were treated with a lethal overdose of pentobarbital (100mg/kg) and rapidly decapitated. The brain was removed and frozen on powdered dry ice. Brain sections were cut on a cryostat at 16 μm , affixed to Plus slides, and stored at -80°C . All steps in the *in situ* hybridization through labeling with the riboprobe were done in an RNase-free manner. The sections were thawed in 4% paraformaldehyde in PBS for 20 min, washed in DEPC-treated water, dehydrated in an alcohol series, and acetylated with acetic anhydride. The slides were washed in DEPC-treated water again, dehydrated in an alcohol series, allowed to air dry, and then covered with the radioactively labeled Cre riboprobe (~400 bp) in hybridization solution [50% formamide, 5x SSC, 1.25mg yeast RNA, 2.4 mg salmon testes DNA, 10x Denhardt's, in 5mL DEPC-

treated water]. The hybridization solution was covered with a coverslip and the slides were placed in a humidified chamber (with 50% formamide in DEPC-treated water) in a 60°C oven overnight. The following day the slides were washed in 60°C 5x SSC for 30 min, 60°C 2x SSC + 50% formamide for 30 min twice, 2x SSC for 5 min, 0.2x SSC for 5 min, and 0.1x SSC for 5 min. The slides were allowed to air dry and then exposed to photographic film (Kodak) for 7 days.

The protocol for the *TrkB in situ* hybridization with the digoxigenin-labeled riboprobe was previously described in section 3.2.4. Briefly, a ~450 bp riboprobe directed against the kinase domain of the full-length *TrkB* mRNA was used to probe sections from BAC *Syn-CreER TrkB^{-/-}* mice and wild type mice. An HRP-coupled anti-digoxigenin antibody (1:200, DakoCytomation) was used to detect the probe and the enzymatic activity of the HRP was used to deposit a cyanine-3 fluorophore (Cy3) onto the tissue using a TSA-Cy3 amplification system (PerkinElmer). The fluorescent signal was visualized using a Leica confocal microscope, with excitation at 543 nm. Images are maximum projections of an image stack that spanned the section thickness.

6.2.5 Tamoxifen treatment

Mice were treated with tamoxifen (7.5mg in sunflower oil (50mg/mL), Sigma) by oral gavage daily for 5 days. Tamoxifen was put into solution by continuous rotation in a 55°C oven for about 1 hour. The tamoxifen was filtered (0.2µM) and kept at 37°C until used. Fresh tamoxifen solution was made each day. For gavage, mice were restrained

by hand and placed in a supine position with the neck extended. A slightly bent feeding needle (22 gauge, Harvard Apparatus) was marked for the proper length (from the mouth to the bottom of the rib cage) was gently inserted with minimal pressure and the tamoxifen solution was delivered slowly using a 1cc syringe. The mice were treated at 2 months of age.

6.3 Results

6.3.1 Generation of *BAC Synapsin1-CreER* mice

Blastocyst injection and transplantation into pseudopregnant females resulted in 9 founders which carried the *BAC Synapsin1-CreER* transgene (F0). Upon a cross to C57/B6 mice, two founders failed to transmit the transgene to the F1 generation and an additional two lines failed to transmit the transgene to the F2 or F3 generation. The remaining 5 lines were back-crossed to *TrkB^{lox/lox}* and *Rosa26-lacZ* lines in a pure C57/B6 background (Charles River) for further characterization. In the course of characterization, 4 of 5 lines had some inducible Cre activity and a single line had significantly more recombination than the other lines. This line was chosen for more extensive examination.

6.3.2 Cre mRNA expression in the *BAC Synapsin1-CreER* mice

An important first step in evaluating the newly generated transgenic lines is to determine expression of the transgene, both the anatomical distribution and the level of

expression. Since antibodies against the Cre protein suitable for immunohistochemistry or immunoblot are not available, mRNA expression of the transgene was examined. The *Cre in situ* hybridization revealed levels of *Cre* mRNA expression comparable to those seen in the *Act-CreER* mouse and greater or equal to the levels seen in the *Syn-Cre* mouse (Figure 31). Importantly, the BAC *Syn-CreER* also has equivalent *Cre* mRNA as the *Act-CreER* mouse, which has been shown to effectively excise a floxed transcriptional and translational *STOP* cassette in the *Rosa26-lacZ* reporter and a floxed *TrkB* allele, we were hopeful that the BAC *Syn-CreER* mouse would have equivalent efficacy.

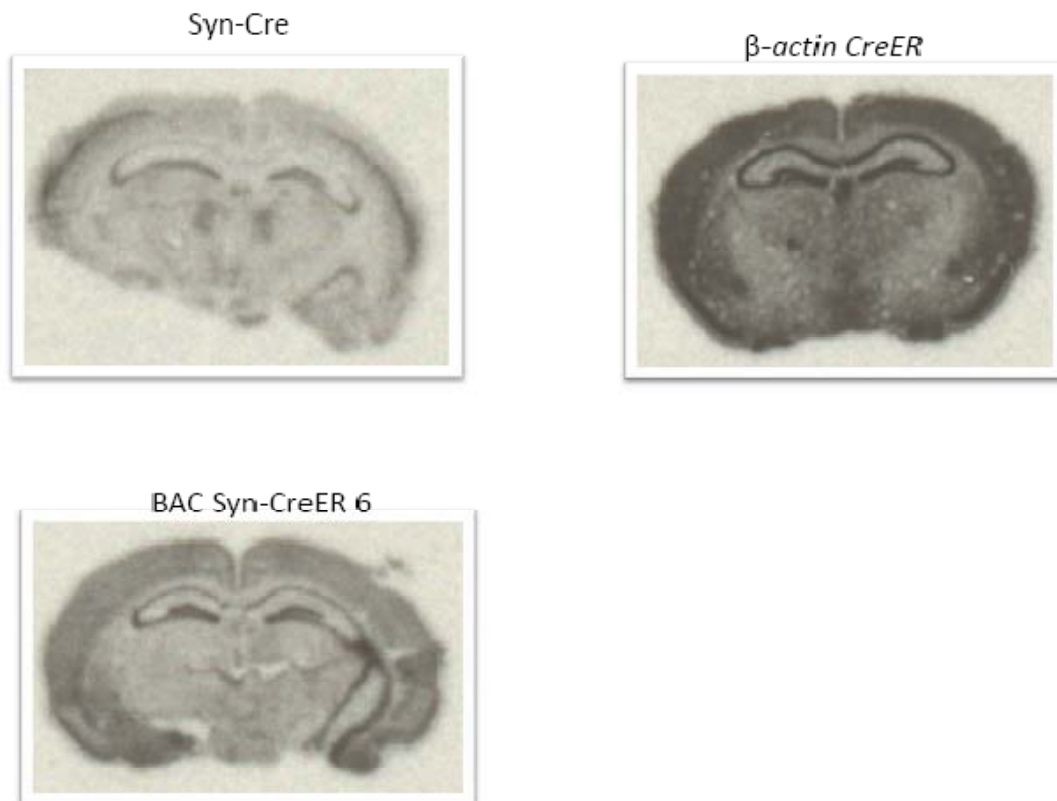


Figure 29: *Cre in situ* hybridization.

A radioactive *in situ* hybridization for *Cre* mRNA was done to compare relative levels of *Cre* expression in the *Syn-Cre*, *Act-CreER*, and BAC *Syn-CreER* mouse. The *Syn-Cre* section shows the expected pattern with *Cre* mRNA in the dentate gyrus and CA3 pyramidal cells of the hippocampus as well as layer 4 of the neocortex. The *Act-CreER* section shows intense signal throughout the cortex and hippocampus. The BAC *Syn-CreER* sections has *Cre* mRNA in the hippocampus, of an intensity intermediate to the *Act-CreER* and *Syn-Cre*, and fainter signal throughout all layers of neocortex.

6.3.3 Recombinase activity in the *BAC Synapsin1-CreER* mice

6.3.3.1 BAC *Syn-CreER Rosa26-lacZ* mice

To determine the anatomical distribution and degree of inducible recombination, the BAC *Syn-CreER* line was crossed to a line of *Rosa26-lacZ* reporter mice. BAC *Syn-CreER Rosa26-lacZ* mice were treated with tamoxifen and the pattern of Cre recombinase activity was identified by X-gal staining. X-gal staining was seen sporadically throughout the brain, with the most intense activity seen in the hippocampus, in particular in the dentate gyrus with some staining in the CA3 pyramidal cells (Figure 32 top), and in the hypothalamus, in particular in the arcuate nucleus (Figure 32 bottom). No recombinase activity was seen in the absence of tamoxifen.

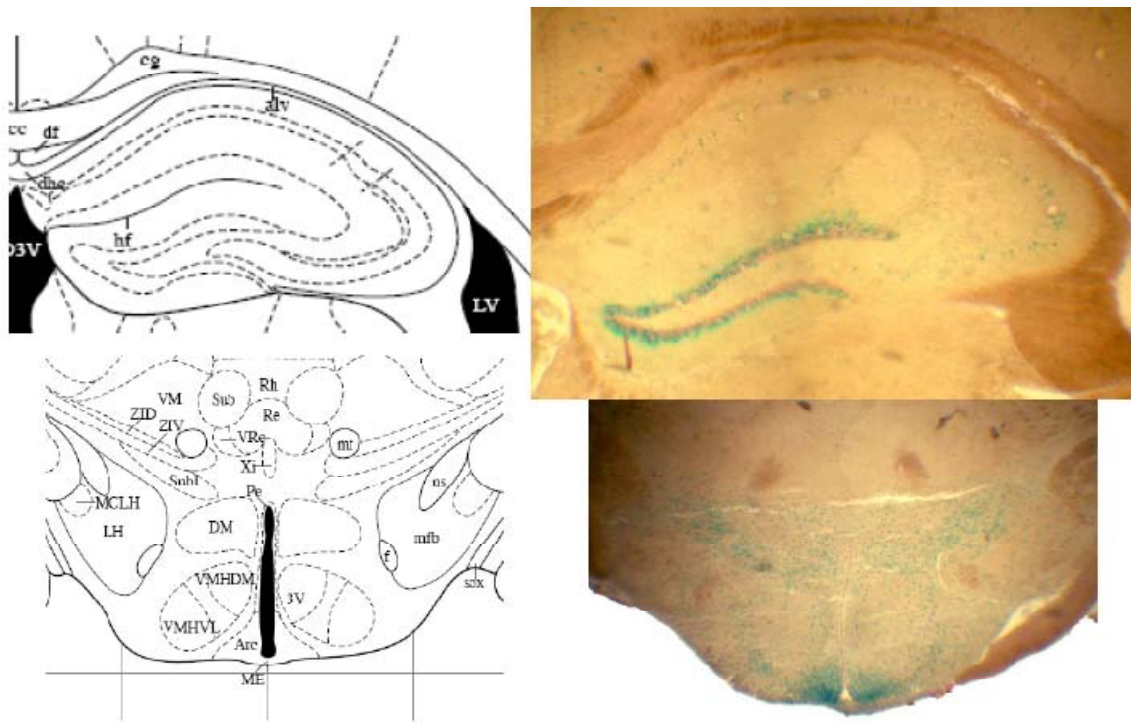


Figure 30: β -galactosidase staining in a tamoxifen-treated BAC *Syn-CreER Rosa26-lacZ* mouse.

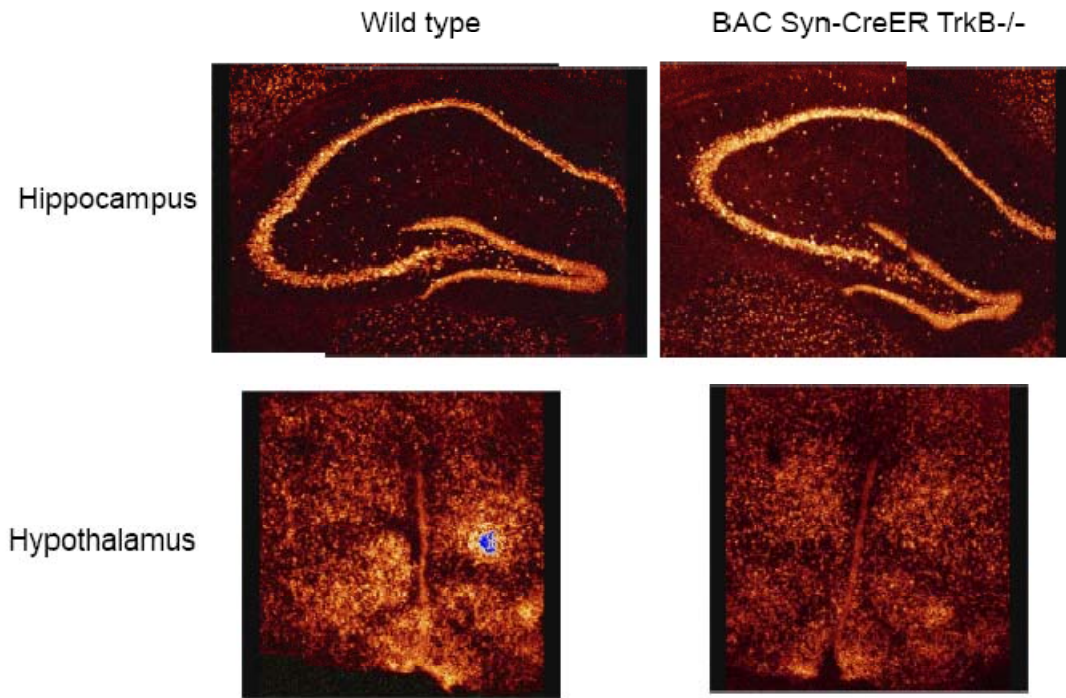
For analysis of inducible Cre activity, *Syn-CreER* mice were crossed to a *Rosa26* reporter line in which β -galactosidase expression reflects Cre activity and treated with tamoxifen. Overall, β -galactosidase activity (blue) is widespread, but very sparse. The highest concentration of activity is seen in the dentate gyrus of the hippocampus, with some cells in the CA3 and CA1 pyramidal layers as well (top right). Staining was seen in the hypothalamus, in particular in the arcuate hypothalamic nucleus and more sparsely throughout the tuber cinereum area (bottom right). No β -galactosidase activity was seen in the absence of tamoxifen.

6.3.3.2 BAC *Syn-CreER TrkB*^{-/-} mice

The final characterization of the BAC *Syn-CreER* mouse is to determine the extent of reduction of *TrkB* that can be induced. Detectable recombination in the tamoxifen-treated BAC *Syn-CreER TrkB*^{flx/flx} mice (hereafter BAC *Syn-CreER TrkB*^{-/-} mice) was limited to the hypothalamus. An *in situ* hybridization for *TrkB* mRNA showed

equivalent signal in the hippocampus in wild type and BAC *Syn-CreER TrkB^{-/-}* mice (Figure 33 A top row). *TrkB* mRNA was moderately reduced in the hypothalamus of the BAC *Syn-CreER TrkB^{-/-}* mouse as compared to the wild type mouse (Figure 33 A bottom row). Examination of TrkB protein levels by immunoblotting did not reveal a decrease in the BAC *Syn-CreER TrkB^{-/-}* mice in the hippocampal homogenate as compared to untreated BAC *Syn-CreER TrkB^{flx/flx}* mouse (Figure 33 B left). In agreement with the *in situ* result, TrkB protein levels in the hypothalamus were moderately reduced in the BAC *Syn-CreER TrkB^{-/-}* mouse as compared to the untreated BAC *Syn-CreER TrkB^{flx/flx}* mouse (Figure 33 B right).

A



B

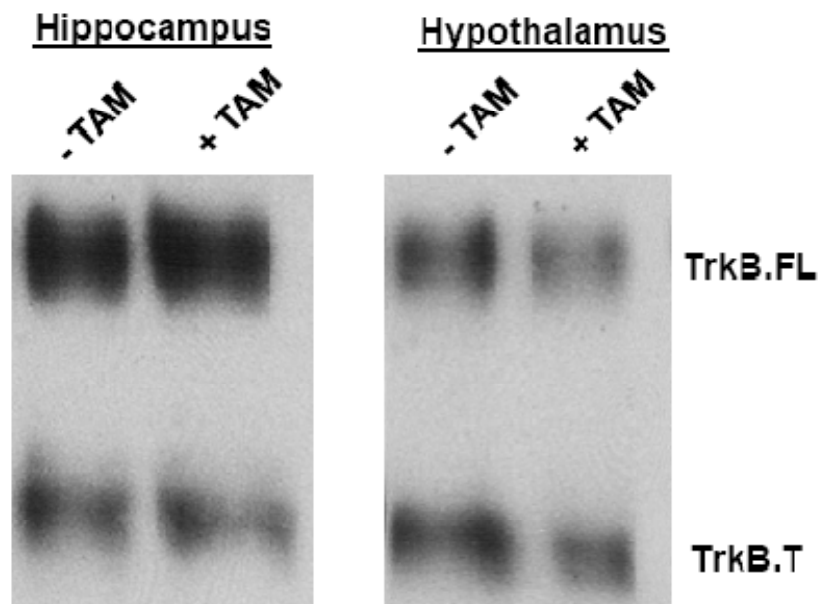


Figure 31: TrkB expression in the BAC Syn-CreER TrkB^{-/-} mice.

A) *In situ* hybridization in the wild type and BAC *Syn-Cre TrkB*^{-/-} mouse shows equivalent expression of TrkB in the hippocampus (top) and a moderate reduction of *TrkB* mRNA in the BAC *Syn-CreER TrkB*^{-/-} mouse in the hypothalamus and in particular in the arcuate hypothalamic nucleus, as compared to the wild type mouse (bottom). B) Immunoblot probed for TrkB showed no difference in hippocampal TrkB protein between treated and untreated BAC *Syn-Cre TrkB*^{flox/flox} mice (left). A moderate reduction in hypothalamic TrkB, both full length (TrkB.FL) and truncated (TrkB.T), is seen in the tamoxifen-treated BAC *Syn-Cre TrkB*^{flox/flox} mouse as compared to an untreated BAC *Syn-Cre TrkB*^{flox/flox} mouse (right).

6.4 Discussion

6.4.1 Summary and Conclusions

In this project BAC transgenic techniques were used to place an inducible Cre recombinase under the control of the *Synapsin1* promoter with the full complement of associated regulatory elements, and the resultant transgenic mice had inducible recombination. In the course of generating this mouse important information was gained: 1) BAC techniques can be used to create an inducible CreER^{TM2} mouse; 2) Tamoxifen-induced reduction of TrkB was seen in the hypothalamus and Cre activity in the absence of tamoxifen has not been seen, although robust and wide spread recombination activity with tamoxifen treatment was also not seen; 3) *Cre* mRNA is expressed throughout the cortex and hippocampus in the BAC *Syn-CreER* mouse, indicating that the cause of the limited recombination activity is after transcription. Finally, the generation of a transgenic mouse expressing an inducible Cre recombinase using BAC transgenic techniques is an important technical step in the search for the structures critical for TrkB actions in epileptogenesis.

6.4.2 Implications for BAC transgenic mice

The limitations of the BAC *Syn-CreER* mouse are useful to facilitate the generation of future, improved BAC *CreER* mice. While the cause of the limited Cre activity is not known, sufficient *Cre* mRNA appears to be present when compared to the *Act-CreER* mouse. However, the comparison of the *Act-CreER* and BAC *Syn-CreER* may miss important differences between the two lines if the signal has reached saturation in the *Act-CreER* mouse. Implications for recombination from comparison between the BAC *Syn-CreER* mouse and the *Syn-Cre* mouse are limited since recombination in the *Syn-Cre* mouse represents the summation of all Cre activity throughout the life of the mouse, while the BAC *Syn-CreER* mouse will only have recombination during tamoxifen treatment.

The level of CreER protein in the BAC *Syn-CreER* mouse is an important factor for the evaluation of the mouse that is unknown at this time. The apparent mismatch between *Cre* mRNA levels and induced Cre activity suggest that insufficient CreER protein may be to blame for the lack of induced recombination. Attempts at determining the level of CreER protein in the BAC *Syn-CreER* mice are currently underway.

Finally, it should be noted that given the high cell density in the principal cell layers and the relatively sparse labeling in the hippocampus of the BAC *Syn-CreER* *Rosa26-lacZ* mouse, even with complete correspondence between recombination in the

Rosa26-lacZ and in the *TrkB^{lox/lox}* mouse the loss of TrkB may not be detectable.

Furthermore, evidence from the *Act-CreER TrkB^{lox/lox}* mouse indicates that recombination in the *TrkB^{lox/lox}* mouse is less than in the *Rosa26-lacZ* mouse in some cases.

6.4.3 Future plans

The BAC *Syn-CreER TrkB^{-/-}* mouse has the potential to be useful for future studies into the role of TrkB in epileptogenesis. In particular the limited recombination could be useful in that TrkB could be eliminated from a small subset of cells in an otherwise normal brain. However, this approach will require that the cells with eliminated TrkB can be identified. Using available techniques, *in situ* hybridization for TrkB could be used to isolate TrkB^{-/-} cells in histological studies. For *in vitro* experiments to study the physiology of TrkB^{-/-} cells, a different indicator of recombination is needed. If a fluorescent reporter mouse (similar to the *Rosa26-lacZ* line, but expressing GFP instead of β -galactosidase) was demonstrated to accurately identify cells with both floxed *TrkB* alleles eliminated, identification of TrkB^{-/-} cells in an *in vitro* preparation would be possible. Using this approach the BAC *Syn-CreER TrkB^{-/-}* mouse could undergo epileptogenesis and the differences in structure and function between TrkB^{-/-} cells and neighboring wild type cells could be compared.

7. Conclusions

7.1 Summary

The work presented in the dissertation has investigated the hypothesis that TrkB is a central factor in the plasticity underlying pathological increases in net excitability in animal models of epilepsy. Loss of TrkB was demonstrated to impair sustained increases in excitability underlying status epilepticus. Loss of TrkB has also been shown to act in the mature mouse to delay the development of hyperexcitability resulting from repeated evoked seizures (epileptogenesis) as well as prevent the persistence of the undiminished hyperexcitable state. One possible hypothesis to unify these observations is that TrkB is required for persistence of increases in net excitability. The role of TrkB in persistence of hyperexcitability can be over hours (sustained seizure activity in status epilepticus), over days (kindling development), or even longer (maintenance of the kindled state) (Figure 34).

Conditional deletion of TrkB in the *Syn-Cre TrkB^{-/-}* mice prevents normal sustained seizure activity induced by pilocarpine in a normal mouse. Furthermore, the *Syn-Cre TrkB^{-/-}* mice may also retain their sensitivity to diazepam following status epilepticus. The finding of a molecule which, when eliminated, regularly prevents the persistence of sustained seizure activity following pilocarpine injection is unique to my knowledge. Additionally, the rapid response to diazepam seen in 2 of 3 *Syn-Cre TrkB^{-/-}*

mice that sustained seizure activity for 3 hours suggests that increased surface GABA_A receptors may be underlying the spontaneous termination of status epilepticus, and is an important finding in itself.

Conditional deletion of TrkB in the *Syn-Cre TrkB^{-/-}* mouse prevented nearly all epileptogenesis in the kindling model, while reduction of TrkB *de novo* in the mature mouse delayed epileptogenesis in the kindling model. While both of these discoveries support TrkB as an important factor in epileptogenesis and as a target for therapeutic interventions, understanding the causes of the different phenotypes in the *Syn-Cre TrkB^{-/-}* mice and the *Act-CreER TrkB^{-/-}* mice is critical in determining if TrkB is required for epileptogenesis or if TrkB is a regulator of epileptogenesis.

Conditional deletion of TrkB in the *Syn-Cre TrkB^{-/-}* mouse and reduction of TrkB *de novo* in the mature *Act-CreER TrkB^{-/-}* mouse impaired persistence of the hyperexcitable state following kindling. However, the impairment in persistence of the kindled state was seen following impaired development of kindling because of induced reductions of TrkB expression. To circumvent this, the inducibility of the *Act-CreER TrkB^{lox/lox}* mouse was used to reduce TrkB expression only after the fully kindled state had been reached and demonstrated that loss of TrkB after completion of kindling impairs persistence of the hyperexcitable state. While the pathophysiology underlying the persistence of hyperexcitability following kindling are poorly understood, this finding identifies a window of opportunity for treatment even after the hyperexcitable

state has been established. An improved understanding of the persistence of hyperexcitability following kindling, which will be facilitated by the identification of a new molecular mechanism, is necessary before informed hypotheses can be made about the cellular mechanisms by which loss of TrkB impairs persistence of the hyperexcitable state.

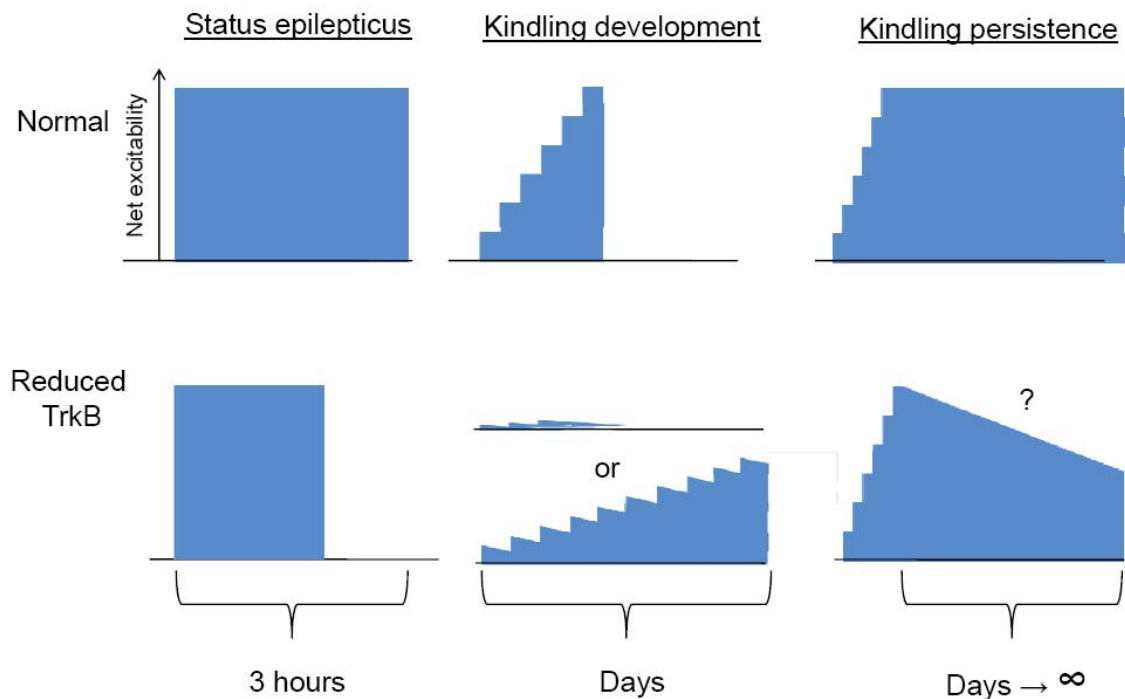


Figure 32: TrkB is required for increases in excitability over multiple time courses.

Loss of TrkB has been shown to impair the persistence of continuous seizure activity in pilocarpine-induced status epilepticus (left column). Furthermore loss of TrkB can cause a complete lack of epileptogenesis (*Syn-Cre TrkB*^{-/-} mice) or delay kindling development following *de novo* loss in the mature mouse (*Act-CreER TrkB*^{-/-} mice) (middle column). Of note, the delay in epileptogenesis in the *Act-CreER TrkB*^{-/-} mice prevents completion of the kindling protocol in only some mice (20% with hippocampal kindling). Finally, loss of TrkB impairs persistence of the kindled

state, even following normal kindling development in the presence of TrkB (right column). The course of the decay in the kindled state is not known.

7.2 Considerations for TrkB-targeted therapeutics

How might a TrkB inhibitor be used to treat epilepsy? The evidence in this dissertation supports the hypothesis that TrkB is involved in plasticities underlying hyperexcitability on different time courses. The chief considerations for a therapeutic approach aimed at inhibiting TrkB signaling are: 1) identifying the pertinent population, including the disease stage at which TrkB inhibition may be effective; 2) determining whether the treatment will be temporary or chronic; 3) consideration of potential side effects.

For status epilepticus, the current findings suggest that inhibition of TrkB before the initiation of status epilepticus reduces the intensity of the electrographic seizure activity and confers the ability to spontaneously terminate status epilepticus in some of the transgenic mice (Chapter 5). The chief concern regarding a therapeutic approach based on inhibition of TrkB for status epilepticus is the time course required for TrkB inhibition in relation to status epilepticus that is required for this effect. A common difficulty with status epilepticus is failure of initial treatments, such as benzodiazepines, which occurs in 40-80% of cases (D. M. Treiman et al., 1998; D. M. Treiman, 1999). Undoubtedly the difficulty in treating status epilepticus contributes to the significant mortality and morbidity associated with status epilepticus (A. R. Towne et al., 1994; R. J. DeLorenzo et al., 1996). If TrkB inhibition after the initiation of status epilepticus was

sufficient to allow spontaneous termination of status epilepticus or the enhanced response to diazepam seen in 2 of 3 *Syn-Cre TrkB^{-/-}* mice, then such a treatment would be immensely useful for improving the care for refractory status epilepticus.

Furthermore, previous work (H. P. Goodkin et al., 2005; S. A. Hewitt and J. S. Bains, 2006) has suggested a mechanism by which loss of TrkB could prevent endocytosis of GABA_A receptors during status epilepticus, which would likely improve the response to benzodiazepines. Even if TrkB inhibition is required before the initiation of status epilepticus, such a treatment may be applicable to individuals prone to develop status epilepticus (R. J. DeLorenzo et al., 1996). However, in this instance the relevant population would be smaller and the concern for side effects would be much greater.

For epileptogenesis the prospect for a therapeutic approach to inhibit TrkB signaling is most promising. The evidence for TrkB being an important component in epileptogenesis is strong (M. Kokaia et al., 1995; D. K. Binder et al., 1999b; X. P. He et al., 2004) and has been advanced by the work presented in this dissertation (Chapters 2 and 3). At least some forms of epilepsy, such as temporal lobe epilepsy, are progressive (A. T. Berg et al., 2006), and progression of disease in these patients likely involves BDNF/TrkB signaling (K. D. Murray et al., 2000). The seizures themselves may play a causative role in the progression of epilepsy since seizures are known to activate TrkB (D. K. Binder et al., 1999a; X. P. He et al., 2002). The advantages of inhibiting TrkB to treat epileptogenesis are, first and foremost, that the risk of developing epilepsy may last

only as long as the inciting stimulus, for instance until a brain tumor is surgically resected or for a limited time after head trauma, and therefore the duration of treatment may be limited. Secondly, the inhibition of epileptogenesis in the kindling model utilizes a supra-threshold stimulation to evoke the electrographic seizures. Therefore TrkB inhibition delays epileptogenesis even after electrographic seizures are being induced, and these electrographic seizures may be detectable in the target population. Finally, loss of TrkB has been shown to delay kindling development with either an amygdaloid or a hippocampal focus, suggesting that reduction of TrkB may be generally useful for treating temporal lobe epilepsy.

For established epilepsy, the evidence presented in this dissertation (Chapter 4) suggests that early inhibition of TrkB following acquisition of the kindled state impairs persistence of the hyperexcitable state. Potentially, this therapeutic approach is the most wide-reaching. As the prevalence of epilepsy is greater than the incidence, the opportunities for treating established epilepsy are great. Additionally, some of the findings in this dissertation suggest that TrkB at the seizure focus may play an important role in persistence of the hyperexcitable state. The most robust reversal of the hyperexcitable state was seen with loss of TrkB at the seizure focus (*Syn-Cre TrkB^{+/-}* mice and hippocampal kindled *Act-CreER TrkB^{-/-}* mice) (Figures 19, 23, 25). If additional studies using viral RNAi techniques conclusively demonstrate that TrkB at the seizure focus is critical, the virally induced reduction in TrkB could be quickly translated into a

clinical treatment. Such a treatment would only reduce TrkB in a select region, and side effects that may plague global reduction of TrkB would be less likely. Furthermore, many other pathological conditions, such as addiction and chronic pain, likely involve abnormal activity in altered networks within a particular structure within the CNS and may be amenable to such an approach. Finally, the discovery of a role for TrkB in the undiminished persistence of the hyperexcitable state is a novel finding and further study is required to fully elucidate the actions of TrkB in this instance. The most important remaining questions are: 1) when in the course of maintain TrkB is required and; 2) whether inhibition of TrkB results in a single reduction in the hyperexcitability or a continuous decay. Answering these questions will identify those individuals for whom inhibition of TrkB may alleviate the hyperexcitable networks underlying their condition and the time course over which TrkB inhibition would be needed.

The hope for an effective and selective inhibitor of TrkB kinase activity that may be clinically useful was greatly advanced by the discovery of imatinib (Gleevec) (M. W. N. Deininger and B. J. Druker, 2003). Imatinib is an agent to treat chronic myelogenous leukemia (CML), gastrointestinal stromal tumors (GISTs), and a variety of other malignancies. The response rate of these malignancies to imatinib is unprecedented (98% with complete histological remission) (M. W. N. Deininger and B. J. Druker, 2003). The pharmacological mechanism of imatinib is to block the activity of the kinase domain of bcr-abl fusion protein resulting from the Philadelphia chromosome translocation,

which is present in 95% of CML cells, and prevent the pathological, continuous activation of the downstream signaling cascades. While imatinib also inhibits the normal abl kinase, as well as c-kit and PDGF-R kinases, its side effects are remarkably mild. The current hypothesis to explain how such a powerful inhibitor does not adversely affect normal cell function is that, while the cancerous cells are dependent on continued activity by the bcr-abl kinase for their pathological proliferation, normal cells have other tyrosine kinases that can compensate for the inhibition of abl kinase to maintain the normal functioning of the cell.

An increased understanding of the pathology underlying epilepsy may identify targets such as bcr-abl that the pathological processes are completely dependent on, but for which normal cellular processes are able to compensate. TrkB has been proposed as such a candidate, although the evidence supporting TrkB is not yet complete. Seizure activity causes extensive activation of TrkB and the plasticities resulting from seizure activity may be dependent on this intense activation. Robust elimination of TrkB from early in development impairs sustained seizure activity during status epilepticus and prevents the development of behavioral seizures in the kindling model, but neither the substantial reduction in TrkB nor the complete prevention of epileptogenesis have been achieved in the mature mouse. However, the reductions in TrkB produced in the *Act-CreER TrkB^{-/-}* mice suggest a direct role for TrkB in the mature mouse in epileptogenesis and persistence of the kindled state. Furthermore, the partial reduction of TrkB in the

Act-CreER TrkB^{-/-} mice produced no obvious health problems for the mice, despite the deficits in the kindling model.

The most probable side effects with TrkB inhibition in the mature animal are impairments in learning and memory. Normal events such as learning and memory may not depend on TrkB activation as extensively and therefore other mechanisms may be able to compensate to prevent a deficit in normal behavior in the mouse. However, reductions in TrkB signaling have been associated with deficits in learning and memory in mice (L. Minichiello et al., 1999), even when these reductions of TrkB signaling occur only in the mature mouse (P. Bekinschtein et al., 2007), implying that a therapy to inhibit TrkB signaling for the treatment of epilepsy may impair learning. However, if learning requires less activation of TrkB, the degree of TrkB inhibition could be titrated to inhibit epileptogenesis while leaving learning minimally affected. Finally, while the time course of treatment that may be required for a TrkB inhibitor is not known, temporary inhibition of TrkB with the limited treatment with the TrkB receptor body delayed kindling. Consequently some of the potential side effects of inhibition of TrkB would be tolerable if temporary.

A chemical-genetic approach to inhibition of the TrkB kinase has essentially developed a imatinib-like inhibitor for TrkB kinase by altering the TrkB protein to be specifically vulnerable to a modified kinase inhibitor (1NMPP1) (X. Chen et al., 2005). This powerful transgenic approach has the potential to rapidly and reversibly inhibit

TrkB signaling at any time point, and thereby address the potential for robust inhibition of TrkB in the mature mouse to affect the kindling model and to address potential side effects. As evidence of the potential of this approach, LTP at the dentate granule cell mossy fiber-CA3 pyramidal cell synapse is only reduced in the *Syn-Cre TrkB^{-/-}* mice (which have a striking phenotype in the kindling model), while mossy fiber-CA3 LTP is eliminated using the chemical-genetic approach (Huang et al., under review). However, the chemical-genetic approach will only prevent kinase-dependent actions of TrkB. Kinase-independent actions of TrkB have been identified (G. T. Baxter et al., 1997; C. R. Rose et al., 2003) and kinase-independent actions are clearly described for the EphB2 receptor tyrosine kinase (I. C. Grunwald et al., 2001; K. Kullander and R. Klein, 2002), and the absence of a phenotype with the chemical-genetic approach would suggest kinase-independent actions of TrkB may be important.

7.3 Implications for TrkB in the epileptic brain

The means by which reduction in TrkB results in spontaneous termination of status epilepticus, delayed kindling development, and impaired persistence of the kindled state likely all involve some combination of a decrease in excitatory synaptic transmission or an increase in inhibitory synaptic transmission. However, given the different time scales involved in each form of plasticity, different molecular and cellular consequences of TrkB signaling are likely relevant in each instance. Furthermore in

some cases TrkB may be acting in a permissive fashion while it may be instructive in other cases.

BDNF/TrkB signaling is likely acting on excitatory synaptic transmission both acutely and chronically with regards to plasticity in epilepsy. For instance, on the proposed roles for BDNF/TrkB signaling is to maintain the population of docked vesicles in the presynaptic terminal (L. D. Pozzo-Miller et al., 1999), which may be permissive for the prolonged seizure activity of status epilepticus. A deficiency in BDNF/TrkB signaling might be expected to result in a more easily depleted pool of presynaptic vesicles, which would cause excitatory synaptic transmission to fatigue more quickly.

In epileptogenesis, the effect of BDNF/TrkB signaling on excitatory synaptic transmission is more likely to be playing an instructive role. The role of BDNF/TrkB in L-LTP, which is dependent on protein synthesis and gene transcription, may be applicable to effecting the long-term changes of epileptogenesis. In support of this comparison, L-LTP does not occlude further potentiation, which matches with the continued increases in excitability in kindling development. BDNF/TrkB signaling has been shown to have a role in L-LTP (C. R. Bramham and E. Messaoudi, 2005) and may even be able to induce L-LTP by itself (H. Kang and E. M. Schuman, 1995; H. Kang and E. M. Schuman, 1996).

The effects of TrkB signaling on inhibitory synaptic transmission could be important before the treatment, during the seizure activity (either prolonged in status epilepticus or sporadic in kindling), and during persistence of the hyperexcitability. Additionally, there is some evidence that the effect of BDNF/TrkB on excitatory synaptic transmission may actually be indirect; that is the changes BDNF/TrkB evoke in inhibitory synaptic transmission cause compensatory changes in excitatory synaptic transmission (S. B. Elmariah et al., 2004).

The mechanism by which reduction in TrkB impairs persistence of the kindled state requires an understanding of both the disease state and the role of TrkB in the normal brain. Unlike status epilepticus and kindling development, during the maintenance period following kindling no exogenous pathological insult is present. Therefore if TrkB is inhibiting plasticity from pathological activity, the pathological activity is spontaneous and undetected. If TrkB is reversing some pathological condition, then the pathological alternations must be selectively dependent on TrkB, since TrkB does not seem to produce a deficit in initial excitability (Figure 16 A & B, 17 A & B). One possibility is that the pathological alterations are selectively vulnerable because they are still being consolidated from temporary effects during kindling to the permanent changes which will maintain the hyperexcitable state. If loss of TrkB interferes with this consolidation, then reduction of TrkB immediately after kindling would impair persistence of the hyperexcitable state. Furthermore, the time course of

any TrkB-dependent processes after kindling could be determined by reducing TrkB at various time points in a fully kindled mouse.

Finally, one of the most important steps for defining the therapeutic potential of TrkB is examination of the effects of reduced TrkB in models of epilepsy with spontaneous seizures. Using the inducible reduction of TrkB in the *Act-Cre TrkB^{-/-}* mice, the effects of loss of TrkB in a clearly epileptic state with spontaneous seizures, such as after status epilepticus, can be examined. Testing the effects of reduction of TrkB spontaneous seizures in direct models of human epilepsy may also be useful. A mutant mouse strain with a null mutation in the *Kv1.1* potassium channel is a homologous model for a human genetic disease of complex partial seizures (S. L. Smart et al., 1998; S. M. Zuberi et al., 1999). The *Kv1.1* null mouse has severe tonic-clonic seizures, episodes of status epilepticus, and often dies at an early age (50% die between the 3rd and 5th week of life). Genetic crosses of the transgenic mice with reduced TrkB mice and the *Kv1.1* null mice would reveal if loss of TrkB prevents the lethal seizures caused by the *Kv1.1* null mutation. Long-term monitoring could also detect changes in seizure frequency. More detailed analysis of these mice, both the *Kv1.1* mutation alone and the *Syn-Cre TrkB^{-/-}* and *Kv1.1* double mutants, may result in an increased understanding in the role of TrkB in the progression of epilepsy and perhaps acutely in status epilepticus.

7.3.1 Implications for TrkB in other diseases

Given the actions of TrkB to convert the fleeting changes evoked by a seizure into the long-term changes in the brain, it is not surprising that TrkB may be involved in other pathologic conditions that entail pathological plasticities. Addiction, like epilepsy, results from repeated insults (exposure to the addicting substance) causing long-term changes to the brain. For instance, an increase in BDNF and activation of TrkB in the nucleus accumbens, a brain structure important for addiction, have been reported following cocaine administration in rats (D. L. Graham et al., 2007). As during epileptogenesis, BDNF/TrkB signaling during repeated administrations of cocaine likely increase the net excitability and foster the strengthening of aberrant connections that eventually lead to permanent changes in the brain. In a study which may have implications for the reversal of the pathological changes in the epileptic brain, Graham and colleagues (D. L. Graham et al., 2007) demonstrated evidence for attenuation of the response to the stimulus (cocaine) following reduction of BDNF *de novo* in mice with multiple previous exposures to cocaine, without impact on motivation to acquire a natural reward (sucrose pellet). This study implies that the pathological changes in the brain's circuitry may continue to be dependent on BDNF/TrkB signaling even after their acquisition and that inhibition of BDNF/TrkB signaling, with perhaps an anatomical restriction to the relevant brain structures, can treat the disease without impairing normal brain functioning.

7.4 *TrkB* in the normal mature brain

Knowledge of roles for BDNF/TrkB signaling in the normal brain suggests that it is ideally suited to convert the fleeting seizure activity induced during kindling stimulations into the long-term changes in excitability. The normal brain function that BDNF/TrkB is most clearly implicated in is learning and memory. This function is consistent with the roles of BDNF/TrkB signaling during development, for example in the formation of ocular dominance columns (R. J. Cabelli et al., 1995; R. A. Galuske et al., 1996; R. J. Cabelli et al., 1997). Similar identified functions in support of this mechanism are the role of TrkB in causing axonal sprouting and synapse formation *in vivo* in the optic axons of *Xenopus* (S. Cohen-Cory and S. E. Fraser, 1995; B. Alsina et al., 2001). Additionally, TrkB has a demonstrated role in the maintenance of synapses in the adult mouse, in the visual cortex (S. Chakravarthy et al., 2006) and at the neuromuscular junction, where continued TrkB signaling is required to maintain post-synaptic receptor clustering, and thereby the maintenance of the synapse itself (M. Gonzalez et al., 1999). BDNF/TrkB signaling is important not only for the physical persistence of the synapses in some cases, but also in the maintenance of the efficacy of the synaptic transmission in the face of changing levels of neuronal activity (homeoplasticity) (L. C. Rutherford et al., 1997; L. C. Rutherford et al., 1998).

Mutations of the *TrkB* gene have been identified in humans and lead to severe obesity, global developmental delay, and significant short-term memory deficits (G. S.

Yeo et al., 2004). The phenotype is thought to be due to loss of TrkB in the hypothalamus, since similar a similar obesity phenotype is seen in some BDNF/TrkB mutant mice (REFs). Humans with a mutation in the *Bdnf* gene have been identified and have poorer episodic memory and reduced hippocampal activation by fMRI analysis (M. F. Egan et al., 2003). These findings are suggestive that BDNF/TrkB are playing a similar role in humans as in mice.

7.4.1 TrkB in learning and memory

Learning and memory are the physiologic processes most similar to the pathologic processes of epilepsy. In the course of learning and memory brief experiences need to be stored in the networks within the brain, the connections are strengthened with repetition of the experience, and the alternations within the brain are permanent. Furthermore the temporal lobe is an important structure for both processes. One view of epileptogenesis could be that the brain is “learning” to have a seizure, which is then permanently “remembered”. With the obvious similarity to the development and persistence of epilepsy, it is not surprising that learning and memory and epilepsy have common cellular and molecular mechanisms. In fact, BDNF/TrkB signaling has been shown to impair learning in organisms from Aplysia (S. K. Sharma et al., 2006) to transgenic mice (L. Minichiello et al., 1999) and perhaps in humans (M. F. Egan et al., 2003).

The study of learning and memory has also identified that the process by which a single episode is permanently stored is an extended process, occurring over days. The hippocampus is an important structure throughout this process, with hippocampal activity (G. Riedel et al., 1999) and in particular activation of NMDA receptors in the CA1 pyramidal cells (E. Shimizu et al., 2000) required up to 5 days after the initial episode. BDNF/TrkB signaling has also been shown to be involved in this extended process, in that reduction of BDNF protein in the hippocampus 12 hours after a training episode impaired memory retention after 7 days (P. Bekinschtein et al., 2007). However, the reduction of BDNF did not affect memory retention at 2 days (P. Bekinschtein et al., 2007), demonstrating that different processes underlie retention after 2 days and after 7 days.

7.6 Concluding remarks

This dissertation has presented a strong case that TrkB is a central factor in hyperexcitability over multiple time scales and in different models of epilepsy. While the fundamental pathophysiology causal for epilepsy is still in the early stages of discovery, linking of molecular pathways to the disease state will bring the extensive knowledge of already acquired into our understanding of the disease. The use of transgenic mice has unambiguously implicated TrkB in the pathology of two animal models of epilepsy, kindling and status epilepticus. Further efforts will define the role of TrkB in the brain both broadly, in additional models of epilepsy and in models of

other diseases involving pathological plasticities, and deeply, as the upstream and downstream constituents of TrkB signaling are identified. A fuller comprehension of the disease process of epilepsy will lead to improved therapeutics and further our understanding of the wonderful and mysterious brain.

8. Appendix

8.1 PCR Primers

Table 1 PCR Primers

Primer Name	Primer Sequence (5' – 3')	Comments
TrkB <i>in situ</i> probe	AATTAACCCTCACTAAAGGGGAATTCCTGTGAAGACGCTGAAG GTAATACGACTCACTATGGGCGAATTCGTGCTGTACACATCTC	Directed against kinase domain (specific for TrkB.FL)
Rosa26 genotyping	GCGAAGAGTTTGCCTCAACC (reverse) GGAGCGGGAGAAATGGATATG (reverse) AAAGTCGCTCTGAGTTGTTAT (forward)	
TrkB flox genotyping	ACACACACAGTATATTTTACC CAAGAAGTCAGAGACCAGAGAGA	
Cre genotyping (both internal)	CGCCGCATAACCAGTGAAAC AATTTACTGACCGTACACCA	
Cre Real-time PCR	Forward CCC GGC AAA ACA GGT AGT TA Reverse GAA CGA AAA CGC TGG TTA GC	

8.2 MATLAB code for spectrogram

```
[Y,F,T,P] = spectrogram(X,1000,400,(.1:1:70.1),200,'yaxis');  
  
surf(T,F,10*log10(abs(P)),'EdgeColor','none');  
  
axis xy; axis tight; colormap(jet); view(0,90);  
  
xlabel('Time (sec)');  
  
ylabel('Frequency (Hz)');
```

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