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BDNF signaling in epilepsy: TRKB-induced JAK/STAT pathway and phosphorylation of LSF in neurons

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BDNF SIGNALING IN EPILEPSY: TRKB-INDUCED JAK/STAT PATHWAY AND PHOSPHORYLATION OF LSF IN NEURONS

by

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DEDICATION

To my family and friends, my dog, and my late father

For their unconditional love and support

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To my late father, John, who was always there for me. I love and miss you.

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BDNF SIGNALING IN EPILEPSY: TRKB-INDUCED JAK/STAT PATHWAY AND PHOSPHORYLATION OF LSF IN NEURONS

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ABSTRACT

Epilepsy is a neurological disorder that causes recurrent and unprovoked seizures due to imbalances in synaptic transmission in distinct regions of the brain. In both human patients and animal models of epilepsy, there is a marked increase in brain-derived neurotrophic factor (BDNF), a critical signaling molecule in the brain that contributes to two divergent pathways important to disease pathology: 1) the regulation of type A receptors for the major inhibitory neurotransmitter GABA ($GABA_ARs$), and 2) aberrant neurogenesis with ectopic expression of new neurons from progenitor cells that disrupt neural network activity in the hippocampus. The first part of my thesis addresses how neurons regulate levels of α1-containing $GABA_ARs$ through BDNF signaling at its receptors, tropomyosin receptor kinase B (TrkB) and p75 neurotrophin receptor (p75NTR). I hypothesized and showed that BDNF, working at TrkB, rapidly

activates the Janus kinase and signal transducers and activators of transcription (JAK/STAT) pathway in neurons and identified a novel intracellular receptor signaling complex composed of p75NTR and JAK2 that is present in neuronal processes, cell body, and nucleus. Based on this finding, we suggest that an intracellular p75NTR/JAK2 signalsome recruits STAT3, a transcriptional activator of the gene coding for the cAMP inducible early repressor (ICER) that blocks synthesis of α1 subunits reducing synaptic $GABA_A$Rs in response to status epilepticus. This model is consistent with our collaborative studies that show a JAK2 inhibitor, WP1066, inhibits development of spontaneous seizures in an epilepsy model and my observation that WP1066 degrades JAK2 protein in primary neurons. The second part of my thesis addresses BDNF regulation of the Late SV40 Factor (LSF), a ubiquitous transcription factor that regulates cell cycle progression and survival. I show that BDNF through the mitogen-activated protein kinase pathway selectively phosphorylates LSF at serine 291 (p291LSF) and that p291LSF is present throughout neurogenesis, increases with status epilepticus in the hippocampus, and is highest in structures associated with neurogenesis (such as olfactory bulb and hippocampus when compared to cortex). Taken together, these results suggest LSF may play an important role in neuronal development and potentially in epilepsy, providing an additional target for future therapeutic intervention.

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ABBREVIATIONS

Aβ	Amyloid beta
ACA	Acetoacetate
AD	Alzheimer's disease
AEDs	Antiepileptic drugs
AICD	Amyloid precursor protein intracellular C-terminal domain
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APP	Amyloid precursor protein
BDNF	Brain derived neurotrophic factor
BHB	β-hydroxybutyrate
BrdU	Bromodeoxyuridine
BZ	Benzodiazepine
Ca^{+}	Calcium ion
cAMP	Adenosine 3', 5'-monophosphate
Cl^{-}	Chloride ion
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CP2	CCAAT binding protein-2
CPEB-1	Cytoplasmic polyadenylation element binding protein 1
CREB	cAMP-response element binding protein
CTF	Carboxyl-terminal fragment
CR	Cysteine-rich

DCX	Doublecortin
DG	Dentate gyrus
DGCs	Dentate granule cells
E2F	Adenovirus E2 promoter factor
ECD	Extracellular domain
EdU	5-ethynyl-2´-deoxyuridine
EGF	Epidermal growth factor
Egr3	Early growth response factor 3
EPSCs	Excitatory postsynaptic currents
EPSPs	Excitatory postsynaptic potentials
Erk	Extracellular-signal-regulated kinase
ES	Electroshock induced seizures
FGF2	Fibroblast growth factor 2
FQI1	Factor quinolinone inhibitor 1
GABA	γ-aminobutyric acid
$GABA_AR$	Type A γ-aminobutyric acid receptor
GAD	Glutamic acid (glutamate) decarboxylase
GAT	γ-aminobutyric acid transporter
GFAP	Glial fibrillary acidic protein
GH	Growth hormone
GRH	Grainyhead
GS	Glutamine synthase

HCC	Hepatocellular carcinoma
HDAC1	Histone deacetylase 1
HIV	Human immunodeficiency virus
HVA	High voltage-activated
I_{KM}	M-current
ICD	Intracellular domain
ICER	Inducible cAMP early repressor
IFN-β	Interferon β
IGF-1	Insulin-like growth factor-1
iGluR	Ionotropic glutamate receptor
IL	Interleukin
JAK	Janus kinase
K^+	Potassium ion
KD	Ketogenic diet
kDa	KiloDaltons
KO	Knockout
LBP-1	Leader binding protein-1c
LIF	Leukemia inhibitory factor
LSF	Late Simian Virus 40 promoter factor
LTR	Long terminal repeat
LVA	Low voltage-activated
MAPK	Mitogen activated protein kinase

MCT	Medium-chain triglyceride
MMP	Matrix metalloproteinases
Na^+	Sodium ion
NAA	N-acetylaspartate
NGF	Nerve growth factor
NICD	Notch intracellular C-terminal fragment
NMDA	N-Methyl-D-aspartic acid
NPC	Neural precursor cell
NSC	Neural stem cell
NTFs	Neurotrophic factors
OPN	Osteopontin
P75NTR	P75 neurotrophin receptor
PIAS	Protein inhibitors of activated STATs
PSA-NCAM	Polysialylated neural adhesion molecule
SE	Status epilepticus
SEF1	Serum amyloid A3 enhancer factor-1
SGZ	Subgranular zone
SNP	Single-nucleotide polymorphism
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
SVZ	Subventricular zone
TACE/ADAM17	TNFα-converting enzyme

TBI	Traumatic Brain Injury
TBP	TATA-binding protein
TCA	Tricarboxylic acid
TLE	Temporal Lobe Epilepsy
TM	Transmembrane
TNF	Tumor necrosis factor
tPA	Tissue plasminogen activator
TrkB	Tropomyosin receptor kinase B
TS	Thymidylate synthase
UBP-1	Upstream region binding protein-1
VGAT	Vesicular γ-aminobutyric acid transporter

I. INTRODUCTION

A. Epilepsy

1. Epilepsy Background and Definition

Epilepsy is a neurological disorder that affects over two million people in the United States, with an estimated 150,000 new cases diagnosed every year (Epilepsy Foundation, 2014). Fisher et al. (2005) previously defined epilepsy as a brain disorder characterized by a lasting predisposition to generate epileptic seizures and by the neurobiologic, cognitive, psychological, and social consequences of this condition, requiring the occurrence of at least one epileptic seizure. The operational definition of epilepsy has recently been updated as a brain disorder consisting of either two or more unprovoked seizures occurring more that 24 hours apart, or one unprovoked seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures occurring over the next ten years, or diagnosis of an epilepsy syndrome (Fisher et al., 2014).

Seizures are periods of abnormal, synchronous excitation of a neuronal population likely caused by a disruption of the balance of excitatory and inhibitory neurotransmission, and can last seconds to minutes, or can be continuous and prolonged. A disturbance in the resting membrane potential of neurons could contribute to seizures, as is evidenced by studies showing that seizures can be caused by both the blockage of sodium-potassium ATPase (Vailland et al., 2002) and mutations that modulate the function of voltage-dependent sodium channel

(Meisler et al., 2001). Seizures may also result from changes in synaptic transmission of the major inhibitory and excitatory neurotransmitters, γ-aminobutyric acid (GABA) and glutamine, respectively. Synchronization of neuronal networks, or the abnormal firing of neurons together, is also involved in seizure activity. This synchronization is thought to be a result of glutamatergic interconnections (Brown and Johnson, 1984), gap junctions (Traub et al., 2004), or GABAergic interneurons (Cobb et al., 1996) that are able to connect groups of cortical neurons.

Epilepsy is marked by recurrent seizures, and has a separate neurobiology from seizures due to underlying structural and functional abnormalities. Epilepsy may result from genetic causes (Meisler et al., 2001; Ottman, 2005), developmental disorders or malformations (Lehericy et al., 1995; Baulac et al., 1998; Ho et al., 1998), and a response to injury or insult, none of which are mutually exclusive (Lewis, 2005). In a majority of epilepsy cases, however, the cause is unknown. The most identifiable cause of epilepsy is from an initial insult, such as traumatic brain injury (TBI) or infection. Initial episodes are commonly followed by a latent period before the development of spontaneous seizures. Many studies have aimed to discover the molecular changes that occur during this latent period, as this may help to uncover the mechanism(s) underlying the development of epilepsy, referred to as epileptogenesis. The development of novel therapeutics is critical, considering

that one third of people with epilepsy does not respond to any current treatments and are therefore refractory to intervention.

2. Types of Seizures

There are many different types of seizures and a person with epilepsy can manifest more than one type of seizure. Seizures are classified into two groups: primary, or idiopathic, generalized seizures affect both sides of the brain, while focal, or partial, seizures affect one area of the brain (Kandel et al., 2000). Generalized seizures are genetic in etiology and include absence and tonic-clonic seizures. Absence, or petit mal, seizures cause rapid blinking or staring into space, while tonic-clonic seizures, or grand mal, can cause patients to cry out, lose consciousness, fall, or have muscle jerks and spasms, or tiredness after the seizure event (Brunton et al., 2005). Focal seizures are typically the result of a cerebral insult, which is unknown in a majority of cases. There are three types of focal seizures: simple seizures, which affect a small part of the brain and can cause twitching or a change in sensations such as taste or smell; complex focal seizures, which can cause confusion or daze, during which people are unable to respond to questions or directions for up to a few minutes; secondary generalized, or partial onset with secondary generalization, begin as a focal seizure in one part of the brain, followed by a generalized seizure by spreading to both sides of the brain (Brunton et al., 2005). If left untreated, prolonged seizures with convulsions may cause immediate and permanent injury or death. (Epilepsy: Epidemiology, Etiology, and Prognosis, n.d.)

3. Refractory and Temporal Lobe Epilepsy

About 60-75% of epilepsy cases involve focal seizures and more than half of these patients experience complex partial seizures (Alving, 1978; Banerjee and Hauser, 2008). Half of these cases cannot be controlled with medication while the other half have seizures despite being on antiepileptic drugs, or experience significant side effects from medications. Cases of epilepsy are considered refractory when two are more antiepileptic drugs have failed to control the seizures (Berg and Shinnar, 1991; Kwan and Brodie, 2000).

Temporal lobe epilepsy (TLE) is the most common form of refractory epilepsy and is characterized by complex partial seizures originating in the limbic system and hippocampus (Wieser et al., 1993). There is strong evidence that the major predisposing factor for epileptogenesis is a rise in brain derived neurotrophic factor (BDNF) that signals through two receptors, tropomyosin receptor kinase B (TrkB) and P75 Neurotrophin Receptor (P75NTR). Increased levels of BDNF mRNA and protein occur in the hippocampus and temporal lobe of human epileptic brains (Takahashi et al., 1999), and neocortical tissue from epileptic patients has increased levels of TrkB, the high affinity BDNF receptor, compared to control patients (Wyneken et al., 2003). Protein and mRNA levels of BDNF is also increased in many animal models of epilepsy, including kindling (Ernfors et al., 1991; Elmer et al., 1998), kainic acid (Rudge et al., 1998), and pilocarpine (Mudo et al., 1996; Poulsen et al., 2004; Roberts et al., 2006). Recent studies from the laboratories of Dr. David Ginty (Harvard University) and

Dr. Jim McNamara (Duke University) using a chemical genetic mouse model for the reversible and specific silencing of TrkB (Chen et al., 2005) now provide evidence for a direct role of TrkB signaling in the development of spontaneous seizures in models of TLE (Liu et al., 2013). How BDNF orchestrates the remodeling of the brain into an epileptic phenotype still remains a mystery and is the subject of many research programs.

4. Animal Models of Epilepsy

Chemoconvulsants

Treatment with the chemoconvulsants pilocarpine and kainic acid can lead to spontaneous seizures in rodents (Leite et al., 2002). These are good models for TLE because rodents follow a similar clinical manifestation: An initial insult to the hippocampus and/or temporal lobe, known as status epilepticus (SE), followed by a latent period before the development of chronic partial and tonic-clonic spontaneous seizures, and histopathological changes (Kandratavicius et al., 2013c; Mathern et al., 2002; Lothman and Bertram, 1993).

When injected systemically or intracerebrally, kainic acid, an L-glutamate analog, causes neuronal depolarization and seizures, mainly in the hippocampus (Nadler et al., 1978). Kainic acid causes recurrent secondarily generalized seizures of variable frequency in rodents, as well as histopathological hippocampal sclerosis (Raedt et al., 2009). Kainic acid injuries are restricted to the hippocampus, while pilocarpine produces lesions in neocortical areas

(Sharma et al., 2007), which is similar to human TLE pathology (Bonilha et al., 2010), making pilocarpine a useful chemoconvulsant model.

Pilocarpine, a muscarinic acetylcholine receptor agonist, is also injected systemically or intracerebrally and causes seizures that build into a limbic SE (Furtado et al., 2002; Turski et al., 1983). The pathology and development of spontaneous seizures is similar to human complex partial seizures (Cavalheiro et al., 1991), and antiepileptic drugs (AEDs) that treat complex partial seizures in humans can stop spontaneous seizures in the pilocarpine model (Leite and Cavalheiro, 1995). There are many similarities between human TLE and the pilocarpine model, including interictal activity in the subiculum (Knopp et al., 2005), and upregulation of neurotrophins in the hippocampus of both mesial TLE patients (Kandratavicius et al., 2013a) and pilocarpine-treated rats (Mudò et al., 1996). Cognitive and memory deficits seen in TLE patients (Kandratavicius et al., 2013b; Pauli et al., 2006) are also present in pilocarpine-injected rats (Leite et al., 1990; Faure et al., 2014).

Electrical stimulation

Electrical stimulation can also induce seizures in animal models, but does not provide cell-specificity in the brain (Pitkänen et al., 2006). Electroshock induced seizures (ES) are induced after a single, whole-brain stimulation. Minimal ES is a model of myoclonic seizures, which can cause muscle jerks and twitching, and forebrain epileptiform activity (Löscher and Schmidt, 1988), while maximal ES is a model for generalized tonic-clonic seizures, with hindbrain

seizures (Browning and Nelson, 1985). Single-evoked epileptic afterdischarges are evoked using electrical stimulation in specific brain regions, and resemble complex partial seizures if applied to the limbic system or myoclonic seizures if applied to the sensorimotor cortex (Sloan et al., 2011; Velísek and Mareš, 2004). Afterdischarges (ADs) are commonly induced in the hippocampus (Kotloski et al., 2002; Norwood et al., 2010), which is followed by postictal refractory period before subsequent seizures (Velísek and Mareš, 2004).

Kindling

Kindling is a seizure-induced plasticity caused by repeated afterdischarge induction by electrical stimulation, ultimately leading to increased seizure susceptibility, spontaneous seizures, and chronic epilepsy (Goddard et al., 1969). Unlike kainic acid and pilocarpine induced TLE models, which have initial severe SE that causes temporal and extratemporal damage and rapid development of spontaneous recurrent seizures (Sutula, 2004), kindling produces small and cumulative neuronal loss and cellular alterations in the brain that eventually result in spontaneous seizures (Sayin et al., 2003).

5. Antiepileptic Drugs

Antiepileptic drugs (AEDs) do not cure or prevent epilepsy, but instead treat the major symptom of epilepsy by supressing seizures. AEDs suppress seizures through three main mechanisms: modulating voltage-gated ion channels, enhancing GABA-mediated inhibitory neurotransmission, and attenuating glutamate-mediated excitatory neurotransmission.

Voltage-gated sodium channel modulators

Certain mutations of sodium channels can lead to familial forms of epilepsy (Catterall et al., 2008) and the most commonly used AEDs act on voltage-gated sodium channels. Voltage-gated sodium channels have three basic conformational states: resting, open, and inactivated. During neuron depolarization, channels cycle through these states in the order resting to open, open to inactivated, inactivated to resting, and are unresponsive to further depolarization until most channels have returned to resting state from the inactivated state (Hille, 1992). AEDs are able to block sodium channels due to their high affinity for the channel in the inactivated state, where they bind and slow the conformational recycling process. These drugs reduce channel conductance in a voltage and frequency-dependent manner, limiting repetitive neuronal firing, with little effect on the generation of single action potentials (Levy et al., 2002). There are multiple inactivation pathways that add complexity to the mechanism of action, with most sodium channel blocking AEDs targeting the fast inactivation pathway. Evidence shows that lacosamide and eslicarbazepine acetate may enhance slow inactivation (Hebeisen et al., 2015). Phenytoin, carbamazepine, and valproic acid are sodium channel blockers that effectively reduce frequency of partial seizures (Mattson et al., 1985; Mattson et al., 1992), and share a common mechanism with newer drugs, lamotrigine, felbamate, topiramate, oxcarbazepine, zonisamide, rufinamide, lacosamide, and eslicarbazepine acetate. Tonic–clonic seizures are commonly treated with

valproic acid, phenytoin, or carbamazepine (Levy et al., 2002). Evidence also suggests that gabapentin has inhibitory effects on neuronal sodium channels (Wamill and McLean, 1994). Phenytoin, a popular AED, is able to control convulsive seizures in both idiopathic generalized epilepsy and focal epilepsy with secondarily generalized tonic-clonic seizures (Stern et al., 2008).

Calcium channel modulators

The voltage-gated calcium channel, which is involved in neuronal excitability, burst firing, and controls pre-synaptic neurotransmitter release, is a common target for AEDs. Calcium channels open from their closed state when the membrane potential depolarizes to threshold, conduct ions in the open state, and inactivate with ongoing depolarization. Inactivated channels reopen by hyperpolarized membrane potentials, to a state of de-inactivation, where membrane depolarization can reopen the channels. Calcium channels are generally slower at opening and closing than voltage-activated sodium channels. High voltage-activated (HVA) channels are slower activating, faster deactivating, and slower inactivating than low voltage-activated (LVA), or T-type channels. HVA channels generate long lasting calcium influxes with sustained depolarization, with T-type channels able to conduct more rapid and shorter calcium influxes under depolarization (Reviewed by Cain and Snutch, 2012).

Ethosuximide and zonisamide, treatments for generalized absence epilepsy, block the LVA T-type calcium channel (Hughes, 2009), an effect also shown by sodium valproate (Kelly et al., 1990). Lamotrigine blocks N and P/Q

type HVA calcium channels (Stefani et al., 1996) and partially bocks N-type calcium currents (Wang et al., 1996), which limits neurotransmitter release. Phenobarbital, felbamate, and topiramate affect HVA calcium channel conductance, though this effect is not well characterized (Kwan et al., 2001; Rogawski and Löscher, 2004). Gabapentin and pregabalin also work via HVA calcium channels by binding to an accessory subunit ($\alpha_2\delta$-1), which modulates the function of various channels (Dooley et al., 2007).

Voltage-gated potassium channel modulators

Potassium channels are the major regulators of the electrical properties of neurons and responsiveness to synaptic inputs (Shieh et al., 2000). Increased membrane conductance to potassium (K^+) ions causes hyperpolarization and reduction in firing frequency, which inhibits neuronal excitability. The M-current (I_{KM}), a low-threshold, slowly activating and deactivating, and noninactivating voltage-dependent K^+ current, limits repetitive firing and causes spike frequency adaptation (Rogawski, 2000). The Kv7 (KCNQ) subfamily of K^+ channel genes contains five members, four of which (Kv7.2 through Kv7.5) are expressed in the nervous system. Mutation studies have shown that Kv7.2 and Kv7.3 are involved in brain excitability control and seizure activity (Bellini et al., 1993-2010; Peters et al., 2005; Watanabe et al., 2000). The antiepileptic drug retigabine activates and opens the Kv7 subfamily of voltage-gated potassium channels, which enhances the M-current, increases the rate of activation by depolarization, and decreases the rate of deactivation. It also enhances the M-current at resting membrane

potential, which hyperpolarizes the cell membrane and reduces neuronal excitability (Barrese et al., 2010).

Glutamate receptor modulators

Glutamate, the principal excitatory neurotransmitter in the mammalian brain, binds to three subtypes of ionotropic glutamate receptor (iGluR) in the postsynaptic membrane, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), kainate, and N-Methyl-D-aspartic acid (NMDA), increases cation conductance and depolarizes the neuron (Dingledine et al., 1999). AMPA receptors (GluA1-GluA4) mediate most of fast excitatory synaptic transmission and are permeable to sodium (Na^+) and potassium (K^+) ions, with GluA2-lacking receptors also permeable to calcium (Ca^+) ions. AMPA receptor inhibitors, such as perampanel, may be an effective adjunctive treatment for drug-resistant partial-onset seizures (Krauss et al., 2012). NMDA receptors (GluN1, GluN2A-GluN2D, GluN3A, and GluN3B) are cationic channels permeable to Na^+, K^+, and Ca^{2+} ions (Perez-Otano and Ehlers, 2005). Felbamate is an effective AED (Sachdeo et al., 1992) that inhibits NMDA-evoked responses of GluN2B-containing NMDA receptors (Sofia, 1994), whose expression changes in epilepsy (Mathern et al., 1998). Other NMDA-antagonist AEDs include remacemide (Subramaniam et al., 1996; Norris & King, 1997), losigamone (Srinivasan et al., 1997), and Huperzine A (Zhang et al., 2002). Kainate receptors (GluK1–GluK5) modulate synaptic transmission and neuronal excitability both as ionotropic

receptors and by linking to metabotropic signaling pathways (Rodriguez-Moreno & Lerma, 1998), but there are no AEDs that act mainly on kainate receptors.

GABA receptor modulators

The major inhibitory neurotransmitter in the central nervous system (CNS), γ-aminobutyric acid (GABA) is released into the synapse when neurons depolarize and binds to post-synaptic ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors, causing hyperpolarization (Rowley et al., 2012). The ionotropic $GABA_A$ receptor ($GABA_AR$) contains binding sites for benzodiazepines (BZs), barbiturates, neurosteroids, general anesthetics, loreclezole, and convulsant toxins, picrotoxin and bicuculline. Many AEDs bind and activate $GABA_AR$, enhancing the GABA response. Barbiturates, including phenobarbital and primidone, and BZs, including diazepam, clobazam, and clonazepam, have this effect, but bind to different receptor sites and differentially affect the opening of the chloride (Cl^-) ion pore (Greenfield Jr, 2013). $GABA_ARs$ are made up of five subunits out of 16 different α, β, γ, δ, π, ε, and θ subtypes (detailed in "$GABA_A$ Receptors" section) and composition can determine the specifity of modulators (Pritchett et al., 1989; Wingrove et al., 1994; Draguhn et al., 1990). BZs are agonists of γ subunit-containing (Macdonald and Olsen, 1994) $GABA_ARs$ that increase the frequency of channel opening (Study and Barker, 1981; Rogers et al., 1994), and are most effective against convulsive seizures (Rogawski and Porter, 1990) and kindling (Swinyard and Castellion, 1966). Barbiturates prolong the opening of $GABA_ARs$ (Eadie and Kwan, 2008) and are effective for focal,

myoclonic and convulsive seizures of idiopathic generalized epilepsies (Fincham and Schottelius, 2002). Loreclezole prevents seizures in a variety of models, acts like a barbiturate by prolonging $GABA_AR$ opening (Ashton et al., 1992). Ganaxolone, an analog of the neurosteroid allopregnanolone and a natural metabolite of progesterone, allosterically enhances $GABA_AR$ current (Carter et al., 1997) by increasing the proportion of $GABA_AR$ openings (Twyman and Macdonald, 1992) and is may be effective for partial onset seizures (Monaghan et al., 1999). Newer AEDs, including topiramate, felbamate, retigabine, losigamone, and stiripentol, modulate GABA responses at the $GABA_AR$ and have other anti-seizure targets, but their mechanisms and binding sites are unknown (Greenfield Jr, 2013). GABA synthesis and metabolism may be affected by gabapentin (Taylor, 1997; Löscher et al., 1991), pregabalin (Nemeroff, 2003) and valproic acid (Löscher, 1982). Other mechanisms of AEDs include blockage of GABA reuptake by tiagabine (Frahm et al., 2001), inhibiting GABA catalysis by vigabatrin (Richens, 1991).

Ketogenic Diet

An alternative treatment to AEDs, especially in children, is the high-fat ketogenic diet (KD), which causes production of ketone bodies, such as, β-hydroxybutyrate (BHB), acetoacetate (ACA), and acetone, by the liver (Peterman, 1924). The currently used KD has been modified to the medium-chain triglyceride (MCT) diet, and is used as an alternative dietary option for medically intractable epilepsy (Huttenlocher et al., 1971; Neal et al., 2009).

Ketone bodies are energy metabolites that may act as anti-seizure agents by regulating neurotransmission or ion channels, enhancing mitochondrial function, reducing oxidative stress, and restricting glycolysis (Rho, 2015), which could affect gene expression of BDNF and TrkB (Garriga-Canut et al., 2006).

B. $GABA_A$ Receptors

1. $GABA_A$ Receptor Structure and Function

GABA synthesis from glutamic acid (glutamate) is catalyzed the enzyme glutamate decarboxylase (GAD) (Schousboe et al., 2013a; Saito et al., 1974) and requires the synthesis of glutamine from glucose in astrocytes, which is catalyzed by pyruvate carboxylase, the tricarboxylic acid (TCA) cycle, and glutamine synthetase (GS) (Norenburg and Martinez-Hernandez, 1979; Yu et al., 1983). GABA is packed into vesicles with vesicular GABA transporters (VGAT) (McIntire et al., 1997), which release GABA into the synapse upon depolarization, allowing GABA to bind to post-synaptic $GABA_A$ and $GABA_B$ receptors and hyperpolarize the post-synaptic neuron (Rowley et al., 2012). GABA neurotransmission is inactivated by the uptake of GABA via GABA transporters (GATs) on the presynapse, where a majority of released GABA ends up, or astrocytes (Rowley et al., 2012; Shousboe et al., 2013b).

GABA acts as an agonist on the type A receptor for GABA ($GABA_AR$). $GABA_ARs$, with glycine receptors, are the major inhibitory ligand-gated anion-selective ion channels and belong to the Cys loop ligand-gated ion channel

superfamily (Betz, 1990; Miller and Smart, 2010). The protein structure of $GABA_A$ receptors consists of a pentamer of different subunits (Macdonald and Olsen, 1994; Nayeem et al., 1994). Sixteen subunit subtypes have been cloned in mammals, including six α, three β and three γ subtypes, δ, π (Hedblom and Kirkness, 1997), ε (Davies et al., 1997) and θ (Bonnert et al., 1999), as well as β2 and γ2 subtype splice variants. The expression of these subunits differs based on region, type of cell (Wisden et al., 1992), and stage of development (Brooks-Kayal et al., 1998a), with the most common composition being two α1, two β2 and a single γ2 subunit, and extrasynaptic receptors expression the δ subunit instead of γ. Each subunit is about 450 amino acids, and half of each subunit consists of a hydrophilic extracellular N-terminal domain, which contains the Cys loop, followed by four transmembrane sequences (M1–M4). M2 lines the ion channel, while M3 and M4 is a large intracellular loop involved in modulation by phosphorylation. The subunits surround a water-filled pore, providing a gate to conduct Cl^- ions when two molecules of GABA are bound to the receptor (Sakmann et al., 1983).

A single cell may contain a diverse population of $GABA_A$Rs, and in some cases more than eight subunit isoforms are expressed in one neuron (Sigel and Steinmann, 2012). Receptor diversity depends on alternative splicing and RNA editing (Daniel and Ohman, 2009). There are about 30 possible pentameric receptors and the same subunits may be arranged differently to form different receptors (Sigel and Steinmann, 2012). The α, β, and γ subunits seem to

combine into defined subunit arrangements (Tretter et al., 1997; Baumann et al., 2002; Baur et al., 2006) while the ε and δ subunits have promiscuous assembly properties (Bollan et al., 2008; Kaur et al., 2009; Baur et al., 2009). Subunit composition (Sigel et al., 1990) and arrangement (Minier and Sigel, 2004) determine the functional properties of the receptors.

2. $GABA_A$ Receptor Changes in Epilepsy

Animal models of epilepsy show alterations in $GABA_AR$ expression and function (Treiman, 2001; Olsen et al., 1999; Jones-Davies and Macdonald, 2003). $GABA_AR$ subunit expression is also altered in the hippocampi of animals with recurrent seizures (Kokaia et al., 1994) and TLE patients (Brooks-Kayal et al., 1998b; Loup et al., 2000). Mutations in $GABA_AR$ subunits have been found in number of human syndromes that cause seizures and epilepsy. Seizures are a symptom of Angelman's syndrome, in which there is a deletion on the chromosome region encoding the β3 subunit (DeLorey et al., 1998). Mutations in the γ2 subunit are linked to childhood absence epilepsy and febrile seizures (Bianchi et al., 2002) and a α1 subunit loss-of-function mutation lease to Juvenile Myoclonic Epilepsy (Cossette et al., 2002).

Using the pilocarpine-induced rat model of SE and primary cultured hippocampal neuron system, studies in our laboratories have found that BDNF signaling controls the subunit composition of the type A receptor for GABA (Roberts et al., 2005). We further established that BDNF transcriptionally regulates differential expression of type A GABA receptor ($GABA_AR$) subunits

genes (*Gabrs*) after SE through two signaling pathways. The product of this regulation is the downstream replacement of synaptic α1-containing GABARs with α4-containing GABARs that also contain the γ2 subunit, which enhances excitability of dentate granule cells (Brooks-Kayal et al., 2009; Roberts et al., 2005; Lund et al., 2009).

Epilepsy also alters the trafficking and localization of $GABA_A$Rs in the hippocampus. In the dentate gyrus (DG), Increased endocytosis of synaptic $GABA_A$Rs occurs from decreased phosphorylation of the β3 subunit, leading to increased association with the clathrin adaptor AP2 (Naylor et al., 2005; Goodkin et al., 2005; Terunuma et al., 2008). Aberrent sprouting of GABAergic neurons has also been reported in the dentate gyrus (Coulter, 2001), as well as increases in extrasynaptic expression of α4 and δ subunits (Fritschy et al., 1999; Brooks-Kayal et al., 1998b; Schwarzer et al., 1997). Overall, these changes are thought to cause dysfunction in inhibitory signaling in the hippocampus of epileptics.

C. Adult Neurogenesis

1. Adult Neurogenesis and Maturation

Neurogenesis is the generation of newborn neurons and subsequent integration of these neurons into neuronal networks (Ming and Song, 2005; Balu and Lucki, 2009; Aimone et al., 2014). While neurogenesis happens most extensively during development, it also continues through adulthood, but is limited to three areas of the brain: the subgranular zone (SGZ), subventricular

zone (SVZ), and the olfactory neuroepithelia (Ming and Song, 2011). Neurons produced in the SGZ migrate short distance to the granule cell layer of the dentate gyrus (DG) of the hippocampus, becoming dentate granule cells (DGCs). About 6% of the granule cell layer is made of up adult-born neurons (Cameron and McKay, 2001), which integrate into hippocampal circuitry and acquire characteristics of mature DGCs (Ge et al., 2006; van Praag et al., 2002). These DGCs are very stable and may replace DGCs that were born during development (Dayer et al., 2003). SVZ neurons migrate through the rostral migratory system and become granule neurons and periglomerular neurons for the olfactory bulb (Lledo and Saghatelyan, 2005). The olfactory neuroepithelia produces excitatory sensory neurons whose axons project to the olfactory bulb. It is not known whether neurogenesis occurs in other adult mammalian brain areas, besides the SVZ and SGZ (Gould, 2007; Rakic, 2002).

Once in the DG, new neurons go through developmental stages both physiologically and morphologically (Esposito et al., 2005; Ge et al., 2006; Overstreet Wadiche et al., 2005; Zhao et al., 2006). Newborn granule cells initially depolarize in response to GABA because of high intracellular concentration of chloride ions, which is important given that knockdown of chloride ion channel NKCC1 causes a defect in neuronal maturation (Ge et al., 2006). Around two to four weeks after neuronal birth, when dendritic spines have grown and respond to glutamate, the response to GABA switches from depolarization to hyperpolarization. During this time, new neurons have lower

thresholds for long-term potentiation (Ge et al., 2007; Schmidt-Hieber et al., 2004) and do not show activity-dependent expression of immediate early genes (Jessberger and Kempermann, 2003), but experience during this time can affect the expression of activity-dependent immediate early genes at six weeks of age or older (Kee et al., 2007; Tashiro et al., 2007). After about four weeks, newborn neurons show a mature phenotype, yet still undergo changes in LTP amplitude and spine density (Ge et al., 2007; Toni et al., 2007; Zhao et al., 2006). When they have matured, granule cells receive similar glutamatergic and GABAergic inputs as existing neurons (Laplagne et al., 2006; Laplagne et al., 2007). Newborn neurons in the olfactory bulb also go through distinct stages of development (Lledo et al., 2006; Petreanu and Alvarez-Buylla, 2002), developing functional GABA receptors before glutamate receptors or dendritic spines (Lledo and Saghatelyan, 2005). Many newborn neurons die within 4 weeks and their survival is regulated by different mechanisms. Survival of one- to three-week-old newborn SGZ neurons is dependent on experience, such as spatial learning and enriched environment (Kee et al., 2007; Tashiro et al., 2007) and NMDA signaling is important at three weeks. Survival of newborn granule neurons in the olfactory bulb also depends on sensory input (Petreanu and Alvarez-Buylla, 2002).

2. Regulation of Adult Neurogenesis

Adult neurogenesis is regulated by physiological and pathological activity, such as, adult neural stem cell (NSC) or progenitor proliferation, progenitor cell

differentiation and fate determination, and the survival, maturation, and integration of newly born neurons. These cells may also be needed for brain functions involving the olfactory bulb and hippocampus, which is important for learning and memory. Adult NSCs within the nervous system are able to self renew and differentiate into any type of neural cells, including neurons, astrocytes, and oligodendrocytes (Gage, 2000). Neural progenitor describes any dividing cells with the capacity to differentiate. Within the SGZ, two types of progenitors have been identified based on morphology and expression of different markers. Type 1 hippocampal progenitors express nestin, glial fibrillary acidic protein (GFAP), and the Sry-related HMG box transcription factor, Sox2, and have radial processes that span the granule cell layer, branching out to the inner molecular layer (Fukuda et al., 2003; Garcia et al. 2004; Suh et al., 2007). While these cells express GFAP, they have a distinct morphology and function from astrocytes. Type 2 hippocampal progenitors do not express GFAP and have short processes. Studies *in vivo* show that type 2 Sox2-positive cells are able self-renew and a single Sox2-positive cell may give rise to a neuron and an astrocyte (Suh et al., 2007). This study also suggests that the type 1 and type 2 cells have a reciprocal relationship.

The SVZ contains three types of precursor cells, type B GFAP-positive progenitors, type C transit amplifying cells, and type A migrating neuroblasts. Type B GFAP-positive neural progenitors are thought to be relatively quiescent, and do not respond to antimitotic treatment (Doetsch et al., 1999). Type C and A

cells are identified by bromodeoxyuridine (BrdU) and 3H-thymidine labeling and by the molecular markers, Dlx2, doublecortin (DCX) and polysialylated neural adhesion molecule (PSA-NCAM). The potential of SVZ progenitor cells is limited, however, because their fate is determined by positional information established in early CNS development (Merkle et al., 2007).

The microenvironment, or neurogenic niche, of the SGZ and SVZ can contribute to their neurogenic capacity. In the SGZ, adult hippocampal progenitors are close to a dense layer of granule cells, which contains both mature and newborn immature neurons, as well as astrocytes, oligodendrocytes, and other types of neurons. Hippocampal astrocytes are thought to play a role in SGZ neurogenesis by promoting neuronal differentiation and integration of adult hippocampal progenitor cells *in vitro* (Song et al., 2002). Wnt signaling is likely involved, since blocking Wnt signaling inhibits the neurogenic activity of astrocytes *in vitro* and SGZ neurogenesis *in vivo* (Lie et al., 2005). Adult neurogenesis declines with age in the SGZ and SVZ (Rossi et al., 2008), with aged animals showing decreases in both the population of progenitors and their ability to self-renew in the SVZ (Molofsky et al., 2006). Whether the number of neural progenitors in the SGZ also declines with aging in unknown, but voluntary exercise can restore neurogenesis in aged animals to a certain extent, suggesting extrinsic affects on these cells (van Praag et al., 2005).

Neurotransmission and growth factor signaling can also affect neurogenesis. In the DG, NMDA receptor-mediated activity is inversely

correlated with proliferation in the hippocampus and neuronal differentiation is increased in hippocampal progenitors in response to glutamate treatment (Jang et al., 2007). Whether adult hippocampal progenitors express functional NMDA receptors is still uncertain. The NR1 NMDA receptor subunit is not expressed in proliferating cells *in vivo* (Deisseroth et al., 2004; Nacher and McEwen, 2006). The inhibitory neurotransmitter GABA depolarizes type 2 progenitors in the adult hippocampus, causing calcium ion influx and increased expression of NeuroD, a neuronal differentiation factor, suggesting that GABAergic input promotes type-2 hippocampal progenitor differentiation (Tozuka et al., 2005). Calcium ion channel antagonists and agonists decrease and increase neuronal differentiation in the adult hippocampus, respectively (Deisseroth et al., 2004).

Adult neurogenesis within the hippocampus is thought to be a key feature of adult brain plasticity (Sahay et al., 2011), which underlies cognitive processing and is fundamental for specific brain functions such as spatial learning, pattern discrimination, contextual memory and mood regulation (Clelland et al., 2009; Denny et al., 2014). Neurogenesis in the SGZ can be influenced genetics and environment (Kempermann and Gage, 2002; Olson et al., 2006). Exercise increases cell proliferation and environmental enrichment promotes survival in the SGZ (Kee et al., 2007; Tashiro et al., 2007). Aging and stress can negatively affect SGZ neurogenesis (Klempin and Kempermann, 2007; Mirescu and Gould, 2006). Other factors, such as structural plasticity, neurotrophins, and hormone

levels may contribute to the environmental influence on learning and memory (Olson et al., 2006).

3. BDNF and Neurogenesis

Neurotrophic factors (NTFs), whose genes are responsive to neuronal activity, support neuronal survival and finely modulate network construction, from neuronal migration to experience-dependent refinement of local connections (Poo, 2001). During nervous system development, neuron survival depends on the secretion of NTFs from target cells (Huang and Reichardt, 2001). NTFs also mediate neuronal plasticity in the adult brain, via axonal and dendritic growth and remodeling, membrane receptor trafficking, neurotransmitter release, synapse formation and function (Lu et al., 2005).

Brain derived neurotrophic factor (BDNF) is an important mediator of neuronal plasticity, due to its abundance in brain regions relevant to plasticity and activity-dependent expression and secretion (Bramham and Messaoudi, 2005). Transcriptional regulation of the rat BDNF gene is complex and regulated by specific signaling pathways (Pruunsild et al., 2011). Nine distinct transcripts can be generated through alternative splicing of 5' un-translated exons to a common 3' exon (IX), which encodes the BDNF protein (Aid et al., 2007). Each transcript has a different distribution and/or translation efficacy and may serve different functions, such as transcripts localized to dendrites may sustain local neurotrophin production, regulating synaptic structure and function (An et al., 2008; Wu et al., 2011). Neurogenesis requires precise spatial and temporal

control, but can be modulated by internal and external stimuli, including BDNF. Several studies have shown that BDNF increases adult neurogenesis in many areas of the brain, including the striatum, septum, thalamus, hypothalamus, and hippocampus (Pancea et al., 2001; Lee et al., 2002; Sairanen et al., 2005; Scharfman et al., 2005; Gass and Riva, 2007; Bergami et al., 2008; Chan et al., 2008; Li et al., 2008; Waterhouse et al., 2012).

BDNF is involved in mood regulation and, along with SGZ neurogenesis, can be regulated by stress and antidepressants (Duman and Monteggia, 2006). BDNF mimics antidepressants and increases neurogenesis when infused into the DG, and mice with impaired BDNF signaling do not have increased neurogenesis with antidepressant treatment. Exercise, dietary restriction, and enriched environment, are able to both induce BDNF expression and increase SGZ neurogenesis. Both BDNF heterozygous mice (BDNF+/−) and mice expressing a dominant negative form of the TrkB receptor show decreased survival of newborn cells. An enriched environment is unable to enhance the survival of newborn cells in BDNF+/− mice (Rossi et al., 2006), suggesting BDNF signaling may enhance the survival of newborn neurons.

4. Aberrent Neurogenesis in Epilepsy

Seizure activity has been shown to increase neurogenesis in both the SGZ and SVZ. Altered neurogenesis in the hippocampal causes a number of cellular abnormalities, such as, increased proliferation of neural progenitors, production of ectopic granule cells (EGCs), mossy fiber sprouting (MFS), dentate

granule cell (DGC) layer dispersion, neuronal hypertrophy and persistence of hilar basal dendrites on adult-generated granule neurons (Parent et al., 1997; Scharfman, 2002; Pun et al., 2012). Rats with pilocarpine-induced TLE have increased neurogenesis in the rostral SVZ, and more rapid migration of neuroblasts from the SVZ to the olfactory bulb, with some premature exiting of the migratory stream (Parent et al., 2002). SE also increases cell proliferation in the SGZ in both pilocarpine and kainic acid rat models (Parent et al., 1997; Jessberger et al., 2005) and persists for weeks after a latent period of several days (Parent et al., 1997; Gray and Sundstrom, 1998). Proliferation rates return to baseline around three to four weeks after SE induction (Parent et al., 1997). Chronic TLE results in decreased neurogenesis five months after SE induction (Hattiangady et al., 2004), which is also seen in children with frequent seizures, where decreased proliferating cells and immature neurons have been seen in the DG (Mathern et al., 2002).

MFS occurs when mossy fiber axons abnormally branch out of the dentate hilus and project onto the dendrites of granule cells of the inner molecular layer. Most of these connections are excitatory, resulting in hyperexcitable recurrent loops in the DG (Koyama and Ikegaya, 2004). Recent studies, however, point to a complexity in the role of adult hippocampal stem cells in epilepsy and suggest that more information is needed to better understand how they are differentiated in the diseased brain. This is especially important because genetic ablation of adult rat hippocampus neurogenesis prior to SE reduces the frequency of

spontaneous recurrent seizures but does not completely prevent seizures (Cho et al., 2015). There is also a decrease rather than increase in neurogenesis reported after kainite-induced hyperactivity due to the increased production of astrocytes rather than neurons from neural stem cell progenitors (Sierra et al., 2015).

5. Markers of Neurogenesis

Kempermann and colleagues proposed a model of developmental milestones in adult neurogenesis identified by morphology, proliferative ability, and expression of markers (Kempermann et al., 2004). The division of a stem cell (type-1), which has radial glia and astrocytic properties (Seri et al., 2001), gives rise to three consecutive stages of putative transiently amplifying progenitor cells (type-2a, type-2b, type-3) differing in proliferative potential and increasing differention. These stages lead to exit from the cell cycle into a transient postmitotic stage, marked by a calretinin (CR)-expressing immature granule cell, which establishes network connections and is selected for long-term survival. The final stage results in a terminally differentiated, calbindin (CB)-expressing mature granule cell. Figure 1 summarizes these stages and indicates when these cellular markers can be identified (Knoth et al., 2010).

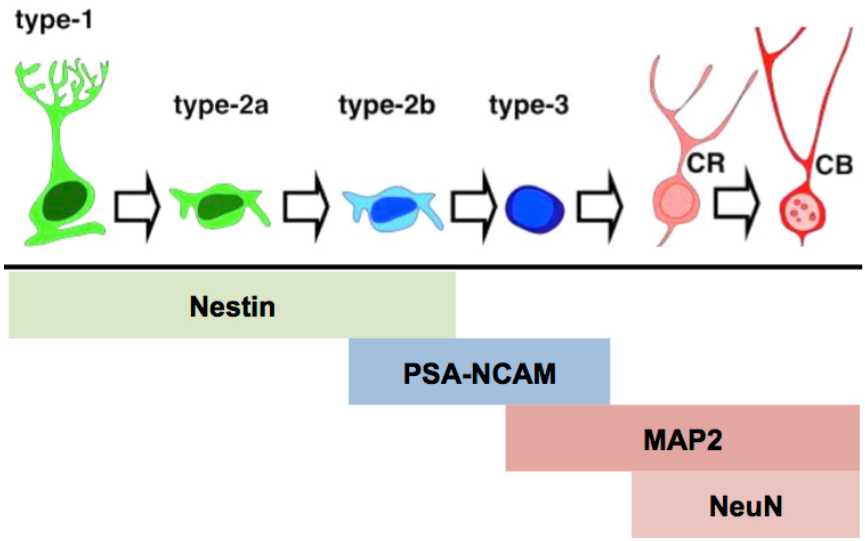


Figure 1: Markers of neurogenesis in the human hippocampus related to adult hippocampus neurogenesis in rodents

(adapted from Knoth et al., 2010).

D. BDNF Signaling

1. ProBDNF and Mature BDNF

Brain derived neurotrophic factor (BDNF) is a member of the neurotrophin family, which also contains nerve growth factor (NGF), neurotrophin-3 (NT-3), and NT-4. Nerve growth factor, BDNF, and NT-3 are all expressed in neurons of the mammalian brain, with the highest levels observed in the adult hippocampus (Ernfors et al., 1991). BDNF is made and secreted from neurons, microglia and astrocytes (Lindholm et al., 1992; Rudge et al., 1992; Coull et al.; 2005; Lu et al., 2009a; Trang et al., 2011) and plays an essential role in brain development that is reflected in its regulation over cellular differentiation and survival; as well as key aspects of neuronal plasticity (Bibel and Barde, 2000; Korte et al., 1995;

Korte et al., 1996; Zigova et al., 1998). BDNF is widely expressed throughout the brain in a similar pattern to TrkB (Binder, 2007) with the highest mRNA and protein expression in the granule and pyramidal cells of the hippocampus (Bibel and Barde, 2000; Bengzon et al., 1993; Ernfors et al., 1990) consistent with a critical role in long-term potentiation (LTP), neuronal survival, and axonal growth (Minichiello et al., 1999; Xu et al., 2000).

Neurotrophins arise from precursors called proneurotrophins about 30-35 kDa in size, which are cleaved into mature proteins about 12-14 kDa in size (Seidah et al., 1996). Intracellular proneurotrophins are cleaved either intracellularly or extracellularly after being secreted, or can be secreted without subsequent cleavage. Mature BDNF (matBDNF; 14 kDa) is cleaved from proBDNF (35 kDa) both intracellularly, by furin and protein convertases (protein convertase 1 or PC1), and extracellularly by zinc-activated matrix metalloproteinases (MMP) MMP3 and MMP7 (Lee et al., 2001), or plasmin, which is converted from inactive plasminogen by tissue plasminogen activator (tPA) (Plow et al., 1995). Plasminogen and tPA are widely expressed in the CNS, with plasminogen being exclusively expressed in neurons, the extracellular space, and at the synaptic cleft (Tsirka et al., 1997). High frequency neuronal activity (Gualandris et al., 1996), causing secretion of tPA into the extracellular space from axon terminals (Krystosek and Seeds, 1981), suggesting that tPA is the main trigger for the tPA/plasmin/proneurotrophin cascade.

Studies results vary as to whether proBDNF is secreted intact after being synthesized in the endoplasmic reticulum (Matsumoto et al., 2008; Barker, 2009b; Yang et al., 2009; Waterhouse and Xu; 2009; Park and Poo, 2013) or whether it is first cleaved to matBDNF and secreted from dense core vesicles in an activity, Ca^{2+} dependent manner (Lessmann et al., 2003 and Trang et al., 2009). In the rat CNS, proBDNF is expressed in many regions, including the spinal cord, substantia nigra, amygdala, hypothalamus, cerebellum, hippocampus and cortex of rats (Zhou et al., 2004). Proneurotrophins either signal by binding and activating p75 neurotrophin receptor (P75NTR) or be degraded extracellularly. Pro-domains show substantial sequence homology and conservation among vertebrates and play an important role in intracellular processing (Suter et al., 1991; Rattenholl et al., 2001) and intracellular trafficking and secretion (Lu, 2003).

An important single-nucleotide polymorphism (SNP) was discovered in the pro-domain of the human BDNF gene at nucleotide 196 (guanine to adenine), producing an amino acid substitution (valine to methionine) at codon 66 (Val66Met, metBDNF). Humans with this allele have impaired hippocampus dependent episodic memory, low levels of hippocampal N-acetylaspartate (NAA), a measure of neuronal integrity and synaptic abundance, and abnormal hippocampal function (Egan et al., 2003; Hariri et al., 2003). Looking at cultured hippocampal neurons, studies found that overexpression of metBDNF caused impaired intracellular trafficking, especially in distal dendrites, failed synaptic

targeting of vesicles containing BDNF, and impaired activity-dependent BDNF secretion (Chen et al., 2004; Egan et al., 2003), most likely due to difficulty generating BDNF-containing secretory granules. Co-expression of wildtype BDNF (wtBDNF) with metBDNF alters intracellular trafficking and regulates secretion of wtBDNF, and metBDNF forms a heterodimer with wtBDNF (Chen et al., 2004).

2. Neurotrophin Receptors

The way in which BDNF mediates differential effects in neurons is by signaling through two distinct receptors: the tropomyosin related kinase (Trk) receptor B (TrkB) and the p75 neurotrophin receptor (P75NTR). The TrkB receptor is a member of the receptor tyrosine kinase family, which inclues TrkA, TrkB, and TrkC. Each Trk family member has a unique set of amino acids in their extracellular domain, allowing for more selective associations between Trk receptors and their ligands (Friedman and Greene, 1999; Pérez et al., 1995). Most importantly, different neurotrophins show selective activation of Trk receptors that is reflected in their binding affinity: with NGF preferentially binding to TrkA, BDNF and NT-4 to TrkB, and NT-3 to TrkC (Barbacid, 1994).

P75NTR is a cell surface glycoprotein and a member of the tumor necrosis factor (TNF)-related receptor family (Naismith and Sprang, 1998). First identified as a neurotrophin receptor based on molecular recognition by NGF, it is now clear that p75NTR binds all neurotrophins (Rodriguez-Tébar et al., 1990; Rodríguez-Tébar et al., 1992). ProBDNF has a higher affinity for p75NTR than

the mature form (matBDNF) that selectively activates TrkB (Teng at al., 2005). Both TrkB and p75NTR are implicated in neurodegeneration (Ginsberg et al., 2006; Schindowski et al., 2008) and in the etiology of multiple neurological disorders (Chao et al., 2006), including temporal lobe epilepsy (TLE) (Kandratavicius et al, 2014b) and traumatic brain injury (TBI) (Rostami et al., 2014). P75NTR has an extracellular domain with tandem arrays of cysteine-rich (CR) domains, which are required for neurotrophin binding. P75NTR typically binds to soluble dimeric ligands as a coreceptor (Reviewed by Barker, 2004). P75NTR homodimers are able to regulate the activation of receptor signaling (Vilar et al., 2009). P75NTR forms a coreceptor with many other proteins to mediating different cellular responses. Binding with Sortilin induces cell death (Nykjaer et al., 2004; Teng et al., 2005), while interactions with Trk receptors can facilitate neurotrophin binding (Teng and Hempstead, 2004; Esposito et al., 2001). The transmembrane (TM) domain contains two residues that are involved in dimerization, cysteine 257 and glycine 266 (Vilar et al., 2009). The intracellular domain (ICD) can initiate signaling without ligand binding and like other TNF receptors, contains a unique death domain and has been linked to apoptosis (Majdan et al., 1997). The metalloproteinase TNFα-converting enzyme (TACE or ADAM17) can cleave P75NTR to produce an extracellular domain and a 24-kDa carboxyl-terminal fragment (CTF) (Zampieri et al., 2005; Kenchappa et al., 2010). The CTF is further processed in the TM domain by γ-secretase to release the 19-kDa ICD into the cytosol (Kenchappa et al., 2006; Kenchappa et al., 2010. The

mature form of p75NTR contains O-glycoside-linked oligosaccharides clustered in a serine/threonine-rich stalk domain adjacent to the TM domain (Johnson et al., 1986; Radeke et al., 1987), as well as an N-glycosylation site (Large et al., 1989). The structure of P75NTR is illustrated in Figure 2.

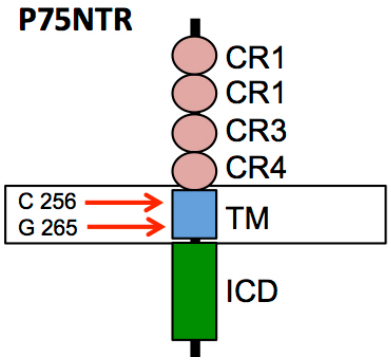


Figure 2: Structure of P75 neutrophin receptor

3. BDNF in Epilepsy

In the hippocampus, BDNF is able to rapidly modulate excitatory and inhibitory synaptic transmission. In cultured hippocampal neurons, two to three minutes of BDNF treatment causes an increase in spontaneous firing rates, increasing both the amplitude and frequency of excitatory postsynaptic currents (EPSCs) (Levine et al., 1995). BDNF also increases field excitatory postsynaptic potentials (EPSPs) at Schaffer collateral-CA1 pyramidal cell synapses in adult hippocampal slices (Kang and Schuman, 1995). BDNF treatment inhibits $GABA_A$ receptor-mediated evoked and spontaneous inhibitory postsynaptic currents (IPSCs), effectively increasing the neural excitability of CA1 pyramidal cells, an

effect blocked by the tyrosine kinase inhibitor k252a, suggesting TrkB receptor activity mediation (Tanaka et al., 1997). Using entorhino-hippocampal slices, Scharfman (Scharfman, 1997) demonstrated that granule cell layer stimulation after an hour of BDNF treatment increased amplitude of CA3 population spikes, and after two hours of BDNF treatment, causes multiple population spikes in CA3. These effects do not occur in the absence of BDNF, suggesting that upregulation of BDNF in epileptic brains may cause abnormal enhancement of excitability in CA3 pyramidal cells (Scharfman, 1997).

In the pilocarpine-induced rat model of epilepsy, Scharfman and colleagues (1999) found that in hippocampal slice preparations with sprouting and BDNF up-regulation, BDNF enhances the excitability of granule cells in the DG by TrkB activation, an effect confirmed by whole-cell patch clamp recordings from dentate granule cells in slices from human TLE patients (Zhu and Roper, 2001). They also found that BDNF, when co-treated with $GABA_A$ receptor blocker picrotoxin, rapidly increased the frequency and amplitude of spontaneous EPSCs and lasted up to an hour after removing BDNF, as well as BDNF-induced increase in size of EPSCs evoked by perforant pathway stimulation. Zhu and Roper (2001) also found that BDNF does not effect spontaneous IPSCs in granule cells in the presence of the glutamate receptor antagonists CNQX and AP5, but it decreases the amplitude of perforant pathway evoked IPSCs.

Koyama and colleagues (Koyama et al., 2004) found, in cultured hippocampal slices that were treated with picrotoxin for 10 days, robust MF

sprouting, abnormal excitability of dentate granule cells, and up-regulation of BDNF in the MF pathway, similar to that observed in both epilepsy patients and rat models, and was blocked by K252a or an anti-BDNF antibody. MF sprouting occurs when a BDNF-including bead is placed on the dentate hilus, even without neural activity, suggesting that BDNF induces the branching out of hilar axonal shafts.

The valine 66 to methionine (Val66Met) mutation in BDNF, which prevents cleavage from proBDNF to matBDNF, has been associated with many diseases (Sonali et al., 2013; Borroni et al., 2012; Fehér et al., 2009; Yu et al., 2008; He et al., 2007), but its association with epilepsy is conflicting. Recent evidence supports an association between this Val66Met polymorphism and epilepsy, with studies showing Val66Met may be associated with seizure susceptibility in small ethnic populations (Sha'ra et al., 2015), and that the Met66 allele may be protective against TLE (Shen et al., 2016).

E. JAK-STAT Signaling

1. Cytokine-Induced JAK-STAT Signaling

Cellular signaling pathways involving Janus kinase (JAK) and the signal transducer and activator of transcription (STAT) are known as JAK-STAT signaling. This pathway mediates proliferation, differentiation, migration, apoptosis, and cell survival, depending on the signal, tissue, and cellular context. JAK-STAT signaling is also necessary for numerous developmental and

homeostatic processes, such as hematopoiesis, immune cell development, stem cell maintenance, organismal growth, and mammary gland development (Ghoreschi et al. 2009). Polypeptide ligands, such as hormones, growth factors, or cytokines, bind to a receptor and induce receptor subunit multimerization. The ligands erythropoietin and growth hormone bind to receptor homodimers while interferons and interleukins bind to receptor heteromultimers. Signal propagation occurs when the cytoplasmic domain of these receptor multimers associated with JAK tyrosine kinases. JAK is activated when ligand-mediated receptor multimerization brings two JAKs in close together, allowing trans-phosphorylation. JAKs have distinct tandem kinase-homologous domains at the C-terminus: a non-catalytic regulatory domain and a second with tyrosine kinase activity. JAK subsequently phosphorylates STATs, latent transcription factors in the cytoplasm, at a conserved tyrosine residue on the C-terminus. Phopshorylated STATs then dimerize through a conserved SH2 domain and translocate to the nucleus via importin α-5 (or nucleoprotein interactor 1) and the Ran nuclear import pathway, where is binds to DNA and regulates transcription (Nicolas et al., 2013). STAT dimers recognize an eight to ten base pair (bp) inverted repeat DNA element with consensus sequence 5'-TT(N4-6)AA-3'. Binding affinity of STAT dimers for a target DNA sequence is determined by the nucleotide sequence (Ehret et al., 2001). Once bound to their target promoter, STAT dimers induce transcription by recruiting nuclear co-activators, which mediate chromatin modifications and communication with core promoters. In

some cases, however, STAT binding can cause transcriptional repression (Ramana et al., 2001; Gil et al., 2001; Ivanov et al., 2001).

Protein kinases, including several mitogen-activated protein kinases (MAPKs), can phosphorylate STATs, which allows other cellular signaling pathways to potentiate the STAT-activating stimulus (Decker and Kovarik, 2000). Additional serine phosphorylation sites or posttranslational modifications may also regulate attenuation of STAT activity (Mowen et al., 2000; Ahmed and Ivashkiv, 2000). The JAK-STAT pathway is negatively regulated through receptor internalization and degradation, by protein tyrosine phosphatases acting on the receptor-kinase complex in membranes (David et al., 1995; Myers et al., 2001; Irie-Sasaki et al., 2001) or dephosphorylation of activated STAT dimers in the nucleus and recycling of STAT monomers to the cytoplasm (David et al., 1993). Suppressor of cytokine signaling (SOCS) proteins, whose expression can be stimulated by cytokines that cause STAT activation, can directly bind to and inactivate JAKs (Krebs and Hilton, 2001). Protein inhibitors of activated STATs (PIAS) can bind to phosphorylated STAT dimers and prevent DNA recognition (Shuai, 2000). Deregulation of JAK-STAT signaling is associated with cancers, immune disorders and cardiovascular diseases. There are four different JAK tyrosine kinases (JAK1, JAK2, JAK3, and TYK) and seven different STAT transcription factors (STAT1, STAT2, STAT3a, STAT3b, STAT4, STAT5a, STAT5b, STAT6).

2. JAK-STAT Signaling in the Central Nervous System

In the CNS, JAK-STAT signaling is associated with gene regulation during development, hormone release, inflammation or tumorigenesis (Nicolas et al., 2013). While JAK-STAT expression in the CNS is lower than other regions, these proteins are expressed in many brain areas, including the cerebral cortex, hippocampus, hypothalamus and cerebellum. Expression of these proteins varies during development, with high levels during embryonic stages (particularly JAK2, JAK1, STAT1, STAT3, and STAT6), and gradually decreases in expression during growth to adulthood (De-Fraja et al., 1998).

The JAK-STAT pathway is able to regulate the proliferation of NSCs. The cytokine interleukin 15 (IL-15) is expressed in adult NSCs in the SVZ, and induces activation of STAT1, STAT3 and STAT5, while JAK inhibitors block proliferation (Bauer, 2009; Gómez-Nicola et al., 2011). Leptin, through activation of STAT3 and Akt, regulates neuroproliferation in the DG of adult mice both *in vitro* and *in vivo* (Garza et al., 2008). Interferon β (IFN-β), the primary treatment for multiple sclerosis, activates STATs, but studies have shown varied results: inhibition (Lum et al., 2009), no affect (Hirsch et al., 2009) or enhancement of NPC proliferation (Arscott et al., 2011). JAK2 and STAT3 are involved in hippocampal synaptic plasticity independently of gene regulation (Nicolas et al., 2012).

Glial differentiation is mediated by JAK-STAT signaling. Activation of ciliary neurotrophic factor (CNTF) receptor in embryonic cortical precursor cells

activates JAK1, STAT1 and STAT3, causing differentiation into astrocytes (Bonni et al., 1997), an effect also caused by microglia-derived IL-6 and leukemia inhibitory factor (LIF) cytokines (Nakanishi et al., 2007), and prolactin via JAK2, STAT1 and STAT3 (Mangoura et al., 2000). Numerous studies have found that STAT3 is involved in glial differentiation (Nakaniski et al., 2007; Cao et al., 2010; He et al., 2005), and conditional knockdown of STAT3 in mice enhanced embryonic neurogenesis and inhibited astrogliogenesis (Cao et al., 2010). STAT3 activation is also needed for neurogenesis in adult dentate gyrus (Müller et al., 2009), a divergence that could be explained by the embryonic and adult NSCs (Chao et al., 2006). JAK-STAT inhibitory proteins SOCS2, 3 and 6, can negatively regulate neuronal differentiation and neurite outgrowth induced by growth hormone (GH) and insulin-like growth factor-1 (IGF-1) (Gupta et al., 2011; Turnley et al., 2002). SOCS2 is expressed in neurons, and SOCS2-/- mice show impaired neurogenesis, while overexpression of SOCS2 increases neuronal differentiation (Turnley et al., 2002). JAKs isoforms have different roles, with JAK1 involved in astrocyte differentiation (Bonni et al., 1997), JAK2 involved in NSC proliferation (Garza et al., 2008; Kim et al., 2010), and JAK3 involved in NPC differentiation (Kim et al., 2010).

JAK-STAT also plays a role in neuron survival, apoptosis, and synaptic plasticity. Apoptosis can be induced by activation of STAT1 and STAT3 with IFN-β (Dedoni et al., 2010), or activation of STAT1 alone by IFN-γ (Mäkelä et al., 2010). IL-9, a JAK-STAT activator of STAT1 and STAT3, however, can protect

neonatal neurons (Fontaine et al., 2008). This is likely because STAT activation causes rapid caspase activation, and later transcription of non-apoptotic proteins (Schindler et al., 2007). GH-induced increases in AMPA and NMDA receptor-mediated EPSPs are reduced by AG490, a JAK2 inhibitor (Mahmoud and Grover, 2006). Cytoplasmic polyadenylation element binding protein 1 (CPEB-1) knockout (KO) in mice causes impaired synaptic plasticity and reduced activation of JAK2 and STAT3 (Mahmoud and Grover, 2006). JAK inhibition blocks the induction of NMDA receptor-dependent long-term depression (LTD) in rat hippocampal slices and STAT3 is activated after NMDA receptor-dependent LTD (Nicolas et al., 2012; Hofman and Kirsch, 2012).

Evidence shows that JAK-STAT is involved in several brain pathologies, including brain tumors, inflammation, and neurodegeneration. Human brain tumors have high levels of STAT activation, which causes impaired tumor recognition by the immune system (Gu et al., 2008). Anti-inflammatory cytokines, such as interleukin 10 (IL-10), activate STAT3, while pro-inflammatory cytokines, such as IFN-γ, causes JAK2-dependent STAT1 phosphorylation (Regis et al., 2008). JAK2 and STAT3 may also protect neurons from degeneration. Neurotoxicity of Aβ can be reduced through the activation of JAK2 and STAT3 by nicotinic Acetylcholine receptors (Buckingham et al., 2009) or humanin (Chiba et al., 2009).

3. BDNF-Induced Activation of JAK/STAT Signaling

Studies in our laboratory have found that BDNF induces an increase in α4 subunit levels through TrkB activation of the mitogen activated protein kinase (MAPK) pathway, which regulates synthesis of the transcription factor early growth response factor 3 (Egr3) (Roberts et al., 2005). BDNF signaling decreases $GABA_AR$ α1 subunit synthesis, and is dependent upon a TrkB-mediated activation of the JAK-STAT pathway (Hokenson et al., in review; Lund et al., 2008). BDNF-induced activation of JAK-STAT signaling eventually leads to STAT3-mediated synthesis of inducible adenosine 3', 5'-monophosphate (cAMP) early repressor (ICER), which binds to *Gabra1* and attenuates its transcription. Interestingly, ICER is a repressor of signaling mediated by the cAMP-response element binding protein (CREB), and mediates the activity of higher order processes that underlie learning and memory (Borlikova and Endo, 2009). How BDNF-signaling activates the JAK-STAT pathway to attenuate the synthesis of synaptic GABARs, as well as other essential CREB-mediated gene products, remains unknown and is the subject of this manuscript.

F. Late SV40 Factor

1. Background and Function

The Late Simian Virus 40 promoter factor (LSF) was first identified as an activator of the Late Simian Virus 40 promoter (Kim et al., 1987; Huang et al., 1990; Shirra et al., 1994) and is a transcriptional regulator ubiquitously expressed in mice and in all human cell lines, where it regulates cell cycle progression, cell

growth, and development (Hansen et al., 2009; Bruni et al., 2002; Powell et al., 2000). It has been given other names based on independent discovery of its binding to DNA and transcriptional regulation, including leader binding protein-1c (LBP-1) or upstream region binding protein-1 (UBP-1), both on the HIV long terminal repeat (Jones et al., 1988; Wu et al., 1988; Yoo et al., 1994), CCAAT binding protein-2 (CP2), on the murine α-globin promoter (Barnhart et al., 1988; Kim et al., 1988; Lim et al., 1992), and serum amyloid A3 enhancer factor-1 (SEF1), on the murine serum amyloid A3 promoter (Huang et al., 1994). LSF is a 502 amino acid long protein (about 57 kDa) that is part of a small, conserved family of transcription factors that includes two subfamilies, LSF/CP2 and grainyhead (GRH) (Venkatesan et al., 2003; Wilanowski et al., 2002; Traylor-Knowles et al., 2010). Genes encoding for LSF/GRH have been identified in both mammalian and non-mammalian species (Swendeman et al., 1994; Traylor-Knowles et al., 2010). Mammals have three to four members of each family in each genome, while other species have one gene of each subfamily.

The human LSF subfamily contains LSF (chromosome 12q13), LBP-1a/b (chromosome 3), and LBP-9 (chromosome 2), (Swendeman et al., 1994; Cunningham et al., 1995). LSF (Ramamurthy et al., 2001; Swendeman et al., 1994) and LBP-1a/b (Ramamurthy et al., 2001) mRNAs are ubiquitously expressed in developing and adult mice, and in all human cell lines examined (Veljkovic and Hansen, 2004). Disruption of the LSF/CP2 gene in mice does not seem to cause any harm, which is likely a result of redundancy between LSF and

paralog LBP-1a/b (Ramamurthy et al., 2001), which is ubiquitously expressed and binds to the same DNA motifs and partner proteins as LSF (Zhou et al., 2000; Tuckfield et al., 2002).

There are a number of identified LSF splice variants. LSF-Internally Deleted (LSF-ID or LBP-1d) (Shirra et al., 1994; Uv et al., 1994; Yoon et al., 1994), lacks part of the DNA interacting domain, amino acids 189 to 239, which prevents DNA binding (Shirra et al., 1994; Uv et al., 1994) and inhibits binding of full-length LSF *in vitro* (Yoon et al., 1994; Zhong et al., 1994), and can function as a mutant dominant-negative LSF when expressed in cells (Drouin et al., 2002; Yoon et al., 1994). Another common splice variant of the LBP-1a/b gene is an alternative LBP-1b isoform, which contains an insertion of 36 amino acids relative to LBP-1a (Huang et al., 2000; Yoon et al., 1994), following the DNA-interaction region, which is important for transcriptional activation and nuclear localization in erythroid cells (Kang et al., 2005; Kang et al., 2010). The third splice variant, LBP9/CRTR-1, is less widely expressed and thought to be in involved in pluripotent stem cell development (Pelton et al., 2002). LBP-9 contains a unique transcriptional repression region (Rodda et al., 2001) and can reverse LBP-1b-mediated transcriptional activation (Huang and Miller, 2000).

LSF is localized to the nucleus in every cell type examined (Drouin et al., 2002; Zambrano et al., 1998; Veljkovic and Hansen, 2004) except for differentiated rat B103 neuroblastoma cells (Kashour et al., 2003), but lacks an obvious nuclear localization signal. LSF-ID is exogenously expressed in the

cytoplasm (Drouin et al., 2002; Zambrano et al., 1998) suggesting residues 189 to 239 are important for nuclear localization (Veljkovic and Hansen, 2004).

2. DNA Binding and Transcriptional Activity

GRH and LSF DNA-binding domains are highly conserved and they bind to similar DNA motifs. LSF binds to a DNA motif of directly repeated half sites on consecutive turns of the DNA helix (Huang et al., 1990) with the consensus sequence CNRG N_6 CNRG (Lim et al., 1993; Shirra, 1995). LSF binds to DNA as a homotetramer through its DNA binding domain (residues 67-260) (Shirra and Hansen, 1998; Murata et al., 1998), while GRH binds a similar single DNA motif (Venkatesan et al., 2003) as a dimer (Attardi and Tjian, 1993; Shirra and Hansen, 1998). LSF is primarily a dimer in solution (Shirra and Hansen, 1998; Zhong et al., 1994), suggesting that LSF dimers must form a tetramer to recognize and bind DNA. Residues 133-383 are required for DNA binding (Shirra and Hansen, 1998). The C-terminal region of LSF is responsible for oligomerization (residues 266-403) with residues 280-501 responsible for homooligomerization (Uv et al., 1994; Zhou et al., 2000). A LSF-LSF interaction region is also present in the DNA-binding domain (residues 211–213, 235–237), which is likely involved in tetramerization (Shirra et al., 1994; Shirra, 1995). LSF and GRH are unable to oligomerize, while members of the same subfamily can interact with one another (Ting et al., 2003b; Uv et al., 1994; Wilanowski et al., 2002; Yoon et al., 1994). LSF can bind with other subfamily members, such as LBP-1a/b, to bind LSF recognition sites, or bind with different proteins to create novel DNA-binding

moieties. Mutations of the DNA binding domain have lead to the discovery of Q234L/K236E LSF (dnLSF), which is unable to bind DNA and oligomerizes with wild-type LSF (wtLSF), as well as other LSF subfamily proteins (Shirra et al., 1994). This mutated LSF has been a helpful dominant-negative in many biological studies (Drouin et al., 2002; Kashour et al., 2003; Powell et al., 2000).

LSF typically activates transcription in mammalian tissue culture cells (Bing et al., 1999; Bing et al., 2000; Casolaro et al., 2000; Lim et al., 1993; Murata et al., 1998; Powell et al., 2000; Yoon et al., 1994; Zheng et al., 2001), as does LBP-1a/b (Huang et al., 2000; Yoon et al., 1994), but it can also repress transcription. The transcriptional activation regions are amino acids 1 to 65 and 164 to 277, which can each activate transcription on their own (Veljkovic and Hansen, 2004). LSF also interacts with TATA-binding protein (TBP) and transcription factor TFIIB (Veljkovic and Hansen, 2004), which are components of basal transcriptional machinery. LSF increases the rate of association of TFIIB with a DNA *in vitro*, and can increase the total number of transcription complexes (Sundseth et al., 1992). LSF represses transcription from the HIV LTR via steric hindrance, which prevents TBP access to TATA (Kato et al., 1991; Coull et al., 2002), and transcriptional elongation (Parada et al., 1995), by forming a heteromeric complex with YY1 and histone deacetylase 1 (HDAC1) (Coull et al., 2000; Romerio et al., 1997). Interleukin-2 (IL-2) promoter is the only published cellular promoter that LSF downregulates (Casolaro et al., 2000). LSF amino

acids 266 to 396 are necessary for transcriptional repression (Veljkovic and Hansen, 2004).

LSF can be regulated at a number of steps, including dimerization, tetramerization, and DNA interaction (Whitmarsh and Davis, 2000). Signal transduction pathways also regulate LSF. Extracellular-signal-regulated kinase (Erk) rapidly enhances DNA-binding of LSF in primary human T cells (Pagon et al., 2003; Volker et al., 1997). Activation of class switching in primary murine splenic B cells decreases DNA-binding of LSF to heavy chain immunoglobulin class switch regions (Drouin et al., 2002). Jurkat cell induction via calcium ionophores increases DNA-binding to the interleukin-4 (IL-4) promoter (Casolaro et al., 2000). Interleukin 1 (IL-1) signaling-induced inflammation increases LSF-mediated stimulation of a serum albumin promoter in liver cells (Bing et al., 2000).

3. Phosphorylation and Regulation of Cell Cycle

The cell cycle is the duplication of DNA and subsequent division into two genetically identical daughter cells, and requires DNA duplication (S, or synthesis, phase), chromosome segregation and cell divisions (M, or mitosis, phase). Gap phases are added into the cell cycle to allow time for growth, including G_1 phase between M and S phase, and G_2 between S phase and mitosis. Eukaryotic cell cycle contains the four phases; G_1, S, G_2, and M, with phases G_1, S, and G_2 collectively called interphase. G_0 is a special resting phase that cells may remain in for long periods of time, and proliferative conditions can

cause G_0 cells to pass through a start (or restriction) point in late G_1 and re-enter the cell cycle (Reviewed by Alberts et al., 2002).

Studies have found that LSF regulates cell cycle progression and may be important for cell survival (Veljkovic and Hansen, 2004). The re-entry of cells from G_0 to G_1 phases requires activation of signal transduction pathways, including the Erk family of MAP kinases, which target many transcription factors (Johnson and Lapadat, 2002; Yang et al., 2003; Zhang and Liu, 2002). G1 cyclin-dependent kinases are required for cells to pass through the restriction point, commit to proliferation, and enter S phase. Cyclin D/cdk4 and cyclin D/cdk6 complexes target pRb, which activates adenovirus E2 promoter factor (E2F) transcription factors. Cyclin E/cdk2 targets transcription factors, including NPAT, which stimulates histone gene expression (Ma et al., 2000; Zhao et al., 2000).

Both Erk and cdk2 phosphorylate LSF (Powell, 1999; Volker, 1998; Pagon et al., 2003; Saxena et al., 2009). The major phosphorylation site is serine 291 (S291), which can be phosphorylated within minutes of mitogenic stimulation (Pagon et al., 2003; Volker, 1998; Volker et al., 1997). MEK inhibition prevents S291 phosphorylation, suggesting Erk phosphorylates S291 (Pagon et al., 2003). Cyclin/cdk2 complexes also specifically phosphorylate LSF (Fecko, 2001; Powell, 1999; Pagon et al., 2003; Saxena et al., 2009). LSF targets the thymidylate synthase (TS) gene by binding to cell cycle regulatory regions in promoter and intron sequences, and upregulating TS mRNA levels at the G_1/S transition, promoting cell cycle re-entry (Powell et al., 2000). Expression of dnLSF blocks

the G_1/S induction of TS expression and causes mouse fibroblasts and human prostate cancer cells to undergo apoptosis during S phase (Veljkovic and Hansen, 2004), an effect reversed by addition of thymidine (Powell et al., 2000). Overexpression of the interacting protein Fe65, which inhibits transactivation of LSF, causes cells to arrest at the G_1/S transition (Bruni et al., 2002), an effect that can also be blocked by thymidine treatment (Powell et al., 2000). Another site of phosphorylation by cyclin C/Cdk2, is serine 309 (S309), which occurs in mouse fibroblasts in early G1, one to two hours after mitogenic stimulation (Saxena et al., 2009). Phosphorylation at both S291 and S309 inhibits the transcriptional activity of LSF. In late G_1, dephosphorylation occurs at both sites, causing LSF activation and TS induction, suggesting that early G_1 phosphorylation inhibits LSF and prevents premature induction of LSF genes (Saxena et al., 2009).

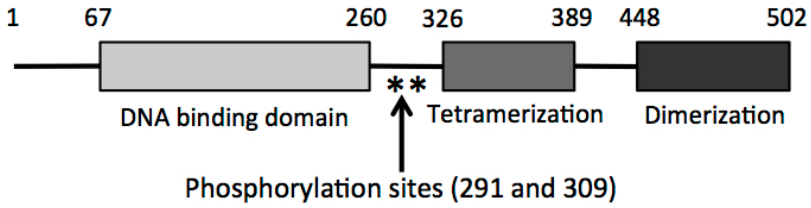


Figure 3: Structure and domains of LSF

4. Role of LSF in Disease

LSF has been implicated in a number of diseases. LSF regulates gene expression of human immunodeficiency virus (HIV) through three half-site motifs in the HIV long terminal repeat (LTR), causing transcriptional repression (Coull et

al., 2000; Coull et al., 2002; He and Margolis, 2002; Jones et al., 1988; Kato et al., 1991; Romerio et al., 1997). LSF has also been identified as an oncogene in hepatocellular carcinoma (HCC), promoting tumorigenesis and upregulating osteopontin (OPN), a gene that regulates tumor progression (Yoo et al., 2010). Treatment with factor quinolinone inhibitor 1 (FQI1), a small molecule inhibitor of LSF DNA-binding activity, reduces tumor growth and cytotoxicity (Grant et al., 2012).

LSF has been linked to Alzheimer's disease (AD) through a number of findings. Amyloid precursor protein (APP) is a transmembrane protein (Coulson et al., 2000) that is protealytically cleaved near the membrane (Hardy and Selkoe, 2002; Steiner and Haass, 2000) by either α and β secretase, which releases the extracellular domain. The carboxy-terminal (C-terminal) is subsequently cleaved by γ secretase within the membrane to produce APP intracellular C-terminal domain (AICD), which is released into the cell. The N-terminal fragment of this product is secreted, and when first cleaved by β secretase, the peptide product is amyloid beta (Aβ). Aβ is correlated with plaque formation in the AD brain, while AICD may enhance apoptosis (Kögel et al., 2012; Kim et al., 2003; Passer et al., 2000). Notch, like APP, can be cleaved by α and γ secretase and the Notch intracellular C-terminal fragment (NICD) translocates to the nucleus to mediate transcription. AICD localizes to the cytoplasm and nucleus (Cupers et al., 2001; Gao and Pimplikar, 2001; Kimberly et al., 2001) and interacts with a number of intracellular adaptor proteins (Neve et

al., 2000; Russo et al., 1998), including Fe65, which is expressed in the brain and shuttles between the cytoplasm and nucleus. Fe65 expression promotes nuclear localization of AICD (Kim et al., 2003; Kimberly et al., 2001). Fe65 can interact with a histone acetyltransferase Tip60, leading to a ternary AICD/Fe65/Tip60 complex (Cao and Südhof, 2001) or Fe65 can interact with LSF (Zambrano et al., 1998) and form a nuclear ternary AICD/Fe65/LSF complex (Kim et al., 2003). Exogenous overexpression of Fe65 causes increased nuclear localization and repression of LSF activation of the TS promoter (Bruni et al., 2002). These findings suggest that LSF transactivation may be inhibited or activated by its association with Fe65/AICD. These complexes may compete for formation, given that Tip60 and LSF interact with the same domain of Fe65 (Cao and Südhof, 2001; Zambrano et al., 1998). Overexpression of APP protects neurons from apoptosis and in APP-lacking rat neuroblastoma B103 cells, expression of wild-type LSF enhanced protection from apoptosis, while dnLSF expression increased apoptosis, suggesting that LSF in involved in APP-mediated cell survival (Kashour et al., 2003). In epidemiological studies, a polymorphism in the 3' untranslated region of the LSF gene has been linked to AD, with the A allele (compared to the G allele) being correlated with protection against AD (Lambert et al., 2000; Taylor et al., 2001; Luedecking-Zimmer et al., 2003; Lendon and Craddock, 2001) while another study found that A allele was associated with an increased risk of early onset AD (Panza et al., 2004).

G. Major Hypothesis and Specific Aims of Thesis

Extensive studies have resulted in two predominant theories of epileptogenesis: The altered expression of γ-aminobutyric acid (GABA) receptor subunits, causing a shift from α1-containing GABARs to rapidly desensitizing α4-containing GABARs at the inhibitory synapse; and abherrent neurogenesis, mainly in the hippocampus, resulting in increased excitability. The detailed regulation of these mechanisms still requires further study and could provide more therapeutic targets and help to develop new therapies. The major hypothesis of our studies is that BDNF signaling mediates a change in inhibitory neurotransmission by activating JAK/STAT and MAPK pathways whose downstream targets are critical determinants of epileptogenesis. Therapies aimed at regulating specific elements of these pathways may be effective in the treatment of a large number of cases of intractable epilepsy, for which no current therapies are effective.

Our studies are focused on understanding TLE, the most common from of refractory epilepsy. While studies have uncovered many underlying mechanisms of this disorder, including increased BDNF signaling through TrkB and P75NTR (Takahashi et al., 1999), the complexities of these pathways have yet to be elucidated. We previously demonstrated (Lund et al., 2008.) that recombinant matBDNF increases pSTAT3 levels, which induces ICER, a potent inhibitor of genes containing cAMP response elements (CRE sites). We also found that STAT-dependent synthesis of ICER, in response to matBDNF, downregulates

the number of α1 containing synaptic GABARs. We now further report that both TrkB and p75NTR are required for BDNF-induced JAK-STAT signaling in neurons: TrkB at the membrane and p75NTR as part of an intracellular JAK2 signalsome ((i)p75NTRJ2). An essential role for rapid TrkB activation (within 15 minutes) as a requirement for JAK/STAT pathway signaling in neurons is discussed in light of biochemical findings that suggest BDNF induces the formation of p75NTR oligomers that include JAK2. Taken together, these recent findings suggest the existence of a new pathway in synaptic plasticity and the brain's response to injury.

The neuronal cell cycle is unique because a majority of adult neurons have long been thought to be post-mitotic or quiescent. The expression of cell cycle regulators, including cyclin D1, Cdk4, Rb proteins, E2Fs, and CKI, are detected in the adult brain at both a transcriptional and translational level (Koeller et al., 2008; Müller et al., 2010; Okano et al., 1993; Schmetsdorf et al., 2007; Ting et al., 2014; Cunningham et al., 2002). The role of these cell cycle regulators in neurons has been attributed to neuronal maturation, migration, and synaptic plasticity (Frank and Tsai, 2009; Lim and Kaldis, 2013). Some neuronal cell types, including sympathetic and cortical neurons, may upregulate cell cycle makers and try to re-enter the cell cycle in response to an acute insult, such as neurotrophic factor deprivation, activity withdrawal, DNA damage, oxidative stress, and excitotoxicity, only to die at the G_1/S checkpoint. This is a process known as "abortive cell cycle re-entry" (Becker and Bonni, 2004; Liu and Greene,

2001). Because the cell cycle regulator LSF is uniquely poised to regulate movement of cells through the G_1/S transition and has been previously identified as a candidate gene in Alzheimer's disease (Taylor et al., 2001), we hypothesized that it might also be a target for BDNF regulation in neurons with the potential of regulating cell fate in response to SE.

We show that BDNF induces phosphorylation specifically at serine 291 in primary neuronal cultures and the hippocampus of rats in the pilocarpine model of TLE. Actively dividing neurons do not express p291 LSF, however, p291 LSF is expressed in neurons throughout the stages of adult neurogenesis and persists in mature neurons, as identified using markers of cell differentiation. Phosphorylation of LSF at p291 is highest in brain tissue where adult neurogenesis is most active, such as the olfactory bulb and hippocampus, when compared to cortex. Whether LSF has a role in the determination of neuronal fate requires further study, however, association with both BDNF signaling and SE suggests that it has a novel role in the brain and may provide a unique target for future therapeutic intervention.

II: MATERIALS AND METHODS

1. Primary neuronal cell culture

Primary cortical and hippocampal neuronal cultures were prepared from embryonic day 18 (E18) Sprague-Dawley rats (Charles River Laboratories, MA). Pregnant female rats were euthanized (95% CO_2/5% O_2) and the embryos were removed. In ice-cold CMF solution [35mg/mL Bovine serum albumin, Ca^+/Mg^+ free Hank's Buffered Saline Solution (HBSS), 1mM sodium pyruvate, 4.2mM sodium bicarbonate, 20mM hydroxyethyl piperazineethanesulfonic acid (HEPES)], the hippocampus and cortex were dissected from the embryonic brains. Cortical tissue was trypsinized in 0.05% trypsin/EDTA (10 minutes, 37°C, 5% CO_2) and the reaction was quenched with pre-warmed (37°C, 5% CO_2) neuron plating medium [Neurobasal® Medium (NBM, GIBCO Life Technologies #21103-049), 10% fetal bovine serum (FBS), 100U/mL L-glutamine, 100U/mL peniclillin/streptomyocin]. Cells were pelleted, washed twice with CMF, and triturated in 1mL of neuron plating medium before resuspending in more media (10 mL total). Cells were counted using a hemocytometer and plated on poly-L-lysine-coated plates (Nunc) at a density of 0.5 million cells per 1mL. Hippocampal tissue was triturated in 1mL of CMF, pelleted, and resuspended in neurobasal plating medium (5mL) before counting and plating. Plated cells were incubated (one hour, 37°C, 5% CO_2) before media was replaced with neuron feeding media (NBM, 2% B27 supplement, 100U/mL L-glutamine, 100U/mL

peniclillin/streptomyocin). Neuron cell cultures were incubated (37°C, 5% CO_2) until use.

2. Human embryonic kidney cell line

Human embryonic kidney cell lines (HEK 293; ATCC #CRL-1573) were started by thawing frozen cells in a water bath (37°C, 2 minutes) and transferred to a vial with 9mL pre-warmed HEK feeding media [Dulbecco's Modified Eagle Medium (DMEM; GIBCO Life Technologies #11965-092), 10% FBS, 1X MEM non-essential amino acids (GIBCO Life Technologies #11140-050)]. Cells were centrifuged (125XG, 5-7 minutes), the pellet was resuspended in HEK feeding media, plated on a 75cm^2 flask, and incubated (37°C, 5% CO_2) to allow cell growth. Cells were maintained until 90-100% confluency before splitting. To split HEK cells, cells were washed once with 1X PBS, trypsinized with 0.05% trypsin/EDTA (5 min, 37°C, 5% CO_2) to remove cells, quenched with HEK feeding media, and centrifuged (2500RPM, 5 minutes) to pellet cells. Cells were resuspended in 10 mL HEK feeding media and triturated well before plating in a new 75cm^2 flask flask at a 1/10 or 1/20 dilution of the cell suspension. HEK cell plating for experiments differed based on the type of experiment.

3. Adult rat brain dissection

For adult brain tissue analysis, brains were removed from pregnant adult female Sprague-Dawley rats used for embryo dissection. Brains were kept on ice while the prefontal cortex, hippocampus, and olfactory bulb were bilaterally

dissected. Tissue was homogenized in Radioimmunoprecipitation assay (RIPA) lysis buffer [1X RIPA (Millipore 20-188), 10mM Tris pH 7.4, 1% NP40, 150mM NaCl, 0.1% SDS, 1X protease inhibitor cocktail, 1X phosphatase inhibitor, 1mM phenylmethyl-sulfonyl fluoride (PMSF), 1mM sodium orthovanadate, 1mM sodium fluoride] using an electronic pestle and passed through a fine tipped syringe for further homogenization. Samples were incubated (on ice, 5 minutes), centrifuged (high speed, 10 minutes), and the supernatant was saved for total protein analysis.

4. Recombinant BDNF and Drug Treatments

Neuron treatments were done on 7 or 8 days *in vitro* (div) and in a controlled environment (37°C, 5% CO_2). Recombinant human brain derived neurotrophic factor (matBDNF; Millipore GF029), mutant (cleavage resistant) human proBDNF (Alomone; B-256), were treated alongside vehicle (water) control samples. Mutant proBDNF contains a single-nucleotide polymorphism (SNP), valine 66 to methionine, in the pro-domain that impairs intracellular trafficking and regulated secretion of BDNF in primary cortical neurons (Chen et al., 2004). Time and concentration of treatment varied based on protein or mRNA of interest. For drug pre-treatments, TrkB inhibitor K252A (Calbiochem 420298; 100nM), JAK/STAT inhibitor Ruxolitinib (Selleckchem S1378; 100nM), MEK inhibitor U0126 (Calbiochem 662005; 20μM), JAK/STAT inhibitor Pyridone 6 (Calbiochem 420099; 500nM), or vehicle (dimethyl sulfoxide; DMSO), were added to cells for one hour before treatment. For treatment with potassium

chloride (KCl, Boston BioProducts MT-250), neurons were treated with either KCl (20mM) or vehicle (water) for 2 hours.

5. Transfection

Calcium phosphate

Calcium phosphate transfection was used for HEK 293 cells and in some neuron culture experiments. Transfection was done 6 to 7 days in vitro (div) for neurons. Conditioned media was removed from cells, filtered (0.2μm), and saved (37°C, 5% CO_2). Cells were washed once with pre-warmed DMEM (GIBCO Life Technologies #11960-044 for neurons, #11965-092 for HEK), and fresh DMEM was added to each well. Plates were incubated (37°C, 5% CO_2; 15 minutes for neurons, 1 hour for HEK) until DNA was added. Amount of plasmid DNA used was dependent on the number of cells being transfected (8ug DNA for 1 million cells, 20ug DNA for 5 million cells). Plasmid DNA was diluted in water before adding calcium chloride (2.5M $CaCl_2$), and transferred drop-wise with constant mixing into 2X HEPES-buffered saline (HeBS; 274mM NaCl, 10mM KCl, 1.4mM $Na_2HPO_4.7H_2O$, 15mM dextrose, 42mM HEPES). DNA solution was incubated (20 minutes, dark, room temperature) before adding to cells and incubating (37°C, 5% CO_2; 45 minutes for neurons, 3 hours for HEK). Olates were washed twice with DMEM, and filtered conditioned media was added.

Electroporation

Electroporation transfection of neurons was done on div 0. After determining the neuron count and concentration, the volume of cells needed (2

million cells per 30mm dish) were removed and centrifuged (80xg, 5 minutes, room temperature) to obtain a pellet. Two million cells were resuspended in 100ul Rat Neuron Nucleofector® Solution (with added supplement) from the Amaxa® Rat Neuron Nucleofector® Kit (Lonza VPG-1003). DNA (3ug per 2 million cells) was added to the cell solution, tranferred into a cuvette, placed in the Nucleofector® II, and program C-009 was applied. DMEM (500ul, 10% FBS) was added to the cuvette with a pipette and the entire volume was transferred to pre-warmed plates with neurobasal feeding media. After incubating (37°C, 5% CO2; 2 hours), media was replaced with fresh neurobasal feeding media.

NeuroMag Magnetofection™

NeuroMag transfection was done on primary neuron cultures at 6 or 7 div, using a NeuroMag Magnetofection™ kit (Oz Biosciences #KC30800). Plasmid DNA was diluted in NBM and added to NeuroMag Transfection Reagent. The DNA solution was incubated (20 minutes, room temperature) before being added to neuron dishes and incubated on a magnetic plate (20 minutes, room temperature) provided in the kit. Cells were removed from the plate and incubated (37°C, 5% CO2) for 24 hours before assaying.

6. P75NTR constructs and mutations

Phillip Barker (McGill) generously provided us with human p75NTR:Flag cDNA in a pcDNA 3.0 vector. To make human transmembrane mutants comparable to previously studied rat p75NTR constructs (Vilar et al., 2009), we did a BLAST alignment of rat and human p75NTR and determined that rat

cysteine 257 aligned to human cysteine 256, and rat glycine 266 aligned to human glycine 265. Two mutants were generated (Mutagenix) from the human p75NTR:Flag construct: cysteine 256 to alanine, p75NTR(C256A):Flag; and glycine 265 to isoleucine, p75NTR(G265I):Flag.

7. RNA extraction and Real-Time PCR

To generate a standard curve for ICER mRNA expression, neurons were treated with Forskolin (4 hours, 20uM). For all RNA extractions, an RNeasy Micro Kit (Qiagen #217084) was used. After treating, cells were washed with ice-cold 1X PBS and buffer RLT (containin 2-mercaptoethanol) was added to each well. Cells were scraped, collected, vortexed (1 minute, high) and passed through a fine-tipped needle (20 gauge) 10-15 times. Ethanol (70%) was added to the samples and transferred to a microcolumn. DNase I was added (15 minutes) and RNA was eluted in 14ul RNase-free water. Nanodrop was used to measure RNA levels. Taqman one-step RT-PCR master mix (Applied Biosystems 4309169) was used for RT-PCR reactions. RT-PCR reactions were performed using the ABI PRISM 7900HT (Applied Biosystems), with a cycle of 50°C for 30 minutes, 1 cycle of 95°C for 10 minutes, and then 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. ICER mRNA was detected using Taqman Rat Crem kit (Life Technologies 4331182). Primers and probes for cyclophilin (cyc) and $GABA_AR$ α1 were designed previously in our laboratory using primer express software (PE Biosystem, see table below). All mRNA was normalized to rat cyclophilin (cyc).

Gene	Sequence
Cyclophilin	Forward: 5'-TGCAGACATGGTCAACCCC Reverse: 5'- CCCAAGGGCTCGCCA Taqman Probe: 5'-CCGTGTTCTTCGACATCACGGCTG
$GABA_AR$ α1	Forward: 5'-CCCCGGCTTGGCAACTA Reverse: 5'-CGGTTTTGTCTCAGGCTTGAC Taqman Probe: 5'-TGCTAAAAGTGCGACCATAGAACCGAAGA

8. Preparation of Whole Cell Protein Extracts

Cells were washed once, scraped, and collected from plates in ice-cold PBS-EDTA (1X, pH 7.4) containing protease inhibitor (1X, Roche #11697498001), phosphatase inhibitor (1X, Roche #04906837001) and 1mM PMSF. Samples were centrifuged (1500 RPM, 5 minutes) to pellet cells, and resuspended in RIPA lysis buffer. The resuspended cells were vortexed (10 seconds, high) and incubated on ice (20 minutes). Samples were centrifuged again (13500 RPM, 10 minutes) and the supernatant containing total protein was removed and saved. Total protein concentration was measured via Bradford Assay (Bio-Rad Laboratories #500-0006). For storage, protein extracts were kept at -80°C.

9. Western Blot Analysis

Total protein samples containing 30ug protein (unless otherwise noted), non-reducing lane maker sample buffer (1X, Thermo Scientific #39001), and Dithiothreitol (DTT, 100mM) were boiled (95°C, 5 minutes), loaded and run on Novex® 10% tris-glycine gels (Invitrogen #EC6078). For non-denaturing

conditions, samples were made without DTT. Gels weretransferred to nitrocellulose membranes, and membranes were blocked at room temperature in 5% milk/tris-buffered saline with 0.05% tween-20 (TBS-T) for one hour. Membranes were incubated in primary antibodies overnight at 4°C. Polyclonal rabbit antibodies were raised against phospho-TrkB Tyr490 (Cell Signaling Technology 9141; 1:1000), phospho-STAT3 Tyr705 (Cell Signaling Techonology 9131; 1:2000), p75ICD (Phil Barker Lab; 1:500), or GFP (Sigma Aldrich G1544; 1:1000). Monoclonal rabbit antibodies were raised against phospho-JAK2 Tyr1007/1008 (Cell Signaling Technology 3776; 1:1000), JAK2 (Cell Signaling Technology 3230; 1:1000), STAT3 (Cell Signaling Technology 4904; 1:2000), or p75NTR (Novus Biologicals NB110-58000; 1:1000). Primary mouse monoclonal antibodies were raised against TrkB (BD Biosciences 610101; 1:1000), Sortilin (BD Biosciences 612100 1:1000), and β actin (Sigma Aldrich A5441; 1:5000). Dr. Ulla Hansen (Boston University) generously supplied the antibodies we used against LSF. Polyclonal rabbit antibodies were raised against phospho-LSF S291 (1:3000), phospho-LSF S309 (1:300), and total LSF (1:500). Primary mouse monoclonal antibodies were raised against β actin (Sigma Aldrich A5441; 1:5000). Primary antibodies were diluted in either 5% milk TBS-T or 5% bovine serum albumin (BSA)/TBS-T (including phospho-specific antibodies). Blots were incubated in the proper secondary antibodies (in 5% milk/TBS-T) for 1 hour at room temperature. Secondary antibody goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology 2004; 1:2000) was used against rabbit primary antibodies.

Secondary antibody peroxidase horse anti-mouse IgG (Vector Laboratories PI-2000; 1:2000) was used against mouse primary antibodies. Protein bands were visualized with ECL chemiluminescence (GE Healthcare #RPN2106). ImageQuant software was used to conduct densitometry analysis and protein bands were normalized to loading control β-actin. For phospho-specific antibodies, protein was normalized to total protein bands.

10. siRNA knockdown

Ambion was used to design and synthesize siRNAs for TrkB and p75NTR. Negative control siRNA was also obtained from Ambion (#AM4620). The followed sequences were used:

Gene	Sequence
TrkB(Ntrk2)-1	Sense 5'-CAACCAUCACAUUUCUCG Antisense 5'-UCGAGAAAUGUGAUGGUUG
TrkB(Ntrk2)-2	Sense 5'-CAAGGACUUUCAUCGCGAA Antisense 5'-UUCGCGAUGAAAGUCCUUG
p75NTR(Ngfr)-1	Sense 5'-GGCUGAUGCUGAAUGCGAA Antisense 5'-UUCGCAUUCAGCAUCAGCC
p75NTR(Ngfr)-2	Sense 5'-GCCUGGACAAUGUUACAUU Antisense 5'-AAUGUAACAUUGUCCAGGC

For p75NTR siRNA transfection, RNAifect transfection reagent (Qiagen) was used on primary neuron cultures 5 div. siRNA (1ug per well) were dileted in 100uL of buffer EC-R, and complex formation was initiated with the addition of RNAiFect transfection reagent (12uL). Complex solution was incubated (15 min, room temperature), and added to the cells and incubated for 24 hours ((37°C, 5%

CO_2). The conditioned media was replaced with NBM and siRNA transfection was repeated and incubated overnight. Neurons were then treated with matBDNF (50ng/mL) for 4 hours and collected for analysis. For TrkB siRNA knockdown studies, the neurons were transfected via electroporation, using the Amaxa Rat Neuron Nucleofector kit (Lonza, #VPG-1003), as described in Methods section 5.

11. Co-Immunoprecipitation

HEK cells were co-transfected via calcium phosphate precipitation of p75NTR-Flag or p75NTR-flag mutants C256A or G265I in combination with JAK2:eGFP (gift of Dr. Ross Levine, Mount Sinai School of Medicine). For inhibitor studies, ruxolitinib (100nM) was added right after transfection. For all transfected cells, protein was collected 24 hours after transfection. Protein was extracted using a Universal Magnetic Co-IP kit (Active Motif 54002). Cells were washed and scraped in 1X PBS (with protease inhibitor cocktail, phosphatase inhibitor, and deacetylase inhibitor), centrifuged (1500 RPM, 5 minutes) to pellet cells, and resuspended in whole cell lysis buffer (Active Motif). The cell suspension was vortexed (10 seconds, high) and incubated on ice (30 minutes). Samples were centrifuged again (13500 RPM, 10 minutes) and the supernatant containing total protein was removed and saved. Total protein concentration was measured via Bradford Assay. For immunoprecipitation, total protein (500ug) was incubated overnight at 4°C on a rotating platform with a rat Flag primary antibody (anti-Flag) (Sigma Aldrich 7425; 6.4ug) or normal rabbit IgG (Santa Cruz

Biotechnology sc-2027) control. Protein G magnetic beads were added and incubated for 1 hour at 4°C on a rotating platform. Inputs were made from either collected flow through from first wash or original protein. Magnetic beads were washed three times before eluting in SDS-Buffer (with DTT). Samples were analyzed via Western blot and probed for presence of GFP. A Veriblot for IP secondary antibody (abcam #ab131366) was used for IP immunoblots.

For endogenous co-immunoprecipitation (Co-IP), protein was extracted from treated primary neuronal cultures. Protein was collected using RIPA lysis buffer.

12. Immunocytochemistry

Primary cortical and hippocampal neurons were fixed for 10 minutes in fixing solution [4% paraformaldehyde (Electron Microscopy Sciences #15710-S), 25mM Hepes, 150mM NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$] at room temperature, and washed and permeabilized in 1% BSA/0.1% Triton-X/1X PBS for 20 minutes. Plates were incubated overnight at 4°C with primary antibodies to either p291 LSF, p309 LSF, or total LSF and either NeuN (1:250; mouse polyclonal, Chemicon), the polysialylated embryonic form of the neural cell adhesion molecule (PSA-NCAM; 1:200; Millipore #MAB5324), Nestin (1:200; abcam #ab6142), MAP2 (1:750; abcam #ab11267), or p75NTR (1:500, Promega #G323A) in 1% BSA/1X PBS. The following secondary antibodies were used: goat anti-rabbit Alexa Fluor 488 (Life Technologies A-11008) and/or anti-mouse IgG Texas Red (Vector TI-2000) in 1% BSA/1X PBS, for two hours at room

temperature in the dark. For all immunocytochemistry, cells were mounted with ProLong Gold Antifade Mountant with DAPI (Thermo Fisher P36941) for microscopy. Cells were visualized on a Nikon Eclipse TE2000-S microscope at 10X or 20X magnification. Imaging was done with Nikon Elements software and analyzed using ImageJ software.

For colocalization studies, primary cortical neurons were transfected via calcium phosphate precipitation using JAK2:eGFP. Twenty-four hours after transfection, cells were fixed in fixing solution (10 minutes), washed and permeabilized in 5% BSA/0.1% Triton-X/1X PBS (20 minutes). Plates were incubated overnight at 4°C with primary antibody P75NTR (Promega G3231) in 5% BSA/1X PBS. Secondary antibody goat anti-rabbit Alexa Fluor 568 (Life Technologies A-11011) in 5% BSA/1X PBS was added for 1 hour at room temperature in the dark.

13. Proximity Ligation Assay

For proximity ligation assay (PLA), neurons were transfected via Neuromag magnetofection (Oz Biosciences) with JAK2:eGFP alone or with p75NTR:Flag. Cells were fixed, permeabilized, and blocked (30 minutes, 37°C) in the blocking solution provided with the Duolink In Situ PLA probes (Sigma Aldrich). Cells were incubated overnight at 4°C in primary mouse anti-GFP (Abcam 1218; 1:500) and rabbit anti-Flag (Sigma Aldrich F7425; 1:250). After washing, cells were incubated in Duolink In Situ PLA Probes anti-rabbit PLUS (Sigma Aldrich DUO92002) and anti-mouse MINUS (Sigma Aldrich DUO92004)

for 1 hour at 37°C. Using red Duolink In Situ Detection reagents (Sigma Aldrich DUO92008), cells were incubated in ligation solution containing ligase (30 minutes, 37°C), and incubated in amplification solution containing polymerase (80 minutes, 37°C), protected from light. Cells were washed and mounting media with DAPI was added to the dish. Confocal images (1 µm thick slices) were taken on a Zeiss LSM 710 microscrope (Zen Software) to visualize red particles indicating presence of (i)p75NTRJ2. ImageJ software was used for visualization. Schematic of PLA that detects p75NTR:Flag and JAK2:eGFP association (predicted distance <40 nm).

14. Enzyme-linked immunosorbent assay (ELISA)

Primary cortical neurons were co-treated with Ruxolitinib (0.05, 0.5, 5, 25, 50 nM) and BDNF (100nM, 30 minutes). Protein was extracted using standard RIPA lysis buffer technique. Equal amounts of protein were added to each well for ELISA detection. Phospho STAT3 levels were measured using a specific STAT3 (pY705) ELISA kit (Life technologies #KHO0481).

15. EdU staining

The Click-iT® EdU (5-ethynyl-2´-deoxyuridine) Alexa Fluor 594 Imaging Kit (Invitrogen C10339) was used to stain proliferating neurons. Live cells were incubated with EdU (10uM) for 2-4 hours (37°C, 5% CO_2). Cells were fixed in 3.7% formaldehyde (15 minutes, room temperature) and permeabilized in 0.5% Triton X-100 in 1X PBS (20 minutes, room temperature). The cells were

incubated in Click-iT® reaction cocktail (1X Click-iT® tris-buffered saline reaction buffer, 100mM $CuSO_4$, Alexa Fluor® 594 azide, and 1X reaction buffer additive) for 30 minutes at room temperature, protected from light. Immunocytochemistry was continued with incubation overnight at 4°C with primary antibody to p291 LSF in 1% BSA/1X PBS. Secondary antibody goat anti-rabbit Alexa Fluor 488 in 1% BSA/1X PBS was added for 1 hour at room temperature in the dark. ProLong Gold Antifade Mountant with DAPI (Thermo Fisher P36941) was used to mount the cells for imaging.

16. Luciferase Promoter Assay

GABRA1 promoter luciferase construct was previously made in lab by cloning GABRA1 promoter fragments (-894/+70) upstream of the luciferase gene in a pGL2 vector (Promega). The promoter fragment confers full promoter activity in primary cortical neurons. Neurons were co-transfected via calcium phosphate precipitation with an alpha1 promoter luciferase plasmid construct and full-length rat BDNF or empty vector pcDNA. Treatment with anti-BDNF (Calbiochem GF35L) to quench extracellular BDNF or a control normal mouse IgG (Millipore 12-371) was started after transfection (16-24 hours). The general protocol for the Luciferase Assay System (Promega E1500) was used. Twenty-four hours after transfection, cells were lysed in the plates by adding Passive Lysis Buffer (Promega E153A) and shaking (20 minutes, room temperature). Cells were scraped, collected, and 20ul of each sample was loaded, in duplicates, onto a 96-well plate. Using a Glomax (Promega) machine, Luciferase Assay Substrate

(Promega E151A/E152A) was added to each well and luminescence was measured for each sample. To normalize these measurements, protein concentration for each sample was determined using Coomassie Plus™ Protein Assay (Thermo Scientific #1856210).

17. Pilocarpine-Induced Model of Status Epilepticus

The laboratory of Dr. Amy Brooks-Kayal performed the pilocarpine-induced SE model in adult rats according to their protocols (Brooks-Kayal et al., 1998b). All rats treated with a full dose of pilocarpine (350mg/kg; ip) were confirmed to have prolonged Stage 5 seizures on the Racine scale (Racine, 1972). Control animals received a sub-convulsive dose of pilocarpine (35mg/kg; ip) and were confirmed to not have seizure activity. Rats were sacrificed 24 hours after induction of SE and a bilateral dissection of the hippocampus was done. Protein extraction was done using RIPA lysis buffer.

18. Data Analysis and Statistics

Statistical analysis was done using Microsoft Excel or Prism software. Significance was evaluated using a 95% confidence interval. Data are expressed as fold change or percentage fold change over control, with control defined as 100% or 1. All data are expressed as mean ± standard error of the mean (SEM), which is the standard deviation divided by the square root of the number of observations per group. For data sets with two groups, data was analyzed using a Student's t-test. For data sets containing three or more groups, a one-way

analysis of variance (ANOVA) was performed, followed by Tukey's multiple comparision post-hoc test for significance between the groups.

For statistical notation in the text, mean is referred to as "M" and standard error of the mean is referred to as "SEM." For T-tests, the t-value, a measure of the size difference relevative to the variation in my data sample, is reported in the form t(degrees of freedom) = t-value. For one-way ANOVAs, F ratio, the ratio of two mean square values, is reported in the form F(degrees of freedom between groups, degrees of freedom within groups) = F ratio.

III: RESULTS: The relationship between BDNF and JAK/STAT signaling in neurons.

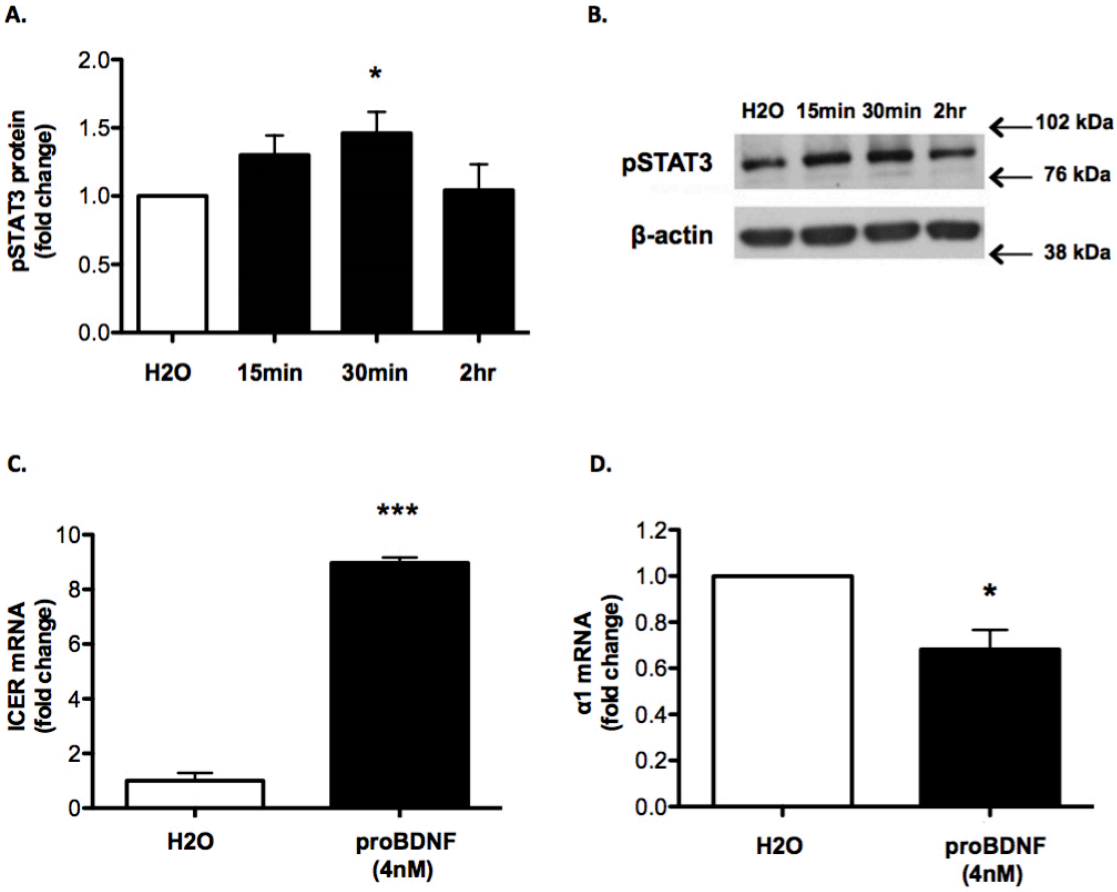
A. Neurotrophin specific activation of ICER synthesis in neurons.

Increased levels of BDNF mRNA and protein have been observed in animal models of TLE; however, it is not clear whether proBDNF and matBDNF and their receptors contribute to this effect. Previous research in our laboratories (Lund et al., 2008) has demonstrated that recombinant matBDNF increases pSTAT3 levels, which induces ICER, a potent inhibitor of genes containing cAMP response elements (CRE sites). We also found that STAT-dependent synthesis of ICER, in response to matBDNF, downregulates the number of α1 containing synaptic GABARs. This may be relevant to changes in brain inhibition reported for multiple disease endpoints, including models of TLE and human epilepsy patients. As BDNF exists in two isoforms in neurons, a precursor form (proBDNF) and a cleaved, mature form (matBDNF), we asked whether ICER induction and subsequent downregulation of α1 subunit gene (Gabra1) expression is specific to matBDNF signaling or could also be produced by proBDNF. In collaboration with Rebecca Benham, we show that, at the same concentration of matBDNF (50 ng/ml or 4 nM) previously used by our labs to identify BDNF-induced JAK/STAT signaling in neurons (Lund et al., 2008), that recombinant proBDNF (i.e. missing the cleavage site necessary for production of matBDNF) activates STAT3 in a time-dependent manner (Figure 4A and B), induces ICER (Figure 4C), and downregulates levels of α1 transcript (Figure 4D).

Figure 4: ProBDNF activates JAK/STAT signaling and alters ICER and Gabra1 levels.

In collaboration with Rebecca Benham, Ph.D.

(A) Quantification of densitometry analysis of pSTAT3 protein in primary cortical neurons after 30 minutes of stimulation with vehicle or recombinant proBDNF (4nM). Western blot analysis was used to measure pSTAT3 expression, and is normalized to β-actin and total STAT3. Data are expressed relative to vehicle as fold change ± SEM (One-way ANOVA followed by Tukey's Multiple Comparison Test, n = 3, *p < 0.05). (B) Representative Western Blot of proBDNF-induced changes in pSTAT3 protein levels. (C) Quantification of RT-PCR analysis using Taqman primers and probe specific to ICER in primary cortical neurons after 4 hours of stimulation with vehicle (H_2O) or proBDNF (4nM). ICER mRNA expression was normalized to cyclophilin mRNA and expressed relative to vehicle as a fold change ± SEM (Student's t-test, n = 3, ***p < 0.001). (D) RT-PCR analysis quantification using Gabra1 primers and probes specific changes in gene expression after 24 hours of stimulation with vehicle (H2O) or recombinant proBDNF. Gabra1 expression was normalized to cyclophilin, and expressed relative to vehicle treatment as a fold change ± SEM (Student's t-test, n = 3, *p < 0.05).

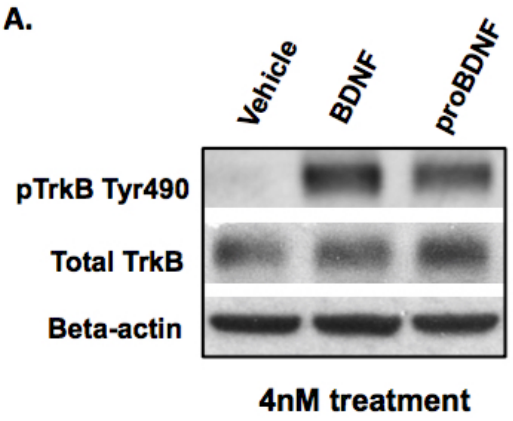

A.
pSTAT3 protein (fold change)
2.0
1.5
1.0
0.5
0.0
*
H2O
15min
30min
2hr
B.
H2O 15min 30min 2hr
pSTAT3
β-actin
102 kDa
76 kDa
38 kDa
C.
ICER mRNA (fold change)
10
8
6
4
2
0
***
H2O
proBDNF (4nM)
D.
α1 mRNA (fold change)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
H2O
proBDNF (4nM)

B. Concentration-dependent affect of proBDNF on TrkB activation.

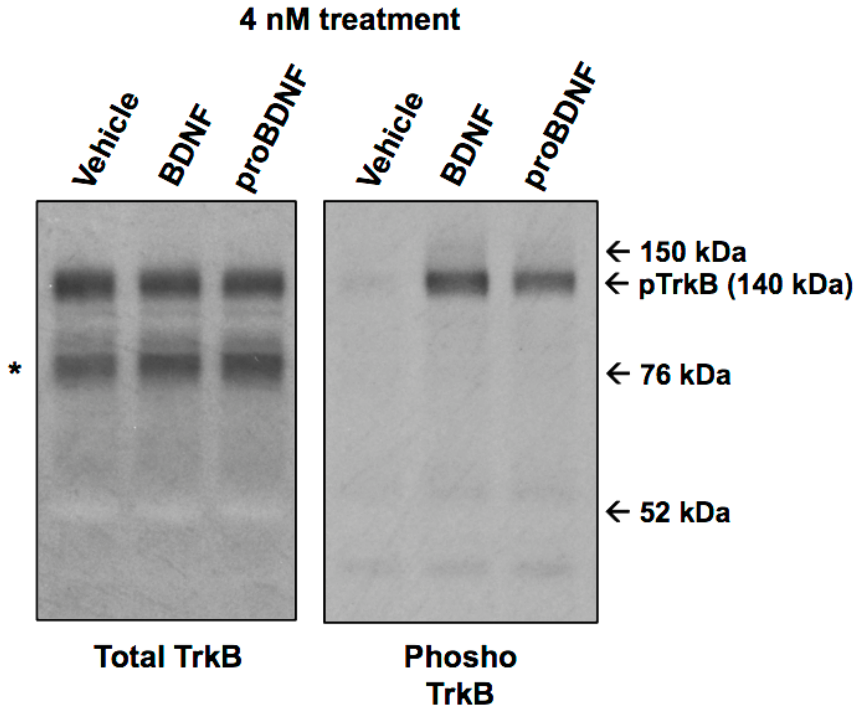
Since proBDNF binds with higher affinity to p75NTR than TrkB, we asked whether TrkB was differentially activated upon exposure to either proBDNF or BDNF or whether it was activated by both proBDNF and BDNF to signal through the same cascade. At 4 nM, both matBDNF and recombinant proBDNF activate TrkB as detected using a TrkB phospho-specific antibody (Tyr490; Cell Signaling Technology) (Figure 5A). Decreasing the concentration about 10-fold (0.7 nM), there is a marked difference between the effects of the two BDNF signaling molecules (Figure 5B). This is consistent with an effect of 0.7 nM matBDNF, rather than proBDNF or nerve growth factor (NGF), in ICER induction, which is used as a downstream indicator of BDNF-induced JAK/STAT activation (Figure 5C). We also show that co-treatment with the TrkB antagonist K252A attenuates ICER induction (Figure 5D). Based on these observations, we conclude that extracellular activation of JAK/STAT signaling in neurons is dependent upon the activity of matBDNF at TrkB and not the action of proBDNF or NGF at p75NTR. Based on this result, matBDNF was used as the signaling molecule to investigate the function of JAK/STAT signaling in primary neurons. It is possible, however, that marked increases in proBDNF in response to brain injury may itself activate the JAK/STAT pathway via TrkB. Total TrkB full Western blots (Figure 5A and 5B) show a truncated form around 76 kDa (indicated by *), which has been reported in the literature (Kryl et al., 1999).

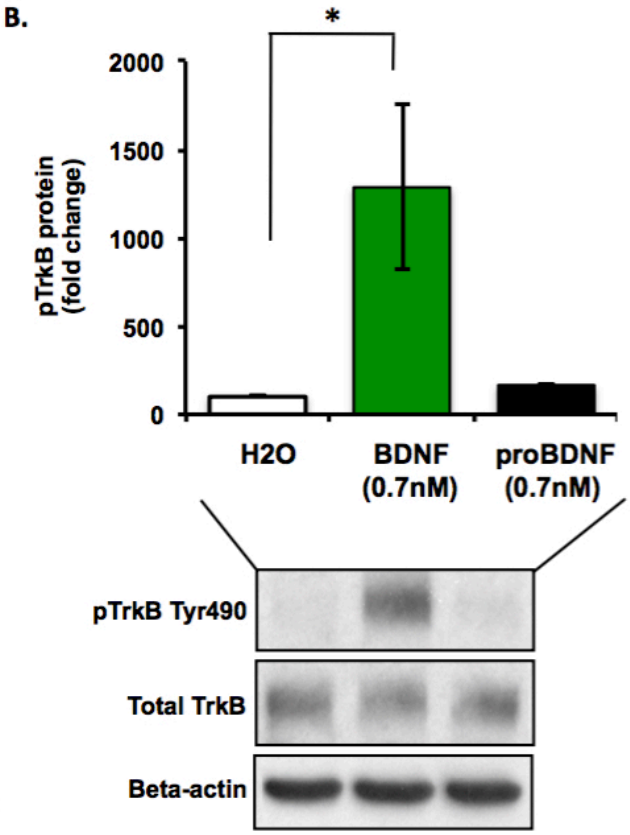
Figure 5. Role for TrkB in JAK/STAT signaling.

Primary cortical neurons were treated for 15 minutes with vehicle (H_2O), recombinant proBDNF, or matBDNF at (A) 4 nM, or (B) 0.7 nM. Whole cell protein extracts from treated cells were analyzed via Western blot. Antibodies against pTrkB (Tyr490) (1:1000, Cell Signaling #9194), total TrkB (1:2000, BD Biosciences #610101), and β-actin were used. ProBDNF at 4nM activates TrkB indicated by pTrkB ($n = 3$, $^*p < 0.05$), while at 0.7 nM it is without effect. *Note the faster migrating band in the full blots for total TrkB in (A) and (B) is the truncated form of TrkB. (C) Neurons were treated with matBDNF, proBDNF, or NGF (0.7 nM, 4 hours) in the presence and absence of TrkB agonist K252A (100 nM, 1 hour) which was applied as a pre-treatment. ICER mRNA levels were then detected in RNA extracts via RT-PCR. matBDNF induces ICER at 0.7 nM ($n = 4$, $^{***}p < 0.001$), while proBDNF and NGF do not. (D) ICER induction is blocked by TrkB agonist K252A ($n = 3$, $^*p < 0.05$), indicating TrkB-dependent activation of JAK/STAT signaling.

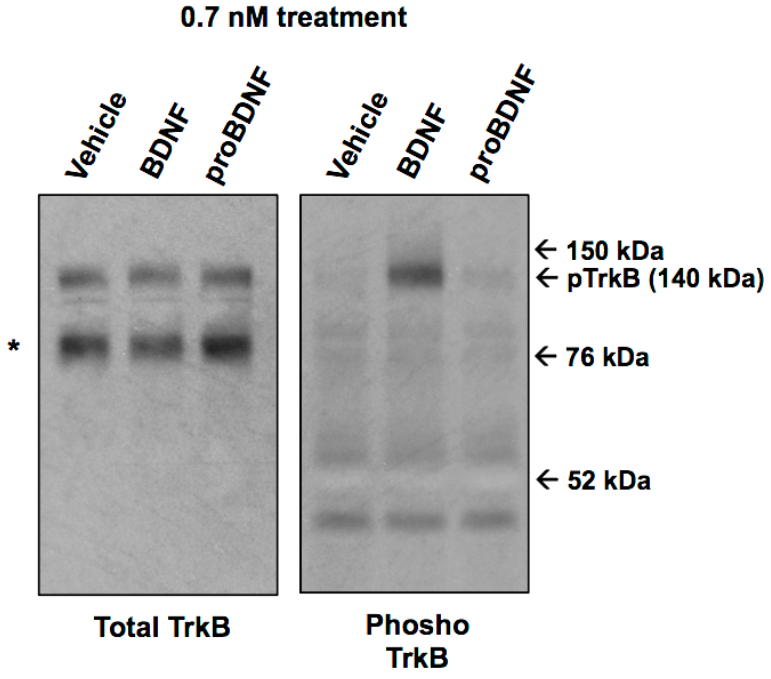

A.
Vehicle
BDNF
proBDNF
pTrkB Tyr490
Total TrkB
Beta-actin
4nM treatment

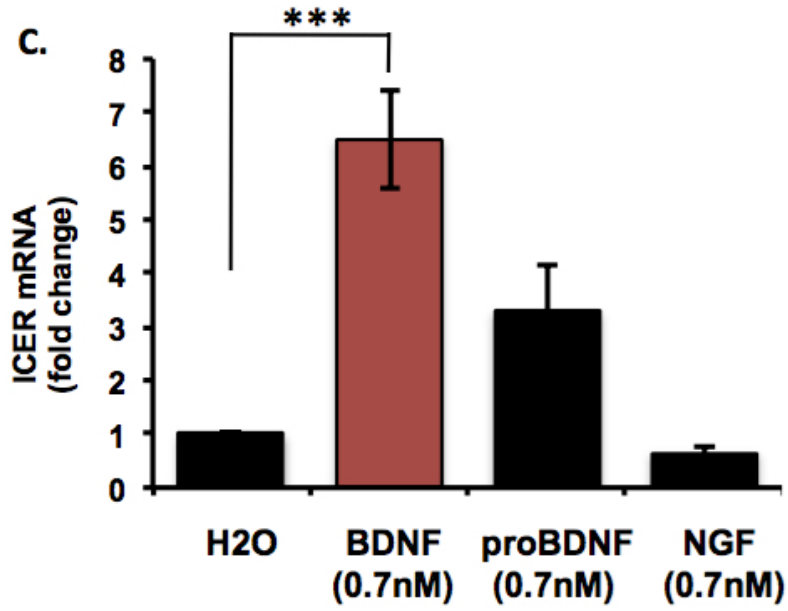
Full blots:

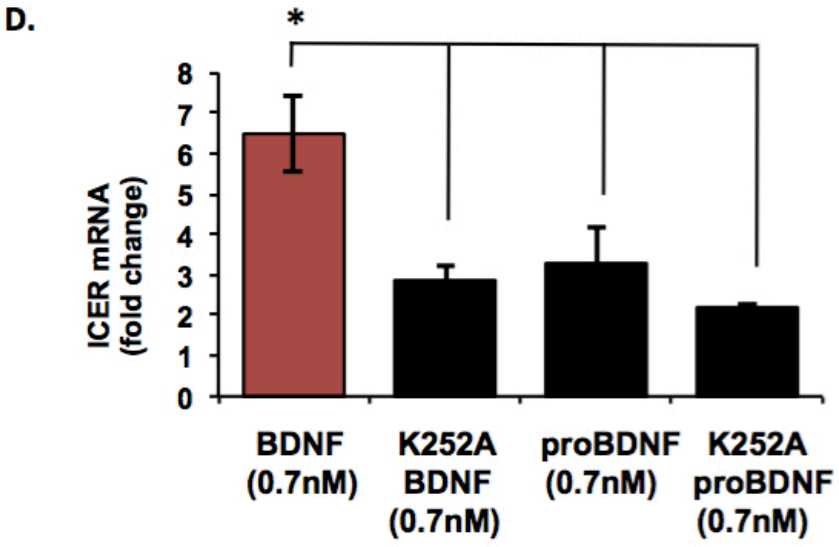

4 nM treatment
Vehicle
BDNF
proBDNF
Vehicle
BDNF
proBDNF
*
← 150 kDa
← pTrkB (140 kDa)
← 76 kDa
← 52 kDa
Total TrkB
Phosho TrkB


B.
*
2000
1500
1000
500
0
pTrkB protein (fold change)
H2O
BDNF (0.7nM)
proBDNF (0.7nM)
pTrkB Tyr490
Total TrkB
Beta-actin

Full blots:


0.7 nM treatment
Vehicle
BDNF
proBDNF
Vehicle
BDNF
proBDNF
*
← 150 kDa
← pTrkB (140 kDa)
← 76 kDa
← 52 kDa
Total TrkB
Phosho TrkB


C.
***
ICER mRNA
(fold change)
8
7
6
5
4
3
2
1
0
H2O
BDNF
(0.7nM)
proBDNF
(0.7nM)
NGF
(0.7nM)


D.
*
ICER mRNA
(fold change)
8
7
6
5
4
3
2
1
0
BDNF
(0.7nM)
K252A
BDNF
(0.7nM)
proBDNF
(0.7nM)
K252A
proBDNF
(0.7nM)

C. Fast activation of JAK/STAT Signaling by BDNF.

Our previous studies show decreased levels of *Gabra1* transcripts and α1 subunit protein, as well as α1-containing GABARs, 24 hours after primary neocortical or primary hippocampal cultures are treated with matBDNF (Lund et al., 2008). These changes are dependent on JAK/STAT signaling and consistent with epilepsy models. Whether or not these changes in receptor expression are due to an initial activation of JAK/STAT signaling by matBDNF had yet to be determined. We found that activation of the JAK/STAT pathway by BDNF occurs within minutes, where activated JAK2, indicated by the presence of phosphorylated JAK2 (pJAK2), is detected at 15 minutes after matBDNF treatment (Figure 6A). Fast activation of JAK2 is also seen in an animal model of SE (Figure 6B), where endogenous levels of BDNF substantially increase over time. To determine whether it is the initial activation of JAK/STAT signaling that drives changes in ICER gene expression, primary cortical neurons in culture were treated with matBDNF for 15 minutes, washed, and incubated in serum free media for 4 hours. Sister dishes were treated with matBDNF for the full 4 hours to serve as a positive control for ICER mRNA synthesis that we have previously reported. Stimulation for 4 hours with matBDNF induces a 5.3-fold increase in ICER expression as compared to vehicle control (H_2O) whereas shorter BDNF stimulation (15 minutes), known to induce peak pJAK2 levels (Figure 6A), followed by incubation in serum free media for 3 hours 45 minutes, increases

ICER mRNA levels by 7.6-fold (Figure 6C). Treatment with matBDNF (4 hours, 4 nM) also caused a decrease in full-length TrkB protein levels (Figure 6D).

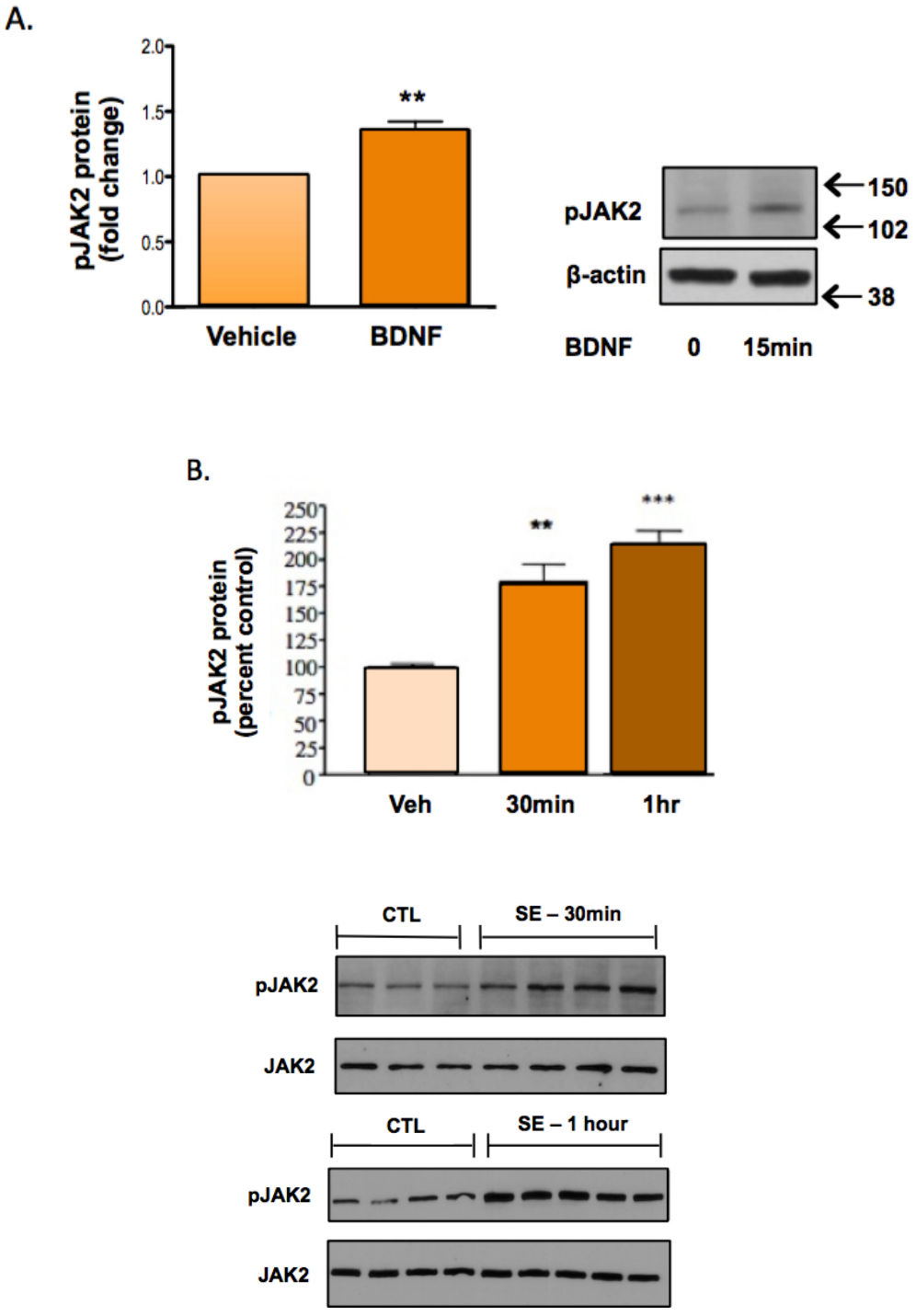
D. A role for both TrkB and p75NTR in JAK/STAT signaling.

Further identification of a specific role for TrkB in neuronal JAK/STAT signaling came from our studies using RNA silencing to deplete the levels of TrkB transcripts. Compared to scrambled controls, siRNAs selective for TrkB decrease endogenous TrkB mRNA levels by 31% in primary cortical cultures, consistent with the transfectional efficiency of the knockdown approach ($p = 0.04$; Figure 7A). Following 4 hours of treatment with matBDNF, selective knockdown of TrkB decreases levels of ICER mRNAs by 32% as compared to scrambled control ($p = 0.0049$; Figure 7B). Although levels of full-length p75NTR are low in the absence of BDNF as detected by western blot, marked increases in levels of p75NTR protein occur upon exposure to matBDNF (Figure 7C, right panel), suggesting that TrkB may regulate the synthesis and/or stabilization of p75NTR in neurons. Surprisingly, siRNAs specific to p75NTR, that reduce endogenous levels by 40% ($p = 0.0012$; Figure 7D), as compared to scrambled controls, produced a small (20%) but significant reduction in ICER mRNA expression ($p = 0.0336$; Figure 7E). We were unable to monitor effects of gene silencing on protein levels so whether p75NTR protein was efficiently downregulated by decreasing its mRNA pool remains unknown; but provides the rationale to further pursue a contribution of p75NTR to the neuronal JAK/STAT response.

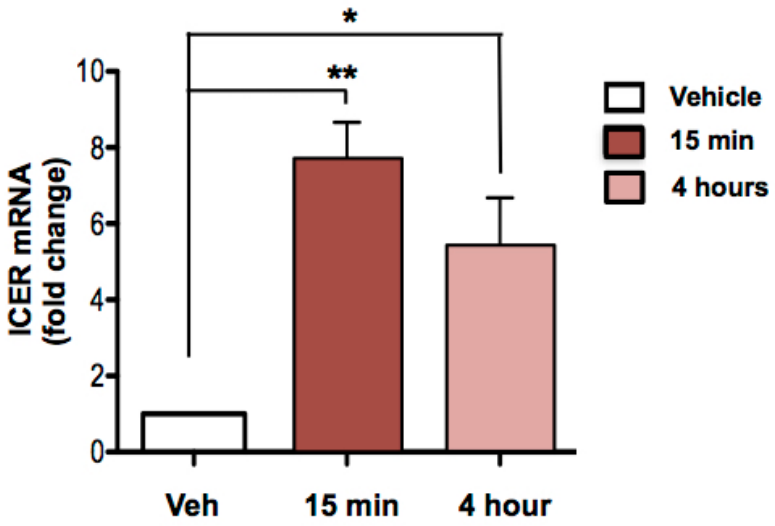
Figure 6. BDNF-induced JAK/STAT pathway activation.

In collaboration with Rebecca Benham, Ph.D.

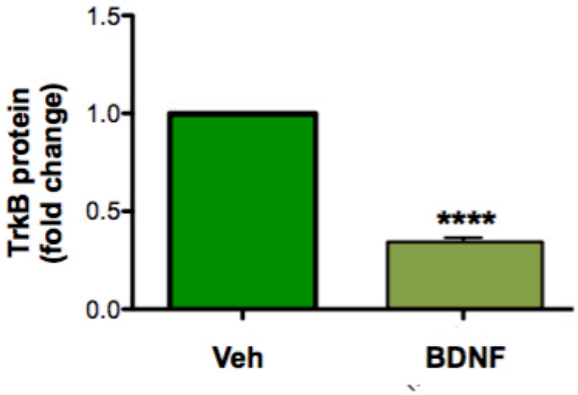
Primary neurons treated with (A) matBDNF (4 nM, 15 minutes) display increased pJAK2 protein levels as seen by Western blot analysis (n = 3, **p < 0.01). (B) Rat hippocampal protein extracts have elevated levels of pJAK2 30 min and 1 hour post-SE induction (n = 3, **p < 0.01, ***p < 0.001). (C) To determine long-term effects of fast activation of JAK/STAT signaling, neurons were treated with matBDNF for 15 minutes, washed, and collected 4 hours later. ICER mRNA is induced 7.6-fold after the 15-minute treatment, greater than the 5.3-fold increase in ICER after 4 hours of matBDNF treatment (n = 3, *p < 0.05, **p < 0.01). (D) TrkB protein levels decrease after 4 hours of matBDNF treatment (n = 3, ****p < 0.0001).


A.
pJAK2 protein (fold change)
2.0
1.5
1.0
0.5
0.0
**
Vehicle
BDNF
pJAK2
β-actin
150
102
38
BDNF 0 15min
B.
pJAK2 protein (percent control)
250
225
200
175
150
125
100
75
50
25
0
**
***
Veh
30min
1hr
CTL
SE – 30min
pJAK2
JAK2
CTL
SE – 1 hour
pJAK2
JAK2

C.


*
**
10
8
6
4
2
0
ICER mRNA
(fold change)
Vehicle
15 min
4 hours
Veh
15 min
4 hour

D.


1.5
1.0
0.5
0.0
TrkB protein
(fold change)
****
Veh
BDNF

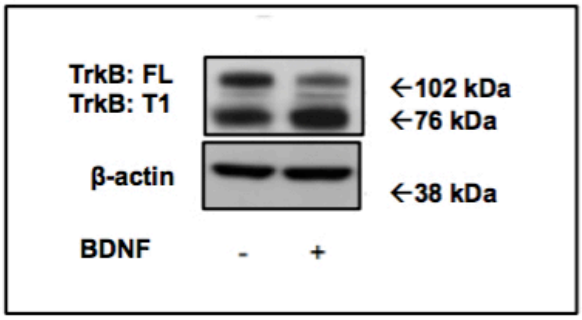
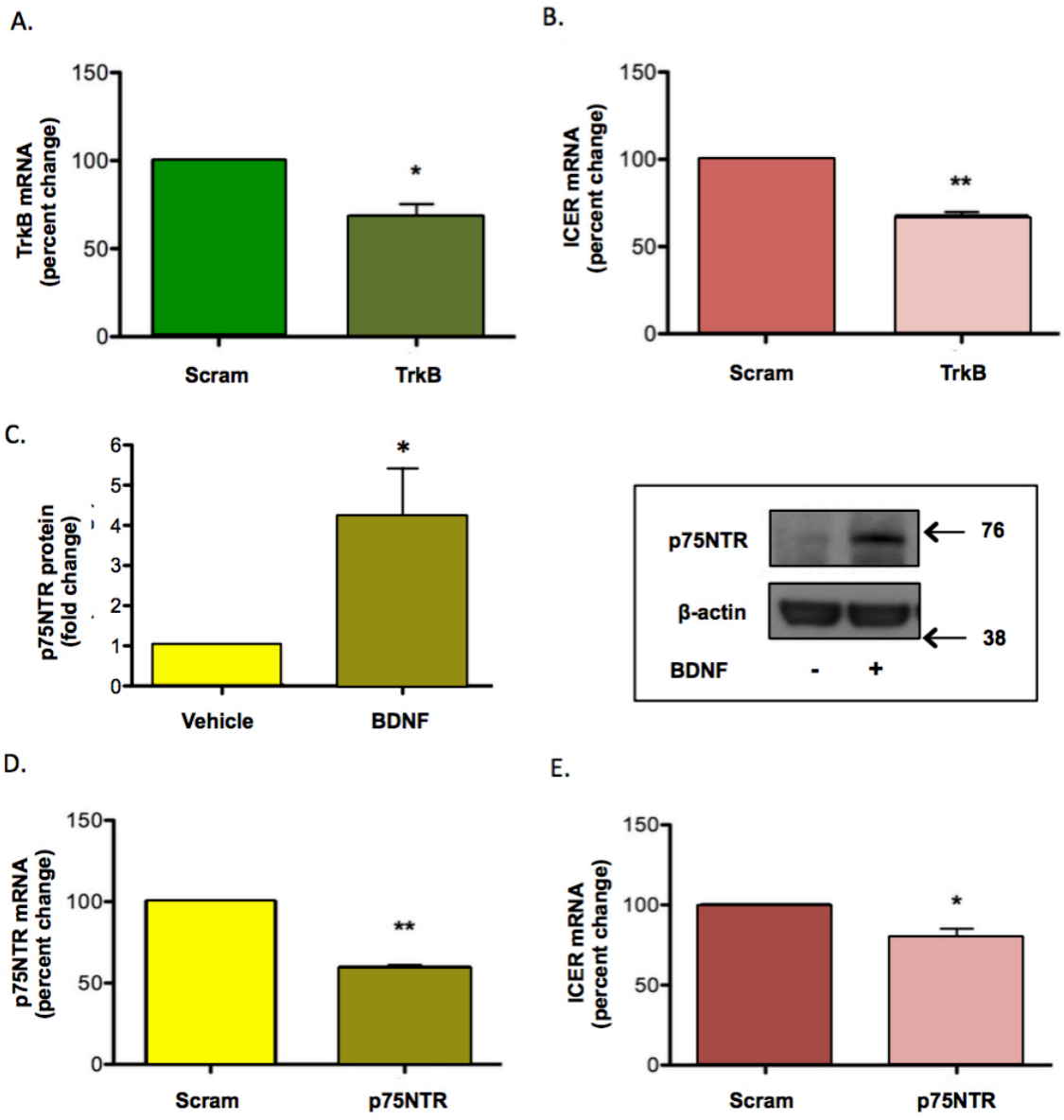

TrkB: FL
TrkB: T1
←102 kDa
←76 kDa
β-actin
←38 kDa
BDNF
-
+

Figure 7: Gene Silencing of TrkB and P75NTR.

In collaboration with Rebecca Benham, Ph.D.

Gene silencing using siRNA targeted against TrkB decreased (A) TrkB protein levels (Student's t-test, *$p < 0.05$) (B) and decreased ICER mRNA expression (Student's t-test, **$p < 0.01$). (C) BDNF treatment of primary neurons resulted in increased p75NTR protein (Antibody: Novus Biologicals NB110-58000; 1:1000) via Western blot analysis. Gene silencing of p75NTR with siRNA (D) decreased P75NTR protein expression (Student's t-test, **$p < 0.01$) and (E) decreased ICER mRNA expression (Student's t-test, *$p < 0.05$).


A.
TrkB mRNA (percent change)
150
100
50
0
*
Scram
TrkB
B.
ICER mRNA (percent change)
150
100
50
0
**
Scram
TrkB
C.
p75NTR protein (fold change)
6
5
4
3
2
1
0
*
Vehicle
BDNF
p75NTR
76
β-actin
38
BDNF
-
+
D.
p75NTR mRNA (percent change)
150
100
50
0
**
Scram
p75NTR
E.
ICER mRNA (percent change)
150
100
50
0
*
Scram
p75NTR

E. Evidence for a p75NTR/JAK2 complex in neurons and HEK.

To further delineate the respective roles of p75NTR and TrkB in JAK/STAT signaling, co-immunoprecipitation (co-IP) was performed to test the potential association of these receptors with JAK proteins in cell lines and in primary neuronal cultures. Employing antibodies (Abs) to the C-terminal region of p75NTR, generously provided by the Phil Barker laboratory (McGill) and referred to as p75ICD-Ab, as well as those purchased from Novus Biologicals (CAT# NB110-58000), we found that neuronal extracts purified using p75NTR Abs are enriched in JAK2 proteins and most importantly that the level of activated JAK2 (pJAK2) within such complexes increases (90%) after 15 min of matBDNF stimulation ($p = 0.031$; Figure 8A), consistent with a role for p75NTR in JAK/STAT signaling. As p75NTR is considered promiscuous in its ability to dimerize with many other receptor types causing opposite cellular responses (Barker, 2009), we determined whether extracts from primary cortical neurons precipitated with JAK2 also contain either TrkB or sortilin. No evidence was found for an association with either protein using conditions where JAK2 antibodies precipitate p75NTR but was found for STAT3 (Figure 8B). Therefore, when taken together with the results of TrkB siRNA knockdown, our results strongly suggest that there is a functional rather than structural link between the two BDNF receptor subtypes (TrkB and p75NTR) in neuronal JAK/STAT signaling.

F. Residues in p75NTR transmembrane domain modulate JAK2 association.

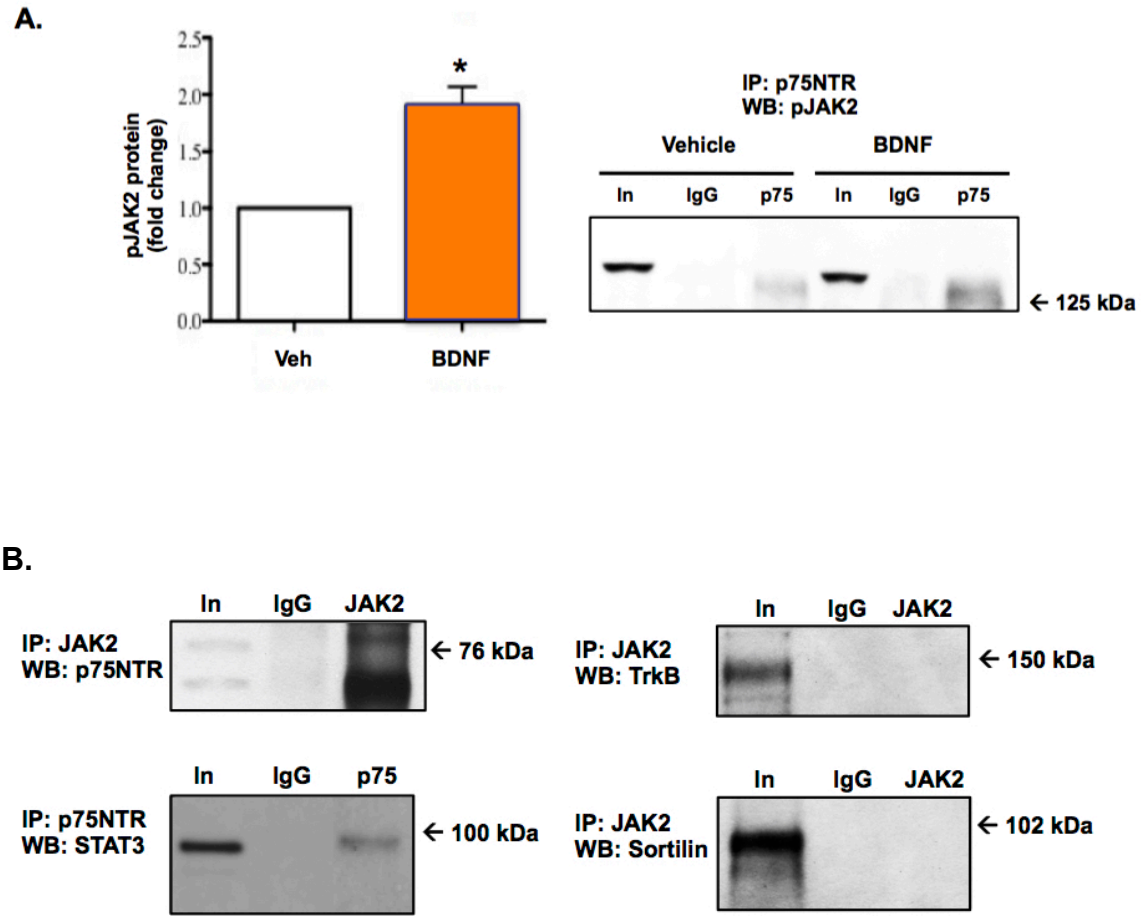
The validity of the JAK2/p75NTR interaction can best be seen, however, using co-IP of fusion proteins JAK2:eGFP and p75NTR:Flag (gift of Phil Barker, McGill) after co-expression in HEK cells. As shown in Figure 8C, JAK2:eGFP is detected with a GFP-Ab upon co-IP using an Ab to the Flag tag in the p75NTR fusion protein, consistent with the association of these two endogenous proteins in neurons as presented in Figure 8A and 8B. To determine whether the transmembrane domain of p75NTR is required for its association with JAK2, we made two mutations in this region of the human protein: cysteine 256 and glycine 265. These residues aligned with rat p75NTR cysteine 257 and glycine 266, which have been previously shown to attenuate p75NTR dimerization at the cell surface as well as receptor coupling to particular intracellular signaling cascades (Vilar et al., 2009). Interestingly, these investigators report that the mutations do not affect the ability of p75NTR to form higher order oligomers in cytoplasm. Indicated in Figure 9A, there is a dramatic increase in the amount of JAK2 fusion protein that is precipitated by Flag Ab from transfected HEK that co-express mutant p75NTR:Flag proteins. Greatest precipitation of JAK2 is seen in extracts that contain p75NTR(G265I):Flag, suggesting that residues in the transmembrane domain, while critical for dimerization of p75NTR at the cell surface, may also regulate its association with JAK2 in the cytoplasm.

To determine if JAK2 expression influences the oligomerization of p75NTR, we transfected HEK cells with either p75NTR alone, JAK2 alone, or co-transfected with both p75NTR and JAK2, and analyzed p75NTR protein levels via Western blot under denaturing (+DTT) as well as non-denaturing (-DTT) conditions, to retain the oligomers. Transfection of wildtype p75NTR with JAK2 in HEK cells increases the formation of p75NTR oligomers (Figure 9B) suggesting that JAK2 may promote the formation of a distinct intracellular p75NTR-containing complex. We do not know at this time whether such complexes come from the glycosylated form or presumed non-glycosylated form (see full gel in lower panel of Figure 9B). Whether or not the signal in lanes 2 and 6 of Figure 9B reflect the presence of endogenous JAK2 and its influence over the formation of p75NTR oligomers, still remains to be determined.

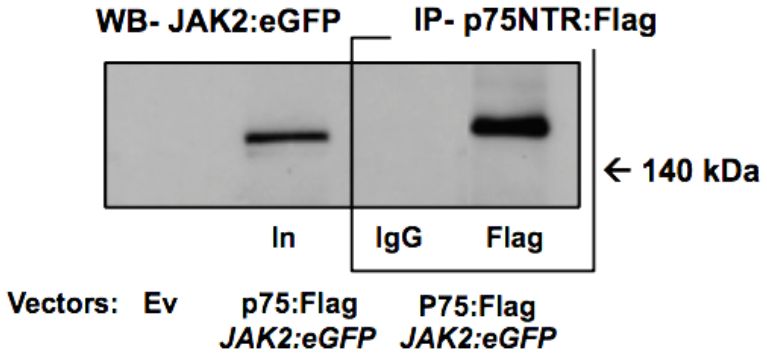
Figure 8. A P75NTR/JAK2 complex is found in neurons and HEK.

In collaboration with Rebecca Benham, Ph.D.

(A) Co-IP of protein extracts from primary neuronal cultures treated with BDNF show increased association of endogenous p75NTR and pJAK2 (Student's t-test, *$p < 0.05$). Pull down was done with p75NTR antibody and immunoblot was probed for pJAK2. (B) Further co-IPs found endogenous association of JAK2 with p75NTR, and p75NTR with STAT3; with no association between JAK2 and TrkB, or JAK2 and Sortilin, even after chemical crosslinking with DSP/DTSSP. (C) CoIP of protein extracts from HEK cells co-transfected with p75NTR:Flag and JAK2:eGFP constructs. Pulldown of proteins with anti-Flag and Western blot detection with anti-GFP identifies presence of p75NTR:Flag/JAK2:eGFP complexes. EV: Empty vector transfected negative control. In: Input sample made from total protein extract of p75NTR/JAK2:eGFP transfected cells. IgG: Control normal rabbit IgG pulldown. Flag: Pulldown with anti-Flag antibody. *Note that the strong bands around 50 and 20 kDa represent heavy and light chains of IgG.


A.
pJAK2 protein (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
*
Veh
BDNF
IP: p75NTR
WB: pJAK2
Vehicle
BDNF
In
IgG
p75
In
IgG
p75
← 125 kDa
B.
In
IgG
JAK2
IP: JAK2
WB: p75NTR
← 76 kDa
In
IgG
JAK2
IP: JAK2
WB: TrkB
← 150 kDa
In
IgG
p75
IP: p75NTR
WB: STAT3
← 100 kDa
In
IgG
JAK2
IP: JAK2
WB: Sortilin
← 102 kDa

C.


WB- JAK2:eGFP
IP- p75NTR:Flag
← 140 kDa
In
IgG
Flag
Vectors: Ev
p75:Flag
JAK2:eGFP
P75:Flag
JAK2:eGFP

Full blot:

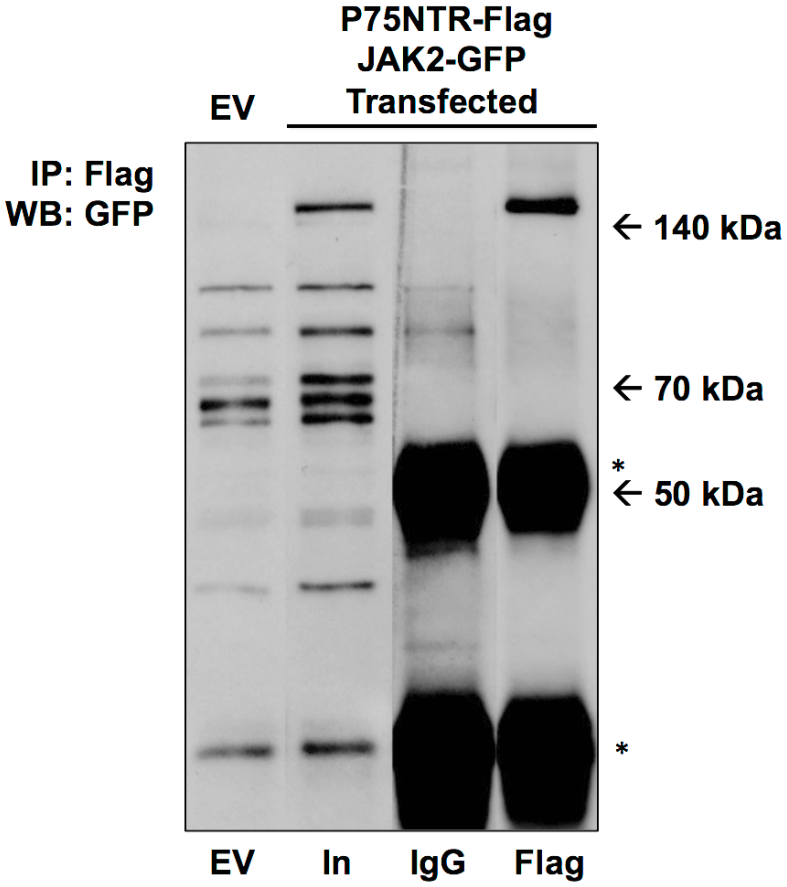
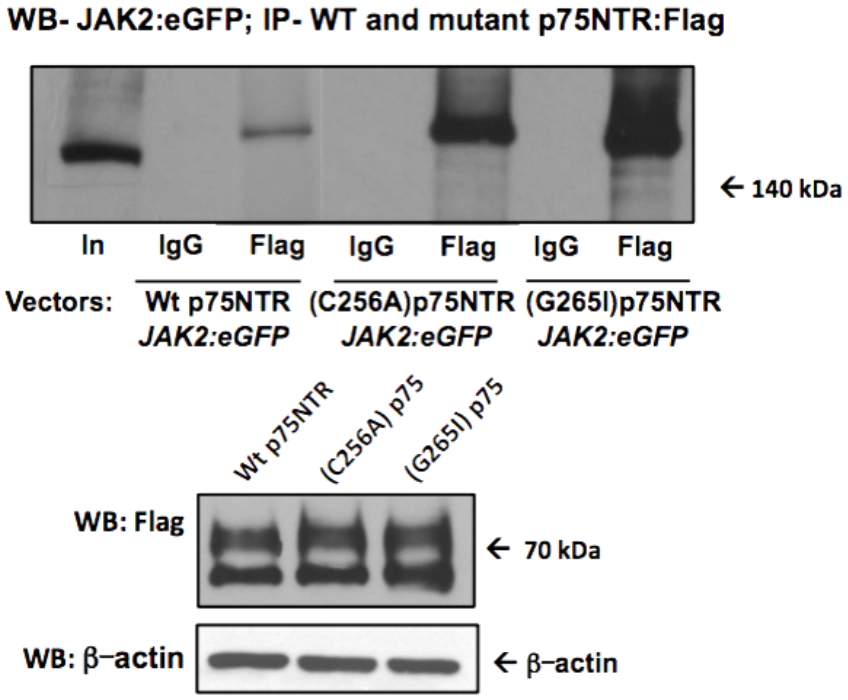

P75NTR-Flag
JAK2-GFP
Transfected
EV
IP: Flag
WB: GFP
← 140 kDa
← 70 kDa
*
← 50 kDa
*
EV
In
IgG
Flag

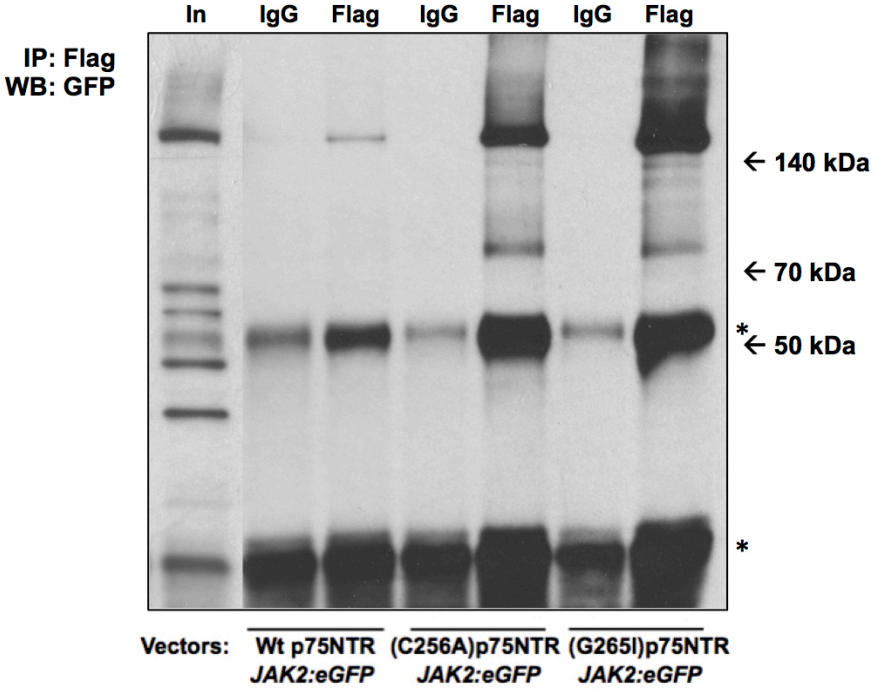
Figure 9: Transmembrane domain p75NTR residues modulate JAK2 association.

(A) CoIP of HEK cells transfected with JAK2:eGFP and either (C256A)p75NTR:Flag or (G265I)p75NTR:Flag. Pulldown of proteins with anti-Flag and Western blot detection with anti-GFP indicates that mutations in the p75NTR:Flag transmembrane domain increase JAK2:eGFP precipitation. Lane labels: In: Input sample made from total protein extract of p75NTR/JAK2:eGFP transfected cells. IgG: Control normal rabbit IgG pulldown. Flag: Pulldown with anti-Flag antibody. Inputs samples (A middle panel) were via Western blot for Flag and β-actin to check equal transfection. *Note that the strong bands around 50 and 20 kDa on the full blot represent heavy and light chains of IgG. (B) Protein samples from HEK cells transfected with either p75NTR:Flag (P75), JAK2:eGFP (JAK2), or both p75NTR:Flag and JAK2:eGFP (P75/JAK2) were compared to non-transfected (NT) controls. The Western blot was probed with a p75NTR antibody (p75ICD, gift of P. Barker). Samples were made with denaturing (+DTT) as well as non-denaturing (-DTT) conditions to visualize dimer and oligomer formation. Transfection of wildtype p75NTR with JAK2 in HEK cells increases the formation of p75NTR oligomers.

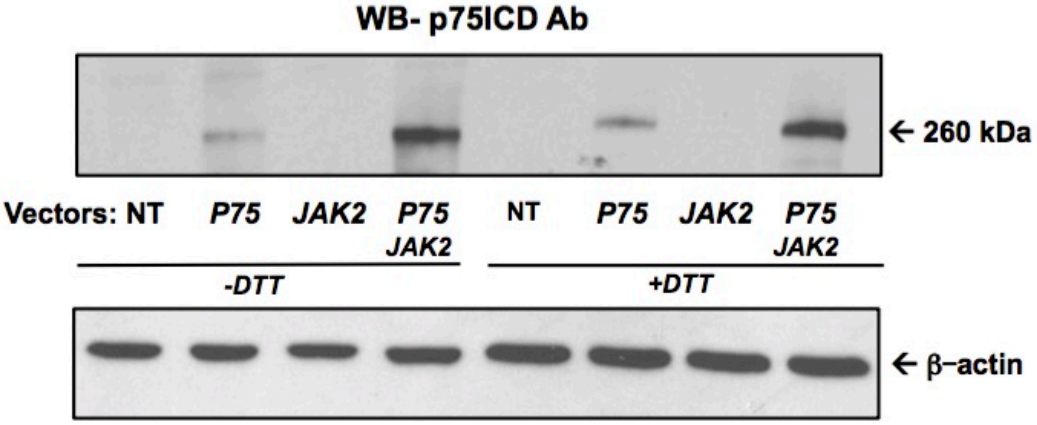
A.


WB- JAK2:eGFP; IP- WT and mutant p75NTR:Flag
← 140 kDa
In
IgG
Flag
IgG
Flag
IgG
Flag
Vectors:
Wt p75NTR JAK2:eGFP
(C256A)p75NTR JAK2:eGFP
(G265I)p75NTR JAK2:eGFP
Wt p75NTR
(C256A) p75
(G265I) p75
WB: Flag
← 70 kDa
WB: β–actin
← β–actin

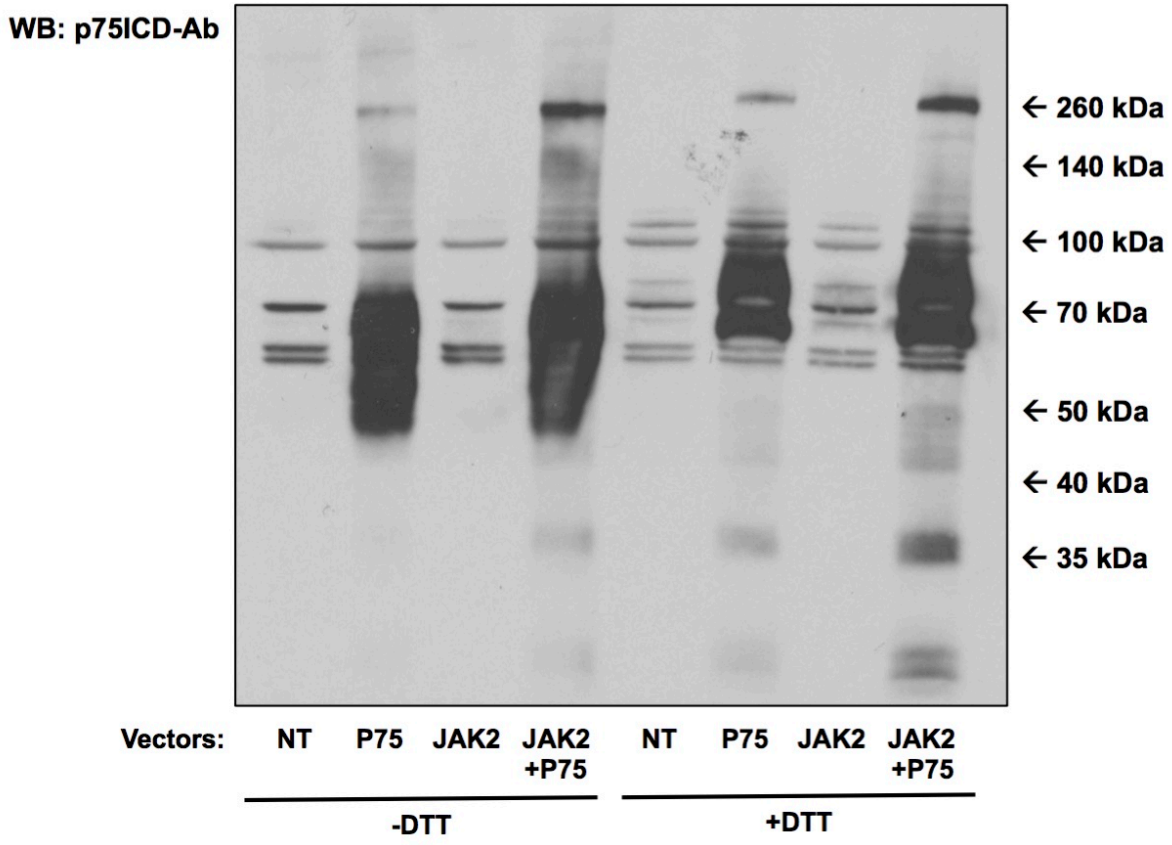
Full blot:


In
IgG
Flag
IgG
Flag
IgG
Flag
IP: Flag
WB: GFP
← 140 kDa
← 70 kDa
*
← 50 kDa
*
Vectors:
Wt p75NTR JAK2:eGFP
(C256A)p75NTR JAK2:eGFP
(G265I)p75NTR JAK2:eGFP

B.


WB- p75ICD Ab
← 260 kDa
Vectors: NT P75 JAK2 P75 JAK2 NT P75 JAK2 P75 JAK2
-DTT
+DTT
← β−actin

Full blot:


WB: p75ICD-Ab
← 260 kDa
← 140 kDa
← 100 kDa
← 70 kDa
← 50 kDa
← 40 kDa
← 35 kDa
Vectors: NT P75 JAK2 JAK2 +P75 NT P75 JAK2 JAK2 +P75
-DTT
+DTT

G. Detection of p75NTR/JAK2 complexes in neuronal processes, cell body, and nucleus.

As a first step to examine where p75NTR/JAK2 complexes are located in the cell, primary cortical neurons were transfected with JAK2:eGFP fusion proteins and immunocytochemistry was performed using antibodies to endogenous p75NTR. As can be seen in Figure 10A-10C, where a single z slice of two representative neurons are displayed, neurons can differ in the localization of both JAK2:eGFP and p75NTR. In addition, not all JAK2:eGFP is associated with p75NTR. There are discrete pockets of co-localization of p75NTR and JAK2:eGFP seen in a single Z slice (Figure 10C) and Z-sum (Figure 10D), as well as in Figure 10E where highest intensity of co-localized points is pseudocolored in green across the Z stack.

H. Detection of p75NTR/JAK2 complexes in neuronal processes, cell body, and nucleus using Proximity Ligation Assay (PLA).

To test for a direct association between p75NTR and JAK2, the proximity ligation assay (PLA, see Methods) was performed using primary cortical neurons transfected with the expression constructs p75NTR:Flag and JAK2:eGFP in intact cells. PLA can detect association between proteins if they are within 30-40 nanometers (nm) using secondary antibodies conjugated to complementary oligonucleotides and detected by in situ hybridization to the products of rolling circle PCR (Figure 11A). As can be seen in Figure 11B and 11D, PLA reveals

that p75NTR/JAK2 complexes (red signals) can form in neurons and that their co-localization and distribution is consistent with detection of endogenous p75NTR and JAK2:eGFP (Figure 10). Detection of native fluorescence from JAK2:eGFP in the cell body of the z-stack always occurs prior to detection of PLA signals, suggesting that the majority of the PLA detected interactions are in the cytoplasm rather than close to the cell membrane, as expected for a transmembrane receptor. Also note the presence of red signals in the nucleus (blue stain) of the magnified Z slice (1 micron thickness) in Figure 11B and the right panel orthogonal views of the full stack that show the red signal sandwiched between layers of the native fluorescence from JAK2:eGFP (Figure 11B, right panel). No red signal, however, is detected when neurons are transfected with an eGFP transgene instead of p75NTR:eGFP (Figure 11C). Detection of PLA signals specific to two transfected primary cortical neurons with z-slice summation is also shown (Figure 11D). The constellation of evidence including co-IP, overlapping fluorescent signals specific to endogenous p75NTR and JAK2:eGFP, and the close proximity of p75NTR:flag and JAK2:eGFP as detected by PLA, strongly suggest that neurons contain an intracellular p75NTR/JAK2 complex that is a component of the JAK/STAT pathway in neurons. We shall refer to this complex as the signalsome (i)p75NTRJ2 throughout the remainder of the manuscript where (i) represents the intracellular presence of the complex and (J2) the association of p75NTR with JAK2.

Figure 10. Confocal analysis of p75NTR/JAK2-eGFP complexes.

Primary neuron cultures were transfected with JAK2:eGFP, fixed, permeabilized, and labeled for endogenous p75NTR with a rabbit primary antibody to p75NTR (1:500, Promega #G323A), followed by secondary with goat anti-rabbit Alexa Fluor 568 (Molecular Probes #A-11011). Images for separate (A) JAK2:eGFP (green) and (B) endogenous p75NTR (red) combined in (C) to show co-localization (white arrows). (D) Z-sum (1 μm thick slices) of confocal image stack and (E) Z slice-summation of co-localized points. JAK2:eGFP: green. p75NTR: red. Co-localization: yellow. Magnification: 63x. Scale bar = 50μm

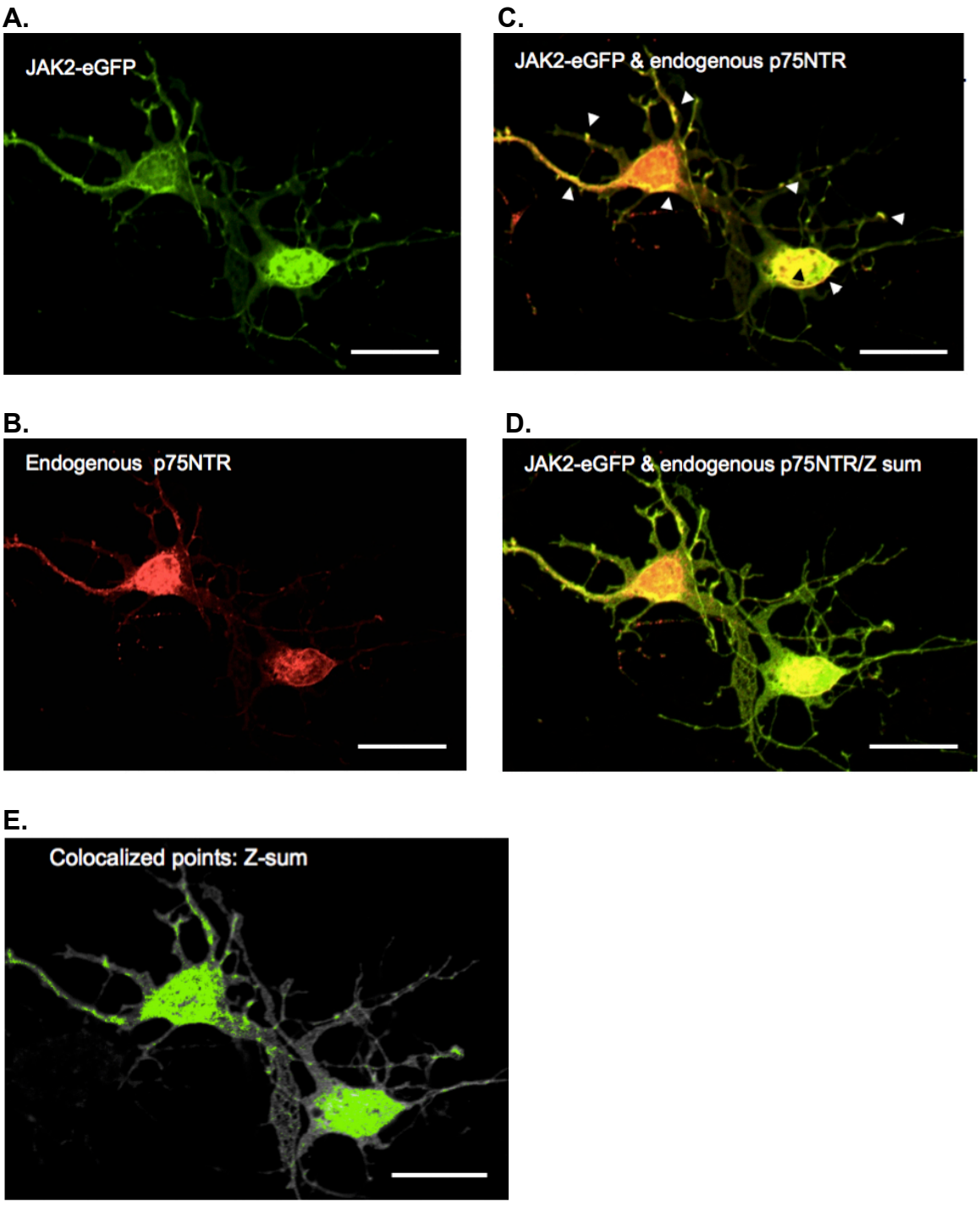
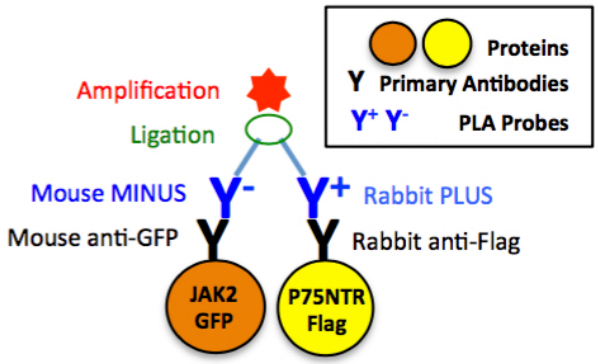

A.
JAK2-eGFP
C.
JAK2-eGFP & endogenous p75NTR
B.
Endogenous p75NTR
D.
JAK2-eGFP & endogenous p75NTR/Z sum
E.
Colocalized points: Z-sum

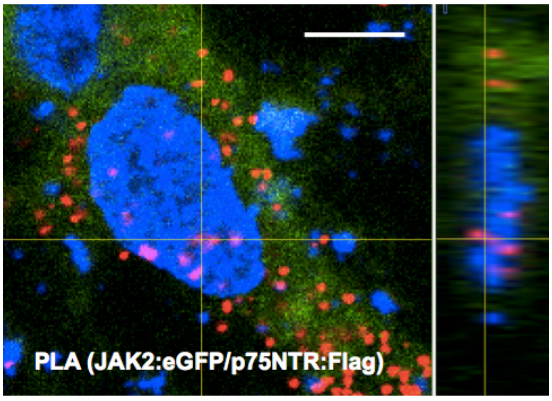
Figure 11. p75NTR-Flag and JAK2-eGFP complex *in situ*.

Neurons were cotransfected with JAK2:eGFP and p75NTR-Flag, fixed, permeabilized, and PLA assay (A) was conducted with anti-GFP and anti-Flag antibodies to visualize interaction between JAK2:eGFP and p75NTR-Flag. (B) Single slice, PLA of neuron transfected with JAK2:eGFP (green) and p75NTR-Flag; PLA complex is visualized by red dots. DAPI: blue. Right; orthogonol view showing all slices. (C) Single slice, negative control transfected with non-specific pmaxGFP and p75NTR-Flag. Note that there are no red puncta. Right; orthogonol view showing all slices. (D) Detection of PLA signals specific to two transfected primary cortical neurons with z-slice summation (set at signal intensity, ImageJ) Scale bar for (B) and (C) = 20 μm. Scale bar for (D) = 50μm. Magnification: 63x.

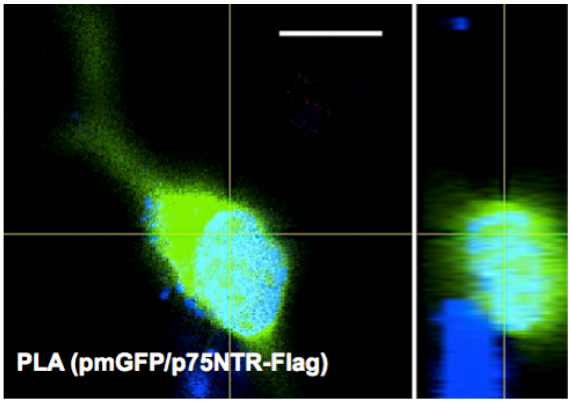
A.


Proteins
Primary Antibodies
PLA Probes
Amplification
Ligation
Mouse MINUS
Rabbit PLUS
Mouse anti-GFP
Rabbit anti-Flag
JAK2
GFP
P75NTR
Flag

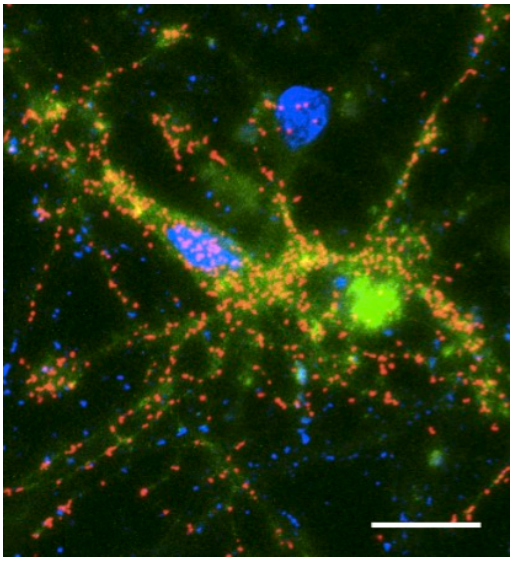
B.


PLA (JAK2:eGFP/p75NTR:Flag)

C.


PLA (pmGFP/p75NTR-Flag)

D.



I. Inhibition of JAK2 reduces the formation of (i)p75NTRJ2.

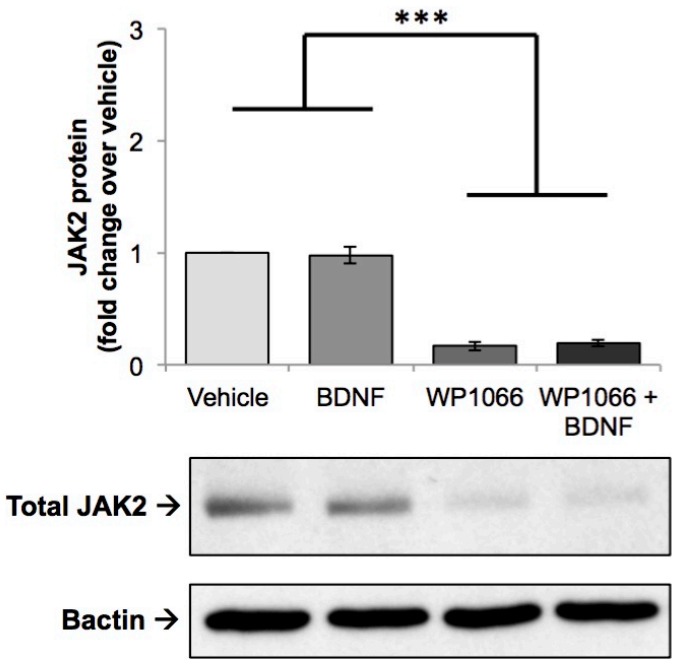
Collaborative studies in our laboratory previously reported that treatment with the JAK/STAT inhibitor WP1066 reduces the frequency of spontaneous seizures in pilocarpine-induced SE rats (Grabenstatter et al., 2014). SE rats treated with WP1066 also had reduced mRNA expression of STAT3-target genes, including ICER, in the DG compared to control-treated SE rats. Since JAK/STAT inhibition can occur through either deactivation or degradation of JAK, we wished to determine the mechanism of action of WP1066 as seen in our primary neuron cultures. Since ICER expression is increased after just 4 hours of BDNF treatment, we expected to see an effect of WP1066 at a similar time period. Indeed, after 5 hours treatment, WP1066 significantly degraded total JAK2 levels, and this effect could not be rescued with BDNF treatment (Figure 12A). Interestingly, a more potent JAK/STAT inhibitor, ruxolitinib, did not degrade JAK2 levels (Figure 12B). These results suggest that WP1066 inihibits JAK mainly through degradation.

We previously showed that pyridone 6, a selective JAK2 inhibitor, blocks STAT3 phosphorylation, ICER induction by BDNF, and the subsequent downregulation of α1 subunit mRNA levels *in vitro* and *in vivo* (Lund et al., 2008) and demonstrated that treatment in vivo with an additional JAK2 inhibitor, WP1066, attenuates the development of spontaneous seizures after SE (Grabenstatter et al., 2014). Here, we employ ruxolitinib (RUXO), a high affinity JAK2 inhibitor, currently used in the clinical treatment of myeloproliferative

neoplasms (Mascarenhas and Hoffman, 2012), and found that it blocks STAT3 phosphorylation in primary neurons with high-affinity (Ic50 = 10 nM; Figure 13A). We also asked whether RUXO interferes with (i)p75NTRJ2 formation, either because activated JAK2 is necessary to build the complex or because RUXO binds to the surface that is important for JAK2/P75NTR interactions, an area of ongoing investigation in our laboratories. As can be seen in Figure 13B, there is a marked decrease in JAK2-GFP precipitated with the Flag antibody after 100nM RUXO treatment of HEK cells, when compared to DMSO vehicle ($n = 3$, normalized to input levels of JAK2:eGFP, $^*p < 0.05$). These results suggest that RUXO not only inhibits JAK2 phosphorylation, as a well described JAK2 inhibitor (Grabenstatter et al., 2014), but also formation of (i)p75NTRJ2 in transfected HEK cells and primary cultured neurons (Figure 13C).

Figure 12: Long-term treatment with WP1066 degrades JAK2 protein. Primary rat cortical neurons were pretreated for 1 hour with WP1066 (10μM, Millipore #573097), Ruxolitinib (10μM), or vehicle (DMSO), before being treated with BDNF (5ng/ml) or vehicle (water) for 4 hours. Cells were collected for protein extraction and analysis via Western blot. Blots were probed with JAK2 antibody (1:1000, Cell Signaling #3230) and βactin. JAK2 levels were normalized to βactin and expressed as fold change over vehicle (DMSO/Water). (A) WP1066 treatment significantly reduced JAK2 protein levels, even in the presence of BDNF. One-way ANOVA was conducted with Tukey's post-hoc test between groups [$F(3,11) = 135.7$, $p < 0.0001$]. (B) Ruxolitinib did not degrade JAK2 protein levels ($n = 3$). RUXO alone: $M = 2.09$, $SEM = 0.67$; RUXO and BDNF: $M = 2.43$, $SEM = 1.43$. Data are expressed as mean ± SEM.

A.


***
JAK2 protein (fold change over vehicle)
3
2
1
0
Vehicle
BDNF
WP1066
WP1066 + BDNF
Total JAK2 →
Bactin →

B.

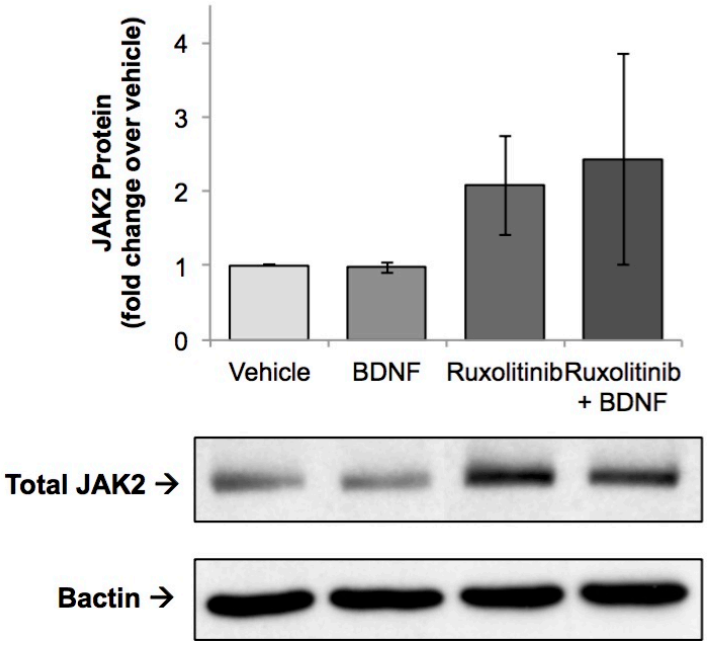
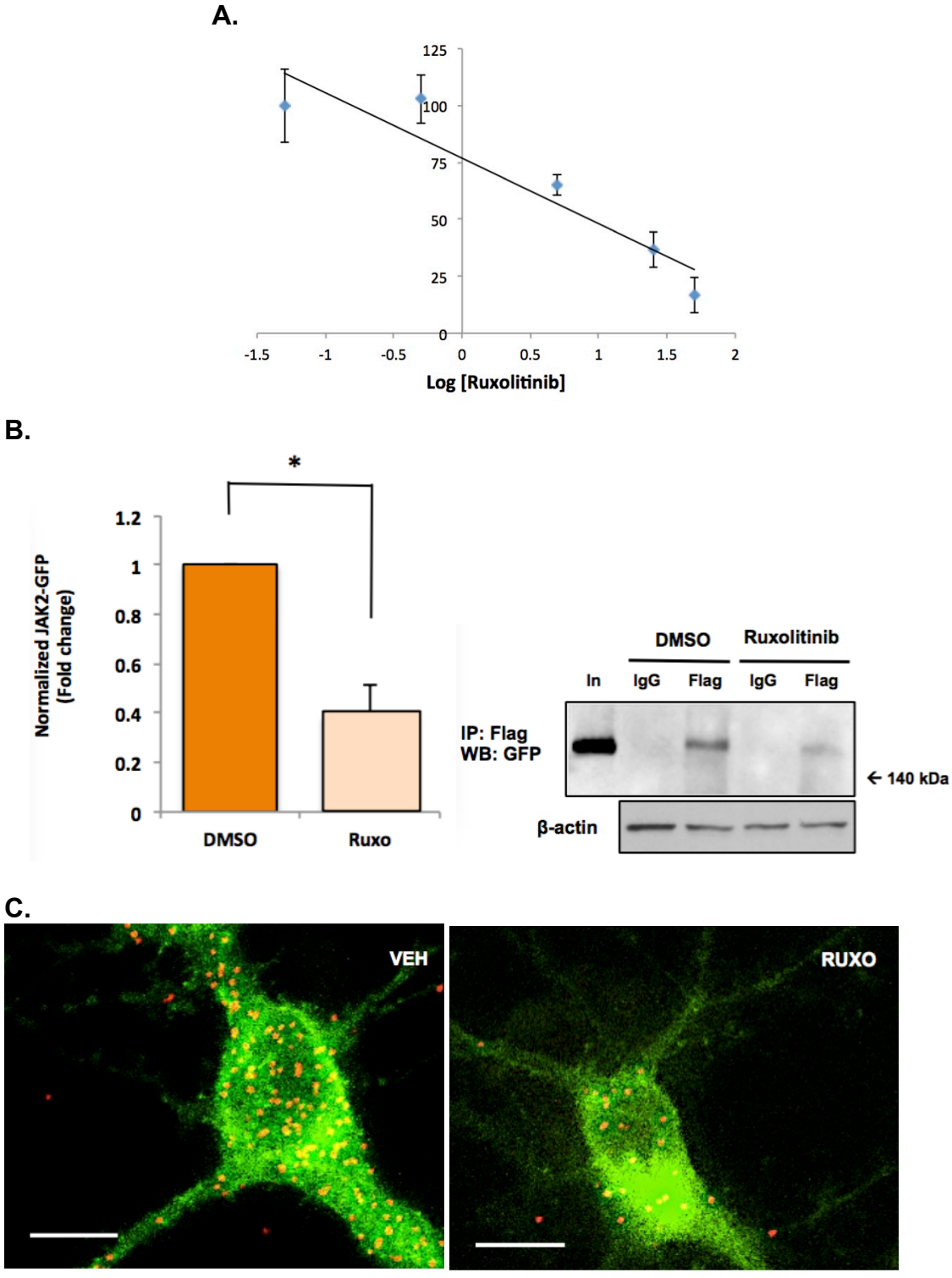

JAK2 Protein (fold change over vehicle)
4
3
2
1
0
Vehicle
BDNF
Ruxolitinib
Ruxolitinib + BDNF
Total JAK2 →
Bactin →

Figure 13: JAK inhibitor ruxolitinib (RUXO) blocks (i)p75NTRJ2 formation.

(A) Determination of Ic50 *in vitro.* Primary cortical neurons were co-treated with RUXO (0.05, 0.5, 5, 25, 50 nM) and BDNF (100 nM, 30 minutes). Phospho STAT3 levels were measured using a STAT3 (pY705) ELISA kit (Life technologies #KHO0481). pSTAT3 levels are expressed as a percentage of the full response to BDNF treatment alone, and plotted against the log[RUXO]. Ic50 calculated = 8.58 nM. Data are expressed as mean ± SD of duplicate samples. (B) Co-IP of proteins from HEK cells transfected with JAK2-eGFP and p75NTR:Flag either vehicle (DMSO) or RUXO (100 nM). Treatment specifically decreases JAK2:eGFP/p75NTR:Flag association (n = 3, normalized to input levels of JAK2:eGFP, *p < 0.05). (C) PLA with vehicle (DMSO) treatment versus RUXO (100nm), which attenuates complex formation. Scale bar = 20 μm. Magnification: 63x


A.
125
100
75
50
25
0
-1.5
-1
-0.5
0
0.5
1
1.5
2
Log [Ruxolitinib]
B.
*
Normalized JAK2-GFP (Fold change)
1.2
1
0.8
0.6
0.4
0.2
0
DMSO
Ruxo
DMSO
Ruxolitinib
In
IgG
Flag
IgG
Flag
IP: Flag
WB: GFP
← 140 kDa
β-actin
C.
VEH
RUXO

J. A role for intracellular proBDNF in the JAK/STAT pathway of neurons.

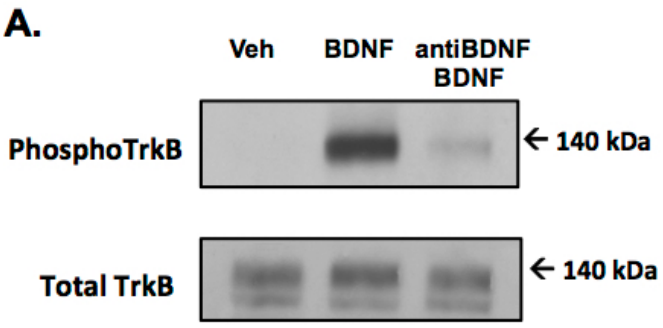
Primary neurons increase their levels of matBDNF mRNAs in response to chronic matBDNF treatment in culture suggesting that intracellular levels of matBDNF can be quite high during brain insult (Greenberg et al., 2009). However, in collaboration with our colleagues in the Dr. Brooks-Kayal laboratory (U. of Colorado) we have just reported that proBDNF rather than matBDNF is increased after seizure activity in a TLE model of epilepsy. Our laboratories reported that proBDNF levels are elevated in the hippocampus of pilocarpine-induced SE mice within 3 hours after onset, persisisting through 24 hours, and peaking at around 3 days after SE onset (Thomas et al., 2016). There is also a reduction in tPA and increased PAI-1, two enzymes involved in the proteolytic processing of proBDNF (Thomas et al., 2016) suggestive of a mechanism for increased proBDNF rather than matBDNF *in vivo.*

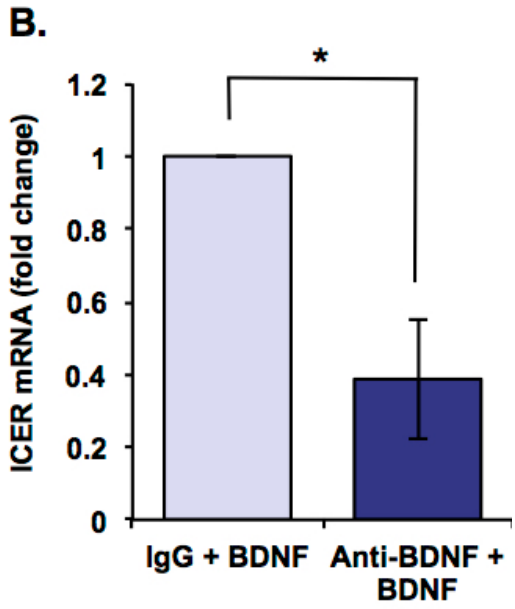
At the higher doses of signaling molecule used in our studies, we showed that proBDNF acts on the matBDNF high affinity receptor, TrkB (Figures 4 and 5). As a first step to understand the importance that intracellular BDNF may play in prolonged BDNF-induced JAK/STAT activation and to test the increase in intracellular proBDNF seen *in vivo,* we asked whether overexpression of proBDNF in transfected neurons would affect the activity of the GABRA1 promoter as assayed in primary cortical cultures. Cultures were co-transfected with GABRA1p-luciferase constructs with either empty vector or a pCAG promoter driven transgene that codes for full length (proBDNF). Application of

anti-BDNF blocking antibody, intended to block extracellular BDNF, blocks both TrkB activation (Figure 14A) and induction of ICER [$t(2) = 3.7$, $p = 0.03$] (Figure 14B) as assayed by real-time PCR when BDNF is applied to the media and serves as a positive control for the promoter studies with the BDNF transgene. Using a promoter assay (Figure 14C), our results clearly indicate that proBDNF delivered in this context is able to downregulate GABRA1 promoter activity [$t(4) = 9.2$, $p = 0.0008$], a hallmark of JAK/STAT activation in neurons (Figure 14D) and that such downregulation occurs even when antibodies to BDNF block activation of cell surface BDNF receptors [$t(4) = 2.7$, $p = 0.03$] (Figure 14E).

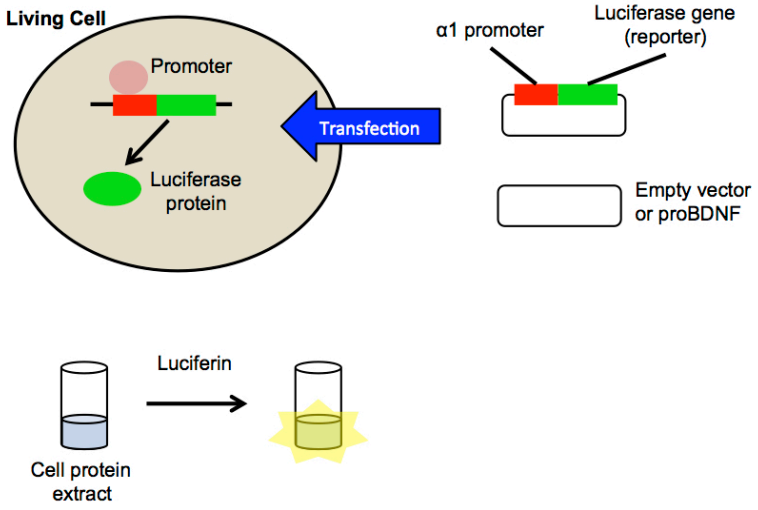
Figure 14. Overexpression of full length BDNF decreases *GABRA1*promoter activity.

(A) Anti-BDNF treatment overnight prevented activation of TrkB receptor, even when treated with recombinant BDNF, as observed by protein levels of phosphoTrk in Western blot. (B) In total RNA extracts from neurons co-treated with anti-BDNF or vehicle (normal mouse IgG) and matBDNF (4 nM, 4 hours), anti-BDNF significantly reduced BDNF-induced ICER expression (Student's t-test, *p < 0.05). (C) Promoter assay (schematic shown) found that (D) *GABRA1* promoter luciferase activity decreased in primary neurons co-transfected with the full length BDNF transgene when compared to empty vector (Student's t-test, ***p < 0.001). (E) Anti-BDNF treatment, which quenches extracellular BDNF in the media, of neurons transfected with the BDNF transgene does not reverse downregulation of *GABRA1* promoter luciferase activity, suggesting a role for intracellular BDNF (Student's t-test, *p < 0.05).

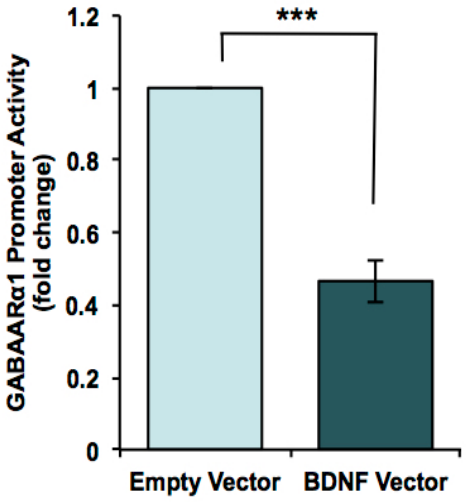

A.
Veh
BDNF
antiBDNF BDNF
PhosphoTrkB
140 kDa
Total TrkB
140 kDa


B.
*
ICER mRNA (fold change)
1.2
1
0.8
0.6
0.4
0.2
0
IgG + BDNF
Anti-BDNF + BDNF

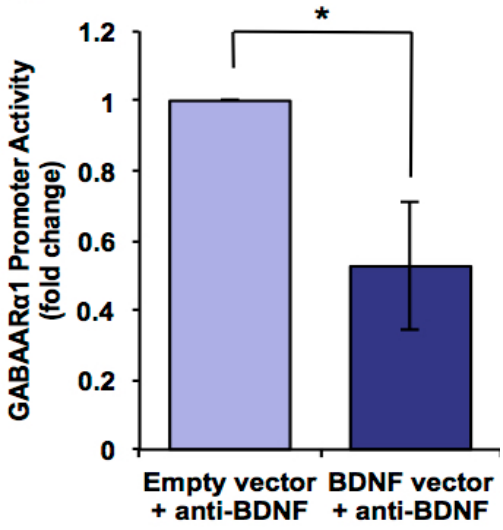
C.


Living Cell
Promoter
Luciferase protein
Transfection
α1 promoter
Luciferase gene (reporter)
Empty vector or proBDNF
Luciferin
Cell protein extract

D.


GABAARα1 Promoter Activity (fold change)
1.2
1
0.8
0.6
0.4
0.2
0
***
Empty Vector
BDNF Vector

E.


GABAARα1 Promoter Activity (fold change)
1.2
1
0.8
0.6
0.4
0.2
0
*
Empty vector + anti-BDNF
BDNF vector + anti-BDNF

IV. RESULTS: BDNF-induced selective phosphorylation of LSF and its association with neurogenesis and status epilepticus.

A. BDNF induces phosphorylation of p291 LSF through MEK signaling.

Molecular changes that occur in epilepsy models and in human patients include a robust increase in BDNF signaling. Since BDNF is involved in neuronal survival, differentiation, and neurogenesis, we hypothesized that BDNF signaling is linked to the activation of LSF, a ubiquitous cell cycle regulator. To examine this possibility, primary cortical and hippocampal neurons were treated with mature recombinant human BDNF (50ng/ml, Millipore) for 4 hours, a time point that we previously reported produces a peak induction in BDNF-induced *ICER* transcription. As shown in Figure 15A, under these same conditions, BDNF markedly increases levels of p291 LSF (cortical M=158%, SEM=14%; hippocampal M=174%, SEM=17%) over vehicle (M=100%, SEM=0%) in cortical [t(6)=4.014, p=0.007] and hippocampal [t(3)=4.270, p=0.024] neurons with no change in p309 or total LSF, suggesting that phosphorylation of LSF at serine 291 is controlled by the same BDNF signaling that regulates the expression of GABARs after SE. The antibody for p291 LSF also recognizes higher molecular weight bands right above 60 kDa, and a large band above 225 kDa, as seen in the full Western blot (indicated by *). While we did not quantify this change, these bands are specific to p291 LSF and seem to increase with BDNF treatment. The ladder pattern of the bands right above 60 kDa may indicate LSF is conjugated to small ubiquitin-like modifiers (SUMOs), a modification known as SUMOylation,

since LSF is a reported substrate of SUMO-2 in U2OS cells, and is increased in response to heat shock (Golebiowski et al., 2009).

To determine whether increases in p291 LSF are specifically dependent on BDNF signaling, we tested whether phosphorylation of LSF at this site would also occur in a cellular model of generalized neuronal depolarization utilizing potassium chloride (KCl) (Toescu, 1999). Primary neuronal cultures were treated with KCl (20 mM) for 2 hours followed by total protein extraction. Western blot analysis revealed no change in p291 LSF protein levels between the vehicle (water) and KCl treatment groups (Figure 15B), suggesting that presence of p291 LSF is dependent on BDNF signaling and not on a general change in the depolarization state of the neuron.

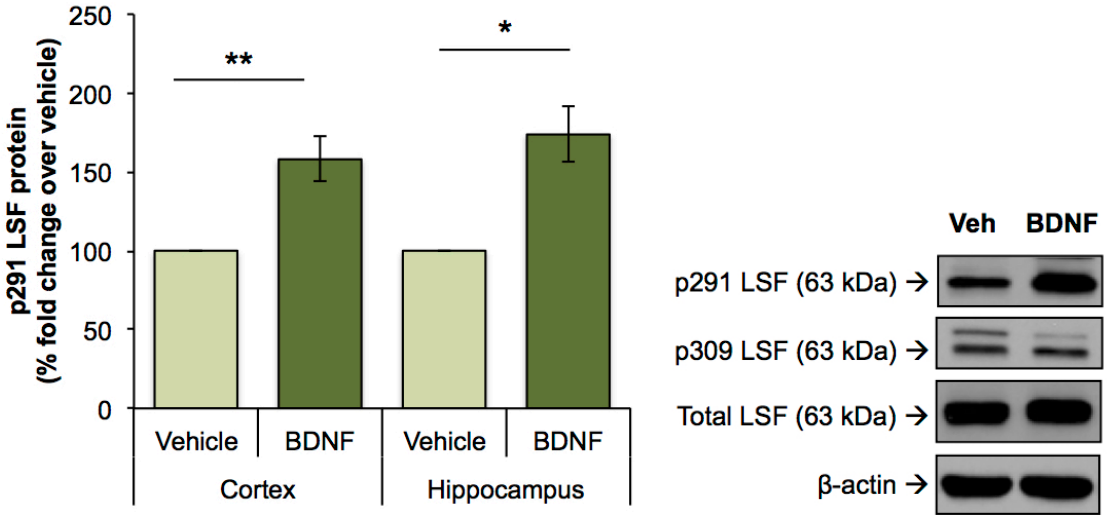
From our previous studies, and those of our colleagues, we know that BDNF signaling occurs through two major pathways that may be relevant for epileptogenesis, MAPK/ERK and JAK/STAT. In order to determine which downstream pathway is responsible for inducing p291 LSF, neurons were pretreated with a MEK inhibitor, U0126 (20 μM), or a JAK/STAT inhibitor, P6 (500 nM) prior to addition of recombinant mature BDNF. Both MEK1 and MEK2 are inhibited by U0216, a MAPK/ERK kinase inhibitor, and pre-treatment before BDNF application blocks BDNF-induced phosphorylation of LSF at p291 [$F(3,20)=8.37$, $p=.0008$] (Figure 15C). Pyridone 6 (P6) is a potent ATP-competitive inhibitor of JAK1, 2, and 3. Unlike U0126, JAK/STAT inhibition with P6 did not block BDNF-induced phosphorylation of LSF at p291 (Fig. 15D),

suggesting that this site-specific post-translational modification does not require BDNF activation of JAK/STAT signaling but rather activation of the MAPK/ERK pathway already identified as required in cancer cell lines (Pagon et al., 2003).

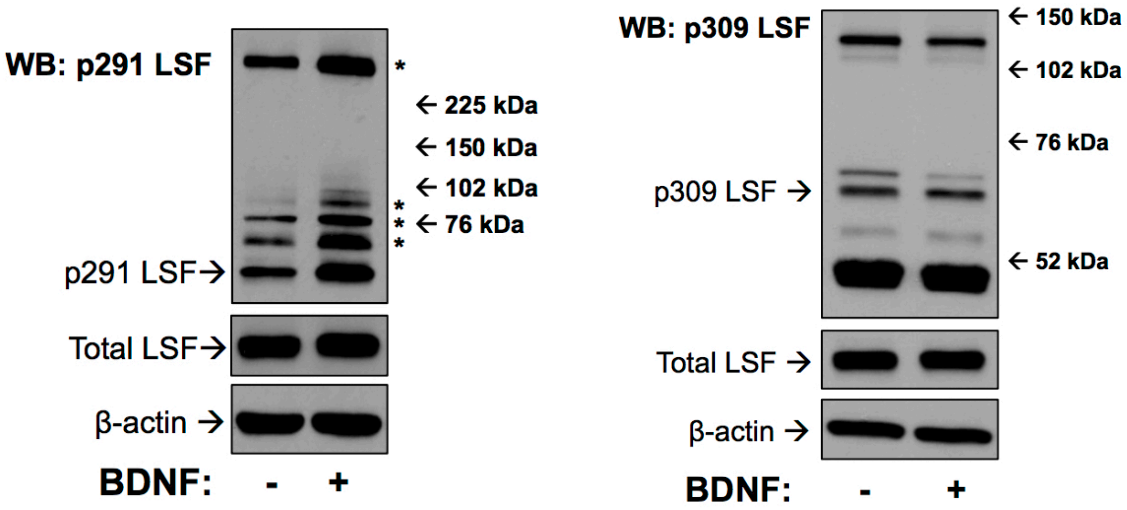
Figure 15: BDNF induces phosphorylation of p291 LSF.

(A) Primary neuron cultures treated with BDNF showed an increase in p291 LSF compared to vehicle, but no increase in total LSF or p309 LSF. Student's t-test was used to analyze cortical (n=7, **p<0.01) and hippocampal (n=4, *p<0.05), cells separately and data are expressed as mean ± SEM. Full blot shows higher molecular weight bands in a ladder pattern right above 60 kDa, and a large band above 225 kDa (indicated by *), which seem to increase with BDNF treatment. (B) Potassium chloride (KCl, 20 mM; Boston BioProducts MT-250) treatment did not induce p291 LSF. Representative data is for two hours treatment at DIV 7. Four hour treatment at DIV 14 also shows no change in p291 LSF. (C) MEK inihibitor (U0216) pretreatment blocked p291 LSF induction by BDNF. Data were analyzed by one-way ANOVA and are expressed as mean ± SEM (**p<0.01 ***p<0.001). (D) JAK inhibitor (P6) pretreatment did not block p291 LSF induction. Total protein extracts analyzed via Western blot and probed with polyclonal rabbit antibodies raised against phospho-LSF S291 (1:3000), phospho-LSF S309 (1:300), and total LSF (1:500). Bands were normalized to β actin (Sigma Aldrich A5441; 1:5000). Full blots are shown for each antibody in this figure and following figures. Full blots for Total LSF and β actin are not shown because only one band was detected.

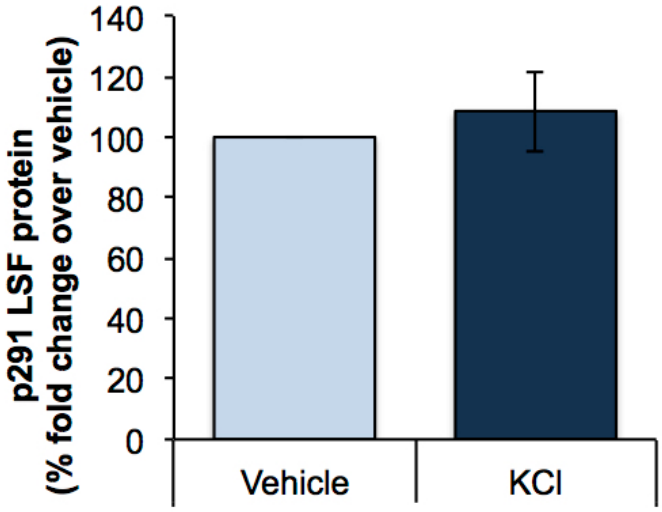
A.

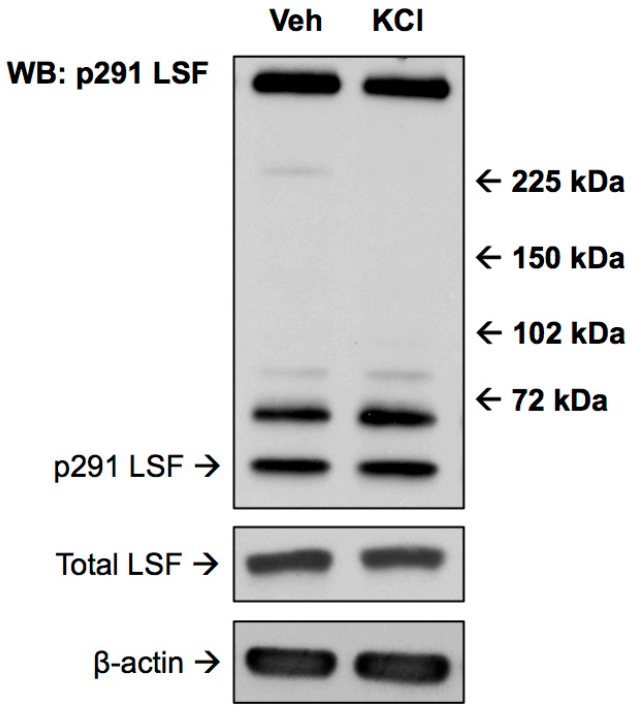

p291 LSF protein
(% fold change over vehicle)
250
200
150
100
50
0
**
*
Vehicle
BDNF
Vehicle
BDNF
Cortex
Hippocampus
Veh
BDNF
p291 LSF (63 kDa) →
p309 LSF (63 kDa) →
Total LSF (63 kDa) →
β-actin →

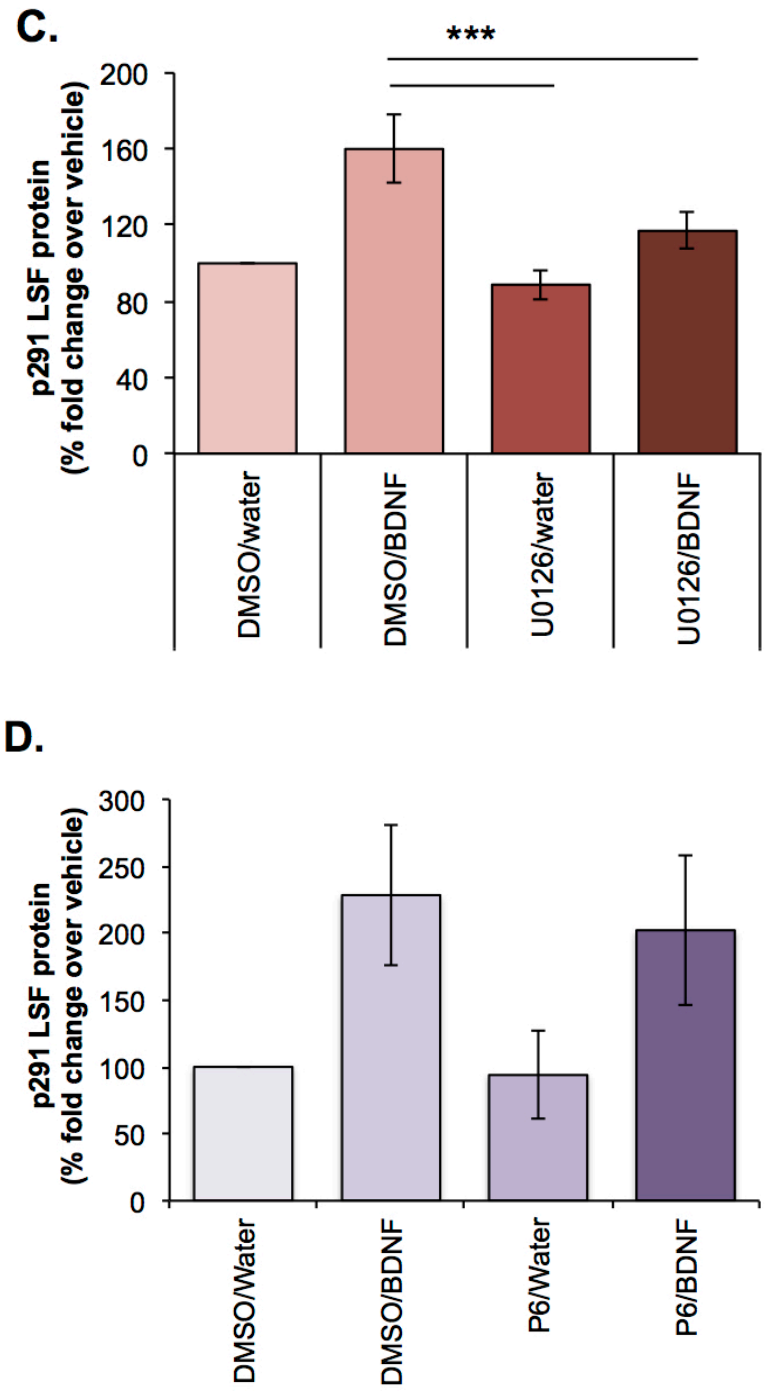
Full blots:


WB: p291 LSF
*
← 225 kDa
← 150 kDa
← 102 kDa
*
*
← 76 kDa
*
p291 LSF→
Total LSF→
β-actin →
BDNF: - +
WB: p309 LSF
← 150 kDa
← 102 kDa
← 76 kDa
p309 LSF →
← 52 kDa
Total LSF →
β-actin →
BDNF: - +

B.


p291 LSF protein
(% fold change over vehicle)
140
120
100
80
60
40
20
0
Vehicle
KCl


Veh
KCl
WB: p291 LSF
← 225 kDa
← 150 kDa
← 102 kDa
← 72 kDa
p291 LSF →
Total LSF →
β-actin →


C.
***
p291 LSF protein
(% fold change over vehicle)
200
160
120
80
40
0
DMSO/water
DMSO/BDNF
U0126/water
U0126/BDNF
D.
p291 LSF protein
(% fold change over vehicle)
300
250
200
150
100
50
0
DMSO/Water
DMSO/BDNF
P6/Water
P6/BDNF

B. P291 LSF is not expressed in proliferating cells.

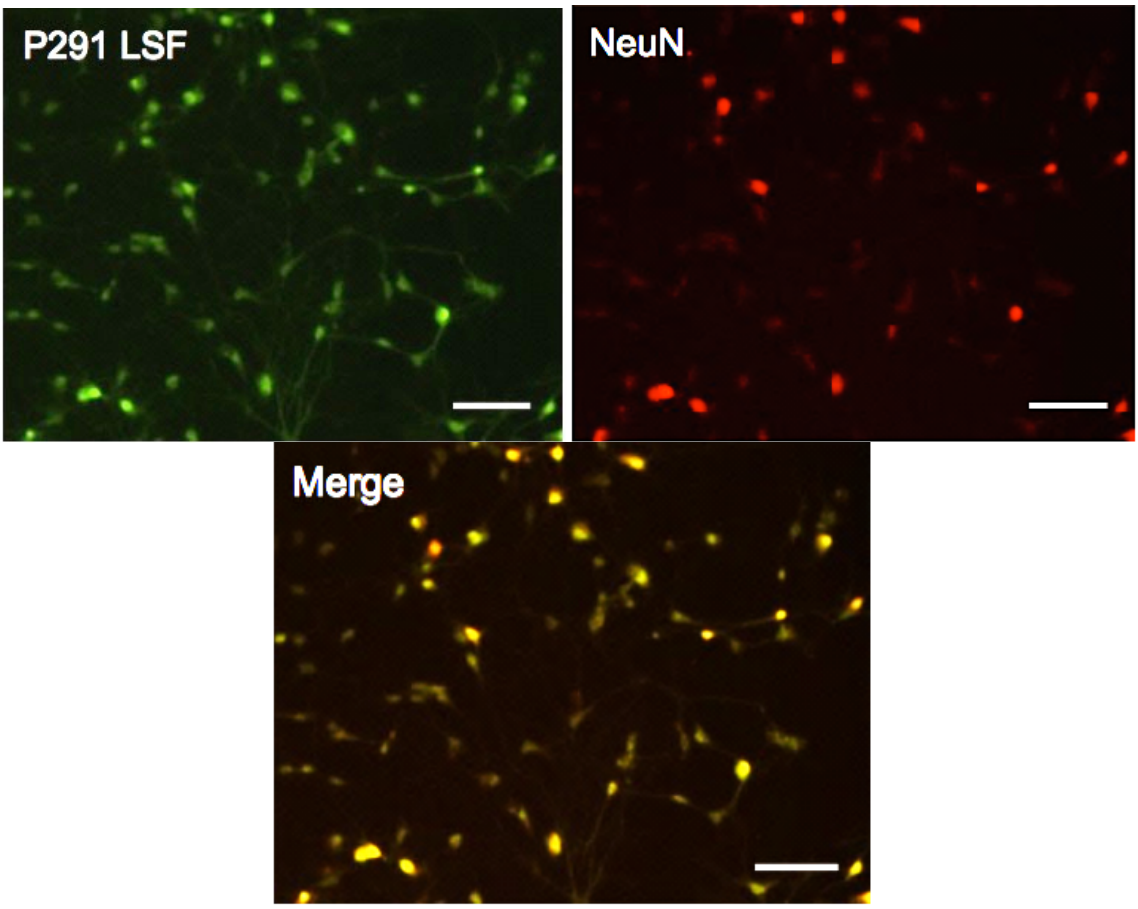
Since phosphorylation of LSF has not been studied in neurons, we set out to characterize the expression of p291, p309, and total LSF, which includes phosphorylated and unphosphorylated LSF, in primary neuronal cultures. We neurons labeled with an antibody to NeuN and an antibody to either p291 LSF (Figure 16A), p309 LSF (Figure 16B), or total LSF (Figure 16C). As expected, total LSF is found in all cells as it is ubiquitously expressed. However, p291 LSF is not found in every cell that contains LSF, suggesting that its presence is post-translationally regulated in primary neurons (Figure 16). Given that LSF is a regulator of the cell cycle and survival (Hansen et al., 2009; Veljkovic and Hansen, 2004) we hypothesized that a change in the state of LSF in the brain may contribute to the regulation of neurogenesis. To address the potential link between neurogenesis and LSF, LSF expression in neurons was examined during different states of cellular differentiation. Using immunocytochemistry, neurons were co-labeled with LSF and a variety of neuronal markers for proliferating and mature neurons.

To mark proliferating or dividing neurons, we used an EdU immunocytochemistry assay (Invitrogen), as an alternative to the use of BrdU. EdU is a nucleoside analog of thymidine and is incorporated into DNA during active DNA synthesis (Salic and Mitchison, 2008). To obtain a signal *in vitro*, a copper catalyzed covalent reaction occurs between a picolyl azide containing an Alexa Fluor 594 (red) dye and an alkyne containing the EdU. Labeling primary

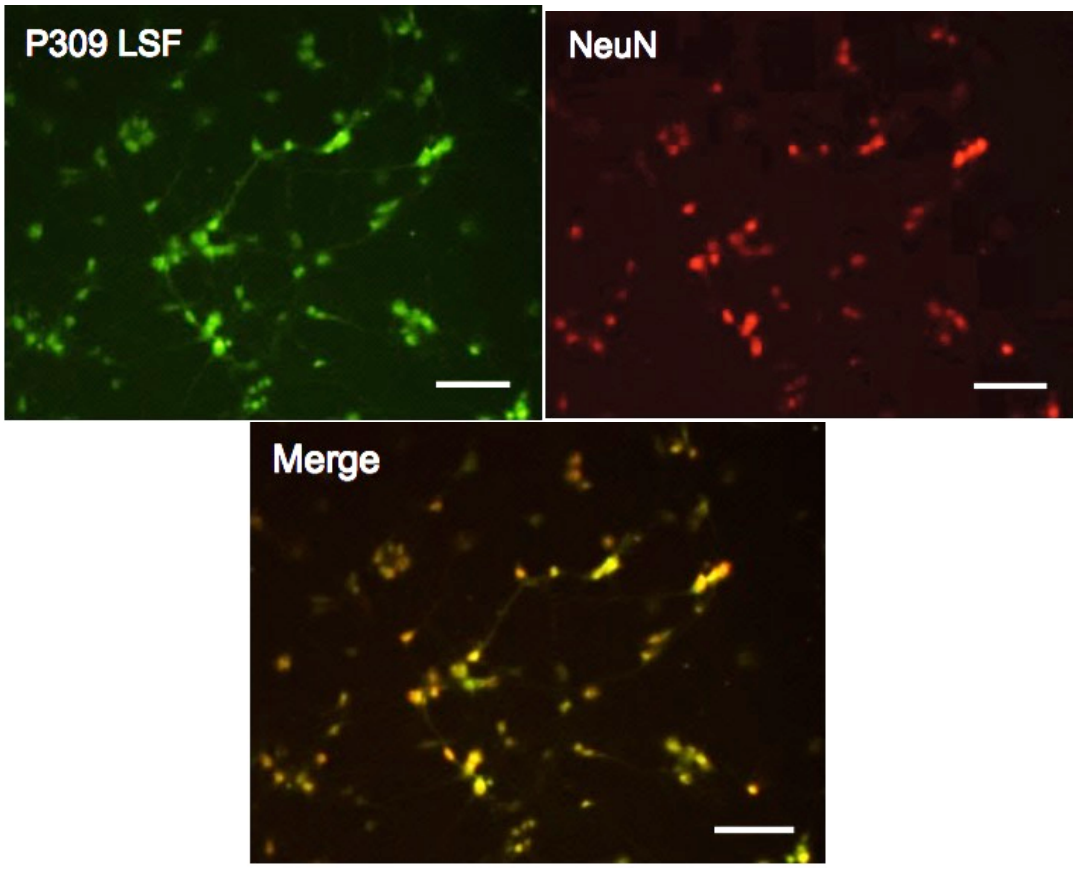
hippocampal and cortical neuronal cultures with p291 LSF and EdU (Figure 17), EdU-positive cells were imaged that contain barely detectable levels of p291 LSF when compared to EdU-negative cells. These results suggest that phosphorylation at serine 291 does not occur in actively dividing neurons. There are also populations of cells, indicated by DAPI staining alone, that do not have p291LSF or EdU. While we only show this in the merged image of Figure 17, we did see this population throughout our ICC experiments.

Figure 16: p291 LSF, p309 LSF, and Total LSF are present in neurons. Immunocytochemistry in primary cortical neuron cultures used antibodies to NeuN (1:250; mouse polyclonal, Chemicon, red) and either (A) p291 LSF, (B) p309 LSF, or (C) Total LSF (green, all from Ulla Hansen's lab) overnight. All forms of LSF co-localized with NeuN-positive neurons. Secondary antibody goat anti-rabbit Alexa Fluor 488 (Life Technologies A-11008) and anti-mouse IgG Texas Red (Vector TI-2000) in 1% BSA/1X PBS was added for two hours at room temperature in the dark. Images were taken at 20x magnification. Scale bar = 100 μm.

A.



B.



C.

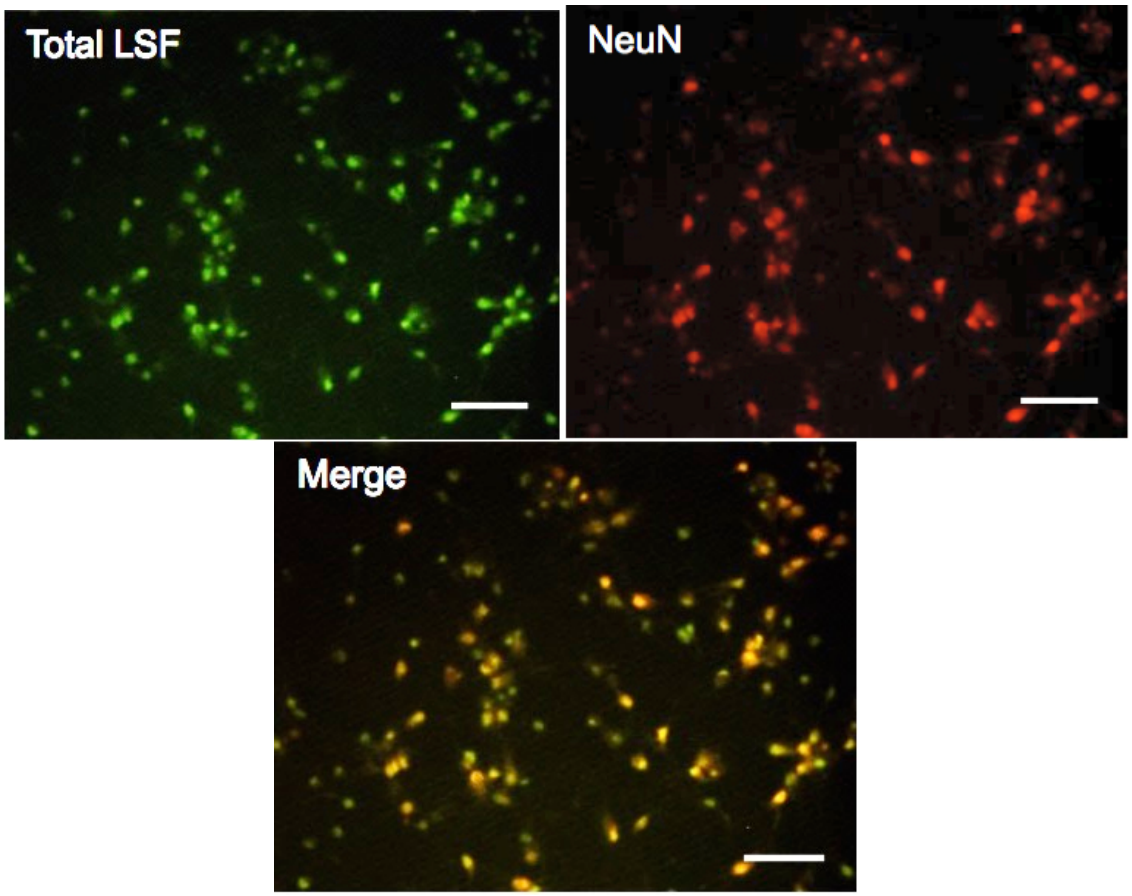
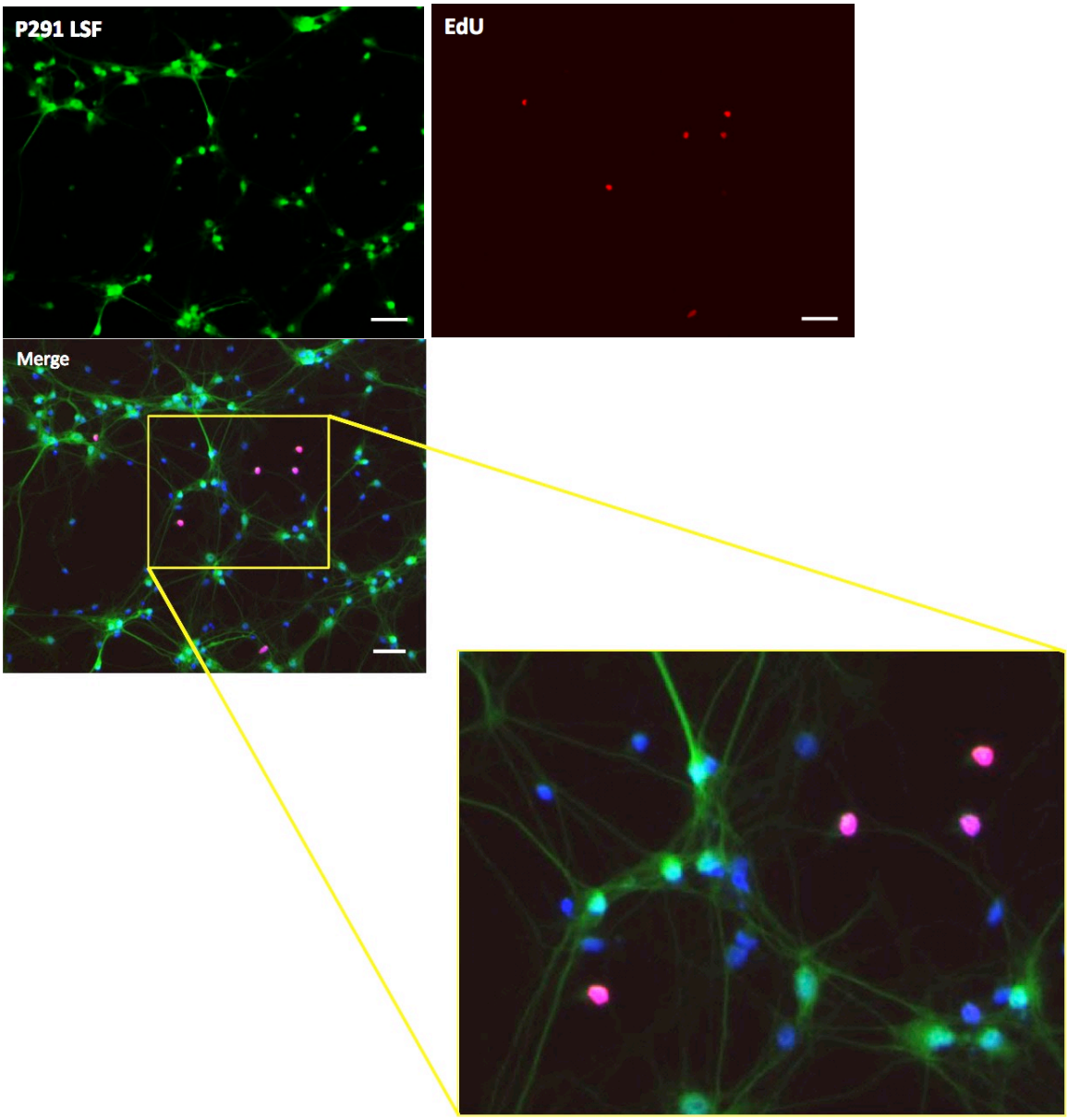


Figure 17: p291 LSF is not associated with proliferating neurons.

Live primary neuron cultures were stained for EdU before fixing, permeabilizing, and incubating with primary antibody to p291 LSF. EdU-positive, proliferating neurons (red) did not co-label for p291 LSF (green). Secondary antibody goat anti-rabbit Alexa Fluor 488 (Life Technologies A-11008) 1% BSA/1X PBS was used to recognize p291 LSF antibody and visualize. ProLong Gold Antifade Mountant with DAPI (Thermo Fisher P36941) was used to mount the cells for imaging. Images were taken at 20x magnification. Scale bar = 100 μm. EdU: red. DAPI: blue. P291 LSF: green.


P291 LSF
EdU
Merge

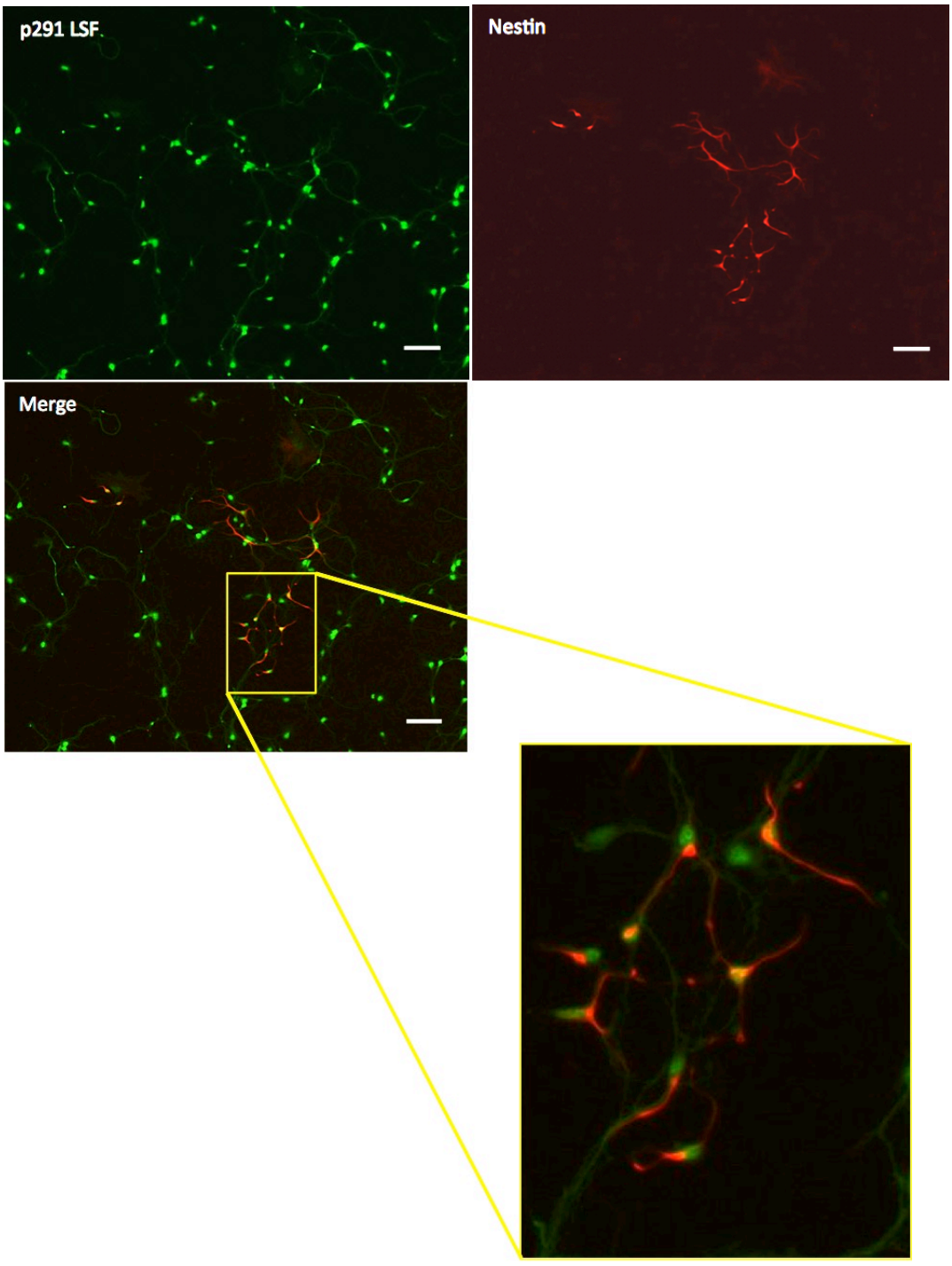
C. P291 LSF is expressed in neurons throughout neurogenesis.

Nestin is an intermediate filament expressed in neural precursor cells (Lendahl et al. 1990) as well as proliferating neurons in the adult brain that develop into mature neurons and astrocytes (Reynolds and Weiss 1992). Using immunocytochemistry to detect Nestin-positive cells, we found that p291 LSF was present in Nestin-positive neurons (Figure 18A), suggesting that phosphorylation at serine 291 is expressed in immature neurons or precursors. PSA-NCAM is highly expressed in newly generated and developing granule cells in the dentate gyrus of the hippocampus (Seki and Arai, 1991) and persists in young post-mitotic neurons (Seki 2002a). Primary neurons were labeled for PSA-NCAM and p291 LSF, and p291 LSF co-labels with PSA-NCAM-positive cells (Figure 18B). As mitogen activated protein 2 (MAP2) is expressed predominantly in the dendrites of mature neurons (Cáceres et al., 1986) we asked whether p291 levels might be high in those cells where MAP2 is present. Primary neuronal cultures labeled for both MAP2 and p291 LSF display the highest levels of p291 LSF (Figure 18C), further suggesting that presence of p291 promotes a post-mitotic, mature neuronal phenotype. Together, the results of immunocytochemistry strongly suggest that phosphorylation of LSF at serine 291 is suppressed during proliferation, but exists in precursor neurons, newly generated neurons, and persists throughout neuronal maturity.

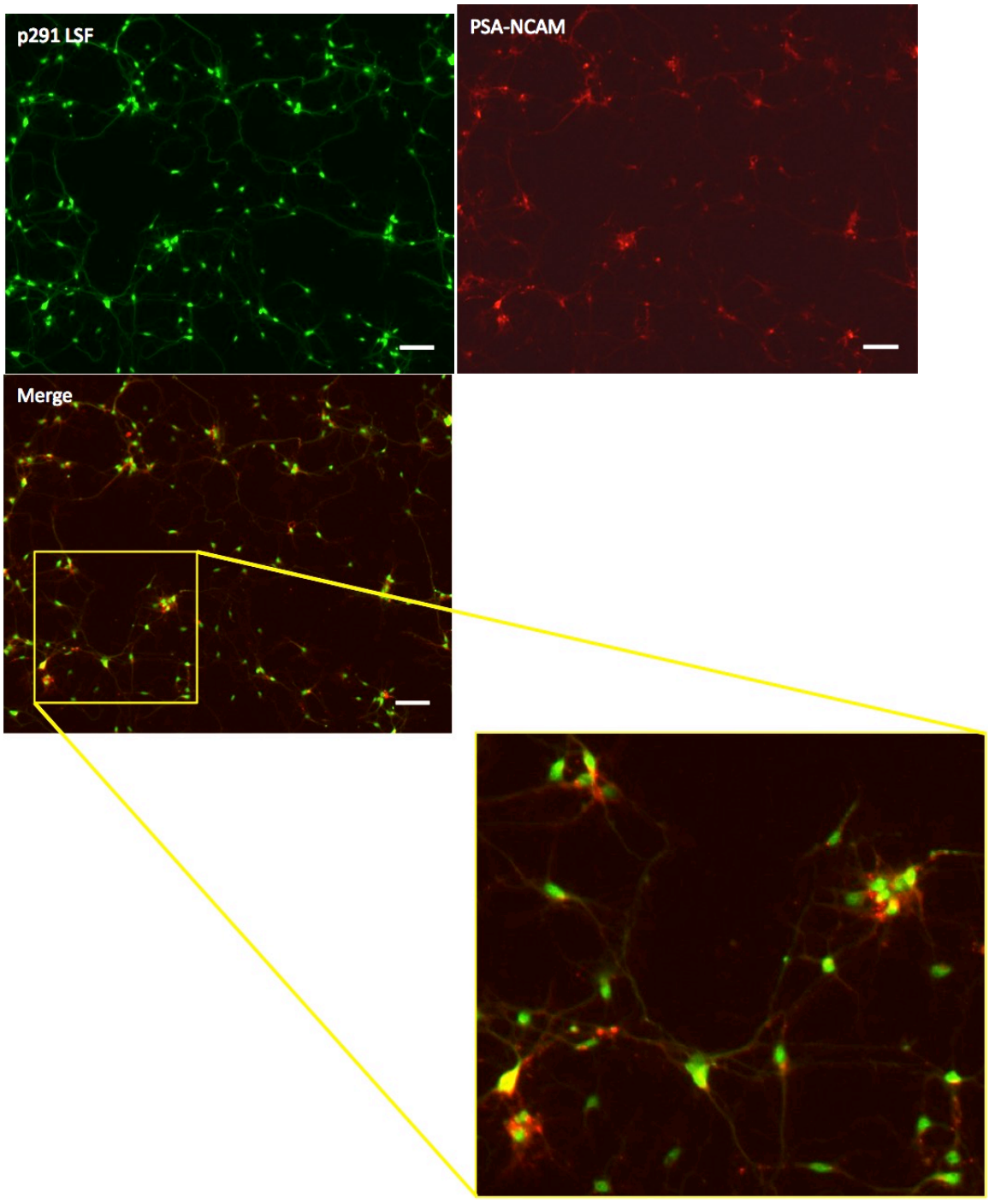
Figure 18: p291 LSF expression persists throughout neurogenesis.

Primary neurons were stained for different proliferation markers. Secondary antibody goat anti-rabbit Alexa Fluor 488 (Life Technologies A-11008) and/or anti-mouse IgG Texas Red (Vector TI-2000) in 1% BSA/1X PBS was added for two hours at room temperature in the dark. ProLong Gold Antifade Mountant with DAPI (Thermo Fisher P36941) was used to mount the cells for imaging. DAPI: blue. P291 LSF: green. (A) Nestin-positive, neural precursor cells (red), contained p291 LSF (green). (B) PSA-NCAM positive, newly generated neurons (red), co-labeled for p291 LSF (green). (C) Microtubule associated protein 2 (MAP2)-positive, mature neurons (red) co-labeled for p291 LSF (green). Images were taken at 10x magnification. Scale bar = 100 μm.

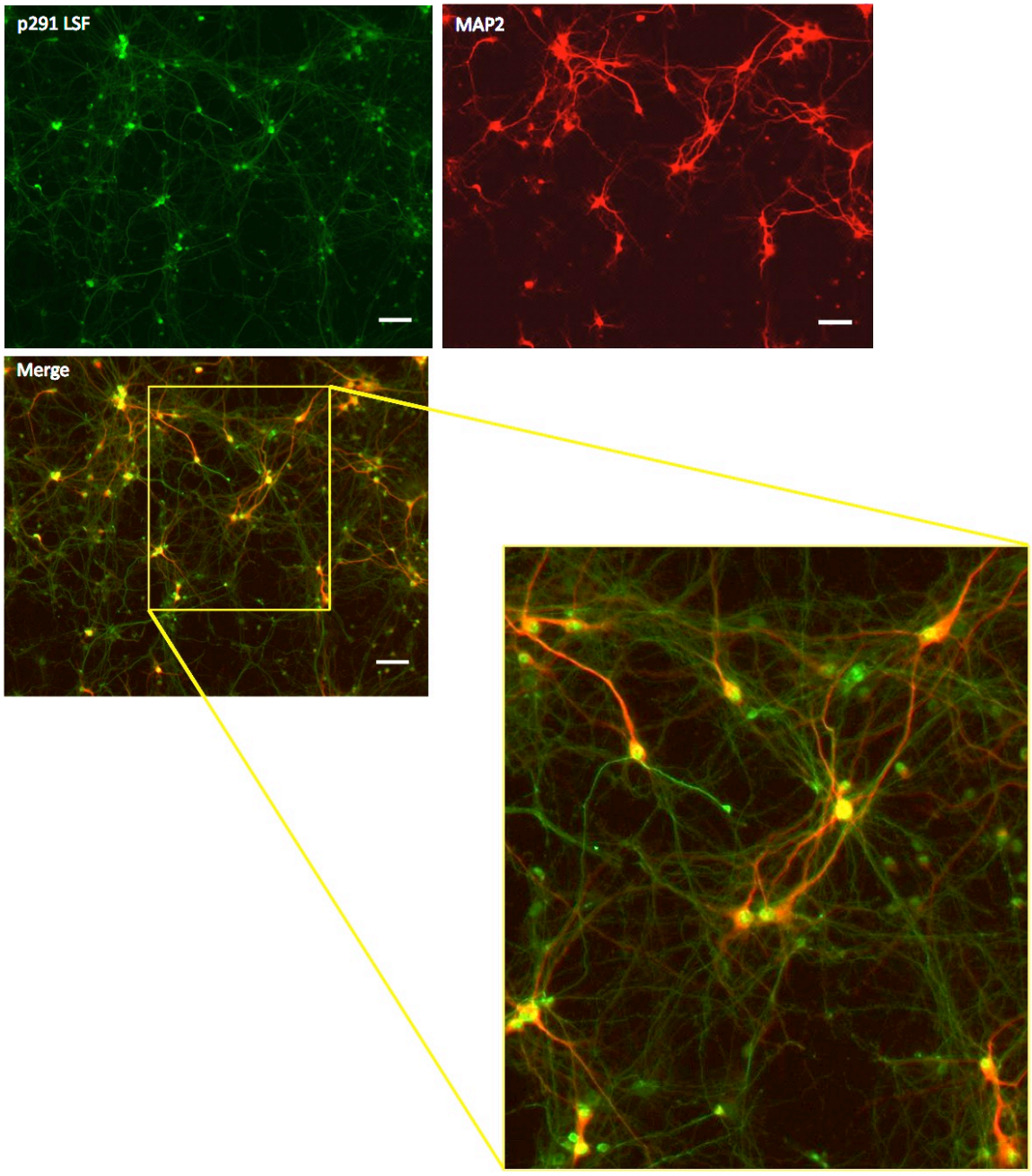
A.



B.



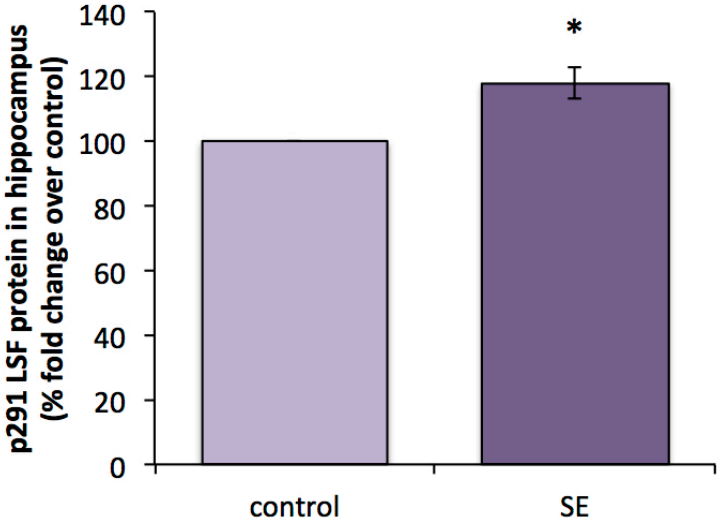
C.

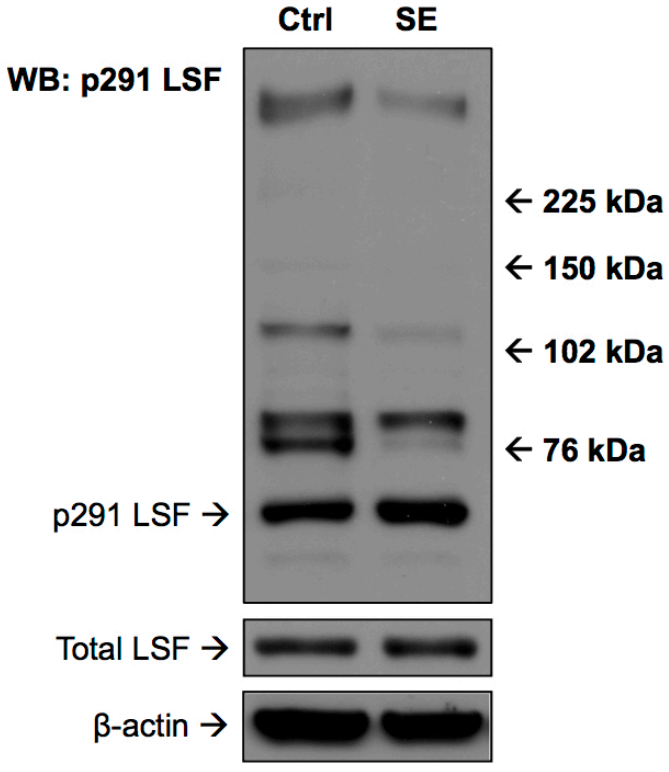


C. Increased p291 LSF in the hippocampus of the pilocarpine-induced model.

To determine whether p291 LSF may be linked to epileptogenesis, given its regulation by BDNF in primary cell cultures, LSF protein levels were examined 24 hours after pilocarpine-induced SE in rats. Levels of LSF were detected in hippocampal tissue microdissected by our colleagues in Dr. Amy Brooks-Kayal's laboratory at University of Colorado. Compared to control animals (n=4, M=100% SEM=0%), animals 24 hours after SE exhibit higher levels of p291 LSF (n=4, M=118% SEM=5%) in the hippocampus [$t(3)=3.6$, $p=0.036$] (Figure 19), suggesting that specific regulation of LSF phosphorylation induced by a rise in BDNF is also associated with the hyperactive state that is the product of prolonged seizure activity. Interestingly, the protein samples from the hippocampal tissue do not have the larger kDa ladder pattern of bands, which may be SUMOylation, seen in Figure 15A.

Figure 19: Elevated levels of p291 LSF in hippocampus of SE model rats. The Amy Brooks-Kayal laboratory performed the pilocarpine-induced SE in adult rats according to standard protocols. All rats treated with a full dose of pilocarpine (350 mg/kg; ip) were confirmed to have prolonged Stage 5 seizures on the Racine scale. Control animals received a sub-convulsive dose of pilocarpine (35 mg/kg; ip). Rats were sacrificed 24 hours after induction of SE and a bilateral dissection of the hippocampus was done for protein extraction. Total hippocampal protein was analyzed via WB and probed for p291 LSF, total LSF, and β actin loading control. Levels of p291 LSF were normalized to total LSF. Hippocampal protein from SE animals has increased levels of p291 LSF normalized to total LSF, when compared to control animals ($n=4$, $^{*}p<0.05$).

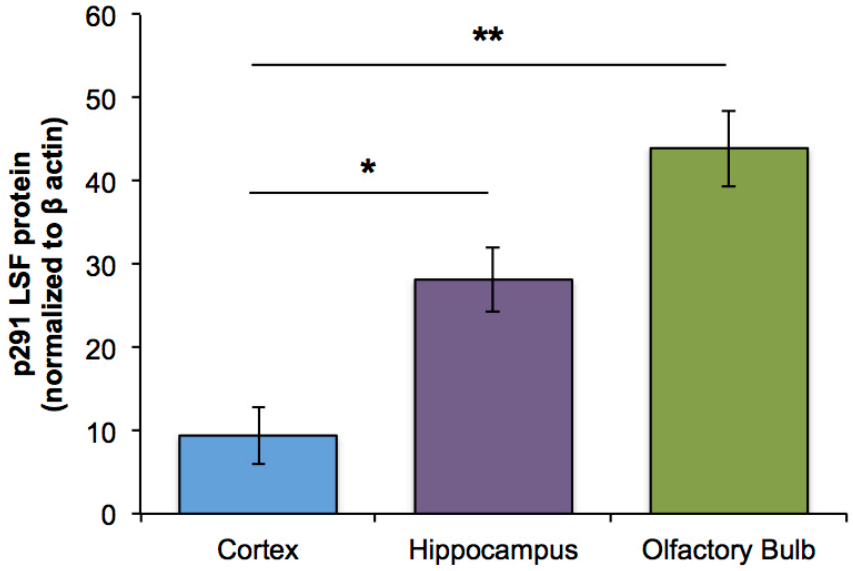

p291 LSF protein in hippocampus
(% fold change over control)
140
120
100
80
60
40
20
0
*
control
SE

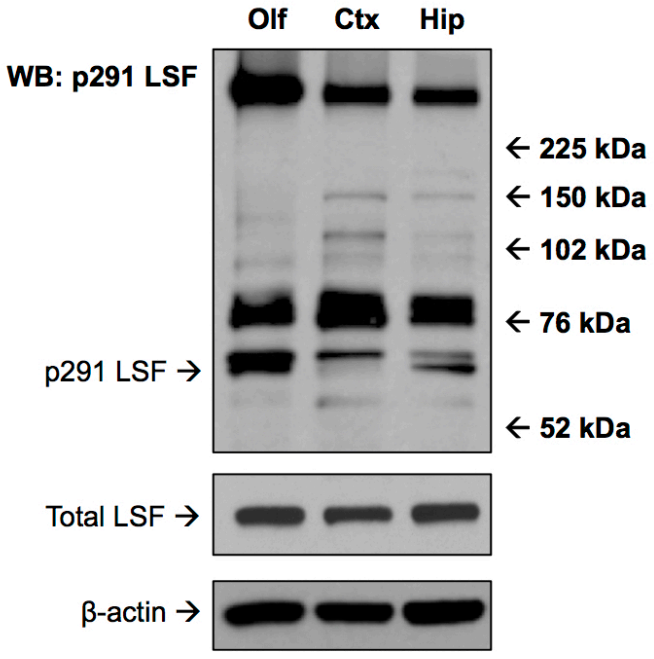

Ctrl
SE
WB: p291 LSF
← 225 kDa
← 150 kDa
← 102 kDa
← 76 kDa
p291 LSF →
Total LSF →
β-actin →

D. P291 LSF is higher in adult brain regions containing newly generated neurons.

To learn more about LSF's association with adult neurogenesis, levels of phosphorylated LSF were compared across brain regions where adult neurogenesis has been reported, such as the hippocampus, and where newly generated neurons are found to migrate, the olfactory bulb. We compared the levels of p291 LSF in these two brain regions to an area that contains mainly mature neurons, the prefrontal cortex. Although not understood at this time, p291 LSF in adult rat brain extracts appears as a doublet around 57 kDa in the hippocampus and olfactory bulb, where there are high levels of neurogenesis but not in cortex or in extracts of primary neuronal cultures. We analyzed p291 LSF at the faster migrating band of the doublet (see full blot in Figure 20) because it aligned with the total LSF single band. Levels of p291 LSF were highest in the olfactory bulb, followed by the hippocampus, with lowest levels in the prefrontal cortex [$F(2,6)=18.85$, $p=.002$] (Figure 20). This is surprising given that p291 is selectively found in neurons that express MAP2 and are post-mitotic, suggesting that it may play a role in transitioning cells into maturity rather than maintaining the mature phenotype.

Figure 20: Expression of p291 LSF in adult rat brain areas of neurogenesis. For adult brain extracts, we saved the brains from adult female rats used for embryo dissection. Protein was extracted from bilateral dissections of the prefrontal cortex, hippocampus, and olfactory bulb tissue. Total protein was analyzed via WB and probed for p291 LSF and β actin loading control. P291 LSF levels were highest in olfactory bulb, followed by hippocampus, while lowest levels were in the cortex. P291 LSF in the adult rat brain extracts show a doublet around 57 kDa, which is not present in primary neuron cultures. Data were analyzed via one-way ANOVA followed by Tukey's post-hoc test for differences between groups (*$p<0.05$, **$p<0.01$).


60
50
40
30
20
10
0
p291 LSF protein
(normalized to β actin)
**
*
Cortex
Hippocampus
Olfactory Bulb


Olf
Ctx
Hip
WB: p291 LSF
← 225 kDa
← 150 kDa
← 102 kDa
← 76 kDa
p291 LSF →
← 52 kDa
Total LSF →
β-actin →

V: DISCUSSION

A. Neurotrophin signaling controls JAK/STAT activation in neurons.

Results of our studies suggest that BDNF signals through two distinct neurotrophin receptor mediated pathways to regulate levels of GABAR subunit expression. Such BDNF-induced changes in receptor subunit levels occur in humans and in animal models of TLE, where an upregulation of α4 and a downregulation of α1 subunit mRNA levels and protein are observed in dentate granule cells after prolonged seizures (Brooks-Kayal et al., 1998b; Lund et al., 2008). Mature BDNF (matBDNF) signals through the TrkB receptor to activate MAPK, which increases the synthesis and binding of Egr3 to early growth response elements in the Gabra4 promoter (Roberts et al., 2006; Roberts et al., 2005). Concurrently, we now suggest that BDNF activates the JAK/STAT pathway, potentailly through a novel p75NTR/JAK2 complex ((i)p75NTRJ2) that induces ICER expression, which in turn heterodimerizes with pCREB reducing the expression of the most abundant GABAR subunit, α1 (Lund et al., 2008; Hu et al., 2008). Given our previous findings that inhibition of the JAK/STAT pathway prevents seizure induced changes in ICER and Gabra1 expression (Lund et al., 2008), it was critical to understand how BDNF mediates this pathway that contributes to altered GABAR expression in the diseased brain (Brooks-Kayal et al., 1998b).

Traditionally, activation of the JAK/STAT pathway has been associated with cytokine receptor signaling. As part of the inflammatory pathway, cytokines

contribute to the fast acting innate immune response by activating signaling cascades that mediate a number of downstream genes involved in cell proliferation, differentiation, immune response, and apoptosis (Darnell et al., 1994; Harrison, 2012; Vezzani and Granata, 2005). The JAK/STAT pathway has distinct roles in the CNS, where it is involved indevelopment, hormone release, inflammation or tumorigenesis (Nicolas et al., 2013). JAK/STAT signaling can also influence glial differentiation (Bonni et al., 1997), proliferation of NSCs (Bauer, 2009; Gómez-Nicola et al., 2011), neurogenesis (Müller et al., 2009), and neuronal survival (Schindler et al., 2007). Studies in our laboratory have identified another role for JAK/STAT signaling pathway in the CNS, where we have have previously demonstrated that BDNF-induced STAT signaling is a critical mediator of Gabra1 expression following SE (Lund et al., 2008).

In these studies, we provide further evidence and characterization of BDNF-induced JAK/STAT signaling, as fast and transient increases in pJAK2, the most abundant JAK isoform in the brain, occur following BDNF treatment and SE (Figure 3A-B). While glial-specific increases in JAK/STAT activation have previously been reported in a kainate model of TLE (Choi et al., 2003) our cultures are predominately neuronal, suggesting that BDNF-induced increases in JAK/STAT signaling are attributed at least in part to neurons. Furthermore, these short-term increases in JAK/STAT activation detected in response to BDNF stimulation are sufficient to induce the downstream changes in ICER expression we observe hours later (Figure 3C). This suggests that minutes of exposure to

matBDNF can leave its imprint on genome expression in a manner relevant to the remodeling of brain systems, even as levels of TrkB are downregulated (Figure 3D) and consistent with our *in vivo* findings that transient blockade of JAK2 after SE by WP1066 causes profound effects on seizure susceptibility (Grabenstatter et al., 2014).

Although still controversial in the field, the fate of a neuron may be controlled by the BDNF isoform, either the translated precursor proBDNF or the cleaved post-translational matBDNF, and the receptor that is activated. A yin and yang theory for BDNF action has developed over the years, suggesting that a balance is struck between matBDNF signaling through TrkB to promote cell survival, while proBDNF signals through p75NTR, complexed with sortilin, to promote apoptosis (Teng et al., 2005). Alterations in proBDNF and matBDNF have been reported in a pilocarpine model of TLE (Unsain et al., 2008), although limitations in interpretation may remain based on antibody specificity, region selective expression and release. With increases in both BDNF isoforms reported in response to SE, we investigated whether proBDNF could also activate JAK/STAT signaling employing a standard concentration reported in the literature (50ng/ml, or 4nM). Using a mutant form of proBDNF, which has a valine 66 to methionine SNP (Chen et al., 2004) that prevents cleavage, we found transient increases in pSTAT3 levels, as well as downstream changes in ICER and Gabra1 mRNA expression, suggesting that stimulation with either BDNF isoform is capable of activating the JAK/STAT pathway (Figure 1). However, as we have

demonstrated, proBDNF does so via the TrkB receptor. This effect of proBDNF is diminished when we drop the concentration of proBDNF and matBDNF 10-fold (to 5ng/mL, or 0.7nM), a concentration at which only matBDNF significantly activates JAK/STAT signaling. These findings suggest that TrkB indeed is coupled functionally to JAK/STAT activation at the membrane. Interestingly, during the course of our studies it was reported that only proBDNF activates the JAK/STAT pathway in two-week old hippocampal neurons. It was also suggested that such activation was due to p75NTR acting at the membrane (Riffault et al., 2014). It is difficult to compare our findings with those of Riffault and colleagues as they did not directly measure whether proBDNF was acting at TrkB, such as could be accomplished by examining phospho-TrkB levels after proBDNF treatment in their system. Instead, lack of TrkB in the pathway was attributed to no effect of proBDNF on levels of phospho-CREB that increase using matBDNF. This does not rule out TrkB-mediated JAK/STAT responses as the two signaling molecules, proBDNF and matBDNF, may differentially regulate the pharmacodynamics of TrKB, and therefore, the effector pathway to which they are coupled. Whether or not their results identify a distinct property of JAK/STAT signaling in mature neurons versus developing neurons remains to be determined. These findings add another dimension to the complexity of this important BDNF-induced signaling pathway in the nervous system and the need to carefully evaluate each recombinant BDNF reagent for its receptor selectivity in control assays.

Along with SE-induced increases in BDNF, changes in TrkB and p75NTR expression are also reported in animal models of epilepsy (Bengzon et al., 1993; Unsain et al., 2008; Kaplan and Miller, 2000). As studies in primary neuronal cultures reported that chronic stimulation with BDNF for several days led to a decrease in TrkB mRNA and protein expression (Knusel et al., 1997), we hypothesized that altered neurotrophin receptor expression in neurons may be mediated by BDNF. We examined this phenomenon at a time point when BDNF-induced changes in ICER expression are observed (4 hours), and found that matBDNF regulates the levels of its own receptors, by increasing p75NTR and decreasing TrkB (Figures 3D and 4C). Again, such feedback regulation by BDNF was ascribed by Riffault and colleagues (Riffault et al., 2014) to p75NTR rather than TrkB, suggesting that they either missed the participation of TrkB in the JAK/STAT signaling cascade, or that BDNF receptors can swap their roles in the JAK/STAT pathway dependent upon the expression levels of different BDNF isoforms. This intriguing possibility will be explored in future studies.

Having observed that TrkB and p75NTR protein expression is altered following BDNF stimulation, the roles of TrkB and p75NTR in the BDNF-induced JAK/STAT signaling pathway were examined in more detail. We hypothesized that siRNA knockdown of either receptor TrkB or p75NTR would influence the expression of ICER. We found that siRNA knockdown of either mRNA species TrkB or p75NTR reduces BDNF-induced ICER synthesis (Figure 4), suggesting that both receptors may play a role in a1 subunit decreases. Whether there is a

difference in the relative contribution of each receptor to BDNF-induced ICER synthesis remains to be determined. However, our current results strongly suggest that both receptor systems are part of the JAK/STAT pathway that regulates transcription of *Gabra1* in neurons.

We hypothesized that an association between JAK2, a cytokine signaling molecule, and a neurotrophin receptor could occur in response to BDNF signaling and that such interaction would happen at the cell membrane. Like neurotrophin receptors, cytokine receptors bind to their ligands as dimers, which helps to activate JAK proteins by bringing them in close proximity. The neurotrophin receptor p75NTR is also known to form a dimer with a number of different proteins and receptors. It is therefore possible that BDNF induces activation of JAK/STAT signaling by binding its receptor, which recruits JAK protein. Results from our Co-IP studies in both primary neurons (Figure 5B) and HEK (Figure 5C), and mutational analysis in HEK (Figure 6), reveal a novel association between full-length p75NTR and JAK2. Interestingly, JAK2 does not associate with TrkB, and p75NTR also associates with STAT3 (Figure 5B). Furthermore, the presence of pJAK2 in samples precipitated with an anti-p75NTR antibody was found to increase in primary neurons following stimulation with BDNF (Figure 5A), as would be expected for traditional JAK/STAT signaling through a growth factor. To our surprise, however, when we used confocal microscopy to localize the complex in neurons we found that it was predominantly in cytoplasm, then nucleus, and processes (Figures 7 and 8).

One of the assumptions made from our studies is the potential use of JAK/STAT inhibitors in the treatment of epilepsy. There are many JAK/STAT inhibitors on the current market, with most being used to treat forms of cancer. Collaborative studies in our laboratories have looked at JAK/STAT inhibition in the pilocarpine-induced SE rat, and found WP1066, an inhibitor of JAK2 and STAT3, (also STAT5 and ERK1/2) reduces the frequency of spontaneous seizures (Grabenstatter et al., 2014). We now show that WP1066 inhibits JAK/STAT through degradation of JAK2 (Figure 9A). Interestingly, ruxolitinib (RUXO), an inhibitor of JAK/STATs currently used in the clinical treatment of myeloproliferative neoplasms (Mascarenhas and Hoffman, 2012), does not degrade JAK2 (Figure 9B), but potently blocks pSTAT3 (Figure 10A). Furthermore, the complex of JAK2:eGFP and p75NTR-Flag in HEK was disturbed when neurons were treated with RUXO both in cultured neurons (using PLA, Figure 10C) and in transfected HEK cells (Figure 10B). Further studies using JAK/STAT inhibitors will help to not only understand the mechanism of JAK/STAT activation through BDNF, but could also help discover potential therapeutics for epilepsy.

Our results from fluorescence microscopy suggest that an endogenous association between JAK2 and p75NTR occurs specifically in these cellular compartments (Figures 7 and 8). Because of the predominantly cytoplasmic localization of the JAK2/p75NTR complex, we have referred to it as the signalsome (i)p75NTRJ2. At this time, we have not found evidence to link

association of JAK2 to p75NTR at the plasma membrane, although we can not rule out that (i)p75NTRJ2 gets trafficked to the membrane at some point for coupling with downstream effectors. In addition, whether or not (i)p75NTRJ2 can be activated by intracellular BDNF also remains a mystery especially fueled by our promoter studies and the fact that intracellular levels of BDNF rise in SE.

Studies have found that neurons increase their levels of matBDNF mRNAs in response to chronic matBDNF treatment in culture, suggesting that intracellular levels of matBDNF may be high during brain insult (Greenberg et al., 2009). Recent studies have also found that proBDNF levels are elevated in the hippocampus of pilocarpine-induced SE mice very quickly (Thomas et al., 2016). This increase in intracellular levels of BDNF may have a number of downstream effects that could contribute to the development of epilepsy. Indeed, in our studies in primary neuron cultures, overexpression of the full-length, translated form of BDNF (non-mutated proBDNF) caused decreased promoter activity at *GABRA1*, which is consistent with findings that seizure activity leads to downregulation $GABA_AR$ subunit α1. These results suggest that intracellular BDNF levels may be a driving factor in changes in gene expression that occur in epilepsy. It is possible that BDNF at the cell surface activates TrkB, leading to increased BDNF transcription via MAPK activation of CREB, increasing intracellular BDNF. The intracellular BDNF could activate an intracellular BDNF receptor coupled to JAK/STAT activation that turns on ICER and turns off GABRA1.

B. LSF is a novel target of BDNF signaling.

LSF acts as a cell cycle regulator in many different cell types and its dysregulation can lead to oncogenesis and unchecked cellular proliferation (Grant et al., 2012). Its role in neurons, however, has yet to be characterized beyond its potential involvement in APP-mediated cell survival (Kashour et al., 2003). Since neurons do not readily divide throughout their lifetime, and cell cycle regulators are involved in neuronal maturation, migration, and synaptic plasticity (Frank and Tsai, 2009; Lim and Kaldis, 2013), LSF likely plays a unique role in the brain. Neurogenesis occurs throughout brain development, and to a lesser extent through adulthood in three brain regions: the subgranular zone (SGZ), subventricular zone (SVZ), and the olfactory neuroepithelia. Adult neurogenesis is closely linked to seizure activity, given that seizure activity leads to aberrant hippocampal neurogenesis characterized by increased proliferation of neural progenitors, although whether this leads to more or fewer new neurons is under question (Sierra et al., 2015). The results of our study now suggest that a specific phosphorylated form of LSF, p291, is present in select neuronal populations and may be linked to seizure activity and neuron fate.

Our present studies *in vitro* with protein extracts from primary neuronal cultures found that BDNF signaling induces phosphorylation of LSF specifically at serine 291, an effect that is blocked with the MEK inhibitor, U0126. General depolarization of neurons through KCl treatment did not produce the same effect, suggesting that BDNF signaling regulates LSF activity through selective

phosphorylation at this residue. Studies from the literature with non-neuronal cell types show that mitogenic stimulation causes quick (five minutes) phosphorylation at serine 291 and later (one to two hours) phosphorylation at serine (Pagon et al., 2003; Saxena et al., 2009). In our studies with primary neuronal cultures, however, BDNF stimulation for four hours selectively phosphorylates serine 291, while there is no effect on phosphorylation of serine 309. These results suggest that phosphorylation at serine 291 of LSF may be a neuron-specific post-translational modification that BDNF uses to move them into the mature state. Because of this BDNF specificity, we chose to focus on p291 LSF rather than p309 for our examination of LSF in developing neurons.

The protein band pattern in p291 LSF western blots from our primary neuron cultures show a ladder of bands of slower moving, larger sized protein species. These bands may represent SUMOylation of LSF, which has been reported in response to heat shock (Golebiowski et al., 2009). This intensity of the bands increases with BDNF treatment and is specific to p291 LSF blots, since it is seen not in p309 LSF or total LSF blots. SUMOylation is a major regulator of neuronal processes, including protein trafficking, neuronal differentiation, synapse formation, and cell survival (reviewed by Henley et al., 2014). It is possible that SUMOylation may be an important step in the neuronal function of LSF. Future experiments should work to determine if these bands are indeed SUMOylated LSF.

It is well established that SE leads to alterations in subunit composition of the type A GABA receptor (Brooks-Kayal et al., 1998b) and that BDNF transcriptionally regulates expression of two subunits, α4 and α1. BDNF activates TrkB and MAPK signaling to regulate synthesis of Egr3, leading to increased α4 subunit levels (Roberts et al., 2005) and also activates JAK/STAT signaling through TrkB (revision in preparation Hokenson et al. 2015) leading to STAT3-mediated synthesis (Lund et al., 2008). To determine whether JAK/STAT signaling contributes to the rise in BDNF-induced p291 LSF levels, cultures were pre-treated with P6, a pan-JAK inhibitor prior to BDNF stimulation. P6 did not block BDNF-induced phosphorylation of LSF at serine 291, suggesting that p291 LSF phosphorylation may be part of the signaling cascade that affects downstream α4 subunit levels rather than α1. Future studies should address whether phosphorylation of LSF at serine 291 affects GABAR subunit expression in dentate granule cells of seizure susceptible rats.

While BDNF treatment of primary neuronal cultures *in vitro* is a limited molecular model of epilepsy, it is hard to draw conclusions about epileptogenesis *in vivo*. These results, however, do suggest that LSF is an attractive target for future research using animal models of TLE. As a first step to link LSF phosphorylation to epileptogenesis, we used an *in vivo* pilocarpine-induced SE rodent model to assess whether p291 LSF levels increase upon SE, as seen in culture after BDNF treatment. We found that hippocampal protein extracts from SE animals do indeed contain increased levels of p291 when compared to

controls. We hypothesize that this increase in p291 levels is a result of increased levels of BDNF known to occur in the hippocampus of SE animals and specifically associated with the process of epileptogenesis.

In addition, we found that total, p291, and p309 LSF, are all expressed in NeuN-positive neurons. Due to the ubiquitous expression of LSF, it is not surprising that total LSF is present in neurons. Interestingly, the majority of total LSF staining appears to be contained within the nucleus or soma of the neuron, the expected localization for a transcription factor, while p291 and p309 LSF also appear in neuronal processes. It is therefore possible that phosphorylated forms of LSF may play another role in neuronal cell types or that phosphorylation may be linked to activities occurring at a distance from the soma. It is also possible that phosphorylated forms, which are inhibitory of LSF activity, may keep LSF outside of the nucleus, where is it most active.

Absence or barely detectable levels of p291 LSF in EdU-positive, dividing neurons is consistent with findings in non-neuronal cells types that phosphorylation of LSF at serine 291 occurs in early G1 and dephosphorylates at the end of G1, before entering S phase (Pagon et al., 2003). EdU incorporates into cells during S phase of the cell cycle, at a point when LSF has been desphosphorylated. However, BDNF is known to promote neurogenesis and so it would be expected that more EdU positive cells would accompany the increase in p291 LSF, as seen in our primary cultured neurons. This does not seem to be the case, at least on an individual cell basis, in our studies, and suggests that

BDNF may promote neurogenesis by stabilizing the mature neuronal state by increasing the number of neurons detected rather than producing more precursor cells. We also saw cells that only stained for DAPI, but did not stain for LSF or any marker we used. Future studies will aim to characterize these populations.

Our immunocytochemistry studies with neuronal maturation markers suggest that p291 LSF may persist throughout neurogenesis, with the highest levels are greatest in newly generated neurons (PSA-NCAM positive) and those with a post-mitotic and mature phenotype (MAP2 positive). We did not look, however, at neurons beyond seven days in vitro, where they may still be undergoing differentiation, so at this time we do not know if p291 expression persists after neurons age. Future studies with older primary cultures, where MAP2 is persistently detected, will be needed to determine if p291 LSF plays a role in maintaining a post-mitotic cellular state over time. The results of our studies, while suggestive that p291 LSF may have a long-term role in post-mitotic neurons of the brain, rather than a short-term regulation of the cell cycle, is attractive, however, its presence in neurons may be an indication of its role as an effector of neuronal fate rather than maintenance. This is consistent with our studies of the adult rat brain regions, which show further correlation between high levels of p291 LSF and areas containing newly generated neurons or undergoing persistent neurogenesis when compared to those with low neurogenesis.

It is possible that phosphorylation of LSF at serine 291 occurs at a certain point during neurogenesis, like a switch that turns on after cell division. Based on

our markers of cell proliferation and different stages of neuronal maturation, we can predict that p291 LSF is turned off during proliferation and turns on when cells are differentiating.

In what appears to be an additional paradox, p291 LSF levels are increased in the hippocampus of animals with epilepsy, where increased neurogenesis is expected. It is possible, however, that the increase in p291 LSF reflects the greater number of post-mitotic cells that occur as a result of increased neurogenesis. In our TLE model, hippocampal dissections are performed twenty-four hours after SE induction, where there is plenty of time for aberrant neurogenesis to produce a substantial increase in post-mitotic neurons. In future studies, it may help to look at how p291 LSF protein levels change at a longer time point after SE, in animals with chronic epilepsy, where neurogenesis is decreased. It would be interesting to see if LSF phosphorylation at serine 291 causes the same abherrant neurogenesis seen after seizure activity in the hippocampus, such as increased proliferation of neural progenitors, production of EGCs, MFS, DGC layer dispersion, neuronal hypertrophy and persistence of hilar basal dendrites on adult-generated granule neurons (Parent et al., 1997; Scharfman, 2002; Pun et al., 2012).

It is also possible that p291 LSF is involved in abortive cell cycle re-entry, in which the initial insult of SE could cause upregulation of cell cycle markers and attempted cell cycle re-entry (Becker and Bonni, 2004; Liu and Greene, 2001). In this case, neurons may specifically show in upregulation in p291 LSF, rather than

total LSF or p309 LSF, the result of which may lead to neuronal cell death. It would be worth investigating if LSF is one of the cell cycle regulators involved in neuronal abortive cell cycle re-entry, and specifically if phosphorylation of LSF occurs in such states.

Increased expression of the Notch intracellular domain 1 (NICD1) and LSF protein have been reported in liver cancer tissue from human hepatocarcinogenesis and an upregulation of LSF protein has been reported in cells overexpressing Notch1 supported by a role for LSF in Notch synthesis and regulation (Fan et al., 2011). Notch genes also encode for heterodimeric transmembrane receptors that help regulate cell proliferation, differentiation, and apoptosis (Artavanis-Tsakonas et al., 1999; Osborne and Miele, 1999) and aberrant Notch signaling may contribute to carcinogenesis (Weijzen et al., 2002). Interestingly, Notch signaling is also important in the central nervous system, where it helps maintain neural stem cells, determine cell fate, and regulate neurite outgrowth in post-mitotic neurons (Ables et al., 2011; Pierfelice et al., 2011). Sha et al (2014) found persistently elevated levels of both Notch1 and NICD1 post-SE in a kainic acid (KA) mouse model of TLE, and activation of Notch signaling increased seizure frequency. Importantly, increased NICD1 protein levels are found in the sclerotic hippocampus from mesial temporal lobe epilepsy (MTLE) patients compared to controls and Notch signaling is required to maintain neural stem cells (NSCs) in the adult brain (Pierfelice et al., 2011) and to drive differentiation towards a post-mitotic state (Chapouton et al., 2010). We

hypothesize that BDNF-induced phosphorylation of LSF at serine 291 in culture and after SE may influence Notch signaling, and is an area of investigation that would be of interest in future studies.

Further study into the mechanism underlying the phosphorylation and regulation of LSF in response to seizure activity is worthwhile, due to the availability of a small molecule inhibitor of LSF, FQI1. LSF inihibition has been shown to reduce tumor growth and cytotoxicity (Grant et al., 2012) and may also serve a dual purpose in the prevention or reduction of the effects of seizure activity in the brain.

C. New Therapeutic Directions in the Treatment and Prevention of Epilepsy

The development of new therapeutics for epilepsy is important, given that one-third of epilepsy cases are refractory and don't respond to current AEDs (French et al., 2013). Current drugs also treat the symptomatic seizures of epilepsy, rather than the underlying mechanisms of the disease (Loscher and Schmidt, 2011). One major challenge to the treatment of epilepsy is about 40% of epilepsy cases result from an insult and could be prevented (Banerjee et al., 2009), but not all brain injuries lead to the development of epilepsy (Annegers et al., 1998). Studies in our lab and others suggest that use of BDNF receptor antagonists or intracellular pathway regulators may be effective for epilepsy treatments.

The JAK/STAT signaling pathway may be a good target for epilepsy treatment. Collaborative studies in our lab have shown that the JAK/STAT

pathway is activated upstream of changes in $GABA_AR$ subunit expression (Lund et al., 2008), and *in vivo* treatment with a STAT3 inhibitor in rats with SE alleviated seizures (Grabenstatter et al., 2014). Use of drugs that are already on the market, or development of more efficacious JAK/STAT inhibitors may help refractory forms of epilepsy.

While BDNF and epilepsy are closely linked, targeting BDNF for anti-epileptic therapeutics has proved to be more complicated. Based on our findings, we would assume reducing levels of BDNF would alleviate epilepsy, however, studies show that continuous administration of low dose BDNF (200-300 pg per hour) in a kainic acid model of epilepsy amelioriated seizure activity (Kuramoto et al., 2011). It is possible that low doses of BDNF downregulate TrkB receptors. Paradiso and colleagues (Paradiso et al., 2009) used a replication-defective herpes simplex virus-1 (HSV-1) vector to deliver BDNF and fibroblast growth factor-2 (FGF-2) into the hippocampus of rats 4 days after SE onset, and while they found signs of aberrant neurogenesis, there was a reduction in spontaneous recurrent seizures. The mechanism of action for this is difficult to know, since treatment was given after epileptogenesis. BDNF application may be difficult to use due to the mechanistic changes in response and therefore intracellular pathway. We want to understand the surface of the interaction between JAK2 and p75NTR and to disturb the interaction as a novel antagonist.

Many experiments inhibiting BDNF activity have targeted its receptor, TrkB, with novel antagonists (McNamara and Scharfman, 2012). TrkB may be a

better target, since treatment of mouse TLE model with a novel peptide, pY816, which uncouples TrkB from its dominant effector phospholipase Cγ1, has an anti-epileptic effect (Gu et al., 2015). There was also a reported anxiolytic effect, which was also seen in mice treated with a TrkB inhibitor, cyclotraxin-B (Cazorla et al., 2010). Given that BDNF and TrkB are important for long-term potentiation (LTP), neuronal survival, and axonal growth (Minichiello et al., 1999; Xu et al., 2000), targeting TrkB may lead to negative side effects. Interestingly, studies have revealed that treatment with AEDs phenytoin and lamotrigine cause decreased levels of serum BDNF (Soysal et al., 2015). There is clearly a causative relationship between increased levels of BDNF and epileptogenesis (Scharfman, 2005), but how to target this pathway for the development of efficacious therapeutics is unknown and is of great interest to our laboratory.

D. Conclusions and Future Directions

We show that BDNF signals through a TrkB-mediated p75NTR/JAK2 signaling pathway to induce ICER expression as relevant to the control of α1-containing GABAARs. Through our detailed characterization of this pathway, we hope to identify novel targets to test as future therapeutics for the treatment and/or cure of epilepsy, as well as other disorders of the nervous system where an alteration in BDNF signaling and GABAR expression has been associated. Taken together, these studies propose a potential mechanism by which BDNF activates the JAK/STAT pathway in neurons, an active area of investigation given the role of TrkB, p75NTR and JAK/STATs in normal processes of learning and

memory (Bibel and Barde, 2000; Korte et al., 1995; Korte et al., 1996; Zigova et al., 1998; Nicolas et al., 2012).

Our data strongly suggest that specific phosphorylation of a cell cycle regulator, at serine 291 of LSF, occurs in response to BDNF signaling and in an animal model of status epilepticus. It is likely that BDNF signaling regulates LSF activity through phosphorylation at serine 291. Elevated levels of p291 LSF are in the hippocampus of SE animals, and may be a result of increased BDNF from seizure activity. The phosphorylation of LSF at serine 291 may also play a role in neurogeneis, given that expression occurs throughout the stages of neurogensis. The relationship between adult neurogenesis and p291 LSF suggests a more complex role for BDNF in the production of neuronal populations in the normal and diseased brain. Whether this phosphorylation of LSF is an adaptive response to help produce new neurons or a part of the disease pathology requires further study.

In summary, we were able to further elucidate the previously studied link between the neurotrophin BDNF and cytokine signaling of JAK/STATs, and identified the cell cycle regulation of LSF as a novel target of BDNF signaling. Continued study of both these pathways will help to further understand the mechanisms underlying the development of epilepsy and provide new targets for therapeutic intervention.

We propose that induction of BDNF after SE binds to and activates TrkB, leading to downstream activation of signaling pathways, including JAK/STAT

phosphorylation and MAPK signaling. JAK/STATs form a complex with p75NTR, either separate complexes of JAK2 and p75NTR, p75NTR and STAT3, or potentially in a complex containing JAK2, STAT3, and p75NTR. Along with JAK/STAT activation of pCREB, which binds with ICER to inhibit *Gabra1* expression, the complex of p75NTR/JAK2 may also translocate to the nucleus and induce changes in gene expression. Activation of MAPK signaling, which leads to Egr3 binding and inducing expression of *Gabra4*, can also cause phosphorylation of LSF at serine 291, and potentially mediate cell fate. Increases in intracellular proBDNF caused by SE, may enhance the formation of a p75NTR/JAK2 complex and promote inhibition of *Gabra1* expression (see figure below).

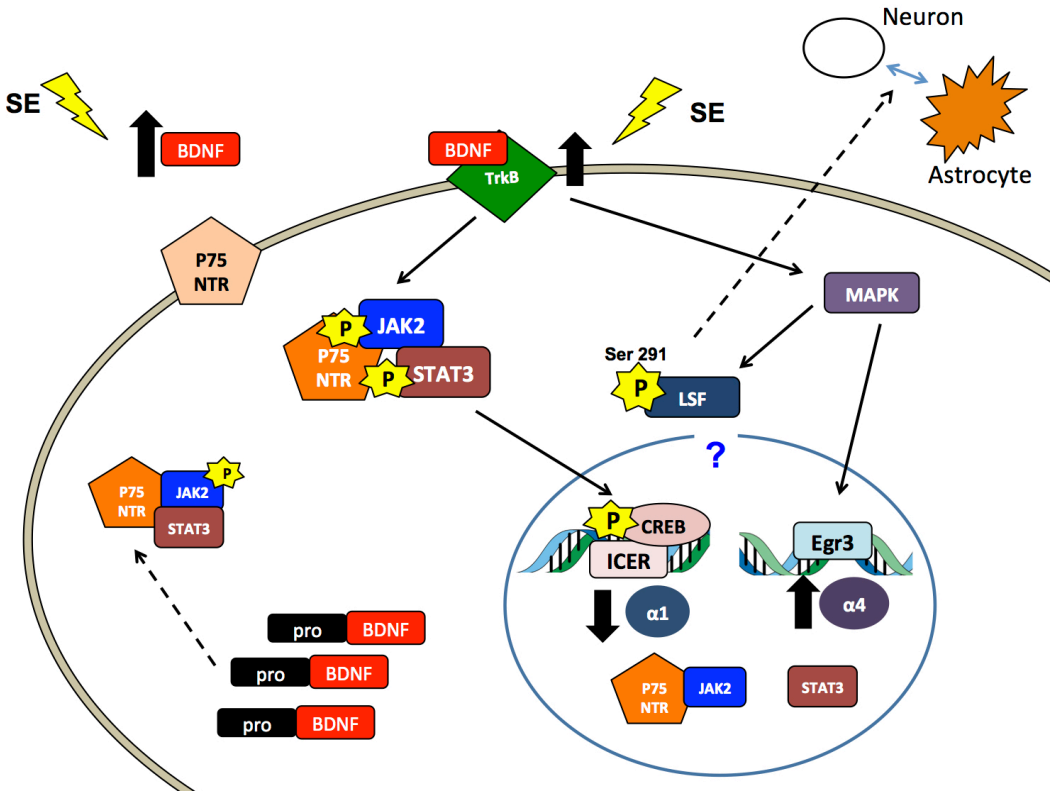


Figure 21: Schematic of proposed cellular responses to SE and BDNF signaling

VI. APPENDIX

A1. Dark-exposure decreases GABAR δ subunit levels in visual cortex of mice.

Data contributed to:
Huang, S., Hokenson, K., Bandyopadhyay, S., Russek, S.J., and Kirkwood, A. (2015) Brief Dark Exposure Reduces Tonic Inhibition in Visual Cortex. *Journal of Neuroscience.* 35, 15916-15920.

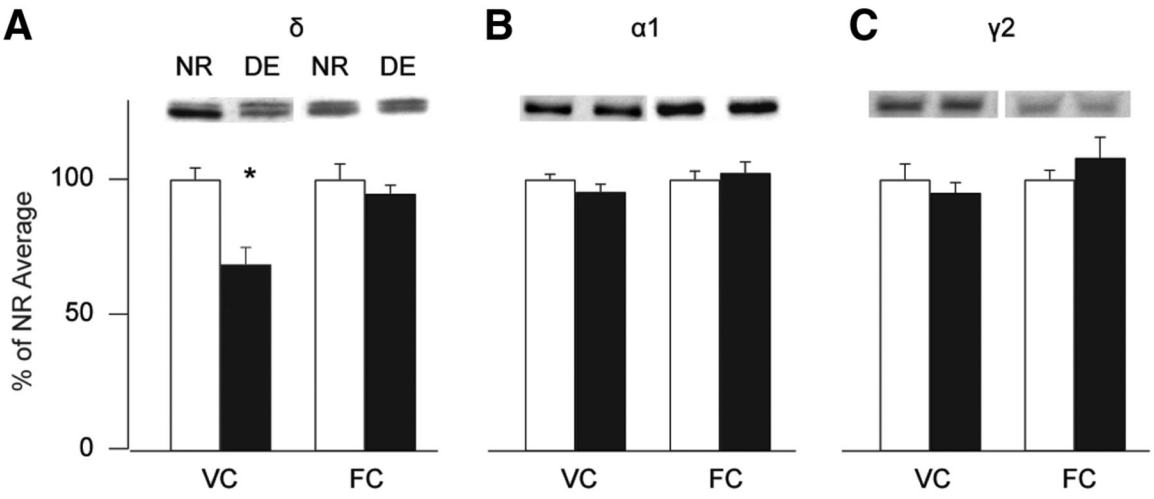


Figure 1A: Brief dark exposure (2-day, starting at P21–P22) selectively decreases GABAR δ subunit levels, but not γ2 or α1 subunit levels, in visual cortex. Shown are the results of Western blot analysis of δ (A), α1 (B), and γ2 (C) subunit expression levels as sampled in the visual cortex (VC) and frontal cortex (FC) of normal-reared (NR) and dark-exposed (DE) mice. The bars indicate the average normalized intensity values for the three subunits in NR (open bars, n = 9 mice) and DE (filled bars, n = 10 mice) mice. *$p < 0.05$. Example blots are shown on top.

VII. REFERENCES

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VIII. CURRICULUM VITAE

KRISTEN ELIZABETH HOKENSON
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Year of Birth: 1985

EDUCATION

Boston University School of Medicine, Boston, MA 2008 to 2016
Doctor of Philosophy, Biomedical Neuroscience and Pharmacology

Smith College, Northampton, MA 2003 to 2007
Bachelor of Arts, Major: Neuroscience

HONORS AND MEMBERSHIPS

Member, Society for Neuroscience, *since 2012*
Member, American Association for the Advancement of Science, *since 2008*
Member, Sigma Xi, *since 2007*
Smith College Special Studies Funding Grant Recipient, *2006*
Smith College Dean's List

RELATED EXPERIENCE

Boston University School of Medicine, Boston, MA 2010 to 2016
Graduate Research, Department of Pharmacology

- Studied the relationship between JAK/STAT and BDNF signaling and its relevance to epilepsy (Advisor: Shelley Russek, Ph.D.)
- Screened novel JAK/STAT inhibitors as therapeutics for epilepsy
- Determined a novel relationship between a cell cycle regulator, neurogenesis, and epilepsy
- Trained and mentored an undergraduate honors thesis student and two high school summer students

Pfizer Pharmaceuticals (formerly Wyeth), Cambridge, MA Summer 2009
Contract Research Associate, Immunopharmacology Research Group

- Analyzed changes in gene expression of inflammatory cytokines in spinal cords of mice with experimental autoimmune encephalomyelitis following treatment with an S1P1 agonist or an mTor inhibitor (Supervisor: Cheryl Nickerson-Nutter, Ph.D.)

Kessler Foundation Research Center, West Orange, NJ 2007 to 2008
Clinical Research Assistant, Stroke Research Lab Summer 2005, 2006

- Conducted a retrospective study on rehabilitative outcomes of right-hemisphere stroke patients with left-sided spatial neglect (Principal Investigator: Anna M. Barrett, M.D.)

- Standardized an experiment on gait trajectory in control patients versus spatial neglect patients
- Examined how donepezil (Aricept) and a self-generation behavior strategy may be used to improve memory in post-stroke patients with vascular dementia
- Studied the spontaneous recovery of spatial neglect and the potential benefits of prism goggles to improve spatial neglect in post-stroke patients

Smith College, Northampton, MA 2006 to 2007
Student Research Assistant, Department of Neuroscience

- Assesed neuroteoxicity of anesthetics on developing murine neuronal tissue with a focus on dose, exposure time, and cell death pathways (PI: Adam C. Hall, Ph.D., *see publications*)

MENTORSHIP
Greg Salimando, undergraduate honors thesis student

PUBLICATIONS
Huang S, **Hokenson K**, Bandyopadhyay S, Russek SJ, Kirkwood A. (2015) Brief Dark Exposure Reduces Tonic Inhibition in Visual Cortex. J Neurosci. 35, 15916-20.

Campbell LL, Tyson JA, Stackpole EE, **Hokenson KE**, Sherrill H, McKeon JE, Kim SA, Edmands SD, Suarez C, Hall AC (2011) Assessment of general anaesthetic cytotoxicity in murine cortical neurones in dissociated culture. Toxicology 283, 1-7.

SUBMITTED MANUSCRIPTS
Hokenson K, Benham RS, Grabenstatter HL, Cruz del Angel Y, Kim JH, Bandyopadhyay S, Brooks-Kayal AR, Russek SJ (2015) Tyrosine kinase B (TrkB) activation controls JAK/STAT signaling in neurons via an intracellular signalsome of JAK2 and the p75 neurotrophin receptor (p75NTR).

POSTER PRESENTATIONS
Hokenson KE, Hansen U., Brooks-Kayal AR, Russek SJ. Title: Probing the novel relationship between a transcriptional regulator of the cell cycle, Late Simian Virus 40 Factor, neurogenesis and epilepsy. Program No. 572.12. Neuroscience 2015 Abstracts. Chicago, IL: Society for Neuroscience, 2015.

Hokenson KE, Benham R, Brooks-Kayal AR, Russek SJ. Intracellular P75NTR: A gateway to TrkB-mediated JAK/STAT signaling in neurons. Program No. 121.06. Neuroscience 2014 Abstracts. Washington, DC: Society for Neuroscience, 2014.

Hokenson KE, Benham R, Brooks-Kayal AR, Russek SJ. Evidence for a TrkB activated P75/JAK2/STAT3 complex in neurons. Program No. 130.24. Neuroscience 2013 Abstracts. San Diego, CA: Society for Neuroscience, 2013.

Stackpole EE, **Hokenson KE**, McKeon JE, Hall AC (2007) Assessment of neurotoxicity of anaesthetic cocktails on primary cultures of dissociated murine cortical neurones. British Neurosci. Assoc. Abstr., Vol 19, PXX. Harrogate, England, UK: British Neuroscience Association Meeting, 2007.

Hokenson KE, Anfang RR, Fellus J, Barrett AM. (2006) Can we improve care plans for right hemisphere stroke and spatial neglect? *JINS, 12*, 286, DOI: 10.1017/S1355617706069918, http://journals.cambridge.org/ Boston, MA: International Neuropsychological Society Meeting, 2006.