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Kevin Ding April 3rd, 2017

Characterization of brain-derived neurotrophic factor following acute, pentylenetetrazol-induced seizures in rats

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Abstract

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Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family and plays diverse roles in enhancing excitatory neural transmission, long term potentiation, axonal growth, and dendritic development. In the context of epilepsy, BDNF protein levels in the hippocampus have repeatedly been shown to increase following status epilepticus and kindling seizures in rodents. Moreover, inhibiting downstream BDNF signaling pathways effectively prevents the development of spontaneous seizures. To our knowledge, BDNF expression has not been characterized following acute, pentylenetetrazol(PTZ)-induced non-status epilepticus seizures. As PTZ is one of the most commonly used chemiconvulsants for anti-epileptic drug screening, it is important to understand the relevance of BDNF and neuroplasticity in this model. If we can show that hippocampal BDNF protein levels increase following acute, PTZ seizures, quantification of BDNF post-seizure can also serve as a physiologically relevant biomarker for seizure intensity. We hypothesized that following acute, PTZ-induced seizures in rats, BDNF levels will increase 2 hours post-seizure. To test our hypothesis, we injected rats intraperitoneally with a convulsive PTZ dosage and sacrificed them at 1, 2, and 24 hours post-seizure. We used an enzyme-linked immunoabsorbance assay (ELISA) to quantify hippocampal and cortical BDNF protein levels. PTZ-induced acute seizures did not lead to any significant changes in hippocampal or cortical levels of BDNF protein compared to saline treated controls. However, we found significantly different levels of BDNF amongst the three times points in both saline and PTZ-treated rats. We followed up with immunohistochemical staining for BDNF and c-fos at the 2-hour time point, where we saw the largest, albeit nonsignificant, increase in BDNF. Qualitative analysis of immunohistochemical staining revealed that BDNF remained primarily somatic in both saline and PTZ treated rats, and was localized to the CA1-CA3 pyramidal layers. C-fos staining showed that neuronal activation from PTZ seizures occurs primarily in the granule cell layer of the dentate gyrus. Together, these results suggest that acute, PTZ-induced seizures may not be sufficiently relevant to the pathophysiology of human epileptic seizures to be used as the first-line screening model for the development of anti-epileptic drugs.

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Table of Contents

Abbreviations

BDNF – Brain derived neurotrophic factor

Trk – Tyrosine receptor kinase

TLE – Temporal lobe epilepsy

SE – Status epilepticus

AED – Anti-epileptic drug

LTP – Long-term potentiation

PTZ – Pentylenetetrazol

Shc – Src homology 2 domain containing

MAPK – Mitogen-activated protein kinase

PI3K – Phosphoinositide 3-kinase

Ras – Rat sarcoma

Raf – Rapidly accelerated fibrosarcoma

Mek – MAPK/ERK kinase

Erk – Extracellular signal-regulated kinase

Akt – AKT8 virus oncogene cellular homologue

CREB – cAMP response element binding protein

PKB – Protein Kinase B

PLC-γ1 – phospholipase C-γ1

EEG – Electroencephalogram

GABA – Gamma amino butyric acid

RIPA – radioimmunoprecipitation assay

UTR – Untranslated region

Introduction

Background and Pathophysiology of Epilepsy

Epilepsy is a chronic disease that is characterized by recurrent, spontaneous epileptic seizures, which are defined as episodes of behavioral abnormalities caused by hyperexcitation in the brain (Fisher et al., 2005). Because epilepsy is such a heterogeneous disease and can vary based on cause, localization, and behavior, its classification is complex and constantly evolving. In terms of causal etiology, epilepsy can be categorized into idiopathic, symptomatic, and cryptogenic epilepsy. Idiopathic epilepsy occurs due to underlying genetic factors and is generally less severe (Morimoto, Fahnestock, & Racine, 2004). Because neuronal activity is regulated by the behavior of voltage and ligand-gated ion channels, imbalance in their type, number, distribution, and activity due to genetic abnormalities can result in neuronal hyperexcitation (2006). On the other hand, symptomatic epilepsy often results from a variety of insults or injuries to the brain, including traumatic brain injury, tumors, infection, birth complications, stroke, and abnormal neuroanatomical development (Morimoto et al., 2004). Cryptogenic epilepsy is used to classify epilepsies of unknown cause. Epilepsy can then be characterized by different types of seizures. Seizure types are broadly classified into partial (focal) seizures that are localized to specific regions of one brain hemisphere, or generalized seizures that spread throughout the entirety of the brain. Within partial seizures, there are simple partial seizures that do not result in a loss of consciousness, and complex partial seizures that do. Symptoms that occur as a result of partial seizures depend on the anatomical location of hyperexcitation, and can include motor, autonomic, sensory, or psychic symptoms (Morimoto et al., 2004). As a result, epilepsy can also be classified based on seizure locus—temporal lobe epilepsy (TLE) is the most common and refractory (drug resistant) form of the epilepsy, involving spontaneous, recurrent complex partial seizures originating from structures

in the mesial temporal lobe. Generalized seizures are usually classified based on their behavioral manifestations, and include absence, atonic, tonic, myoclonic, and tonic-clonic seizures (Shin & McNamara, 1994).

Seizures often become spontaneous and recurrent due to brain injury, tumors, stroke, or an episode of status epilepticus (SE), a prolonged seizure that lasts over 30 minutes or the occurrence of multiple seizures within the 30-minute period without recovery (Cherian & Thomas, 2009). The excitotoxicity of status epilepticus often results in neuronal death, and the loss of inhibitory interneurons in postsynaptic target cell populations can result in hyperexcitation due to insufficient inhibitory feedback (Kobayashi & Buckmaster, 2003; Morimoto et al., 2004; Staley & Dudek, 2006). Moreover, neurodegeneration of target cell populations can initiate the sprouting of new excitatory collaterals and the formation of excitatory synapses on the networks of origin, leading to synchronous hyperexcitation of a cell population such as the dentate gyrus (Morimoto et al., 2004; Shin & McNamara, 1994; Staley & Dudek, 2006). At the network level, repeated excitatory output from one population of neurons to another in an epileptic network leads to a strengthening of excitatory glutamatergic synapses in a long term potentiation (LTP)-mediated fashion (Leite et al., 2005). In the hippocampus, LTP can mediate the strengthening of glutamatergic CA3 pyramidal cells that project onto other within-network pyramidal cells, resulting in pathologically sustained, synchronous firing (Bains, Longacher, & Staley, 1999; Miles, Traub, & Wong, 1988). It is speculated that the sprouting of aberrant axonal projections, activity-dependent strengthening of hyperexcitable networks, and progressive cortical, hippocampal, and parahippocampal atrophy due to excitotoxicity act synergistically as the mechanisms underlying the progressive worsening of the epileptic condition (McNamara & Scharfman, 2012).

Current Treatments

Despite the surge in pharmacological drug development for seizures in the past few decades, anti-epileptic drugs (AEDs) remain ineffective for about one-third of patients. Uncontrolled epilepsy puts an individual at greater risk for physical and psychological comorbidities, sudden and unexplained death, and lower quality of life (Golyala & Kwan, 2017). Although new AEDs, including first-line treatments for epileptic seizures like levetiracetam and lamotrigine, have been introduced to the market with improved tolerability and safety, the rate of effectiveness for patients is still comparable to old AEDs like phenobarbital and carbamazepine (French & Gazzola, 2011). This occurrence might stem from the fact that treatments for epilepsy have been focused on symptomatic treatment of seizure hyperexcitation through generalized enhancement of neuronal inhibition in the brain. For example, levetiracetam (Keppra), a first-line treatment for epileptic seizures, binds to synaptic vesicle glycoprotein SV2A to inhibit presynaptic release of Ca^{2+}, thereby suppressing the release of neurotransmitters and acting as an inhibitory neuromodulator (Vogl, Mochida, Wolff, Whalley, & Stephens, 2012). While the inhibitory effects of these drugs can effectively inhibit hyperexcitation, their chronic, generalized inhibition of the central nervous system can have damaging neurological and cognitive effects. Research for the development of new, more effective AEDs is now focusing on targeting the underlying pathophysiology of epilepsy for a more targeted effect. Thus, investigators have been critically reevaluating the current animal models that are used to screen for AED efficacy and favoring models that better represent the pathophysiology of human epileptic seizures (Loscher, 2011). This objective ultimately requires a better understanding of the physiological mechanisms of epileptogenesis in both human patients and animal models of epilepsy.

Rodent Models of Seizures and Epilepsy

Throughout the years, a multitude of animal models have been developed to investigate seizure and epilepsy pathology. These models use different chemical compounds and metrics of intra-cerebral electrical stimulation to either induce acute seizure behavior or precipitate a chronic epileptic state. Kindling, status epilepticus, and electrical lesion seizure models are generally used to induce epileptogenesis, where animal subjects develop either chronic spontaneous seizures or a permanently increased susceptibility to electrical or chemical seizure induction. On the other hand, certain acute models can mimic single, isolated seizures and do not generate an epileptic state (Loscher, 2011). The Racine seizure scale was developed as a qualitative measure of seizure intensity by correlating EEG patterns with different classes of motor characteristics. In the rodent model, hese classes are: I) mouth and facial movements; II) head nodding; III) forelimb clonus; IV) tonic-clonic seizures with rearing; and V) tonic-clonic seizures with rearing and falling (Racine, 1972).

Kindling Model

The kindling model of epilepsy involves the induction of seizures by administrating repeated, low doses of chemical convulsants or low amplitude electrical stimulations of limbic structures via implanted depth electrodes. With each successive stimulation, animals develop more intense seizures until a Racine standard class V seizure is obtained (Morimoto et al., 2004). At the fully kindled state, animals have permanently increased seizure susceptibility, accompanied by many of the structural alterations seen in human temporal lobe epilepsy (Loscher, 2011). Continued repeated evocation of around 100 seizures following the initial development of class V seizures leads to epileptogenesis, or the development of spontaneous recurrent seizures (Morimoto

et al., 2004). Chemical agents used for kindling include pentylenetetrazol (PTZ), bicuculline, and picrotoxin, amongst others (Cain, 1987; Dhir, 2012; Uemura & Kimura, 1990).

Status Epilepticus Model

The status epilepticus (SE) seizure model involves a single, high dose systemic injection of a convulsant drug, usually kainic acid or pilocarpine, or a prolonged, electrical stimulation of limbic structures and pathways. SE can also be induced via an electrical lesion of specific hippocampal structures, such as the hilus of the dentate gyrus (Nawa, Carnahan, & Gall, 1995). These SE episodes often result in neurodegeneration and lesions due to the toxicity of the drug or the duration of the electrical stimulation (Kandratavicius et al., 2014). In addition to the Racine class V motor behaviors, SE is characterized by prolonged seizure duration with seizures that last over thirty minutes and tend to lead to the development of spontaneous, recurrent seizures after a latent period of about 40 days (Glien et al., 2001; Klitgaard, Matagne, Vanneste-Goemaere, & Margineanu, 2002; Morimoto et al., 2004). The length of the latent period between SE and the development of spontaneous seizures (chronic period) is inversely correlated with the seizure duration and drug dosage (Morimoto et al., 2004). Many of the structural changes seen in patients with temporal lobe epilepsy are also observed in the animal hippocampus during the chronic period following SE (Morimoto et al., 2004).

Acute Model

Acute, non-SE seizures can be induced by single doses of a chemical convulsant such as pentylenetetrazol (PTZ) or bicuculline. A single intraperitoneal injection of PTZ at a convulsive dosage can result in an isolated, generalized tonic-clonic seizure episode without the development of spontaneous, recurrent seizures. PTZ is a competitive inhibitor of the $GABA_A$ ($\alpha1\beta2\gamma2$)

receptor, propagating neuronal excitation by inhibiting GABA activated Cl^- currents in a concentration dependent but voltage independent manner (Huang et al., 2001). Compared to kindling and SE seizure models, acute PTZ-induced seizures are more consistent and easier to reproduce, which is the reason for their role as a first-line screening of the inhibitory effectiveness of AEDs (Loscher, 2011).

Brain Derived Neurotropic Factor & the TrkB Receptor

A molecule that has been heavily implicated in epileptogenesis is brain-derived neurotrophic factor (BDNF), a member of the neurotrophic family of proteins that promotes cell survival and growth throughout the nervous system during development and throughout adulthood (Binder & Scharfman, 2004). The human *Bdnf* gene contains 11 exons (10 noncoding 5' exons, 1 coding 3' exon) and 9 promoters that direct tissue and region-specific BDNF expression, while the rodent gene contains 9 exons (Pruunsild, Kazantseva, Aid, Palm, & Timmusk, 2007). When BDNF is transcribed, alternative splicing gives rise to various mRNA transcripts that contain one of the 10 non-coding exons spliced with the coding exon. These mRNA transcripts are translated into the same mature BDNF protein. BDNF gene expression is regulated by a multitude of factors, many of which are dependent on neural activity. For example, whisker stimulation leads to increased BDNF expression in the somatosensory cortex, osmotic stimulation increases expression in the hypothalamus, visual stimulation in the visual cortex, and exercise in the hippocampus (Binder & Scharfman, 2004). Activity dependent expression of BDNF non-coding mRNA in an immediate early gene-like fashion appears to be specific to 5' exons III and IV, but not exons I & II (Binder & Scharfman, 2004; Lauterborn et al., 1996).

BDNF binds with the highest affinity to the tyrosine receptor kinase B (TrkB) receptor, which in turn leads to the auto-phosphorylation of the receptor's tyrosine residues.

Autophosphorylation of the tyrosine residues then activates a series of downstream cell signaling pathways, many of which are depicted in Figure 1. Upon activation, the receptor initiates signaling via phospholipase Cγ1 (PLC-γ1) and Src homology 2 domain containing (Shc) proteins (Patapoutian & Reichardt, 2001). PLC-γ1 activation ultimately leads to activation of several Ca^{2+} mediated pathways, including activation of mitogen-activating protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K) (Segal, 2003). Signaling via Shc results in the downstream activation of Ras, which then activates a MAPK cascade involving Raf, Mek, and Erk. Activation of Erk in turn phosphorylates transcription factors such as CREB (Segal, 2003). Ras-mediated signaling can also activate PI3K signaling, which has been implicated in neuronal survival through activation of Akt and protein kinase B (Crowder & Freeman, 1998; Segal, 2003). However, the specific pathways activated by the Trk receptors depend on the location of the receptors, rate or "tempo" of activation, and the type of ligand that is binding to the receptor (Segal, 2003). BDNF binding to TrkB has specifically been shown to activate PLC-γ1 mediated increases in Ca^{2+}, resulting in Hebbian-like synaptic potentiation and plasticity (Gottschalk, Pozzo-Miller, Figurov, & Lu, 1998; Kleiman et al., 2000; Segal, 2003). Moreover, it has been shown that lack of BDNF in genetically mutated mice leads to an impairment in long term potentiation (LTP), which is restored with a viral transfer of the BDNF gene (Korte et al., 1996). It has been proposed that BDNF's role in LTP can be either *permissive* or *instructive* (Bramham & Messaoudi, 2005). Its permissive role of BDNF is to maintain presynaptic release machinery, while its instructive role is to modulate post-synaptic Ca2+ influx and suppress of synaptic fatigue (Bramham & Messaoudi, 2005).

BDNF and TrkB in Epilepsy

BDNF has been shown to play a role in potentiating neuronal excitation in several *in vitro*

studies. Incubation of adult rat hippocampal slices in BDNF has been shown to potentiate excitatory glutamatergic transmission and decrease inhibitory GABAergic transmission (Binder & Scharfman, 2004) (Kang & Schuman, 1995). This BDNF induced hyperexcitation was specifically seen in the CA3 mossy fibers and was blocked upon presentation of generic Trk receptor antagonist K252a (Scharfman, 1997). *In vivo* studies have shown that acute infusions of BDNF into the hippocampus induces spontaneous limbic seizures in 25% of rats tested (Scharfman, Goodman, Sollas, & Croll, 2002). While acute infusion of BDNF increases hippocampal excitability, chronic infusion of BDNF to the hippocampus has the opposite effect and inhibits electrical seizure kindling (Osehobo et al., 1999). Eliminating BDNF either through intraventricular infusion of TrkB receptor bodies that bind to and scavenge endogenous BDNF protein or through mutation of the BDNF gene also results in the suppression of kindling (Binder, Routbort, Ryan, Yancopoulos, & McNamara, 1999; Kokaia et al., 1995).

In addition to BDNF's effects on potentiating hyperexcitatory transmission and kindling, a substantial amount of research has been conducted regarding the role of BDNF and TrkB signaling during epileptogenesis. Theories on BDNF's role in epileptogenesis began with the discovery of elevated nerve growth factor mRNA following limbic seizures, ischemia, and brain injury, suggesting that neurotrophic factors might play a role in the development of spontaneous seizures (C. M. Gall, 1993). Since then, many independent studies have shown that BDNF and TrkB mRNA levels in the brain increase as early as 30 minutes following kainic acid status epilepticus, electrical kindling, and chemical kindling models (Dugich-Djordjevic et al., 1992; Ernfors, Bengzon, Kokaia, Persson, & Lindvall, 1991; C. Gall, Lauterborn, Bundman, Murray, & Isackson, 1991; Humpel, Wetmore, & Olson, 1993; Lindvall, Kokaia, Bengzon, Elmer, & Kokaia, 1994). A study performed on adult hippocampal sections showed that following pilocarpine-

induced seizure-like activity, there was a time and dose-dependent expression of exon V of BDNF mRNA, with maximal transcription occurring at 6 hours in the dentate gyrus and at 24 hours in the CA1-CA3 pyramidal cell layers (Poulsen, Lauterborn, Zimmer, & Gall, 2004). Immunohistochemistry studies have also shown that there are high levels of TrkB activation (seen through phosphor-TrkB immunoreactivity) in the molecular layer of the dentate gyrus and stratum lucidum of the CA3 (Binder, Routbort, & McNamara, 1999; Lau et al., 2010). Although full length TrkB protein levels seem to be unchanged following kainic acid induced SE, truncated TrkB protein levels increase at 12 hours post-seizure (Rudge et al., 1998). Elevated expression of mature BDNF protein in the hippocampus has been observed using a two-site enzyme immunoassay as soon as 100 minutes post-SE seizure, and persists to 4 days post-seizure (Nawa et al., 1995). On the other hand, BDNF mRNA levels fall back to baseline 46 hours post-seizure (Elmer et al., 1998; Nawa et al., 1995). In a separate study, it was shown that following electrical kindling seizures, BDNF protein levels measured by an enzyme-linked immunoabsorbance assay (ELISA) increased in the dentate gyrus, CA1, CA3, piriform cortex, and striatum 2 hours post-seizure (Elmer et al., 1998). Studies that quantified BDNF using immunohistochemistry showed that BDNF protein immunoreactivity increases in the CA3 stratum lucidum after pilocarpine-induced SE seizures (Lau et al., 2010). Finally, more recent studies have shown that downstream of the TrkB receptor, the PLC-γ1 pathway is specifically responsible for epileptogenesis following kainic acid-induced SE. Uncoupling TrkB receptor from PLC-γ1 using peptide inhibitor pY816 effectively prevents the development of spontaneous seizures (Gu et al., 2015).

The plethora of evidence implicating BDNF-TrkB signaling in both the potentiation of excitatory transmission in the hippocampus and in the development of spontaneous seizures suggest that BDNF is a key player in the pathophysiology of epilepsy. Considering PLC-γ1

mediated signaling pathways are ultimately responsible for LTP-driven synaptic potentiation and neuroplasticity, it seems likely that epileptogenesis involves both a BDNF-dependent strengthening of excitatory synapses and a synaptic rewiring of limbic circuitry (Minichiello, 2009).

Human studies, BDNF as a biomarker

Outside of studies involving animal models of seizures and epilepsy, clinical studies have shown that BNDF is relevant to epilepsy in human patients. BDNF mRNA are elevated in hippocampal tissue surgically resected from patients with intractable temporal lobe epilepsy (LaFrance, Leaver, Stopa, Papandonatos, & Blum, 2010). In infants and children with epilepsy, serum levels of BDNF range from normal to increased (Ismail, Babers, & El Rehany, 2015; LaFrance et al., 2010). However, contrary to what might be expected, separate studies of adult patients with epilepsy have shown decreased levels of serum BDNF (Hong et al., 2014; LaFrance et al., 2010). Moreover, a multiple linear regression analysis revealed that seizure frequency and duration are negatively correlated with serum levels of BDNF (Hong et al., 2014). This suggests that clinically, BDNF might be used as a biomarker for assessing severity levels of epileptic conditions.

Rationale

BDNF expression and TrkB activation increase extensively following limbic seizures, specifically in kindling and SE models of epilepsy. However, to the best of our knowledge, BDNF expression following acute, non-status epilepticus seizures has not been characterized. In order to better understand the pathophysiological significance of acute, PTZ-induced seizures, we proposed to characterize BDNF protein expression in a seizure model that does not lead to the generation of an epileptic state. Characterizing the expression of BDNF would provide us insight into the

physiological relevance of the PTZ seizure model, and whether this model shares the involvement of BDNF that is seen in kindling and SE models. Moreover, quantification of BDNF following acute PTZ seizures would allow us to better understand the involvement of BDNF expression prior to epileptogenesis. Finally, if acute PTZ-induced seizures lead to increased BDNF protein expression, quantification of BDNF following acute PTZ-seizures can also serve as a physiologically relevant biomarker for seizure intensity in AED drug-efficacy studies.

Hypothesis

We hypothesized that following acute, PTZ-induced seizures in adult rats, hippocampal BDNF expression will increase at 2 hours compared to saline treated controls. We also hypothesized that immunohistochemical analysis of protein expression will show increased BDNF immunoreactivity primarily in the dentate gyrus, CA1-3 pyramidal cell layers, and mossy fiber layers of the stratum lucidum 2 hours post-seizure compared to saline treated animals.

Materials and Methods

Animals were cared for and utilized in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health. Experimental protocols are approved by the Emory University Institutional Animal Care and Use Committee.

Animals

Sprague-Dawley male rats (250g-350g) were obtained from Charles River Laboratories, raised in pairs and housed in 12-hour light/dark cycles with ad lib access to food and water.

Pentylenetetrazol acute seizure model

Each rat was injected intraperitoneally (i.p.) with a single 60mg/kg dose of PTZ, freshly dissolved in 500μL of 0.9% saline. Control animals received a 500μL i.p. injection of 0.9% saline. All rats

used in seizure experiments experienced Racine class 5 generalized tonic-clonic seizures. Seizure onset occurred within 2 minutes after PTZ injection, beginning with myoclonic jerks and building up to forelimb clonus with rearing and falling (Racine class V). Observable seizure motor behavior were approximately 5 minutes in duration. Rats were sacrificed 1, 2 and 24 hrs after the end of observable seizure behavior or saline injection. For each time point, 4 animals were treated with PTZ and 4 animals were treated with saline.

ELISA BDNF quantification

An ELISA assay (ab212166, Abcam, Cambridge, MA, USA) was used to measure BDNF levels in the entire hippocampus and in the occipital cortex at each time point according to the manufacturer's recommendation. At the time point post-seizure or post-saline injection, rats were anesthetized with isoflurane and sacrificed using a guillotine. Brains were immediately extracted and placed in PBS on ice. The hippocampus and posterior cortex were dissected from the right hemispheres, placed in a microcentrifuge tube, and snap frozen. The left hemispheres were placed in 4% PFA overnight for follow-up histological analysis. To prepare for the ELISA, hippocampal tissues were weighed and homogenized in lysis buffer (1x protease inhibitor cocktail, 1 mL Tris-EDTA buffer (100x), 0.1M EDTA, 4mL of 0.5M EDTA, 0.5% IGEPAL (50µl), and 5mL molecular grade water) at a 1:10 weight/volume ratio. After the homogenates were centrifuged (13000rpm, 4ºC), the supernatants were collected and diluted in sample diluent at a 1:10 dilution. Eight successive dilutions from 2000μg/mL to 7.8125μg/mL of human BDNF lyophilized recombinant protein were used to construct the standard curve. The standard stock solutions used to construct a standard optical density (OD) vs. protein concentration (pg/mL) reference curve were prepared using 1:10 concentration of lysis buffer to control for its possible effects on OD readings. According to the kit protocol, human BDNF has the same affinity for the ELISA antibody

as rat and mouse BDNF. After loading 50µL of the diluted sample and standard into a 96 well tray, 50µL of the BDNF capture antibody + detector antibody cocktail was added. After a 1 hour incubation period on a shaker at 400rpm at room temperature, the wells were decanted and washed with 350µL wash buffer three times. For visualization, 100mL of TMB substrate was added to each well and incubated at 400 rpm at room temperature for 10 minutes. Finally, stop solution was added and the wells were shaken for 1 minute. Plates were read at 450nm with a microplate reader (Epoch, BioTek, Winooski, VT, USA) for colorimetric absorbance using Gen5 software (Version 3, Biotek, Winooski, VT, USA). Duplicate wells were prepared for each sample lysate. BDNF quantities were extrapolated from the standard curve equation.

BCA protein assay for total protein concentration

A bicinchoninic acid (BCA) protein assay (23225, ThermoFisher Scientific, Waltham, MA, USA) was used as another method of standardizing the concentration of proteins analyzed in the BDNF ELISA. The BCA assay provided total protein concentrations in sample lysates, which were used to normalize BDNF concentrations as a percentage of total sample protein concentration. Sample lysates were diluted 1:20 in 0.9% saline to minimize interference from the lysis buffer. 25μL of each sample was loaded in duplicates into a 96-well tray. A standard curve was created using dilutions of albumin protein ranging from 1600 μg/mL to 0 μg/mL. 200 μL of BCA reagent cocktail (10:1 concentration of BCA reagent A to BCA reagent B) was loaded into each sample well, and shaken for 30 seconds before being placed in an incubator at 37ºC for 30 minutes. Plates were read at 562 nm using Gen5 software using a BioTek microplate reader. Readings from duplicate wells were averaged. Standardized BDNF percentages were obtained by dividing ELISA BDNF quantities by total protein quantities x100.

BDNF and c-fos Immunohistochemistry

The left hemispheres of each animal were post-fixed in 4% PFA for overnight before being placed in a 30% sucrose solution for cryoprotection. After sinking, brains were cryosectioned into 40 μm free-floating coronal sections through the hippocampus and immunohistochemically stained with BDNF antibody (H-117, sc-20981, rabbit polyclonal IgG; Santa Cruz Biotechnology, Dallas, TX, USA) at 2μg/mL. A separate series of brain sections were stained with c-fos primary antibody (sc-52, rabbit polyclonal antibody; Santa Cruz Biotechnology, Dallas, TX, USA) at 1μg/mL to compare localization of BDNF expression and cellular activation. Sections were mounted on glass slides with mounting medium containing DAPI nuclear marker (VectorShield, Vector Laboratories, Burlingame, CA) and imaged with fluorescence microscopy. Mosaic images of the anterior, medial and posterior dorsal hippocampus experimental and control rats were taken at 10x magnification using a Nikon fluorescent microscope equipped with a camera and NIS-Elements Basic Research software (Nikon Instruments Inc., Melville, NY, USA) and merged using Adobe Photoshop software. Images were qualitatively compared for BDNF and c-fos immunoreactivity.

Statistical Analysis

After ELISA BDNF quantities from each well were extrapolated from the standard curve equation using the absorbance readings, values from duplicate wells were averaged. A Two-way ANOVA was used to evaluate the effects of treatment (saline vs PTZ) and time (1 hr, 2 hr, 24 hr), alone and in combination. When the level of significance was 0.05 or less, *post-hoc* multiple comparisons using Bonferroni's test was performed. Values of $P < 0.05$ were considered to be statistically significant. All statistical analyses were performed using Prism software (GraphPad Software Inc, La Jolla, CA, USA).

Results

Hippocampal BDNF Expression

We first analyzed BDNF levels using a weight to volume standardization method(Figure 2A)(Elfving, Plougmann, & Wegener, 2010). Acute, PTZ-induced seizures did not lead to significant increases in hippocampal BDNF expression at 1, 2, or 24 hrs post seizure compared to saline treated animals ($F(1,18)=2.764$, $p=0.1137$). However, there was a significant effect of time on hippocampal BDNF expression for both treatments($F(2,18)=8.636$, $p=0.0023$). Post-hoc analysis with a Bonferroni correction showed significant increases in BDNF expression in the hippocampus from 1 hour to 24 hours post-injection (88.29 to 151.7 μg/mL, respectively) in rats treated with saline, and from 1 hour to 2 hours post-seizure (112.1 to 173.3 μg/mL, respectively) in rats treated with PTZ. There was no significant time-treatment interaction effect ($F(2,18)=1.589$, $p=0.2314$).

Analyzing ELISA quantities by standardizing BDNF quantities to total sample protein concentrations obtained from a BCA protein assay yielded similar results (Figure 2B)(Lexmond et al., 2011). Again, acute PTZ-induced seizures did not lead to a significant increase in hippocampal BDNF levels compared to saline treated animals ($F(1,18)=0.09641$, $p=0.7597$). However, there was a significant effect of time ($F(2,18)=16.12$, $p<0.0001$), with BDNF increasing over time for both PTZ and saline treated rats. Post-hoc multiple comparisons with a Bonferroni correction showed an increase in BDNF from 1 to 24 hours post-injection (1.79% to 4.03%, respectively) and 2 to 24 post-injection (2.485% to 4.028%, respectively) in rats treated with saline, and from 1 to 24 hours (1.841% to 2.866%, respectively) in rats treated with PTZ. There was no significant time-treatment interaction effect ($F(2,18)=0.2421$, $p=0.7875$).

Immunohistochemical (IHC) staining for BDNF expression in saline control and PTZ rats showed no qualitative differences in the hippocampus at the two-hour time point (Figure 4-7). Micrographs were taken of the anterior dorsal (Figure 4), medial dorsal (Figure 5), and posterior dorsal (Figure 6) hippocampus. Expression was primarily concentrated in the pyramidal cell layers (CA1-CA3), with lower immunoreactivity in the dentate gyrus. On the other hand, IHC staining showed that c-fos increases substantially in the dentate gyrus granule cell layer, and to a lesser degree in the pyramidal cell layers in rats treated with PTZ. Rats treated with saline showed little to no nuclear expression of c-fos. Higher magnification images showed that BDNF expression was primarily somatic and non-nuclear in the pyramidal cell layer (Figure 7).

Cortical BDNF Expression

Cortical BDNF protein levels were also evaluated using a weight-to-volume standardization (Figure 3A). Acute PTZ-induced seizures did not lead to significant increases in cortical BDNF expression at 1, 2, or 24 hrs post seizure compared to saline treated animals ($F(1,18)=2.582$, $p=0.1255$). There was a significant effect of time on cortical BDNF expression ($F(2,18)=42.64$, $p<0.0001$). Post-hoc multiple comparisons with a Bonferroni correction showed that saline treated rats had a significant increase in BDNF expression from 1 to 2 hours post injection (18.13 to 53.86 μg/mL, respectively) and 1 to 24 hours post-injection (18.13 to 39.08 μg/mL, respectively), while from 2 to 24 hours, there was a significant decrease in BDNF expression (53.86 to 39.08 μg/mL), respectively. PTZ-treated rats showed significant increases in cortical BDNF from 1 to 24 hours post seizure (23.47 to 54.37 μg/mL, respectively) and 2 to 24 hours post-seizure (23.47 to 47.73 μg/mL, respectively). There was no significant time-treatment interaction effect($F(2,18)=0.6187$, $p=0.5497$)

Analyzing ELISA quantities by standardizing BDNF quantities to total sample protein concentrations obtained from a BCA protein assay again yielded similar results (Figure 3B). There was no significant effect of treatment on BDNF levels ($F(1,17)=0.03316$, $p=0.8577$), and a significant effect of time ($F(2,17)=7.216$, $p=0.0054$). Post-hoc analysis showed a significant decrease in cortical BDNF levels between 1 and 24 hours post seizure (1.32% to 0.44%, respectively) in rats treated with PTZ. There was no significant time-treatment interaction effect ($F(2,17)=0.1567$, $p=0.8562$).

Discussion

Maintenance of BDNF levels post-seizure

In this study, we show that acute-PTZ induced seizures do not lead to an increase in BDNF protein expression in the rat hippocampus or cortex as compared to saline treated animals. These results contradict our original hypothesis, where we predicted an increase in BDNF protein expression as soon as 2-hours post seizure, compared to rats administered saline at the respective time point post-injection. This is particularly surprising considering the extensive number of studies showing that BDNF expression increases with neuronal activation, and that BDNF protein levels increase following chemically induced SE and electrical kindling seizures (Binder, Croll, Gall, & Scharfman, 2001). In studies on the role of BDNF in activity-driven LTP, it has been shown that BDNF mRNA expression in the rat hippocampus increases with electrical stimulus-induced LTP (Patterson, Grover, Schwartzkroin, & Bothwell, 1992). More recent studies have specifically shown that calcium-responsive transcription factor (CaRF) mediates activity-dependent transcription of BDNF exon III (Tao, West, Chen, Corfas, & Greenberg, 2002). Separate studies have found that distinct 3' untranslated regions (UTRs) of BDNF mRNA regulate transport and localization of BDNF mRNA as well as the activity-dependent translation of BDNF

protein (Zheng, Zhou, Moon, & Wang, 2012). Because the BDNF gene contains two poly-adenylation sites, the resulting BDNF mRNA transcripts have either a short (0.35 kb) or long (2.85 kb) 3'UTR. While short 3'UTRs shuttle BDNF mRNA to the cytoplasm of neurons, long 3'UTRS localize mRNA transcripts to the dendrites and axons. At rest, the long 3'UTRs suppress translation of BDNF while the short 3'UTRs maintain basal levels of BDNF translation. Following neuronal activity, these roles are reversed—the long 3'UTR activates BDNF translation while mRNAs containing short 3'UTRs are aggregated into ribosome free messenger ribonucleoprotein complexes (Lau et al., 2010). Therefore, long 3'UTRs mediate expression in the dendrites during neuronal activation, while short 3'UTRs mediate expression in the soma at rest. In our experiments, qualitative histological observation of BDNF immunoreactivity two-hours post-seizure shows BDNF primarily localized in the soma of the dentate gyrus and pyramidal cell layers. This finding suggests the possibility that acute, PTZ-induced seizures do not provide sufficiently prolonged or repeated activation to induce increased long 3'UTR-mediated activation of BDNF translation. Meanwhile, studies have repeatedly shown that kindling and status epilepticus seizures, both of which are models of prolonged and repeated seizure activity, result in increased BDNF protein levels in the mossy fiber layer of the CA3 (Binder et al., 2001; Elmer et al., 1998; Nawa et al., 1995).

Since BDNF plays a crucial role in mediating synaptogenesis, neurite outgrowth, and dendritic formation, it is speculated that the structural changes that occur in epileptogenesis are BDNF dependent (McNamara & Scharfman, 2012; Pruunsild et al., 2007). In acute, PTZ-induced seizures, hyperactivity of hippocampal neurons does not appear to be sufficient to increase BDNF protein expression, suggesting that isolated, class V non-SE seizures do not induce epileptogenic structural changes seen in SE and kindling seizure models.

BDNF changes over time

There are many factors that may have contributed to the unexpected significant effect of time. This finding may have been due to the variability in rat age and weight amongst the groups of animals that were used in the experiments. Animals used for the 24-hour time point weighed approximately 350g on average, while the animals used for the 1 and 2 hour time points weighed approximately 250g on average. This accounts for over a two-week age difference. With evidence suggesting age-dependent variations in brain BDNF levels, age consistency may have been a confounding variable that appeared to produce a significant effect of time (Erickson et al., 2010). Another explanation for the significant time effect is the contextual learning that may have occurred from introducing novel stimuli settings in the laboratory. Rats were often brought to the laboratory from the animal housing facility in the morning to be handled and weighed, and remained in the laboratory with various external stimuli until being administered a PTZ or saline injection in the late afternoon. Because contextual learning also increases hippocampal BDNF expression (Hall, Thomas, & Everitt, 2000), it is possible that novel contexts and sensory stimuli played a role in increasing BDNF expression regardless of treatment.

Future directions

To follow up with this study, it would be interesting to characterize the expression of BDNF protein throughout the process of seizure kindling. Understanding the number of repeated kindling sessions necessary to induce activity-dependent translation of BDNF mRNA transcripts would allow us to better understand the kinetics of epileptogenesis, and possibly use the threshold of kindling sessions that produce increased BDNF protein expression as a metric to evaluate the effectiveness of anti-epileptic treatments in the laboratory. Another possible direction would be to investigate the expression of BDNF mRNAs following acute PTZ seizures, and characterize these

mRNAs based on 3' UTR lengths and 5'UTR transcripts. Observation of the types of mRNAs that are expressed following acute seizure activity would provide insight into the metrics of their downstream translation into mature BDNF protein.

Conclusion

The goal of this study was to characterize the expression of BDNF in acute, PTZ induced seizures in an attempt to understand the role of neuroplasticity in single, non-SE seizures. Although studies suggest that BDNF protein levels increase following kindling and SE limbic seizures, our findings show that acute, non-SE PTZ-induced seizures do not lead to increased BDNF protein expression. Follow-up studies would be targeted at understanding the thresholds of activation necessary to induce increased BDNF protein expression. As the pursuit and discovery of new, more effective treatments for epilepsy continues, scientists are pushed to re-evaluate the translatability of certain seizure models in the AED screening process. More recent studies using kindling and SE spontaneous seizure models of epilepsy have suggested that the structural alterations in the brain may be more important than the mode of seizure induction in the process of screening for antiepileptic drugs (Golyala & Kwan, 2017). As we demonstrate in the present study, the inability of acute PTZ seizures to recruit increased levels of BDNF in the hippocampus may suggest that the acute PTZ model, which has long been used for first-line screening of AEDs, lacks pathophysiological translatability to the human epileptic condition.

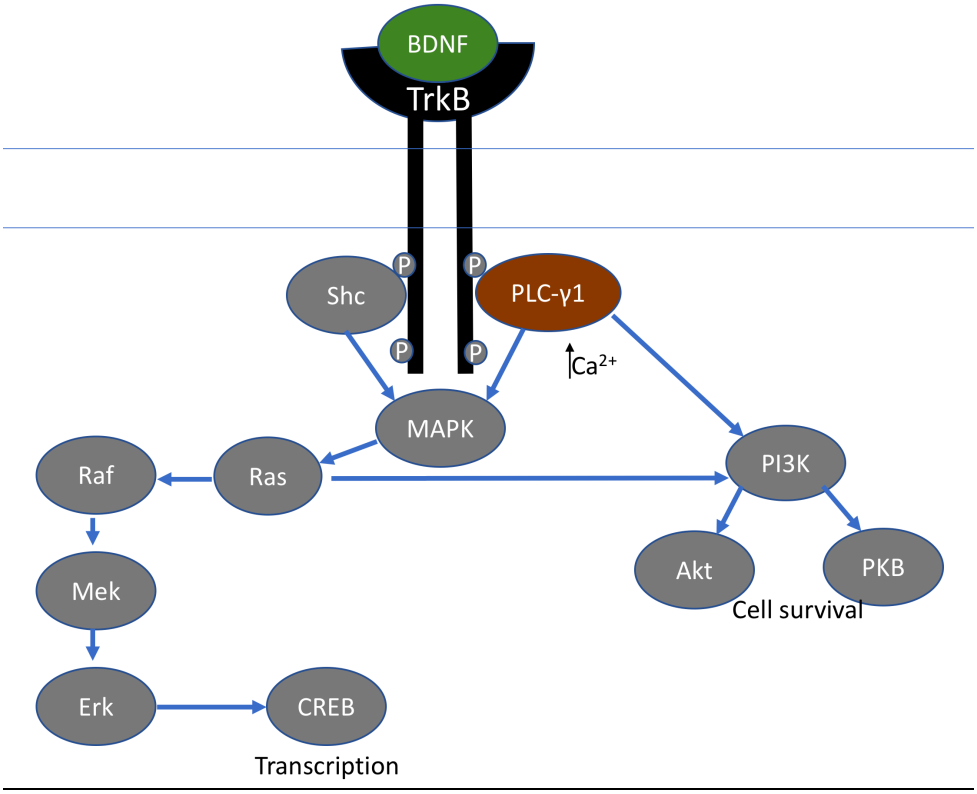

Figure 1

Schematic of BDNF-TrkB signaling pathways. Adapted from Patapoutian & Reichardt, 2001.

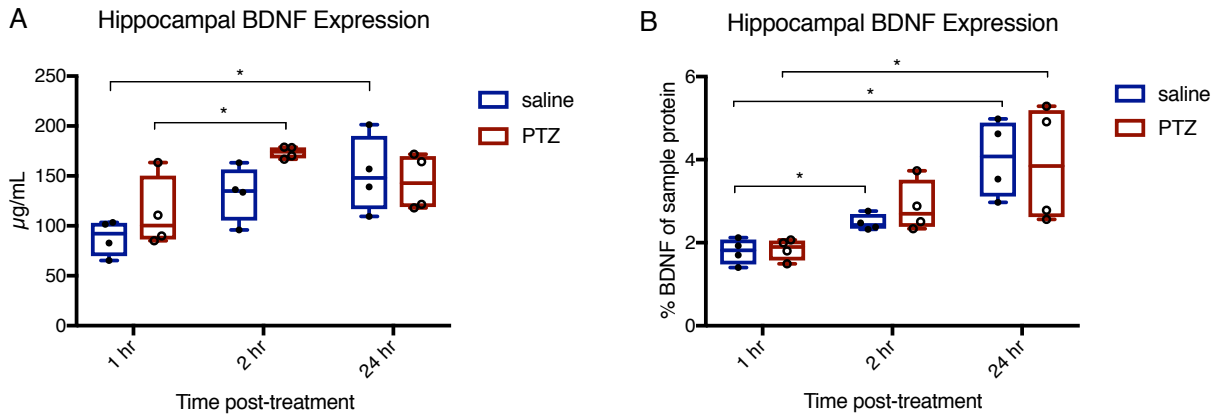

Figure 2

Box-and-whisker plots showing median, 1st and 3rd quartile, minimum, and maximum values of hippocampal BDNF protein quantities in μg/mL standardized by weight to volume ratio (A), and as a percent of total protein (B) are shown for rats treated with saline (blue) or PTZ (red) at 1, 2, and 24 hours post-seizure/post-saline injection. There is no significant effect of treatment on hippocampal or cortical BDNF ($p>0.05$, two-way ANOVA), but a significant effect of time ($p<0.05$, two-way ANOVA) in both standardizations. * indicates $p<0.05$, post-hoc Bonferroni's multiple comparison's test.

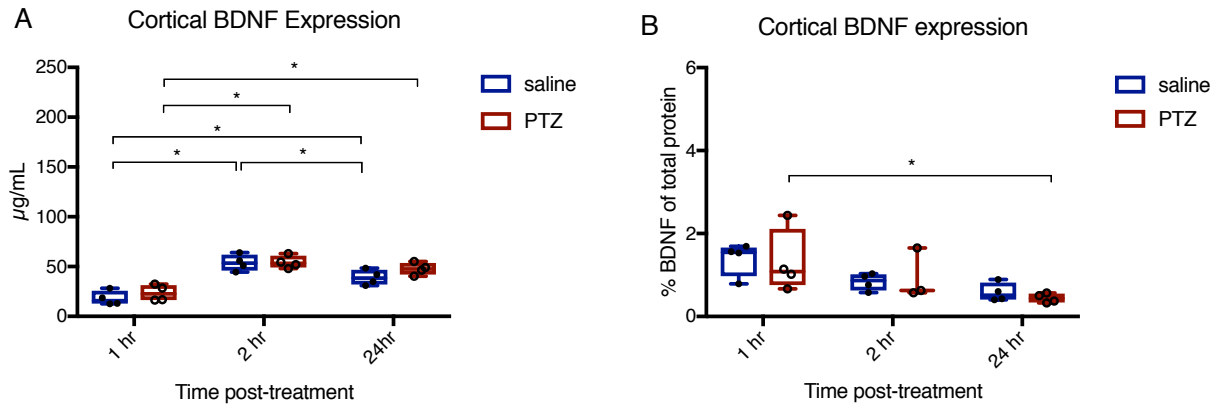

Figure 3

Box-and-whisker plots showing median, 1st and 3rd quartile, minimum, and maximum of cortical BDNF protein quantities in μg/mL standardized by weight to volume ratio (A), and as a percent of total protein (B) are shown for rats treated with saline (blue) or PTZ (red) at 1 hour, 2 and 24 hours post-seizure/post-saline injection. There was no significant effect of treatment on hippocampal or cortical BDNF ($p>0.05$, two-way ANOVA), but a significant effect of time ($p>0.05$, two-way ANOVA) in both standardizations. * indicates $p<0.05$, post-hoc Bonferroni's multiple comparison's test.

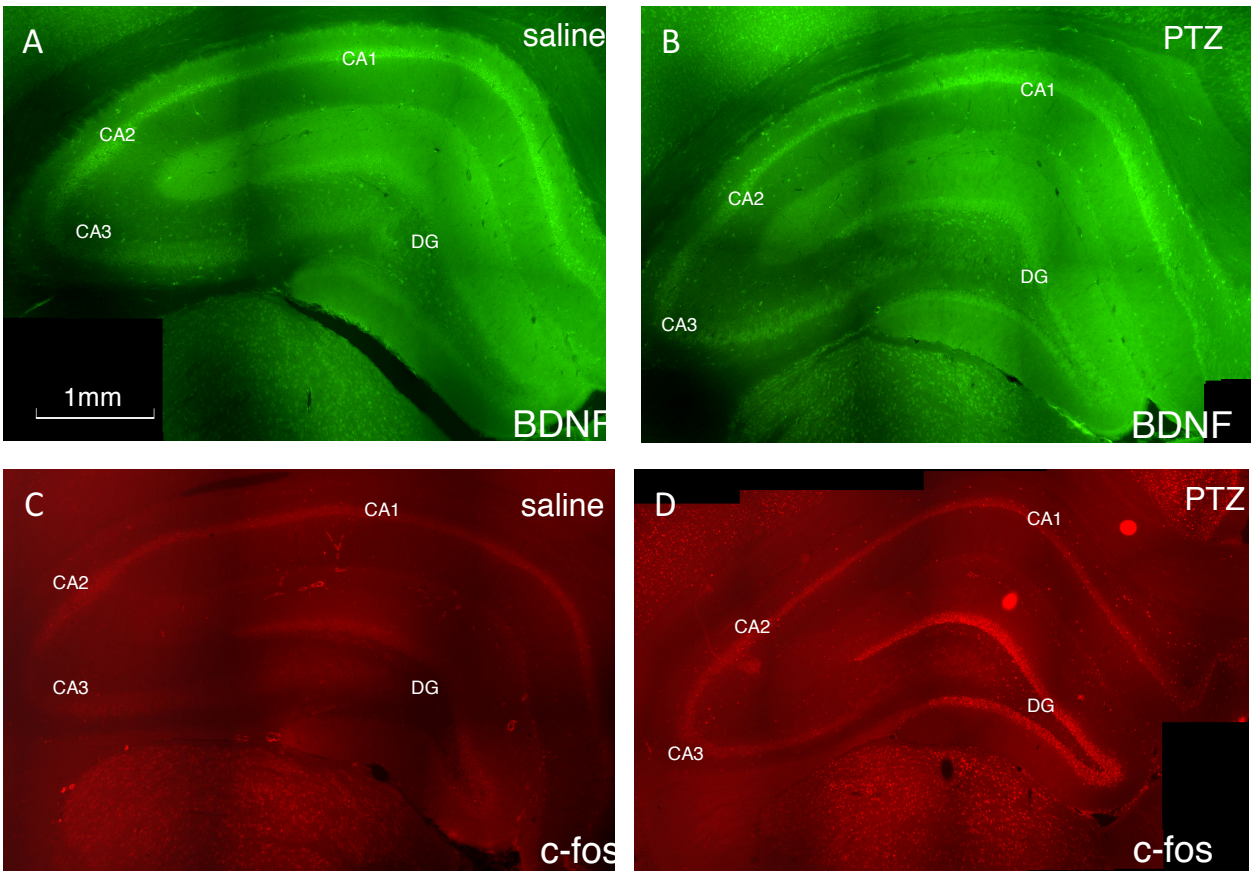

Figure 4

BDNF and c-fos in anterior dorsal hippocampus at Bregma -3.60. Sections from rats sacrificed 2-hours post seizure (B and D) or post-saline injection (A and C) were stained for BDNF (A and B) or c-fos (C and D). Micrographs were taken at 10x magnification and merged using Adobe Photoshop. Qualitative comparison of BDNF expression in the saline treated rat (A) versus the PTZ-treated rat (B) shows no differences in BDNF expression. BDNF immunoreactivity was qualitatively greater in the CA1-3 pyramidal cell layers than the dentate gyrus (DG). C-fos IHC showed that PTZ-treated rat (D) shows higher levels of c-fos immunoreactivity, particularly in the dentate gyrus, compared to the saline-treated rat (C).

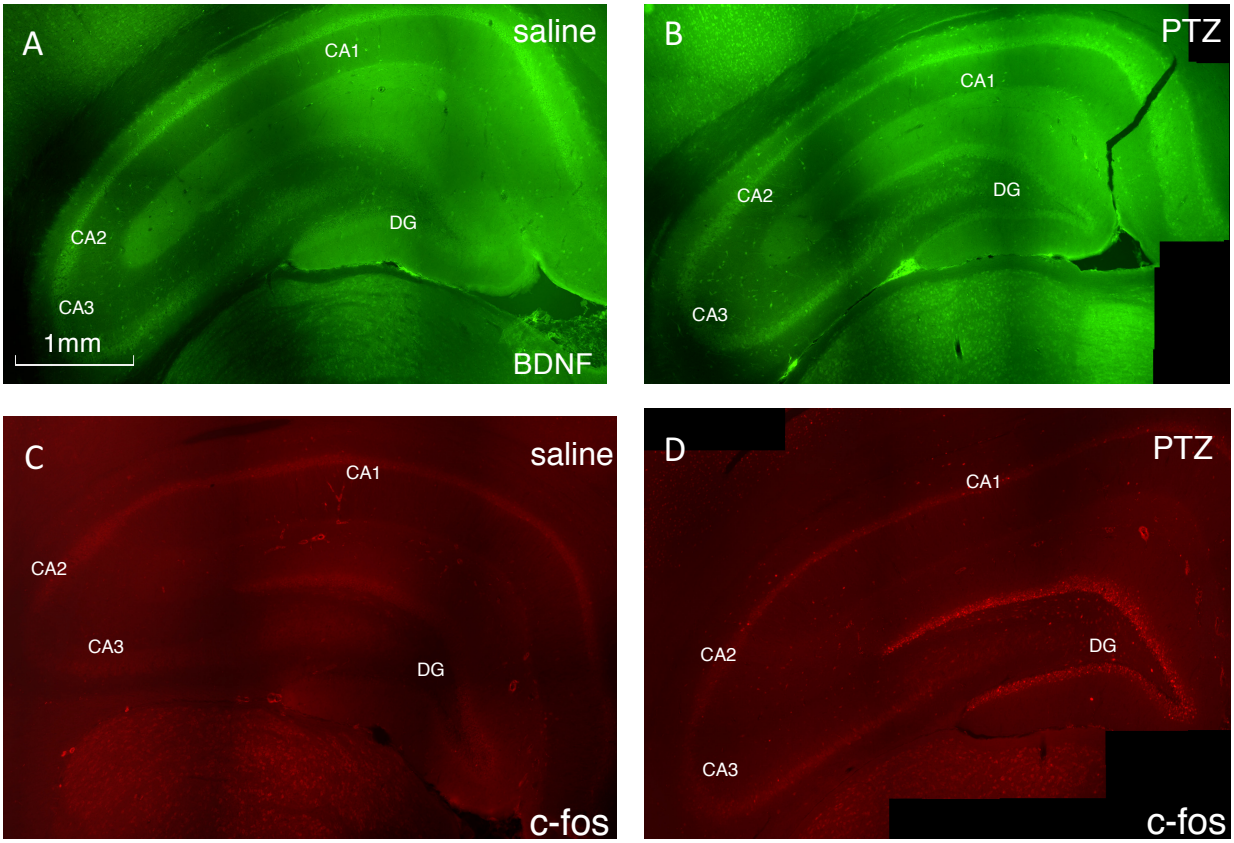

Figure 5

BDNF and c-fos in medial dorsal hippocampus at Bregma -4.30. Sections from rats sacrificed 2-hours post seizure (B and D) or post-saline injection (A and C) were stained for BDNF (A and B) or c-fos (C and D). Micrographs were taken at 10x magnification and merged using Adobe Photoshop. Qualitative comparison of BDNF expression in the saline treated rat (A) versus the PTZ-treated rat (B) shows no differences in BDNF expression. BDNF immunoreactivity was qualitatively greater in the CA1-3 pyramidal cell layers than the dentate gyrus (DG). C-fos IHC showed that PTZ-treated rat (D) shows higher levels of c-fos immunoreactivity, particularly in the dentate gyrus, compared to the saline-treated rat (C).

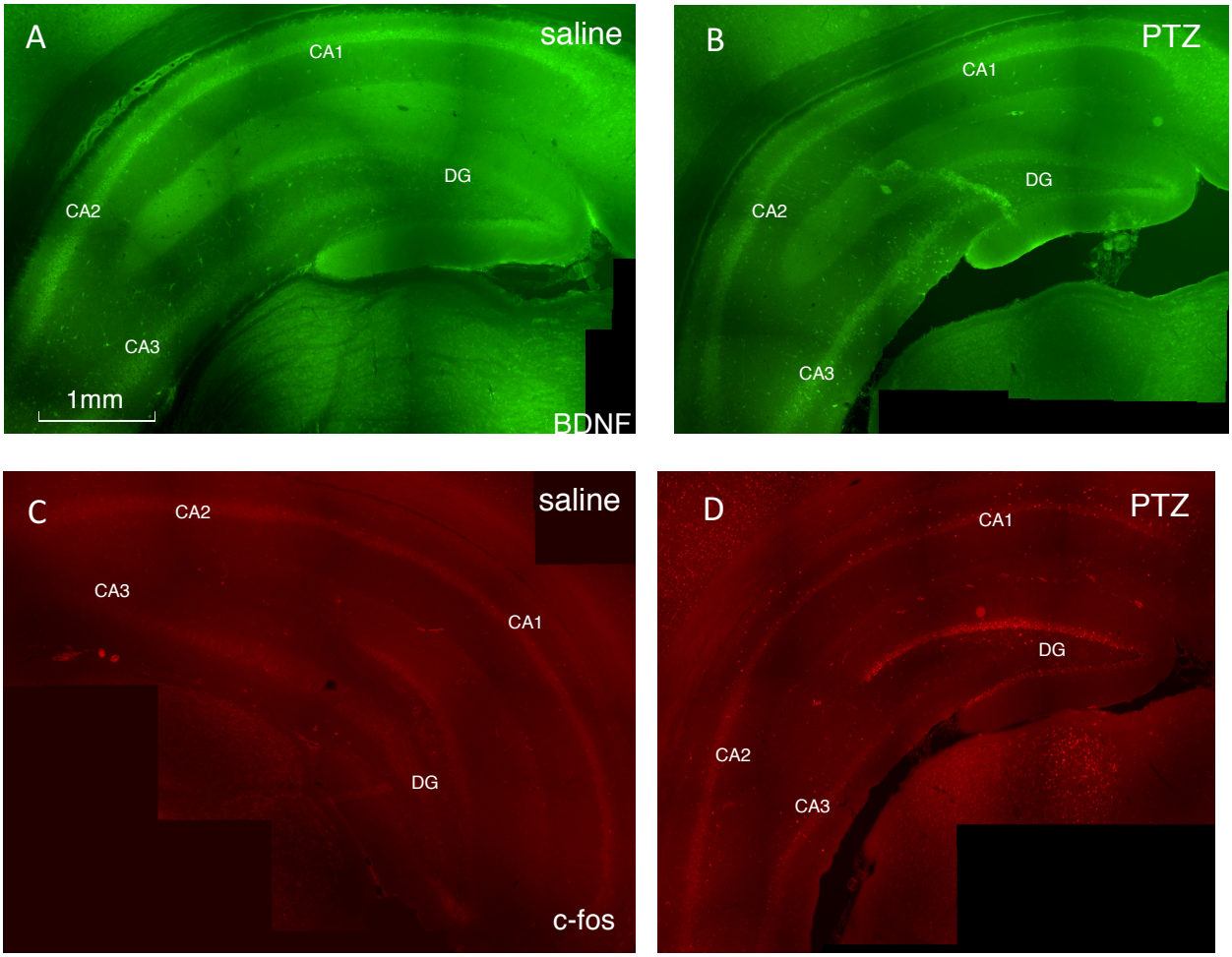

Figure 6

BDNF and c-fos in posterior dorsal hippocampus at Bregma -4.80. Sections from rats sacrificed 2-hours post seizure (B and D) or post-saline injection (A and C) were stained for BDNF (A and B) or c-fos (C and D). Micrographs were taken at 10x magnification and merged using Adobe Photoshop. Qualitative comparison of BDNF expression in the saline treated rat (A) versus the PTZ-treated rat (B) shows no differences in BDNF expression. BDNF immunoreactivity was qualitatively greater in the CA1-3 pyramidal cell layers than the dentate gyrus (DG). C-fos IHC showed that PTZ-treated rat (D) shows higher levels of c-fos immunoreactivity, particularly in the dentate gyrus, compared to the saline-treated rat (C).

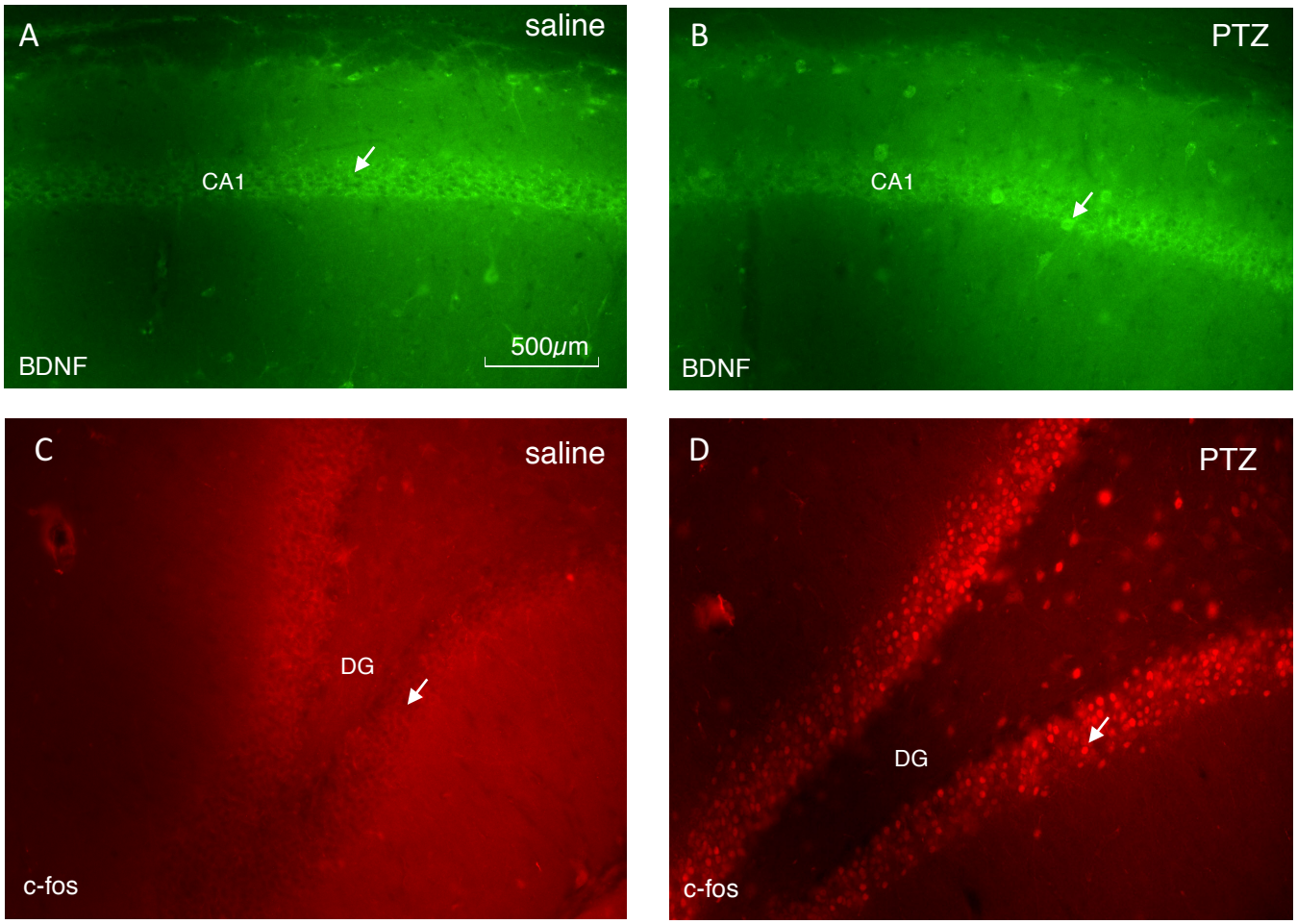

Figure 7

Higher magnification of anterior dorsal hippocampus at Bregma -3.60. Sections were stained with BDNF (A & B), and cfos (C & D). Qualitative evaluation shows somatic localization of BDNF expression in the CA1 for both PTZ and saline treated animals (arrows in A and B), nuclear localization of c-fos expression in the dentate gyrus for PTZ treated rats (arrow in D), and somatic localization for saline treated rats (arrow in C).

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