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BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Dissertation

MAPPING THE TRANSCRIPTOME OF NEURONAL JAK/STAT SIGNALING IN RESPONSE TO STATUS EPILEPTICUS

by

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B.S., Brigham Young University, 2011

Submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

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DEDICATION

I would like to dedicate this work to my supportive husband, Nathan and our beautiful son, Elliott.

ACKNOWLEDGMENTS

To Nathan, thank you for joining me as I fulfilled my lifelong dream of moving to Boston. I know you have sacrificed so much as you have supported me in this endeavor. Thank you for listening to me practice all my talks- despite not understanding them- for editing my writing, and for the unconditional support and love that has carried me through to this achievement. I could never have done it all without you as a partner in parenting our Elliott, thank you for all you do for us!

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MAPPING THE TRANSCRIPTOME OF NEURONAL

JAK/STAT SIGNALING IN RESPONSE TO STATUS EPILEPTICUS

KATHRYN MAURENE HIXSON

Boston University School of Medicine, 2019

Major Professor: Shelley J. Russek, PhD, Professor of Pharmacology and Biology,

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ABSTRACT

Epilepsy, a disease characterized by recurring spontaneous seizures, affects over 65 million people, 2% of the world's population. Over 30% of patients are refractory to all current medical therapy, and for those that can be treated, many suffer from severe drug side-effects. Understanding the molecular basis of epilepsy is vital to the advancement of better therapeutic options and an eventual cure. Upregulation of brain-derived neurotrophic factor (BDNF) is highly associated with epileptogenesis in human patients, as well as animal models.

Our laboratory discovered that BDNF induces the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway in neurons and that inhibition attenuates spontaneous seizures in a temporal lobe epilepsy model. The mechanism behind JAK/STAT signaling in neurons and its relationship to epilepsy still remains to be elucidated and is the subject of my thesis.

Surprisingly, even though BDNF is such a major signaling molecule, its full genomic impact has never been assessed. We conducted a high-density RNA-sequencing analysis of the BDNF transcriptome in cortical neurons and probed such regulation with selective JAK inhibitors. Results suggest that 68% of BDNF-induced changes in gene expression implicated in epilepsy are regulated by JAK/STAT signaling. Eighty percent of BDNF-induced changes coding for proteins involved in synaptic neurotransmission (receptor subunits and ion channels) involve JAK/STATs. Additionally, these datasets include genes that have never been associated with BDNF regulation (such as Dopamine Receptor D5 and Galanin Receptor 1). Most interestingly, the datasets reveal that BDNF-induced JAK/STAT signaling in neurons is non-canonical, as STAT3 phosphorylation at tyrosine 705 is not required for action.

To directly examine STAT3's role in epileptogenesis, we studied the transcriptome of transgenic mice that express lower levels of STAT3 specifically in neurons. Using the intrahippocampal kainic-acid (KA) model of epilepsy, our datasets suggest that STAT3 knockdown *in vivo*, and selectively in neurons, protects mice from KA-induced dysregulation of the sphingolipid metabolism pathway that is associated with the trafficking, sorting, and stability of membrane-bound proteins, including neurotransmitter receptors and ion channels. Finally, we discuss a model for JAK/STAT signaling in neurons that includes structural aspects of an intracellular BDNF receptor (p75NTR) associated with JAK2.

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LIST OF ABBREVIATIONS

4EBP1 ..... Eukaryotic translation initiation factor 4E binding protein 1

AMPA..... α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ARC ..... Activity-regulated cytoskeleton-associated protein

ART3 ..... ADP-ribosyltranferase 3

BDNF..... Brain-derived Neurotrophic Factor

CA1/3..... Cornu Ammonis 1/3

CACNB4..... Tris-buffered Saline-Tween

CCND1 .....cyclin D1

ChIP ..... Chromatin immunoprecipitation

CHRNA4 ..... Cholinergic Receptor Nicotinic Alpha 4 Subunit

CMF.....Calcium Magnesium Free

CMV ..... cytomegalovirus

CNS ..... Central Nervous System

CNTF.....Ciliary Neurotrophic Factor

CO .....Carbon Monoxide

CONTRA .....Contralateral

COX2..... Cyclooxygenase 2

CRD .....Cysteine Rich Domain

CREB..... cAMP response elements binding protein

CXCR4 ..... C-X-C chemokine receptor type 4

DBS ..... Deep Brain Stimulation

DG ..... Dentate Gyrus

DIV ..... Days In vitro

DMSO ..... dimethyl sulfoxide

DNA ..... Deoxyribonucleic acid

DRD5 ..... Dopamine Receptor D5

DTT ..... Dithiothreitol

ECD ..... Extracellular Domain

EEG ..... Electroencephalogram

EGR ..... Early Growth Response

EP2 ..... Prostaglandin E2 receptor 2

ERK ..... extracellular-signal-regulated kinase

FC ..... Fold change

GABA ..... Gamma-Aminobutyric acid

GABAR ..... Gamma aminobutyric acid type A receptor

GABRA1, 3, etc. ..... GABAR alpha subunit 1,3, etc.

GABRG2 ..... Gamma aminobutyric acid type a receptor subunit gamma2

GALR1 ..... Galinin Receptor 1

GFP ..... Green Fluorescent Protein

GnRH ..... Tris-buffered Saline-Tween

GPCR ..... G-protein Coupled Receptor

GRIN1 ..... Glutamate Ionotropic Receptor NMDA Type Subunit 1

GRM1 ..... Glutamate Metabatropic receptor 1

H3 ..... Histone 3

HDAC ..... Histone Deacetylase

HEK ..... Human Embryonic Kidney

HIV ..... Human Immunodeficiency Virus

HP1 ..... Heterochromatin Protein 1

HRP ..... Horse Radish Peroxidase

ICD ..... Intracellular Domain

ICER ..... Inducible cAMP early repressor

IHKA ..... Intrahippocampal Kainic Acid

IL ..... Interleukin

ILAE ..... International League Against Epilepsy

IP ..... Immunoprecipitation

IPA ..... Ingenuity Pathway Analysis

IPS ..... Ipsilateral

JAK ..... Janus Kinase

JNK ..... c-Jun N-terminal kinase

KA ..... Kainic Acid

KD ..... Knockdown

KLB ..... Klotho Beta

LTD ..... Long-term Depression

LTP ..... Long-term Potentiation

LY6G6D ..... lymphocyte antigen 6 complex, locus G6D

MAPK.....Mitogen-Activated Protein Kinase

MCL1..... Induced myeloid leukemia cell differentiation protein

MMP..... Matrix Metalloproteases

MRI.....Magnetic Resonance Imaging

mTOR.....Mammalian Target of Rapamycin

NADE..... p75NTR-associated cell death executor

NF-KB .....nuclear factor kappa-light-chain-enhancer of activated B cells

NF1-C2 ..... Nuclear Factor 1-C2

NGF .....Nerve Growth Factor

NGFR.....nerve growth factor receptor

NMDA .....N-Methyl-D-aspartic acid

NT-3,4,5..... Neurotrophin 3,4,5

OMIM.....Online Mendelian Inheritance in Man

P75NTR ..... p75 Neurotrophin Receptor

PI3K.....Phosphoinositide 3-kinases

PIAS .....Protein inhibitor of activated STAT

PIK3C2B..... Phosphatidylinositol-4-Phosphate 3-Kinase Catalytic Subunit Type 2 Alpha

PILO ..... Pilocarpine

PKC ..... Protein Kinase C

PLA.....Proximity Ligation Assay

PLC-gamma .....Phospholipase C gamma

PPAR .....Peroxisome proliferator-activated receptors

PPM1L ..... Protein Phosphatase, Mg2+/Mn2+ Dependent 1L

PPP2R1B ..... Protein Phosphatase 2 Scaffold Subunit A-beta

PRMT5 ..... protein arginine methyltransferase 5

PRSS23OS ..... protease, serine 23, opposite strand

PTK ..... protein tyrosine kinases

PTP ..... Protein tyrosine phosphatase

PTZ ..... Pentylenetetrazole

REST ..... RE1 Silencing Transcription Factor

RhoA ..... Ras homolog gene family, member A

RIN ..... RNA integrity number

RIP2 ..... Receptor interacting protein-2

RIPA ..... Radioimmunoprecipitation assay buffer

RNA ..... Ribonucleic Acid

RNS ..... Responsive Nerve Stimulation

RUXO ..... Ruxolitinib

S6K1 ..... Ribosomal Protein S6 Kinase Beta-1

SCN1A ..... Sodium Voltage-Gated Channel Alpha Subunit 1

SCN3A ..... sodium channel, voltage-gated, type iii, alpha subunit

SE ..... Status Epilepticus

SEZ6L ..... Seizure 6 Like Homolog

siRNA ..... small interfering RNA

SLC6A12 ..... solute carrier family 6 protein

SMPD4 ..... Sphingomyelin Phosphodiesterase 4

SOCS .....suppressor of cytokine signaling proteins

SPHK ..... Sphingosine Kinase

STAT .....Signal Transducer and Activator of Transcription

StIP ..... Stat-interacting Protein

TBI..... Traumatic Brain Injury

TBST .....Tris-buffered Saline-Tween

TGF..... Transforming Growth Factor

TLE.....Temporal Lobe Epilepsy

TLR.....Toll-like receptor

TM ..... Transmembrane Domain

TNFR..... Tumor necrosis factor receptor

TRAF .....TNF receptor associated factors

TRKB..... Tropomyosin receptor kinase B

VNS .....Vagus Nerve Stimulation

WHO.....World Health Organization

CHAPTER I: INTRODUCTION

A. EPILEPSY

1. OVERVIEW AND SIGNIFICANCE

In 2018, the World Health Organization (WHO) determined that epilepsy impacts over 50 million people (WHO, 2018) and, in the United States alone, the cost of medical care for these patients is around $15.5 billion dollars (Epilepsy Foundation, 2015). Most importantly, 30% of those suffering from the disorder are characterized with intractable epilepsy, a condition where current drug therapy is without effect. This is especially disturbing because these patients, both young and old, are at high risk of subsequent brain damage that affects both cognitive and emotional processes, severely impacting the quality of life. Additionally, where successful management of seizures is possible through the use of current therapeutic options, the therapies are not curative as would be expected for an anti-epileptic treatment.

Seizures that occur directly in response to a brain insult should not be confused with the condition of epilepsy as seizures can resolve without epileptogenesis, the process by which the normal brain acquires the disease. Once established, however, epilepsy is a chronic disorder characterized by re-occurring and unprovoked seizures, and an epilepsy diagnosis is given to anyone with 2 or more of these episodes. There are hundreds of epilepsy subtypes. They are characterized based on the age of onset, the region of the brain that contains the seizure focus (i.e., the area where the seizures originate), the genetic mutations that produce the seizure susceptibility, and a collection of additional clinical manifestations.

Epileptogenesis can occur with or without prior evidence of status epilepticus (SE), a condition where the seizure itself is prolonged, occurring greater than 5 min, or where individual seizures occur rapidly with loss of consciousness between them. A spontaneous seizure is defined as a transient and abnormally excessive or synchronous neuronal activity that occurs at unpredictable times. In some cases, the cause is genetic (due to mutations or chromatin abnormalities in a particular epilepsy allele) or the result of a brain injury such as a stroke, head trauma, infection or tumor. These precipitating factors can often occur years before the manifestation of the epilepsy symptoms. When epilepsy is attributed to a specific cause it is referred to as *symptomatic epilepsy*. However, in *idiopathic epilepsy*, which consists of 60% of all cases, the cause is unidentifiable (WHO, 2018).

Spontaneous seizures can have various symptoms and can originate from any part of the brain. Due to the assortment of distinct seizure types a comprehensive classification system was implemented by the International League Against Epilepsy (ILAE). This diagnostic system assists healthcare professionals and patients to better understand what may have precipitated the epilepsy and the best course of its potential treatment (Shneker and Fountain 2003).

2. SEIZURE CLASSIFICATION

Historically, seizures were classified as either petit mal (no convulsions) or grand mal (with convulsions). This terminology became insufficient to characterize the wide array of symptoms and began to be used loosely to indicate the severity of the seizure. In order to standardize and accommodate the growing understanding of different types of

seizures, ILAE Classification of Epileptic Seizures was published in 1981 (ILAE, 1981), and followed up with the expanded Classification of Epilepsies and Epileptic Syndromes, in 1989 (ILAE, 1989). In 2017, it was again updated (Fisher et al. 2017).

The current classification system uses the following parameters to categorize seizures: a) where seizures begin in the brain, b) level of awareness during a seizure, and c) other features of seizures. Each will be discussed in turn.

a. Where seizures begin

This parameter is used to determine whether the onset of the seizure is focal-- meaning the seizure activity initiates in a localized region of one side of the brain, or generalized-- meaning networks on both sides of the brain are engaged at the onset of the seizure. Prior to this, the term partial was often used instead of focal, but classifications such as simple partial or complex partial have been replaced in order to decrease confusion regarding whether the word "partial" referred to the regions of the brain where the seizures originated or the level of conscious awareness during the seizure.

In addition to these designations, "unknown onset" is now used if no other category is sufficient, and "focal to bilateral seizure" is used to identify a seizure that begins on one side of the brain but spreads to both sides (previously known as a secondary generalized seizure) (Epilepsy Foundation, 2016).

b. Level of awareness

Rather than use consciousness as a measure, which has both subjective and objective aspects, patients can be categorized by their awareness of self and their surrounding environment. Generalized seizures all presumably impair the level of

awareness of the patient, so no additional description is needed. However, focal seizures may or may not involve a change in the level of awareness. “Focal aware” is used when awareness remains intact, (a term that replaces simple partial), and “focal impaired awareness” is used if any changes in awareness occur. It is also possible that the state of awareness is unknown, for example, if they were asleep or alone, in which case it is simply called an “awareness unknown” seizure.

c. Other features

Another common trait of seizures is their effect on movement. These symptoms are described as the final component to the seizure classification as either motor or non-motor. Examples of motor symptoms include atonic, clonic, or tonic movements. Non-motor symptoms may include changes in sensation, cognition or experiences. Figure 1 represents the expanded version of the seizure classification system that brings all three of these elements together.

Figure 1. The expanded ILAE classification system of seizure types from 2017. The expanded ILAE 2017 operational classification of seizure types. Focal, generalized or unknown is selected based on the location of seizure onset. For focal seizures, the specification of awareness level is optional. Focal aware or impaired awareness seizures can be further characterized by motor-onset or nonmotor-onset symptoms A focal seizure name also can omit the mention of awareness when awareness is not applicable, or is unknown, and thereby classify the seizure directly by its motor onset or nonmotor-onset characteristics. Atonic seizures and epileptic spasms usually do not have specified awareness level. Cognitive seizures imply impaired language or other cognitive domains or positive features such as déjà vu, hallucinations, illusions, or perceptual distortions. Emotional seizures involve anxiety, fear, joy, other emotions, or the appearance of affect without subjective emotional context. An "absence" seizure is atypical because of its slow onset or termination, or significant changes in tone supported by atypical, slow, generalized spike and wave activity on the electroencephalogram (EEG). Adapted from (Fisher et al. 2017).

ILAE 2017 Classification of Seizure Types Expanded Version [1]

Focal Onset

Aware | Impaired Awareness

Motor Onset
- automatisms
- atonic [2]
- clonic
- epileptic spasms [2]
- hyperkinetic
- myoclonic
- tonic

Non-Motor Onset
- autonomic
- behavior arrest
- cognitive
- emotional
- sensory

focal to bilateral tonic-clonic

Generalized Onset

Motor
- tonic-clonic
- clonic
- tonic
- myoclonic
- myoclonic-tonic-clonic
- myoclonic-atonic
- atonic
- epileptic spasms

Non-Motor (absence)
- typical
- atypical
- myoclonic
- eyelid myoclonia

Unknown Onset

Motor
- tonic-clonic
- epileptic spasms

Non-Motor
- behavior arrest

Unclassified [3]

[1] Definitions, other seizure types and descriptors are listed in the accompanying paper and glossary of terms

[2] Degree of awareness usually is not specified

[3] Due to inadequate information or inability to place in other categories

3. EPILEPSY CLASSIFICATION

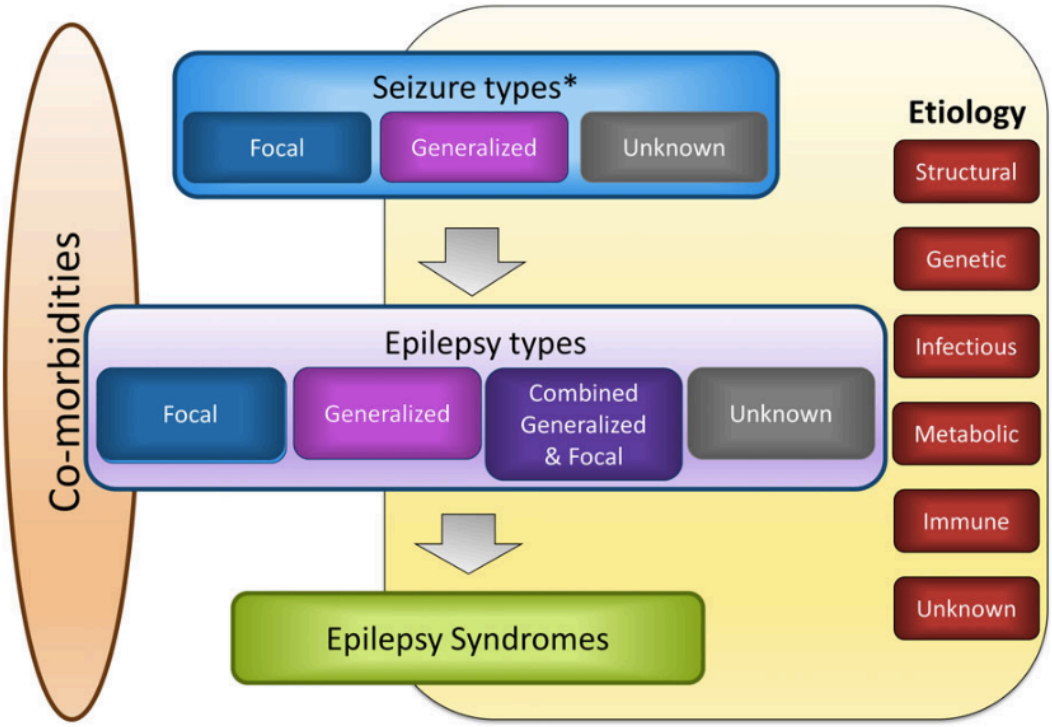
Once the seizure type(s) has been classified (as described above), a specific epilepsy subtype can then be identified. Factors that contribute to epilepsy classification include epilepsy type (one of the following: focal, generalized, combined generalized and focal, unknown), etiology, and comorbidities. These factors together lead to the diagnosis of a specific epilepsy syndrome, of which there are hundreds, each with a specific name. To choose the best classification for an epilepsy syndrome, the physician considers the patient's family history, which could indicate a heritable form of the disease, and genetic testing is performed to determine if known mutations are causing seizure susceptibility. In addition, age of seizure onset (and possibly age of remission), seizure triggers, time of day for onset, seizure frequency, characteristic EEG patterns, results of human brain imaging, associated comorbidities (such as mood disorders and cognitive impairment), and drug responsive or non-responsive state can all assist in identifying the etiology of epilepsy in an individual patient and its classification.

There are five types of epilepsy etiology. Magnetic resonance imagining (MRI) can usually pinpoint if the epilepsy is structural in nature. Outside of a structural etiology, there are five additional etiologic groups: genetic, infectious, metabolic, and immune, as well as an unknown group. Some epilepsies may have multiple etiologies. The most common of these worldwide is the infectious etiology, which could be caused by meningitis, Zika virus, Human immunodeficiency virus (HIV), and many other regional infections (Scheffer et al. 2017). Genetic epilepsies are some of the few where the etiology is clear and well understood. For those with other types of epilepsy, the cause is

often more difficult to pinpoint. Figure 2 displays the framework for using the information available to make an epilepsy syndrome diagnosis.

Figure 2. Framework for classification of epilepsies.

Seizure type, as determined by following ILAE guidelines, leads to the selection of an epilepsy type, this paired with comorbidities and etiology can point to a specific epilepsy syndrome. *Denotes onset of seizures. Figure from (Scheffer et al. 2017).


Co-morbidities
Seizure types*
Focal
Generalized
Unknown
Epilepsy types
Focal
Generalized
Combined Generalized & Focal
Unknown
Epilepsy Syndromes
Etiology
Structural
Genetic
Infectious
Metabolic
Immune
Unknown

a. Genetic epilepsies

For those cases where the etiology is genetic, there are many different types of epilepsy that arise, dependent on which gene is mutated. In the Online Mendelian Inheritance in Man database (OMIM) there are 84 genes classified as epilepsy genes, meaning a mutation in the gene causes pure epilepsies, i.e., syndromes with epilepsy as the core symptom (J. Wang et al. 2017). Mutations in these genes cause 23 various epilepsy phenotypes, meaning that most of these syndromes involve multiple genes that can cause that particular form of epilepsy. A list compiled by epilepsy syndrome with each of the mutated genes is included in Table 1. These syndromes range from mild to extremely severe forms of epilepsy and also affect patients of various ages.

Epilepsy genes have a range of functions, but the majority of them code for ion channels or neurotransmitter receptors, other common gene types include enzymes and transporters. Table 2 includes a list that categorizes each of the epilepsy genes by function.

There are also many genes associated with epilepsy that are not directly causative of a pure epilepsy. 893 genes fall into this category (284 of them are only putatively associated and need further verification) (J. Wang et al. 2017).

Table 1. Causative Epilepsy Genes

Adapted from (J. Wang et al. 2017)

Phenotype (in order of the onset age)	**Gene**
Neonatal	
Pyridoxamine 5'-phosphate oxidase deficiency (PNPOD)	PNPO
Pyridoxine-dependent epilepsy (EPD)	ALDH7A1
Benign familial neonatal seizures (BFNS)	KCNQ2, KCNQ3
Infantile and childhood	
Familial infantile myoclonic epilepsy (FIME)	TBC1D24
Benign familial infantile seizures (BFIS)	PRRT2, SCN2A, SCN8A
Amish infantile epilepsy syndrome (AIES)	ST3GAL5
Early infantile epileptic encephalopathy (EIEE)	CACNA1A, GABRA1, GABRB3, KCNQ2, KCNT1, SCN2A, SCN8A
Dravet syndrome (DS)	SCN1A, SCN9Ab
Familial febrile seizures (FFS)	GABRG2, GPR98, SCN1A, SCN9A
Generalized epilepsy with febrile seizures plus (GEFS +)	GABRD, GABRG2, SCN1A, SCN1B, SCN9A, STX1B
Generalized epilepsy and paroxysmal dyskinesia (GEPD)	KCNMA1
Myoclonic-atonic epilepsy (MAE)	SLC6A1
Childhood-onset epileptic encephalopathy (COEE)	CHD2
Focal epilepsy and speech disorder (FESD) with or without mental retardation	GRIN2A
Childhood absence epilepsy (CAE)	GABRG2
Juvenile and later	

Juvenile absence epilepsy (JAE)	CLCN2a, EFHC1
Juvenile myoclonic epilepsy (JME)	CACNB4, CLCN2a, EFHC1, GABRD
Idiopathic generalized epilepsy (IGE)	CACNB4, CLCN2a, GABRD, SLC12A5, SLC2A1
Familial adult myoclonic epilepsy (FAME)	ADRA2B
Familial temporal lobe epilepsy (FTLE)	CPA6, GAL, LGI1
Not specific	
Progressive myoclonic epilepsy (PME)	KCNC1
Nocturnal frontal lobe epilepsy (NFLE)	CHRNA2, CHRNA4, KCNT1
Familial focal epilepsy with variable foci (FFEVF)	DEPDC5

Table 2. Functional classification of epilepsy genes

Encoded protein function	**Gene and phenotype (OMIM phenotype)**
Sodium channel	SCN1A, SCN1 B, SCN2A, SCN8A, SCN9A
Potassium channel	KCNA2, KCNB1, KCNC1, KCNMA1, KCNQ2, KCNQ3, KCNT1
HCN channel	HCN1
Calcium channel	CACNA1A, CACNA1H, CACNB4
Chloride channel	CLCN2a
GABA-A receptor	GABRA1, GABRB1, GABRB3, GABRD, GABRG2
NMDA receptor	GRIN2A, GRIN2 B, GRIN2D
Acetylcholine receptor	CHRNA2, CHRNA4, CHRNB2
Enzyme	AARS, ALDH7A1, ALG13, CDKL5, CERS1, CHD2, CPA6, DNM1, EPM 2A, GNAO1, GUF1, ITPA, NHLRC1, PLCB1, PNPO, PRDM8, SIK1, ST3 GAL3, ST3GAL5, UBA5, WWOX
Enzyme modulator	ARHGEF9, CSTB, DOCK7, TBC1D24
Transporter	SLC1A2, SLC12A5, SLC13A5, SLC25A12, SLC25A22, SLC2A1, SLC6A 1
Receptor	ADRA2 B, CASR, FRRS1L , GPR98, SCARB2
Cell adhesion molecule	CNTN2, PCDH19
Signal transduction/molecule	EFHC1, FGF12
Membrane trafficking	GOSR2, STX1 B, STXBP1
Cytoskeletal protein	LMNB2, SPTAN1
Nucleic acid binding	EEF1A2, GUF1

Unclassified	ARV1, DEPDC5, GAL, KCTD7, LGI1, NECAP1, PRICKLE1, PRRT2, SZT2

Table adapted from (J. Wang et al. 2017).

4. CURRENT EPILEPSY TREATMENT

Some epilepsy syndromes resolve for their patients, however, in many cases, epilepsy patients need long-term intervention, usually pharmacological, to treat their symptoms by reducing seizure occurrence. Current epilepsy drugs treat around 70% of patients, however in 50% of those patients there are severe side effects. In the remaining 30% of patients, no drug or combination of drugs is able to ameliorate the seizures (Schmidt and Schachter 2014), which puts these patients at risk for severe brain damage and ultimately death from status epilepticus, a condition of either prolonged seizures or an increase in their frequency within a narrow time period.

The group of drug-resistant patients often has to seek less efficacious or more invasive alternatives (Saxena and Nadkarni 2011; Hirsch 2012), such as hippocampal resection an invasive surgical procedure for those suffering from temporal lobe epilepsy (TLE). Even for those patients who are responsive to current drugs, the drug treats only the seizures and occasionally boosts mood, but they are ineffective in modifying the course of the disease. Comorbidities of epilepsy including depression, anxiety, and cognitive impairment can also be exacerbated by the drug itself (Noe, Locke, and Sirven 2011); thereby, decreasing the quality of life for the patient along with their compliance to take the much needed medication.

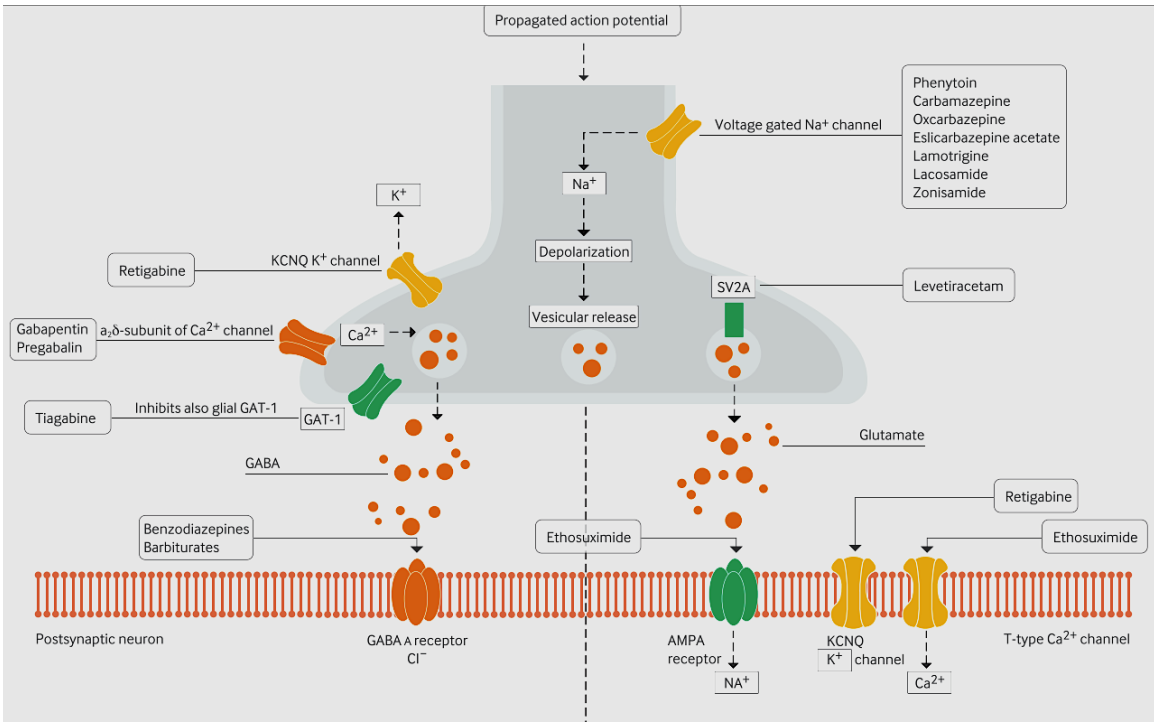
a. Pharmacological treatments

Currently available anti-seizure medications all reduce seizures by directly targeting neurotransmission. They accomplish this through various mechanisms of action. Common mechanisms include the modulation of ion channels to reduce the probability of

action potential firing, the potentiation of the γ-aminobutyric acid (GABA) response, and the inhibition of the glutamate response by targeting their respective receptors and transporters (Schmidt and Schachter 2014). The mechanisms of several common drugs working at the neuronal synapse to improve neurotransmission and reduce seizures are seen in Figure 3.

Figure 3. Mechanisms of action of antiepileptic drugs.

Drugs act through diverse mechanisms, mainly involving modulation of voltage activated ion channels, the potentiation of GABA receptors, and the inhibition of glutamate receptors. Approved antiepileptic drugs have effects on inhibitory (left-hand side) and excitatory (right-hand side) nerve terminals. Taken from (Schmidt and Schachter 2014)**.**


Propagated action potential
Voltage gated Na+ channel
Phenytoin
Carbamazepine
Oxcarbazepine
Eslicarbazepine acetate
Lamotrigine
Lacosamide
Zonisamide
Na+
K+
Depolarization
Retigabine
KCNQ K+ channel
SV2A
Levetiracetam
Vesicular release
Gabapentin
Pregabalin
a2δ-subunit of Ca2+ channel
Ca2+
Tiagabine
Inhibits also glial GAT-1
GAT-1
Glutamate
GABA
Retigabine
Benzodiazepines
Barbiturates
Ethosuximide
Ethosuximide
Postsynaptic neuron
GABA A receptor
Cl−
AMPA
receptor
NA+
KCNQ
K+ channel
Ca2+
T-type Ca2+ channel

b. Non-pharmacological treatments

For those patients with refractory epilepsy, where current treatment in ineffective, or as a complementary therapy, there are some additional options. The most drastic of these treatments is a brain surgery that involves resecting the focal area of seizure onset. This surgery is becoming more common for those with focal epilepsy and is especially successful when there is a visible brain abnormality or sclerosis. Common areas of resection are in the temporal lobe and frontal lobe. The surgery is permitted only for those where the brain location is not necessary for basic functions. A long-term study of the results of these surgeries revealed that only about 50% of patients who undergo the surgery become seizure free for 10 years (Hirsch 2012).

Outside of surgery, a ketogenic diet is another non-pharmacological option. Intractable childhood epilepsies, in particular epileptic encephalopathies are particularly responsive to a ketogenic diet. The diet involves eating four parts of fat to one part of protein and carbohydrates combined (Saxena and Nadkarni 2011). Results from a study of patients on the ketogenic diet at 3 months report that 3% of the patients were free from seizures, 31% had a greater than 90% reduction in seizure frequency, and 26% had a 50–90% reduction in seizures. After 12 months, 7% of these patients were seizure free, 20% had greater than 90% reduction in seizures, and 23% had a 50–90% reduction in seizures (Vining 1999). However, the underlying mechanism of why the ketogenic diet reduces seizures is still unknown, although it has been suggested that it increases mitochondrial glutathione levels which enhance mitochondrial antioxidant status protecting neurons from damage (Jarrett et al, 2008).

Neurostimulation is also an increasingly used method for refractory epilepsy treatment. Three stimulation devices have been approved. The first one to be developed, Vagus Nerve Stimulation (VNS), can now be delivered in a non-invasive way. It was early reported that a 50% seizure reduction occurred in 24.5%–46.6% of patients (Uthman et al. 1993). Recently in 2018, Deep Brain Stimulation (DBS) was approved in the United States for epilepsy. DBS involves putting an electrode in the brain that is able to disrupt seizure-triggering electrical activity by delivering continuous intermittent stimulation. A seven year study revealed that people had a 75% reduction in seizure frequency (Salanova et al. 2015). The last approach, Responsive Neurostimulation (RNS) is a very similar approach but differs in that it senses when electrical activity is altered in the brain that could cause a seizure and then initiates within milliseconds stimulation to interfere with seizure generation. An RNS study showed 44% decrease in seizures after 1 year, 53% at 2 years, and up to 66% after 3 to 6 years of using RNS (Bergey et al. 2015).

There are also some alternative medicine strategies that are offered to patients with varying levels of success. Many improve the quality of life with and without the reduction of seizures: Yoga, acupuncture, meditation, and herbal treatments (Saxena and Nadkarni 2011). Despite the multitude of treatment options, the debilitating disease of epilepsy and the harsh side effects of drugs remain a pressing problem for most epilepsy patients. Some, especially the young, have a very low quality of life due to multiple seizures a day that occur at unpredictable times. It is, therefore, important to identify drugs that will reduce seizure frequency and severity and, moreover, be curative to alter the progression of the disease. Making a real difference can only occur once we have a

clearer understanding of the molecular basis of epilepsy, as characterized by the susceptibility to spontaneous seizures.

5. TEMPORAL LOBE EPILEPSY

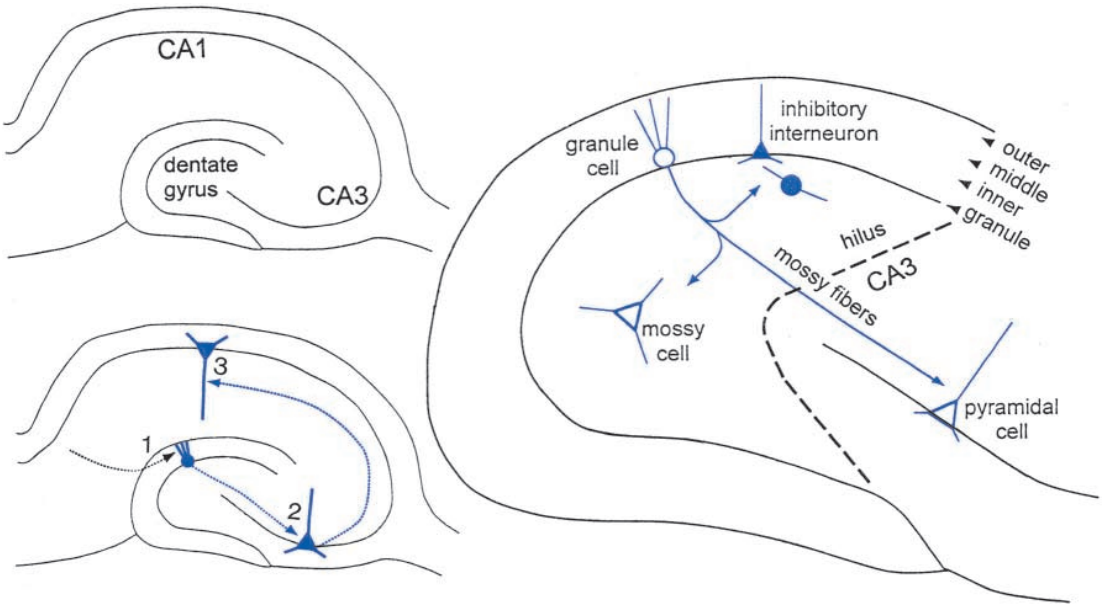
One epilepsy syndrome that has particularly high rates of drug resistance is Temporal Lobe Epilepsy (TLE). TLE is the most common form of focal epilepsy, and it affects approximately 615,600 people in the United States (Asadi-Pooya et al. 2017). Although the cause in many cases is unknown, often a brain insult such as stroke (Menon and Shorvon 2009), traumatic brain injury (TBI) (Pitkänen and Bolkvadze 2012), infection (Fotheringham et al. 2007; Donati et al. 2003), or a seizure (Theodore et al. 1999) occurs years prior to the development of the spontaneous seizures. After the injury occurs, molecular cascades, morphological changes, and inflammatory pathways are initiated that alter brain circuitry over the course of months to years and can ultimately result in a brain with increased susceptibility to spontaneous seizures.

The characteristic pathophysiology of TLE includes the onset of the seizures occurring in the temporal lobe (see Figure 4, a diagram of the hippocampus), and in 75% of cases (No et al. 2017) hippocampal sclerosis, which includes neuronal loss in the cornu Ammonis 1 and 3 (CA1 and CA3) region of the hippocampus, damage to inhibitory interneurons in the hilar region of the hippocampus and seizure-induced excitotoxic death in granule cells of the dentate gyrus (Nihal C. de Lanerolle, Lee, and Spencer 2012; Mathern et al. 1996). Mossy fibers, the axons of the granule cells that are being damaged, which project into the dentate gyrus (DG) and CA3 region also sprout new aberrant

connections resulting in synaptic reorganization that can affect both excitatory and inhibitory pathways (Sutula et al. 1989; Nadler 2003; Babb et al. 1991).

Figure 4. Organization of the hippocampus.

Left: Top. A schematic of the rodent hippocampus illustrates the major subfields: area CA1, area CA3, and the dentate gyrus. Bottom. The trisynaptic pathway is illustrated. Afferents from the entorhinal cortex (the perforant path) innervate the outer two-thirds of the granule cell dendrites (synapse 1). Granule cell axons (the mossy fibers) project to the proximal apical dendrites of area CA3 pyramidal cells (synapse 2). CA3 pyramidal cell axon collaterals (the Schaffer collaterals) innervate the proximal apical dendrites of area CA1 pyramidal cells (synapse 3). Right A schematic of the rat dentate gyrus indicates the major cell types and the laminar organization. The molecular layer is subdivided into three distinct lamina, the outer, middle, and inner molecular layers. The granule cell layer is a packed layer of granule cells and some inhibitory neurons ("interneurons"). Interneurons are a heterogeneous population of neurons that contain GABA as well as many other peptides, and are distributed throughout the dentate gyrus. Glutamatergic "mossy" cells are located in the hilus and project primarily to the inner molecular layer. Although the major afferent input to the region is the perforant path, many other afferent systems, for example, CA3 pyramidal cell axons and various subcortical nuclei (e.g., dorsal raphe, locus coeruleus, septum), also innervate distinct areas of this region. Figure from (Helen E. Scharfman 2002).


CA1
dentate
gyrus
CA3
3
1
2
granule
cell
inhibitory
interneuron
outer
middle
inner
granule
hilus
CA3
mossy fibers
mossy
cell
pyramidal
cell

Seizures for those with TLE can be focal aware or focal with impaired awareness, they can also spread bilaterally. Focal aware seizures in the temporal lobe often last less than sixty seconds and symptoms can include abnormal sensations like déjà vu, unwarranted fear, hallucinations, amnesia, or nausea. Many with TLE experience auras which is an indescribable sensation that is usually predictive of an upcoming seizure. An aura is actually a small focal aware seizure itself and it can mean a focal seizure with impaired awareness is coming. Since this type of seizure is sometimes subtle, it can often go undiagnosed for extended periods of time.

Focal impaired awareness seizures have spread to larger regions of the temporal lobe and include motionless staring, automatic movement like lip smacking, or transient aphasia. They typically last around 2 minutes but can leave the patient confused for 15 minutes to days later. Although the seizure itself usually is not dangerous, if the patient is mid activity such as driving, it can lead to serious injury. When a focal seizure spreads bilaterally it can lead to a tonic-clonic seizure involving jerking and these can last up to 3-5 minutes or more (Bob and Neckar 2016; French et al. 1993).

6. ANIMAL MODELS

In order to study TLE, animal models are used to discover the molecular determinants of neuropathology that is responsible for spontaneous seizure development, the hallmark of the disease. Animal models recreate an initial injury that has a latent period prior to the onset of spontaneous seizures. Several methods are used to accomplish this, each presents with a slightly different version of the disease and has its

own specific advantages and disadvantages. The leading approaches in the field will be discussed below.

a. Chemoconvulsants

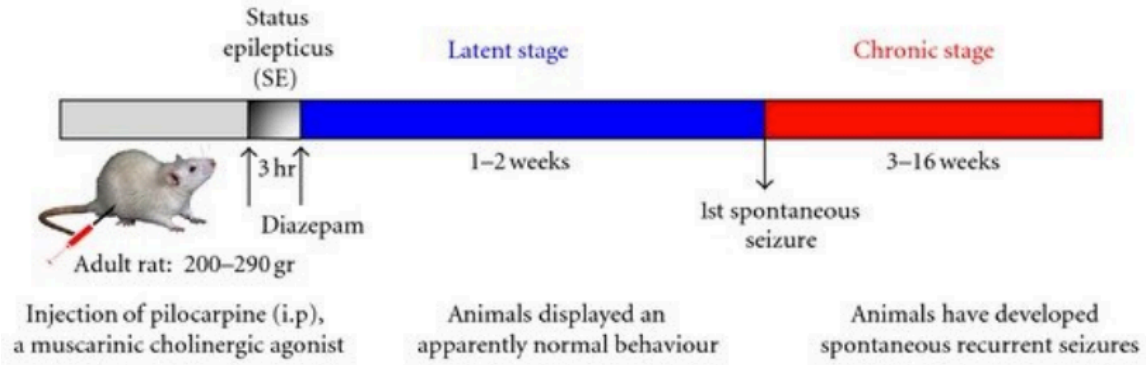
Two major approaches are used to invoke chronic acquired epilepsy in rodents. The first is termed “kindling”. The kindling methodology involves the use of a low, sub-seizure threshold dose of convulsant agent, often pentylenetetrazol (PTZ). In the initial dose, no seizure activity will be seen. The animal is then given the same dose repeatedly at varying time points, but usually 1 or more times per day, after several doses, the excitability of the brain circuitry shifts such that the small dose is able to invoke a seizure, and as the administration of the drug proceeds, the seizures become increasingly severe. Pathology of this method mimics TLE with mossy fiber sprouting, hippocampal damage, and glucose hypometabolism (Samokhina and Samokhin 2018).

The other major approach is to give one large dose of the convulsant, large enough to cause SE. After a set amount of time (45 min-1hr) the seizure is halted by administration of an anti-convulsant. After SE, the animals may be seizure free for several days to weeks, and many go on to develop spontaneous seizures, thus mimicking the human condition (see Figure 6). Two main drugs are used to invoke the SE, Kainic Acid and pilocarpine. Kainic Acid is an l-glutamate analog that causes seizure activity localized in the hippocampus. It mirrors human TLE with significant neuronal loss, neurogenesis and mossy fiber sprouting (Raedt et al. 2009). A major downside, however, is that mortality rates are high, there is variable frequency and severity of spontaneous seizures, and the chemical itself can cause confounding damage so that not all deleterious

effects can be ascribed to the acute seizures (Kandratavicius, Balista, et al. 2014). The other chemoconvulsant, pilocarpine, is a muscarinic acetylcholine receptor agonist. SE induced by pilocarpine causes seizure activity in both the limbic regions and the neocortex. Neurotrophin upregulation similar to that characteristic in humans is seen in the pilocarpine model (Kandratavicius, Balista, et al. 2014). Pilocarpine symptoms also include mossy fiber sprouting and cell loss (Mello et al. 1993). In this model, mortality is high and the development of spontaneous seizures is variable, although combining a pre-treatment with lithium at 30 mg/kg and multiple doses of pilocarpine at 10 mg/kg until the occurrence of status epilepticus can reduce mortality rates to 7% and a high proportion of animals (85%) will develop spontaneous seizures (Glien et al. 2001). While other agents can be used, they don't imitate the human development of chronic epilepsy as well.

Figure 5. Rodent status epilepticus model of temporal lobe epilepsy.

Timeline and protocol for pilocarpine-induced status epilepticus with subsequent spontaneous seizures. (Similar for Kainic Acid.) Following the injection of drug, a one to two-week latent period with normal behavior occurs, followed by chronic epilepsy symptoms. In this model, a dynamic reorganization of the glutamatergic network, including neurodegeneration, neurogenesis, neo-spinogenesis, spine morphogenesis, and neo-synaptogenesis associated with an aberrant sprouting of granule cell axons, is well established in the dentate gyrus. Adapted from (Ferhat 2012).


Status
epilepticus
(SE)
Latent stage
Chronic stage
3 hr
1–2 weeks
3–16 weeks
Diazepam
lst spontaneous
seizure
Adult rat: 200–290 gr
Injection of pilocarpine (i.p),
a muscarinic cholinergic agonist
Animals displayed an
apparently normal behaviour
Animals have developed
spontaneous recurrent seizures

b. Electrical stimulation

Electrical stimulation is generally more laborious and expensive for chronic studies. There are three main types of electrical stimulation. The first is electroshock-induced seizures. This method is a whole-brain, one-time shock. The shock can be at various strengths which can determine if a focal or generalized seizure occurs. Usually a small shock leads to a forebrain focus. This method can be good for drug screening in some cases, but in others it has shown low predictability (Kandratavicius, Balista, et al. 2014).

The second electrical stimulation approach is afterdischarges. Afterdischarges are able to stimulate more precise regions, and is most commonly used to stimulate limbic regions, including the hippocampus. In particular, this focal ability is important for the study of TLE. One disadvantage of the approach is that although the stimulation is region specific, it is not exciting the subtype of cells that are responsible for human seizures, but rather exciting all the cells (Kandratavicius, Balista, et al. 2014). This is, however, a problem that is integral to all of the models currently in use.

Lastly, there is the kindling model of electrical stimulation. This is similar to the chemoconvulsant kindling model but used more often. It involves repeated electrical stimulations rather than drug administrations. This approach eventually leads to neuronal loss and a number of cellular alterations in brain circuits that result in spontaneous seizures. One major disadvantage is its high cost and labor intensity (Kandratavicius, Balista, et al. 2014).

7. EPILEPTOGENESIS

Since TLE is an acquired epilepsy, there is a latent period of time prior to spontaneous seizures where the previously normal brain undergoes changes that make it susceptible to epilepsy. These changes can be spurred on by a brain insult like those listed above that increase risk for TLE, or they can be initiated by an unknown cause. The process whereby epilepsy-promoting changes lead to neural network hyperexcitability is referred to as epileptogenesis. The pathophysiology in the temporal lobe described above as characteristic for TLE patients is a result of this process. What follows is a review of the various components involved in epileptogenesis and how they contribute to seizure susceptibility.

a. Molecular signaling pathway regulation

Brain-Derived neurotrophic factor (BDNF) is markedly increased after SE and its levels change during the course of epileptogenesis. It is also associated with a change in the expression of epilepsy relevant gene products through activation of several major molecular cascades that include protein kinase C/mitogen-activated protein kinase (PKC)/(MAPK) and Janus kinases/Signal Transducer and Activator of Transcription (JAK/STAT) (Brooks-Kayal, Raol, and Russek 2009). A thorough discussion of the involvement of BDNF in epileptogenesis will be presented in later parts of this introduction.

Altered gene regulation during epileptogenesis has also been attributed to changes in the mechanistic target of rapamycin (mTOR) pathway. mTOR is activated after brain injuries such as TBI and chemoconvulsant-induced seizures in animal models (Goldberg

and Coulter 2013). When rapamycin, an mTOR inhibitor, is administered prior to the insult, it prevents the development of spontaneous seizures in a subset of animals. mTOR inhibition also prevents mossy fiber sprouting in epileptogenesis animal models (L.-H. Zeng, Rensing, and Wong 2009) suggesting that it may play an important role in the etiology of epilepsy. mTOR pathway signaling leads to the activation of the gene p70 ribosome S6 kinase1 (S6K1) and eukaryotic initiation factor 4E-binding protein 1 (4EBP1) which both promote protein synthesis and have been implicated in epilepsy (Ling-Hui Zeng et al. 2010).

Another important transcriptional regulator that is implicated in epileptogenesis is the repressor element 1 silencing transcription factor (REST). SE in animals increases the expression of REST in the hippocampus (Palm et al. 1998). REST binds to co-repressors and recruits histone deacetylases (HDACs) to the gene. These HDACs tighten the chromatin structure and inhibit transcription. Many genes involved in epilepsy have REST binding sites and in animal models, those genes are significantly enriched in differentially expressed gene sets between SE and control animals (Roopra, Dingledine, and Hsieh 2012). Also, a conditional REST knockout mouse modulated the rate of seizure progression in a kindling model of TLE (Hu et al. 2011). It has also been suggested that the ketogenic diet exerts its effects through the manipulation of RESTs (Garriga-Canut et al. 2006).

b. Cellular reorganization

Several types of cellular reorganization that occur in the hippocampus coincide during the epileptogenic phase. Which of these are caused by seizures vs. cause the seizures is still unclear. They include cell death, the birth of new cells, strengthening and weakening of existent synapses, and rerouting of axons. It is believed that the product of these changes is hyperexcitability in the temporal lobe (Goldberg and Coulter 2013).

Neuronal death occurs in the dentate hilus and the hippocampal pyramidal cell layers in CA1, and CA3, as well as some in CA2 (Chang and Lowenstein 2003). The type of cells lost include somatostatin-containing interneurons and in some cases parvalbumin-containing interneurons as well (Gorter et al. 2001), hilar neurons (Paul S. Buckmaster 2012), and neuropeptide Y-immunoreactive interneurons (N C de Lanerolle et al. 1989), whereas dentate granule cells are relatively preserved (Chang and Lowenstein 2003). Cell death has also been seen in layers II and III of the entorhinal cortex and in the amygdala (Du et al. 1993). The neuronal loss leads to astrocytosis which causes scarring that can often be seen in MRI or in resected tissue and is referred to as hippocampal sclerosis (Johnson et al. 2016). These changes have been seen in humans and animals models after SE (Fujikawa et al. 2000; P S Buckmaster and Dudek 1997) as well as after several smaller seizures and the cell death progresses with frequency of seizures (Cavazos and Sutula 1990).

While some cells are dying, other cells are going through neurogenesis. Neurogenesis occurs in the dentate gyrus throughout life and appears to be beneficial in

the normal brain, and after some injuries. However, in animal models of epileptogenesis, neurogenesis increases dramatically for several weeks (Helen E. Scharfman 2002; Danzer 2018; J M Parent et al. 1997). Some of these newborn neurons appear to be functioning normally and migrate to the granule cell layer. However, other cells migrate ectopically to the molecular layer in the hilus (H. Scharfman et al. 2005). One study showed ablation of neurogenesis prior to SE in rats slowed the progression of spontaneous seizures in those animals demonstrating these cells may form aberrant connections that contribute to overexcitation (Cho et al. 2015).

Another process that contributes to network reorganization during epileptogenesis is mossy fiber sprouting. Mossy fibers are the axons of glutamatergic dentate granule cells that synapse onto the dendrites of hilar mossy cells, pyramidal cells in the CA3 region of the hippocampus, and inhibitory interneurons (Paul S. Buckmaster 2012). Aberrant growth of these axons, and synapses onto their own granule cell dendrites in the inner molecular layers is called mossy fiber sprouting. Most patients with TLE have mossy fiber sprouting, although there are a few exceptions (Paul S. Buckmaster 2012). Most animal model of epileptogenesis also display the phenomenon, although even if it is prevented by rapamycin, an mTOR inhibitor, seizures may still develop (P. S. Buckmaster and Lew 2011). One potential cause of this abnormal axonal growth is the loss of mossy fiber target cells, the hilar mossy cells. The death of these mossy cells may lead the mossy fibers to reroute, setting up a recurrent circuit in the dentate (Cavazos and Sutula 1990). Mossy fiber sprouting can also increase synapses on to inhibitory interneurons, potentially as a protective mechanism (Ribak and Peterson 1991). Outside

of mossy fibers, sprouting of GABAergic cell axons has also been identified (Christenson Wick et al. 2017).

Axonal sprouting is a hallmark of changes in synaptic plasticity that are key to processes of learning and memory. Synaptic plasticity is the ability of synapses to strengthen or weaken over time as a response to the activity level of the cell. Long-term plasticity (LTP) occurs at excitatory synapses and can last for hours or longer. LTP is an increased response of a neuron to repeated stimulation such that a subsequent equal intensity stimulus will now generate a greater depolarization (Purves, 2001). Interestingly, LTP is found to be suppressed in both SE and kindling models of epileptogenesis (Suárez et al. 2012; Leung and Wu 2003). It has been suggested that increases in long-term depression (LTD), which weaken synaptic strength, also contributes to LTP decline in the pilocarpine model of epilepsy (unpublished results from the Brooks-Kayal laboratory). This is especially important since it has been shown that blocking LTD enhances memory retrieval (Wong et al., 2007).

c. Inflammation

Brain inflammation is also a hallmark of TLE that initiates prior to the onset of spontaneous seizures. Within 30 minutes of an epileptogenic injury (such as SE, stroke or TBI), receptors for cytokines and prostaglandins are activated creating a prolonged inflammatory response both in the central nervous system (CNS) and peripheral nervous system (Riazi, Galic, and Pittman 2010). This inflammatory response is not sufficiently counteracted by endogenous anti-inflammatory mechanisms. Three main inflammatory pathways are believed to be involved in the response to injury: 1) Interleukin (IL)-1

receptor (R)/Toll-like receptor (TLR), 2) Transforming Growth Factor (TGF)-β, and 3) Cyclooxygenase-2 (COX-2) (Annamaria Vezzani, Friedman, and Dingledine 2013).

IL-1/TLR signaling activates innate immunity and is thus the first immune pathway to be activated after a stressor. IL-1R as well as several TLRs are expressed in the brain by microglia, astrocytes and neurons. After a seizure or other brain injury, there is a rapid upregulation of these inflammatory receptors that is associated with a change in hippocampal neuronal excitability. The molecular mechanisms underlying such changes is under investigation by multiple labs. In particular, it has been shown that activation of IL-1B alters synaptically-mediated GABA inhibition in the CA3 (S. Wang et al. 2000) and reduces N-Methyl-D-aspartic acid (NMDA) receptor and voltage-gated calcium channel-induced outward currents, leading to an increase in CA1 neuronal excitation (R. Zhang et al. 2010). IL-1/TLR signaling can also inhibit glutamate reuptake and affect permeability of the blood brain barrier (Annamaria Vezzani, Friedman, and Dingledine 2013; A. Friedman, Kaufer, and Heinemann 2009). In addition, IL-1β has been associated with the aberrant positioning and function of cells born from post-injury neurogenesis (Bernardino et al. 2005).

The TGF-β signaling pathway can be activated by serum albumin that can enter the brain through a damaged blood brain barrier that occurs during or in response to SE or other brain injuries. TGF-β signaling is activated in many epileptic conditions and results suggest that it may also contribute to brain resident microglial activation (Cacheaux et al. 2009; Plata-Salamán et al. 2000).

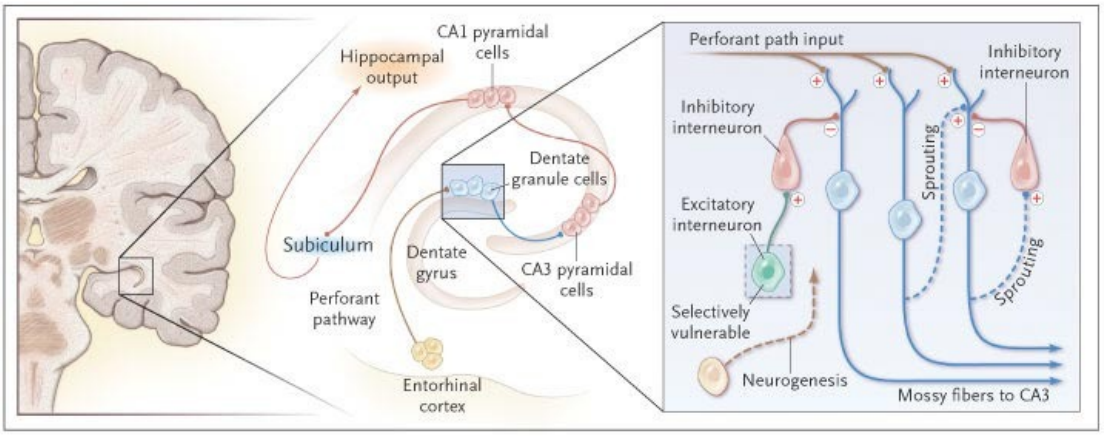
The third inflammatory pathway, COX2, is also markedly increased after acute seizures and is associated with an increase in hippocampal granule cell excitability and increases in LTP (Annamaria Vezzani, Friedman, and Dingledine 2013; Marcheselli and Bazan 1996). COX2 pathway stimulation is long known to cause free radical release that potentiates glutamate-mediated excitotoxicity (V. L. Dawson et al. 1994) and plays an important role in the aberrant neurogenesis involved in epileptogenesis (Jung et al. 2006). Although COX2 is involved in the inflammatory response in epilepsy, COX2 inhibition as a therapeutic approach has shown only inconclusive results (Dhir 2019). Recent research in this area has identified a specific role for COX2 induction of prostaglandin E2 synthesis and subsequent activation of the E prostanoid receptor 2 (EP2). EP2 activation contributes to microglial cell death, an autoregulatory response which can resolve the inflammatory phase of microglia and prevent tissue damage after a seizure (Fu et al. 2015). Targeting EP2 could offer a more selective and potentially more efficacious COX2-pathway treatment approach.

Taken together, the emerging association of inflammatory signaling with epilepsy etiology suggests that selective targeting of these pathways in the future may offer new options in the treatment of the intractable epilepsies. In fact, manipulation of each of these pathways (IL-1/TLR,TGF-B, and COX2) during the epileptogenic phase can delay or prevent development of spontaneous seizures in animal models of epileptogenesis (Maroso et al. 2011; A. Vezzani et al. 2000; Annamaria Vezzani, Friedman, and Dingledine 2013; Serrano et al. 2011). The key will be to identify the most selective targets within these pathways to produce therapies with the least amount of side effects so

that they can make it to the marketplace and improve the quality of life for epilepsy patients.

Figure 6. Hippocampal reorganization with mossy fiber sprouting.

Hippocampal sclerosis is the most common identified pathological feature in cases of mesial temporal-lobe epilepsy. A close-up of the dentate granule-cell layer reveals several morphologic changes characteristic of hippocampal sclerosis that may play a part in epileptogenesis. Newly sprouted mossy fibers from dentate granule cells can synapse on dendrites of neighboring dentate granule cells, resulting in a recurrent excitatory circuit. They can also sprout onto inhibitory interneurons. Excitation interneurons, which normally activate inhibitory interneurons, may be selectively vulnerable to brain insults. Finally, neurogenesis of new dentate granule cells continues into adult life, and these neurons may integrate themselves into abnormal circuits. Figure and caption from (Chang and Lowenstein 2003).


CA1 pyramidal cells
Hippocampal output
Dentate granule cells
Subiculum
Dentate gyrus
CA3 pyramidal cells
Perforant pathway
Entorhinal cortex
Perforant path input
Inhibitory interneuron
Inhibitory interneuron
Sprouting
Excitatory interneuron
Sprouting
Selectively vulnerable
Neurogenesis
Mossy fibers to CA3

B. BDNF SIGNALING IN THE BRAIN

1. OVERVIEW

One factor known to play a role in the epileptogenic process is Brain-derived neurotrophic factor (BDNF). BDNF is a member of the neurotrophin family along with nerve growth factor (NGF), involved in the regulation of growth, maintenance, proliferation, and survival of certain target neurons, neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4), which support survival of existing neurons and maturation of new neurons. BDNF was first isolated in 1982 (Barde, Edgar, and Thoenen 1982) and the structure was determined in 1989 (Leibrock et al. 1989). The protein, composed of 11 exons, can be spliced to produce various biologically active forms that have distinct functions in multiple tissue types throughout the entire brain and other areas of the body (Kowiański et al. 2018). BDNF is particularly expressed at high levels in the hippocampus (Conner et al. 1997). In the endoplasmic reticulum it is synthesized and undergoes its first cleavage from its first precursor form, preproBDNF, to its second precursor form, proBDNF. Next it is translocated to the golgi apparatus to be packaged into vesicles. ProBDNF can be cleaved intracellularly by furin in the golgi or in secretory vesicles by convertases (Mowla et al. 1999). It can also be cleaved extracellularly by matrix metalloproteases (MMP) 2, 3, 7 and 9 (Mizoguchi et al. 2011). ProBDNF and BDNF are secreted by both neurons and glial. In neurons, its release is activity-dependent (Jianmin Yang et al. 2009).

The actions of BDNF are very important during brain development but continue to have important functions throughout adulthood. BDNF plays a crucial role in

neurogenesis (Foltran and Diaz 2016; H. Scharfman et al. 2005), gliogenesis and synaptogenesis (Park and Poo 2013). It can also regulate short and long synaptic plasticity that is important for learning and memory (Edelmann, Lessmann, and Brigadski 2014). In the peripheral nervous system (PNS), BDNF prevents regulated cell death (Sendtner et al. 1992). BDNF can act both pre- and post-synaptically (Sasi et al. 2017; Zakharenko et al. 2003).

2. BDNF RECEPTORS

The various isoforms of mature BDNF and proBDNF can have different and even opposing actions through acting on distinct receptors. BDNF can increase neuronal excitability and synaptic strength while proBDNF can decrease neuronal excitability and facilitate synaptic depression (Sasi et al. 2017). BDNF has high affinity for the tropomyosin receptor kinase B (TrkB) receptor (dissociation constant ~10^{-11} M) it can also bind the p75 neurotrophin receptor (p75NTR), although with lower affinity. (dissociation constant 10^{-9} M). proBDNF binds the p75NTR preferentially. Each receptor and its functions will be discussed below.

a. TrkB receptor

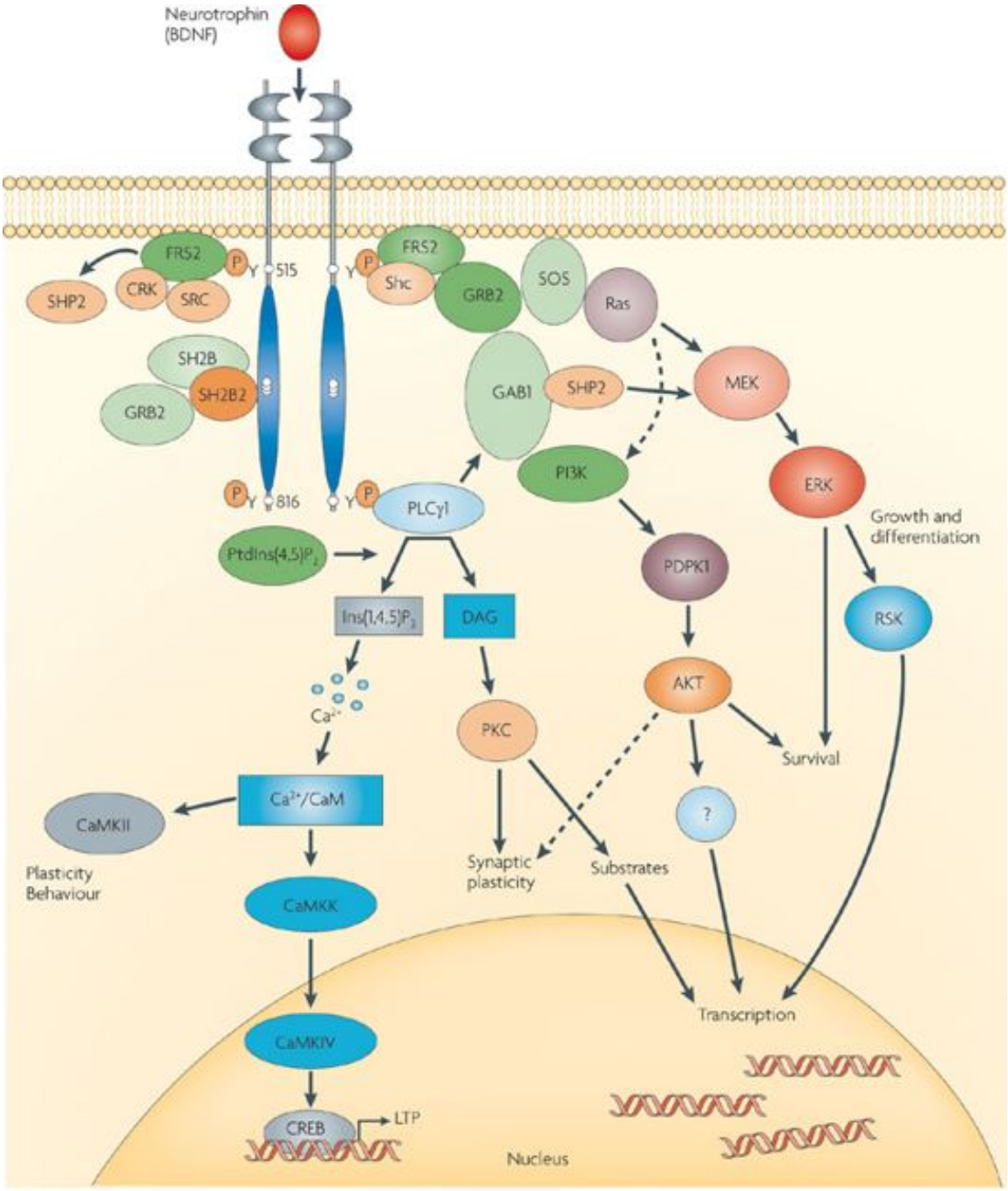
BDNF-activated TrkB signaling promotes synaptic efficiency and LTP. Upon BDNF binding, TrkB dimerizes and autophosphorylates which leads to the activation of three main signaling cascades: the Ras–mitogen-activated protein kinase (Ras/MAPK) pathway that promotes neuronal differentiation and growth, the phosphatidylinositol 3-kinase -Akt (PI3K/Akt) pathway that regulates the survival and growth of neurons and glia, and the Phospholipase C-γ (PLCγ) pathway (Minichiello 2009) that orchestrates the

release of internal Ca^{2+} stores and the activation of calcium/calmodulin dependent kinases along with the cyclic AMP-responsive element-binding protein (CREB), a transcription factor that is crucial to neuroplasticity (Figure 7).

TrkB activation is required for the phenomenon of LTP. Both homozygous and heterozygous BDNF knockout mice show substantial reductions in LTP (Korte et al. 1995a) that can be rescued by applying recombinant BDNF (Patterson et al. 1996). Even in wild-type animals, application of additional BDNF is sufficient to increase LTP (Kang et al. 1997; Figurov et al. 1996). BDNF's control over LTP was shown to be dependent upon the TrkB receptor using TrkB mutant mice (Minichiello et al. 2002) and the blockade of BDNF/TrkB interactions (Kang et al. 1997). Both of these manipulations result in decreased LTP. Moreover, when the TrkB mutation specifically disrupted the docking site on the receptor for PLCγ, LTP was abolished. This blockade was not seen for the other TrkB mutations, suggesting that the PLC pathway may play a more specific role in learning and memory (Huang and Reichardt 2003; Gärtner et al. 2006).

Figure 7. Major TrkB related signaling pathways

The interaction between the receptor tyrosine kinase TrkB and neurotrophins activates three main intracellular signaling pathways. Activation of the MAPK signaling cascade promotes neuronal differentiation and growth, activation of the PI3K cascade promotes survival and growth of cells, and activation of PLCγ1 may control LTP. Adapted from (Minichiello 2009).



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b. p75NTR receptor

The other BDNF receptor, p75NTR, binds mature BDNF with low affinity but proBDNF with high affinity. Activation of p75NTR by proBDNF produces a variety of effects: from neuronal survival and neurogenesis to the inhibition of growth, reduction of neuron excitability, decreased synaptic efficiency, synaptic depression, growth cone retraction, dendritic spine shrinkage and even apoptosis (Jianmin Yang et al. 2014). The downstream outcome of the response is determined in large part by the ligand and the binding partner of the p75NTR receptor. p75NTR can facilitate pro-survival effects by binding to Trk receptors and increasing the affinity of the neurotrophins for those receptors. It can also bind to sortilin. Binding of p75NTR to Sortilin increases the affinity of the receptor for proBDNF and often promotes cell death through apoptosis (Meeker and Williams 2015). The p75NTR/sortilin complex signals through activation of c-Jun amino terminal kinase (JNK), Ras homolog gene family member A (RhoA), and nuclear factor kappa B (NF-κB) (Kowiański et al. 2018). In addition to binding to sortilin and Trk receptors, p75NTR also forms oligomers and can function independently. The most abundant form is a trimer, but monomers also exist at the cell surface. Trimers are not critical for p75NTR activation, but the oligomerization state may be important in determining which of its effects prevail, survival or death (Anastasia et al. 2015).

While the TrkB receptor mediates the effects of BDNF on LTP, p75NTR exerts its effects on synaptic plasticity by regulating long-term depression (LTD). In particular, mice with deletion of p75NTR display impaired NMDA-dependent LTD, and a decrease in the expression of the NMDA receptor subunit NR2B that is known to be involved in

LTD. This is consistent with the observation that proBDNF activation of p75NTR enhances LTD (Woo et al. 2005). Also, in mice with cleavage-resistant proBDNF (where mature BDNF cannot be generated), LTD is enhanced in the CA1 pyramidal neurons of the hippocampus (Jianmin Yang et al. 2014).

P75NTR structure is similar to other receptors in the family of tumor necrosis factor receptors (TNFRs). It contains an extracellular domain with 4 cysteine rich domains (CRD3 is thought to be the ligand binding site), a small transmembrane domain that is known to be vital for oligomerization, and an intracellular domain which consists of a palmitoylation site at cysteine 279, two potential TNF receptor associated factor (TRAF)-binding sites, a Type II death domain, a potential G -activating domain, and a PDZ domain binding motif. The death domain is the prominent feature of the intracellular domain (Roux and Barker 2002).

Full length p75NTR has many ligands that bind to its extracellular domain. They include nerve growth factor (NGF), BDNF, NT3, NT4/5 proneurotrophins. In disease, it can also bind the rabies virus, prion protein, and Aβ-peptide involved in Alzheimer's. The extracellular ligands are thought to bind to the CRD3 region. In addition to signaling molecules, p75NTR has many other binding partners that include: Trk receptors, the Nogo receptor, neurotrophin receptor-interacting melanoma antigen gene (NRAGE), and RhoA (see Figure 8). Most of the cytoplasmic interactions of p75NTR with these partners occurs close to its juxtamembrane domain or death domain (Roux and Barker 2002) and many of them have been shown to be ligand-dependent interactions (Hempstead 2002)

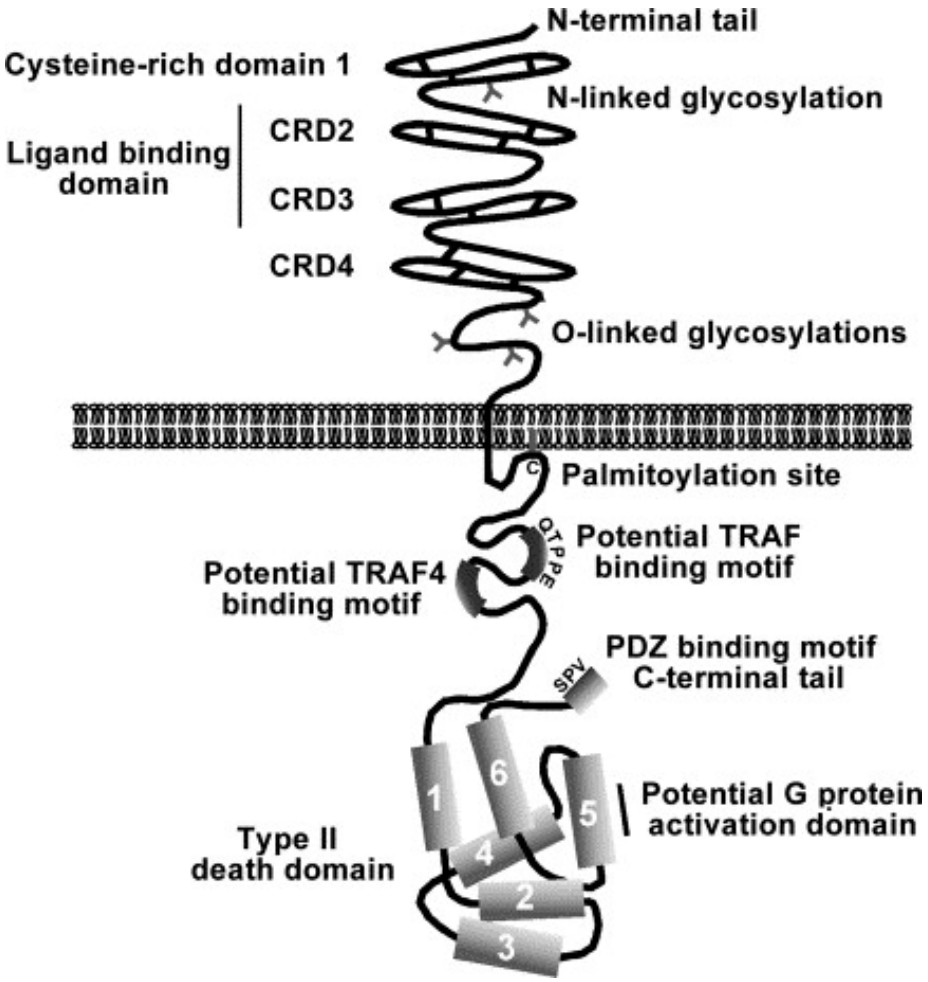
that occur when the receptor is membrane-bound. However, the interaction between sortilin and p75NTR is at the extracellular domain (Skeldal et al. 2012).

Figure 8. p75NTR structure and binding partners

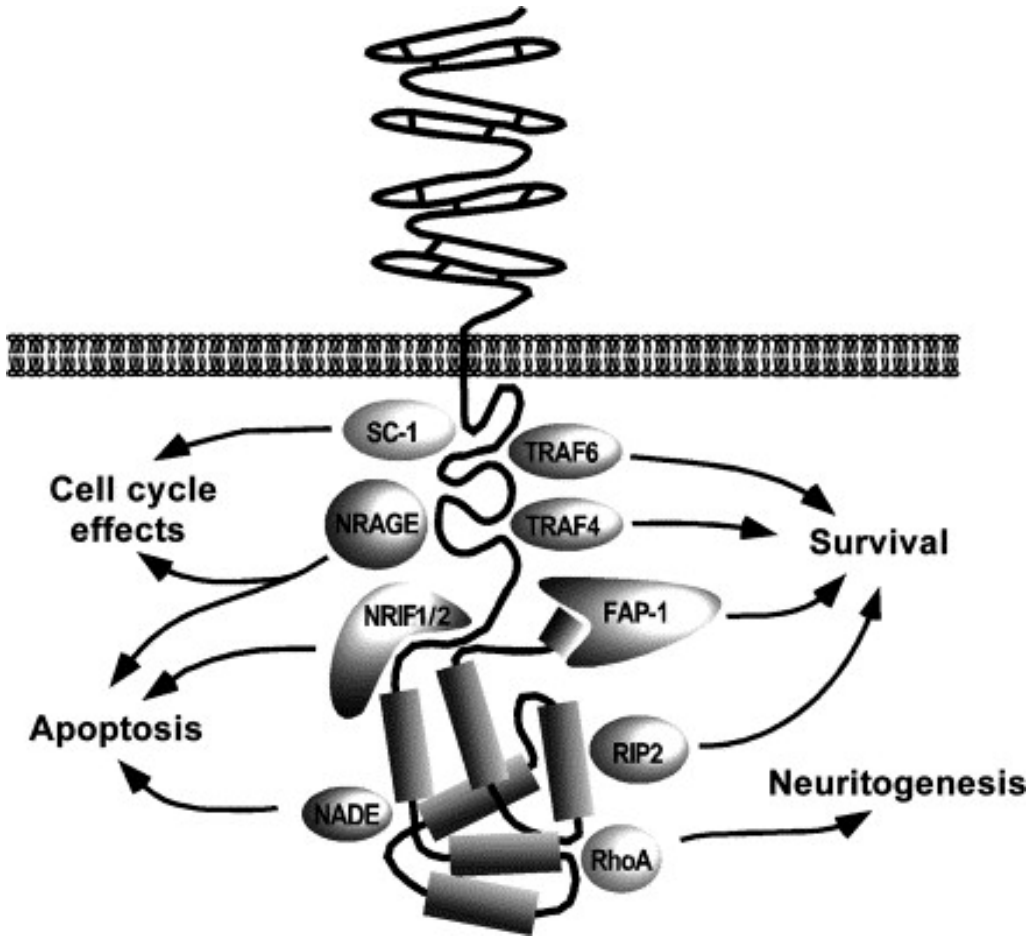
A) Schematic representation of the structure of the p75NTR protein. The extracellular domain contains four cysteine-rich domains (CRDs), and multiple O- and N-linked glycosylation sites. The intracellular domain contains a palmitoylation site, two potential TRAF-binding sites, a Type II death domain, a potential G protein activating domain, and a PDZ domain binding motif.

B) Schematic representation of the different p75NTR interactors identified to date, in contact with the reported intracellular region of p75NTR. NRAGE, SC-1, TRAF4, and TRAF6 which bind to the p75NTR juxtamembrane domain. NRIF interacts with both the juxtamembrane and death domains. At this time, p75NTR-associated cell death executor (NADE), Receptor interacting protein-2 (RIP2), and RhoA have only been shown to bind to the death domain. A and B Figures and legends are adapted from (Roux and Barker 2002).

A.


N-terminal tail
Cysteine-rich domain 1
N-linked glycosylation
Ligand binding domain
CRD2
CRD3
CRD4
O-linked glycosylations
C
Palmitoylation site
OTPPE
Potential TRAF binding motif
Potential TRAF4 binding motif
SPY
PDZ binding motif
C-terminal tail
1
6
5
4
2
3
Potential G protein activation domain
Type II death domain

B.


SC-1
TRAF6
Cell cycle
effects
NRAGE
TRAF4
Survival
NRIF1/2
FAP-1
Apoptosis
RIP2
Neuritogenesis
NADE
RhoA

p75NTR and other neurotrophin receptors are known to be internalized when activated by their ligand through both clathrin-dependent and additional endocytosis mechanisms (Winckler and Yap 2011). p75NTR is found both in endosomes with Trk receptors as well as without Trk receptors. Interestingly, it has been shown that p75NTR that is found in endosomes can bind to its partners and participate in intracellular signaling from the cytoplasm (Bronfman et al. 2003). These endosomes are eventually recycled but it has also been reported that they can be exocytosed into the extracellular space, although the function of exocytosed p75NTR is still unclear (Escudero et al., 2014). An alternative route of intracellular movement of p75NTR is through cleavage of the intracellular domain (ICD) by gamma secretase. This cleaved form of p75NTR contains the death domain and can translocate into the nucleus to bind DNA altering transcription, however, the full-length form has not yet been shown to migrate into the nucleus (Parkhurst, Zampieri, and Chao 2010).

3. ROLE OF BDNF IN DISEASE

Due to the beneficial effects of BDNF in learning and memory, and its essential cellular functions, increasing one's BDNF is thought to be beneficial. Many recent studies have shown that exercise can boost BDNF levels and may improve memory (Coelho). However, due to the interplay of TrkB and p75NTR, with their distinct affinities for mature and proBDNF, and sometimes opposing actions, a change in the abundance of BDNF can also interfere with brain homeostasis and contribute to disease.

a. Epilepsy

One of the diseases where BDNF is believed to play a prominent role is epilepsy. Increased levels of BDNF transcript and protein are found in human patients of TLE in localized areas involved in epileptogenesis, mainly the dentate gyrus, and CA1/CA3 regions of the hippocampus (Martínez-Levy et al. 2017; Gall 1993; Isackson et al. 1991). In addition, BDNF levels rise after a brain insult like a seizure or TBI, events that can increase the risk for developing TLE (Lindvall et al. 1994).

Various animal studies manipulating BDNF signaling have shown that it can modulate the development of spontaneous seizures. In BDNF heterozygous animals the rate of kindling was slowed two-fold (Kokaia et al. 1995). Similarly, animals with BDNF overexpression have more severe seizures in response to Kainic Acid and some even spontaneously seize (S.D. Croll et al. 1999). Animals infused with an inactive receptor body for TrkB that sequestered BDNF also kindled at a slower rate (Susan D. Croll et al. 1998). Strikingly, a conditional knockdown of TrkB led to complete protection in a kindling model of mouse TLE (He et al. 2004).

After SE, TrkB phosphoreactivity is significantly increased, peaking at 24-hr post seizure, in the dentate hilus and CA3, an area coinciding with the mossy fiber pathway (Devin K. Binder, Routbort, and McNamara 1999). BDNF immunoreactivity is particularly increased after the epileptogenic phase in pilocarpine-treated rats in newly sprouted mossy fibers (H E Scharfman, Goodman, and Sollas 1999). Increased BDNF in the hippocampus has also been directly related to hyperexcitability in those cells (Helen

E. Scharfman 1997). This increase in hyperexcitability may reflect BDNF's ability to modulate inhibitory synaptic transmission (Tanaka, Saito, and Matsuki 1997).

Although substantial evidence points to the upregulation of BDNF as critical for epileptogenesis, there are also some confounding reports that it can be neuroprotective. In fact, a recent study used BDNF delivery as a therapeutic for epilepsy in a rodent model and saw 80% decreases in spontaneous seizures and improvement in cognitive performance (Falcicchia et al. 2018). Improving our understanding of BDNF biology, and the heterogeneity that exists in the intracellular pathways that it controls, will most certainly contribute to the development of more selective antiepileptogenic options in the future.

b. Additional diseases

In addition to epilepsy, BDNF dysregulation has been identified in multiple other disorders:

- **Neuropsychiatric disease:** Major depressive disorder is associated with reduced levels of BDNF and TrkB in the hippocampus and prefrontal cortex, while it is increased in the nucleus accumbens and amygdala (Autry and Monteggia 2012). Treatment with anti-depressants normalizes BDNF levels (Duman and Monteggia 2006), and BDNF infusion into depressive-like animals is associated with anti-depressive behavior (Shirayama et al. 2002). Dominant negative TrkB expression can lead to loss of antidepressant efficacy (Saarelainen et al. 2003). Similar effects are seen in bipolar disorder. In schizophrenia, conflicting studies of dysregulation of BDNF levels have been seen, some

showing an increase and others a decrease in BDNF in the prefrontal cortex and the hippocampus (Autry and Monteggia 2012).

- **Neurodegenerative disorders:** BDNF levels are reduced in patients of Alzheimer's, Parkinson's, and Huntington's disease (Chiara Zuccato and Cattaneo 2009). In an animal model of Alzheimer's, injection of a BDNF AAV restored BDNF level, alleviated neuronal loss, and reduced behavioral deficits (Jiao et al. 2016). In a rat model of parkinsonism, transplantation of BDNF astrocytes significantly attenuated symptoms (Yoshimoto et al. 1995). In Huntington's disease, the huntingtin gene is mutated, and the mutant form of the protein loses the ability to upregulate transcription of BDNF that occurs with wild-type huntingtin. In addition, the loss of the neurotrophic support of BDNF leads to cell death in striatal neurons (C Zuccato et al. 2001). In an animal model of Huntington's disease, BDNF overexpression rescues the disease phenotype (Xie, Hayden, and Xu 2010).
- **Addiction:** A single dose of cocaine results in an increase in BDNF in the nucleus accumbens, but not the hippocampus (Russo et al. 2009). Cocaine use can lead to a decreased threshold for LTP in the reward pathway thought to be associated with the increase in BDNF. Infusion of BDNF into the reward pathway results in potentiated addictive behavior (Graham et al. 2007). The role for BDNF in opiate addiction is also important, although it differs from that of cocaine. Human heroin addicts have significantly lower serum levels of BDNF. In animals, a morphine injection will result in a lowering of BDNF in the ventral tegmental

area, followed by an increase after withdrawal for a short period before it decreases again. In postnatal BDNF knockdowns, morphine responses are reduced (Akbarian et al. 2002).

- **TBI:** BDNF levels on the day of injury post-TBI are lowered, and the amount of BDNF in the serum can be used as a biomarker to indicate prognosis for severity of TBI and information regarding their recovery trajectory (Korley et al. 2016). BDNF levels inside the brain differ by region and CSF levels are increased (Failla, Conley, and Wagner 2016). Regions with the least damage have increased BDNF expression. In animal models, BDNF administration was able to reduce the cell death (G. B. Kaplan, Vasterling, and Vedak 2010), and use of the TrkB agonist, 7, 8- dihydroxyflavone has shown promise in many TBI animal models in improving neuroprotection and attenuating motor deficits (Wurzelmann, Romeika, and Sun 2017).
- **Cancer:** BDNF and TrkB are found at increased levels in many types of tumors. The prosurvival effects of BDNF through the PI3K/Akt pathway act to protect the tumor from the body's mechanisms to suppress tumors and induce cell death (Radin and Patel 2017). Although in general, BDNF's role is oncogenic, regional specific BDNF from the hypothalamus may support an anti-tumor immune response (Radin and Patel 2017). Targeting the TrkB pathway has been promising in preclinical studies (Roesler et al. 2014).

4. BDNF AS A MASTER REGULATOR

a. BDNF target genes and immediate early gene expression

Each of the BDNF-induced signaling pathways leads to altered gene expression. Some of the genes that have been identified as targets of BDNF include immediate early genes. Immediate early genes are activated transiently and rapidly in response to stimuli and require no protein synthesis functions for expression. Some immediate early genes that respond to BDNF stimuli are early growth response gene 1 and 3, (Egr1, Egr3), c-fos, neuritin, activity-regulated cytoskeletal-associated protein (Arc), and the BDNF gene itself (El-Sayed, Hofman-Bang, and Mikkelsen 2011; Daniel S. Roberts et al. 2006; J. H. Kim et al. 2012; Zheng, Luo, and Wang 2009; Alder et al. 2003) The TrkB-MAPK pathway mediates immediate early gene activation (Roberts et al. 2006; Zheng, Luo, and Wang 2009). By regulating the activation state of these gene products, BDNF is able to act as a master regulator, especially since many of these transcription factors then regulate the downstream synthesis of additional transcription factors that promote long-lasting changes in synaptic plasticity, necessary for learning and memory.

b ICER: a BDNF-induced repressor protein

In addition to the immediate early genes listed above, BDNF also induces the phosphorylation of CREB at Serine 133 (pCREB). This activation of CREB is associated with an increase in the transcription of CREB target genes. One of these CREB family members is the inducible cAMP early repressor gene (ICER). ICER is transcribed from an internal promoter in the cAMP response element modulator (CREM) gene that codes for four proteins. The majority of transcripts specific to the CREM gene locus code for

transcriptional activators (Lund et al. 2008). Our laboratory has demonstrated that ICER can heterodimerize at CREs with pCREB to repress CREB-induced transcription (Lund et al. 2008; Grabenstatter et al. 2014). In addition to BDNF inducing ICER transcription through pCREB in primary neuronal cultures (Lund et al. 2008), seizures in animal models of epilepsy lead to increased levels of ICER in the hippocampus (Lund et al. 2008; Kojima et al. 2008).

c. Control over GABAR subunit genes

The altered transcription of the immediate early genes and ICER modulate synaptic transmission by controlling the expression of GABA and glutamate receptor subunit genes. Changes in the expression of these genes can play a profound role in the alteration of neural circuitry in the hippocampus that contributes to TLE development. In fact, GABA receptor subunit genes mutations are responsible for a number of genetic epilepsies- mutations of GABRB3 and GABRA1 are associated with childhood absence epilepsy and juvenile myoclonic epilepsy, while mutations and variants of GABRG2 and GABRD are associated with febrile seizures and dravet syndrome. Altered composition of the type A GABA receptors (GABARs), the ionotropic mediator of fast inhibitory synaptic transmission, is seen in both human epilepsy patients and in animal models. GABARs are pentameric with a chloride pore through the center, and are composed of 2 α subunits, 2 β and either a γ or δ subunit (or various less common subunits whose function in GABARs is yet to be described) (Campagna-Slater and Weaver 2007). The γ2 subunit plays a role in synaptic localization (Essrich et al. 1998) while δ limits the receptor to the extrasynaptic and presynaptic space (Nusser, Sieghart, and Somogyi

1998). The γ2 subunit is also required for the classical benzodiazepine allosteric response and along with the α (2/3) subunit is part of the binding interface for many anti-convulsant drugs.

Multiple studies using epilepsy models show increased presence of the α4 subunit and decreased α1 at the synapse (Brooks-Kayal et al. 1998; Roberts et al. 2006; Houser et al. 2012). Under normal conditions, α4 is assembled with δ subunits and found in perisynaptic and extrasynaptic sites of the post-synaptic membrane. Our lab was the first to show that 24 hours after SE, there is a redistribution of α4 containing receptors to the synapse but now instead of association with δ, they are assembled with γ2 (Lund et al. 2008). We also showed that rescue of α1 levels using AAV virus attenuated seizure frequency and severity even in the presence of increased α4 (Raol et al. 2006).

Altered GABAR composition is, at least in part, driven by rising levels of BDNF in epilepsy models. Our lab showed that Egr3 mediates the increased transcription of the α4 gene (*GABRA4*) by BDNF activation of TrkB and via a direct interaction with the core *GABRA4* promoter (Roberts et al., 2006). Decreased α1 subunit levels are also a response to BDNF signaling. BDNF induction of ICER leads to the binding of the ICER repressor to the α1 core promoter, an action that has been associated with regulation by the JAK/STAT pathway (Lund et al. 2008).

In addition, GABA's actions can be modulated by BDNF and proBDNF through alterations in receptor internalization and thereby access to its binding site. TrkB pathways can inhibit internalization of GABARs through activation of the PI3K/PKC

pathway. Conversely, p75NTR activation can lead to receptor internalization through dephosphorylation of the β3 subunit (Porcher, Medina, and Gaiarsa 2018).

d. Control over Glutamate receptor subunit genes

Excitatory modulation and altered LTP also occurs as a response to BDNF. TrkB-induced pCREB and EGR3 upregulation results in the binding of pCREB to CRE elements in the glutamate ionotropic receptor NMDA type subunit 1 gene, *GRIN1*. This regulatory process is dependent on BDNF, pCREB, and EGR3, and both factors bind to distinct sites on the *GRIN1* promoter (J. H. Kim et al. 2012). Interestingly, there are three functional sites on the promoter for pCREB with one embedded in the core promoter region adjacent to the EGR3 binding site.

The GRIN1 gene encodes for the NR1 subunit protein and is found in all NMDA receptors. It can partner with multiple NR2 subunits, such as NR2A, B, or C and NR3 subunits. NMDA receptors are critical for excitation in the adult nervous system and LTP. There are higher levels of NR1 in animal models of epilepsy and in humans with TLE. Increased activity of NMDA receptors can lead to excitotoxic cell death (J. H. Kim et al. 2012).

BDNF-induced Arc expression is one of the few immediate early genes that is not a transcription factor, but rather a cytosolic protein that acts in several downstream signaling cascades known to be important for synaptic plasticity. Arc modulates glutamate signaling by affecting the trafficking of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Like NMDA receptors, AMPA receptors play a critical role in both LTP and LTD. Arc also modulates plasticity by increasing

spine density and the distribution of thin spines known to be involved in learning and memory. Arc can also activate notch signaling which plays a distinct role in neuronal plasticity (Korb and Finkbeiner 2011).

The datasets contained within my thesis suggest an even greater role for BDNF in the modulation of excitatory/inhibitory balance at the transcriptional level that can impact synaptic transmission to alter disease progression in multiple contexts.

C. THE JAK/STAT SIGNALING PATHWAY

The JAK/STAT signaling pathway is a molecular cascade preserved across species from drosophila to humans. It is important in modulating cell proliferation, differentiation, migration and apoptosis. It is critical for normal hematopoiesis, immune development, lactation, adipogenesis, and other growth processes. Mutations in this pathway can lead to inflammatory disease, cancer, and gigantism (Rawlings, Rosler, and Harrison 2004). In mammals, the JAK family is comprised of four members that were discovered in the early 1990s: JAK1, JAK2, JAK3, and Tyk2. JAKs are members of the protein tyrosine kinases (PTK) family, but differ from other members in that they have an additional kinase domain. The term Janus kinase stems from this idea as it is named after a two-faced Roman God (Rane and Reddy 2000). The STAT family includes seven members: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. The diverse set of family members can tailor the cascade based on tissue type and a variety of signaling properties. (Aaronson and Horvath 2002). In addition, even though there is a heterogeneity in ligands and receptors, they have also been shown to activate a similar

pathway. The ligands for receptors that drive JAK/STAT signaling are generally extracellular polypeptides such as hormones or cytokines.

1. CANONICAL SIGNALING PATHWAY

a. JAKs and STATs

Mechanistically, the JAK/STAT pathway has few partners that move the signal from the membrane to the nucleus. In most cases, ligand binding to a membrane bound receptor leads to receptor multimerization either as homodimers, heterodimers, or heteromultimers. The cytoplasmic domain of the receptor then associates with the N-terminus of the JAK protein, and multimerization of the receptor allows for two JAKs to be in close proximity, allowing trans-phosphorylation. The activated JAK (pJAK) can then phosphorylate additional targets including STATs.

STATs were first discovered a few years after JAKs, in 1994 (Darnell, Kerr, and Stark 1994). Inactive STATs generally reside as homodimers in the cytoplasm, through interaction with JAK in the SH2 domain, they become phosphorylated and rearrange into an antiparallel dimer. The activated dimer then translocates into the nucleus through the Ran nuclear import pathway. Inside the nucleus they are free to bind target genes to activate or repress expression. This mechanism relies on the phosphorylation of STAT to invoke transcriptional changes. Additional effector proteins, including the stat-interacting protein (StIP), may be involved in the pathway in order to facilitate or inhibit the activation of STATs.

b. Inhibition of the JAK/STAT pathway

Suppression of the pathway generally occurs through activation of suppressors of cytokine signaling (SOCS) proteins, protein inhibitors of activated stats (PIAS) and protein tyrosine phosphatases (PTPs). The SOCS are stimulated by activated STAT to create a negative feedback loop. SOCS can inhibit the pathway in three main ways: binding pJAKs to inhibit the recruitment of STATs, inhibiting JAK kinase activity, and facilitating the ubiquitination of JAKs to foster proteasomal degradation. The PIAS bind STAT and prevent them from binding DNA. PTPs simply reverse the activity of the JAK tyrosine kinases (Rawlings, Rosler, and Harrison 2004).

c. Crosstalk with other signaling pathways

The JAK/STAT pathway can also crosstalk with other signaling pathways. The MAPK pathway is known to be further activated by JAK activation through its upstream effector Ras and can also potentiate JAK/STAT activity by phosphorylating STAT (Rane and Reddy 2000; Malik, Pal, and Gupta 2017). Reciprocal activation has also been identified with the PI3K, Notch/Hes, Peroxisome proliferator-activated receptor-α (PPARα) and TGF-B pathways (Y. C. Zhou and Waxman 1999; Rawlings, Rosler, and Harrison 2004; Kamakura et al. 2004).

2. NON-CANONICAL SIGNALING

Although the mechanism listed above is conserved throughout the animal kingdom and is thought to be among the most well understood signaling pathway, recent discoveries have revealed additional complexities that remain unsolved. Findings that JAK can act to alter gene transcription independently of STAT, and that STAT can be

present in the nucleus without phosphorylation and regulate chromatin stability in an unphosphorylated state revealed what has become termed the *non-canonical JAK/STAT signaling pathway*. This non-canonical pathway categorization includes several alternative pathways that deviate from the canonical mechanism and which are still being investigated to enhance understanding of its mechanism and function.

a. Non-canonical role of JAK

Although originally thought to be only a cytosolic protein with its actions occurring when associated with membrane-bound receptors, JAK has been identified in the nucleus since as early as 1996 (Lobie et al. 1996), although its nuclear function remained largely uncharacterized until more recently. The receptor binding region of JAK is thought to have a nuclear localization signal that permits its translocation (Ragimbeau et al. 2001). Nuclear JAK has been shown to directly complex with STAT (Kamakura et al. 2004), but can also function independently of STAT to modify the activity of transcription factors including nuclear factor 1-C2 (NF1-C2) and RUSH (M. A. Dawson and Bannister 2012). JAK can also act as a kinase in the nucleus acting at Histone 3 (H3) tyrosine 41, an important location that determines nucleosome structure and stability. H3 phosphorylation disrupts the association of heterochromatin protein 1 (HP1) with chromatin (M. A. Dawson et al. 2009) which can modify mitosis by de-repressing gene expression (Panteleeva et al. 2007; Yamagishi et al. 2008). Cancerous tissue can increase the levels of nuclear JAK to produce cell cycle alterations that HP1 inhibits and are likely oncogenic. Oncogenic JAK2 mutations, particularly JAK2V617F are constitutively active, and can phosphorylate the protein arginine methyltransferase 5 (PRMT5) in the

nucleus inhibiting its methyltransferase activity. This inhibition is believed to produce the myeloproliferative phenotypes that are associated with JAK2 mutations in humans (Zouein, Duhé, and Booz 2011). In addition to a novel nuclear function, JAK can also have non-canonical roles in the cytoplasm such as a participation in kinase-independent scaffolding (Majoros et al. 2017).

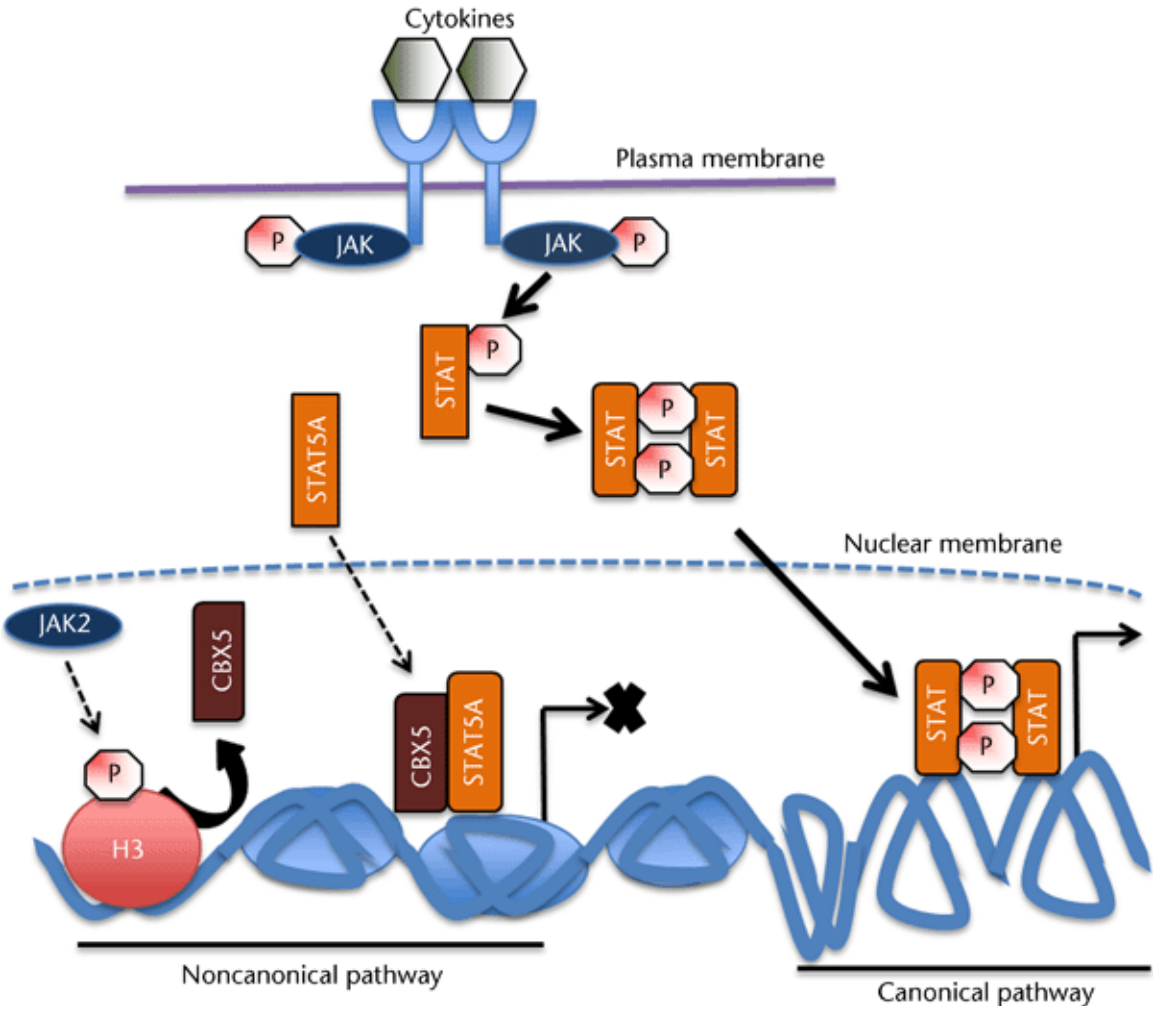
b. Non-canonical role of STAT

STAT proteins can act non-canonically when unphosphorylated (uSTAT3) to regulate transcription in a variety of important ways. After the pSTAT3 responsive genes are activated, transcription of a host of uSTAT3 responsive genes follow. uSTAT3 has been shown to translocate into the nucleus (Liu, McBride, and Reich 2005). Once there it can bind to unphosphorylated NFκB (uNFκB) in the nucleus and activate a host of NFκB-dependent genes. A chromatin immunoprecipitation assay (ChIP) performed upon overexpression of a Y705F-STAT3 mutant (that cannot be phosphorylated) revealed that uSTAT3 binds to DNA of several genes including IL-6, IFN-B, IL-8. The promoters that bound uSTAT3 all had NFκB sites (Jinbo Yang et al. 2007). uSTAT3 can either bind to the DNA in the same regions as pSTAT3 as a monomer or a dimer, acting to compound the effects of pSTAT3 (Timofeeva et al. 2012), or it can bind to HP1 which acts to stabilize chromatin, an effect in direct opposition to pSTAT3 (Brown and Zeidler 2008). Studies which genetically manipulate STAT to reduce amounts of uSTAT3 result in DNA damage demonstrating it has a protective role(Yan et al. 2011). See Figure 9.

The investigation of the non-canonical JAK/STAT signaling pathway appears to differ based on cell type and surely additional mechanisms are yet to be discovered which will add even more complexity to this intracellular signaling pathway in the brain.

Figure 9. Schematic of canonical and non-canonical JAK/STAT signaling

Canonical and noncanonical JAK-STAT signaling. In the canonical mode of signaling, cytokines bind to receptor that leads to phosphorylation of the JAK kinase. Activated JAK phosphorylates STAT. Phosphorylated STAT dimerizes and translocates to the nucleus to bind sequences in the target gene promoters. Dimeric STAT binding results in gene transcription. In the noncanonical pathway, unphosphorylated STAT in the nucleus physically interacts with heterochromatin protein1 HP1α thereby promoting heterochromatin formation or stability. In addition, it is also shown in human cells that JAK2, in the noncanonical context, can act as a histone kinase and phosphorylated tyrosine 40 of histone 3. Histone 3 phosphorylation displaces HP1α from chromatin. Figure from (Dutta and Li 2013).


Cytokines
Plasma membrane
P
JAK
JAK
P
STAT
P
STAT
P
P
STAT
STAT5A
Nuclear membrane
JAK2
CBX5
P
H3
CBX5
STAT5A
STAT
P
P
STAT
Noncanonical pathway
Canonical pathway

3. IN THE CNS

The JAK/STAT pathway plays an important role in the brain. It is involved in development, hormone release, plasticity, neuro- and glio-genesis, inflammation and tumorigenesis. Expression of JAK/STATs are lower in the adult brain than other areas, but can be found in the cerebral cortex, hippocampus, hypothalamus and cerebellum, in development several of the proteins are found in higher concentrations (Celine S. Nicolas et al. 2013).

a. Neurogenesis and gliogenesis, proliferation and differentiation

Proliferation of neural stem cells is regulated by the JAK/STAT pathway. IL-15 can induce activation of STAT1, STAT3 and STAT5 in the subventricular zone and proliferation is blocked by JAK inhibitors (Bauer 2009; Gómez-Nicola et al. 2011). Leptin can activate STAT3 and induce proliferation in the DG (Garza et al. 2008).

JAK/STATs also participate in glial differentiation through the ciliary neurotrophic factor (CNTF), prolactin, IL-6 and leukemia inhibitor factor (LIF) which activates JAK1, STAT1 and STAT3 to trigger astrocyte differentiation (Peterson et al. 2000; Bonni et al. 1997; Nakanishi et al. 2007; Mangoura et al. 2000). STAT3 knockdown mice have dysfunctional astrogliogenesis (Cao et al. 2010). In the adult DG STAT3 is critical for neurogenesis (Müller et al. 2009), particularly after an injury, while STAT1 is important in activating caspases and inducing apoptosis (Schindler, Levy, and Decker 2007).

Differential regulation is also ascribed to the JAK proteins. JAK1 is implicated in gliogenesis while JAK2 is associated more with neurogenesis. JAK3 also plays an

important role by controlling differentiation in neurons and oligodendrocytes (Celine S. Nicolas et al. 2013).

b. Hormone regulation

Hormone regulation is also influenced by the JAK/STAT pathway in the hypothalamus. Leptin signaling activates STAT3 and STAT5 controlling satiety (Villanueva and Myers 2008). JAK2 also plays a role in the production of gonadotropin releasing hormone (GnRH) and secretion of prolactin, which play roles in reproduction and lactation respectively (Magni et al. 2007; Ma et al. 2005).

c. Synaptic plasticity

Synaptic plasticity and modulation of receptor signaling is an important function of JAK/STATs in the CNS. JAK inhibition can block NMDA-dependent LTD (but not LTP or AMPA-mediated synaptic transmission) and knockdown of JAK abolishes LTD. NMDAR-mediated LTD activates JAK2 which is located at the synapse. Interestingly, while STAT3 is also activated, its movement to the nucleus is not required for LTD to occur (Céline S. Nicolas et al. 2012).

d. Disease

In neurological disease, JAK/STAT signaling contributes to brain tumors much as it does to other types of cancer, due to its role in cytokine signaling. In addition, it plays a large role in inflammatory associated brain disorders including epilepsy, ischemia, and neurodegenerative disease (Celine S. Nicolas et al. 2013).

4. THE BDNF-INDUCED JAK/STAT PATHWAY

a. Gene expression changes

Studies from our laboratory found that in addition to the CNS functions described above, BDNF can activate the JAK/STAT pathway and affect synaptic transmission via transcriptional regulation. We demonstrated for the first time that BDNF activates the JAK/STAT pathway both *in vivo* in the rat pilocarpine model of epilepsy and *in vitro* in BDNF-treated primary cultured neurons (Lund et al. 2008). Activation involves the binding of pSTAT3 to the ICER promoter and the induction of transcription. As mentioned above, ICER represses transcription at genes containing CRE elements. Our laboratory found that one of the major effects of ICER synthesis is the downregulation of α1 GABAR subunit genes (*GABRA1),* which decreases the number of synaptic GABARs limiting GABA's inhibitory effect in the hippocampus (Lund et al. 2008). A reduction in efficient GABARs at the synapse contributes to epilepsy susceptibility and rescue of animals in the TLE model is seen when a1 is replaced via viral delivery (Raol et al. 2006). A reduction in α1 subunit expression is also seen in epilepsy patients (Brooks-Kayal et al. 1998) as well as in other disorders of the nervous system (Petryshen et al. 2005; Raud et al. 2009; Bossers et al. 2010). Moreover, use of the JAK/STAT inhibitor, WP1066, in the pilocarpine rat model of epilepsy reduces the number of spontaneous seizures after the latent period (Grabenstatter et al. 2014), suggesting that BDNF-induced JAK/STAT activation may be relevant to disease .

b. BDNF receptor signaling and JAK/STAT activation

Research to determine the identity of the BDNF receptor, TrkB or p75NTR, involved in the activation of the neuronal JAK/STAT pathway is an active area of research in the Russek Laboratory. While TrkB has been found crucial to the onset of pathway activation, it is not coupled to JAK proteins as expected. Rather, a novel interaction of Jak2 with an intracellular p75NTR suggests that there is a more complicated relationship of JAK signaling as mediated by BDNF.

Proximity ligation assay with transfected constructs of JAK2-GFP and p75NTR-flag have also shown that the localization of the p75NTR/JAK2 complex is not at the membrane, where JAK usually interacts with its receptors, but in the cytoplasm and nucleus of the cells, indicating that the BDNF-induced JAK/STAT pathway may operate using a non-canonical mechanism with a signalsome that converts membrane action of the TrkB receptor into gene regulation on chromatin in the nucleus. Evidence for this unique relationship comes from additional experimentation that shows siRNA knockdown of TrkB, p75NTR and STAT3 all inhibit ICER induction as does treatment with JAK inhibitors pyridine 6 and WP1066 (Benham, Hokenson, unpublished results.)

Table 3. BDNF-induced JAK/STAT related findings

Related Findings	**Reference**
BDNF activates JAK through phosphorylation within 15 minutes	(Lund et al. 2008)
BDNF activates STAT through phosphorylation within 30 minutes	(Lund et al. 2008)
BDNF causes an induction of ICER transcription	(Lund et al. 2008)
BDNF causes a reduction in α1 protein levels in 24 hours	(Lund et al. 2008)
pSTAT3 binds to the promoter of ICER, which increases after Status Epilepticus	(Lund et al. 2008)
ICER binds to *GABRA1* promoter	(Lund et al. 2008)
WP1066 reduces the severity of epilepsy in pilocarpine-treated rats	(Grabenstatter et al. 2014)
WP1066 prevents ICER induction	(Grabenstatter et al. 2014)

E. MAJOR HYPOTHESIS AND SPECIFIC AIMS

Our laboratory has identified that activation of the JAK/STAT pathway of primary cultured neurons (both cortical and hippocampal) can downregulate synaptic GABARs. This is also seen *in vivo* in response to pilocarpine-induced status epilepticus. Additionally, JAK/STAT inhibitor administration at the time of pilocarpine-induced status epilepticus is protective in rats. Despite these discoveries, the mystery of how the JAK/STAT pathway in neurons can remodel the nervous system to produce hyperactivity remains unsolved.

One mode of JAK/STAT activation identified by our lab is its activation through BDNF. As a master regulator of information processing in the brain, BDNF is known to dynamically activate multiple intracellular signaling pathways with diverse functions (from neurogenesis to programmed cell death) and with interrelated endpoints. Different functional roles have been described for the two receptors (TrkB and p75NTR), that can bind to the same BDNF signaling molecules (mature and pro-BDNF). P75NTR has been found in complex with JAK proteins but the role of this complex in BDNF-induced JAK/STAT signaling and a comprehensive account of the pathway's effects on the transcriptome remain unidentified.

Based on this evidence, we developed the following overarching hypothesis that drives the direction of my thesis: *The JAK/STAT pathway has far reaching effects on the transcription of the neuronal genome in response to injury, such as status epilepticus, and selective targeting of this pathway may lead to disease modifying therapeutics for multiple brain disorders.* As a first step to better identify the extent and mechanism of

JAK/STAT pathway function in neurons, the following Specific Aims were conducted in my thesis research:

Aim 1. To determine the genome response of neurons to BDNF-induced JAK/STAT signaling *in vitro* using deep RNA-sequencing in the presence and absence of JAK/STAT pathway inhibitors.

Aim 2: To determine the genome response *in vivo* to Status Epilepticus as produced by an intrahippocampal injection of kainate in wildtype and transgenic neuronal STAT3 (knockdown) mice.

Aim 3: To determine the region of p75NTR that interacts with JAK2 in order to test its role as a signalsome for BDNF-induced JAK/STAT signaling in neurons.

The datasets from the presented work highlight the complexities of the JAK/STAT pathway in neurons and suggest the presence of a non-canonical JAK/STAT mechanism that may be activated upon increased synthesis and release of BDNF as it occurs in temporal lobe epilepsy. *Such results provide the basis for future studies aimed at determining the role of the JAK/STAT-induced alterations in sphingolipid metabolism in epileptogenesis and informing the design of disease-modifying therapeutics targeting the non-canonical JAK/STAT pathway for TLE and its associated comorbidities of cognitive impairment, learning and memory disorders, and depression.*

CHAPTER II: MATERIALS AND METHODS

A. MATERIALS AND METHODS SPECIFIC TO CHAPTER III

1. MATERIALS

Antibody Name	**Primary incubation**	**Washes (1) after blocking 2) after primary, 3) after secondary**	**Secondary antibody (all 1 hr. RT)**	**Blocking (1 hr. RT or O/N 4ºC)**
STAT3 CST 4904	1:2000 5% milk in TBS-T O/N	1) no washes 2) 3x10' in 1% milk 3) 3x10' in 1% milk in TBS-T	1:6000 goat anti-rabbit Life technology AP 123P in1% milk in TBS-T	5% non-fat dry milk in TBS-T
P-STAT3 CST 9131	1:1000 5% BSA in TBS-T O/N	1) 3x5' in TBS-T 2) 3x10' in TBS-T	1:5000 goat anti-rabbit Life technology	5% non-fat dry milk in TBS-T (Do

3) 3x10' in 1% milk in TBS-T	AP 123P in 1% milk in TBS-T	Not use BSA)

2. METHODS

a. Cell culture and drug treatments

Primary neocortical neurons were dissected from neocortex of E18 Sprague-Dawley rat embryos (Charles River Laboratories). Pregnant dams were euthanized by CO 2. Embryonic brains were removed and placed in ice-cold Ca 2+ /Mg 2+ free (CMF) media [Ca 2+ /Mg 2+ free Hanks BSS, 4.2mM sodium bicarbonate,1mM pyruvate, 20mM HEPES, 3mg/mL BSA, pH 7.25-7.3]. Cortices were dissected under a dissection microscope, trypsinized centrifuged, and triturated in plating media [Neurobasal media (Invitrogen), 10% fetal bovine serum (Gibco), 100 U/mL penicillin, 100 µg/mL streptomycin, 200mM glutamax (Gibco)]. Dissociated neurons were plated on Poly-L-lysine coated 6-well plates ($1x10^6$cells/well) and placed in an incubator (37ºC/5% CO 2) for attachment to plate. 1hr later, plating media was removed and replaced with 2mL of defined medium [Neurobasal media (Invitrogen), B27 serum-free supplement (Gibco), 100 U/mL penicillin, 100 µg/mL streptomycin, 200mM glutamax (Gibco)]. Neurons were cultured in the incubator for 9 days until use (9 days in vitro [DIV]). Treatments: At 9DIV cells were pretreated for 1-hr. with vehicle dimethyl sulfoxide (DMSO) at 0.1%, Ruxolitinib (Sellckchem S1378; 100nM or 10uM), or WP1066 (EMD Millipore 573097;

10uM). Nuclease-free water vehicle or Brain-derived neurotrophic factor was then added (EMD Millipore GF029; 0.7nM) for 4 hours before collection.

b. RNA extraction and library preparation

At the end of the 4-hr treatment period, cells were collected and RNA extracted using the Qiagen RNeasy Micro Kit with DNAase treatment (Cat. 74004). RNA was run in the Agilent Bioanalyzer to determine RNA integrity number (RIN). RIN of all samples was ≥ 9.0. mRNA was selected by using the NEBNext® Poly(A) mRNA Magnetic Isolation Module (E7490S) before using the NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (E7420S) with Agencourt AMPure XP Clean up beads (A63881) to make RNA into strand specific cDNA libraries with multiplexing barcodes from the NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 1) kit (E7335S).

c. Illumina sequencing

Sequencing was performed at the Boston University Microarray Core and University of Chicago Genome Center. Fragmentation and concentration were analyzed on the Agilent Bioanalyzer and Fragment analyzer prior to sample pooling. Runs were either sequenced at 75 bp single-end reads on the Illumina NextSeq sequencer to a depth of 100-120 million reads per sample or at 50 bp single-end reads on the Illumina HiSeq sequencer to a depth of 90-100 reads per sample. All reads were conducted in one lane using barcodes to separate biological replicates.

d. RNA-seq bioinformatics

Data analysis was performed using Strand NGS software, Version 3.2, Build 237248. © Strand Life Sciences, Bangalore, India. Proprietary Strand NGS algorithms

were used to conduct read alignment to the rat genome build rn6 RefSeq Genes (2016.05.11). Reads were filtered based on the following parameters: Max number of novel splices: 1, Min alignment score: 90, Number of gaps allowed: 5, number of matches to be output for each read: 1, exclude reads with alignment length less than: 25, ignore reads with matches more than: 5, Trim 3' end with average base quality less than: 10. RNA quantification was performed by the Strand NGS software, which counts the total number of reads that map to each gene and exon in the genome, and is reported as raw counts. Normalization of the raw counts was achieved using the DESeq method, which accounts for difference in the total number of reads between samples and log transforms the data (Anders and Huber 2010). R version 3.1.2 was used with DEseq2 version 1.6.1 to determine differential expression FDR=0.05, Wald test type. The list of significant genes was then used in subsequent analyses. Disease involvement data was analyzed through the use of IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com /products/ingenuitypathway-analysis). Cytoscape 3.5.1 software was used with GeneMANIA for functional network generation with (Shannon et al. 2003; Warde-Farley et al. 2010). EnrichR was used for GO-term analysis and KEGG pathway analysis (Kuleshov et al. 2016; Chen et al. 2013).

e. Real-time quantitative reverse transcriptase PCR (qRT-PCR)

Primary neurons were prepared, treated, and RNA extracted, as described above for RNA-seq. The Applied Biosystems 1-step RNA to Ct (cat. 4392938) kit was used and protocol was followed as directed: 50 ng of RNA template, 10 µL of 2x mastermix, 0.5 µL of each 40x primer/probe kit (Ppia and the gene of interest), 0.5 µL of the enzyme

mix, and nuclease free water was added to each 20 μL reaction. Each reaction was performed with 2 technical replicates in a 384-well plate. All taqman primer/probe kits were ordered from Thermo Fisher Scientific.

Table 4. Thermofisher assay IDs for taqman primers and probes

Gene Symbol	**Assay ID**
Ppia (housekeeping)	**Rn00690933_m1 VIC-MGB**
Galr1	**Rn04932425_m1 FAM-MGB**
Drd5	**Rn00562768_s1 FAM-MGB**
Jak2	**Rn00676341_m1 FAM-MGB**
Gabrg2	**Rn01464079_m1 FAM-MGB**
Grm1	**Rn00566625_m1 FAM-MGB**
Icer	**Rn00569145_m1 FAM-MGB**
Ccnd1	**Rn00432359_m1 FAM-MGB**
Icer	**Rn00569145_m1 FAM-MGB**
Socs3	**Rn00585674_s1 FAM-MGB**
Mcl1	**Rn00821024_g1 FAM-MGB**

The Applied Biosystems 7900 HT Real-Time PCR system was used to run with the following cycling parameters: 48ºC 15 min. hold (Reverse Transcription), 95ºC 10 min. hold, 40 cycles: 95ºC 15 sec, 60ºC 1 min. After run completion, the ΔΔCT method was used to calculate the relative values of the transcripts.

f. Western Blot

Primary cortical cultures used for Western Blot were prepared and treated with drugs the same as described for RNA-seq. After the 4-hr period of BDNF treatment, cells were washed once with ice-cold PBS solution (PBS+EDTA, 1x PhosStop (Roche) and 1x Protease Inhibitor cocktail (Roche cOmplete protease inhibitor)) and centrifuged in 1.5 mL tubes at 5000RPM for 4 min at 4ºC. PBS was removed and c0ell pellet was resuspended in 1x Radioimmunoprecipitation assay buffer (RIPA) buffer (with 1x Protease and Phosphatase inhibitors). Lysates were incubated on a rotator at 4ºC for 15 min, then centrifuged at 13000 RPM for 10 min. Supernatant was transferred to a fresh tube and aliquoted for Western Blot. 35µg of sample was diluted to 10µL in RIPA buffer and 10µL of 2x SDS Tris-Glycine loading buffer (Invitrogen LC2676) with 50mM Dithiothreitol (DTT). Samples were boiled at 95ºC for 5 min, centrifuged and loaded into a Novex 10% Wedgewell gel (Invitrogen XP00100BOX). The gel was run at 220V for 40 mis and wet transfer was performed at 33V for 2 hrs to a nitrocellulose membrane. Membrane was quickly washed in Tris-buffered Saline-Tween (TBST) then blocked in 5% milk in TBST for 1 hr at room temperature. Three 5 min washes in TBST were performed. Membrane was incubated overnight at 4ºC in primary antibody then washed 3x10 minutes in TBST. Horse radish peroxidase (HRP)-conjugated secondary was added

for one hour at room temperature, then 3 washes in 1% milk were performed prior to imaging the blot with ECL (BioRad Clarity #1705060). Membrane was stripped using the GM Biosciences One Minute Advance Stripping buffer #GM6031. Antibodies were used as follows: 1:1000 pSTAT3 (CST #9131S) in 5% BSA, 1:5000 anti-rabbit HRP-conjugated secondary antibody (EMD Millipore AP132P) in 1% milk in TBST. 1:2000 total STAT3 (CST #4904S) in 5% milk in TBST. 1:6000 β-actin (Sigma A5441) in 1% milk in TBST, 1:2500 Anti-mouse HRP-conjugated secondary antibody (Vector Labs PI2000) in 1% milk in TBST.

B. MATERIALS AND METHODS SPECIFIC TO CHAPTER IV

1. METHODS

a. Tamoxifen treatment

Camk2a-cre/ERT2-Stat3-flox mice from jax (https://www.jax.org/strain/016923) were used to create CAMK2a promoter specific Tamoxifen induced knockdown. These mice have a deletion of exons 18-20 of Stat3(encoding the SH2 domain) in cre-expressing forebrain excitatory neurons. Kainic Acid and tissue harvesting procedures were performed by the Amy Brooks-Kayal Lab, but information about those protocols are included here. Tamoxifen (Sigma, with corn oil vehicle) or sterilized Corn oil (Sigma) is injected IP at 8 weeks old, 100mg/kg, 20 mg/ml, once per day for 5 consecutive days, and separated 1 per cage. For complete elimination of tamoxifen, mice wait an additional 14 days before implantation for VEEG recording and Kainate or saline (ctrl) injections.

b. Kainic Acid injection

Adult mice will be first anesthetized with isoflurane (3%) and then transferred to a stereotaxic frame and maintained under 1-2% isoflurane. Animals will be placed on a heat blanket to maintain normothermic conditions during the procedure, and eye lubricant applied to minimize desiccation. A small midline sagittal incision of approximately 1 -1.5 cm will be made in the skin on the scalp to expose the skull. Up to seven small holes (.8 mm) will be drilled over the areas of interest (bilateral frontal cortices, bilateral hippocampal CA1 regions, one anterior and medial to the bilateral hippocampal CA1 regions, and bilaterally behind lambda for reference and ground electrodes). A 32G 0.5ul microsyringe will be lowered below the dura at 2.0mm posterior to bregma, 1.3mm lateral from midline and 1.5mm dorsally into the hippocampus, and injected with 40 - 100nL of 20mM Kainic Acid in phosphate buffered saline or normal saline into the dorsal hippocampus via an infusion pump at 50nL/min. The syringe will be left in place for 5 minutes to facilitate complete perfusion around the injection site and minimize tissue damage. Bilateral hippocampal electrodes are placed 2.8mm posterior of bregma, +/- 2.0 mm from midline and 2.0mm dorsally into the hippocampus. Then the entire electrode assembly is fixed in place with dental cement or kwik-sil and anesthesia discontinued. Mice will be moved to the EEG Core to evaluate the resulting status epilepticus. Status epilepticus is described as initiating after the effects of anesthesia subside. In short, electrodes will be connected to a swivel commutator enabling free movement and EEG recordings. The animals will be monitored by the experimenter throughout the duration of SE. In order to attenuate behavioral status epilepticus to improve survival, all mice will

be given an i.p. injection of 6mg/kg lorazepam 3 hours after initiation of status epilepticus. If necessary a second dose of 6mg/kg lorazepam will be administered 60 minutes after the first dose. 24 Hrs after KA is administered, the animals are sacrificed and the tissue is harvested.

c. Tissue harvesting

Right and left hippocampus was harvested 24 hrs. after KA injection. Tissue was placed directly into RNALater (Thermofisher AM7020) and kept at 4ºC overnight, then the excess liquid was removed and tissue was stored at -80ºC until shipment in dry ice to Russek lab.

d. RNA extraction

Tissue was homogenized using either an electric mortar and pestle (ipsilateral hippocampus) or with the Covaris tissue pulverizer (contralateral hippocampus) in 500uL Trizol (Thermofisher, Cat. 15596018) reagent and incubated for 5 minutes at room temperature. 100 uL Chloroform was then added and samples were vigoursly mixed for 15 seconds and incubated at room temperature for 2-3 minutes. Samples were centrifuged at 4ºC for 15 minutes at 12,000xg. Upper aqueous phase was carefully removed from tube and placed in new 1.5mL centrifuge tube. 1 volume of 70% ethanol was added. QIAGEN RNeasy mini columns were then used to purify the RNA and remove the DNA using DNAse I according to the kit protocol (QIAGEN, Cat. 74804). Quantity and quality of RNA was then verified using the Bioanalyzer at the Harvard Biopolymers Facility (ipsilateral) or Boston University Microarray and Sequencing Core (contralateral).

e. RNA library prep

For the ipsilateral side, 3 samples had a RIN slightly below 7 (and above 6). Due to the potentially damaged polyA tails of this RNA, the NEB Next rRNA depletion kit (E6310) had to be used to select for mRNA rather than the polyA selection kit that is preferable (due to cost), and for consistency, this kit was used for all samples, despite the majority of samples having a RIN >8. After rRNA depletion, the NEBNext Ultra II Directional Kit (E7765) was used to create RNA libraries and NEB Multiplex Oligos (Kits E7335, E7710) were used to barcode the samples. After library prep, samples were analyzed at the Harvard Biopolymers facility by Tapestation to determine if proper fragmentation was achieved and by qPCR using the KAPA Quant assay. KAPA Quant uses the P5/P7 adapters that allow successfully prepped libraries to adhere to the flowcell for sequencing as priming sites, such that the concentration value generated by this assay reflects only material that will actually sequence. Using these concentration values, they created an equimolar pool for an equal distribution of reads across samples.

f. RNA sequencing

All samples were pooled together and read in 4 lanes (ipsilateral) or 3 lanes (contralateral). Note: more lanes were needed for contralateral due to the addition of other unrelated samples to the mix. The NextSeq Illumina system was used and 70-80 million reads per sample were generated by the Harvard Biopolymers Facility.

g. RNA sequencing analysis

Data analysis was performed using Strand NGS software, Version 3.2, Build 237248. © Strand Life Sciences, Bangalore, India. Proprietary Strand NGS

algorithms were used to conduct read alignment to the rat genome build rn6 RefSeq Genes (2016.05.11). Reads were filtered based on the following parameters: Max number of novel splices: 1, Min alignment score: 90, Number of gaps allowed: 5, number of matches to be output for each read: 1, exclude reads with alignment length less than: 25, ignore reads with matches more than: 5, Trim 3' end with average base quality less than: 10. RNA quantification was performed by the Strand NGS software, which counts the total number of reads that map to each gene and exon in the genome, and is reported as raw counts. Normalization of the raw counts was achieved using the DESeq method, which accounts for difference in the total number of reads between samples and log transforms the data (Anders and Huber 2010). R version 3.1.2 was used with DEseq2 version 1.6.1 to determine differential expression FDR=0.05, Wald test type. The list of significant genes was then used in subsequent analyses. EnrichR was used for GO-term analysis and KEGG pathway analysis (Kuleshov et al. 2016; Chen et al. 2013).

C. MATERIALS AND METHODS SPECIFIC TO CHAPTER V

1. MATERIALS

a. Plasmids

1. p75 flag: 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.

2. JAK2GFP: the JAK2 GFP is contained in the DEST40 vector under the CMV promoter. The construct was received as a gift from Dr. Ross Levine, Mount Sinai School of Medicine.

3. pcdna3.1- commercially available from Invitrogen

4. Gateway pcDNA Dest40- commercially available from Invitrogen

5. p75 ICD- Received as a generous gift from Phillip Barker (McGill University),

6. ΔICD p75 and ΔECD p75- Received as a generous gift from Moses Chao (New York University). Both contain HA tag. ΔICD (deleted intracellular domain, amino acids 275–425), ΔECD (deleted extracellular domain, amino acids 30–250).

7. Mutant and constitutively active JAK2 ?

b. Antibodies

Antibody Name	**Primary incubation**	**Washes (1) after blocking 2) after primary, 3) after secondary**	**Secondary antibody (all 1 hr RT)**	**Blocking (1 hr RT or O/N 4ºC)**	**Amount if used in IP**

P75NTR Millipore 07-476 Rabbit	1:1000 in 5%BSA in TBS-T O/N	1) 3x5' in TBS-T 2) 3x10' in TBS-T 3) 3x10' in 1% milk in TBS-T	1:5000 goat anti-rabbit Life technology AP 123P in 1% milk in TBS-T	5% non-fat dry milk in TBS-T	For HEK cells 3uL in one IP For neurons:8-10uL per IP
P75NTR Cell Signaling Technology (CST) 8238 Rabbit	1:1000 in 5%BSA in TBS-T O/N	1) 3x5' in TBS-T 2) 3x10' in TBS-T 3) 3x10' in 1% milk in TBS-T	1:5000 goat anti-rabbit Life technology AP 123P in 1% milk in TBS-T	5% non-fat dry milk in TBS-T	
JAK2 CST 3230	1:2000 in 5%BSA in TBS-T O/N or RT 1 hr.	1) 3x5' in TBS-T 2) 3x10' in TBS-T 3) 3x10' in 1% milk in TBS-T	1:6000 goat anti-rabbit Life technology AP 123P in1% milk in TBS-T	5% non-fat dry milk in TBS-T	

STAT3 CST 4904	1:2000 5% milk in TBS-T O/N	1) no washes 2) 3x10' in 1% milk 3) 3x10' in 1% milk in TBS-T	1:6000 goat anti-rabbit Life technology AP 123P in1% milk in TBS-T	5% non-fat dry milk in TBS-T
P-STAT3 CST 9131	1:1000 5% BSA in TBS-T O/N	1) 3x5' in TBS-T 2) 3x10' in TBS-T 3) 3x10' in 1% milk in TBS-T	1:5000 goat anti-rabbit Life technology AP 123P in 1% milk in TBS-T	5% non-fat dry milk in TBS-T (Do Not use BSA)
P-JAK2	1:1000 5% BSA in TBS-T O/N	1) 3x5' in TBS-T 2) 3x10' in TBS-T 3) 3x10' in 1% milk in TBS-T	1:5000 goat anti-rabbit Life technology AP 123P in 1% milk in TBS-T	5% non-fat dry milk in TBS-T (Do Not use BSA)

HP1a CST 2616	1:1000 in 5%BSA in TBS-T O/N	1) 3x5' in TBS-T 2) 3x10' in TBS-T 3) 3x10' in 1% milk in TBS-T	1:5000 goat anti-rabbit Life technology AP 123P in 1% milk in TBS-T	5% non-fat dry milk in TBS-T	15 uL for IP of 500uL total volume
H3Y41P Thermofisher PA5-40142	1:2000 in 5% BSA in TBS-T RT 1 hr or O/N	1) 3x5' in TBS-T 2) 3x10' in TBS-T 3) 3x10' in 1% milk in TBS-T	1:7500 goat anti-rabbit Life technology AP 123P in 1% milk in TBS-T	5% non-fat dry milk in TBS-T	
β -actin CST 3700	1:1000 in 1% milk in TBS-T 1 hr RT	1) none 2) 3x10' in 1% milk in TBS-T 3) 3x10' in 1% milk in TBS-T	1:5000 anti-mouse Vector Labs PI2000 in 1% milk in TBS-T	5% non-fat dry milk in TBS-T	

β -actin Sigma A5441	1:6000 in 1% milk in TBS-T 1 hr RT	1) none 2) 3x10' in 1% milk in TBS-T 3) 3x10' in 1% milk in TBS-T	1:2500 anti-mouse Vector Labs PI2000 in 1% milk in TBS-T	5% non-fat dry milk in TBS-T	
CREB CST 9197	1:1000 5% BSA in TBS-T O/N or 1 hr RT	1) 3x5' in TBS-T 2) 3x10' in TBS-T 3) 3x10' in 1% milk in TBS-T	1:5000 goat anti-rabbit Life technology AP 123P in 1% milk in TBS-T	5% non-fat dry milk in TBS-T	
HA-tag CST 3724	1:1000 5% BSA in TBS-T O/N or 1 hr RT	1) 3x5' in TBS-T 2) 3x10' in TBS-T 3) 3x10' in 1% milk in TBS-T	1:5000 goat anti-rabbit Life technology AP 123P in 1% milk in TBS-T	5% non-fat dry milk in TBS-T	8-10 uL in 500uL Total Volume

2. METHODS

a. Cell culture

1. Human Embryonic Kidney (HEK293) cells

HEK293 cells were maintained in Dulbecco's Modified Eagle Medium (high glucose/L-glutamine) containing 0.1 mM non-essential amino acid medium and 10% fetal bovine serum. Cells were incubated at 37ºC and 5% CO2. At 80-90% confluency, cells were trypsinized and centrifuged for 5 min. followed by a wash and trituration with HEK media. Cells were split 1:5into 35mm or 100mm cell culture dishes.

b. Primary neuronal culture

Primary neocortical neurons were dissected from neocortex of E18 Sprague-Dawley rat embryos (Charles River Laboratories). Pregnant dams were euthanized by CO 2. Embryonic brains were removed and placed in ice-cold Ca 2+ /Mg 2+ free (CMF) media [Ca 2+ /Mg 2+ free Hanks BSS, 4.2mM sodium bicarbonate,1mM pyruvate, 20mM HEPES, 3mg/mL BSA, pH 7.25-7.3]. Cortices were dissected under a dissection microscope, trypsinized centrifuged, and triturated in plating media [Neurobasal media (Invitrogen), 10% fetal bovine serum (Gibco), 100 U/mL penicillin, 100 µg/mL streptomycin, 200mM glutamax (Gibco)]. Dissociated neurons were plated on Poly-L-lysine coated 6-well plates ($1x10^6$cells/well) and placed in an incubator (37ºC/5% CO 2) for attachment to plate. 1hr later, plating media was removed and replaced with 2mL of defined medium [Neurobasal media (Invitrogen), B27 serum-free supplement (Gibco), 100 U/mL penicillin, 100 µg/mL streptomycin, 200mM glutamax (Gibco)]. Neurons were cultured in the incubator for 9 days until use (9 days in vitro [DIV]).

b. Transfection

1. HEK transfection

HEK were plated in 35 mm dishes such that their confluency was approximately 70—90% the following day at the time of transfection. Cells were transfected with Lipofectamine 2000 (Life technologies). For a 35 mm dish, 2500 ng of total DNA was added into Optimem. In the case of co-transfection, equal molar amounts of each DNA were added unless otherwise specified. 8 uL of lipofectamine was added into optimum for dilution, then the diluted lipofectamine and diluted DNA were combined and incubated at room temperature for 5 min. Mixture was added dropwise to cells and dishes were replaced in incubator for 24 hours before use.

c. Molecular subcloning

Constitutively active JAK2 construct and the corresponding wild-type JAK2 (both with HA tags) were provided to us in a storage vector, Sabita Bandyopadhyay moved them into the a pcdna3.1 CMV (cytomegalovirus) promoter vector. Other vectors were in the lab or we received them from other labs (as listed above).

d. Protein extraction

1. Whole-cell extractions

Cells were washed once with ice-cold PBS (pH 7.4). Additional PBS was added and cells were scraped and spun down at 5000rpm for 4 min. at 4ºC. Supernatant was discarded and the cell pellet was resuspended with an appropriate volume of RIPA lysis buffer (1x RIPA buffer (Millipore), 1x protease inhibitor cocktail (Roche), and 1X phosphatase inhibitor cocktail (Roche). Cell were briefly vortexed and incubated for 10-

15 min rotating at 4ºC. Cell lysates were centrifuged at maximum speed at 4ºC for 10 min. and supernatant was collected. Protein sample concentrations were determined with the Bradford assay (Bio-Rad Laboratories) and stored at -80ºC.

2. Nuclear and cytoplasmic fractionated extractions

Cells were washed once with ice-cold PBS (pH 7.4). Additional PBS was added and cells were scraped and spun down at 500g for 2.5 min. at 4ºC. Supernatant was discarded and the cell pellet was resuspended with an appropriate volume of Cytoplasmic Extraction Reagent I (1x Cytoplasmic Extraction Reagent I (ThermoFisher), 1x protease inhibitor cocktail (Roche), and 1X phosphatase inhibitor cocktail (Roche). Cells were vortexed for 15 sec. and incubated on ice for 10 min. Appropriate volume of Cytoplasmic Extraction Reagent II (ThermoFisher) was added. Cells were vortexed for 5 sec. and incubated on ice for 1 min. then vortexed again for 5 sec. Cell lysates were centrifuged at maximum speed at 4ºC for 5 min. and supernatant (cytoplasmic extract) was collected in separate tube. Insoluble (pellet) fraction was suspended in appropriate volume of Nuclear Extraction Reagent (1x Cytoplasmic Extraction Reagent I (ThermoFisher), 1x protease inhibitor cocktail (Roche), and 1X phosphatase inhibitor cocktail (Roche)), then vortexed for 15 sec. Sample was incubated on ice then vortexed every 10 min. for 40 min. Cell lysates were centrifuged at maximum speed at 4ºC for 10 min. and supernatant (nuclear extract) was collected in separate tube. Protein sample concentrations were determined with the Bradford assay (Bio-Rad Laboratories) and stored at -80C.

e. Coimmunoprecipitation

Active Motif Magnetic co-immunoprecipitation kit (Cat.54002) was used for all coimmunoprecipitations. Generally, 200-500 ug of protein was incubated overnight with recommended amount of each antibody in the kit provided CoIP/Wash buffer with 1x protease, deacetylase and phosphatase inhibitor. The following morning, 25 uL of magnetic bead slurry is added to each IP for 1 hr. Samples then undergo 4 washes with the CoIP wash buffer mix before being eluted into 20 uL Tris Glycine buffer (2x) with 10% DTT. Samples were immediately loaded into gel or else stored at -20C.

f. Western Blot

Protein extracts were boiled at 95C for 5 minutes then loaded into 10% Tris-glycine SDS-PAGE Wedgewell gels (Invitrogen) under reducing conditions and transferred to nitrocellulose membranes (Invitrogen), along with Life Technologies Spectra Broad Range marker. Membranes were blocked in 5% non-fat dry milk in Tris-buffered saline with .05% tween (TBS-T) for 1 hr. at room temperature. Immunoblots were then washed 3x for 5 min. and incubated with primary antibodies (typically in 5% BSA unless otherwise directed) overnight at 4ºC or 1 hr. at room temperature (depending on the antibody), washed 3 times for 10 minutes in TBS-T and incubated with secondary Ab in conditions described in Table X for 1 hr. at room temperature then washed 3x for 10 minutes in 1% milk. Proteins were visualized suing enhanced chemiluminescence if HRP-conjugated or imaged directly on LI-COR Odyssey system if infrared (IR) dye-conjugated. Anti-β-actin was used for protein normalization. Immunoblot films were scanned and quantified using Image J for film or Image Studio Lite for LI-COR images.

g. Statistics

For immunoprecipitation in figure 24 quantitation, significance was tested using One-Way ANOVA followed by Tukey's test for multiple comparisons. (*$p<0.05$, **$p<0.01$, ***$p<0.0005$, ****$p<.0001$). In the immunoprecipitation quantitation in figure 26, significance was tested with a student's unpaired, two-tailed, t-test, $p<0.05$.

CHAPTER III: TRANSCRIPTOMIC ANALYSIS OF THE BDNF-INDUCED JAK/STAT PATHWAY IN NEURONS: A WINDOW INTO EPILEPSY-ASSOCIATED GENE EXPRESSION

A. ABSTRACT

Many anti-seizure medications target neurotransmission to increase inhibition via membrane- bound receptors and ion channels but over 30% of epilepsy patients are refractory to them. Understanding the molecular basis of acquired epilepsy, as seen through the lens of intracellular signaling, may provide a new opportunity to break through this treatment barrier. Brain-derived neurotrophic factor (BDNF) is a major signaling molecule that controls a vast network of intracellular cascades fundamental to properties of learning and memory, and epilepsy development. Neurons chronically exposed to BDNF display activation of the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway and treatment with a JAK/STAT inhibitor reduces the frequency of spontaneous seizures in animal models of temporal lobe epilepsy (TLE). RNA silencing of STAT3 prevents BDNF-stimulated decreases in type A GABA receptor α1 subunits, indicating a role for JAK/STATs in brain inhibition. In this study, we discover new BDNF-regulated genes using deep RNA-sequencing of primary cortical neurons treated in the presence and absence of the JAK/STAT inhibitors WP1066 and Ruxolitinib. Expression of BDNF-responsive genes reversed by JAK/STAT inhibition map to neurological diseases, with the top four categories relevant to epilepsy. Pathways involved in epileptogenesis: synaptic transmission, plasticity, and neurogenesis, are enriched in the BDNF-induced JAK/STAT gene set and include the novel targets:

Dopamine receptor D5 (*Drd5),* Glutamate metabotropic receptor 1 *(Grm)1,* Galinin Receptor 1 *(Galr1),* and the Sodium Voltage-Gated Channel Alpha Subunit 1 (*Scn1a)*. We also discover that canonical activation of STAT3 is not required for BDNF-induced JAK/STAT gene regulation, providing evidence for a non-canonical role of JAK/STATs in neurons. The emerging landscape of the transcriptome for BDNF-induced JAK/STAT regulation suggests that this intracellular signaling pathway may play a broader role than originally expected and discovering its novel mechanism may provide new targets for the future treatment of intractable epilepsies.

B. INTRODUCTION

Epilepsy, characterized by recurrent spontaneous seizures, affects 1% of the world's population, making it the third most common form of neurological disease. Although many anti-seizure drugs are available, most target membrane-bound receptors or ion channels and while they reduce neuronal activity, they do not treat the underlying pathology that causes the spontaneous seizures. In addition, at least 30% of patients are refractory to all currently available anti-seizure drugs (Kwan, Schachter, and Brodie 2011). Lack of therapies that modify the course of epileptogenesis could reflect the complexity of disease etiology as well as the heterogeneity of molecular changes that occur throughout the course of disease.

Of the acquired epilepsies, temporal lobe epilepsy (TLE) is the most common form in adults. Some cases of TLE develop years after a brain injury, infection, status epilepticus (SE), or stroke; and, ongoing epileptogenic reorganization of neural circuits likely occurs before seizures begin (Mathern et al. 1996; Prince et al. 2009). Therefore, a

number of laboratories have focused their efforts on identifying novel therapeutics that target the activation of molecular cascades during the latent period after injury in the hopes of attenuating the early activation of intracellular signals that may have far-reaching effects on the expression of multiple gene families involved in epileptogenesis.

In the injured brain, it has been suggested that neurogenesis (Danzer 2018), mossy fiber sprouting (Sutula et al. 1989), and hippocampal cell death (Mathern et al. 1996) contribute to aberrant neuronal circuit reorganization. One manifestation of this reorganization is thought to be altered long-term potentiation (LTP)-induced synaptic plasticity (Kullmann, Asztely, and Walker 2000), which is associated with altered levels of particular neurotransmitter receptors and ion channels (Brooks-Kayal et al. 1998; L. K. Friedman et al. 1994). As the functional properties of neurons change, a state of unchecked overexcitation may occur and is accompanied by decreased GABA-mediated inhibition (Cossart et al. 2001). Many believe that a major mechanism for this altered brain plasticity is the increased synthesis and release of Brain-derived Neurotrophic Factor (BDNF), a major brain signaling molecule that plays an important role throughout life. BDNF is a neurotrophin ubiquitously expressed in the whole brain with a major role in activity-dependent alterations in neuronal morphology and synaptogenesis. Protein and mRNA levels of BDNF are markedly increased after seizures in brain regions involved in epileptogenesis (Murray et al. 2000; Takahashi et al. 1999) and mutations in *BDNF* can reduce the development of spontaneous seizures in animal models (Kokaia et al. 1995). Moreover, a conditional deletion of the high-affinity BDNF receptor TrkB in a subset of neurons, is sufficient to completely eliminate all behavioral evidence of the

progression of epilepsy in the kindling model (He et al. 2004) and it has been shown that enhanced TrkB signaling can exacerbate epileptogenesis (Heinrich et al. 2011).

In addition to the TrkB-mediated BDNF response, the p75 Neurotrophin Receptor (p75NTR, also referred to as NGFR) is a low-affinity receptor for the cleaved mature form of BDNF while a high-affinity receptor for the uncleaved form, proBDNF. p75NTR levels are elevated after SE in animals and increased activation by proBDNF, like mature BDNF, increases susceptibility to seizures (Riffault et al. 2018; VonDran et al. 2014). Interestingly, our labs have shown that increased synthesis of proBDNF, and not mature BDNF, is the first response of the injured brain in the pilocarpine (PILO) model of epilepsy prior to SE-induced increases of p75NTR (Thomas et al. 2016). This finding suggests that high levels of proBDNF may exert their effects through TrkB rather than p75NTR.

In the normal brain, BDNF activation of its receptors regulates multiple signaling pathways: mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK), phospholipase C-γ (PLCγ), phosphoinositide 3-kinase (PI3K), c-Jun N-terminal kinase (JNK), and NFkB (D. R. Kaplan and Miller 1997). It is well established that BDNF modulates long-term potentiation (LTP) at Schaffer collateral-CA1 and mossy fiber-CA3 hippocampal synapses by acting through TrkB (Devin K Binder and Scharfman, n.d.; Yamada and Nabeshima 2004; B. Xu et al. 2000; Muller et al. 2000; Minichiello et al. 2002). Although p75NTR is not implicated in LTP, it plays a significant role in learning and memory through its modulation of hippocampal long-term depression (LTD) by altering AMPA receptor expression (Rösch et al. 2005).

Studies from our laboratories demonstrate that in addition to the signaling pathways described above, increased levels of BDNF activate the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway both *in vivo* in the rat PILO model of epilepsy and in vitro in BDNF-treated primary cultured neurons (Lund et al. 2008). The JAK/STAT pathway is a signaling cascade that has a prominent role in immune function and cancer development. The canonical pathway involves the binding of JAK to its target (such as a cytokine or hormone receptor), subsequent JAK phosphorylation that stimulates the recruitment and activation of STAT proteins (phospho-STAT (p-STAT)) and their movement as dimers into the nucleus where they function as transcriptional activators. Although JAK/STATs regulate transcription through this canonical pathway, more recently, non-canonical JAK/STAT signaling has been discovered where JAKs can act independently of STATs to regulate transcription in the nucleus, and STATs can function independently of their phosphorylation state to alter genome stability (Li 2008). While the role, and mechanism, of JAK/STAT signaling in the brain is still not completely understood, it is clear that it is an important part of how the brain regulates its synaptic connections (Céline S. Nicolas et al. 2012).

Research from our laboratory has demonstrated that activation of the JAK/STAT pathway results in the expression of the inducible cAMP early repressor (ICER) via STAT3-mediated gene regulation. Moreover, we show that ICER represses the gene (*Gabra1/GABRA1*) coding for the α1 subunit of the type A γ-aminobutyric acid (GABA) receptor (GABAR) and that such repression reduces the number of α1 containing GABARs in neurons (Lavoie et al. 1997). Most importantly, reduced α1 subunit gene

expression also occurs in epilepsy patients (Brooks-Kayal et al. 1998) as well as in other disorders of the nervous system (Petryshen et al. 2005; Raud et al. 2009; Bossers et al. 2010). In addition, use of the JAK/STAT inhibitor, WP1066, in the PILO rat model of epilepsy reduces the number of spontaneous seizures after the latent period (Grabenstatter et al. 2014). We now hypothesize that in addition to controlling the gene regulation of *Gabra1/GABRA1*, the BDNF-induced JAK/STAT pathway controls the expression of diverse gene products that are involved in synaptic plasticity as well as in the epileptogenic process that follows brain injury. We also hypothesize that attenuating this pathway may provide a gateway to new treatments for refractory epilepsy.

To test these hypotheses, and to gain a comprehensive understanding of the genome impacted by BDNF-induced JAK/STAT activation, we exposed primary cultured neurons to BDNF with and without JAK/STAT inhibitors and performed deep RNA-sequencing (RNA-seq) in order to determine the gene set within the BDNF response that could be ascribed to JAK/STAT activation (as defined by WP1066 and Ruxolitinib (Ruxo), a potent inhibitor of JAK1/2 activation (T. Zhou et al. 2014)). We now report evidence that suggests BDNF acts through the JAK/STAT pathway to execute changes in: the expression of ion channels, neurotransmitter and G-protein coupled receptors (GPCRs), and the expression of key modulators of synaptic plasticity, neurogenesis, and axonal remodeling. Many of these functions were previously ascribed to BDNF, however, the role of the JAK/STAT pathway in their manifestation has never been previously explored. Our studies also reveal the presence of many new BDNF target

genes providing a broader landscape for BDNF mediated gene regulation of the impaired nervous system.

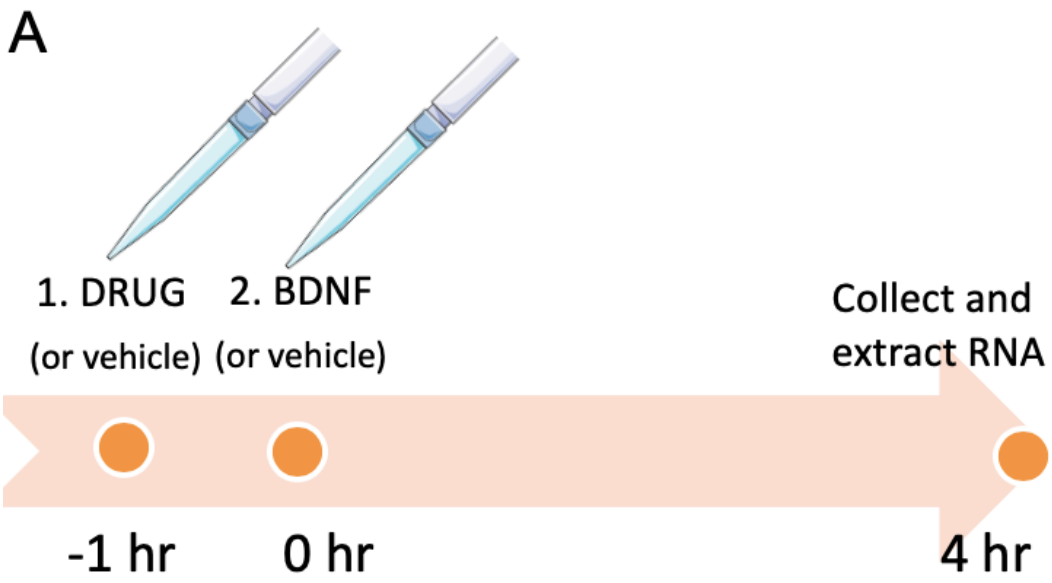
C. RESULTS

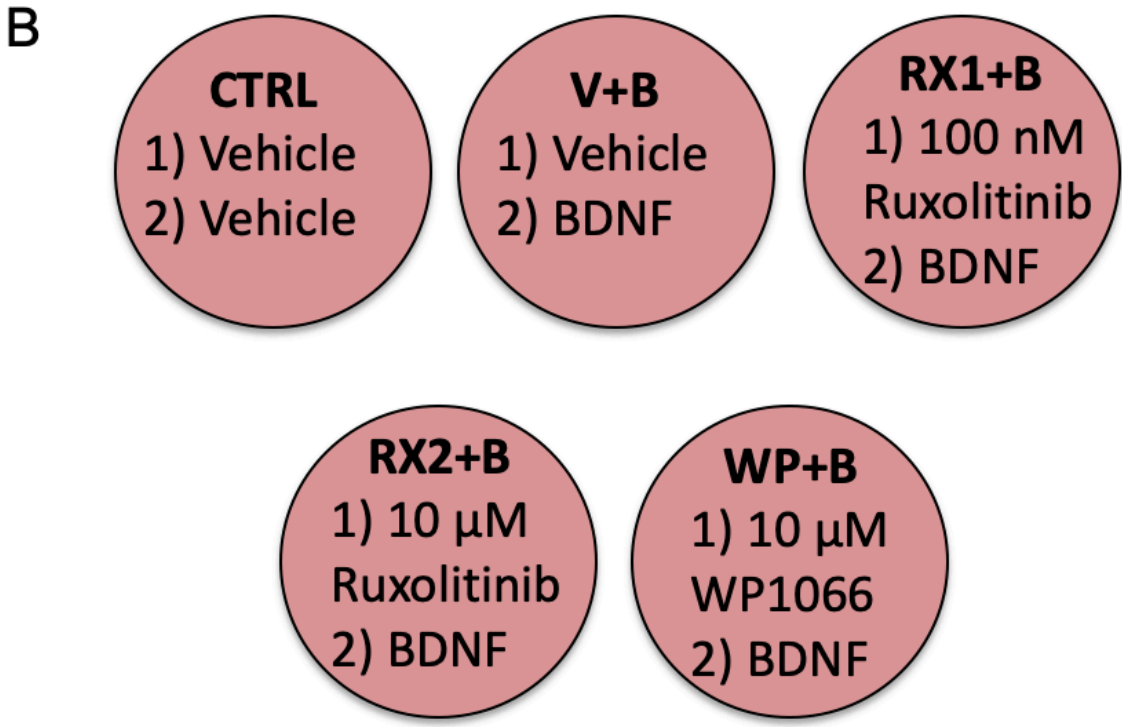
1. BDNF SIGNALING AND THE IDENTIFICATION OF EPILEPSY-ASSOCIATED GENE NETWORKS.

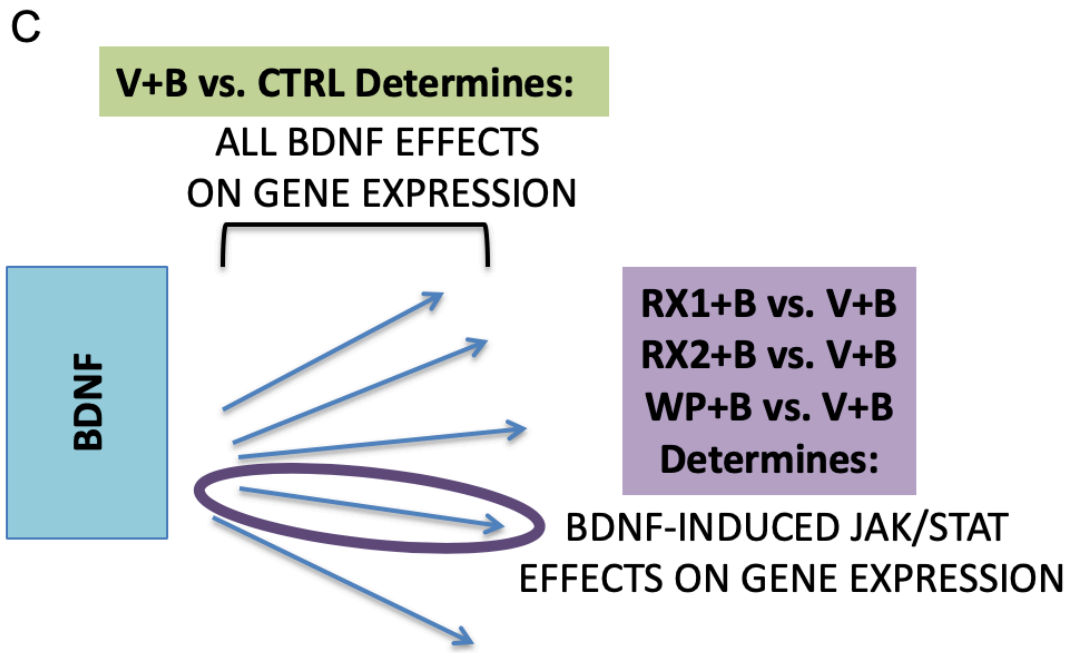
Primary cultured neurons were treated with BDNF or vehicle control (water) to determine the genome response to prolonged receptor activation (4 hours) (Fig. 10). Using RNA-sequencing for open discovery, we identified a total of 2869 differentially expressed genes (DEGs) whose levels change (increase or decrease) in response to BDNF signaling (Fig. 11A) (CTRLvsV+B gene list, supplemental excel sheet). Inspection of the gene set using Ingenuity Pathway Analysis (IPA) (QIAGEN Inc., n.d.) revealed that only 83 of these genes have been previously associated with BDNF (as determined using the Ingenuity Knowledge Base, a curated database of all historical scientific information in IPA databases), suggesting that the majority of genes identified in this study are novel in their association with the BDNF signaling pathway. A subset of 194 potential BDNF regulated genes are associated with “seizure disorders” (BDNF and epilepsy, supplemental excel sheet), an umbrella category in IPA that includes all epilepsy classes as represented in the curated knowledge base. Moreover, seizure disorder is the primary most significant neurological disorder identified by enrichment analysis of the gene pool (p= 3.29E-27).

Figure 10. Schematic of experimental design and analysis.

(A) Timeline representation of the drug treatment protocol, including the 1 hr drug or DMSO (vehicle, V) pretreatment (initiated at -1 hr), administration of BDNF (B) or water (vehicle) treatment (Time=0 hr) and time of cell collection and RNA extraction (4 hr). (B) 6-well plate showing each one of the 5 treatment groups and the abbreviations by which they will be referred to in the text: DMSO+Water (CTRL), DMSO+BDNF (V+B), 100nM Ruxolitinib+BDNF (RX1+B), 10μM Ruxolitinib+BDNF (RX2+B), 10μM WP1066+BDNF (WP+B). (C) Diagram represents the approach taken to identify differential gene expression (DEG) in response to JAK/STAT pathway inhibition. BDNF through its receptors activates multiple signaling pathways (represented by blue arrows) that impact transcription. Differential expression analysis comparing V+B vs. CTRL treatment groups (green box) will reveal the total set of genes that are regulated by BDNF-induced intracellular signaling pathways. To identify BDNF DEGs that are specific to JAK/STAT signaling, comparisons are made between the groups pretreated with JAK inhibitors (RX1+B, RX2+B, WP+B) and the group pretreated with vehicle (V+B) (purple box); DEGs from this comparison (where JAK/STAT inhibition reverses BDNF stimulation or inhibition) are thought to be associated with the JAK/STAT pathway.


A
1. DRUG
(or vehicle)
2. BDNF
(or vehicle)
Collect and
extract RNA
-1 hr
0 hr
4 hr


B
CTRL
1) Vehicle
2) Vehicle
V+B
1) Vehicle
2) BDNF
RX1+B
1) 100 nM
Ruxolitinib
2) BDNF
RX2+B
1) 10 µM
Ruxolitinib
2) BDNF
WP+B
1) 10 µM
WP1066
2) BDNF

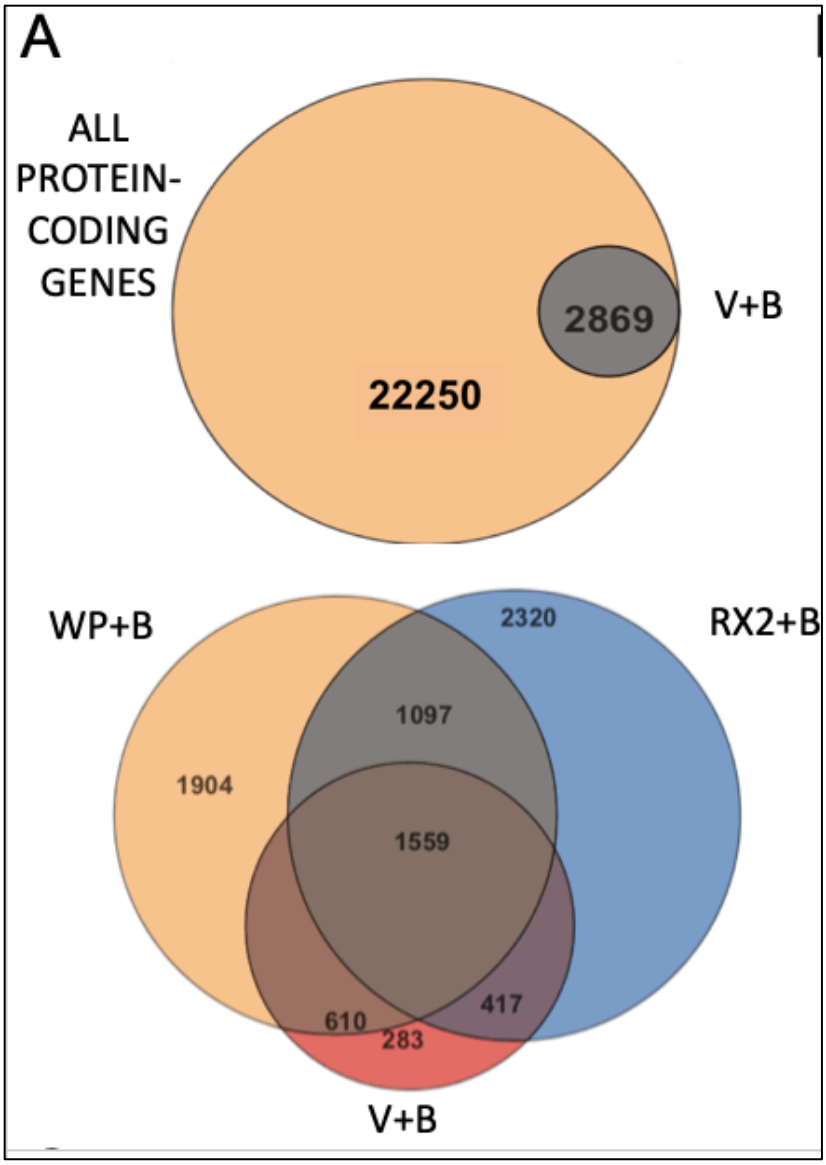

C
V+B vs. CTRL Determines:
ALL BDNF EFFECTS
ON GENE EXPRESSION
BDNF
RX1+B vs. V+B
RX2+B vs. V+B
WP+B vs. V+B
Determines:
BDNF-INDUCED JAK/STAT
EFFECTS ON GENE EXPRESSION

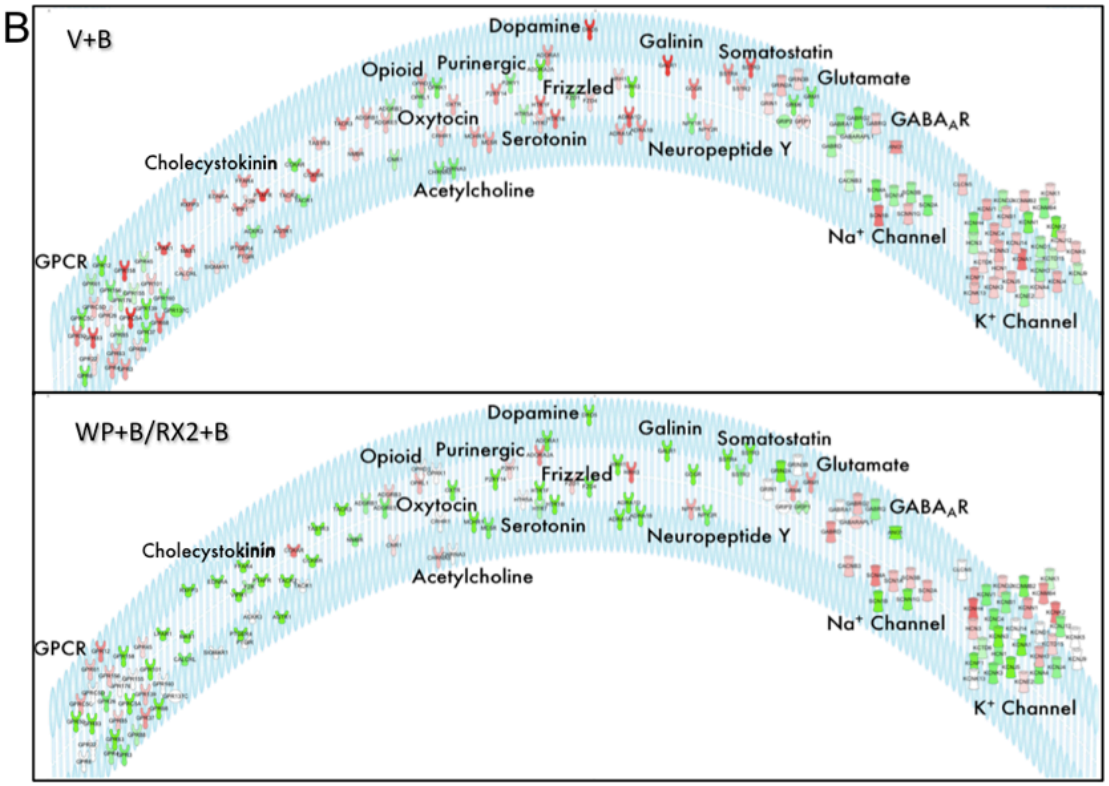
In addition to the 194 epilepsy-associated genes, pathway analysis suggests that increased levels of BDNF alters the expression of essential neurobiological gene sets that are required for brain development and that are dysregulated during epileptogenesis in animal models and human patients. For instance, the differentially expressed set of transcripts from BDNF-treated (0.7nM) primary neurons is highly enriched for genes essential to axonal guidance (p=1.09E-11); further, there is a striking enrichment that is specific to all major classes of ion channels and neurotransmitter/neuropeptide receptors, with 133 of 599 total genes related to one of these categories regulated by prolonged BDNF exposure (4 hr) (p=2.66E-11) (Fig. 11B). Specifically, the differentially expressed transcripts for receptors include those activated by GABA, glutamate, acetylcholine, dopamine, opioids, serotonin, galanin, and neuropeptide Y, covering both metabotropic and ionotropic receptor subtypes. Most classes of ion channel genes are also represented, including those for potassium, sodium, and calcium, where mutations have been shown to underlie specific genetic epilepsies (Claes et al. 2001; Biervert et al. 1998; Jouvenceau et al. 2001). There is also a specific decrease (1.73 fold) in the expression of *Gabra1* that is a member of the GABAR gene cluster which we have previously demonstrated to be regulated by BDNF using qPCR in extracts of BDNF-treated primary neurons and hippocampal tissue extracted from the *in vivo* PILO model of temporal lobe epilepsy (Lund et al. 2008). As expected, the neuronal RNA-seq dataset revealed that prolonged exposure to BDNF increases BDNF transcript levels, a finding that is well established in the literature (Tuvikene et al. 2016). Given our previous identification of a novel JAK/STAT pathway in neurons that is regulated by BDNF, we

asked whether any members of the JAK/STAT pathway might be regulated at a transcriptional level in response to BDNF receptor activation. Our dataset indicates significant enrichment for transcripts linked to JAK/STAT signaling (p=6.03E-5) and in particular, an enrichment of *Jak1* (1.6-fold enrichment) and *Jak2* (1.8-fold enrichment).

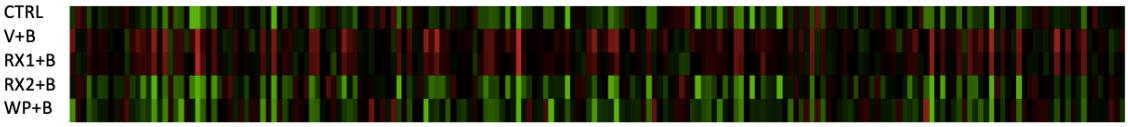
Figure 11. JAK/STAT inhibitors reverse BDNF-induced gene expression.

(A) TOP: Venn diagram representation of all protein-coding genes in the rat genome (in accordance with Strand NGS notation) with all differentially expressed genes (DEGs) between primary neurons treated with DMSO+BDNF vs. DMSO+Water. BOTTOM: Venn diagram representation of all DEGs in DMSO+Water vs. DMSO+BDNF (BDNF, Red, 2869), WP1066+BDNF vs. DMSO+BDNF (WP1066, Orange, 5170 total) and 10µM Ruxo+BDNF vs. DMSO+BDNF (RX2, Blue, 5393 total genes). (B) Receptor and Ion Channel expression is altered by BDNF and rescued by JAK inhibition. TOP: List of Ion Channels and Receptor Subunits whose expression is altered by 4-hr BDNF treatment. Color represents direction and degree of fold change (Red: up, Green: down) sorted by receptor or channel type. BOTTOM: Receptor and Ion Channel reversal in expression by addition of WP or RX2 (white receptors are not affected by JAK/STAT inhibitors). Response to BDNF in presence of WP was used as the basis for coloring receptors depicted in the diagram. Red: Upregulated, Green: Downregulated. (C) Heatmap of all DEGs (columns) associated with Epilepsy (IPA) that are affected by exposure to BDNF. DW: DMSO+Water, DB: DMSO+BDNF, RX1: 100nM Ruxo+BDNF, RX2:10µM Ruxo+BDNF, WP: 10µM WP1066+BDNF. Green: low expression, red: high expression.


A
ALL PROTEIN-CODING GENES
22250
2869
V+B
WP+B
2320
RX2+B
1097
1904
1559
610
417
283
V+B


B
V+B
Dopamine
Galinin
Somatostatin
Opioid
Purinergic
Glutamate
Frizzled
Oxytocin
GABA$_A$R
Serotonin
Neuropeptide Y
Cholecystokinin
Acetylcholine
Na$^+$ Channel
GPCR
K$^+$ Channel
WP+B/RX2+B
Dopamine
Galinin
Somatostatin
Opioid
Purinergic
Glutamate
Frizzled
Oxytocin
GABA$_A$R
Serotonin
Neuropeptide Y
Cholecystokinin
Acetylcholine
Na$^+$ Channel
GPCR
K$^+$ Channel

C.


CTRL
V+B
RX1+B
RX2+B
WP+B

2. NEUROPHARMACOLOGICAL DISSECTION OF BDNF-INDUCED JAK/STAT SIGNALING REVEALS A NON-CANONICAL PATHWAY IN NEURONS

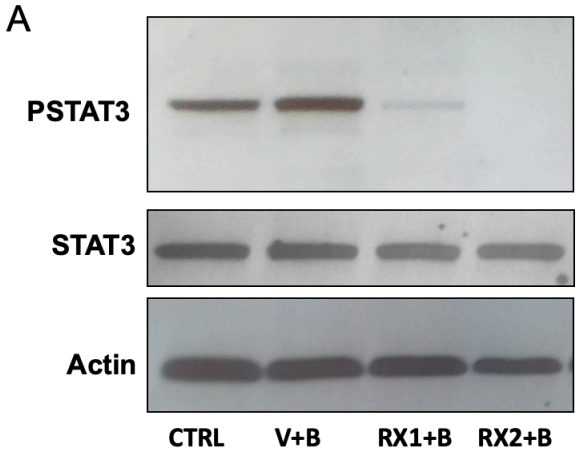
Primary cultured neurons were pre-treated with agents known to inhibit the JAK/STAT pathway (Fig. 10) by interfering with JAK kinase activity, either directly by inhibiting JAK phosphorylation or stimulating JAK degradation. Two different antagonists were employed in these studies: the small molecule WP1066 that has been shown to inhibit JAK2/STAT3 signaling and degrade total JAK2 (Verstovsek et al. 2008) and Ruxolitinib (Ruxo), currently in use as a cancer therapeutic, which acts on JAK1 and JAK2 by forming two hydrogen bonds with the hinge region of the JAK protein (the segment that connects the N-lobe to the C-lobe of the kinase domain) and reduces phosphorylation of the JAK2 protein at Tyr1007/1008 (T. Zhou et al. 2014). Based on our earlier discovery that siRNA knockdown of STAT3 reverses BDNF-induced downregulation in the levels of *Gabra1* subunit mRNAs (Lund et al. 2008), as does WP1066, we used RNA-seq to identify the full set of genes responsive to JAK inhibition. This strategy enables us to unmask those genes in the BDNF transcriptome that may be JAK/STAT dependent.

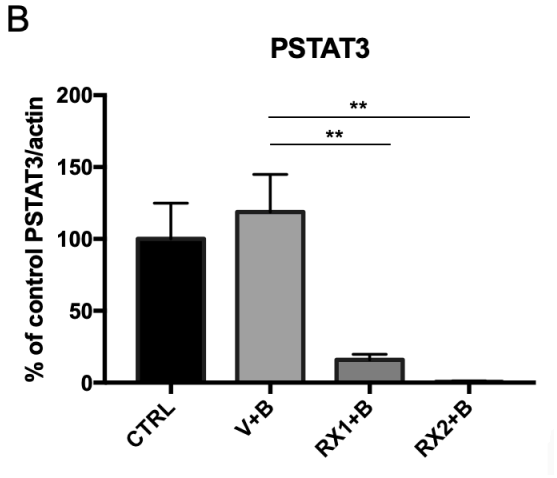
Comparing the transcriptomes BDNF (V+B) or 100nM Ruxo + BDNF (RX1+B), we were surprised to find that there was no difference between these two transcriptomes, as statistically assessed using DSEQ2 (0 DEGs). To make sure that Ruxo treatment at 100nM was active in the primary cultures of our studies, Western blot was performed using the same drug stock as used in cultures that generated the RNA-seq libraries. In this control experiment, Ruxo (100nM) potently inhibited STAT3 phosphorylation at 30

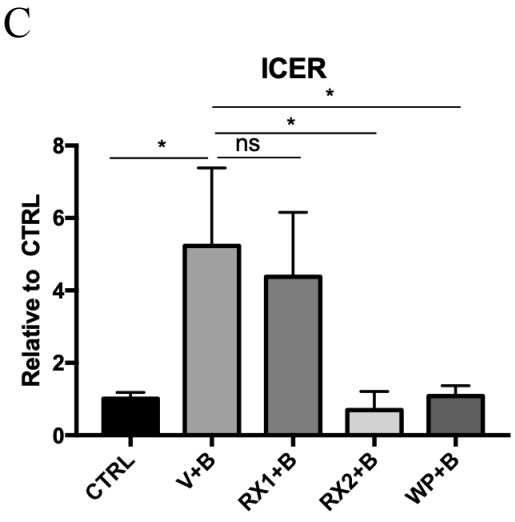
min (80% +- 3.07 reduction of BDNF signal) and at 4 hr (73% reduction of BDNF signal, Fig. 12). Therefore, despite reducing STAT3 phosphorylation at Tyr705, our data taken together demonstrate that 100nM Ruxo does not alter BDNF-induced gene regulation, including changes in ICER expression (Fig. 12C). This finding suggests that the BDNF-induced JAK/STAT pathway is not STAT3 phosphorylation-dependent even though knockdown of STAT3 with siRNAs prevents ICER induction and *Gabra1* downregulation (Lund et al. 2008). Note that in our current studies BDNF does not significantly increase STAT3 phosphorylation but Ruxo still inhibits the basal state of its activation. These observations strongly favor the hypothesis that neurons use a non-canonical mechanism of JAK/STAT signaling which may be relevant to epilepsy, and potentially to learning and memory where BDNF is a critical signaling molecule.

Figure 12. RX1 and RX2 reduce levels of phospho-STAT3 but only RX2 blocks ICER induction.

(A) Representative Western Blot analysis of whole-cell protein extracts from primary neurons 9 DIV pretreated with100nM Ruxolitinib (RX1), 10uM Ruxo (RX2), or DMSO for 1 hr before the addition of BDNF. Cells were collected 30 min after BDNF administration and probed with anti pSTAT3, STAT3 and β-Actin. (B) Quantitation of signals from densitometry is displayed as mean percent change (±SEM) relative to the DMSO+Water control group (**$p<0.01$). Ruxolitinib significantly reduces the levels of P-STAT3 with no change in total STAT3. (C) Graphical representation of results from real time PCR analysis using specific Taqman probe and primers. RNA was extracted from cells collected 4 hr after BDNF administration. Transcript levels are shown as the mean values (±SEM) of the ratio relative to the DMSO+Water control group for *Icer* n=6. Note, that while RX1 blocks pSTAT3, it does not block the effect of BDNF treatment on *ICER* induction while WP and RX2 do. *$p<0.05$. One-way ANOVA with Tukey's multiple comparison test.


A
PSTAT3
STAT3
Actin
CTRL
V+B
RX1+B
RX2+B


B
PSTAT3
% of control PSTAT3/actin
200
150
100
50
0
**
**
CTRL
V+B
RX1+B
RX2+B


C
ICER
Relative to CTRL
8
6
4
2
0
*
*
*
ns
CTRL
V+B
RX1+B
RX2+B
WP+B

3. USING PHARMACOLOGY TO REDUCE THE DIMENSIONALITY OF THE DATASET AND IDENTIFY POTENTIAL BDNF-INDUCED JAK/STAT TARGET GENES

While we discovered that co-treatment of neurons with BDNF (V+B) vs. RX1+B produced similar transcriptomes, we found that a 10-fold increase in the concentration of Ruxo (10μM) plus BDNF (RX2+B) attenuated the expression of multiple genes that were altered in response to BDNF, and most importantly, those that we previously identified in the PILO model of epilepsy. We next compared the RX2 DEG to those generated from cultures treated with 10μM WP1066 plus BDNF (WP+B) and generated a subset of genes whose BDNF-induced expression was reversed back to vehicle control by both drug treatments. Using these JAK/STAT inhibitors as probes for unmasking potential genes that are regulated by the BDNF-induced JAK/STAT pathway, we identified 1559 of the 2659 BDNF target genes that may be most associated with JAK/STAT signaling in neurons. Interrogation of IPA networks specific to this shared gene set revealed that 131 genes are epilepsy-related and seizure disorder is still the top most significant neurological disease (P=7.31E-22). Considering that 131 of the 194 BDNF-regulated epilepsy-linked genes are reversed by JAK/STAT inhibitors (68%), it suggests that JAK/STAT signaling may be a large component of BDNF-related epileptogenesis. This can be best appreciated by looking at the heat map displayed in Figure 11C which depicts the expression levels of all 194 epilepsy-linked genes that are changed by BDNF treatment. The results clearly show that WP+B (10μM) and Ruxo (RX2+B, 10μM) block BDNF-induced changes in neuronal gene expression for epilepsy-linked genes and both

appear to have a similar gene expression signature to that of DMSO+Water control. Note that Ruxo at its lower concentration (RX1+B, 100nM) looks similar to BDNF alone.

4. GENE ONTOLOGY AND PATHWAY ANALYSIS

Gene Ontology (GO) term analysis of the WP+B/RX2+B dataset, performed using EnrichR (Kuleshov et al. 2016; Chen et al. 2013), reveals that the 1559 overlapping genes are significantly associated with molecular functions related to receptor and channel activity (found in 8/10 top molecular functions) (Fig. 13A, top panel). All of the top ten biological process GO terms include the words “synaptic transmission” (Fig. 13A, middle panel). These consistent findings strongly suggest that both WP and Ruxo have the capacity to regulate synaptic transmission at the gene expression level in the brain. When compared with the total list of receptor/ion channel related genes that were regulated by BDNF (as depicted in Figure 11B, top panel), 107/133 genes (80%) were reversed by treatment with JAK/STAT inhibitors (Fig. 11B, bottom panel). This is best seen by the color reversal in the receptors for all but a few that were not significantly affected by pre-treatment with the inhibitor prior to BDNF activation (displayed in white).

In addition, neurotransmitter receptors, axonal guidance, GPCR and c-AMP signaling were found to be amongst the top KEGG pathways associated with the shared gene set of WP+B and RX2+B (Figure 13A, bottom panel). Members of the JAK/STAT signaling pathway were also identified as significant gene targets (P=1.044E-3) suggesting that BDNF may also participate in the feedback regulation of the pathway.

Figure B is a schematic representation showing many of the component molecules that make up the JAK/STAT pathway, with expression of all the colored genes being affected by BDNF treatments and all the ones with a purple border reversed by JAK/STAT inhibitor treatments WP+B and RX2+B.

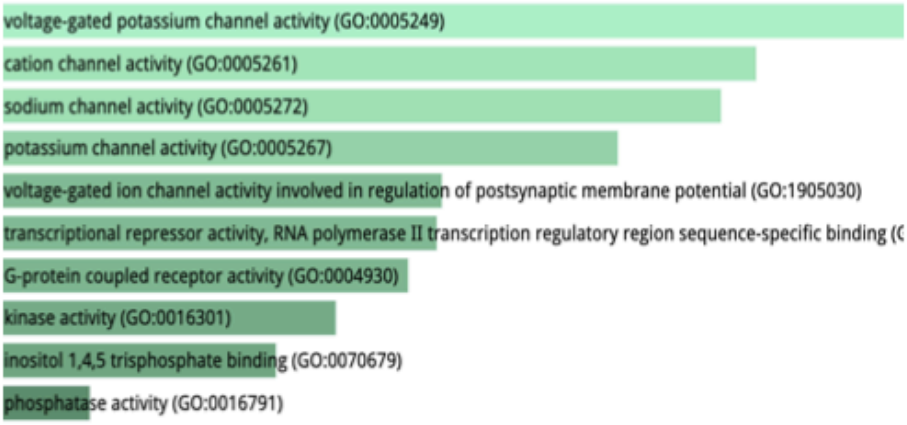
In addition to the significant relationship to epilepsy identified by IPA, the overlapping WP/RX2 gene set had a significant link to neuroinflammation despite the neuronal cultures being largely devoid of any glial cells, 41 genes of the BDNF-induced set of 56 neuroinflammation-linked genes were reversed by WP+B or RX2+B treatment (73% of the gene set) (Fig. 13C).

Figure 13. Pathway Enrichment and Gene Ontology analysis of the overlapping RX2/WP dataset.

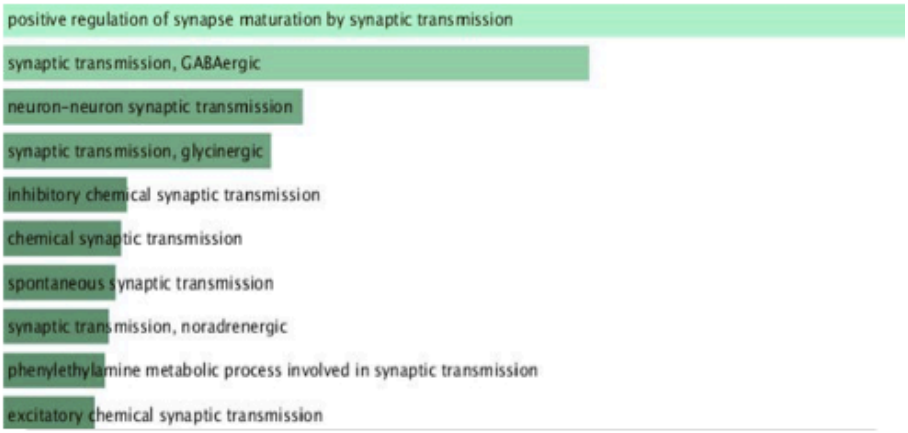
(A) Pathways and functions enriched in the list of 1559 genes altered by WP and 10μM Ruxo (RX2). Gene Ontology and KEGG enrichment analysis performed using EnrichR. (B) Genes involved in the canonical JAK/STAT signaling pathway with functional representation. Genes are colored by degree of fold change with BDNF alone (see panel legend). Purple border signifies that WP+B and RX2+B reverse effects of BDNF on gene expression. (C) List of DEGs involved in neuroinflammatory signaling and degree of fold change of WP+B vs V+B. Listed in order of FC most negative to most positive.

A

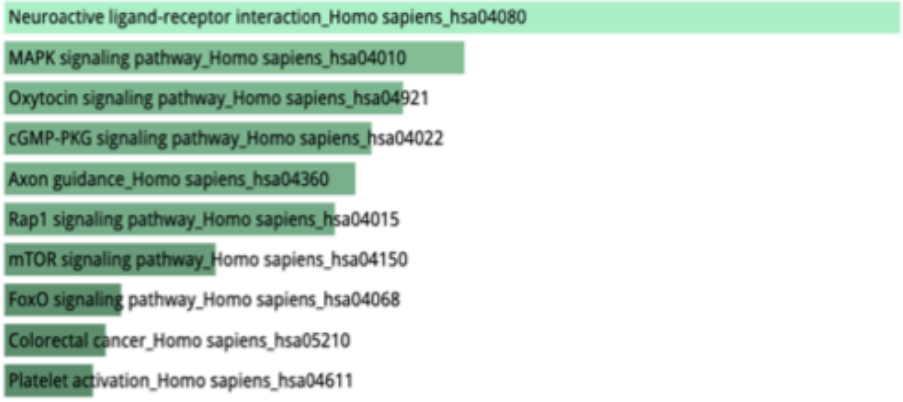
GO: Molecular Component



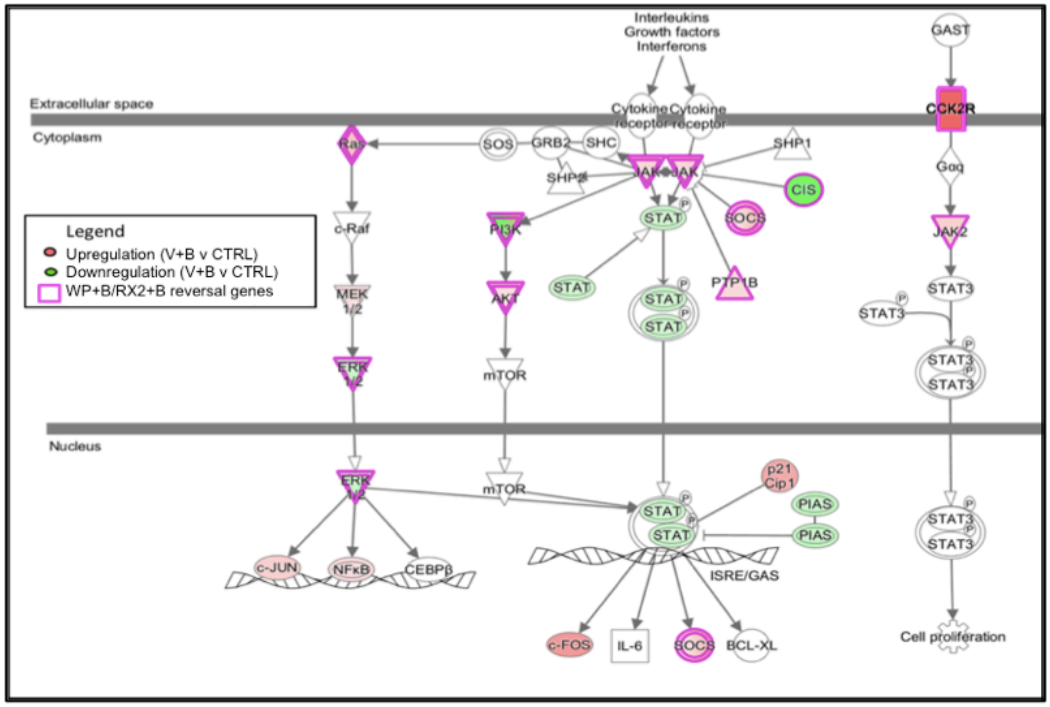
GO: Biological Process



Pathway Analysis: KEGG



B



C

Neuroinflammation Signaling Pathway- IPA					
Symbol	**FC w/WP**	**Symbol**	**FC w/WP**	**Symbol**	**FC w/WP**
KL	-12.15	BDNF	-2.9	FZD1	1.73
MMP3	-10.37	NFATC1	-2.71	PPP3CC	1.73
GRIN2A	-8.8	JAK2	-2.11	IFNGR2	1.74
IL6R	-6.8	CFLAR	-2.07	PIK3C3	2.04
TGFBR2	-5.47	TGFB1	-1.93	GABRG2	2.24
PTGS2	-4.99	GABRQ	-1.89	CD200	2.26
FGFR1	-4.97	ACVR1	-1.86	MAPK3	2.32
IFNG	-4.13	PIK3CB	-1.86	SNCA	2.45
ACVR1C	-4.06	MAP2K4	-1.86	PSENEN	2.53
KCNJ5	-3.98	AKT2	-1.65	GABRD	3.47
GDNF	-3.65	CX3CL1	-1.59	PIK3CD	4.01
NGF	-3.61	JAK1	-1.5	IL34	4.23
PLA2G4A	-3.38	SYK	-1.43	MAPK15	5.82
IRS2	-3.35	NCSTN	1.28		

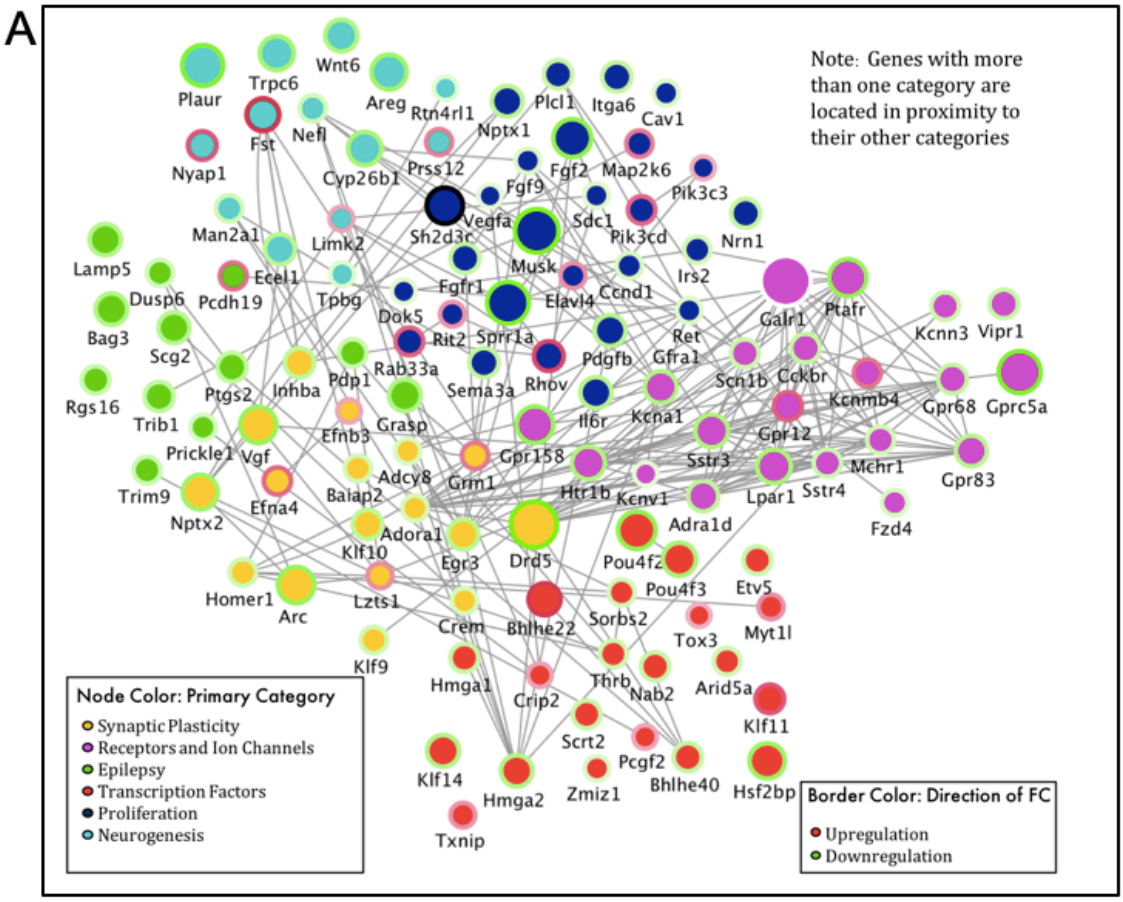
5. FUNCTIONAL RELATIONSHIPS BETWEEN GENES AS DESCRIBED IN THE LITERATURE

Pathway and GO analysis identified the following epileptogenic processes, in addition to inflammation, as being enriched in the WP+B/RX2+B overlapping data sets: synaptic plasticity, receptors and ion channels, epilepsy, transcription factors, proliferation, and neurogenesis. In order to visualize the functional connectivity of the genes involved in epileptogenesis, we created a functional network of the most significant genes as determined by a p-value of <1x10^10 and a fold-change >2 (Figure 14). Genes that met these conditions were separated into the lists of the epileptogenic processes, as described above. In order to relate these genes to one another and their epileptogenic functions, we created a network that portrays their function, expression fold change, direction, and functional protein relationships in Cytoscape (Fig. 14A) (Shannon et al. 2003). Using GENEMania, a Cytoscape tool containing a database of experimental data showing protein-protein interactions (Warde-Farley et al. 2010), we determined potential functional relationships. Each node in the network represents a gene while the edges represents a protein interaction. Node color is determined by the epileptogenic category. Due to the interplay between these functions, many genes within one category overlap with another category. For instance, Egr3 plays a role in 5 of the 6 categories: synaptic plasticity, epilepsy, transcription factors, proliferation, and neurogenesis. The color in this case is determined by whichever category comes first in this list: synaptic plasticity, receptors and ion channels, epilepsy, transcription factors, proliferation, and neurogenesis, where the order is determined by the number of genes in that category from

smallest to largest. This was done to avoid having the majority of the nodes colored for the largest category (neurogenesis). For Egr3, it is yellow representing synaptic plasticity, however, its location in the network is near the center because the proximity of the node to other categories represents its link to those secondary categories. In addition, the Venn diagram in 14B also shows the degree of overlap for each category. For instance, there are 108 total genes related to receptors and ion channels. In the list, 61 of those did not belong to any other category, 11 were also linked to neurogenesis, 5 to receptors, neurogenesis and proliferation,14 to receptors and epilepsy, 6 to receptors, epilepsy and neurogenesis, and 2 to receptors, neurogenesis, epilepsy and proliferation.

Figure 14. Functional Network of DEGs involved in epileptogenic-related processes.

(A) Node color represents primary category, determined by order indicated in panel legend. Node size indicates degree of fold change (FC). Border color indicates direction of FC. Gray lines show functional connectivity determined by GeneMania in Cytoscape. Location in network roughly determined by category and multiple associations with neighboring and other categories. (Genes located near the center are involved in multiple categories). (B) Venn diagram of genes contained in network diagram in A demonstrating overlap of the genes in each category, neurogenesis (blue), receptors (red), epilepsy (gray), synaptic plasticity (yellow), and proliferation (green). Gene associations provided by IPA.


A
Note: Genes with more than one category are located in proximity to their other categories
Plaur
Trpc6
Wnt6
Areg
Rtn4rl1
Plcl1
Itga6
Cav1
Nptx1
Fst
Nefl
Nyap1
Cyp26b1
Prss12
Fgf2
Map2k6
Fgf9
Pik3c3
Vegfa
Sh2d3c
Sdc1
Pik3cd
Nrn1
Man2a1
Limk2
Lamp5
Musk
Irs2
Ecel1
Fgfr1
Ccnd1
Dusp6
Pcdh19
Tpbg
Elavl4
Ptafr
Galr1
Bag3
Dok5
Rit2
Sprr1a
Ret
Kcnn3
Vipr1
Scg2
Pdgfb
Gfra1
Rab33a
Pdp1
Inhba
Scn1b
Cckbr
Rgs16
Ptgs2
Sema3a
Rhov
Kcnmb4
Gpr68
Gprc5a
Trib1
Grasp
Il6r
Kcna1
Efnb3
Gpr12
Prickle1
Vgf
Gpr158
Sstr3
Mchr1
Adcy8
Grm1
Htr1b
Kcnv1
Gpr83
Trim9
Baiap2
Lpar1
Sstr4
Efna4
Nptx2
Adora1
Adra1d
Fzd4
Klf10
Egr3
Drd5
Pou4f2
Homer1
Lzts1
Pou4f3
Etv5
Arc
Crem
Bhlhe22
Sorbs2
Tox3
Myt1l
Klf9
Hmga1
Thrb
Nab2
Arid5a
Crip2
Klf11
Scrt2
Pcgf2
Klf14
Hmga2
Zmiz1
Bhlhe40
Hsf2bp
Txnip
Node Color: Primary Category
Synaptic Plasticity
Receptors and Ion Channels
Epilepsy
Transcription Factors
Proliferation
Neurogenesis
Border Color: Direction of FC
Upregulation
Downregulation

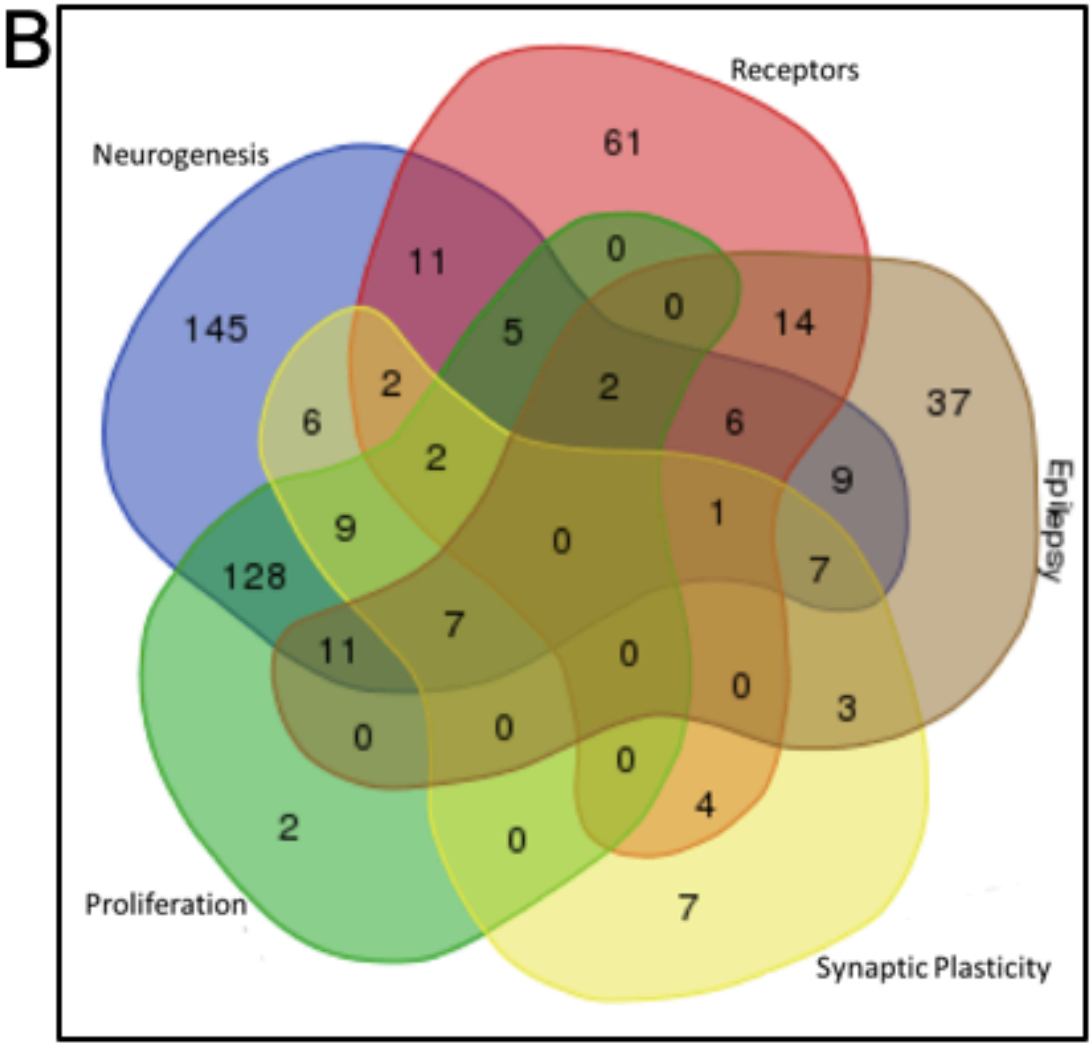
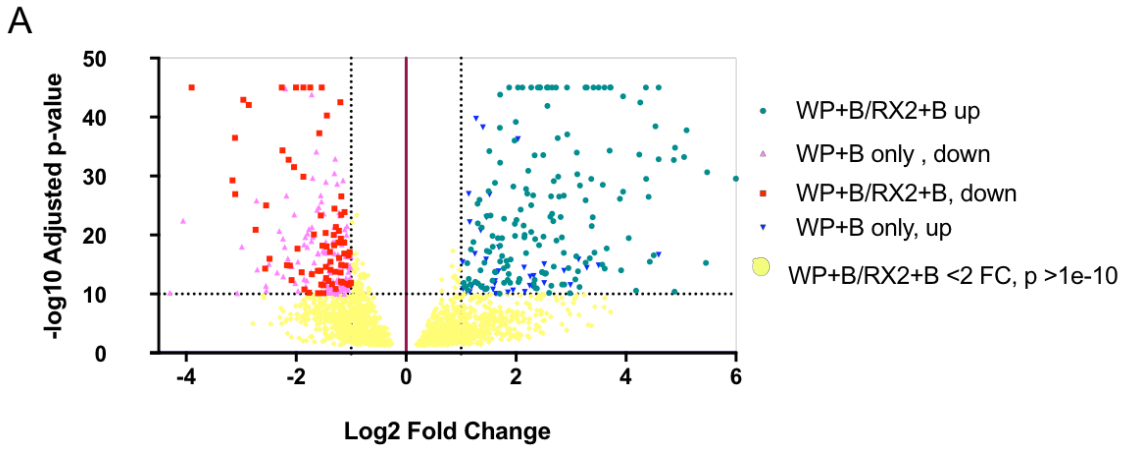

B
Receptors
Neurogenesis
61
11
0
145
5
0
14
2
2
37
6
6
2
9
Epilepsy
9
1
0
128
7
7
11
0
0
3
0
0
0
4
2
0
7
Proliferation
Synaptic Plasticity

Figure 15. Volcano Plot of BDNF regulated genes whose response is reversed by WP+B/RX2+B

(A) Representation of degree of fold change and significance used to narrow down the list of top genes to include in the Figure 14 network. Teal-genes upregulated by both WP+B and RX2+B; Blue: genes upregulated by WP+B only; Red: genes downregulated by both WP+B and RX2+B; Pink: genes downregulated only by WP+B; Yellow: genes that did not meet cutoffs of >2 FC, p<1e-10. Teal and Red gene list used for Figure 3 network. Maximum –log10 p value detected was 45, p values more significant than 45 are represented as –log10 of 45.


A
-log10 Adjusted p-value
50
40
30
20
10
0
-4
-2
0
2
4
6
Log2 Fold Change
WP+B/RX2+B up
WP+B only , down
WP+B/RX2+B, down
WP+B only, up
WP+B/RX2+B <2 FC, p >1e-10

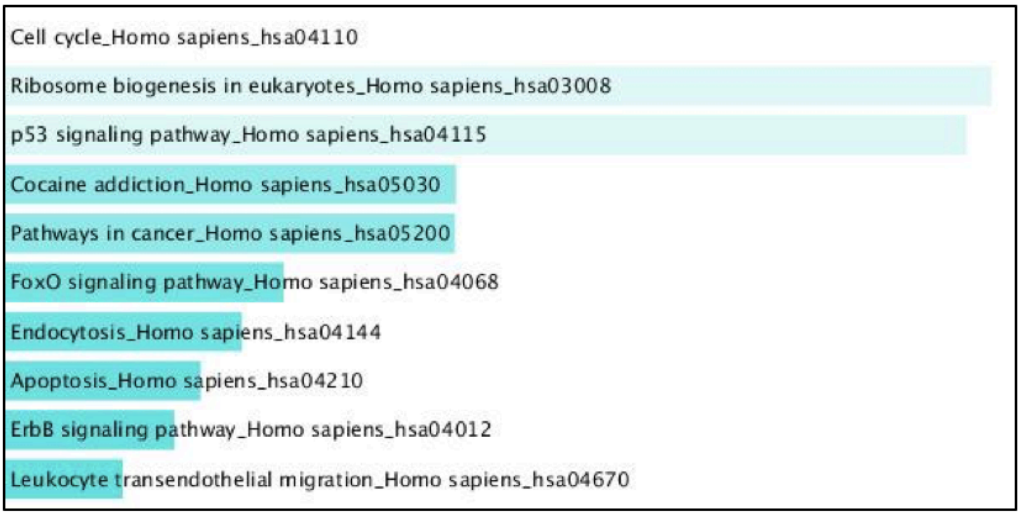
6. DIFFERENTIALLY EXPRESSED GENES EXCLUSIVE TO BDNF/RX2 CONTAIN A RICH SET OF EPILEPSY-ASSOCIATED TARGETS NOT PRESENT IN THE WP1066 EXCLUSIVE LIST

To further dissect the regulatory contribution of the two JAK/STAT inhibitors, we examined the list of DEGs that differed between WP+B and RX2+B treatments. 417 DEGs are reversed by RX2+B treatment that are absent with WP+B and 610 DEGs are reversed by WP+B that are not by RX2+B (Fig. 11A, bottom panel). Interestingly, the genes whose expression is exclusively reversed by RX2+B are significantly related to neurodegeneration, and brain-related movement disorders (i.e., Huntington's disease) as well as to epilepsy. On the other hand, those exclusively regulated by WP+B were not highly associated with epilepsy or other brain disorders. Supplemental Figure 2 contains the EnrichR generated KEGG pathway analysis for this gene set (Figure 16A). 9 of the 10 top related canonical pathways differ from the KEGG list of overlapping WP/RX2 DEGs (Fig. 13B). Top genes involved in the KEGG pathways listed are also shown in a clustergram (Fig. 16B). The list of genes exclusively regulated by RX2+B that are linked to epilepsy, Huntington's disease, and neurodegeneration (E) are also presented (Fig. 16C-E).

Figure 16. Enrichment Analysis of BDNF regulated genes whose expression is reversed exclusively by 10uM Ruxo

Enrichment analysis was performed on the set of 417 DEGs between RX2+B vs. V+B that were not overlapping with WP+B. (A) KEGG pathway analysis as generated in EnrichR ranked and colored by p value. (B) Top gene clusters involved in the KEGG canonical pathways from A. (C-E) Neurological diseases significantly associated with the RX2 exclusive gene set for (C) Epilepsy, (D) Huntington's disease, (E) Neurodegeneration.

A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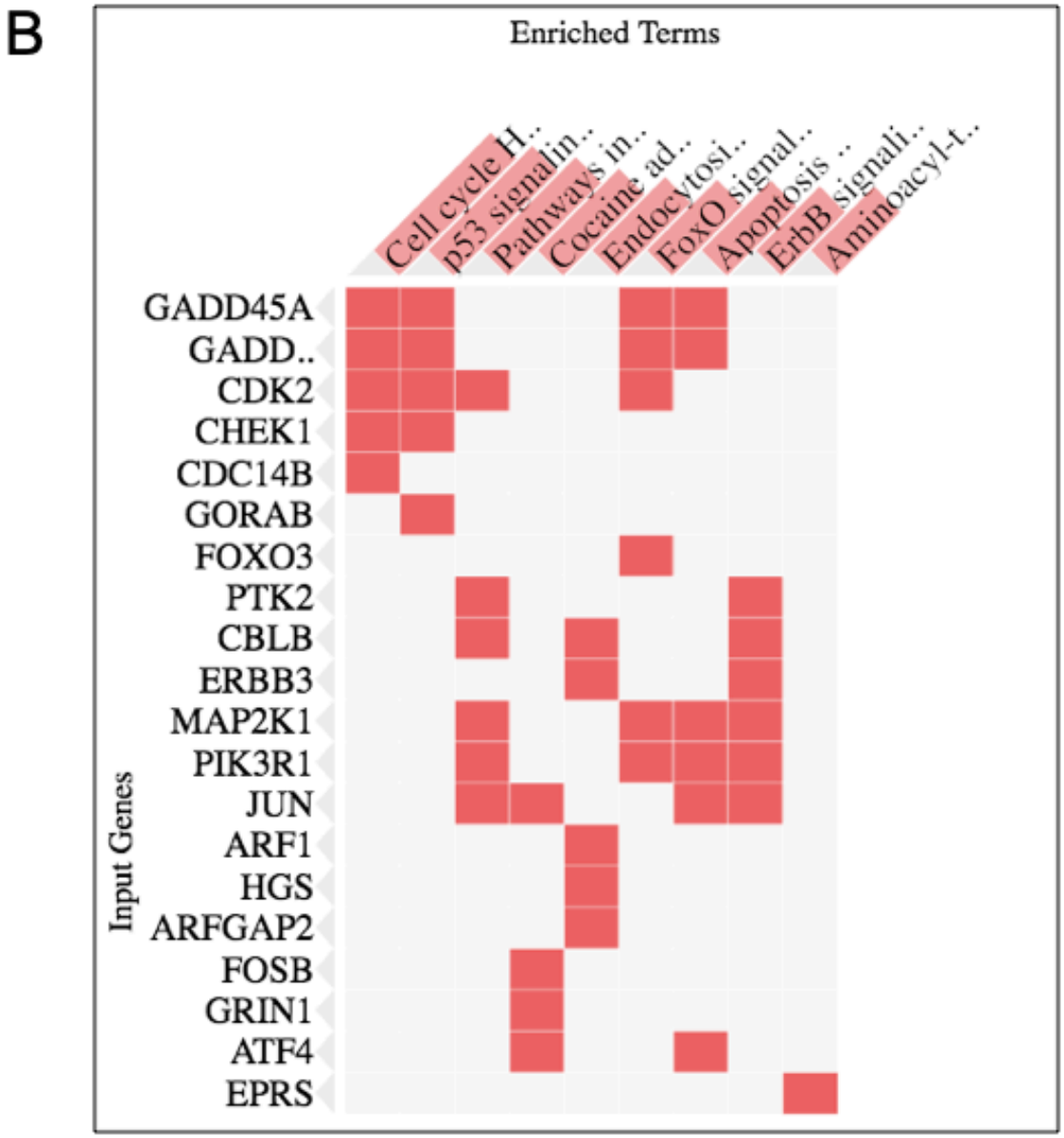
Ribosome biogenesis in eukaryotes_Homo sapiens_hsa03008
p53 signaling pathway_Homo sapiens_hsa04115
Cocaine addiction_Homo sapiens_hsa05030
Pathways in cancer_Homo sapiens_hsa05200
FoxO signaling pathway_Homo sapiens_hsa04068
Endocytosis_Homo sapiens_hsa04144
Apoptosis_Homo sapiens_hsa04210
ErbB signaling pathway_Homo sapiens_hsa04012
Leukocyte transendothelial migration_Homo sapiens_hsa04670


B
Enriched Terms
Cell cycle H..
p53 signalin..
Pathways in..
Cocaine ad..
Endocytosi..
FoxO signal..
Apoptosis ..
ErbB signali..
Aminoacyl-t..
GADD45A
GADD..
CDK2
CHEK1
CDC14B
GORAB
FOXO3
PTK2
CBLB
ERBB3
MAP2K1
PIK3R1
JUN
ARF1
HGS
ARFGAP2
FOSB
GRIN1
ATF4
EPRS
Input Genes

C

Epilepsy	
GENE	FC
EGR4	8.40
EGR1	7.14
RAG1	7.09
PNOC	4.08
MMP9	3.88
GFRA2	3.56
FOSB	3.51
DUSP2	3.06
IER2	2.94
SLC2A1	2.09
JUN	1.93
PNKP	1.83
ADCYAP1	1.79
GADD45G	1.67
PLK3	1.64
ADARB1	1.63
GADD45A	1.47
GRIN1	1.42
PPP1R1B	1.34
RAPGEF3	1.27
MCM6	-0.85
FKBP1A	-0.84
ST8SIA4	-0.70
AKAP5	-0.69
LDLR	-0.67
GABRA1	-0.54
ARRDC3	-0.49
MBNL2	-0.49

D

Huntington's Disease	
GENE	FC
EGR4	8.40
EGR1	7.14
DDIT3	2.75
MLF1	2.13
HDAC5	2.02
JUN	1.93
NGEF	1.81
USP13	1.76
ICAM5	1.65
PRKX	1.49
GADD45A	1.47
PCDH7	1.45
GRIN1	1.42
CHAF1B	1.40
NDRG1	1.38
PPP1R1B	1.34
FBL	1.27
ATP6V1B2	1.26
ENPP5	-0.67
LDLR	-0.67
MAP3K5	-0.65
UGCG	-0.57
HMGCR	-0.57
GABRA1	-0.54
FOXO3	-0.49
MBNL2	-0.49
HBP1	-0.41
ST8SIA2	-0.39

E

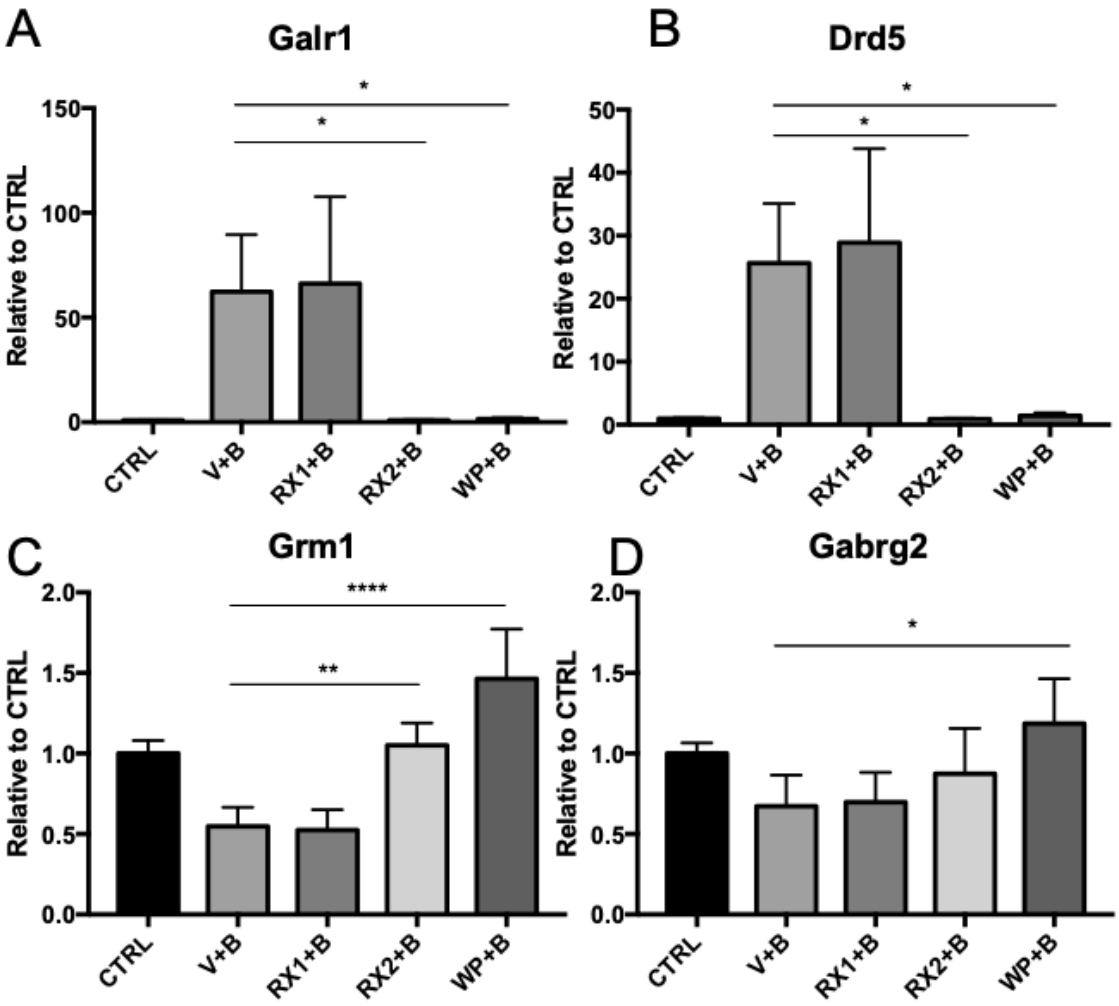
Neurodegeneration	
Gene	FC
EGR1	7.14
CRHR1	3.40
SERPINE1	3.01
DDIT3	2.75
CXCL12	2.38
RASSF5	2.28
SLC2A1	2.09
JUN	1.93
NCS1	1.89
ADCYAP1	1.79
HSF1	1.72
GRIN1	1.42
MAP2K1	1.41
PAWR	1.33
RAPGEF3	1.27
ATF4	-1.24
CDK2	-1.41
CDC25A	-1.47
MAP3K5	-1.54
HDAC9	-1.86
FOXO3	-2.03

7. QUANTITATIVE RT-PCR GENE VALIDATION OF DATASETS

Many of the genes identified in these RNA-seq studies were originally reported by us in the literature (*Icer*, *Gabra1*, *Egr3*), using a variety of molecular approaches (Lund et al. 2008; Grabenstatter et al. 2014; Daniel S. Roberts et al. 2006). To further validate the new datasets, we generated an additional set of cultures, originating from the embryos of multiple pregnant rats, and chose the following genes for qRT-PCR because of their interest in the field of epilepsy: Dopamine Receptor D5 (*Drd5),* Galinin Receptor 1 *(Galr1),* γ2 subunit of the GABAR (*Gabrg2),* Glutamate metabotropic receptor 1 (*Grm1)*, and their relationship to the JAK/STAT pathway: Inducible cAMP early repressor (*Icer),* myeloid leukemia cell differentiation protein *(Mcl1),* Suppressor Of Cytokine Signaling 3 *(Socs3),* Cyclin D1 *(Ccnd1),* and C-X-C Motif Chemokine Receptor 4 *(Cxcr4)* (Fig. 17). In most cases, findings from qRT-PCR were consistent with those of RNA-seq. Quantitated results from select validations are shown (Figure 17A). A results table for all candidates where validation was attempted is included in Figure 17B.

Figure 17. qPCR validation of BDNF regulated genes in presence of JAK/STAT inhibitors

Primary neurons 9 DIV were pretreated with100nM Ruxolitinib (RX1), 10uM Ruxo (RX2), 10uM WP1066 (WP) or DMSO for 1 hr before the addition of BDNF. (A-D) Graphical representation of selected results of real time PCR analysis using Taqman probe and primers. RNA was extracted from cells collected 4 hr after BDNF administration. mRNA levels are shown as the mean values (±SEM) of the ratio relative to DMSO+Water control group for (A) Galr1 n=3, (B) Drd5 n=3, (C) Grm1 n=4, (D) Gabrg2 n=4. (E) Compilation of all genes tested for validation with qPCR represented alphabetically. Values are the ratio relative to DMSO+Water for RNAseq (top row) and qPCR (2nd row) for comparison. Indication of a validating result (3rd row) is marked in green. Results marked with Yes(Y) followed by the level of significance indicate a significant result consistent with RNA-seq. Yes- Not significant (Y-NS) seen in all the RX1 is the expected result consistent with our RNA-seq findings. Measurements that did not reach significance are marked in gray as Not Significant (NS). Significance was tested using One-Way ANOVA followed by Tukey's test for multiple comparisons. (*$p<0.05$, **$p<0.01$, ***$p<0.0005$, ****$p<.0001$).


A
Galr1
Relative to CTRL
150
100
50
0
*
*
CTRL
V+B
RX1+B
RX2+B
WP+B
B
Drd5
Relative to CTRL
50
40
30
20
10
0
*
*
CTRL
V+B
RX1+B
RX2+B
WP+B
C
Grm1
Relative to CTRL
2.0
1.5
1.0
0.5
0.0
****
**
CTRL
V+B
RX1+B
RX2+B
WP+B
D
Gabrg2
Relative to CTRL
2.0
1.5
1.0
0.5
0.0
*
CTRL
V+B
RX1+B
RX2+B
WP+B

E

Gene	Technique	CTRL	V+B	RX1+B	RX2+B	WP+B
Ccnd1	RNAseq	1.00	2.85	2.66	1.09	0.77
	qPCR	1.00	1.62	1.67	0.94	1.10
	Validates		Y-**	Y-NS	Y-**	Y-**
Cxcr4	RNAseq	1.00	0.62	0.77	1.67	0.88
	qPCR	1.00	0.77	0.65	1.82	0.89
	Validates		NS	Y-NS	Y-**	NS
Drd5	RNAseq	1.00	68.25	47.84	1.86	0.39
	qPCR	1.00	25.64	28.90	0.90	1.45
	Validates		Y-*	Y-NS	Y-*	Y-*
Galr1	RNAseq	1.00	93.18	51.53	0.93	0.42
	qPCR	1.00	62.34	66.18	0.98	1.54
	Validates		Y-*	Y-NS	Y-*	Y-*
Gamma2	RNAseq	1.00	0.39	0.34	0.67	0.73
	qPCR	1.00	0.59	0.61	0.74	1.07
	Validates		NS	Y-NS	NS	Y-*
Grm1	RNAseq	1.00	0.35	0.36	1.04	0.98
	qPCR	1.00	0.55	0.52	1.05	1.46
	Validates		Y-**	Y-NS	Y-**	Y-****
Icer	RNAseq	1.00	5.81	4.14	1.92	1.98
	qPCR	1.00	5.23	4.38	0.70	1.08
	Validates		Y-****	Y-NS	Y-****	Y-****
Jak2	RNAseq	1.00	1.78	1.37	0.88	0.67
	qPCR	1.00	1.60	1.44	0.75	1.01
	Validates		Y-*	Y-NS	Y-***	Y-*
Mcl1	RNAseq	1.00	1.27	1.31	0.74	0.69
	qPCR	1.00	1.20	1.09	0.75	0.90
	Validates		Y-*	Y-NS	NS	Y-*
Socs3	RNAseq	1.00	3.34	2.30	0.80	0.76
	qPCR	1.00	3.40	2.44	0.83	1.55
	Validates		Y-**	Y-NS	Y-**	Y-*

8. COMPARISON OF DEGS WITH CHIP-SEQ DATASETS

Using a list of STAT3 target genes identified by chromatin immunoprecipitation (ChIP)-sequencing (ChIP-seq) of glioblastoma cells (J.-X. Zhang et al. 2013), we generated a list of 308 genes from the overlapping WP+B/RX2+B dataset that have been previously shown to have functional binding sites for STAT3 (Table 5). We were particularly interested that *BDNF*, Calcium Voltage-Gated Channel Auxiliary Subunit Beta 4 (*Cacnb4)*, *Gabrg2*, *Grm1, Jak1/2*, and Sodium Voltage-Gated Channel Alpha Subunit 1(*Scn1a)* were on the list. They have all been associated with epilepsy models and with human mutations in epilepsy patients (D K Binder et al. 2001; Escayg et al. 2000; Watson et al. 2017; Claes et al. 2001).

Table 5. ChIP-seq identifies multiple genes in the WP+B/RX2+B overlapping datasets that contain validated STAT3 binding sites

List of all WP+B/RX2+B genes (308) that are validated STAT3 ChIP-seq targets (as determined by Zhang, et.al, 2013) in alphabetical order. Highlighted in yellow are genes of special interest.

STAT3 ChIP seq Targets											
ABI1	BBX	CPNE4	EML1	GPRC5A	KCNE2	MAN2A1	NTRK3	PRKCQ	RYBP	ST18	TXNRD1
ACTN1	BCAS3	CPNE8	EMP1	GRIP1	KCNK2	MAP2K5	NVL	PSME4	SAMD4A	ST6GAL1	UBASH3B
ACTR3B	BDNF	CREBL2	EPB41L4B	GRM1	KCNMB2	MAP2K6	OGDHL	PSTPIP2	SAMHD1	SV2C	ULK2
ACVR1C	BHLHE40	CRHBP	ETV5	GSPT2	KCTD15	MAPK3	OSBPL6	PTPDC1	SATB1	SVOP	UNC5B
ADAP1	BMPER	CRYM	EXO1	HAUS8	KLF2	MAS1	OTUD3	PTPN1	SCN1A	SYT10	USP20
ADCY8	BNIP3L	CUBN	FAM102B	HMGA2	KLF7	MAT2B	PAPPA	PTPRR	SDCCAG8	SYT12	USP28
ADM	BRE	CUX2	FAR1	HMGCLL1	KLHL14	MCCC2	PDE12	PUS7	SDK2	SYT6	USP46
AGFG1	CACNB4	CX3CL1	FBXL14	HS3ST5	KLHL25	MED4	PDK1	QPCT	SFMBT2	SYT9	USP49
AHCYL2	CAMKK1	CYLD	FBXL20	HSD11B1	KLHL36	MEIS2	PDSS2	RAMP3	SGMS1	TAC1	UST
AHI1	CAPN2	DAAM1	FBXO31	HSPA4L	KLHL7	MFSD6	PHACTR3	RASA1	SH3BP5	TBC1D17	UTP20
AIG1	CAV1	DCLK1	FBXO41	HTR7	LASP1	MICAL2	PHF14	RASAL2	SHC4	TEAD1	VCL
AKAP7	CCDC148	DDIT4	FGF13	IFT122	LBH	MINK1	PIK3C3	RASSF8	SIAH3	TEX264	VEGFA
AKT2	CCDC53	DIAPH1	FJX1	IGF2BP2	LHX2	MON2	PLA2G4A	RBAK	SIRT5	THRB	VWC2L
ANK1	CD200	DIP2C	FN3KRP	IL1RAP	LIPG	MRM1	PLCL1	RCAN2	SLC22A1	THSD7A	WASH1
ANKMY2	CDC14A	DISC1	FNDC3B	IL1RL2	LMO2	MYO9B	PLK2	REEP1	SLC25A20	TM4SF1	WDR1
ANKRD34C	CDK5	DLC1	FRMD3	INO80C	LPAR1	MYRIP	PLXNA2	RND1	SLC30A10	TMEM163	WNT7A
ANKRD6	CDKN2AIPNL	DLK1	FSTL5	INPP5D	LPL	MYT1L	PMP22	RNF150	SLC41A2	TMEM184B	WSCD2
ANO4	CHD6	DOCK8	GABRG2	INSC	LRCH1	NAGA	PNMAL1	RNF152	SLC6A17	TMTC4	XKR4
AP3S2	CHN2	DOK5	GADD45B	IRF6	LRFN5	NAMPT	PNRC1	RNF180	SLCO3A1	TNFAIP1	XPO4
APBA2	CHODL	DPF3	GCLC	ITGA11	LRRC20	NCALD	POLE4	RNGTT	SMARCA2	TOX3	ZDHHC14
ARHGAP10	CHRM2	DUSP14	GFPT2	ITGA6	LRRC8B	NCOR2	POLR1A	ROCK2	SMPD3	TPK1	ZMYND8
ARHGEF3	CLDND1	DYNC1I1	GLDN	JAK1	LTBP1	NOG	POU4F2	RPH3A	SNRNP40	TRIB1	
ARIH1	CNTN3	EBF1	GLRA2	JAK2	LUZP1	NOL10	PPHLN1	RPS6KA2	SORBS2	TRPC4	
ATP5SL	COBL	EFR3A	GPATCH2	JAKMIP1	MAML3	NR2F1	PPP2R2B	RPS6KA5	SPAST	TSC22D1	
BBC3	COG3	EHBP1	GPR156	KCNB1	MAN1A1	NRF1	PRKAA1	RTF1	SPHKAP	TTC9	
BBS2	COMMD1	ELAVL4	GPR37	KCND2	MAN1C1	NTM	PRKAR2A	RTN4RL1	SS18	TUBGCP3	

D. DISCUSSION

There is a strong body of evidence suggesting that BDNF signaling through its TrkB receptor contributes in a major way to the development of TLE, as suggested by animal models of epilepsy (McNamara and Scharfman 2012; He et al. 2004) and their potential relationship to human epilepsy brain pathology (Lipovich et al. 2012). BDNF plays a critical role in the cell biological processes that regulate the structural plasticity of dentate granule cells and the enhanced excitation associated with changes in LTP and compromised GABA-mediated inhibition (McNamara and Scharfman 2012; Brooks-Kayal and Russek 2010; D K Binder et al. 2001; Devin K Binder and Scharfman, n.d.). Although the role BDNF plays in brain disorders is better understood now than at the time of its discovery, many key aspects of its complex regulatory programs remain a mystery. We designed our study to gather a comprehensive list of genes whose expression is either upregulated or downregulated by increased exposure to BDNF, as is believed to occur in response to seizures and other brain insults such as traumatic brain injury (Raible et al. 2012).

The use of primary rat neurons treated with recombinant BDNF allows us to pinpoint the effects of BDNF without the background of additional signaling pathways that may be activated in TLE models. Our study provides a comprehensive list of the changes in the transcriptome that occur in response to a relatively short but continuous exposure to BDNF (4 hr) at a concentration of 0.7 nM that is relevant to endogenous levels in neurons that activate TrKB receptors and not p75NTR (unpublished data from our laboratory). Many of the BDNF regulated genes within the list have been previously

identified both in treated cultures and *in vivo* by our laboratory and others. They include *Gabra1,* the inducible cAMP early repressor (*ICER*) (which negatively regulates *Gabra1)* (Lund et al. 2008), *BDNF* itself (Tuvikene et al. 2016), and multiple ion channel genes, as well as genes that code for important markers of neurogenesis and cell proliferation (Boles et al. 2014; Qiao et al. 2017).

In addition to being consistent with the findings in the literature, our results reveal new BDNF-induced gene targets that are linked to epilepsy. Continuous exposure to BDNF alters the expression of 194 epilepsy-linked genes identified by IPA. The following genes are of particular interest to us because of their marked level of transcriptional alteration and their involvement in epilepsy:

- Dopamine receptor D5 (*Drd5)- A* D1-type dopamine receptor whose knockdown has been shown to be protective against seizures (O'Sullivan et al. 2008). In our dataset, the neuronal expression of Drd5 increases nearly 15-fold in response to BDNF treatment.
- Galinin receptor 1 (*Galr1*)- Galanin is a neuropeptide in the brain that inhibits glutamate release and galanin agonists delivered to the brain can inhibit seizures (Mazarati 2004). Our dataset reveals a 35-fold increase in *Galr1* expression*, de*monstrating the complex medley of both protective and pathogenic changes in genome expression that are invoked by increasing levels of neuronal BDNF.
- Glutamate metabotropic receptor 1 (*Grm1*)- *Grm1* has been associated with the alterations in synaptic plasticity and glutamate disturbances that

result in neuronal overexcitation, a hallmark of epilepsy (Ichise et al. 2000; Watson et al. 2017). *Grm1* mutations can also cause spinocerebellar ataxia type 44, a genetic disease that can present with seizures. In our dataset, BDNF treatment led to a 2.45-fold increase in the expression of *Grm1*.

- γ2 subunit of the GABAR (*Gabrg2*)- the *Gabrg2* subunit of GABAR is important for benzodiazepine action and mutations lead to epilepsy (Audenaert et al. 2006; Chou et al. 2007; Kananura et al. 2002; Bianchi et al. 2002). In this study, RNA-seq revealed a 2.38-fold decrease in the expression of *Gabrg2* in response to BDNF.

Our new results suggest that sustained levels of BDNF at endogenous concentrations (0.7 nM) can dysregulate a major segment of the epilepsy-associated transcriptome. Recent discovery of the role of BDNF in cancer suggests that understanding how BDNF orchestrates its diverse effects on genome dysregulation will also provide a new window on cancer chemotherapies (Radin and Patel 2017; Yin et al. 2015; S. Zhang et al. 2016). Surprisingly, beyond its activation of downstream targets like the cAMP regulatory element binding (CREB) protein, whose relationship to BDNF has been rigorously described (Finkbeiner et al. 1997; Tao et al. 1998), we still know little about the role of other BDNF controlled transcriptional regulators and their intracellular signaling partners that contribute to disease.

Using the TLE model of epilepsy, we previously showed that during the latent period after pilocarpine-induced SE, there is a marked upregulation in activated CREB

and an induction in the expression of the inducible cAMP early repressor (ICER) (Lund et al. 2008). We also reported that activated CREB interacts with ICER to downregulate the levels of *Gabra1* transcripts leading to a decrease in α1-containing GABARs in hippocampal neurons. In our studies, we discovered that BDNF was linked to the JAK/STAT pathway as 1) STAT3 knockdown blocked ICER induction in response to BDNF, 2) STAT3 was found on the ICER promoter in response to BDNF treatment (as measured by chromatin immunoprecipitation (ChIP), 3) JAK/STAT inhibitors (pyridone 6 and WP1066) blocked ICER induction and *Gabra1* downregulation, and 4) *in vivo* application of WP1066 at the time of pilocarpine-induced SE reduced the frequency of subsequent spontaneous seizure animals from pilocarpine-induced spontaneous seizures after SE (Grabenstatter et al. 2014).

Based on these findings, we asked whether the BDNF transcriptome, as identified in our prior studies, could be partitioned into those where the BDNF response could be blocked by exposure to JAK/STAT inhibitors (Fig. 10). We chose two inhibitors, WP1066 (a JAK2 inhibitor with diverse mechanisms of inhibition (Verstovsek et al. 2008)), that we showed was active in primary cultures and *in vivo*, and Ruxolitinib (Ruxo), a high-affinity JAK1/2 inhibitor that blocks JAK activation and is currently used in the treatment of patients with high-risk myelofibrosis. To our surprise, when we compared the transcriptome of cells exposed to BDNF, and to low dose Ruxo plus BDNF (RX1,100nM+B), we did not see a significant difference between the two datasets. As we show in Figure 12, this was not due to any issue with the ability of the stock drug to block STAT3 phosphorylation. Phosphorylation-independent STAT3 activity, however, has

been described in multiple contexts (Jinbo Yang et al. 2007; S. Shi et al. 2008) suggesting that JAK/STAT signaling in neurons may act in a non-canonical fashion, at least when activated by prolonged BDNF signaling as studied in our research. A role for unphosphorylated STATs in genome stability and for nuclear JAK2 in the regulation of histone 3 Y41 phosphorylation has been described (M. A. Dawson et al. 2009; Zouein, Duhé, and Booz 2011) and is the subject of our future studies.

Despite the lack of response of the transcriptome to BDNF in the presence of the high affinity concentration of Ruxo (RX1, 100nM), WP1066, and a ten-fold increase in Ruxo (RX2, 10μM) produced substantial changes in gene expression (Fig. 11A). By comparing the two datasets (WP1066+B and RX2+B) we were able to decrease the dimensionality of the BDNF transcriptome into those most relevant by their association with epilepsy. In fact, using enrichment analysis, we discovered an association with epilepsy that is more pronounced than any other neurological disease, due to the large proportion of differentially expressed epilepsy-associated genes present within the WP+B and RX2+B subset (68%) (Table 6).

Table 6. Top Neurological disease relationships determined by IPA

(A) Top 10 significantly enriched neurological diseases in the BDNF transcriptome with their p-value (IPA). (B) Top 10 significantly enriched neurological diseases in the WP/RX2 vs. BDNF set of differentially expressed genes listed with their p-value from IPA.

A

TOP 10 Neurological diseases: V+B vs. CTRL	
Disease	**P-value**
Seizure disorder	3.29E-27
Seizures	6.75E-27
Epileptic seizure	7.44E-26
Epilepsy	2.91E-24
Neurological signs	1.55E-16
Dyskinesia	1.57E-14
Motor dysfunction or movement disorder	3.75E-14
Abnormal morphology of nervous system	5.14E-14
Movement Disorders	6.48E-14
Huntington's Disease	1.17E-12

B

TOP 10 Neurological diseases: WP+B/RX2+B reversal of BDNF	
Disease	**P-value**
Epileptic seizure	7.32E-23
Seizures	3.82E-22
Epilepsy	7.57E-22
Seizure disorder	1.24E-21
Neurological signs	8.91E-13
Motor dysfunction or movement disorder	2.12E-11
Movement Disorders	5.01E-11
Dyskinesia	1.67E-10
Huntington's Disease	6.53E-10
Abnormal morphology of nervous system	9.99E-10

Pathway analysis also revealed that JAK/STAT inhibition had a marked effect on reversing the BDNF-induced transcriptional response for genes organized in pathways that control neurogenesis, neuroinflammation, synaptic plasticity, receptor expression, and synaptic transmission. Alterations in these functions exist in the epileptogenic brain, which is known to have altered neurogenesis, mossy-fiber sprouting, inflammation and neuronal pruning leading to increased plasticity, and altered excitation and inhibition at the synapse (Annamaria Vezzani et al. 2011; Mathern et al. 1996; Sutula et al. 1989). They also expand upon the recent findings that JAK/STAT signaling plays an important role in learning and memory (Céline S. Nicolas et al. 2012).

Given that multiple epilepsy-associated genes have been shown to have functional binding sites for STAT3, including BDNF itself (Table 5), and considering the substantial relationship between prolonged exposure to BDNF and the ability of JAK/STAT inhibitors to reverse the differential expression of epilepsy associated genes in our studies, we hypothesize that identifying the mechanism that connects BDNF signaling to this novel non-canonical role of JAK/STATs in neurons has the potential to reveal new strategies in the treatment of intractable epilepsy for human patients and provide a new window on how neuropharmacology can be used to identify the complex cross-talk between different intracellular signaling pathways.

E. CONCLUSION

We have employed RNA-seq to determine the effect of BDNF in neurons, and using pharmacological inhibitors, have identified which of those changes may be mediated by the JAK/STAT pathway. Our results show the impact of JAK inhibitors in

preventing BDNF-regulated changes of many epilepsy related genes. The BDNF-induced JAK/STAT gene set also reveals significant enrichment for genes involved in synaptic transmission, particularly ion channels and membrane receptors. Additionally, the pathway plays a significant role in other functions known to be involved in epileptogenesis including synaptic plasticity, neurogenesis, transcriptional regulation, neuroinflammation and proliferation. Some of the JAK inhibitor-regulated genes are known STAT targets, while others are not, and dephosphorylation of STAT at Tyr705 may not control the observed changes in gene transcription. This finding suggests that the mechanism of BDNF-induced JAK/STAT signaling is non-canonical. Most importantly, the RNA-seq datasets highlight the importance of the JAK/STAT pathway in acquired epilepsies and validate the JAK/STATs as novel therapeutic targets that could modify the course of the disease.

Table 7. Genes of interest in the dataset and their relationship to epilepsy

(A) Several genes of particular interest to the field of epilepsy are listed with the degree of FC from DMSO+Water vs. DMSO+BDNF and the FC of DMSO+BDNF vs. WP1066+BDNF. Many have not been associated with the JAK/STAT pathway or BDNF regulation. The name of the gene, the related function, and a reference to support the potential relationship with epilepsy is listed.

Relationship of Epilepsy Genes with JAK/STAT Inhibition					
Gene of Interest	FC V+B	FC WP+B	Name	Function	References
GALR1	34.36	-115.70	Galinin receptor 1	Protective in Epilepsy	Mazarati, 2004
BHLHE22	-5.1	7.78	Basic Helix Loop Helix E 22	Repressor involved in neurodevelopment	Joshi, 2008
SOCS3	2.89	-3.29	Suppressor of Cytokine Signaling 3	Inhibits JAK signaling, increased in epilepsy	Pernot, 2011
DRD5	14.91	-63.92	Dopamine Receptor 5	Knockout protects from seizures	O'Sullivan, 2008
INHBA	5.26	-5.27	Inhibin Beta A	Reduces excitotoxicity	Lau, 2015
NPTX1	5.01	-6.30	Neuronal Pentraxin 1	Plays role in epileptogenic synaptic remodeling	Sanabria, 2008
GRIN2A	2.18	-8.80	Glutamate Ionotropic Receptor NMDA Type Subunit 2A	Mutations cause epilepsy, involved in LTP	Carvill, 2013
CXCR4	-1.53	1.77	CXC Motif Chemokine Receptor 4	Inhibition protects against epilepsy	Song, 2016
HRH3	-3.93	6.53	Histamine H3 Receptor	Increased Histamine confers protection in epilepsy	Bhowmik,2012
RGS4	4.46	-2.77	Regulator of G protein signaling 4	Inhibition protects against epilepsy	Chen, 2012
SH2D3C	-11.46	14.91	SH2 Domain Containing 3C	Involved in cell migration	Wang, 2012
ELAVL4	-2.04	2.76	ELAV Like RNA Binding Protein 4	Controls mRNA processing, increased in Epilepsy models	Bronicki, 2013
FYN	1.35	NA	FYN Proto-Oncogene, Src Family Tyrosine Kinase	Overactivity leads to epilepsy, activates mTORC1 pathway	Kojima, 1996; Wang, 2015
RPS6K(A2, A3, A5, A6, B1, B2)	A2--2.04	A2-2.59	Ribosomal Protein S6 Kinase	mTOR activated, involved in proliferation and cell growth	Cho, 2011
ARC	33.24	-15.04	Activity Regulated Cytoskeleton Associated Protein	Known to be seizure and BDNF induced, plasticity	Kulpers, 2016
BDNF	3.23	-2.90	Brain Derived Neurotrophic Factor	Increased in epilepsy, neuroplasticity	Binder, 2001
JAK2	1.79	-2.11	Janus Kinase 2	Activated by BDNF and Status Epil.	Lund, 2008
EGR3	5.57	-6.08	Early Growth Response 3	Regulates α4 subunit $GABA_AR$	Roberts et al, 2006

CHAPTER IV: NEURONAL STAT3 REGULATES THE SPHINGOLIPID METABOLISM NETWORK IN THE INTRAHIPPOCAMPAL KAINIC ACID-INDUCED SE MOUSE MODEL OF TLE

A. ABSTRACT

Temporal lobe epilepsy (TLE) develops after a period of ongoing molecular cascades and neural circuit remodeling in the hippocampus resulting in increased susceptibility to spontaneous seizures. Targeting these cascades in TLE patients could reverse their symptoms and have the potential to provide a viable disease-modifying treatment, especially for the large portion of over 30% of TLE patients who do not respond to any available treatments. In recent years, the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway has been implicated in temporal lobe epilepsy (TLE). The JAK/STAT pathway is known to be involved in inflammation and immunity, and only more recently has been shown to be associated with neuronal functions such as synaptic plasticity. When a JAK inhibitor, WP1066, was administered to a rat Pilocarpine Status Epilepticus (SE) model of TLE at the time of SE, it greatly reduced the number of spontaneous seizures those animals went on to develop over time, as well as altered gene expression of key genes associated with epilepsy, including the α1 subunit of the GABAA receptor (GABAR). In this study, we continue to investigate the mechanism of potential JAK/STAT-induced epileptogenic responses by performing deep RNAseq transcriptomic analysis of a Kainic Acid (KA) epilepsy model that expresses neuron-specific Stat3 knockdown (KD) in a transgenic line using tamoxifen-induced CRE expression. Here we show the transcriptomic signature of intrahippocampal KA

injections in both the side of the hippocampus ipsilateral to the injection, and the contralateral hippocampus, identifying and comparing the transcriptome altered by KA in both sides of the brain. Our results indicate that STAT3 knockdown protects many genes from KA-induced changes in gene expression associated with epilepsy, demonstrating a role for STAT3 in the response to status epilepticus, particularly in genes important for sphingolipid metabolism. Lastly, we summarize the differential expression caused by STAT3 neuronal knockdown in saline treated animals without SE to determine the role of STAT3 in a non-pathological hippocampus. The emerging transcriptome for neuronal Stat3 in the context of epilepsy models suggests that it may be useful for identifying potential epileptogenic gene networks that were previously unknown, selecting early-detection biomarkers that inform seizure susceptibility, as well as choosing new targets for the future treatment of intractable epilepsies.

B. INTRODUCTION

Temporal lobe epilepsy (TLE) is the most common form of focal epilepsy, and it affects approximately 615,600 people in the United States (Asadi-Pooya et al. 2017). Development of TLE follows a phase of epileptogenesis during which molecular cascades and morphological changes occur in the hippocampus which cause cell death, axonal sprouting, altered synaptic transmission, and inflammation that result in brains overly susceptible to seizure activity (Paul S. Buckmaster 2012; Jack M. Parent and Kron 2012; N C de Lanerolle et al. 1989). Although available epilepsy medications can control seizures in around 65% of patients, no available therapeutics target epileptogenic

processes and thus are unable to modify the course of the disease. Additionally, over a third of TLE patients are refractory to medications that control seizures.

In order to elucidate the signaling pathways and circuitry changes which progress during the epileptogenic phase, the kainic-acid (KA) intrahippocampal injection (IHKA) model of TLE has emerged as a promising tool that mimics temporal lobe damage, particularly inflammatory damage, similar to human patients. Kainic Acid, a convulsant agent, is used to induce status epilepticus (SE). SE is a severe, generalized seizure of long duration. Spontaneous seizures develop weeks later in many of these animals. Ongoing changes during the latent period prior to the onset of seizures can be investigated to understand the effect of SE in circuitry reorganization (Lévesque and Avoli 2013).

Documented alterations that occur in the IHKA model include pyramidal cell loss, granule cell dispersion, recurrent spontaneous seizures, mossy fiber sprouting, and astrogliosis in the hippocampus (Bouilleret et al. 1999). Degeneration and seizures are seen in both hippocampi despite injection being to a single hippocampus (Arabadzisz et al. 2005; Bielefeld et al. 2017; Miltiadous et al. 2013). Altered sphingolipid metabolism including increased ceramides and sphingomyelinases is also a documented effect of IHKA (Guan et al. 2006; Lee et al. 2010; Heischmann et al. 2016; Mikati et al. 2008) as well as linked to human epilepsy (Mosbech et al. 2014).

In a similar TLE rodent model, the Pilocarpine-induced SE model, a JAK/STAT inhibitor (WP1066) has previously been successfully tested, demonstrating a protective effect by reducing the number of spontaneous seizures after the latent period (Grabenstatter et al. 2014). Research from our laboratory has demonstrated that activation

of the JAK/STAT pathway in neurons can be regulated by the neurotrophin BDNF and results in the expression of the inducible cAMP early repressor (ICER) via STAT3-mediated gene regulation. Moreover, we show that ICER represses the gene (*Gabra1/GABRA1*) coding for the α1 subunit of the type A γ-aminobutyric acid (GABA) receptor (GABAR) and such repression reduces the number of α1-containing GABARs in neurons (Lavoie et al. 1997). Most importantly, reduced α1 subunit gene expression also occurs in epilepsy patients (Brooks-Kayal et al. 1998) as well as in other disorders of the nervous system (Petryshen et al. 2005; Raud et al. 2009; Bossers et al. 2010).

JAK/STATs have also been linked to synaptic plasticity, especially in NMDA-receptor dependent long-term depression (LTD) in the hippocampus at Schaffer collateral-CA1 synapses and temporoammonic-CA1 synapses (Celine S Nicolas et al. 2013; McGregor, Irving, and Harvey 2017). We hypothesize this alteration of synaptic transmission may also contribute to epileptogenesis.

In order to determine the role of the JAK/STAT pathway in the development of epileptogenic changes at the molecular level using the IHKA model, we developed an excitatory neuron-specific STAT3 transgenic inducible knockdown mouse and exposed it to intrahippocampal Kainic Acid injections before transcriptomic characterization. Here, we profile the transcriptomic signature of IHKA injections in wild-type mice in both the side of the hippocampus ipsilateral to the injection, and the contralateral hippocampus, identifying and comparing all the genes altered by KA in both sides of the brain. Additionally, our results show STAT3 knockdown protects many genes from KA-induced gene alteration, demonstrating a role for STAT3 in the early response to status

epilepticus, particularly in genes important for regulating sphingolipid metabolism, an essential component of the trafficking, stability, and function of multiple membrane bound receptors, including ligand- and voltage-gated ion channels. Lastly, we summarize the differential expression caused by STAT3 neuronal knockdown in saline treated animals without SE to determine the role of STAT3 in a non-pathological hippocampus.

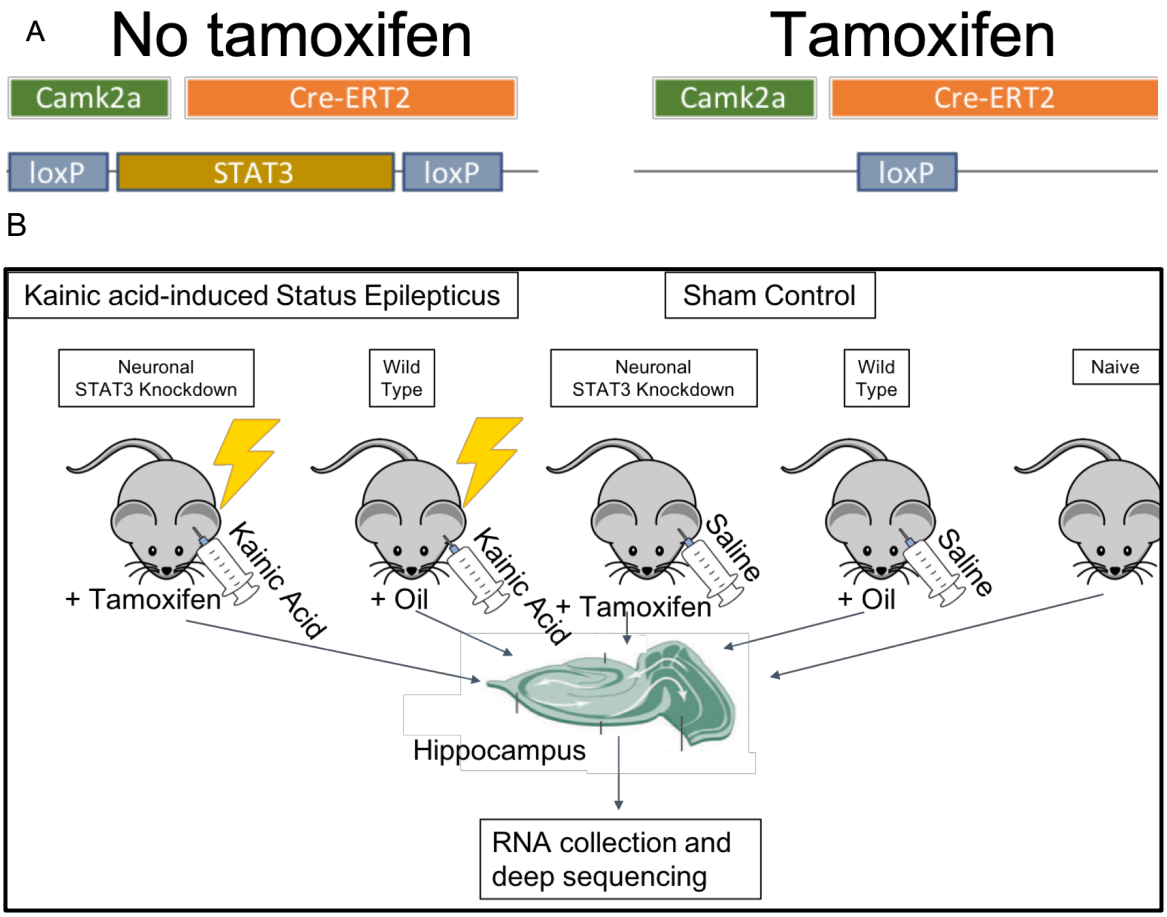
C. RESULTS

1. EFFECT OF KAINIC ACID INJECTION ON THE TRANSCRIPTOME OF THE IPSILATERAL HIPPOCAMPUS

STAT3 neuronal knockdown (KD) under the CamK2a promoter was induced with tamoxifen treatment in mice. In addition, both wild type (WT) and knockdown (KD) mice were treated with a intrahippocampal injection of KA or saline to investigate the effects of status epilepticus (SE) on gene expression and to understand the role of the STAT3 protein, as expressed in neurons, in their response to injury. A day after the KA injection, both ipsilateral (ips) and contralateral (contra) hippocampi were collected for RNA extraction and library preparation (see Figure 18).

Figure 18. Experimental design of STAT3 knockdown and Kainic Acid-induced status epilepticus.

A) Tissue specific Tamoxifen-induced Cre-loxP mice were created using the neuronal-specific Camk2a promoter. Tamoxifen in oil or oil alone intraperitoneal administration every day for five days was done to induce recombination and remove Stat3 expression in neurons. B) Intrahippocampal injection of Kainic Acid, a chemical convulsant, was administered to the right hippocampus in order to induce Status Epilepticus in the knockdown and WT mice, control groups were administered a saline solution. The right (ipsilateral) and left (contralateral) hippocampus were harvested from each animal and RNA was analyzed using RNAseq, comparing each of the five experimental groups.


A
No tamoxifen
Tamoxifen
Camk2a
Cre-ERT2
Camk2a
Cre-ERT2
loxP
STAT3
loxP
loxP
B
Kainic acid-induced Status Epilepticus
Sham Control
Neuronal STAT3 Knockdown
Wild Type
Neuronal STAT3 Knockdown
Wild Type
Naive
+ Tamoxifen
Kainic Acid
+ Oil
Kainic Acid
+ Tamoxifen
Saline
+ Oil
Saline
Hippocampus
RNA collection and deep sequencing

We first examined the differential change in expression that occurred in the ipsilateral hippocampus of WT animals in those administered KA or saline injection. 502 protein-coding genes were found to be differentially expressed (FDR <.05). Ingenuity pathway analysis (IPA) revealed that 20 of these genes are known to be linked to epilepsy (Figure 19A). KEGG pathway and gene ontology analysis highlight the disruption of sphingolipid metabolism caused selectively by KA injection (Figure 19B-C). Genes involved in this pathway include Klotho Beta (*Klb*), Phosphatidylinositol-4-Phosphate 3-Kinase Catalytic Subunit Type 2 Alpha (*Pik3c2a*), Protein Phosphatase, Mg2+/Mn2+ Dependent 1L (*Ppm1l*), Protein Phosphatase 2 Scaffold Subunit Abeta (*Ppp2r1b*), Sphingomyelin Phosphodiesterase 4 (*Smpd4*), Sphingosine Kinase 1 and 2 (*Sphk1* and *Sphk2*) (See figure 19).

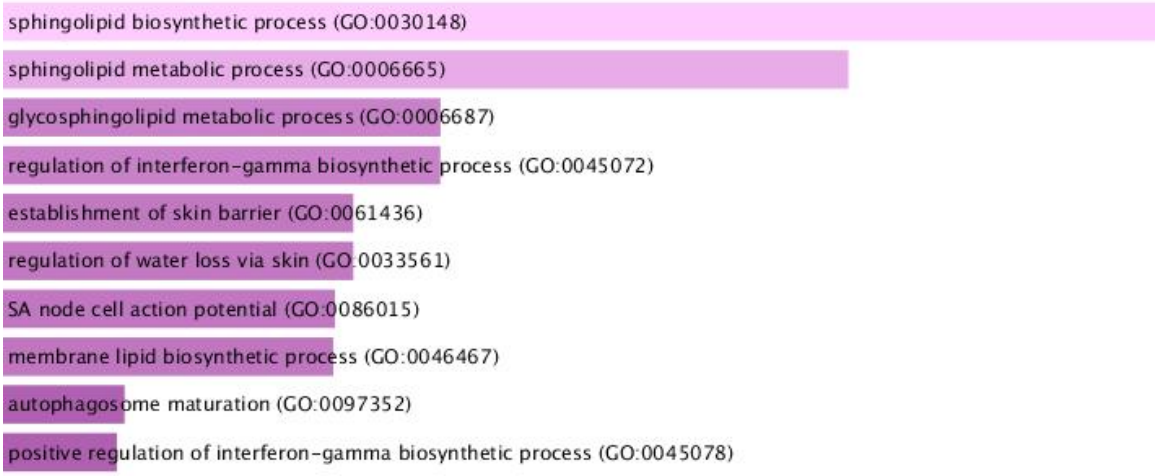
Figure 19. Effect of Kainic Acid on the ipsilateral hippocampus of WT mice

A.) List of genes identified by IPA as related to epilepsy. All 20 were found in the KA v Saline analysis in WT mice. Those highlighted in yellow were protected by STAT3 KD. B.) Gene Ontology Biological Processes from EnrichR. Ordered by p-value. All colored ones are significant $p < 0.05$. C.) KEGG pathway analysis done in EnrichR of gene set. Ordered by p-value. All colored genes are significant $p < 0.05$.

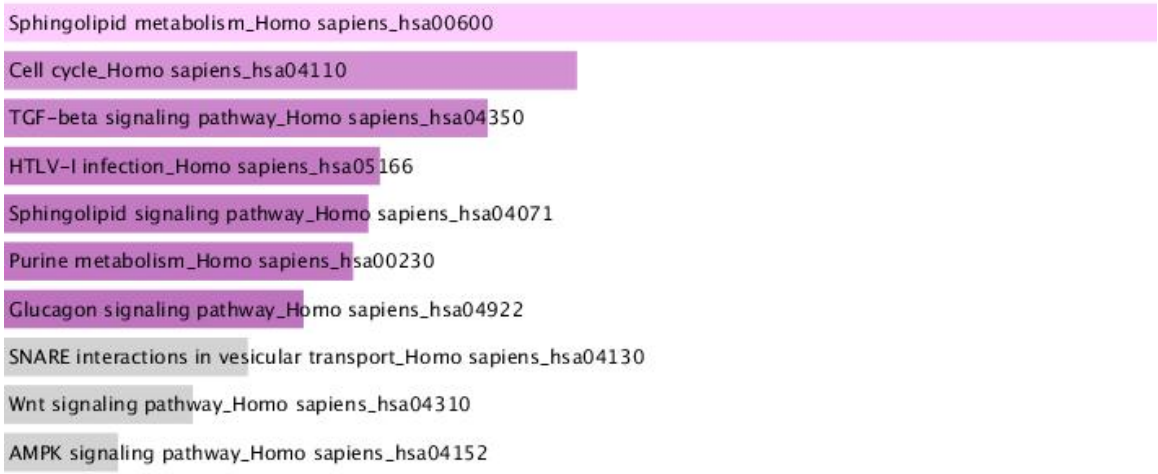
A.

GENE	FC	GENE	FC
SLC6A13	68.64	CHRNA4	4.38
DCX	27.40	PDE4DIP	3.87
TIPARP	15.14	SCG2	3.81
MCM6	14.99	SLC6A12	2.96
GOSR2	10.45	SCN3B	1.74
C12orf57	8.15	JUNB	-2.97
POLG	7.01	MYC	-5.18
NPTX2	5.78	Pou3f1	-8.57
ABCC2	4.93	INHBA	-8.69
CLN3	4.62	HTR1A	-12.34

B. Ipsilateral GO: Biological Process



C. Ipsilateral KEGG 2016 pathway analysis



2. EFFECT OF KAINIC ACID INJECTION ON CONTRALATERAL HIPPOCAMPUS

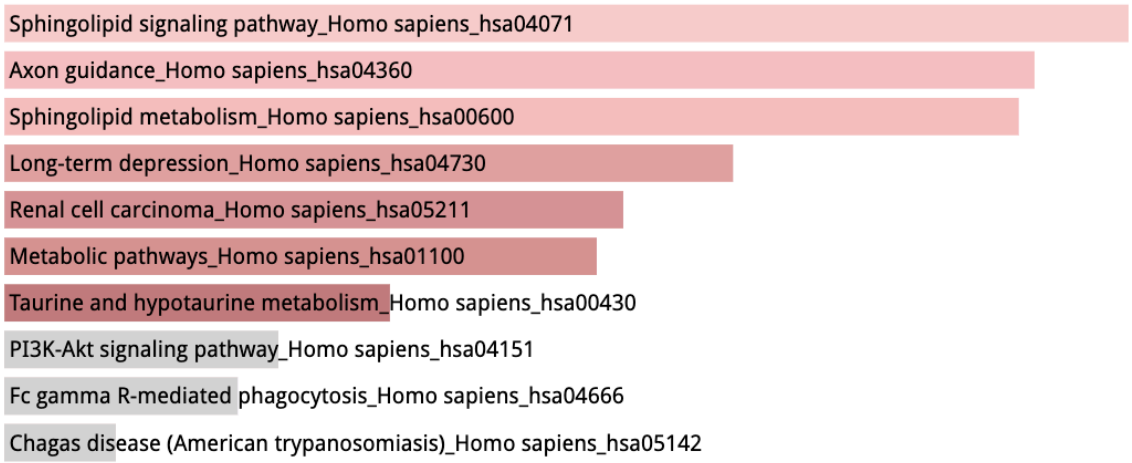
Due to the toxic effects of KA on neuronal survival, some molecular responses to the KA injection on the ipsilateral side may be more related to gene expression changes that occur prior to SE and not the process of disease that occurs after SE. To determine the gene set most likely associated with epilepsy progression, we next examined the contralateral hippocampus which also displays SE and goes on to produce spontaneous seizures after a characteristic latent period (*in vivo* EEG monitoring was performed in the Brooks-Kayal laboratory, UColorado, Denver). Degeneration on the contralateral side, while present, was much less severe than that seen on the ipsilateral side.

In the contralateral hippocampus, the differential effect of KA versus saline injection resulted in 82 differentially expressed protein-coding genes between the two groups, WT and KA. KEGG analysis again revealed that the top candidate genes were in the gene network of sphingolipid metabolism, followed by axon guidance and long-term depression (previously associated with JAK/STAT signaling) (see Figure 20).

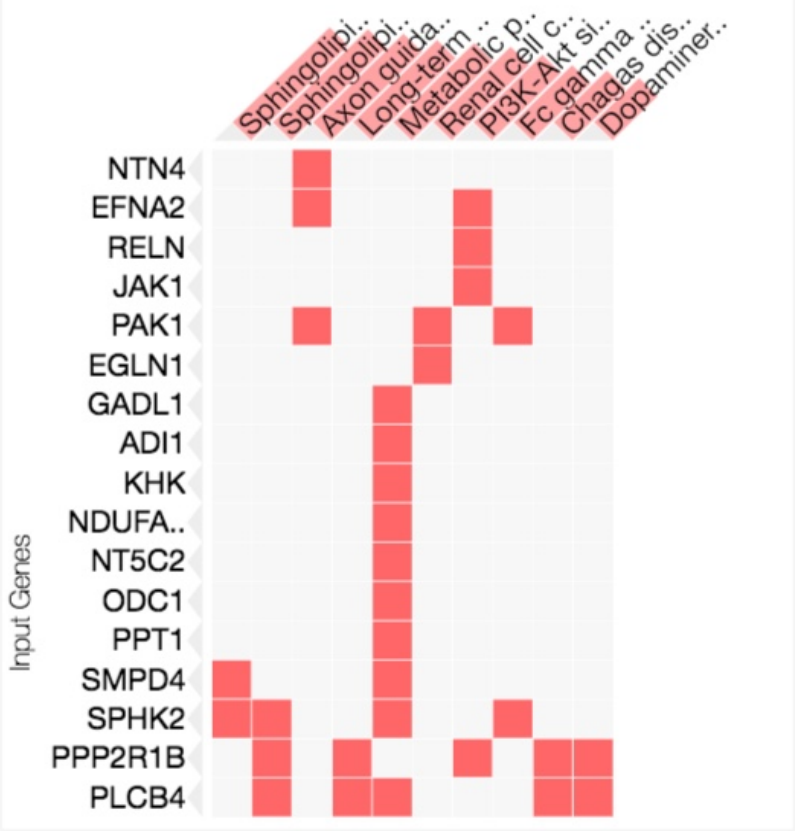
Figure 20. Effect of Kainic Acid in WT animals in the contralateral hippocampus

A.) Contralateral hippocampal KEGG pathway analysis from EnrichR. Ordered by p-value. All colored genes are significant $p < 0.05$. B.) Gene clustering of the pathways using KEGG (A) specification of the genes specific to each pathway. C.) Compilation of all genes altered by KA treatment in both hemispheres of the hippocampus with accompanying fold change.

A. Contralateral KEGG pathway analysis



B. Gene Clustering of KEGG pathway analysis



C. Bilateral Gene changes from KA v Saline in WT animals

Gene Symbol	Ips FC	Contra FC
Acp1	4.67	2.82
Arl5c	5.55	2.80
Art3	29.66	3.60
BC024978	1.91	1.71
Capn12	1.75	1.69
Fam151a	-2.55	-2.28
Jak1	-2.95	-4.45
Khk	8.60	12.60
Letm2	2.93	1.99
Lrp3	-3.08	-1.97
Mcmdc2	2.37	1.71
Mid1	7.01	7.62
Nat8f1	-13.23	-5.12
Nudt6	2.53	3.31
Oraov1	1.85	2.91
Ovca2	6.59	8.10
Plppr4	4.74	3.69
Ppp2r1b	1.88	1.78
Rtl5	-2.31	-2.10
Smpd4	2.64	2.24
Sphk2	-3.96	-3.94
Spopl	1.59	1.40
Tmem132a	3.40	2.19
Tti2	1.60	1.43
Tulp4	1.79	1.59
Zeb2os	-1.94	-2.25

3. BILATERAL EFFECTS OF KAINIC ACID INJECTION

Twenty-five of the gene targets in neurons are consistent bilaterally, suggesting that these genes are most closely related to SE and not the non-specific KA toxicity. See Figure 20C for a list of all genes and their differential responses. Of considerable interest are: *Jak1* (involved in the JAK/STAT pathway), *Smpd4* and *Sphk2* (involved in sphingolipid signaling), and *Art3* (ADP-ribosyltranferase 3 that is increased after TBI and can regulate the inflammatory response) (W. Shi et al. 2012).

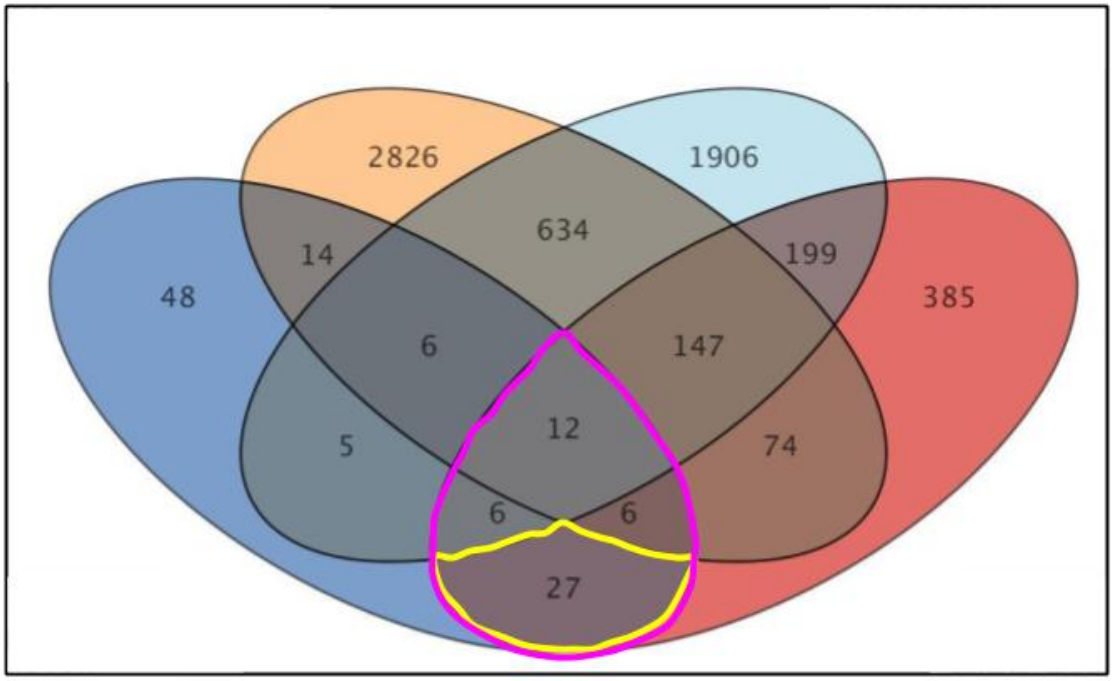
4. STAT3 NEURONAL KNOCKDOWN PROTECTS FROM KAINIC-ACID INDUCED DIFFERENTIAL GENE REGULATION

Comparison of the neuronal STAT3 KD mice to WT mice, with and without KA treatment, uncovered what is most likely a collection of gene targets specifically regulated by STAT3 in neurons. To determine which alterations in the genome are most dependent upon STAT3-mediated transcription, we identified a set of genes where STAT3 knockdown prevents the change in expression that previously occurred in response to KA. This list of targets is composed of genes that are significantly different in WT mice between KA and saline, and do not have a greater than 2-fold change in STAT3 KD KA mice when compared to WT saline mice. For ips hippocampi, the differential expression of 251 genes were protected by STAT3 KD. For the KA-induced gene alteration on the ips side linked to epilepsy, nearly half of these genes were protected by STAT3 KD in neurons, demonstrating an important role for STAT3 in the neuronal response to KA-induced SE and overall toxicity (indicated in yellow in Figure 19). For the contralateral hippocampi, 60 genes were protected from KA by KD of STAT3 in

neurons. Sixteen of the 25 (64%) genes which were altered by KA bilaterally were also protected by STAT3 knockdown bilaterally, indicating an important role for STAT3 signaling in the response to SE as seen in both hemispheres of the hippocampus. The overlapping genes on these lists are listed in Figure 21B.

Figure 21. STAT3 KD Reverses Differential Responses to KA

A) Venn diagram of ipsilateral (ips) and contralateral (contra) KD KA vs WT Saline, and WT KA vs WT Saline, indicates the portion of genes whose alteration is seen in both ips and contra hippocampi upon KA injection as well as those genes where KD of STAT3, specifically in neurons attenuates the KA-induced alterations in transcription. Purple outline denotes the overlapping gene set in ips and contra of KA vs Saline in WT mice and Yellow outline denotes the overlapping gene set in ips and contra of genes protected by STAT3 KD from KA-induced changes in expression., B) List of Genes in the yellow outline, C) mRNA expression levels of genes Sphingomyelin Phosphodiesterase 4 (*Smpd4*), Cholinergic Receptor Nicotinic Alpha 4 Subunit (*Chrna4*), Klotho Beta (*Klb*), Protein Phosphatase 2 Scaffold Subunit Abeta (*Ppp2r1b*).

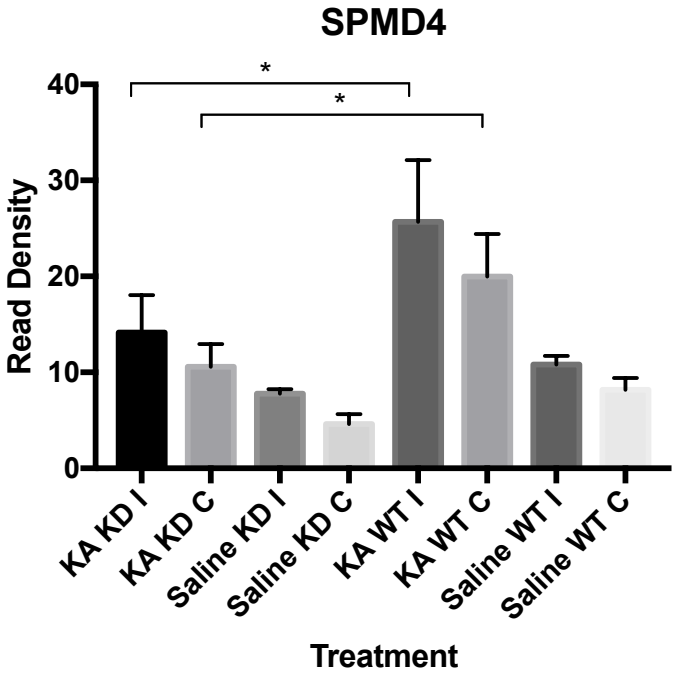


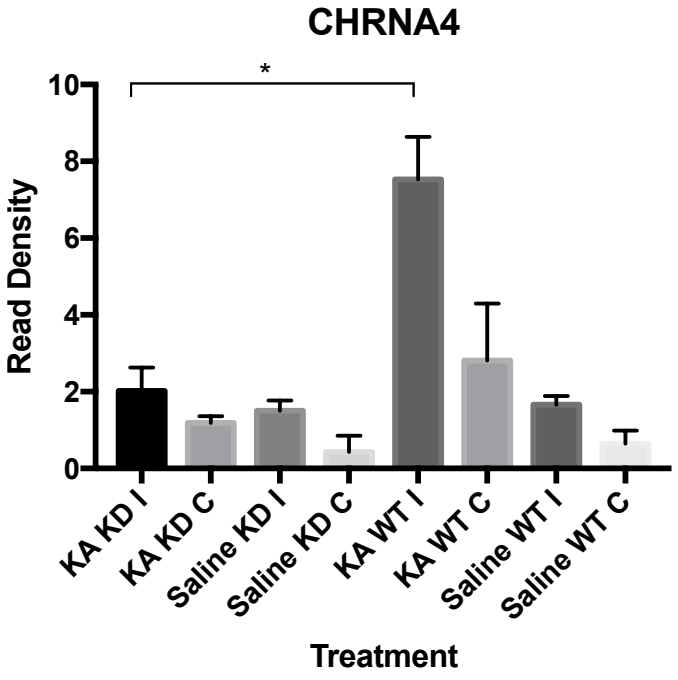
Red: IPS WT KA v WT Saline
Sky blue: IPS KD KA v WT Saline
Orange: CONTRA KD KA v WT Saline
Dark blue: CONTRA WT KA v WT Saline
Purple outline: overlapping genes in KA v Saline IPS and CONTRA
Yellow outline: overlapping genes protected by STAT3 KD

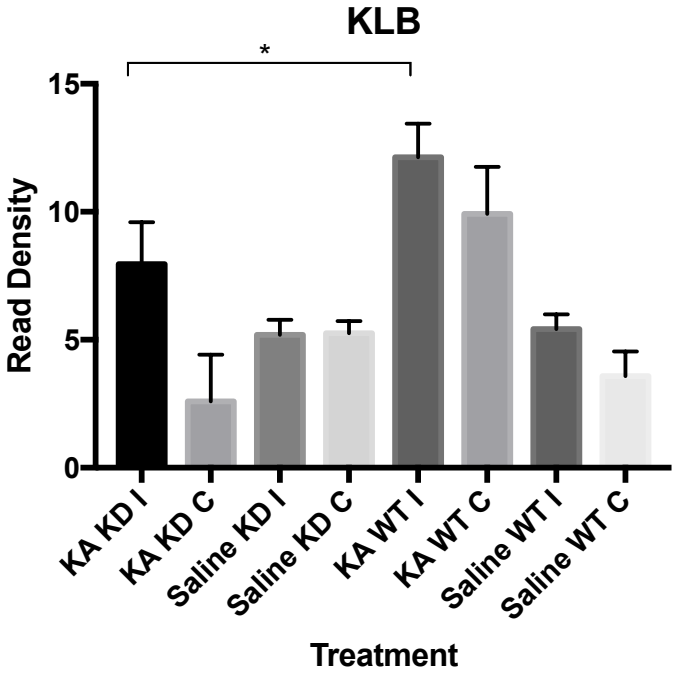
B**. List of genes bilaterally protected from KA by STAT3 KD**

Gene Symbol	Description
Acp1	acid phosphatase 1, soluble
Arl5c	ADP-ribosylation factor-like 5C
Capn12	calpain 12
Fam151a	family with sequence simliarity 151, member A
Letm2	leucine zipper-EF-hand containing transmembrane protein 2
Lrp3	low density lipoprotein receptor-related protein 3
Mcmdc2	minichromosome maintenance domain containing 2
Mid1	midline 1
Nudt6	nudix (nucleoside diphosphate linked moiety X)-type motif 6
Oraov1	oral cancer overexpressed 1
Ppp2r1b	protein phosphatase 2, regulatory subunit A, beta
Smpd4	sphingomyelin phosphodiesterase 4
Spopl	speckle-type POZ protein-like
Tti2	TELO2 interacting protein 2
Tulp4	tubby like protein 4
Zeb2os	zinc finger E-box binding homeobox 2, opposite strand

C. mRNA expression profiles of key KA-induced genes protected by STAT3 KD






KLB
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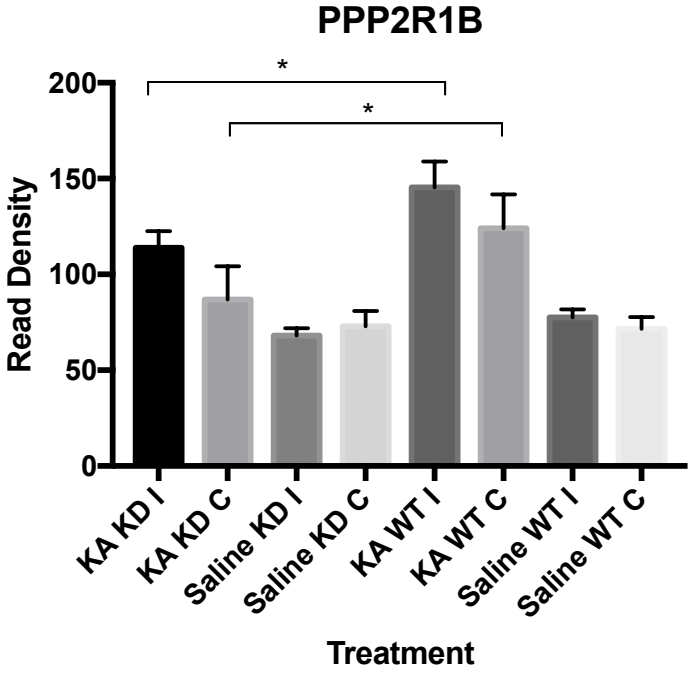
Treatment


PPP2R1B
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Saline WT I
Saline WT C
Treatment

5. EFFECT OF STAT3 NEURONAL KNOCKDOWN IN THE ABSENCE OF KAINIC-ACID INDUCED STATUS EPILEPTICUS

Outside of the effects of KA, the role of STAT3 KD was examined in saline injected animals. It has been suggested that STAT3 has a role in the nonpathogenic state of the hippocampus. We found 28 genes were differentially expressed between WT and STAT3 KD in the ips hippocampus to the saline injection. On the contralateral side, just 3 genes were differentially expressed. The 3 genes, seizure related 6 homolog-like protein (*Sez6l*), protease, serine 23, opposite strand (*Prss23os*), and lymphocyte antigen 6 complex, locus G6D (*Ly6g6d*) (a known STAT3 target) were also altered on the ips side. The differential gene expression suggests that the damaging act of injection alone, even of saline, causes activation of STAT3 signaling in neurons. See Figure 21D.

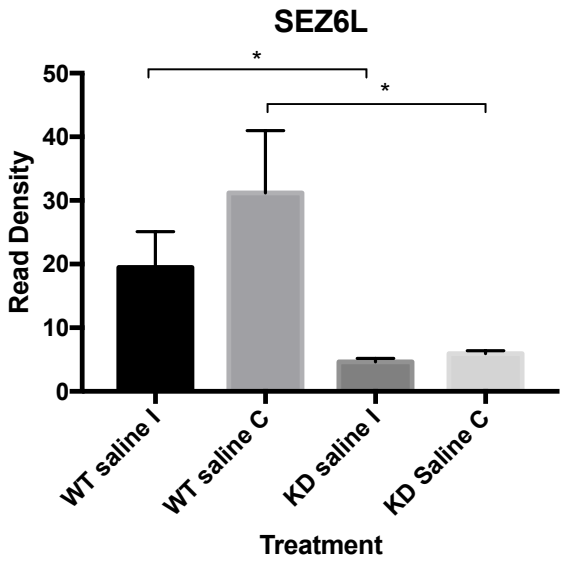
Figure 22. STAT3 Responses in Saline Injected Mice

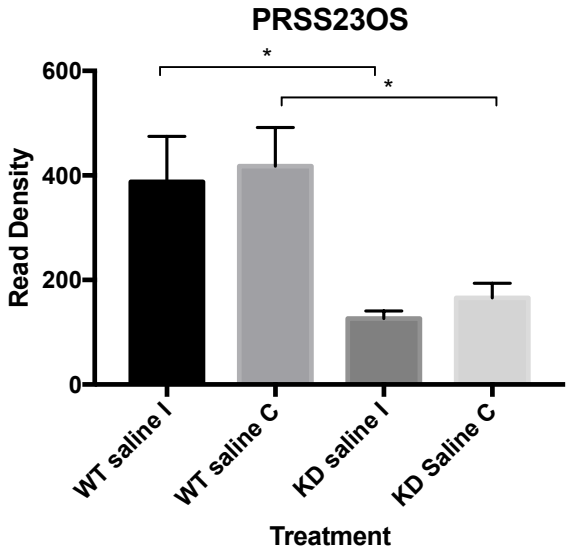
A) List of Genes altered in ips saline injected WT brain when compared to STAT3 KD. B) mRNA expression levels of the 3 genes altered in ips and contra in saline injected WT compared to STAT3 KD: seizure related 6 homolog-like protein (*Sez6l*), protease, serine 23, opposite strand (*Prss23os*), and lymphocyte antigen 6 complex, locus G6D (*Ly6g6d*).

A. Saline responsive genes in IPS side of STAT3 KD vs WT

Ly6g6d	-7.00	Srrm4os	-1.45
Sez6l	-4.20	Fam210a	-1.36
Art3	-3.86	Fars2	-1.35
Tmem132a	-3.31	Tmem55b	-1.33
Gas2l1	-3.28	Ccdc152	1.43
Cd163	-3.14	Itga4	1.60
Prss23os	-3.12	Ppfia2	2.35
Exosc5	-2.59	Zcchc2	2.52
Ssbp2	-2.57	Nat8f1	2.65
Shc2	-1.92	Wwc2	3.30
Abi3	-1.85	Ubxn4	4.46
Trim2	-1.77	Rhbdd2	5.07
Junos	-1.54	Uvrag	7.10
Hectd2os	-1.52		

B.




PRSS23OS
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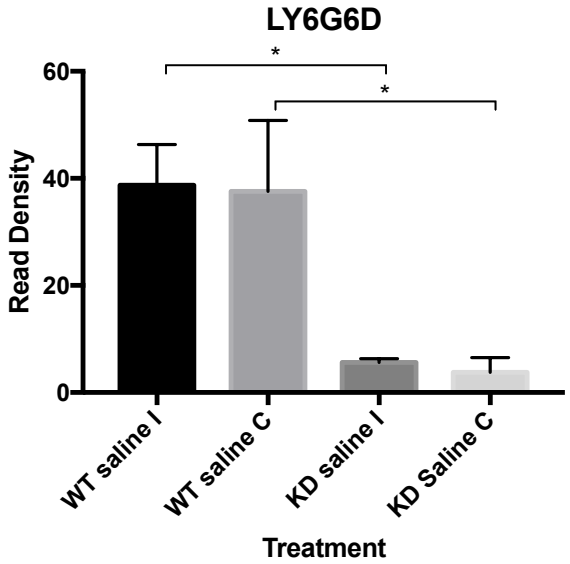
WT saline C
KD saline I
KD Saline C
Treatment


LY6G6D
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Treatment

D. DISCUSSION

Many studies of IHKA focus solely on the changes in the ipsilateral hippocampus, ignoring the contralateral or even using the contralateral side as a control. This study provides a unique and novel transcriptomic comparison of the effects on each side of the hippocampus of an individual brain. Our analysis contains multiple levels of information that include not only the responses of the genome on the ipsilateral side where KA is injected, and where changes in gene expression are robust, but also alterations that are seen bilaterally, and most related to SE as this side is away from the injection site but also displays the hallmarks of epileptogenesis after the characteristic latent period (see Figure 20 below; or for a complete list of genes from each comparison, see Appendix). Key members of this list are *Smpd4*, *Sphk2*, and *Ppp2r1b*, which each participate in sphingolipid metabolism and are described below in more detail.

- ***Smpd4***- sphingomyelin phosphodiesterase 4- catalyzes the hydrolysis of membrane sphingomyelin to form phosphorylcholine and ceramide, activated by DNA damage, cellular stress and tumor necrosis factor. Increases over two-fold with KA injection.
- ***Sphk2***- encodes one of two sphingosine kinase enzymes that catalyze the phosphorylation of sphingosine into sphingosine 1-phosphate. Sphingosine 1-phosphate (S1P) is important in migration, proliferation and apoptosis (Bryan et al. 2008). S1P receptor knockout mice show increased neuroexcitability (MacLennan et al. 2001) and an S1P modulator, Fingolimod, has shown promising anti-seizure effects in

pilocarpine rat models and PTZ mouse models of epilepsy (Leo et al. 2017). *Sphk1* is also affected in just the ipsilateral side. Kainic Acid treatment decreases its expression almost 4-fold.

- ***Ppp2r1b***- encodes Protein Phosphatase 2, Structural/Regulatory Subunit A, Beta, one of four major Ser/Thr phosphatases implicated in negative control of cell growth and division, including in negative regulation of Sphingosine Kinase 1 in sphingolipid metabolism (Barr et al. 2008). It can also deactivate the JAK/STAT pathway. Mutations in this gene can contribute to Myoclonic epilepsy of Lafora, a progressive neurodegenerative disorder with seizures, autism and schizophrenia (Takata et al. 2016). KA injection results in a 1.8-fold increase in expression.

By comparing the transcriptome of WT mice with neuronal STAT3 KD mice, we can for the first time examine the role of neuronally expressed STAT3 in the context of the KA-induced SE model of TLE. Here, we demonstrate that STAT3 knockdown can protect from KA-induced status epilepticus. Our results indicate that the gene network most predominately induced by KA and rescued by STAT3 KD in mice is sphingolipid metabolism. In gene ontology and pathway analysis of KA vs saline in WT animals, sphingolipid metabolism was a top hit. STAT3 KD reversed KA-induced changes in the transcriptome of the hippocampus and an important set of genes was identified for future study: *Klb* (ips), *Pik3c2a* (ips), *Ppp2r1b* (ips and contra), *Smpd4* (ips and contra), SPHK1 (ips) and *Sphk2* (ips and contra).

Sphingolipids play a major role in the synthesis and maintenance of lipid rafts where they assemble with cholesterol in the lipid bilayer, and make up about 45% of the cell surface in some cell types. These rafts play a crucial role in signal transduction by forming concentrated platforms that act as a stable microenvironment for receptor activation protected from enzymes that could affect signaling processes (Simons and Toomre 2000). They also play a role in sorting and vesicle formation that is crucial for synaptic neurotransmission (Ikonen 2001) (Figure 23). In addition, they have an important role in other CNS functions including the interaction of myelin cells with neurons (Posse de Chaves and Sipione 2010), neurogenesis (Mizugishi et al. 2005), intracellular organelles (Siow et al. 2010), and synapse formation (Kanno et al. 2010).

Figure 23. Sphingolipid participation in lipid rafts

Diagram of lipid raft components in the brain at the synaptic cleft demonstrating the trafficking of membrane proteins laterally to foster signaling of ligand activated pathways (BDNF at TrkB receptor as an example). From (Zonta and Minichiello 2013).

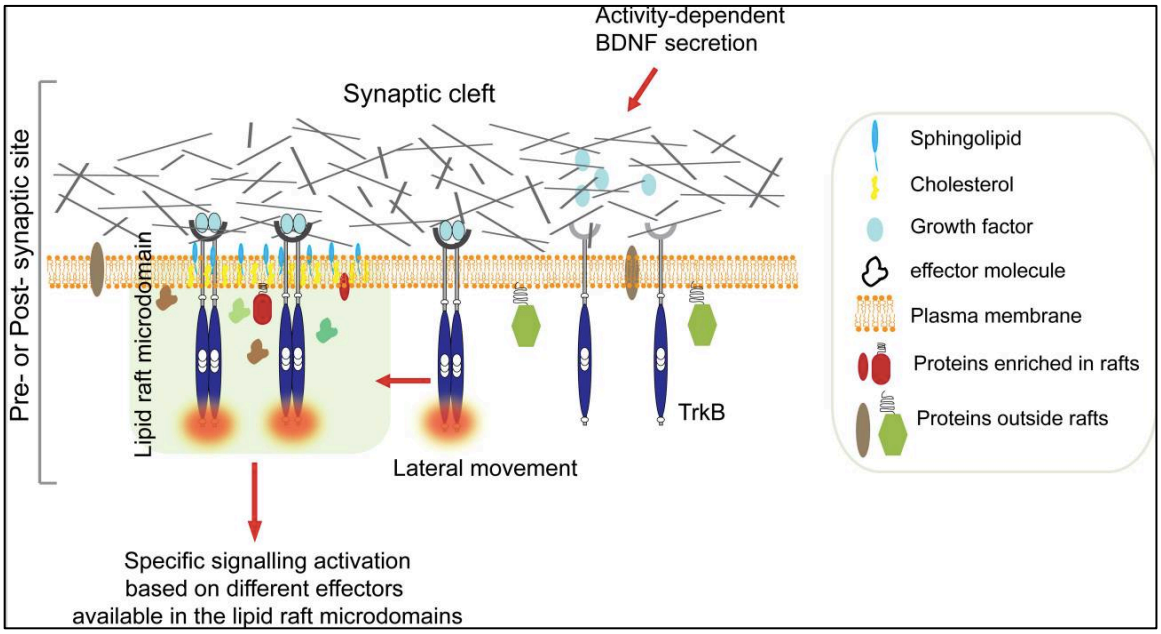


Table 8. List of ion channel and receptors in the brain affected by sphingolipids

Adapted from (Olsen and Færgeman 2017).

Receptor or Channel	Functional Effects
α3β2 nicotinic acetylcholine receptor	removal of cholesterol and hydrolysis of SM into ceramide decreases desensitization half-time
α7 nicotinic acetylcholine receptor	removal of cholesterol and hydrolysis of SM into ceramide slows down the desensitization kinetics including increased agonist affinity
Kir1.1	hydrolysis of SM into ceramide inhibits K^+ conductance and decreases ionic and gating currents
Kv1.3	constitutively localized in sphingolipid-rich microdomains; hydrolysis of SM into ceramide decreases ionic and gating currents
Kv1.5	Co-localizes with caveolin; inhibition of CERS activity induces hyperpolarization shift of the activation and inactivation curve
Kv2.1	hydrolysis of SM into ceramide-1-phosphate induces hyperpolarization shift in the conductance–voltage relation
TRPA1	SM hydrolysis of ceramide decrease AITC-induced Ca^{2+} uptake, which is not due to an increase in ceramide or sphingosine
TRPM8	SM hydrolysis and inhibition of de novo synthesis of ceramide decrease icilin-induced Ca^{2+} uptake
TRPV1	SM hydrolysis as well as inhibition of the synthesis of GSLs and de novo ceramide decrease both capsaicin- and resiniferatoxin-evoked Ca^{2+} uptake
AMPA receptor	disruption of microdomains by simultaneous cholesterol depletion and CERS inhibition results in fewer, but larger receptor clusters; loss of synapses and dendritic spines
GABAA	disruption of microdomains by simultaneous cholesterol depletion and CERS inhibition result in fewer, but larger receptor clusters, meaning reduced synapse number; loss of synapses and dendritic spines

NMDA receptor	localized into PSD-95-rich microdomains and synaptic microdomains, ceramide can stimulate receptor clustering
serotonin1A receptor	inhibition of ceramide synthesis leads to impaired function of the serotonin1A receptor due to reduced ligand binding
serotonin7 receptor	inhibition of ceramide and GSL synthesis reduces maximum agonist binding
Trk A	GM1 directly associates with Trk and enhances neurite outgrowth and neurofilament expression induced by nerve growth factor (NGF)
EGFR	GD3 mediates membrane microdomain localization of EGFR; ablation of GD3 results in reduced level of EGFR expression and accelerates EGF-induced EGFR degradation leading to decreased self-renewal capability
insulin receptor	lack of C22–C24 ceramides inhibits phosphorylation and translocation of the insulin receptor into microdomains upon insulin stimulation

In addition to the modulation of the sphingolipid metabolism response, STAT3 KD protects from alterations of gene expression in many of the epilepsy-linked genes affected by KA.

- ***Chrna4-*** Encodes the cholinergic receptor nicotinic alpha 4 subunit protein. Mutations in the gene can lead to autosomal dominant nocturnal frontal lobe epilepsy (Kurahashi and Hirose 1993). This gene was one of the first genes to be identified that can directly cause epilepsy.
- ***Slc6a12-*** **(AKA *Bgt1*)** Encodes the solute carrier family 6 protein (aka betaine-GABA transporter) which is responsible for the uptake of GABA in the extracellular space. It is a target of certain anti-seizure drugs due to its ability to prolong the effects of GABA at the synapse (Lehre et al. 2011).
- ***Scn3a-*** Encodes the sodium channel, voltage-gated, type iii, alpha subunit, part of an ion channel crucial for action potential generation. Mutations in this gene, along with *Scn1a*, *Scn2a*, and *Scn8a* cause early infantile epileptic encephalopathy (Zaman et al. 2018).

Comparative analysis of the *in vivo* dataset here described with that of Chapter III, where BDNF is the only inducer of transcriptional change to model SE in response to the pilocarpine model of epilepsy, reveals that the genome most likely responds differentially to the manner in which it is injured. Where mice and rats in the pilocarpine model of temporal lobe epilepsy (PILO) show a strong upregulation in BDNF mRNA and protein, that is mimicked by the application of BDNF *in vitro* in primary rat cortical neurons, this

was absent in the IHKA model, as BDNF transcripts do not increase significantly at 24 hrs post SE. One possibility that we have not yet addressed is that BDNF could increase rapidly in the IHKA model and subside by 24 hrs when we did our RNA collection.

Although both datasets of Chapters III and IV demonstrate the transcriptional support for alterations in synaptic transmission, they appear to be mediated by distinct biological processes. Where BDNF treatment and *in vivo* PILO directly alters the transcription of receptors and ion channel genes, the datasets of the IHKA model suggest that there may be an indirect modification of receptors and ion channels based on changes in sphingolipid synthesis that has previously been shown to alter membrane trafficking, stability, and location of the membrane proteins (Olsen and Færgeman 2017).

Many studies have highlighted the similarities and differences between the PILO and KA models of TLE. Both models result in SE and subsequent spontaneous seizures in a similar time line, however, the neuropathological changes in the hippocampus appear to occur faster in PILO than KA injected animals (Lévesque, Avoli, and Bernard 2016). Species also plays a role, as mice injected with KA develop fewer spontaneous seizures than rats (McKhann et al. 2003).

Interestingly, in behavioral studies in collaboration with our colleagues in the Brooks-Kayal laboratory, STAT3 KD in saline-treated animals produced a significantly increased freezing time in a conditional fear memory task, which was absent in KA treated animals. They also showed in earlier studies that rats treated with WP1066, a JAK inhibitor, displayed reduced long-term depression (LTD) in control rats but not in PILO. These results taken together suggest that STAT3 regulation of LTD is disrupted post-

seizures, however follow-up studies are underway to determine whether STAT3KD in neurons displays a similar phenotype.

Our datasets of IKHA *in vivo* also reveal an interesting role for STAT3 KD in the absence of KA. When comparing STAT3 KD datasets to WT, when both are injected with saline, key genes were identified *Sez6l*, *Prss23os,* and *Ly6g6d* as potential STAT3 targets in neurons.

- ***Sez6l-*** Encodes for the seizure related 6 homolog-like protein. SEZ6L is highly expressed in the brain and known to play a role in autism and bipolar disorder (Chapman et al. 2015; C. Xu et al. 2013) Although it has not yet been associated with seizure disorders, it gets its name from a related protein in the same family. It also plays a role in lung cancer (Gorlov et al. 2007). SEZ6L was downregulated by KD 4.2-fold (ips) and 5.3-fold (contra).
- ***Prss23os-*** Encodes for the protease, serine 23, opposite strand protein. A member of the trypsin family of serine proteases. Involved in melanoma (Potrony et al. 2018) and retinopathy. PRSS23OS was downregulated 3.1-fold (ips) and 2.3-fold (contra).
- ***Ly6g6d-*** Encodes the lymphocyte antigen 6 complex, locus G6D protein. It belongs to a cluster of leukocyte antigen-6 (LY6) genes located in the major histocompatibility complex (MHC) class III region on chromosome 6. It is a GPI-anchored cell surface protein thought to be involved in migration, adhesion, and differentiation. Multiple microarray datasets in

GEO (Gene Expression Omnibus) including KA and pilocarpine epilepsy models have shown differential expression of this gene. *Ly6g6d* was downregulated by STAT3 KD 7.0-fold (ips) and 17.7-fold (contra).

The neuron-specific STAT3 knockdown allows us to determine the effect of neuronal JAK/STAT signaling not only in the expression of the neuronal transcriptome, but also, we must keep in mind, that RNA was extracted from hippocampal tissue that contains many different kinds of cells. These cells are most likely responding to the impact of STAT3 removal in neurons. Astrocytes are major mediators of inflammation, and along with microglia, they also have JAK/STAT signaling pathways that are involved in neuroinflammation (O. S. Kim et al. 2002; Kacimi, Giffard, and Yenari 2011; Justicia, Gabriel, and Planas 2000). Astrocytes undergo many changes during epilepsy and can contribute to hyperexcitability of neurons by altering potassium buffering in the interstitial fluid at the synapse, glutamate uptake, gliotransmission, and secretion of proinflammatory cytokines like IL-1B and TNF-alpha. Microglial cells are also activated and release proinflammatory cytokines that can alter neuronal excitability by an unknown mechanism (Devinsky et al. 2013; Heuser, Szokol, and Taubøll 2014). Understanding the role of JAK/STAT signaling in all of the cells of the tripartite synapse, along with the resident and innate immune cells will contribute greatly to our understanding of how a JAK/STAT inhibitor may function as a therapeutic option.

In conclusion, our results taken together demonstrate that differential gene expression occurs in both the ipsilateral and contralateral hippocampus in the IKHA model of epilepsy and that knockdown of the STAT3 transcription factor solely in

neurons selectively attenuates alterations in the KA-induced transcriptome. We also show an important role for STAT3 in sphingolipid metabolism and genes previously associated with epilepsy development. Lastly, we identify a few key genes where STAT3 is important in the non-pathological hippocampus.

E. FUTURE DIRECTIONS

Open-ended research such as deep RNA sequencing directs us in an unbiased way towards findings that may lead research into new areas of exploration and discovery. Some of the findings which inform future studies include the role of STAT3 in sphingolipid metabolism in the context of epilepsy development. Armed with the results presented here and the association of sphingolipids with synaptic transmission, we would hypothesize that KA-induced disruptions in sphingolipids would alter brain receptor populations, and their function, and that STAT3 KD would protect the brain from these changes. To test this hypothesis, we can use slice electrophysiology of hippocampal slices from the transgenic mice and examine the effect of STAT3 KD and KA treatment on the function of key receptors including acetylcholine, GABA, and AMPA receptors. In addition, hippocampal tissue from KA animals and saline animals can be examined with immunohistochemistry to determine if there is altered expression or activity states of key sphingolipid metabolic proteins. As we do not yet know the impact of non-neuronal cell responses to the loss of STAT3 in neurons, single-cell RNAseq could be used to determine whether neuron-specific STAT3 knockdown impacts the transcriptional profile of the brain's interacting cells, ie astrocytes, glia, and even blood vessels. To address the role of STAT3 in glial cells directly, the Brooks-Kayal and Russek labs will be taking a

complementary approach of knocking down STAT3 specifically in astrocytes and comparing these responses to those of STAT3 KD in neurons.

Finally, mice who underwent these studies but were not sacrificed after 24 hours for transcriptomic analysis were allowed to continue through the latent stage of the model and into spontaneous seizures. We are waiting for the datasets from these animals who were studied via *in vivo* EEG monitoring to determine if STAT3 KD in neurons protects against spontaneous seizures after the latent period in the IHKA model.

CHAPTER V: THE STRUCTURE OF THE P75NTR/JAK2 COMPLEX AND ITS ROLE AS A SIGNALSOME IN BDNF SIGNALING

A. ABSTRACT

BDNF-activated signaling pathways have an important role in development and neurogenesis, however, increased levels of BDNF after brain insults leads to aberrant signaling activation and gene expression changes resulting in circuitry remodeling and a disruption in the balance of excitation and inhibition in the brain. Neurons chronically exposed to BDNF display activation of the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway and treatment with a JAK/STAT inhibitor reduces the frequency of spontaneous seizures in animal models of temporal lobe epilepsy (TLE). Our laboratory has discovered what may be a unique intracellular association between JAK2 and the BDNF receptor p75NTR in primary neurons. Canonical JAK/STAT signaling would predict that the interaction between the proteins would take place at the intracellular domain of membrane bound p75NTR, however, our results do not support the classical model and indicate a potential role of the intracellular p75NTR/JAK2 complex as a signalsome. Here, we demonstrate that JAK2 binds to p75NTR on its extracellular domain and that both proteins are present in the nucleus of primary cortical neurons alluding to a direct role in chromatin stability and gene transcription independent of downstream factors. We also show that the complex formation is dependent on the activation of JAK2. A detailed understanding of the role of the p75NTR/JAK2 complex in BDNF-induced JAK/STAT activation could be critical to our understanding of how the JAK/STAT pathway functions in neurons.

B. INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is a neurotrophin whose expression is highest in the hippocampus (Ernfors et al. 1991). BDNF plays an essential role in brain development and neurogenesis, as well as in adult neuroplasticity (Korte et al. 1995). BDNF signaling is mediated through its two different receptors- tropomysin-related kinase B (TrkB) receptor and the p75 neurotrophin receptor (P75NTR). Mature BDNF, formed after cleavage of the proBDNF protein, binds with high affinity to TrkB, in the hippocampus, receptor activation of TrkB by BDNF plays an important role in neuronal survival and axonal growth. In contrast, p75NTR, a cell surface glycoprotein and a member of the tumor necrosis factor family of related receptors binds with higher affinity to proBDNF than mature BDNF and also binds to several other neurotrophins, such as nerve growth factor (NGF), and neurotrophin 3 and 4 (NT-3, NT-4) (W. J. Friedman and Greene 1999).

Both TrkB and p75NTR are implicated in neurological diseases including neurodegeneration, temporal lobe epilepsy (TLE) and traumatic brain injury (TBI) (Chao, Rajagopal, and Lee 2006; Kandratavicius, Hallak, et al. 2014; Rostami et al. 2014). In epilepsy patients, a well-established alteration in the epileptic brain is an altered composition of the type A receptor for γ- aminobutyric acid (GABAR), the major inhibitory neurotransmitter in the adult brain. Our laboratory has shown that BDNF regulates expression of at least 2 GABAR subunits after SE, which results in replacement of synaptic α1- containing GABARs with α4 to enhance excitability of dentate granule cells.

BDNF increases expression of the α4 subunit through TrkB activation via the mitogen activated protein kinase (MAPK) pathway (D. S. Roberts et al. 2005). BDNF can decrease α1 subunit expression through the TrkB receptor (Russek, unpublished results) via the Janus kinase (JAK)/ signal transducer and activator of transcription (STAT) pathway. BDNF activation of the JAK/STAT pathway results in STAT3-mediated induction of the gene coding for the inducible cAMP early repressor (ICER). ICER binds to the *Gabra1* promoter to attenuate transcription. This response can be blocked by JAK/STAT inhibitors (WP1066 and Pyridone 6) and STAT3 small-interfering RNAs (siRNAs) (Lund et al. 2008). Chapters III and IV reveal additional genes whose expression may be regulated by the JAK/STAT pathway in response to status epilepticus and chronic BDNF. Additionally, in a pilocarpine rat animal model, application of WP1066, a JAK/STAT inhibitor reduces the number of spontaneous seizures that develop after the latent period (Grabenstatter et al. 2014) suggesting that JAK/STAT signaling may be important to epileptogenesis.

The mechanism for BDNF-induced activation of the JAK/STAT pathway is still unresolved. Results show that inhibition of TrkB through an inhibitor, K252A, and siRNA knockdown can both block the induction of ICER and *Gabra1* downregulation. Despite its apparent involvement in the pathway, TrkB does not bind to JAK2 in coimmunoprecipitation assays. P75NTR siRNA can also block the induction of ICER and *does* bind to JAK2 *in vitro,* in primary neuronal cultures. Proximity Ligation assays in the laboratory using primary neuronal cultures demonstrate that p75NTR/JAK2 clusters can be found throughout the cytoplasm in neuronal processes as well as in the nucleus. Also,

immunoprecipitation experiments with forms of P75NTR with mutations in the transmembrane domain that prevent dimerization and localization in the membrane increase JAK2 binding to an intracellular and full-length p75NTR (Benham and Hokenson et al. unpublished results) .

JAK/STAT signaling is well studied as it has an important function in the immune system and is implicated in cancer. Recently, many drugs targeting canonical JAK/STAT signaling have emerged to treat various cancers and autoimmune diseases. Canonical JAK/STAT signaling consists of a ligand such as a cytokine or hormone binding to its receptor which leads to multimerization of the receptor molecules either as homodimers or heteromultimers. The cytoplasmic domain of the membrane-bound receptors then associates with the N-terminus of the JAK proteins, multimerization of the receptor molecules allows for two JAK proteins to be in close proximity. This proximity fosters trans-phosphorylation. Activated JAK (pJAK) can then phosphorylate STAT and it arranges into an antiparallel dimer.

The activated dimer then translocates into the nucleus through the Ran nuclear import pathway. Inside the nucleus STATs are free to bind target genes to activate or repress expression (Darnell 1997). Due to the results of research from Chapter III, where transcriptional changes of the BDNF-induced JAK/STAT pathway appear to be occurring independently of the phosphorylation state of STAT3, and evidence from our laboratory that suggest JAK2 and p75NTR are binding within the nucleus, my thesis research findings suggest that the JAK/STAT signaling pathway in neurons is non-canonical

Non-canonical JAK/STAT signaling includes several alternative pathways that deviate from the canonical mechanism and which are still being studied for further understanding. They involve findings that JAK can act in the nucleus to alter gene transcription independently of STAT, and that STAT can be present in the nucleus without phosphorylation and regulate chromatin stability in an unphosphorylated state (M. A. Dawson and Bannister 2012; Brown and Zeidler 2008).

P75NTR signaling also follows a consistent signaling mechanism that deviates in the case of the JAK/STAT pathway of neurons. Usually, p75NTR functions through extracellular binding of the neurotrophic ligand to the membrane-bound receptor followed by intracellular domain binding to all other signaling partners for subsequent actions. Although the intracellular domain of p75NTR is known to be cleaved and subsequently travel into the nucleus, full length p75NTR has not been previously identified in the nucleus, as is seen in these experiments (Roux and Barker 2002).

Here, we investigate a potential role for a p75NTR/JAK2 signalsome in the BDNF-induced JAK/STAT genome response as well as distinguish important structural and trafficking characteristics of the complex. These findings add to the existence of a potential non-canonical JAK/STAT signaling pathway in the brain and provide valuable information for its potential use as a novel epilepsy drug target.

C. RESULTS

1. BINDING DOMAIN OF P75NTR and JAK2 RESIDES IN P75NTR EXTRACELLULAR DOMAIN

Due to the finding that p75NTR and JAK2 co-immunoprecipitate, we further tested where binding might occur between these two molecules to signify a specific interaction. According to what is known regarding both p75NTR and JAK2 classical signaling, it was hypothesized that the interaction would occur in the intracellular C-terminal region. Plasmids containing a truncated form of p75NTR that contains only the intracellular domain (ICD) and full-length WT JAK2 were overexpressed in human embryonic kidney (HEK-293) cells through co-transfection. Cells were transfected with WT p75NTR alone + JAK2, ICD alone + JAK2 or a 50/50 combination of both WT and ICD p75NTR + JAK2. Results of co-immunoprecipitation assays seen in Figure 24 showed that no binding occurred between the ICD and JAK2, while WT Full-length p75NTR showed robust interactions. In the 50/50 combination of transfected expression constructs, less than 50% binding to WT occurred, demonstrating that presence of the ICD inhibited binding to WT p75. In addition, we replicated the interesting finding from the laboratory that binding interactions between p75NTR and JAK2 increase when the transmembrane domain of p75NTR is mutated.

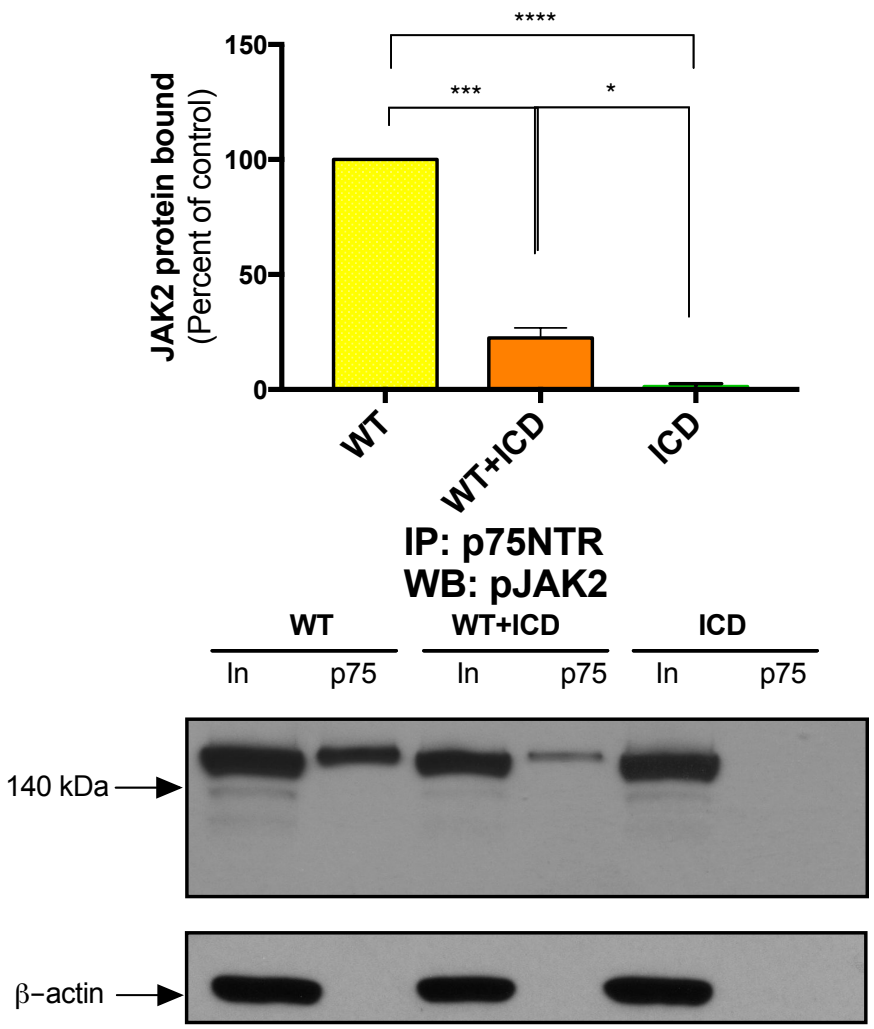
To follow up on the surprising result that the ICD does not bind to JAK2 as is seen in the majority of receptor JAK interactions and to focus our investigation into finding the interacting surface, we used other truncated plasmids: ΔICD which includes the extracellular domain (ECD) and transmembrane domain (TM) but lacks the ICD, and

the ΔECD which includes the TM and ICD but lacks the ECD, in a series of transfection assays. We overexpressed HEK cells with ΔICD, ΔECD, or WT p75NTR and JAK2 and performed coimmunoprecipitation assays to determine the binding surface of the p75NTR/JAK2 interaction. Figure 24 demonstrates that the binding of p75NTR and JAK2 occurs in the ECD, due to lack of binding when only the ECD is removed. Since all of the truncation plasmids include the TM, direct binding of JAK2 to that region of p75NTR can be eliminated because it is also present in the ΔECD which does not bind to JAK2.

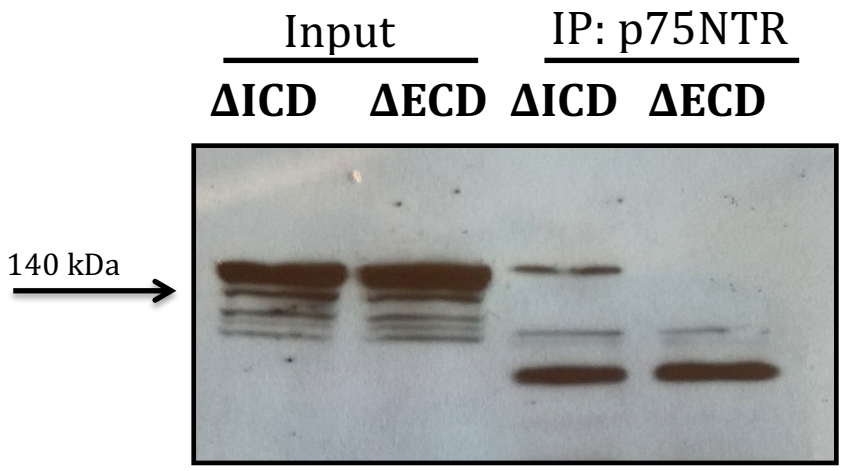
Figure 24. JAK2 binds to extracellular domain of p75NTR

A) Representative Western blot showing co-immunoprecipitation (IP) products of the p75NTR/JAK2 interaction as detected by immunoblotting for JAK2 using whole cell extracts from HEK cells co-transfected with p75NTR-flag + JAK2-GFP, p75NTR-flag+ ICD +JAK2-GFP or ICD+ JAK2-GFP. Quantitation of precipitated JAK2 was determined using the input signal for JAK2 for each IP reaction. The detection signal of JAK2 in input lanes was normalized to β-actin and subsequently used to normalize across IP lanes per sample. B) IP with HA, immunoblot with JAK2 on whole cell extracts from HEK cells co-transfected with truncated p75NTR forms including ΔICD-HA fusion tag or ΔECD-HA fusion tag and JAK2-GFP. C) IP of WT p75NTR and two full-length p75NTR mutants, C256A and G265I, co-transfected with JAK2-GFP. Immunoblotting was performed for JAK2 in all lanes. ****$p<0.0001$, ***$p<0.0005$, *$p<0.05$. Student's t test.

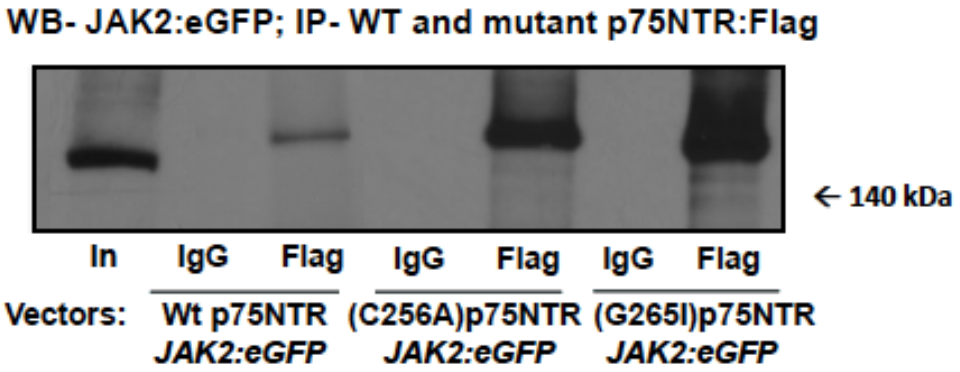
A


****
***
*
150
100
50
0
JAK2 protein bound
(Percent of control)
WT
WT+ICD
ICD
IP: p75NTR
WB: pJAK2
WT
WT+ICD
ICD
In
p75
In
p75
In
p75
140 kDa
β-actin

B


Input
IP: p75NTR
ΔICD
ΔECD
ΔICD
ΔECD
140 kDa

C

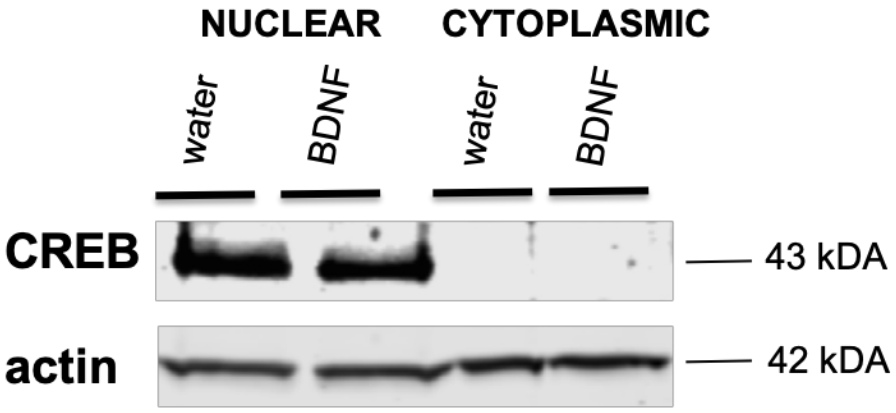

WB- JAK2:eGFP; IP- WT and mutant p75NTR:Flag
← 140 kDa
In
IgG
Flag
IgG
Flag
IgG
Flag
Vectors:
Wt p75NTR
JAK2:eGFP
(C256A)p75NTR
JAK2:eGFP
(G265I)p75NTR
JAK2:eGFP

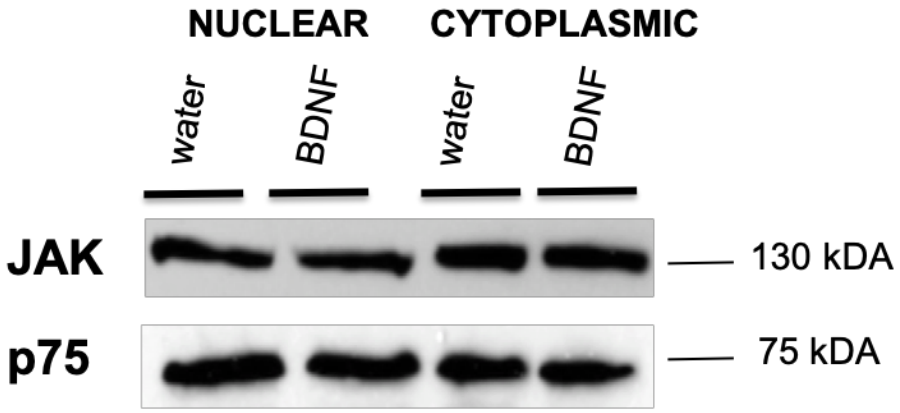
2. P75NTR AND JAK2 ARE PRESENT IN BOTH NUCLEUS AND CYTOPLASM

Proximity ligations assays previously performed in the laboratory with overexpressed p75NTR-flag and JAK2-GFP constructs in primary neurons suggested that the localization of both p75NTR and JAK2 is in the nucleus as well as cytoplasm. To determine whether this is also the case for the endogenous proteins in neurons, soluble nuclear and cytoplasmic fractions of endogenous protein were extracted from primary neuronal cultures and examined by Western Blot for the presence of p75NTR and JAK2. Figure 25 shows the presence of both p75NTR and JAK2 in both nuclear and cytoplasmic fractions demonstrating that there are endogenous levels of both full-length proteins in the nucleus of cortical neurons.

Figure 25. Endogenous JAK2 and p75NTR are found in the nucleus of neurons

Representative Western blots of nuclear and cytoplasmic extracts from primary cortical neurons immunoblotted with CREB and β-actin to verify effective fractionation. Once fractionation was verified, a separate blot was then immunoblotted for p75NTR and JAK2. Both JAK2 and p75NTR are found in the nuclear fraction, treatment with BDNF does not increase the presence of either protein. Order left to right: Nuclear fraction: 1) Nuclear water-treated 2) Nuclear BDNF-treated 3) Cytoplasmic water-treated 4) Cytoplasmic BDNF-treated.


NUCLEAR
CYTOPLASMIC
water
BDNF
water
BDNF
CREB
43 kDA
actin
42 kDA

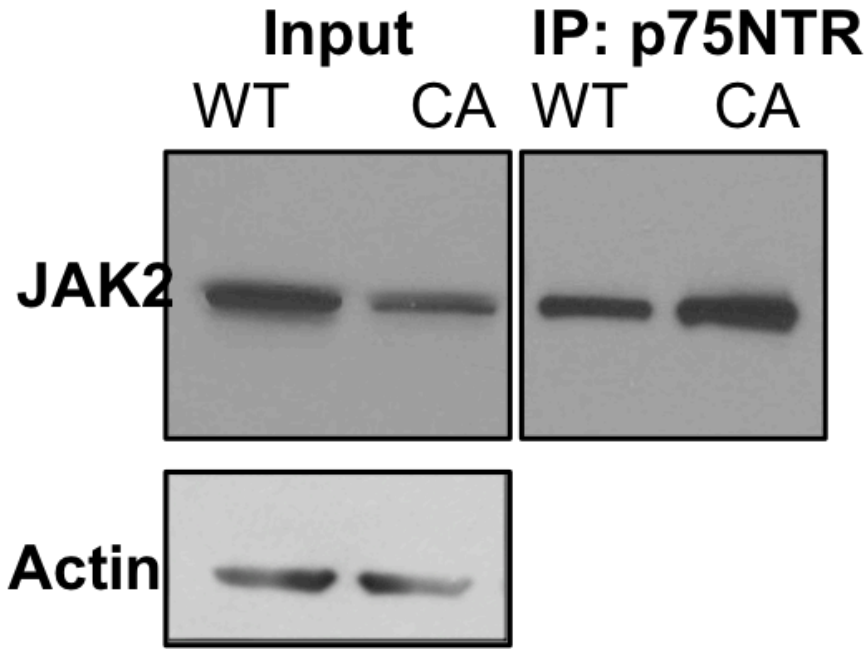

NUCLEAR
CYTOPLASMIC
water
BDNF
water
BDNF
JAK
130 kDA
p75
75 kDA

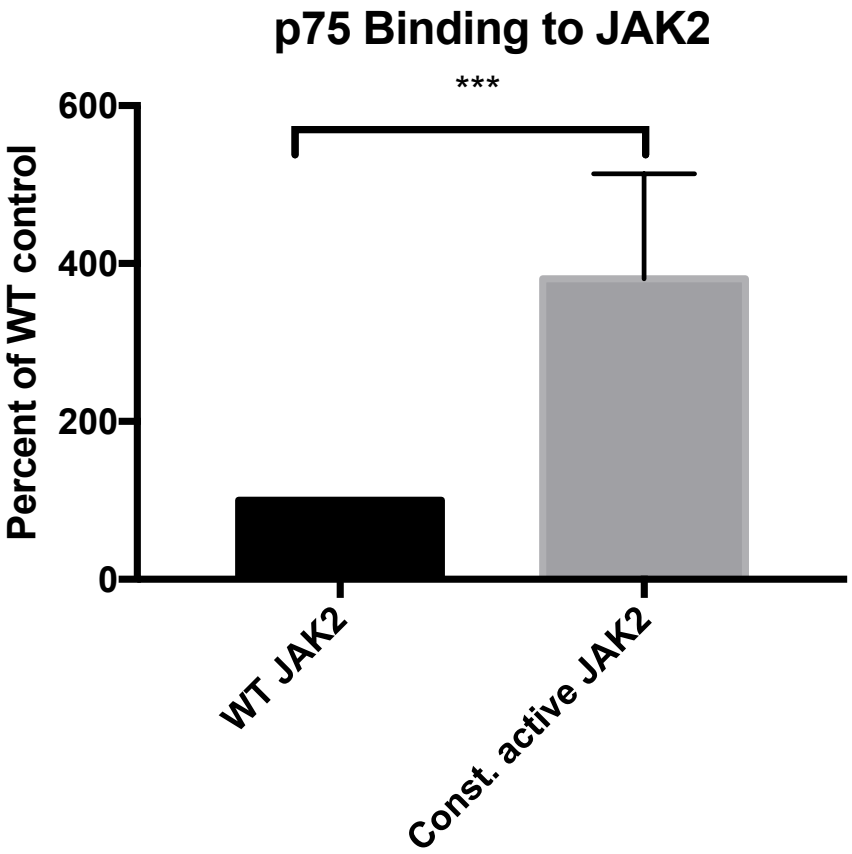
3. P75NTR BINDS PREFERENTIALLY TO ACTIVATED JAK2

To determine if the activation state of JAK2 affects the p75NTR/JAK2 complex formation, a mutant form of JAK2 that is constitutively active (CA) (V617F) was transfected with WT p75NTR to determine by immunoprecipitation whether the activation state of JAK2 determines its interaction with p75NTR. Due to increased stability of the mutant protein, equal molar transfection of the constructs resulted in a much greater expression of the mutant protein. To avoid any bias due to increased amounts of CA JAK2, 1/8 the amount of CA JAK2 DNA was transfected to achieve equal or lower amounts of expressed protein in input samples. Co-immunoprecipitation revealed that when normalized to the amount of JAK2, binding or p75NTR was increased nearly 4-fold to CA mutant in comparison to WT.

Figure 26. p75NTR binds preferentially to the activated state of JAK2

Representative Western Blot of co-immunoprecipitation with p75NTR and immunoblotting for JAK2 of HEK cells co-transfected with WT or constitutively active (CA) JAK2 and p75NTR. For quantitation, input samples were normalized to β-actin expression and IP samples were subsequently normalized to the inputs. (N=3). ***$p<0.0001$ student's t test.


Input
IP: p75NTR
WT
CA
WT
CA
JAK2
Actin


p75 Binding to JAK2
***
Percent of WT control
600
400
200
0
WT JAK2
Const. active JAK2

D. DISCUSSION

Current paradigms of JAK/STAT signaling and p75NTR signaling suggest that BDNF-induced JAK/STAT signaling would likely be occurring due to an interaction at the membrane of ligand-activated and dimerized p75NTR with JAK2 at the intracellular domain. This interaction would lead to the subsequent activation of STAT3 (pSTAT3) followed by gene expression changes in the nucleus that occur through direct pSTAT3 actions at the DNA. However, the results of this study suggest that signaling by this canonical mechanism may not be present in the primary neurons of our culture system where we have reported JAK/STAT activation by BDNF. Our results suggest that the interaction does not occur at the cell membrane but inside the cell in its cytoplasm and nucleus. Moreover, we have evidence that it most likely occurs at the extracellular domain of the p75NTR protein and that the full-length form of both proteins can be found in the nucleus of neurons. We also determined that p75NTR/JAK2 interactions may be dependent upon the activation state of JAK2.

Moreover, taken together with our earlier results from proximity ligation analysis (PLA) (Hokenson K 2016) the ability of what we believe is a p75NTR/JAK2 signalsome to be trafficked throughout the cell suggests an intracellular signaling role that is unique from canonical JAK/STAT signaling. Previous research from our laboratory has shown that BDNF can be present within the cell and presumably activate receptors which are not membrane-bound. Taken together with the fact that TrkB knockdown markedly reduces BDNF-induced JAK/STAT signaling, our working hypothesis is that extracellular BDNF binds TrkB which markedly increases intracellular BDNF levels to form an intracellular

p75NTR/JAK2 complex that has the function of a signalsome to alter transcription in the nucleus.

Outside of ligand binding to the extracellular domain of the membrane bound receptor, p75NTR is not known to interact with other proteins through this domain prior to membrane insertion, but localization inside the cytoplasm may permit unusual interactions that have not yet been characterized. Although endosomal trafficking of neurotrophin receptors including p75NTR has been identified (Bronfman et al. 2003; Escudero et al. 2014), the extracellular domain of the receptor still generally resides inside the endosome, unreachable by the intracellular milieu. Understanding which subcellular organelles participate in the trafficking of the potential p75NTR/JAK2 signalsome and how this might occur in neurons is a goal for our future research.

The identification of JAK2 in the nucleus has been seen before, although its role in this compartment is still largely conjecture and seems to differ by cell type (Zouein, Duhé, and Booz 2011; M. A. Dawson and Bannister 2012; Lobie et al. 1996). Since PLA studies identify presence of the p75NTR/JAK2 complex in the nucleus, which is further supported by the results presented here demonstrating that endogenous p75NTR and JAK2 are present in the nucleus of cortical neurons, the complex is ideally poised to act as a signalsome for regulating interactions at the chromatin and directly impacting gene transcription. Moreover, in hematopoietic cells, nuclear JAK is recognized as a histone 3 protein kinase (M. A. Dawson et al. 2009). H3 phosphorylation by JAK2 can lead to the release of HP1 from the chromatin. Identifying the role of JAK in the nucleus of neurons

in the context of BDNF signaling, as either a kinase at H3 or as a transcription factor, is the subject of ongoing research.

The role of full-length p75NTR in the nucleus is also of interest. Ligand-activated cleavage of the ICD of p75NTR by gamma-secretase and subsequent translocation into the nucleus has been identified in Schwann cells and PC12 cells (Frade 2005; Kanning et al. 2003; Parkhurst, Zampieri, and Chao 2010) and that this process participates in cellular apoptosis (Kenchappa et al. 2006). Despite these numerous studies of nuclear p75NTR in its cleaved form, the full-length p75NTR seen in our studies in primary neurons is novel. Our preliminary studies in HEK cells of chromatin immunoprecipitation reveal that overexpressed p75NTR can bind to DNA suggesting that the nuclear role of the p75NTR/JAK2 signalsome may have DNA-binding capabilities and act to control transcription of several genes (unpublished datasets).

Some limitations of our current findings include the fact that direct linkage has not yet been achieved between p75NTR/JAK2 signalsome formation and functional changes in ICER, GABRA1 or other BDNF-induced JAK/STAT mediated genes. The findings discussed here provide additional tools that can move us forward in being able to address this important question, such as blocking the binding of the complex and analyzing the effect on downstream gene expression.

E. CONCLUSION

Due to the important role of JAK/STAT inhibitors in reducing the occurrence of spontaneous seizures in rodent TLE models, and the epilepsy-related gene expression changes controlled by the BDNF-induced JAK/STAT pathway, understanding the

molecular mechanism of JAK activation and the actions of a potential p75NTR/JAK2 signalsome could lead to crucial progress in identifying the molecular basis of certain epilepsies and developing novel drug targets that can modify the course of the disease, especially for the patients who do not respond to any available pharmacologic treatments.

In the future, to follow up on these studies, more research into: a) the role of STAT3 in the p75NTR/JAK2 complex both in the cytoplasm and in the nucleus, b) the presence of JAK2 or p75NTR at the chromatin, c) whether or not the actions are dependent upon the proteins being bound, and d) the genome response to inhibiting complex formation will be important for progress. Additionally, investigating whether other isoforms of JAK such as JAK1 are equally effective will enables us to determine if there are JAK and STAT isoform specific actions.

In conclusion, the results shown here demonstrate that p75NTR and JAK2 form a complex found not at the membrane, but inside the cytoplasm and nucleus of the cell, that the binding domain responsible for complex formation resides in the extracellular domain of p75NTR, and that complex formation is dependent upon JAK2 activation. These results strongly support a non-canonical form of the JAK and p75NTR signaling pathways but further investigation is needed to determine whether this complex indeed is a feature of BDNF-induced JAK/STAT signaling in neurons.

CHAPTER VI: OVERALL CONCLUSIONS

A. CONCLUSIONS

As described in the above chapters, the work presented in this thesis is aimed at providing a foundation to test my overarching hypothesis that: *the BDNF-induced JAK/STAT pathway has far reaching effects on the transcription of the genome in response to brain insult and that targeting this pathway has the potential for the discovery of disease modifying therapeutics for multiple brain disorders.*

First, using BDNF and JAK inhibitors in primary cortical cultures, I identified a large set of BDNF-induced JAK/STAT mediated genes with significant involvement in the regulation of synaptic neurotransmission, including ion channel and membrane receptors, and modifiers of synaptic plasticity, neurogenesis, and neuroinflammation. Results from the *in vitro* studies using primary neurons also revealed that phosphorylation of STAT3 at Tyr705 may not control the observed changes in gene transcription as originally expected. This finding suggests that the mechanism of BDNF-induced JAK/STAT signaling in neurons may be non-canonical. Most importantly, the RNA-seq datasets highlight the importance that the JAK/STAT pathway may play in the acquired epilepsies and strengthen the hypothesis that identifying the mechanism of JAK/STATs in neurons may lead to novel therapeutic targets for disease modification.

Next, using a neuron-specific STAT3 KD in a mouse KA SE model, I identified unique transcriptomic signatures of IHKA injections in wild-type mice on the side of the hippocampus ipsilateral to the injection and the contralateral hippocampus. Additionally, results show STAT3 knockdown protects many genes from KA-induced gene expression

changes associated with epilepsy, demonstrating a role for STAT3 in neurons as an early responder to status epilepticus, and particular for genes involved in sphingolipid metabolism. I also presented data relevant to the differential expression caused by neuron-specific STAT3 knockdown in saline-treated animals without SE to determine the role of STAT3 in a non-pathological hippocampus.

Finally, my experiments reveal important information regarding the structural determinants of a putative intracellular p75NTR/JAK2 complex found in primary neurons and HEK cells overexpressing P75NTR and JAK2 tagged expression constructs. Taken together with previous research in the laboratory, my results suggest that p75NTR and activated JAK2 form a complex that travels throughout the cytoplasm and its processes, including the nucleus, and that the intracellular interaction of p75NTR with JAK2 is on its N-terminus. These results further suggest that the JAK/STAT signaling pathway in neurons is non-canonical and may have actions in the nucleus independent of classical JAK/STAT signaling.

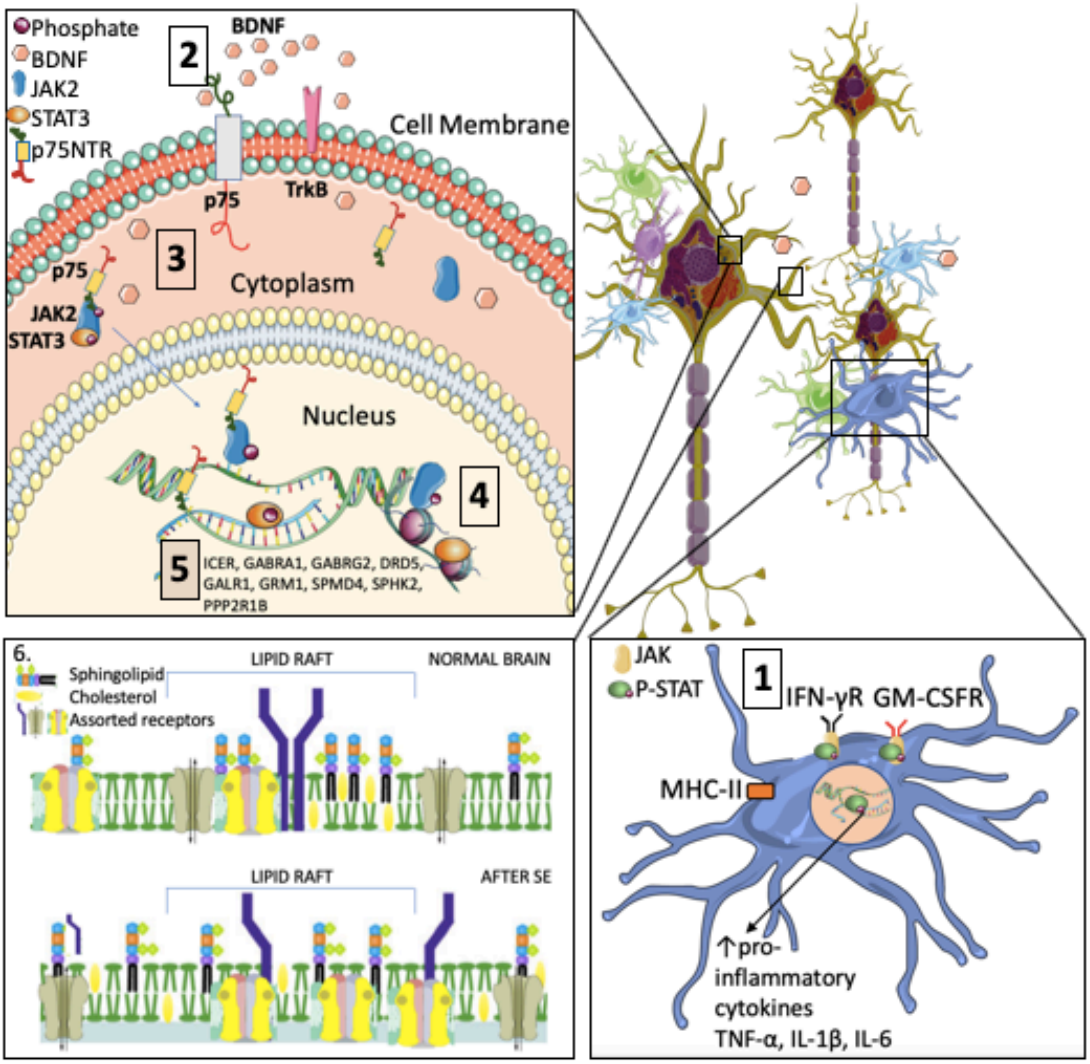
Taken together, the data of my thesis expand upon a role for the JAK/STAT pathway in neurons that may be relevant to the acquired epilepsies where status epilepticus precipitates the development of spontaneous seizures. The open discovery datasets also provide a rich knowledge base that can be mined in the future for determining the mechanism and impact of TrkB-mediated JAK/STAT signaling; and, moreover, the role of neuron-specific STAT3 in the diseased and non-pathological brain. These results expose an unchartered route for epilepsy treatment that could rescue the synaptic transmission dysfunction that develops during epileptogenesis and reduce

seizures as well as comorbidities. A summary of my findings as they relate to one another is seen in Figure 27.

Figure 27. Working model of JAK/STAT signaling in neurons

This schematic displays my working model of the role of JAK/STAT signaling in the brain and includes the data from my thesis (represented in **bold** in the text below) and my hypothesis for the integration of other cellular responses (listed in *italic* in the text below). Numbers 1-5 correspond to the region of interest I have designated in the Figure. **1)** Although the role of JAK/STAT signaling in the context of non-neuronal cells was not studied in my thesis work, it is well known that it plays an important role in microglia and astrocyte signaling. The highlighted blue cell is a microglia, the primary immune cell of the central nervous system. Activation of the interferon-gamma receptor (IFN-γR) and granulocyte -macrophage colony stimulating factor receptor (GM-CSFR) leads to activation of the cell (into M1 state) and recruits JAK and STAT. This results in the secretion of proinflammatory cytokines (TNF-α, IL-1β, IL-6) (Subramaniam and Federoff 2017). These signaling pathways are represented as the first response of the brain to trauma, a major contributor to epileptogenesis (Zhao, Zhu, and Huang 2018; Hiragi, Ikegaya, and Koyama 2018). **2)** Status epilepticus results in increased extracellular BDNF which activates TrkB and *subsequently releases intracellular BDNF to activate intracellular p75NTR*. **3) JAK2 binds the extracellular domain of p75NTR inside the cell and moves into the nucleus.** *STAT may also be bound and become activated upon BDNF signaling.* **4) p75NTR, JAK2 and STAT3 in nucleus** *bind to chromatin and enact changes in transcription. STAT3 binds DNA as a transcription factor, or HP1 as a chromatin stabilizer*. **Genome changes are independent of the STAT3 phosphorylation state. 5) BDNF-induced JAK/STAT signaling regulates the**

expression of the following genes: ion channel/receptors (*Grm1, Galr1, Drd5, Gabrg2*). Neuron-specific STAT signaling affects the following genes: sphingolipid metabolism: *Spmd4, Sphk2, Ppp2r1b*. 6) *At the dendritic membrane, disturbances in sphingolipid metabolism after SE result in altered lipid raft recruitment of receptors and channels. This disturbance alters ion channel conductances at the membrane due to altered distribution of important receptors such as TrkB, GABARs, or AMPA receptors* (Olsen and Færgeman 2017).


Phosphate
BDNF
JAK2
STAT3
p75NTR
2
BDNF
Cell Membrane
p75
TrkB
3
p75
JAK2
STAT3
Cytoplasm
Nucleus
4
5
ICER, GABRA1, GABRG2, DRD5, GALR1, GRM1, SPMD4, SPHK2, PPP2R1B
6.
Sphingolipid
Cholesterol
Assorted receptors
LIPID RAFT
NORMAL BRAIN
LIPID RAFT
AFTER SE
JAK
P-STAT
1
IFN-γR
GM-CSFR
MHC-II
↑pro-inflammatory cytokines
TNF-α, IL-1β, IL-6

B. FUTURE DIRECTIONS

The previous research in our laboratory using the pilocarpine model of TLE demonstrated that status epilepticus (SE) activates the JAK/STAT pathway and alters the number and kind of GABARs on dentate granule cells of the hippocampal formation. We also showed that such affects could be reversed upon delivery of two different JAK/STAT inhibitors, pyridone 6 and WP1066. As levels of BDNF rise after SE, we asked the question of whether BDNF might be the signal that activates the JAK/STAT pathway to alter transcription of the genome promoting dysregulated hippocampal plasticity *in vivo*. In my thesis (Chapter III), using a cell culture model of chronic BDNF exposure, I identified the BDNF transcriptome in neurons, and the component of such a transcriptome that is responsive to JAK/STAT inhibitors. We were surprised to find that there were a number of genes whose expression had previously been ascribed to the activation of the mitogen activated protein kinase (MAPK) pathway, such as Egr3 that we have shown upregulates the expression of *GABRA4*. In future studies of Chapter III (Aim 1) we would like to revisit the use of pharmacological antagonists to probe what may be a cooperative relationship between MAPKs and JAK/STATs in the nervous system using RNA-seq, as well as promoter/reporter assays to interrogate the binding of transcription factors to their respective sites at STAT1/3, NF-kB, and CREB regulatory elements in the ICER gene of neurons.

In addition, the work of my thesis suggests that there is a non-canonical role for the BDNF-induced JAK/STAT pathway in neurons that we hope to explore more fully. The drosophila literature suggests that unphosphorylated STAT3 binds to

heterochromatin to maintain genome stability. With production of pSTAT3, heterochromatin protein 1 (HP1) is believed to dissociate from DNA promoting an active state of gene expression (S. Shi et al. 2008). However, whether this occurs in neurons is unknown as are additional non-canonical forms that include direct phosphorylation of histone protein 3 (H3) by nuclear JAK2 In hematopoietic cells (M. A. Dawson et al. 2009).

To begin to address non-canonical forms of JAK/STATs in neurons, and as seen in Appendix 2, we first generated whole cell extracts of primary cortical neurons and asked whether antibodies to HP1 also precipitate STAT3. Unexpectedly, we found that BDNF substantially increases the association of HP1 with both total STAT3 and pSTAT3. Future studies will examine whether this result is due to cytoplasmic or nuclear HP1/STAT3 association by precipitating chromatin directly and probing for the presence of DNA binding proteins and histone marks that change in the presence of BDNF treatment with and without JAK/STAT inhibitors. The recent identification of HP1 in the cytoplasm suggests that there may be a more complex relationship with this protein than originally described (Charó, Galigniana, and Piwien-Pilipuk 2018).

Finally, analysis of alternative phosphorylation sites outside of Tyr705 on STAT3 could reveal an alternative mechanism (Schuringa et al. 2000) of gene regulation that may be active in neurons; and, as STAT1 and STAT3 bind to the same regulatory elements in genes, we would also like to determine whether such non-canonical pathways may be specific to the STAT3 isoform or accommodate all STAT interactions with chromatin.

For the work described in Chapter IV, the CAMK2a promoter that drove neuron-specific knockdown of STAT3 gave unique information on the role of the JAK/STAT pathway in excitatory neurons *in vivo*, it is expected that the JAK/STAT pathway in non-neuronal cells, including microglia, will play a substantial role in the epileptogenic aspects of JAK/STAT signaling. Abundant research has tied both inflammation and glial cells to the development of epilepsy (Bernardino et al. 2005; Annamaria Vezzani, Friedman, and Dingledine 2013; Johnson et al. 2016). Since it is likely that the JAK/STAT response in these cells is part of the disease process, transcriptomic analysis from the glial-specific knockdown of STAT3 will enhance our understanding of the unique roles of neurons and non-neurons in the epilepsy process. These mice are already being bred in the laboratory of our collaborator, Dr. Amy Brooks-Kayal. Adding the technique of single-cell sequencing could be very useful to identify the direct effects of STAT3 KD in excitatory neurons within the context of their neighboring cells whose transcriptome has not been modified by genetic intervention.

The excitatory neuronal-specific STAT3 KD mice will also offer valuable information about the development of epilepsy once the EEG and behavioral results on those animals (not analyzed by RNA-seq but retained throughout the latent period to generate the spontaneous seizure phase of the model) have been completed.

Lastly, to continue research from Chapter V, it will be critical to develop a molecular tool that disrupts the binding of JAK2 and p75NTR to determine the precise importance of that complex in the BDNF signaling cascade and whether its presence relates to gene expression changes seen in primary cultured neurons and *in vivo*. We also

do not know at this time whether the interaction of p75NTR with JAK2 is JAK-specific, ie specific to only the JAK2 isoform.

My work identifying the N-terminus of p75NTR (ECD) as a potential association domain for JAK protein interactions provides crucial information for moving this work forward. Now the laboratory needs to identify the critical residues within the ECD that are required for JAK recognition via site-directed mutagenesis so that we can develop small molecules to disrupt its potential function in neurons. Once determined, studies can be better designed to link the role of the p75NTR/JAK complex to its potential nuclear function, perhaps with STATs, that when taken together lead to the transfer of information from the cell membrane via TrkB receptors to the genome to alter synaptic neurotransmission.

CHAPTER VII: APPENDICES

A. APPENDIX I

Table A1. List of Genes differentially expressed with WP+BDNF and RX2+BDNF treatment vs. V+BDNF

These genes are protected by JAK/STAT inhibition. Also hypothesized to be the genes regulated by the BDNF-induced JAK/STAT pathway. Log2 Fold Change is listed.

GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC
AATK	1.18	0.70	AFTPH	-0.61	-0.51	ANO1	-2.36	-4.01	ATF1	-0.54	0.36
ABCB10	0.76	0.33	AGBL5	0.69	0.64	ANO4	-1.63	-2.53	ATG10	1.27	0.98
ABCG1	0.97	1.36	AGFG1	-0.32	-0.59	ANO6	-0.77	-0.53	ATG16L1	-0.68	-1.04
ABHD14A	0.98	-0.80	AGFG2	0.85	-0.68	ANXA11	-1.45	-1.39	ATMIN	-0.44	-0.30
ABHD6	0.50	0.89	AGPAT4	0.30	-0.60	ANXA7	-0.34	-0.60	ATP11A	-1.05	-1.22
ABHD8	1.40	1.18	AGTR1A	-2.27	-3.30	AP1S3	-1.35	-1.60	ATP13A3	-1.18	-1.02
ABI1	-0.33	-0.24	AHCYL2	-0.72	-0.66	AP3S2	1.30	0.77	ATP1B1	-0.36	-0.48
ABTB1	1.12	0.41	AHI1	-0.50	-0.28	APBA2	-0.56	-0.36	ATP2B1	-0.34	-0.97
ACAA1	1.11	0.62	AIFM1	0.48	0.27	APBB1	1.16	0.71	ATP5SL	0.81	0.61
ACAN	-1.59	-1.95	AIG1	0.99	0.78	APC2	1.16	1.31	ATP6V0A2	-1.03	-0.58
ACPP	-1.95	-3.08	AIPL1	-1.71	-3.35	APCS	-2.24	-2.67	ATPAF2	1.14	-0.51
ACSL1	-0.26	-0.67	AJAP1	-0.82	-1.07	APOLD1	-1.27	-0.56	ATRAID	1.18	0.69
ACSL4	-0.52	-0.39	AK1	1.23	1.18	AQP11	0.85	0.59	AUH	1.05	0.53
ACTN1	-1.69	-0.82	AK4	-1.56	-0.99	ARC	-3.91	-5.23	B3GNT2	0.64	0.93
ACTN2	1.00	0.51	AKAP7	0.81	0.70	AREG	-3.61	-4.79	B3GNT7	1.72	0.96
ACTR3B	1.15	0.75	AKT2	-0.73	-0.95	ARFGAP3	0.54	0.62	B4GALT7	1.23	0.42
ACVR1	-0.90	-0.25	ALDH1B1	1.51	0.87	ARFIP1	-1.07	-0.40	BAALC	-0.87	-0.69
ACVR1C	-2.02	-1.15	ALG9	0.69	0.54	ARHGAP10	-0.85	-1.95	BACH1	-1.72	-0.53
ADAMTS1	-1.39	-1.57	ALKBH4	-0.54	0.62	ARHGAP17	-0.66	-0.66	BAD	1.30	0.41
ADAMTS15	1.46	1.65	ALOXE3	-1.67	-2.19	ARHGAP20	0.95	1.73	BAG3	-2.88	-2.70
ADAMTS2	1.61	1.63	AMN	3.07	1.49	ARHGAP25	-1.03	-2.21	BAI1	-0.76	-1.21
ADAMTS5	-2.06	-1.43	AMOTL2	0.96	1.30	ARHGEF25	1.35	0.71	BAIAP2	-1.71	-2.79
ADAMTS7	1.23	0.81	AMPD2	0.99	0.62	ARHGEF28	1.01	0.98	BANP	0.29	0.46
ADAP1	1.14	1.03	AMZ2	0.91	0.82	ARHGEF3	-1.71	-1.77	BAZ1A	-1.06	-1.45
ADCK3	1.70	0.98	ANAPC2	1.15	0.43	ARID5A	-1.84	-1.73	BBC3	1.53	1.22
ADCY7	-1.51	-1.66	ANAPC7	0.79	0.45	ARIH1	-0.69	-0.77	BBS2	0.80	-0.63
ADCY8	-1.47	-2.26	ANGPT2	-1.49	-1.48	ARL14EP	-0.94	-0.69	BBX	-2.25	-1.01
ADGRB3	0.91	0.79	ANK1	-0.72	-0.82	ARMC7	0.31	1.26	BCAS3	1.12	0.94
ADGRE5	-1.10	-1.82	ANKEF1	-1.14	-2.33	ARMCX6	1.83	0.86	BCKDHA	0.84	0.38
ADM	-0.84	-1.28	ANKMY2	0.90	0.89	ARPP21	-0.40	-1.04	BCL11A	0.97	1.12
ADORA1	-1.57	-1.52	ANKRD12	0.848	0.592	ARRDC4	0.90	1.63	BCOR	-0.54	-0.87
ADORA2A	2.09	1.51	ANKRD13D	1.23	0.44	ARSJ	-2.64	-2.96	BCORL1	0.89	0.95
ADRA1A	-3.61	-2.40	ANKRD33B	-3.65	-4.88	ASAP1	-1.25	-0.67	BDNF	-1.54	-1.35
ADRA1B	-1.75	-1.86	ANKRD34A	1.29	1.17	ASCC1	1.10	0.53	BEND5	1.41	0.90
ADRA1D	-2.53	-3.01	ANKRD34C	-2.42	-2.41	ASCC2	-0.75	-0.83	BEND7	-1.35	-0.99
ADSS	0.48	0.65	ANKRD52	-1.05	-0.97	ASCC3	-0.68	-0.38	BHLHE22	2.96	3.30
AEN	-0.63	0.55	ANKRD6	1.04	0.83	ASGR1	-2.11	-1.75	BHLHE23	-1.46	-1.74
AFAP1L1	-0.63	-1.65	ANKS1B	-0.76	-0.50	ASUN	-0.64	-0.77	BHLHE40	-2.21	-1.11

GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC
BID	0.84	1.03	CCDC28A	2.01	1.52	CHN2	1.04	1.04	CRIPT	1.14	0.93
BLES03	1.27	0.70	CCDC28B	1.71	0.49	CHODL	2.23	1.75	CRLF3	-0.74	-0.68
BLOC1S5	0.93	0.58	CCDC32	0.84	1.10	CHRAC1	0.64	-0.60	CROT	-0.85	-0.99
BLOC1S6	0.50	0.46	CCDC40	1.05	0.58	CHRM2	-1.69	-2.04	CRY1	-0.63	-0.77
BMPER	-1.19	-1.67	CCDC53	1.46	0.39	CHRNA5	1.18	0.71	CRYM	1.51	1.04
BNIP3	1.08	0.88	CCDC86	-1.25	0.69	CHST10	-0.44	-0.49	CSPG5	0.85	0.71
BNIP3L	1.05	0.57	CCDC92	-0.51	-0.38	CHST12	1.28	0.88	CSRNP1	-1.39	-2.54
BOK	1.68	0.82	CCDC93	-0.62	-0.84	CHST7	-0.50	-0.46	CSRP2	1.73	0.83
BPHL	1.21	0.41	CCKAR	2.46	2.13	CHSY1	-0.34	0.28	CTDP1	-0.30	0.57
BRE	1.04	0.56	CCDC92	-0.51	-0.38	CHURC1	1.98	0.79	CTPS2	0.96	0.77
BRINP3	1.38	1.23	CCDC93	-0.62	-0.84	CIR1	1.56	1.27	CTSF	1.23	0.76
BROX	0.64	0.50	CCKAR	2.46	2.13	CISD3	1.93	0.99	CUBN	-2.38	-2.63
BSDC1	0.70	0.71	CCKBR	-2.22	-2.85	CISH	1.44	0.51	CUL9	1.50	0.56
BTBD6	0.78	1.12	CCND1	-1.54	-1.04	CITED2	-1.59	-2.11	CUX2	0.99	0.82
BTG4	1.83	1.02	CCNG2	1.87	2.11	CLASP2	0.70	0.92	CX3CL1	-0.68	-0.50
BTRC	-0.56	-0.38	CCNL2	-0.53	0.52	CLDN9	1.47	0.95	CXCR4	0.83	1.75
BVES	-1.40	-2.00	CCPG1	1.12	1.36	CLDND1	-0.42	-0.29	CXXC5	-0.89	-0.68
C1QL2	-3.34	-4.40	CD200	1.18	1.19	CLIP2	1.12	0.97	CYB561	1.34	0.89
C1QL3	-1.62	-1.75	CD226	1.88	2.71	CLMP	1.87	1.54	CYB561D2	1.68	0.96
C1S	-1.77	-0.59	CD55	0.74	-1.00	CLN5	-0.88	-1.22	CYB5R1	1.32	0.87
C2CD2L	0.64	0.59	CD59	1.08	0.96	CLN8	0.57	1.08	CYGB	1.80	0.96
CABLES1	0.78	0.69	CDC14A	-1.72	-1.49	CMAS	1.22	1.05	CYHR1	1.54	0.49
CACNB1	-0.37	-1.78	CDC25B	0.92	-0.48	CMPK2	0.86	0.41	CYLD	0.64	0.87
CACNB3	1.04	0.50	CDC37L1	-0.51	-1.39	CMTR2	-1.02	0.57	CYP11B2	-1.75	-2.25
CACNB4	-1.24	-1.65	CDC42EP3	0.96	0.89	CNGA4	-3.43	-4.32	CYP11B3	-2.21	-2.80
CACNG3	-1.32	-1.46	CDC42EP4	1.37	1.03	CNNM1	-0.75	-1.02	CYP26B1	-3.26	-3.30
CALCA	-2.24	-3.28	CDCA4	-0.64	0.42	CNNM2	0.49	1.16	CYP27B1	-2.62	-3.00
CALCOCO1	0.70	0.61	CDCA7L	-0.64	-0.33	CNP	0.97	0.50	CYR61	2.17	1.13
CALCRL	-1.23	-0.70	CDH15	1.67	0.96	CNR1	0.54	0.61	CYS1	1.24	-0.60
CALHM2	0.94	0.97	CDH7	1.45	1.05	CNTFR	-0.87	-0.74	CYTH1	0.56	0.91
CALY	1.34	0.97	CDK19	0.82	1.06	CNTN2	1.43	1.34	D2HGDH	1.05	0.69
CAMK1G	-0.52	-1.27	CDK5	1.12	0.60	CNTN3	1.27	1.72	DAAM1	1.20	1.90
CAMKK1	-0.62	-1.19	CDK5RAP1	1.08	-0.79	COASY	0.92	0.75	DARS2	0.78	-0.55
CAPN2	-0.52	-0.59	CDK5RAP3	1.18	0.62	COBL	0.65	0.37	DBNDD1	2.86	1.75
CAPN5	1.18	1.12	CDK8	-0.37	-0.86	COG3	-0.78	-1.04	DBP	1.01	1.22
CARF	0.78	0.73	CDKN2AIPNL	0.94	0.56	COL26A1	1.10	1.01	DCAF10	-0.70	0.58
CARS	-0.69	-1.05	CDO1	1.28	0.54	COL4A1	-0.59	-0.49	DCAF4	1.12	0.57
CAT	0.48	0.36	CDYL	-1.38	-0.75	COL5A1	-1.45	-1.08	DCAKD	0.60	0.44
CAV1	-1.37	-2.03	CDYL2	-1.35	-1.72	COMMD1	1.66	1.03	DCLK1	-0.53	-0.43
CBFA2T3	-1.15	-0.69	CELF6	0.99	0.73	COMTD1	1.24	0.46	DCLK3	3.16	3.02
CBFB	-0.63	-0.46	CEP57L1	0.59	0.35	COX11	-0.72	0.58	DCPS	0.74	0.39
CBLN1	1.33	0.97	CFB	-1.42	-0.81	COX19	1.22	1.19	DDAH2	1.74	0.72
CBLN2	-0.98	-1.14	CFLAR	-1.05	-0.76	CPNE4	1.01	0.83	DDHD1	-0.57	-0.72
CBX8	0.73	1.64	CH25H	-1.18	-1.44	CPNE8	1.22	0.88	DDIT4	0.81	1.22
CCBL2	1.23	0.35	CHD1	-1.08	0.43	CRAT	0.67	0.46	DDX19B	-0.27	0.30
CCDC129	-1.82	-3.01	CHD6	0.69	0.56	CREBL2	0.47	0.29	DDX20	-0.47	0.26
CCDC148	1.49	0.90	CHML	-2.27	-2.48	CREM	-1.44	-1.11	DDX21	-1.08	-0.46
CCDC175	1.62	0.81	CHMP1A	0.82	0.80	CRHBP	-3.95	-3.96	DDX3X	-0.43	-0.48
CCDC184	1.31	1.30	CHMP6	1.12	0.93	CRIP2	1.19	1.02	DEAF1	0.98	0.73

GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC
DEF6	1.98	1.22	DUSP6	-1.71	-2.12	EXO1	-0.83	0.76	FGF3	-2.56	-4.15
DEPDC5	0.61	0.28	DYNC1I1	0.85	0.87	EXOC3L1	1.60	0.80	FGF8	-2.03	-3.31
DES	-1.39	-2.25	EBF1	1.81	1.79	EXOC5	-0.76	-0.47	FGF9	-1.35	-1.80
DESI1	-0.39	-0.56	EBF3	-1.46	-0.93	EXTL2	0.47	0.44	FGFR1	-2.31	-1.70
DGCR8	-0.29	-1.55	EBPL	1.86	0.98	EZR	-1.58	-0.57	FHL2	-0.58	-1.02
DGUOK	0.75	-1.12	ECEL1	-2.56	-2.96	F2R	-0.68	-0.45	FIBP	1.27	0.47
DHRS9	-4.41	-4.39	ECH1	0.81	0.52	FADS3	0.81	-0.63	FIG4	0.71	0.43
DHX40	0.87	0.96	EDNRA	-1.55	-1.52	FAIM2	1.22	1.12	FILIP1	2.74	2.16
DHX57	0.91	0.54	EEA1	-1.30	-0.76	FAM102B	-0.89	-0.94	FJX1	-0.47	-0.35
DIAPH1	-0.69	-0.87	EEF2K	0.95	0.42	FAM107B	-0.62	-0.92	FKBP5	-0.69	-0.91
DIEXF	-0.45	-0.54	EFNA4	1.98	1.80	FAM110C	-4.87	-5.45	FLOT1	1.47	0.60
DIP2C	-1.25	-0.92	EFNB3	1.12	1.10	FAM110D	2.55	0.58	FLOT2	1.47	0.66
DIRAS1	1.17	1.37	EFR3A	-1.28	-1.04	FAM118A	1.03	0.54	FLRT1	-1.62	-1.48
DIRC2	0.76	1.01	EGR2	-1.31	-3.91	FAM127B	0.98	0.89	FLT1	-2.63	-3.73
DIS3	-0.56	0.54	EGR3	-2.60	-3.39	FAM129B	-0.37	-0.43	FMNL1	-0.79	-1.41
DISC1	-1.47	-1.56	EHBP1	0.50	0.61	FAM129C	1.77	0.77	FN3KRP	0.50	0.42
DISP2	0.91	0.63	EHBP1L1	2.03	0.89	FAM134A	0.82	0.78	FNDC3A	-1.38	-0.56
DKK2	-4.43	-4.48	EHD1	1.20	0.96	FAM136A	1.20	0.87	FNDC3B	-1.88	-0.90
DLC1	-2.87	-1.94	EHD4	-1.35	-2.20	FAM150B	-4.36	-6.15	FOXP2	1.06	0.78
DLEU7	1.97	0.57	EHF	-2.37	-1.15	FAM163B	-0.50	-0.40	FRA10AC1	1.53	0.53
DLG1	-1.56	-0.91	EIF1B	1.03	0.89	FAM184B	2.17	1.84	FRAS1	-1.36	-2.05
DLGAP2	-3.27	-1.92	EIF2B5	0.30	0.26	FAM192A	0.85	1.15	FRMD3	0.56	0.30
DLK1	-1.06	-0.82	EIF5A	0.48	0.44	FAM196A	-1.62	-2.27	FRMD6	-1.45	-1.03
DMP1	-3.08	-3.08	ELAVL4	1.46	1.12	FAM212B	-2.04	-1.89	FRS3	1.50	1.05
DNAJB4	0.95	1.14	ELF3	2.78	1.80	FAM214A	1.17	0.88	FST	3.11	2.69
DNAJB5	-0.77	-0.81	ELMOD1	1.46	0.95	FAM220A	0.81	0.69	FSTL5	0.42	0.80
DNAJC25	-0.40	0.41	ELOVL7	-2.48	-3.22	FAM222B	-0.98	0.34	FTSJ3	-0.36	-0.43
DNASE2B	-3.72	-3.54	ELP2	0.68	0.19	FAM26F	-2.45	-3.37	FUCA2	1.16	0.44
DOC2A	1.26	0.88	EMC9	2.02	0.60	FAM43A	-1.42	-1.08	FUNDC1	0.85	0.70
DOCK8	-1.87	-1.09	EMILIN3	-1.27	-1.74	FAM65B	-0.96	-1.34	FURIN	-0.75	-0.63
DOK4	1.21	1.21	EML1	0.83	0.90	FAM84A	-1.50	-1.76	FUT7	-1.48	-1.81
DOK5	-1.18	-1.83	EML4	-0.95	-0.63	FAR1	-1.33	-0.94	FXYD6	1.02	0.80
DOT1L	-1.49	-1.35	EML5	-1.10	-1.14	FARS2	1.28	0.69	FYTTD1	-0.32	-0.49
DPF1	1.48	0.92	EMP1	-4.59	-4.05	FBN2	-0.93	-1.00	FZD1	0.79	1.29
DPF3	-1.25	-1.37	EMP3	-1.65	-1.95	FBXL14	-0.89	0.41	FZD4	-1.47	-1.45
DPM2	1.26	0.58	ENTPD6	0.90	0.61	FBXL20	0.55	1.38	FZR1	1.04	0.75
DPY19L1	-0.48	-0.30	EPB41L4B	-0.94	-1.21	FBXL6	1.48	0.56	GABARAPL1	0.78	1.03
DPYD	0.57	0.66	EPDR1	-0.72	-0.87	FBXO16	1.76	0.48	GABRD	1.80	0.99
DPYSL2	1.00	1.08	EPHA3	1.08	1.86	FBXO31	1.44	0.99	GABRG2	1.16	1.11
DRD5	-6.00	-4.50	EPHA4	0.99	1.53	FBXO41	1.22	0.47	GABRQ	-0.92	-0.66
DSE	-0.54	-0.51	EPHB6	1.60	0.43	FBXO44	1.10	0.88	GADD45B	-1.48	-3.24
DSG3	-3.31	-2.39	ERAL1	0.82	-0.23	FBXW17	1.38	0.40	GAL	-2.95	-2.93
DTX3	0.93	0.82	ERCC1	-0.50	-1.86	FCHO1	1.36	0.59	GALE	1.56	0.75
DUOX1	-1.18	-1.46	ERRFI1	-1.04	-0.75	FDFT1	1.18	1.17	GALK2	1.32	0.85
DUSP14	-1.98	-1.78	ESR1	-1.14	-1.71	FDXR	1.55	0.45	GALNT3	0.70	0.95
DUSP23	1.68	0.67	ETHE1	1.30	0.52	FEZ1	1.10	0.72	GALNT7	-1.49	-1.00
DUSP26	1.57	1.09	ETV4	-0.70	-0.95	FFAR4	-2.52	-2.93	GALNT9	-1.11	-1.79
DUSP4	-2.83	-2.23	ETV5	-2.09	-1.44	FGF13	1.02	0.87	GALR1	-6.85	-5.99
DUSP5	-2.42	-2.79	EVA1A	-0.94	-1.12	FGF2	-4.24	-3.39	GAS7	0.70	1.08

GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC
GBX1	-1.86	-3.22	GRASP	-2.93	-4.18	HRK	0.69	0.74	ISM1	-3.16	-3.79
GCGR	-1.85	-0.91	GREM1	-1.08	-1.11	HS3ST5	-0.75	-1.72	ISOC1	0.45	0.77
GCK	4.06	2.31	GRHL2	-1.45	-1.44	HSD11B1	-0.91	-1.12	ITGA11	1.85	1.26
GCLC	-1.51	-1.08	GRIN2A	-3.14	-1.61	HSDL1	0.47	0.94	ITGA6	-2.34	-2.43
GCNT1	-3.37	-3.86	GRIP1	-0.63	-0.56	HSF2BP	-3.72	-4.32	ITM2C	1.09	1.11
GDNF	-1.87	-3.29	GRK5	-0.73	-0.60	HSH2D	-2.35	-3.06	ITPR3	-1.21	-1.46
GEM	-1.46	-1.51	GRM1	1.76	1.87	HSPA12A	0.69	0.61	ITSN1	-0.89	-0.82
GEMIN8	1.10	0.48	GRM6	1.58	1.13	HSPA1B	1.39	-1.31	IVD	0.46	0.56
GFOD1	-1.84	-0.82	GRN	0.69	0.62	HSPA4L	-1.16	-0.94	IWS1	-0.49	0.34
GFPT2	1.25	0.84	GRP	1.79	0.91	HSPB11	1.57	-0.60	JADE3	-1.50	-1.05
GFRA1	-2.57	-2.25	GRWD1	-0.57	0.26	HSPH1	-0.59	-0.50	JAK1	-0.66	-0.42
GINS2	-0.75	-0.94	GSPT2	-0.38	-0.78	HTR1B	-3.11	-2.67	JAK2	-1.08	-0.68
GJB2	-1.04	-0.72	GTF2IRD1	0.80	0.87	HTR1F	-3.04	-4.34	JAKMIP1	1.50	1.75
GLDN	-0.81	-1.70	GTF3C5	0.54	0.42	HTR7	-0.67	-1.58	JAKMIP3	0.52	1.22
GLI2	-1.81	-1.08	GTPBP4	-1.08	0.68	ICA1	0.96	0.34	JPH3	0.86	0.88
GLRA2	0.91	0.89	GUCY1A3	1.23	1.53	ICK	0.79	0.55	JPH4	0.83	0.70
GLRB	0.66	0.49	GUCY1B3	1.25	0.63	IDH1	0.74	0.98	JUNB	1.20	-0.64
GLRX	0.94	0.78	GYPC	-3.38	-2.72	IER5	-1.13	-1.43	KATNBL1	-0.85	0.66
GMPPA	1.18	0.34	GZF1	-0.42	-0.65	IER5L	-1.00	-1.66	KBTBD2	-0.71	0.50
GOLGB1	0.60	0.63	H2AFY2	1.06	0.39	IFFO2	-1.53	-0.87	KCNA1	-2.93	-3.16
GOLPH3L	0.27	0.48	HACE1	-1.30	-2.43	IFNG	-2.05	-2.34	KCNA4	-1.14	-1.35
GPANK1	1.29	1.13	HAS1	-2.83	-1.79	IFNGR2	0.80	0.53	KCNB1	-0.91	-0.61
GPAT3	-1.70	-1.11	HAUS8	-2.35	-3.59	IFRD1	-0.88	-2.12	KCNC4	-1.20	-2.24
GPATCH2	-1.04	-0.67	HBEGF	-0.84	-1.12	IFT122	0.85	0.31	KCND2	0.85	1.08
GPC2	1.54	0.83	HCN1	-0.98	-0.52	IFT52	0.86	0.65	KCNE2	0.58	2.28
GPM6A	0.86	1.24	HCN3	1.01	0.83	IGF1	-1.43	-1.57	KCNF1	-1.57	-1.10
GPR101	-2.50	-1.79	HDAC11	1.31	0.54	IGF2BP2	-1.09	-1.18	KCNH3	1.26	0.75
GPR12	2.11	1.81	HDX	-1.62	-0.79	IGSF3	1.22	1.21	KCNH4	2.72	1.12
GPR139	1.13	1.88	HEATR5B	0.73	0.57	IGSF8	1.39	0.91	KCNJ12	-0.90	-0.88
GPR156	0.98	1.49	HECA	-0.65	-0.82	IGSF9	2.19	0.86	KCNJ4	-0.86	-1.20
GPR158	-3.95	-3.57	HECTD2	-2.12	-2.42	IL1RAP	-1.09	-0.92	KCNJ5	-1.99	-2.88
GPR21	0.33	0.30	HEPHL1	-1.88	-3.02	IL1RL2	-2.45	-2.61	KCNK1	-0.40	-0.33
GPR26	-1.10	-2.07	HERC3	0.55	0.24	IL25	1.67	1.77	KCNK2	2.31	1.07
GPR3	-1.15	-1.27	HERPUD2	0.38	0.54	IL34	2.08	1.35	KCNK3	-1.17	-1.19
GPR37	1.85	1.00	HES6	1.67	0.99	IL6R	-2.77	-2.70	KCNMB2	-1.35	-1.71
GPR4	-0.75	-2.29	HIC1	-1.65	-1.98	IMPDH1	1.17	0.63	KCNMB4	1.85	1.24
GPR45	0.54	0.40	HIGD2A	1.52	0.57	ING4	1.33	1.20	KCNN1	1.51	0.65
GPR50	-2.25	-2.87	HIST3H2A	1.75	1.06	INHBA	-2.40	-2.03	KCNN3	-1.98	-1.50
GPR52	1.06	1.43	HMG20A	0.53	0.85	INIP	-0.40	0.35	KCNV1	-1.05	-2.02
GPR61	1.01	1.42	HMGA1	-2.14	-2.04	INO80C	0.64	0.36	KCTD15	0.80	0.72
GPR63	-2.41	-1.76	HMGA2	-2.87	-2.23	INPP5D	-1.46	-1.72	KCTD6	-0.47	-0.37
GPR68	-2.27	-3.53	HMGCLL1	1.35	0.99	INSC	-3.39	-4.41	KDM1A	-0.25	0.49
GPR83	-2.69	-3.98	HOMER1	-1.78	-1.46	IPPK	-1.43	-1.39	KDM4A	0.65	0.80
GPR85	0.89	0.95	HOMER2	-0.79	-0.51	IQSEC3	-0.82	-1.77	KDSR	0.58	0.98
GPR88	-0.57	-0.39	HP1BP3	0.68	0.47	IRF6	-1.33	-1.63	KIF27	0.98	0.50
GPRASP1	0.37	0.64	HPCAL1	1.04	0.86	IRGQ	0.53	0.69	KIFAP3	0.73	0.94
GPRC5A	-5.10	-5.43	HPN	-1.92	-3.23	IRS2	-1.75	-1.20	KITLG	-1.46	-1.01
GPRC5C	1.41	0.91	HRH1	-1.74	-0.58	IRX6	-1.50	-2.73	KL	-3.60	-2.73
GPX3	1.05	0.77	HRH3	2.71	1.76	ISG20L2	-0.82	-1.22	KLC4	1.40	0.37

GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC
KLF10	-2.43	-2.58	LPL	0.85	1.21	MAP7D1	-0.61	-0.78	MON2	-0.68	-0.77
KLF11	2.26	2.53	LRCH1	-0.82	-0.47	MAPK15	2.54	0.94	MORN4	1.30	1.13
KLF13	0.91	1.13	LRFN5	1.12	0.62	MAPK1IP1	0.80	-0.47	MOSPD3	0.75	0.47
KLF14	-2.95	-2.39	LRRC10B	-1.20	-1.48	MAPK3	1.22	0.78	MPDU1	1.14	0.46
KLF2	-1.36	-1.79	LRRC16B	1.17	0.43	MARCH11	-0.93	-1.87	MPEG1	0.99	0.96
KLF5	-0.85	-0.79	LRRC17	-0.84	1.01	MARCH2	1.02	0.66	MPPED1	1.55	1.38
KLF7	1.19	1.98	LRRC20	1.25	0.55	MARCH8	0.56	0.90	MPV17L2	1.11	0.38
KLF9	-1.78	-1.92	LRRC26	-2.55	-3.51	MARCH9	1.28	1.38	MRM1	-0.40	-1.81
KLHDC8A	3.11	1.62	LRRC28	0.93	0.63	MAS1	-1.59	-3.08	MRPL10	0.88	0.61
KLHDC8B	1.52	1.04	LRRC34	1.67	1.29	MAT2B	0.68	0.34	MRPL14	1.63	0.96
KLHL14	0.94	0.71	LRRC4	-0.77	-0.47	MB21D2	0.77	0.82	MRPL34	1.78	0.96
KLHL23	0.76	0.76	LRRC42	0.96	0.61	MBLAC2	0.65	0.62	MRPS12	1.14	0.41
KLHL24	0.92	1.45	LRRC55	0.85	1.17	MC5R	-1.28	-2.69	MRRF	0.70	0.51
KLHL25	0.53	1.19	LRRC56	1.62	0.46	MCCC2	0.78	0.57	MSL3L2	-1.08	-1.47
KLHL29	-0.89	-0.24	LRRC6	0.89	1.02	MCEE	1.34	0.86	MSRB1	1.26	0.63
KLHL36	1.10	0.56	LRRC61	1.63	0.56	MCHR1	-1.83	-2.13	MSTN	1.35	0.94
KLHL5	0.55	0.93	LRRC71	1.30	0.50	MCL1	-0.56	-0.43	MTERF2	1.30	-1.71
KLHL7	0.77	1.06	LRRC8B	-1.51	-1.05	MCM5	-0.34	0.37	MTHFD2	-0.61	-0.39
KMT2E	0.76	1.54	LRRN1	0.79	1.04	MCOLN3	-1.31	-1.30	MTMR12	-2.16	-1.36
KRAS	-0.94	-0.67	LRRN3	1.19	1.28	ME1	-0.73	-0.20	MTPAP	-0.47	0.23
LAMB3	-1.70	-3.27	LRRTM3	-0.68	-0.59	MED15	-0.79	-1.42	MTSS1	1.12	1.21
LAMP5	-2.84	-3.28	LRTM2	1.34	1.32	MED4	0.92	1.23	MTUS2	2.04	1.41
LANCL2	0.60	0.82	LTBP1	-0.54	-0.64	MED9	1.15	0.69	MUSK	-5.45	-5.02
LASP1	0.84	0.76	LUZP1	-0.90	-0.61	MEIS2	-1.14	-0.82	MUSTN1	-0.97	-1.61
LBH	-1.34	-1.59	LYPD1	0.98	0.92	MELTF	1.84	0.95	MVK	1.58	0.88
LCK	1.72	0.57	LYPLA2	0.45	-0.48	MEPCE	0.78	0.83	MXD4	0.58	0.97
LDAH	0.56	0.38	LYRM7	1.14	1.16	MEST	-0.84	-0.41	MXI1	0.50	0.42
LDB1	1.00	0.40	LYRM9	1.85	0.99	METRNL	-1.04	-1.10	MYADML2	2.60	2.16
LDLRAD4	-0.88	-0.61	LYVE1	-1.92	-2.80	METTL18	-0.63	-0.80	MYBPH	-0.96	-1.19
LEF1	-2.29	-1.63	LZIC	0.59	0.97	MFAP2	0.54	-0.40	MYC	-0.72	-0.28
LENG8	-1.19	-1.41	LZTFL1	0.62	0.42	MFAP3L	-2.32	-1.19	MYCL	2.56	3.14
LEXM	-4.36	-3.89	LZTS1	1.46	1.14	MFN1	-0.58	-0.66	MYH1	-2.25	-2.92
LGI1	1.14	1.22	M6PR	-0.55	-0.41	MFSD13A	0.64	-0.58	MYH4	-3.57	-3.30
LHFPL2	-1.40	-0.66	MAF1	1.28	0.68	MFSD14B	-0.84	0.51	MYH7	1.82	1.16
LHX2	1.18	1.20	MAFK	0.61	1.65	MFSD6	0.67	0.50	MYO1E	-2.87	-1.74
LHX4	-0.76	-1.26	MAGED2	1.15	0.59	MFSD9	0.57	0.99	MYO9B	-0.92	-1.06
LIMK2	1.19	1.13	MAGEE2	1.45	0.94	MGAT1	0.59	0.36	MYRIP	-1.15	-0.85
LINGO3	-0.96	-1.03	MAL2	1.18	1.03	MGAT5B	1.19	0.37	MYT1L	1.44	1.08
LIPA	0.52	0.50	MAML3	-2.51	-2.50	MICAL2	-0.93	-1.87	N4BP2L1	0.57	0.87
LIPG	-1.36	-0.73	MAN1A1	-2.66	-3.59	MICU3	-0.74	-0.95	NAA40	-0.40	-0.90
LIX1	1.09	0.97	MAN1A2	-1.00	-0.48	MINK1	-0.51	-0.69	NAAA	1.13	1.08
LMNA	-1.34	-1.15	MAN1C1	-1.46	-1.48	MKRN1	0.75	0.57	NAB2	-2.15	-1.18
LMO1	1.62	0.54	MAN2A1	-1.74	-1.81	MLLT3	-0.93	-0.44	NAGA	0.84	0.30
LMO2	-1.46	-2.05	MAP2K3	-1.86	-2.26	MMP10	-4.51	-5.38	NAMPT	-1.04	-0.70
LMO4	1.68	1.16	MAP2K4	-0.90	-1.01	MMP13	-5.48	-6.33	NAP1L3	0.95	1.00
LOC682102	1.51	1.25	MAP2K5	0.89	0.51	MMP17	-0.74	-0.70	NAPEPLD	-1.51	-1.43
LOC688390	1.02	1.20	MAP2K6	1.74	1.38	MMP3	-3.37	-6.66	NARF	1.28	1.01
LPAR1	-3.29	-3.21	MAP3K12	1.04	0.56	MMS19	0.69	0.26	NAT14	1.63	0.36
LPCAT2	-0.81	-1.44	MAP3K7	-0.47	-0.33	MNT	0.99	1.88	NBR1	0.38	0.53

GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC
NCALD	0.59	1.13	NR1D2	0.41	1.55	PARD6A	1.30	1.36	PIANP	1.13	0.68
NCBP1	-0.46	-0.74	NR1I3	0.70	0.79	PATL1	-0.70	0.56	PIEZO1	-1.17	-2.09
NCBP3	-0.33	-1.07	NR2F1	1.06	1.14	PAX1	-1.13	-1.80	PIGM	-1.54	0.56
NCOR2	-0.65	-0.61	NRF1	-0.96	-0.80	PBX1	0.75	1.10	PIH1D1	1.26	0.29
NCSTN	0.36	0.28	NRIP1	1.03	1.37	PCBP4	0.93	0.56	PIK3C3	1.03	1.12
NDRG4	0.99	0.82	NRIP3	-0.46	-1.02	PCDH19	1.90	1.46	PIK3CB	-0.90	-0.79
NDST3	-1.10	-0.77	NRN1	-2.28	-2.84	PCDH8	-1.31	-0.91	PIK3CD	2.00	1.25
NDST4	1.31	1.73	NRSN2	1.84	0.63	PCDHA10	0.84	1.14	PIK3IP1	1.05	1.90
NDUFAF4	-1.00	-0.38	NTF3	1.42	0.72	PCDHA12	0.84	1.11	PITX1	-3.43	-3.50
NEDD9	-0.98	-1.17	NTM	1.25	1.18	PCDHA2	0.88	1.03	PJA2	0.38	0.90
NEFL	-1.71	-1.62	NTRK3	-0.62	-0.47	PCDHA3	0.69	1.10	PKD2	-1.09	-0.52
NET1	0.48	0.55	NUAK1	0.99	1.42	PCDHA4	0.68	1.02	PKLR	2.10	1.11
NETO1	-1.09	-1.41	NUB1	0.81	1.09	PCDHA5	0.69	0.85	PKNOX1	-1.00	-1.19
NEUROD1	1.05	1.15	NUDT14	1.91	0.87	PCDHA6	0.81	1.07	PKNOX2	0.75	0.87
NEUROD2	0.50	1.01	NUDT16	1.45	0.85	PCDHA7	0.76	0.98	PLA2G4A	-1.76	-1.25
NEUROD4	0.93	1.25	NUDT18	1.29	0.44	PCDHA9	0.59	1.09	PLAGL1	-0.50	-1.15
NFATC1	-1.44	-1.28	NUDT6	-1.11	-1.68	PCDHGA2	0.63	0.90	PLAUR	-4.89	-5.47
NFIB	0.50	1.25	NUTM1	-1.01	-1.27	PCGF2	1.14	1.42	PLCL1	-2.18	-2.09
NFKBIE	0.68	0.67	NVL	-0.43	-0.26	PCGF5	-0.94	-0.86	PLCXD2	1.53	1.65
NFRKB	-0.56	-0.50	NYAP1	2.25	1.47	PCSK1	-1.17	-1.46	PLEKHG5	-1.08	-2.26
NFX1	-0.36	0.32	OGDHL	0.89	0.35	PCSK4	1.92	0.88	PLEKHH2	-2.64	-2.23
NGB	1.23	0.79	OGFOD1	-1.28	-0.51	PDCD6IP	-0.49	0.24	PLK2	-0.87	-0.35
NGF	-1.85	-2.64	OLIG2	-0.65	0.36	PDE10A	-1.50	-0.73	PLK4	-0.31	0.35
NHLH2	1.12	1.41	OPRL1	1.21	0.91	PDE12	-0.81	0.46	PLPPR3	1.31	0.56
NIPSNAP1	1.30	0.62	ORAI2	1.56	1.51	PDE6D	1.54	1.03	PLPPR4	-0.36	-0.48
NIPSNAP3B	1.46	0.76	OS9	0.89	0.68	PDE8A	-3.10	-2.75	PLXNA2	-1.10	-0.46
NIT1	0.86	0.77	OSBP2	-1.14	-2.28	PDE9A	-0.95	-1.85	PLXND1	0.89	1.00
NKAP	1.26	1.24	OSBPL1A	0.47	0.53	PDGFB	-2.76	-3.72	PMP22	-0.66	-0.34
NKD1	1.17	0.50	OSBPL2	0.51	0.50	PDK1	-0.31	-0.56	PMVK	1.86	0.80
NKIRAS2	0.89	0.65	OSBPL6	-1.46	-1.08	PDP1	-2.00	-1.47	PNCK	1.77	0.60
NMBR	-0.97	-1.74	OSCAR	-1.07	-2.14	PDSS2	1.60	1.41	PNMA2	1.23	1.08
NME7	0.62	-1.12	OSCP1	0.87	0.34	PDXK	-2.50	-2.12	PNMAL1	1.44	1.81
NOC3L	-0.67	-0.41	OTUD3	-1.24	-0.91	PDXP	1.20	0.90	PNMAL2	0.62	1.20
NOCT	-0.38	-0.30	OVCA2	0.88	0.46	PDZD8	-0.96	-0.46	PNPO	0.63	0.34
NOG	-0.91	-1.37	OXTR	-1.51	-2.05	PEAK1	-2.51	-1.85	PNRC1	1.24	1.53
NOL10	-0.35	-0.81	P2RX2	1.58	1.42	PECAM1	1.63	1.43	PODXL	0.63	0.69
NOL3	1.47	0.60	P2RY1	0.71	1.37	PENK	-2.98	-3.53	PODXL2	1.23	0.83
NOP2	0.28	-0.73	P2RY14	-3.15	-3.74	PEO1	1.10	0.73	POLD1	0.31	-0.87
NOSIP	1.58	0.41	P4HA1	-0.59	-0.45	PEX14	1.05	0.76	POLE4	1.21	0.84
NOTCH4	1.02	0.47	PACSIN1	-0.70	-0.98	PFKFB3	-2.15	-0.88	POLG	-1.03	-1.05
NOV	1.44	1.28	PACSIN2	-0.68	-0.53	PGD	0.75	0.62	POLL	1.26	0.73
NPAP60	-0.42	0.31	PAFAH1B3	1.49	0.86	PHACTR1	0.76	0.98	POLR1A	-0.62	-0.59
NPL	-2.44	-3.39	PAK6	-0.74	-0.37	PHACTR3	0.97	1.01	POLR3E	-0.65	-0.86
NPLOC4	-0.43	-0.74	PAK7	0.51	0.96	PHEX	-3.10	-2.09	POMK	0.81	0.63
NPTX1	-2.65	-3.31	PALMD	1.18	0.66	PHF14	0.49	0.61	POP1	-0.63	-1.68
NPTX2	-3.61	-3.41	PAM	-1.42	-1.10	PHF20	0.63	0.59	POPDC2	1.42	1.65
NPY1R	1.49	1.06	PANX1	-1.03	-0.91	PHLDA1	-1.04	-1.03	POPDC3	-0.69	-1.02
NPY2R	-1.33	-1.87	PAPOLA	-0.51	-1.78	PHOSPHO2	-0.25	-0.33	POU4F2	-4.18	-4.53
NR1D1	0.51	1.03	PAPPA	-2.38	-1.72	PI16	1.55	0.89	POU4F3	-3.26	-4.39

GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC
POU6F1	-1.14	-0.42	PTPRH	1.98	0.88	RBPMS	-0.87	-0.96	RPS6KB1	-0.62	-1.15
PPEF1	1.23	0.72	PTPRN	-0.87	-0.99	RCAN2	-0.77	-0.84	RRAD	-3.13	-3.59
PPFIA4	1.32	0.87	PTPRR	2.13	1.93	RDH10	-1.58	-1.78	RRAGD	0.99	1.40
PPHLN1	-0.90	-0.54	PURG	-0.60	-0.93	REEP1	0.88	1.06	RRP8	-0.91	-0.63
PPIF	-0.41	-0.40	PUS3	-0.87	-0.37	REEP2	0.92	0.58	RSPO3	1.82	1.55
PPM1H	-0.74	-0.36	PUS7	-0.76	-1.33	REM2	-0.65	-2.38	RSRP1	-0.61	0.87
PPP1CA	0.35	0.28	PVR	-1.52	-1.43	RET	-1.36	-1.35	RT1-S3	-2.68	-2.34
PPP1R12A	-0.46	0.30	PVRL1	-0.62	-0.75	RFXAP	-0.50	-0.68	RTF1	0.81	0.80
PPP1R18	1.58	1.45	PWWP2B	-0.59	-1.25	RGD1307443	0.87	0.83	RTFDC1	0.59	0.83
PPP2R2B	1.08	0.91	PXN	-0.61	-0.54	RGD1309028	1.25	1.09	RTN4RL1	-1.12	-1.86
PPP2R3A	0.94	0.60	QDPR	1.26	0.90	RGD1560108	0.96	1.10	RTN4RL2	-1.19	-0.89
PPP2R5A	-0.53	0.22	QPCT	1.20	0.69	RGS12	1.00	0.62	RUNX1	-2.42	-2.92
PPP3CC	0.79	-0.38	R3HDM1	-0.94	-0.92	RGS14	1.34	0.79	RUNX1T1	0.62	1.14
PPP4R3A	-0.26	0.61	RAB11B	0.88	0.84	RGS16	-2.01	-1.34	RUNX2	-1.89	-3.71
PPRC1	-0.75	-0.65	RAB20	-1.37	-0.76	RGS17	-0.70	-1.49	RXFP3	-2.74	-1.90
PRAGMIN	-1.57	-1.48	RAB27B	-1.70	-0.99	RGS2	-0.40	-1.13	RXRB	1.47	0.43
PRICKLE1	-1.52	-1.67	RAB2B	0.89	0.58	RGS20	-1.52	-0.91	RXRG	2.59	1.70
PRIMA1	-4.53	-5.31	RAB30	1.20	1.88	RGS4	-1.47	-2.11	RYBP	-0.29	-0.20
PRKAA1	-0.53	-0.59	RAB33A	2.08	1.41	RGS5	-2.49	-2.24	RYK	-0.50	-0.23
PRKAR2A	-1.73	-1.02	RAB36	1.07	0.55	RHBDF2	2.34	1.22	SAMD4A	-2.26	-1.31
PRKCQ	0.70	1.18	RAB3A	1.00	0.94	RHBDL1	1.62	0.85	SAMD5	-1.50	-1.16
PRKCZ	0.98	0.79	RAB3D	1.71	1.03	RHOQ	-1.28	-0.70	SAMHD1	-0.70	-1.09
PRKG2	-1.43	-0.37	RAB43	-1.06	-1.39	RHOV	2.48	1.85	SAP30BP	-0.26	-0.41
PRKRA	1.26	0.36	RAB9B	0.65	0.92	RHPN2	0.94	1.04	SATB1	0.88	0.90
PRMT2	1.35	0.78	RAD21	0.47	0.79	RIN1	-2.48	-2.61	SC5D	-0.58	0.82
PRR13	0.84	0.43	RAI14	-1.46	-0.91	RIT1	0.44	1.05	SCG2	-2.73	-2.63
PRR15	-1.01	-2.33	RALGAPA1	-0.49	-0.75	RIT2	1.38	1.25	SCLY	0.80	0.39
PRRT1	1.21	1.04	RALGDS	0.94	0.42	RNASEH2A	1.67	0.63	SCN1A	1.08	1.63
PRRT2	1.00	0.74	RALYL	1.38	0.99	RND1	1.71	1.01	SCN1B	-2.04	-3.07
PRRT4	1.82	0.96	RAMP2	1.58	0.91	RNF111	-0.31	0.50	SCN2A	1.29	0.92
PRSS12	1.68	1.25	RAMP3	2.99	1.95	RNF145	0.69	1.32	SCN3B	0.89	1.18
PRSS23	-2.58	-2.57	RANBP6	-0.21	0.46	RNF150	-0.74	-0.68	SCN4A	2.26	1.82
PRSS35	-2.02	-2.16	RAP1B	-0.72	-0.40	RNF152	1.91	2.65	SCNN1G	-1.56	-1.63
PRUNE	0.67	1.06	RASA1	-0.78	-0.36	RNF167	0.68	0.74	SCRT2	-2.06	-2.52
PSD2	1.26	0.67	RASAL2	-1.38	-1.02	RNF180	0.40	0.58	SDC1	-1.30	-2.16
PSENEN	1.34	0.86	RASGEF1C	1.61	1.16	RNF182	1.58	1.19	SDCBP	-0.39	-0.39
PSME3	-0.26	-0.27	RASGRF1	0.56	0.68	RNF19B	0.67	0.31	SDCCAG8	1.55	0.71
PSME4	-0.71	-0.54	RASGRP2	1.70	0.86	RNF217	-0.65	-0.41	SDHC	0.99	0.85
PSMG1	1.26	0.74	RASIP1	1.80	0.78	RNF34	0.42	0.37	SDK2	-1.95	-2.31
PSTPIP2	-1.28	-0.99	RASL11A	-3.71	-3.77	RNF39	-0.73	-0.61	SDR39U1	1.14	0.59
PTAFR	-4.05	-4.10	RASSF8	-3.70	-2.75	RNF5	1.02	0.56	SEC13	0.34	-0.31
PTBP1	-0.45	0.51	RAVER1	-0.34	-0.99	RNFT2	0.75	0.58	SEC16B	-2.16	-2.56
PTCHD1	-1.86	-1.46	RBAK	-0.73	0.58	RNGTT	0.58	0.66	SEC24B	-0.55	0.30
PTGER4	-2.13	-3.43	RBFA	1.17	0.73	ROCK2	-0.98	-0.51	SEC24D	-1.62	-1.30
PTGS2	-2.32	-3.82	RBM11	2.23	1.39	RPH3A	0.86	1.11	SELE	-1.92	-3.91
PTH2R	-2.45	-3.22	RBM12	-0.82	-1.74	RPS6KA2	1.37	1.35	SEMA3A	-2.35	-1.82
PTPDC1	-0.89	-0.53	RBM24	1.45	1.61	RPS6KA3	-0.87	-0.57	SEMA3D	-1.22	-1.01
PTPN1	-0.38	0.28	RBM46	1.03	-1.23	RPS6KA5	0.63	0.72	SEMA3E	-0.87	-0.77
PTPRE	-0.61	-0.86	RBMS1	-1.90	-2.70	RPS6KA6	-0.65	-0.44	SEMA4F	1.42	0.68

GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC
SEMA4G	1.18	0.81	SLC25A20	0.85	0.42	SOAT1	-1.53	-0.68	STX11	-3.50	-3.90
SEMA6B	1.12	0.82	SLC25A37	-1.89	-1.79	SOCS3	-1.72	-1.68	SULT2B1	1.11	0.51
SEMA6C	2.21	1.45	SLC25A39	1.32	0.66	SOGA3	0.78	0.78	SUN1	-0.84	-1.27
SEP6	1.00	1.06	SLC27A2	1.19	0.78	SORBS2	-1.71	-1.56	SUOX	0.61	-0.42
SEPHS1	-0.42	-0.41	SLC28A3	1.94	2.30	SOX4	1.25	1.18	SV2A	1.02	0.49
SERPINB2	-4.88	-6.63	SLC2A3	-1.55	-1.00	SP7	-4.59	-3.87	SV2B	1.27	1.72
SERPINB8	-2.83	-3.12	SLC30A10	-1.26	-0.51	SPAST	0.56	0.40	SV2C	-3.50	-2.31
SERTAD1	-1.59	-2.58	SLC30A3	-0.73	-1.96	SPATA2L	-0.43	-1.16	SVBP	1.74	0.64
SERTM1	-0.83	-0.97	SLC30A4	-0.84	-0.42	SPCS3	-0.78	-0.49	SVOP	1.19	0.60
SESN1	1.06	1.38	SLC35B4	0.37	0.55	SPHK1	-3.06	-4.48	SYK	-0.53	-0.82
SFI1	0.85	-1.13	SLC35D1	-1.62	-0.60	SPHKAP	1.48	0.86	SYNJ2	-1.72	-2.07
SFMBT1	-0.63	-0.63	SLC35D3	-1.69	-0.77	SPIRE1	-0.45	-0.41	SYNPO	-1.39	-2.06
SFMBT2	-1.64	-1.70	SLC35E3	-0.41	-0.79	SPIRE2	1.35	1.41	SYPL2	-2.38	-2.31
SFRP4	-1.08	-1.11	SLC35F5	0.37	0.53	SPNS2	-0.66	-1.32	SYT10	-3.58	-3.20
SGCA	-1.97	-1.74	SLC37A4	1.13	0.40	SPOP	1.04	0.89	SYT12	-0.96	-1.16
SGMS1	-0.87	-0.42	SLC40A1	-2.23	-1.60	SPRED3	-1.85	-2.12	SYT13	0.61	0.80
SH2B2	1.18	1.23	SLC41A2	-1.28	-1.47	SPRR1A	-5.06	-5.27	SYT3	1.61	0.74
SH2D3C	3.90	3.50	SLC4A11	-1.71	-2.12	SPRTN	-1.17	0.47	SYT6	-0.76	-1.18
SH3BP1	-0.76	-0.43	SLC4A7	-2.08	-2.06	SPRY2	-1.61	-0.95	SYT9	0.76	0.91
SH3BP5	1.11	1.00	SLC5A10	-1.87	-1.93	SPRY4	-2.69	-1.38	SYTL5	-1.75	-2.05
SH3GLB1	-0.40	-0.49	SLC5A3	-1.52	-1.68	SPSB1	-2.02	-1.90	TAC1	-1.18	-1.70
SHC4	-2.42	-3.15	SLC5A8	-1.73	-2.28	SQLE	0.31	0.62	TACC1	-1.16	-0.77
SHISA5	0.86	0.66	SLC6A15	0.71	0.77	SRD5A1	0.85	0.72	TACR2	-2.09	-2.72
SHQ1	-0.90	-1.18	SLC6A17	-1.30	-1.58	SRPK1	-0.41	-0.26	TACR3	-2.05	-2.64
SHROOM2	0.94	1.49	SLC6A7	1.19	0.83	SRPRA	-0.30	-0.69	TADA2B	-0.44	0.25
SHROOM4	-3.29	-1.94	SLC6A8	-0.67	-1.48	SRRM3	0.93	0.51	TAF12	-0.51	-1.02
SHTN1	0.75	1.00	SLC9A2	-1.35	-1.64	SRSF9	0.43	0.79	TAGAP	-1.81	-2.34
SIAH3	-2.74	-1.86	SLC9B2	1.97	0.80	SRXN1	-4.26	-2.64	TARS	-0.58	-0.78
SIDT1	0.91	1.03	SLCO2A1	-4.59	-2.76	SS18	-0.85	-0.51	TAS1R3	-1.86	-1.01
SIK1	-1.64	-1.35	SLCO3A1	-1.38	-0.44	SSR1	-0.20	0.36	TBC1D14	0.71	0.85
SIK3	-1.20	-1.08	SLCO4A1	1.24	0.84	SSTR2	-0.92	-1.43	TBC1D17	1.25	0.56
SIL1	1.59	0.44	SLITRK4	0.98	0.95	SSTR3	-3.10	-3.46	TBC1D25	0.56	0.69
SIM1	-2.92	-5.54	SMAD3	-2.03	-0.73	SSTR4	-1.95	-2.44	TBC1D9	0.79	0.61
SIN3A	0.41	1.28	SMAGP	-2.02	-2.95	ST18	-1.02	-1.41	TBCK	0.83	0.52
SIPA1L2	-0.81	-0.70	SMARCA2	0.89	0.68	ST3GAL2	0.83	0.75	TBX21	-1.78	-3.92
SIRT3	1.12	0.41	SMARCD3	1.46	0.51	ST3GAL3	0.82	1.07	TCEAL1	0.98	0.90
SIRT5	1.80	0.74	SMIM17	1.05	1.11	ST6GAL1	-0.37	-0.80	TCP11L2	0.66	0.39
SKP2	-1.16	0.74	SMIM20	1.14	0.49	ST6GALNAC6	0.96	1.03	TCTN1	0.78	1.49
SLAIN2	-0.46	-0.27	SMIM3	0.82	0.64	ST8SIA5	-2.47	-2.61	TDRD6	2.30	1.58
SLC16A14	-2.19	-1.93	SMOC2	2.27	1.44	STAC2	-0.91	-1.16	TEAD1	-2.51	-1.05
SLC16A8	-1.30	-1.45	SMPD3	1.05	0.85	STAMBP	0.91	0.71	TEF	0.61	1.39
SLC17A5	0.61	0.48	SMPDL3B	-2.79	-3.14	STC2	-1.63	-0.96	TEKT2	1.80	0.81
SLC17A6	-0.97	-0.90	SMR3B	-1.36	-1.83	STEAP1	-1.23	-1.56	TENM3	0.91	1.23
SLC18A2	-0.70	-0.78	SMTN	-1.97	-2.91	STEAP2	-0.30	-1.32	TEX264	1.29	0.70
SLC1A6	1.30	0.92	SMYD5	0.71	0.34	STIM1	0.49	0.37	TFAP4	0.83	1.48
SLC22A1	4.30	2.94	SNCA	1.29	1.17	STK26	-3.24	-3.62	TGFB1	-0.95	-1.37
SLC22A3	2.55	1.77	SNN	0.45	0.79	STOML1	1.39	1.21	TGFBR2	-2.45	-1.41
SLC24A4	-0.37	-0.61	SNRNP40	0.37	0.50	STRADA	0.83	-0.28	TGIF1	-1.10	-0.90
SLC25A14	0.50	-0.38	SNX9	-1.36	-0.94	STT3B	-0.82	-0.86	THBD	-1.78	-2.09

GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC	GENE	WP FC	RX2 FC
THNSL1	0.41	0.38	TPH1	-1.76	-2.70	UOX	-2.72	-4.40	ZBTB16	1.20	1.79
THRB	-1.88	-1.76	TPK1	1.10	0.83	USF1	0.79	0.82	ZBTB22	1.00	1.18
THSD7A	0.64	0.95	TPM4	-0.79	-0.63	USO1	-0.35	-0.84	ZBTB46	-1.41	-1.56
TIGAR	-0.44	-0.40	TPPP3	2.49	1.43	USP11	0.58	0.36	ZBTB5	0.53	1.36
TIMP1	-1.59	-1.15	TRAPPC2B	1.48	0.77	USP20	1.08	0.67	ZBTB9	-0.53	0.36
TLK2	-0.35	0.68	TRAPPC3	1.18	0.73	USP28	-0.79	-1.16	ZC2HC1A	1.06	0.99
TM4SF1	-1.89	-1.95	TRERF1	0.67	1.40	USP46	0.68	0.56	ZC3H18	-0.48	-0.42
TMCC2	1.10	1.06	TRIB1	-2.34	-1.48	USP49	0.68	1.43	ZC3H8	0.77	0.61
TMCC3	-0.96	-0.53	TRIB2	-0.86	-0.60	UST	-1.31	-0.83	ZC4H2	1.32	1.17
TMEM106B	0.32	0.47	TRIM23	0.63	0.39	UTP15	-0.92	0.41	ZDHHC14	-0.55	-1.32
TMEM106C	1.07	0.72	TRIM45	1.01	-1.30	UTP20	-0.92	-1.06	ZDHHC20	-0.73	-0.44
TMEM107	1.56	0.32	TRIM9	-1.87	-1.66	VAC14	0.92	0.70	ZDHHC5	-0.77	-0.41
TMEM141	1.84	0.62	TRIQK	1.44	1.02	VASP	-1.26	-2.21	ZFAND2B	1.19	-0.50
TMEM150C	1.59	0.74	TRMT10C	-0.39	0.44	VCL	-1.68	-0.68	ZFAND6	1.02	0.54
TMEM163	-0.71	-1.22	TRMT6	-0.89	-0.44	VEGFA	-1.07	-1.35	ZFAT	-0.37	-0.64
TMEM179	1.21	-1.34	TRPC1	0.64	0.48	VGF	-3.89	-4.29	ZFP217	-2.16	-1.51
TMEM182	-2.99	-2.45	TRPC4	1.10	1.01	VGLL4	-0.45	0.60	ZFP238	1.11	1.49
TMEM184B	0.40	0.39	TRPC6	-3.41	-4.23	VIPR1	-2.18	-2.55	ZFP358	0.99	0.69
TMEM19	0.86	0.39	TRPC7	1.17	1.37	VKORC1	1.41	0.82	ZFP362	0.49	0.77
TMEM200C	1.03	1.08	TRPM4	1.40	0.74	VOPP1	0.84	0.85	ZFP395	0.55	0.71
TMEM202	-1.22	-1.53	TRPT1	1.09	-0.56	VPS25	0.98	0.33	ZFP414	1.27	-0.92
TMEM218	1.48	0.93	TRPV6	1.32	0.39	VPS53	0.69	0.30	ZFP467	0.88	0.53
TMEM229B	0.89	0.89	TSC22D1	0.62	0.50	VSTM2B	-0.90	-1.16	ZFP513	0.50	0.38
TMEM246	1.44	0.68	TSPAN17	1.59	0.80	VWA1	2.24	1.00	ZFP575	1.45	0.78
TMEM25	1.23	-1.33	TSPAN2	0.83	0.64	VWA5B2	1.10	0.49	ZFP672	-0.68	-0.36
TMEM39A	0.63	0.63	TSPAN5	-0.75	-0.55	VWC2	-0.75	-1.68	ZFP697	-1.33	-0.80
TMEM41A	-0.72	-1.30	TSPYL1	0.56	0.95	VWC2L	2.18	2.16	ZFP709	-0.77	0.40
TMEM42	1.29	0.53	TTC19	-0.53	-0.63	WASH1	1.41	0.33	ZFP775	1.25	1.21
TMEM45A	-2.08	-2.55	TTC9	-0.55	-0.84	WDHD1	-0.54	-0.66	ZFP786	0.84	1.03
TMEM55B	0.97	1.16	TTLL1	1.31	0.68	WDR1	-0.49	-0.44	ZFP90	1.15	1.13
TMEM65	-0.78	-1.21	TTPAL	-1.03	-1.31	WDR34	1.40	0.78	ZFPM1	-1.17	-1.51
TMEM68	0.98	0.86	TUBA8	-1.27	-2.31	WDR43	-0.65	0.31	ZFYVE1	0.55	1.14
TMEM8A	0.99	-0.36	TUBB4B	0.43	0.29	WDR45	1.13	0.42	ZFYVE19	1.45	-0.44
TMIE	2.15	1.41	TUBG2	1.35	0.74	WDR45B	-0.67	-0.44	ZFYVE9	-0.70	-0.62
TMOD1	-0.80	-1.69	TUBGCP3	-0.41	-0.29	WDR6	0.90	0.95	ZGPAT	0.54	0.88
TMPRSS6	-2.13	-1.51	TUSC5	-2.46	-2.84	WFS1	-0.65	-0.45	ZMIZ1	-1.38	-1.19
TMTC4	1.45	1.15	TWIST1	-0.96	-1.17	WIPI2	-0.44	-0.37	ZMYND8	0.61	0.70
TNFAIP1	0.38	0.60	TXNIP	1.41	1.94	WISP1	-2.86	-1.76	ZNRF2	0.74	1.20
TNFRSF11A	0.84	0.66	TXNRD1	-0.75	-0.43	WNT6	-2.98	-3.77	ZPBP2	0.88	-1.03
TNFRSF12A	-1.58	-0.93	TYMS	-0.87	-0.71	WNT7A	0.55	0.68	ZW10	-0.37	-0.28
TNFRSF25	-2.01	-3.19	TYR	2.11	1.62	WSCD1	-0.64	-0.78	ZYX	-0.74	-2.13
TNIP1	0.55	0.42	TYRO3	-0.39	-1.14	WSCD2	1.47	1.55			
TNIP2	-0.99	-0.84	UAP1	-0.38	-0.67	XKR4	0.65	1.07			
TNRC18	0.62	1.24	UBASH3B	-2.50	-1.96	XKR7	-1.95	-1.24			
TOR1AIP1	-0.39	0.40	UBE2D1	1.01	0.89	XKR8	-1.11	-1.69			
TOX3	1.00	1.46	UHRF1BP1L	-0.48	-0.76	XPO4	-0.98	-0.48			
TP73	-1.82	-1.42	ULK2	0.43	0.19	XRCC4	1.13	0.64			
TPBG	-1.22	-1.74	UNC119	1.48	0.80	YPEL3	1.66	1.03			
TPD52L3	2.16	1.54	UNC5B	-2.81	-1.47	YPEL5	0.98	1.13			

For all lists of gene sets from Chapter III, please see this link:

https://docs.google.com/spreadsheets/d/1hRzY5BWQHQtfVJ3ll7cbh_t3EUwURed410UY

UYMReuS/edit?usp=sharing

Table A2. Genes differentially expressed due to KA treatment in WT animals, ipsilateral side.

GENE	LOG2FC	GENE	LOG2FC	GENE	LOG2FC	GENE	LOG2FC	GENE	LOG2FC
ABCC2	-2.30	CCDC8	-1.62	DPH1	-3.07	GPR39	0.74	KLHL34	0.83
ABCC6	-5.48	CCDC88B	1.29	DTNBP1	-2.23	GRCC10	-3.03	KLHL8	-3.30
ACAA1A	-1.14	CDH4	-3.22	DTX1	0.86	GSS	0.88	KLRK1	-4.64
ACADSB	-4.88	CDK17	-1.01	DUSP18	-1.91	GTF2E2	-3.18	LAMC2	-2.36
ACOD1	-4.99	CDK4	-0.64	DYNC1H1	-2.12	GTPBP8	-0.67	LARGE1	-1.85
ACP1	-1.50	CEBPG	1.20	ECM1	-1.19	GYS1	-2.73	LCT	-2.61
ACP6	-4.03	CECR2	-2.77	EHF	-5.09	H2-T22	-1.29	LEFTY1	3.56
ACRV1	-2.68	CHIL5	1.58	EIF2AK4	-1.37	HACD4	1.01	LEMD3	-2.90
ACTL10	1.01	CHN1OS1	0.97	ELMO3	0.50	HCN4	-2.51	LGR5	-4.62
ADCY4	-3.49	CHN1OS3	0.77	EME1	-0.71	HECW1	-2.27	LIMS2	1.65
AHNAK2	-5.45	CHPF2	0.65	EMILIN1	-4.61	HHATL	-1.28	LIN28B	-2.48
AI849053	0.98	CHRNA4	-2.13	EPHA1	-1.12	HIPK4	0.73	LPO	-3.20
ALDH6A1	0.51	CIAPIN1	-2.68	EPM2AIP1	-3.32	HOPXOS	1.21	LRP3	0.97
ANAPC11	-2.34	CKAP5	-2.67	EPS8L3	1.18	HRNR	-3.43	LRP4	-3.99
ANAPC7	-2.73	CLN3	-2.21	ERGIC1	-3.11	HTR1A	1.86	LRRC40	0.65
ANK2	-1.38	CLVS1	-0.93	ERMARD	-0.75	IFITM7	0.59	LRRC7	-1.48
ANKMY1	0.56	CNIH4	-2.01	EVI5L	1.16	IGF2BP2	-4.83	LRTM1	0.66
ANKRD33B	-1.43	CNOT1	-1.74	FAM120B	-3.25	IGSF10	0.64	LYL1	1.09
AP1S3	0.37	COL4A3BP	-3.47	FAM13A	-2.15	IKBIP	-4.00	LZTFL1	-1.67
APC2	-0.92	COQ10B	-4.14	FAM151A	1.18	IL21	-1.94	MACROD2OS2	0.60
APOL6	-2.65	COQ9	-2.97	FAM163A	-4.89	IL23R	-5.80	MAP3K4	-1.74
ARAP3	-5.00	CPNE8	-2.69	FAM168B	0.95	ILDR1	-3.17	MAR1	-1.29
ARFGEF3	1.13	CPTP	-1.92	FAM32A	-1.89	ILKAP	-4.32	MCM6	-3.91
ARL4AOS	-5.30	CREBBP	-1.69	FANCC	-0.60	INHBA	1.64	MCMDC2	-0.78
ARL5C	-1.49	CREBL2	-1.02	FBL	-0.88	INTS9	-1.94	MED8	-4.99
ARMC9	-3.30	CRTAM	-6.23	FBXL22	0.52	ITGA1	-1.75	MEDAG	-4.13
ARMCX2	-2.75	CTC1	-2.38	FBXO7	0.68	ITGAV	-1.50	MEST	0.79
ASH2L	-1.17	CTNND2	-2.64	FLG	-3.04	JAK1	2.15	METTL3	-0.67
ATF7	-1.66	CTSA	0.88	FLG2	-6.86	JHY	-6.20	MFSD8	-1.40
B3GNT8	0.72	CTU2	-1.35	FLRT3	0.61	JMY	-1.19	MGAT5	-1.98
BANP	-1.45	CUL7	-4.62	FMN1	-2.96	JRK	-3.57	MIB2	-2.74
BBIP1	0.79	CXADR	-1.29	FNDC3C1	-5.92	JUNB	0.65	MID1	-2.94
BCAP29	-3.09	CXXC4	1.29	FOSL1	0.74	JUND	0.84	MID1-PS1	-4.20
BHMT	-4.93	CYTH2	-0.98	FPR1	-1.61	KANK3	-0.85	MMP23	-1.45
BICC1	-4.05	DALRD3	0.93	FSCN2	-1.26	KARS	-3.42	MMP24	0.57
BMP2K	0.59	DCST1	0.75	GALE	-1.61	KBTBD2	-1.83	MOB1A	-3.33
BOLA3	-1.01	DCX	-4.78	GCSAM	-3.60	KCNMB2	-2.72	MORN1	-1.38
C1QTNF7	-5.14	DEGS2	-0.67	GDA	-2.67	KCNMB4OS1	0.60	MPG	0.59
C2CD4B	1.08	DESI2	-3.53	GGCX	-1.90	KCNMB4OS2	0.77	MPLKIP	-2.04
CAMK2D	-2.83	DLGAP1	-3.87	GINS2	0.75	KCNQ1OT1	-0.87	MRPS34	-1.52
CAMKK2	-2.41	DLST	-1.87	GJA8	-2.49	KCTD10	1.54	MTAG2	1.54
CAPN12	-0.76	DLX1AS	0.85	GNAT2	-1.23	KDM6BOS	-1.02	MTF1	0.62
CAPN2	2.13	DNAH12	-4.14	GNPDA1	-1.41	KDM7A	-1.69	MTF2	-2.04
CARLR	-1.44	DNAH7A	-4.63	GOLGB1	-2.03	KHK	-3.66	MTG1	0.52
CARMIL3	1.06	DNAJB2	-2.24	GOSR2	-3.39	KHNYN	0.47	MUC16	-4.67
CCDC117	-1.30	DNASE1L3	-5.34	GPATCH8	-2.42	KIF24	-0.56	MUC2	-5.02
CCDC155	-3.23	DNM3	-1.44	GPR137	1.17	KLB	-1.08	MUC5AC	-3.86
CCDC167	0.47	DNMT3L-PS1	-1.30	GPR171	0.68	KLHL20	-2.13	MUC5B	-1.45

GENE	LOG2FC	GENE	LOG2FC	GENE	LOG2FC	GENE	LOG2FC	GENE	LOG2FC
MYC	1.25	PDAP1	-1.15	PRUNE2	-0.91	SLC6A21	-5.70	TMEM175	0.44
MYEF2	2.07	PDE2A	-3.22	PSMD1	-2.62	SLC9A9	-3.44	TMEM184A	-1.41
MYO1H	-0.56	PDE4D	-1.24	PTGIR	-3.80	SMAP1	1.10	TMEM238	-4.89
MYO3B	-3.64	PDE4DIP	-1.95	PTK6	-4.85	SMPD4	-1.17	TMEM54	1.22
MYOC	-4.93	PDE6H	-1.34	PTP4A3	-2.84	SNPH	-1.68	TMEM88	0.59
NAA35	0.77	PDE7B	-3.37	RAP1GDS1	-1.86	SORCS3	-1.95	TMTC2	-3.12
NAIF1	-0.83	PDZD4	-1.18	RASSF8	0.64	SOWAHA	0.92	TNFAIP8	2.72
NARFL	0.81	PEAR1	-2.39	RETREG3	-2.74	SPATA33	-1.34	TOM1L2	-1.70
NAT8F1	2.35	PFDN2	1.71	RFC2	-0.88	SPHK1	-1.89	TOR2A	-3.45
NCAPD2	-2.31	PFPL	-5.56	RIC8A	0.55	SPHK2	1.97	TPSG1	1.09
NDUFS2	-0.94	PHACTR1	-2.16	RLN1	-4.52	SPOPL	-0.50	TRAF3IP2	0.48
NEFH	0.78	PHRF1	-2.64	RNF113A1	-1.53	SPTLC2	-3.37	TRAPPC13	-0.72
NELFA	-1.31	PIGB	0.50	RNF123	-0.87	SRRM3	-2.27	TRIM2	0.87
NEMF	0.51	PIGF	-0.88	RPL18A	-5.18	ST3GAL6	-0.78	TRIM37	-1.09
NEU1	-2.35	PIH1D2	-5.31	RPL28	-1.87	ST8SIA3	-1.79	TRIQK	2.39
NF2	1.34	PIK3C2A	-0.71	RPRD1B	-1.86	ST8SIA3OS	-0.59	TRP53COR1	-3.05
NHSL1	0.99	PLA2G3	1.14	RPS11	1.79	STAT5A	-1.26	TSC22D1	-1.43
NME7	0.50	PLCB2	-2.26	RPS4L	1.09	STK16	0.81	TSFM	-3.65
NOC2L	-2.22	PLEKHH2	1.31	RPS6KB1	-1.11	SWT1	-1.63	TSR1	0.46
NPTX2	-2.53	PLIN3	-1.71	RPS7-PS2	-1.69	SYCP2	0.87	TSTD2	-0.53
NRG3OS	0.78	PLOD3	-0.91	RPTN	-5.30	SYNRG	-1.73	TTC14	0.84
NRM	-1.45	PLPPR3	-0.62	RRM2B	-2.39	SYT1	-2.83	TTC21A	-4.26
NSD1	-1.46	PLXNB3	-2.27	RTL5	1.06	TARDBP	-2.08	TTC9	-2.43
NTM	-2.10	PMM1	-1.68	RTN4	0.70	TBC1D14	-5.56	TTC9B	0.46
NUDT6	-1.73	POGZ	-1.09	RUFY3	-1.63	TBC1D23	-2.18	TTI2	-0.53
NWD1	-4.18	POLG	-2.81	SART3	-1.67	TBPL1	1.56	TTLL11	-1.38
NXPE4	-3.67	POLH	-2.80	SCG2	-1.93	TBX4	-5.12	TTLL13	-2.60
OLFR1342	-5.11	POLQ	0.66	SCN3B	-0.80	TCAF1	-2.47	TTN	-0.75
OLFR1440	-3.57	POLR2B	-1.10	SCUBE3	0.53	TCAF2	-4.05	TULP4	-0.68
OLFR1508	-4.98	POU3F1	1.63	SDCCAG8	0.77	TCAF3	-2.44	TXLNB	-1.42
OLFR218	-5.34	PPAN	-3.59	SDHAF2	-5.40	TCHP	-1.76	UBASH3B	-3.04
OLFR31	-1.98	PPM1L	-1.57	SERPINA12	-1.48	TECPR1	-2.55	UBE2C	-3.22
OLFR418	-5.65	PPP1R18	-1.81	SERPINA1F	-4.44	TEX26	-4.55	USP43	-5.35
OLFR455	-3.07	PPP2R1B	-0.84	SETD5	-3.62	TFDP2	-1.48	VAMP3	1.32
OLFR457	-2.59	PPP2R3C	-0.90	SF3B4	-3.56	THBD	0.96	VAMP4	-2.06
OLFR524	1.92	PPP4C	-2.61	SGK3	-2.25	THOC1	0.49	VCP	-3.37
OLFR681	-3.09	PRCD	1.06	SH3D21	-2.24	THUMPD2	-3.63	VLDLR	1.19
OLFR91	-2.64	PRDM11	-1.54	SHE	-1.99	TIMP3	0.64	VPS33A	-4.96
OLFR92	-5.48	PRDM9	-2.96	SHROOM3	-4.65	TIMP4	0.68	VPS33B	-4.95
ONECUT2	-2.05	PRELID3A	-1.34	SIAE	-0.62	TIPARP	-3.92	VSTM2B	1.41
ORAOV1	-1.55	PREPL	-2.15	SIN3B	-1.30	TKTL1	-3.41	VWA5B1	-3.32
ORC4	0.66	PRKAB2	-3.20	SKIV2L	-2.06	TLN1	-2.18	WDR47	-3.03
OS9	-1.33	PRKAG2OS2	0.56	SLC3A2	-1.86	TMBIM1	0.56	WDR95	-2.93
OVCA2	-3.03	PRLR	-2.96	SLC4A5	-1.75	TMC3	1.05	WNK1	-0.99
P2RY12	0.92	PRMT5	-5.70	SLC4A7	-2.23	TMEM126A	-0.65	ZBTB37	-2.99
P2RY14	0.52	PROX2	0.50	SLC6A12	-1.56	TMEM126B	-2.96	ZC3H12B	-0.92
PCBP2	0.46	PRPF3	-4.26	SLC6A13	-6.10	TMEM132A	-1.14	ZCCHC11	-0.80
PCGF3	-2.50	PRUNE1	-3.41	SLC6A20A	-1.49	TMEM150COS	0.82	ZDHHC20	-1.59

GENE	LOG2FC
ZDHHC6	-0.62
ZEB2OS	1.17
ZFC3H1	-1.31
ZFP113	-1.80
ZFP202	-3.90
ZFP35	-1.88
ZFP451	-4.35
ZFP532	-1.54
ZFP583	-1.39
ZFP606	-3.48
ZFP623	-3.15
ZFP950	-1.23
ZFP964	-4.95
ZFPM1	-1.47
ZHX2	-3.02
ZMAT3	-1.21
ZMIZ1OS1	-0.84
ZMYND11	-1.47
ZMYND15	-1.50
ZSCAN12	-3.90
ZZZ3	0.82

Table A3. Genes differentially expressed due to KA treatment in WT animals bilaterally.

GENE
ACP1
ARL5C
ART3
BC024978
CAPN12
FAM151A
JAK1
KHK
LETM2
LRP3
MCMDC2
MID1
NAT8F1
NUDT6
ORAOV1
OVCA2
PLPPR4
PPP2R1B
RTL5
SMPD4
SPHK2
SPOPL
TMEM132A
TTI2
TULP4
ZEB2OS

Table A4. Genes protected from KA effects by STAT3 knockdown, ipsilateral side.

GENE	GENE	GENE	GENE	GENE	GENE
ACAA1A	DALRD3	KHNYN	OS9	SMAP1	ZDHHC6
ACADSB	DCST1	KIF24	P2RY12	SMPD4	ZEB2OS
ACOD1	DEGS2	KLB	P2RY14	SOWAHA	ZFC3H1
ACP1	DLST	KLHL34	PCBP2	SPOPL	ZFP202
ACTL10	DLX1AS	LRP3	PDE4D	ST3GAL6	ZFP532
ALDH6A1	DNM3	LRRC40	PDE4DIP	ST8SIA3	ZFP583
ANK2	DTX1	LRRC7	PDE6H	ST8SIA3OS	ZFPM1
ANKMY1	DYNC1H1	LRTM1	PDZD4	STAT5A	ZMAT3
ANKRD33B	ECM1	LYL1	PEAR1	STK16	ZMIZ1OS1
AP1S3	EIF2AK4	LZTFL1	PFDN2	SYCP2	ZMYND15
APC2	ELMO3	MACROD2OS2	PIGB	SYT1	ZZZ3
ARAP3	EME1	MCMDC2	PIGF	TARDBP	
ARFGEF3	EPS8L3	MEST	PIK3C2A	TBPL1	
ARL5C	ERMARD	MFSD8	PLA2G3	TBX4	
ASH2L	FAM151A	MID1	PLIN3	THBD	
ATF7	FAM168B	MID1-PS1	PLOD3	THOC1	
B3GNT8	FAM32A	MMP24	PLPPR3	TIMP4	
BBIP1	FANCC	MORN1	POGZ	TMBIM1	
BC024978	FBL	MPG	POLH	TMC3	
BC037039	FBXL22	MRPS34	POLQ	TMEM126A	
BC060293	FBXO7	MTAG2	POLR2B	TMEM132A	
BCAP29	FLRT3	MTF1	POU3F1	TMEM150COS	
BHMT	FOSL1	MTG1	PPP1R18	TMEM175	
BMP2K	FPR1	MUC16	PPP2R1B	TMEM54	
BOLA3	FSCN2	MUC2	PPP2R3C	TMEM88	
C1QTNF7	GINS2	MYC	PRCD	TPSG1	
C2CD4B	GNAT2	MYO1H	PRKAG2OS2	TRAF3IP2	
CAPN12	GOSR2	NAA35	PROX2	TRAPPC13	
CARLR	GPATCH8	NAIF1	PRUNE2	TRIM2	
CCDC117	GPR137	NARFL	RFC2	TRIM37	
CCDC167	GPR171	NDUFS2	RIC8A	TSC22D1	
CDK17	GPR39	NEFH	RNF113A1	TSR1	
CDK4	GSS	NEMF	RNF123	TSTD2	
CEBPG	GTPBP8	NME7	RPL18A	TTC14	
CHN1OS1	HACD4	NOC2L	RPRD1B	TTC9B	
CHN1OS3	HIPK4	NRG3OS	RPS4L	TTI2	
CHPF2	HOPXOS	NRM	RPS6KB1	TTLL11	
CHRNA4	IFITM7	NSD1	RRM2B	TTLL13	
CLN3	IGSF10	NTM	RTN4	TTN	
CLVS1	IL23R	OLFR1342	SART3	TULP4	
CNIH4	JUNB	OLFR1508	SCN3B	TXLNB	
COQ10B	JUND	OLFR218	SDCCAG8	USP43	
CREBBP	KANK3	OLFR31	SGK3	VLDLR	
CREBL2	KCNMB2	OLFR418	SIAE	WDR47	
CTC1	KCNMB4OS1	OLFR524	SIN3B	WDR95	
CTSA	KCNMB4OS2	OLFR92	SLC4A5	WNK1	
CTU2	KCTD10	ORAOV1	SLC6A12	ZCCHC11	
CYTH2	KDM7A	ORC4	SLC6A20A		

Table A5. Genes protected from KA effects by STAT3 knockdown bilaterally.

GENE
ACP1
ARL5C
CAPN12
FAM151A
LETM2
LRP3
MCMDC2
MID1
NUDT6
ORAOV1
PPP2R1B
SMPD4
SPOPL
TTI2
TULP4
ZEB2OS

For all lists from the analysis in Chapter IV please see this link: https://docs.google.com/spreadsheets/d/1WZvFZH_RAD8FHhQdpbBf7Ujw0ZUOCu_BBBYeiIOnB3E/edit?usp=sharing

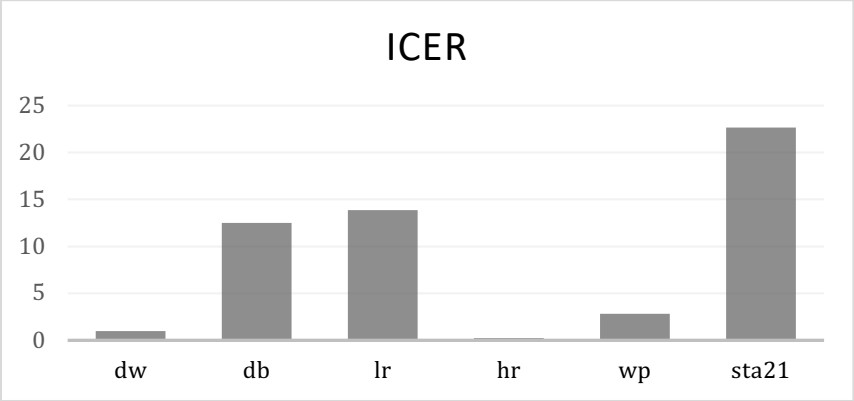
B. APPENDIX II

For the purpose of the Russek lab being able to follow up on preliminary studies of the non-canonical JAK/STAT signaling pathway, I have provided the results from two ongoing experiments: 1) qRT-PCR analysis of neurons treated with STA-21 2) Immunoprecipitation of whole cell neuron extracts pulling down with HP1 and looking for total STAT and pSTAT3 protein.

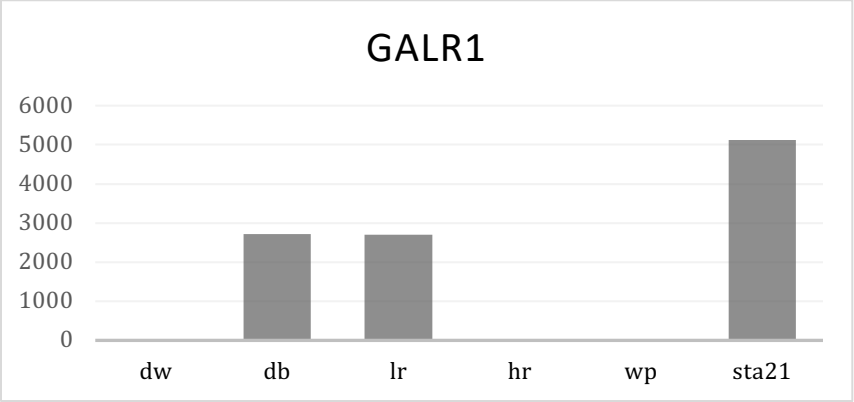
Figure A1. qRT-PCR of STA-21

To follow up on studies showing that gene expression effects in Chapter III are not pSTAT3- dependent, primary cortical neurons were pretreated with STA-21 (20uM), a STAT3 inhibitor that blocks pSTAT3 at Tyr 705 without affecting JAK. Cells were treated identically to the other treatments (1 hr pretreatment, 4 hr BDNF) and RNA was collected. The effect on expression of A) Icer, B) Galr1, and C) Drd5 was assessed, the inhibitor did not have an effect on any of the genes, further promoting the idea that it is not p-STAT3 dependent. N=1.

A.


ICER
25
20
15
10
5
0
dw
db
lr
hr
wp
sta21

B.


GALR1
6000
5000
4000
3000
2000
1000
0
dw
db
lr
hr
wp
sta21

C.

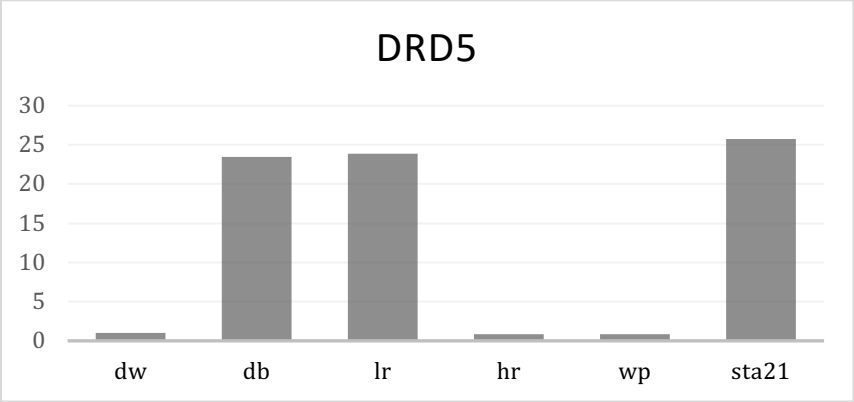
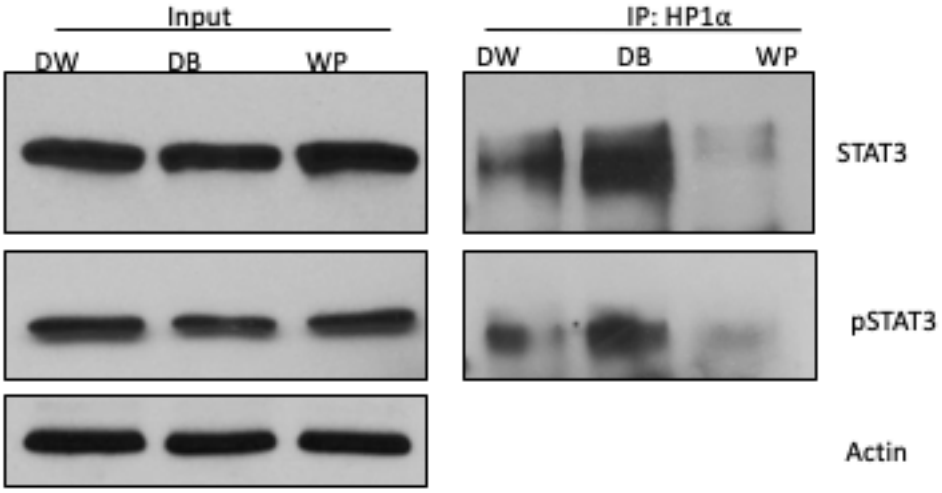

DRD5
30
25
20
15
10
5
0
dw
db
lr
hr
wp
sta21

Figure A2. Localization of STAT3 to Heterochromatin Protein 1- alpha.

Since unphosphorylated STAT3 in *Drosophila* has been identified as regulating HP1 binding to the chromatin (S. Shi et al. 2008), we sought to determine if this was the case in neurons. Using whole cell extracts of primary cortical neurons, we did coimmunoprecipitation (Active motif kit) with HP1α and immunoblotting to determine if there was binding of STAT3. Both pSTAT3 and STAT 3 were found bound to the HP1α. In addition, cells were cotreated for 30 minutes with DMSO+Water, DMSO+BDNF, and WP1066+BDNF. The results demonstrate BDNF is having an effect on the binding of HP1α by causing an increase in STAT3 and pSTAT3 binding, reversed by WP1066 cotreatment. N=3.


Input
DW
DB
WP
IP: HP1α
DW
DB
WP
STAT3
pSTAT3
Actin

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CHAPTER IX: CURRICULUM VITAE

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