Activity-dependent gene regulation in neurons: energy coupling and a novel biosensor

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ACTIVITY-DEPENDENT GENE REGULATION IN NEURONS:
ENERGY COUPLING AND A NOVEL BIOSENSOR

by

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ACTIVITY-DEPENDENT GENE REGULATION IN NEURONS:
ENERGY COUPLING AND A NOVEL BIOSENSOR
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ABSTRACT

Multiple brain disorders are associated with hypoinhibition of neural circuits that are controlled by inhibitory neurons using the neurotransmitter γ-aminobutyric acid (GABA). GABA activates type A receptors (GABARs) to mediate the majority of inhibitory neurotransmission and changes in GABAR subunit composition have profound effects on brain function. In fact, down-regulation of one of the three β isoforms, β1, is associated with alcoholism, autism, epilepsy, schizophrenia, and bipolar disorder. These conditions also present with mitochondrial defects and metabolic dysregulation.

In the first Aim of my thesis, I ask whether the core promoter of the human GABAR β1 subunit gene (GABRB1) can be regulated by the same transcription factor, the nuclear respiratory factor 1 (NRF-1) that controls oxidative phosphorylation and mitochondrial biogenesis in neurons. The ENCODE database of NRF-1 binding in human embryonic stem cells was used to identify an interaction of NRF-1 with GABRB1. Using a variety of approaches: electro mobility shift, promoter/reporter luciferase assays, gene silencing and bioinformatics, we demonstrate that GABRB1 contains a canonical NRF-1 element responsible for the majority of GABRB1 promoter- luciferase activity in transfected primary neurons. Moreover, we show that endogenous NRF-1 is responsible
for a substantial amount of luciferase activity in our studies. Altogether, our results suggest GABRB1 is a target gene for NRF-1, providing a possible link between mitochondria related energy metabolism and transcriptional regulation of β1-containing GABARs in neurological disease.

Synthesis of NRF-1 is regulated by the transcription factor cAMP response element binding protein (CREB), an important memory molecule implicated in multiple brain disorders. The second Aim of my thesis was to develop a molecular sensor that can be used in living neurons to signal the presence of CREB dependent gene regulation. We employ a split complement bioluminescent sensor to monitor interaction of protein surfaces that link CREB with its co-factor CBP and demonstrate that it can detect activation of CREB via its serine 133 phosphorylation site and activation through an undiscovered mechanism. We also show that this sensor can be used to monitor BDNF signaling providing the foundation for its future use in in vivo models of disease where BDNF is implicated.
PREFACE

This thesis is organized in the form of a general introduction to my areas of interest, a methods section that contains information relevant to the complete thesis research, and two research projects that are presented in the form of manuscripts that address the following two specific Aims:

Aim 1. To determine whether the core promoter region of the human GABAR β1 subunit gene (GABRB1) can be regulated by the same transcription factor, the nuclear respiratory factor 1 (NRF-1) that controls oxidative phosphorylation and mitochondrial biogenesis in neurons.

Aim 2. To develop a molecular sensor that can be used in living neurons to signal the presence of CREB dependent gene regulation.
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<th>Abbreviation</th>
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<tbody>
<tr>
<td>2DG</td>
<td>2-deoxy-D-glucose</td>
</tr>
<tr>
<td>ACD</td>
<td>Anticonvulsant drug</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>AP2</td>
<td>Clathrin adaptor protein 2</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BLI</td>
<td>Bioluminescence imaging</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>bZIP</td>
<td>Basic leucine zipper</td>
</tr>
<tr>
<td>CaMK</td>
<td>Calcium-calmodulin-dependent kinase</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COX</td>
<td>Cytochrome c oxidase</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding</td>
</tr>
<tr>
<td>CREM</td>
<td>cAMP response element modulator</td>
</tr>
<tr>
<td>Cycs</td>
<td>Cytochrome c</td>
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DG: Dentate granule
DMEM: Dulbecco’s Modified Eagle Medium
DMSO: Dimethyl sulfoxide
DN: Dominant negative
DNA: Deoxyribonucleic acid
EAAT: Excitatory amino acid transporter
EDTA: Ethylenediaminetetraacetic acid
EMSA: Electrophoretic mobility shift assay
ENCODE: Encyclopedia of DNA elements
ER: Endoplasmic reticulum
ETC: Electron transport chain
EWG: Erect wing
FADH2: Flavin adenine dinucleotide
FMR1: Fragile X mental retardation 1
FSK: Forskolin
GABA: γ-aminobutyric acid
GABRA4: GABA receptor α4 subunit
GABRB1: GABA receptor β1 subunit
gDNA: Genomic deoxyribonucleic acid
HAP1: Huntingtin-associated protein 1
HBSS: Hank’s balanced salt solution
HDAC: Histone deacetylase
HEK ........................................................................................................... Human embryonic kidney
ICER ........................................................................................................... Inducible cAMP early repressor
Inr ............................................................................................................. Initiator element
IVIS ......................................................................................................... In vivo imaging system
KA ............................................................................................................ Kainate
KCC2 ....................................................................................................... K-Cl co-transporter
KID .......................................................................................................... Kinase inducible domain
KIX ........................................................................................................... Kinase interacting domain
LTD .......................................................................................................... Long-term depression
MAPK ...................................................................................................... Mitogen-activated protein kinase
mtDNA ..................................................................................................... Mitochondrial DNA
NADH ...................................................................................................... Nicotinamide adenine dinucleotide
NBM ....................................................................................................... Neural basal medium
NGF .......................................................................................................... Nerve growth factor
NKCC1 ..................................................................................................... Na-K-Cl cotransporter
NLS .......................................................................................................... Nuclear localization signal
NMDA ...................................................................................................... N-Methyl-D-aspartate acid
nNOS ....................................................................................................... Neuronal nitric oxide synthase
NRF-1 ...................................................................................................... Nuclear respiratory factor 1
NT3 .......................................................................................................... Neurotrophin 3
OXPHOS ................................................................................................. Oxidative phosphorylation
p75NTR .................................................................................................. p75 neurotrophin receptor
PBS ................................................................. Phosphate buffered saline
PGC-1α .................................. Peroxisome proliferator-activated receptor γ coactivator 1 alpha
PhF1b ................................................................. polycomb-like protein
PKA ................................................................. Protein kinase A
PLCγ1 ................................................................. Phospholipase Cγ1
pol ................................................................. Polymerase
qPCR ................................................................. Quantitative polymerase chain reaction
RNA ................................................................. Ribonucleic acid
RTK ................................................................. Receptor tyrosine kinase
SE ................................................................. Status epilepticus
shRNA ................................................................. Short hairpin RNA
siRNA ................................................................. Small interfering RNA
TFAM ................................................................. Mitochondrial transcription factor A
TLE ................................................................. Temporal lobe epilepsy
TrkA ................................................................. Tropomyosin receptor kinase A
TrkB ................................................................. Tropomyosin receptor kinase B
TSSs ................................................................. Transcriptional start sites
VDCC ................................................................. Voltage-gated calcium channel
VP16 ................................................................. Viral protein 16
wt ................................................................. Wild type
CHAPTER I. INTRODUCTION

Chapter 1 is organized to introduce the areas of metabolism and neurobiology that I reviewed during the progression of my thesis research. It begins with the role of Nuclear Respiratory Factor 1 (NRF-1) in mediating energy metabolism and neural activity. It continues with an overview of epilepsy and the GABAR subunit changes that most likely contribute to an imbalance in inhibitory neurotransmission. The ideas presented in these two areas form the basis and motivation for Aim 1 of my thesis: *To determine whether the core promoter region of the human GABAR β1 subunit gene (GABRB1) can be regulated by the same transcription factor, the nuclear respiratory factor 1 (NRF-1), that controls oxidative phosphorylation and mitochondrial biogenesis in neurons.*

Next, I review the salient features of activity-dependent gene regulation as mediated by the cAMP regulatory binding protein (CREB) and as informed by biochemical analysis. This provides the background for Aim 2 of my thesis: *To develop a molecular sensor that can be used in living neurons to signal the presence of CREB-dependent gene regulation.*

A. Neuronal activity and energy metabolism

1. Energy metabolism: a coordination between two genomes

Oxidative phosphorylation (OXPHOS) is a cellular process that takes place in the mitochondria, in which ATP is formed from the combustion of glucose into carbon dioxide and water. During OXPHOS, ATP is synthesized by ATP synthase when protons flow across the inner mitochondrial membrane into the mitochondrial matrix down their concentration gradient and transmembrane electrical potential. This pH gradient is
maintained through a set of enzyme complexes, known as the electron transport chain (ETC), that transfers electrons from NADH or FADH$_2$ to molecular oxygen, while pumping protons back across the inner membrane against the established gradient. The last protein complex of the ETC, cytochrome c oxidase (COX) or Complex IV, catalyzes the final transfer of electrons from cytochrome c (Cyt c) to oxygen, returning Cyt c to its oxidized state, reducing oxygen to water, resulting in a net translocation of protons from the matrix into the intermembrane space. The subunits that constitute COX, as well as ETC Complexes I, and III, are encoded by both the nuclear and mitochondrial genomes (Kadenbach, Jarausch et al. 1983, Au, Seo et al. 1999, Scarpulla 2006). As such, it is essential that a proper coordination and communication between the nucleus and the ATP generating organelle, the mitochondria, must occur in order to maintain energy balance. This coordination is especially important in neurons whose energy demands are extremely high and must be met so that they can adapt to the needs of a continually changing extracellular environment.

2. Nuclear-encoded regulators of mitochondrial biogenesis: NRF-1 and PGC-1α

2.1 NRF-1.

One of the workhorses that bridges nuclear-mitochondrial communication is the transcription factor, nuclear respiratory factor 1 (NRF-1). The full length 503-amino acid human NRF-1 is a nuclear-encoded gene, first discovered as a transcriptional regulator of the Cycs gene that encode cytochrome c (Dudek 2007). It has since been characterized as a positive transcriptional regulator of COX subunit genes (Dhar, Ongwijitwat et al. 2008, Wong-Riley 2012) and other respiratory genes (Evans and Scarpulla 1990, Scarpulla...
underscoring its major role in mitochondrial function. The contribution of NRF-1 in cellular respiration goes beyond OXPHOS, as its targets include those responsible for mitochondrial biogenesis, the birth of new mitochondria. Mitochondrial biogenesis occurs in response to energy demand, thermogenesis, and oxidative stress. This cellular program is directed by a limited number of nucleus-encoded factors (Tfam, TFB1M, TFB2M, and mTERF). In preparation for mitochondrial biogenesis, the mitochondrial transcription factor A (Tfam) is synthesized to drive the transcription and replication of mitochondrial DNA (mtDNA) replication (Scarpulla 2008). The results of these studies suggest that the generation and establishment of functioning mitochondria require the expression of both mitochondrial-encoded and nuclear-encoded genes (Evans and Scarpulla 1989, Scarpulla 2006, Scarpulla 2008, Scarpulla 2011).

As a transcription factor that mediates nuclear-mitochondrial interactions, NRF-1 is highly conserved between species, particularly in the amino-terminal half of the protein, where it shares high sequence similarity with the developmental regulatory factors erect wing (EWG) of Drosophila and P3A2 of sea urchin (Virbasius, Virbasius et al. 1993). This particular region of the proteins encompasses the nuclear localization (NLS), as well as the DNA binding and dimerization domains. Juxtaposed to the NLS sequence, there is a conserved inhibitory domain that was revealed through deletion analysis, suggesting that the N-terminus may modulate transcriptional activity (Fazio, Bolger et al. 2001, Ramachandran, Yu et al. 2008). In contrast to the N-terminus, its carboxy-terminal half, where the transcriptional activation domain lies, is divergent across species. Further analysis of the transactivation domain in EWG also showed
conservation with NRF-1 (Fazio, Bolger et al. 2001).

Within the NRF-1 amino-terminus, there are multiple residues for post-translational modification that affects its function. NRF-1 is phosphorylated on serine residues, resulting in enhanced intrinsic binding to DNA and trans-activation function (Gugneja and Scarpulla 1997, Herzig, Scacco et al. (2000). Its binding and activity can also be stimulated by phosphorylation at Threonine 109 through the PI3K/AKT pathway (Piantadosi and Suliman 2006). However, not all phosphorylation sites lead to activation. Phosphorylation at serine 47 by Cyclin D1-dependent kinase leads to repression of NRF-1 expression and activity (Wang, Li et al. 2006). Taken together, these findings highlight the importance of the N-terminal domain in the regulation of NRF-1 activity.

Since dimerization and DNA binding is not required by phosphorylation (Gugneja and Scarpulla 1997), this may suggest that NRF-1 is constitutively bound to its targets (Ramachandran, Yu et al. 2008). NRF-1 binds to a GC-rich element within its target promoters and recognizes the consensus sequence (T/C)GCGCA(C/T)GCGC(A/G). This binding motif has been identified as one of those highly represented in core human promoter sequences, particularly in TATA-less promoters (Xi, Yu et al. 2007). Associated with TATA-less promoters are short interspersed GC-rich DNA sequences, called CpG islands, which are sites of transcription initiation (von Beroldingen, Reynolds et al. 1984, Rozenberg, Shlyakhtenko et al. 2008). This discovery has led to the proposed role of NRF-1 as a transcriptional initiator (Zhang, Yu et al. 2013) in CpG islands, exemplified by NRF-1’s capacity to mediate transcriptional initiation of insulin-degrading enzyme independent of TATA box-binding protein (Zhang, Ding et al. 2012).
Recently, a growing number of studies have reported functional NRF-1 binding to genes within CpG islands (Gonen and Assaraf 2010, Li, Li et al. 2011, Zhang, Ding et al. 2012) suggesting that coordinated control over this region of the genome may link the energy demands of the cell to a rapid and adaptive transcriptional response.

Located on Chromosome 7 in the human genome, NRF-1 is ubiquitously expressed, as assessed by \textit{in vitro} hybridization in rat, with relatively high expression levels in the lungs and testis, and moderate expression in the brain (Gopalakrishnan and Scarpulla 1995). Homozygous NRF-1 knockout mice exhibit peri-implantation lethality between embryonic day E3.5 and E6.5. Upon closer examination, the blastocyst of these animals harbor less mitochondria and mitochondrial DNA content, consistent with NRF-1’s role in regulating TFam (Huo and Scarpulla 2001). In comparison, the heterozygous mice appear phenotypically normal, but failure of blastocysts to develop properly \textit{in vitro} was noted. The NRF-1 homologues EWG in drosophila and not really finished (nrf) in zebra fish are both implicated as important in early development and associated specifically with the central nervous system (CNS) (Huo and Scarpulla 2001). These homologous systems suggest that the lethality observed in knockout mice may be due to a failure in neural development.

\textit{2.2 PGC-1α and NRF-1 in neurons}

In addition to NRF-1, there is a master molecular regulator called peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) that is critical for mitochondrial biogenesis (Puigserver, Wu et al. 1998) and the maintenance of energy balance. PGC-1α is a coactivator of NRF-1 function, as well as an activator of NRF-1
expression. PGC-1α is regulated by various post-translational mechanisms including phosphorylation by AMP-activated protein kinase (AMPK) that leads to its activation in response to increased AMP/ATP ratio, a marker of energy depletion.

The major consumer of oxygen is the brain. For the brain, its source of energy is limited to only glucose and ketone bodies. The nervous system is highly dependent on OXPHOS (Sokoloff and Kety 1960, Hall, Klein-Flugge et al. 2012). Given that the brain is the most energy-dependent organ of the body (Attwell and Laughlin 2001), it is not surprising that NRF-1 may be critical to its development and continued health.

Interestingly, cytochrome c promoter activity is activity-dependent. Neural activity-mediated stimulation of cytochrome c occurs via both NRF-1 and the cAMP response element binding protein (CREB) in response to synaptic stimulation (Mayr and Montminy 2001, Deisseroth and Tsien 2002). NRF-1-mediated expression of cytochrome c is calcium-dependent and requires extracellular-signal-regulated kinases ERK1/2 (Delgado and Owens 2012).

Brain-derived neurotrophic factor (BDNF), known to support neuronal survival, and the growth and differentiation of nascent neurons, stimulates the expression of mitochondrial biogenesis markers such as PGC-1α, Tfam, and NRF-1 in cultured hippocampal neurons. Stimulation of PGC-1α by BDNF is dependent on mitogen-activated protein kinase (MAPK) and CREB. In fact, the number of mitochondria in a neuron correlates with its spine density, and its number of synapses positively correlates with PGC-1α levels (in a model where PGC-1α knock-down reduces spine density) (Cheng, Wan et al. 2012). Moreover, PGC-1α has been implicated in the formation and
maintenance of neuronal dendritic spines. NRF-1 can also regulate neurite outgrowth through its transcriptional regulation of novel genes (Chang and Huang 2004, Chang, Chen et al. 2005, Wang, Tong et al. 2013), also reported in neuroblastoma IMR-32 cells and hippocampal neurons (Tong, Wang et al. 2013).

The transcriptional regulation of NRF-1 in neurons is activity-dependent (Yang, Liang et al. 2006) and mediated through AMPK (Yu and Yang 2010), a key enzyme in cellular energy homeostasis. Interestingly, reports show that NRF-1, under conditions of depolarizing stimulation, regulates expression of receptor subunit genes associated with glutamatergic synaptic neurotransmission, such as NR1, NR2B (Dhar and Wong-Riley 2009), GluR2 (Myers, Peters et al. 1998, Dhar, Liang et al. 2009), and neuronal nitric oxide synthase (nNOS) (Myers, Peters et al. 1998, Dhar, Liang et al. 2009). These findings further emphasize that neuronal activity and energy metabolism are tightly coupled.

NRF-1 and/or mitochondrial biogenesis can be induced by various stimuli associated with brain disorders. In response to transient hypoxia, there is an increase in mitochondrial biogenesis in the brain subcortex of mice, along with an activation of NRF-1 and PGC-1α expression (Gutsaeva, Carraway et al. 2008). The molecular mechanism of such changes in expression is driven by nNOS and the activation of CREB, which through its role as a key transcriptional regulator of synaptic plasticity is also implicated in the dysregulation of plasticity seen in epilepsy (Lund, Hu et al. 2008, Porter, Lund et al. 2008).

Mitochondrial biogenesis also occurs in response to inflammation that is induced
by lipopolysaccharide and depends upon the upregulation of NRF-1 expression that is
controlled by NFκB and CREB (Suliman, Sweeney et al. 2010). These reports taken
together point to a convergence on CREB as a major factor in the control of
mitochondrial biogenesis, achieved through its upregulation of nuclear genes and its
translocation from cytoplasm to mitochondria to activate mitochondrial gene
Rasmo, Signorile et al. 2010).

In addition, a pathway analysis using ENCODE datasets of ChIP-seq, with SK-N-
SH human neuroblastoma cells, suggests a potential role for NRF-1 in Alzheimer’s and
Parkinson’s disease (Satoh, Kawana et al. 2013) and, consistent with its role in
neurodegeneration due to impaired metabolism, PGC-1α and BDNF are both implicated
in the neuropathologies of Alzheimer’s (Arancibia, Silhol et al. 2008, Qin, Haroutunian et
al. 2009, Zheng, Liao et al. 2010) and Parkinson’s disease (Maswood, Young et al. 2004,
Zheng, Liao et al. 2010).

B. Epilepsy

1. General classification

Epilepsy is a disorder characterized by an aperiodic recurrence of seizures in
response to brain injury, a genetic predisposition, or brain malformation. The incidence
of epilepsy is 44 per 100,000 person-years. Each year, about 125,000 new cases are
diagnosed. There is a bimodal distribution in the occurrence of the first seizure in the
population, with one peak occurring in newborn and young children and the second peak
occurring in patients older than age 65 (DiPiro 2005). However, the underlying cause of
the seizures stems from a diversity of etiologies, varying in clinical presentations and consequences. Both genetic and acquired factors can contribute to epilepsy, which lead to the etiological categorization of idiopathic and symptomatic epilepsies, respectively. Due to diverse phenotypes within idiopathic epilepsies, scientists like William Lennox, support the idea that there is a “neurobiological spectrum of epilepsies” (Berkovic, Mulley et al. 2006). At one end of the spectrum are the idiopathic epilepsies associated with a single gene, commonly one that encodes for an ion channel, while the opposite spectrum represents epilepsies with no genetic susceptibility for the disease, but arise from insults to the brain such as head trauma, stroke, lesions and tumors. Falling in between these two extremes are epilepsies that involve multiple genes, those that do not follow Mendelian pattern of inheritance and are referred to as cryptogenic. Currently, the category “cryptogenic epilepsies” refers to epilepsies that are associated with unknown factors. In the perspective of epilepsy being a continuum, “cryptogenic epilepsies” may be accounted for by the “interaction of genetic and acquired factors”, in which a mechanism that has not been elucidated (Berkovic, Mulley et al. 2006).

Despite the ongoing challenge in the etiological framework, the numerous syndromes are categorized into two broad groups: partial and generalized epilepsies. Spontaneous seizures of partial epilepsies have a focal anatomical origin, and can be either idiopathic or symptomatic in etiology. They account for 60% of all epilepsies. The remaining 40% of epilepsies in the general population are generalized. These epilepsies are characterized as having anatomical onsets in both brain hemispheres and, although candidate genes remain to be identified, they are considered idiopathic. Partial epilepsies
are also subdivided based on whether there is a change in consciousness, with simple partial epilepsies being specific to the maintenance of consciousness and complex partial to the impairment of consciousness. Due to the complexity of epilepsy presentations, the treatment and management of symptoms for patients must reflect the specific syndrome, placing emphasis on an accurate diagnosis in order to choose an appropriate pharmacotherapy and to act early to reduce seizure events that can be life threatening.

2. Treatment

The goal of pharmacologic therapy for epilepsy is to properly balance drug efficacy with drug side effects, where the therapy serves to improve the overall quality of life rather than decrease it through disturbances in cognitive function. Effective anticonvulsant drugs (ACDs) reduce the number and severity of spontaneous seizures in patients protecting them from subsequent brain damage.

One may ask, what are the physiological consequences of a seizure? The prolonged abnormal excitation leads to neuronal toxicity due to the over-exposure of neurons to the excitatory neurotransmitter glutamate. As a consequence, there is also an increased risk of the brain to ischemia, a condition that leads to the shortage of oxygen and nutrients to the brain, ultimately resulting in brain damage. Ideally, only a single therapeutic agent should be used to prevent the probability of seizure production in an individual. In reality, approximately 65% of patients suffering from epilepsy can manage the disease with monotherapy (DiPiro 2005), the remaining patients either require two-drug regime or are refractory to any drug therapy that is available. When pharmacotherapy is not possible, surgery, dietary restriction or vagal nerve stimulation
through implantation of a biomedical device is required. Either as a response to disease progression or side-effects of pharmacotherapy, epileptic patients often suffer from severe neuropsychiatric conditions such as anxiety and depression.

In general, ACDs control abnormal neuronal activity by elevating the spike threshold of neurons to stimuli or by limiting the propagation of the seizure discharge from its initial site. The mechanism of action of most ACDs can be categorized into three types: modulation of voltage-gated ion channels; enhancement of synaptic inhibition; and inhibition of synaptic excitation (Rogawski and Loscher 2004). The types of voltage-gated ion channels that current molecular therapies target are the sodium channels and calcium channels. Enhancement of synaptic inhibition is achieved through the modulation of GABAergic neurotransmission. Inhibition of excitation involves the blockade of glutamate receptors such as NMDA, kainate (KA) and AMPA. Some ACDs target one specific molecular entity, but most have a combination of complex or poorly understood mechanisms of action. For example, the drug Topiramate has been shown to affect the function of sodium channels, calcium channels, type A GABA receptors (GABARs), and Kainate and AMPA receptors (Rogawski and Loscher 2004). In contrast, phenytoin, an ACD prescribed for the protection of patients suffering from generalized tonic-clonic (GTC) and partial seizures acts primarily through the inactivation of voltage-gated sodium channels, and specifically by prolonging the refractory period in which sodium channels remain unresponsive to opening.

Along with phenytoin, carbamazepine, oxcarbazepine, lamotrigine, and valproic acid are the first-line drugs for treating partial seizures, although they are associated with
a variety of side effects that can impair the quality of life. Phenytoin has been used for
treatment of partial seizures for over 60 years and also as a first-line drug for the
treatment of status epilepticus, an immediate response of the brain to injury.
Carbamazepine has been well studied and causes minimal cognitive impairment,
although chronic use has been associated with altered bone mineral density and liver
toxicity. Structurally similar to carbamazepine, oxcarbazepine, however, is a prodrug that
is converted to the active 10-monohydrate derivative. Despite similar mechanism of
action with carbamazepine, oxcarbazepine appears to modulate N- and P-type Ca\textsuperscript{2+}
channels (DiPiro 2005). Although rare, oxcarbazepine and carbamazepine can cause
rashes and hyponatremia. Neither drug is effective against “absence” or myoclonic
seizures. In contrast to the seizure specific targets, lamotrigine is a rather broad spectrum
ACD, having efficacy in partial seizures and several types of generalized seizures. The
drug is also useful as an adjunctive treatment in partial seizure patients.

Another broad-spectrum ACD is valproic acid, which is a first-line drug for both
partial and primary generalized seizures, but can be toxic if taken chronically. The
pharmacology and mechanism of action in the treatment of epileptic seizures is unclear.
Initially, valproic acid was thought to assert its anticonvulsant property through altering
the synthesis and degradation of GABA. It has now been shown that it may exert its
affects via the potentiation of postsynaptic GABA responses and may also affect
potassium channels (DiPiro 2005). In addition, Valproate alters expression of vesicular
 glutamate transporter 1 (VGLUT1) and presynaptic glutamate release; increases
expression of the excitatory amino-acid transporter (EAAT), and alters the function of
glutamate receptors (Sanacora, Zarate et al. 2008). Valproate has also been used in the treatment of other brain conditions such as migraine headaches and bipolar disorder. As Valproate is a histone deacetylase (HDAC) inhibitor, it remains to be determined whether its long-term effects are due to a change in genome expression. Major side effects that lead to a lack of patient compliance include significant weight gain, multiple drug-drug interactions, tremor, pancreatitis, and polycystic ovary disease.

Augmentation in inhibitory neurotransmission includes increasing CNS concentrations of GABA and potentiating the effects of GABA at its receptor. The latter is achieved through a class of drugs known as benzodiazepines that modulate GABARs by increasing their affinity for GABA. There is also evidence of felbamate and topiramate acting as positive modulators of GABARs (Rogawski and Loscher 2004). The concentration of GABA in the brain is controlled by the enzymes glutamate decarboxylase (GAD) and GABA transaminase (GABA-T). The GABA analogue, vigabatrin (γ-vinyl GABA) irreversibly inhibits GABA-T, reducing GABA degradation, leading to a large increase in brain GABA levels. The ACD tiagabine also decreases the removal of GABA from the synapse, however, it does this by inhibition of GABA reuptake at the GABA transporter (GAT1).

Efforts to decrease excitatory neurotransmission are primarily focused on decreasing (or antagonizing) glutamate and aspartate neurotransmission. The specific receptors of interests are the NMDA receptor, AMPA receptor and Kainate receptors. These mechanisms are reflected in drugs like felbamate (inhibits NMDA receptors), topiramate, phenobarbital (AMPA inhibition) and both kainite and AMPA receptors.
Drugs that reduce corticothalmic T-type calcium currents, like valproic acid and ethosuximide, are effective against generalized absence seizures.

Overall, the greatest issue with drug therapy for epilepsy is the side effects that influence patient compliance. The non-life-threatening side effects are generally dose-dependent. Other adverse effects include sedation, dizziness, altered vision, difficulty focusing. Long-term adverse effects lead to bone disorders such as osteomalacia and osteoporosis. A proposed etiology suggests that treatment interferes with vitamin D metabolism, reflected in laboratory tests in increased alkaline phosphatase concentration, decreased blood calcium and 25-OH vitamin D concentration (DiPiro 2005).

Again, one must emphasize that the current ACDs do not prevent the development or progression of epilepsy. In addition, there are patients who are resistant to all drugs. Therefore, there is an urgent need to develop new therapeutic targets for this group of patients, working towards prevention of epilepsy in patients at risk and disease modification drugs, as well as symptomatic treatment (Bethmann, Brandt et al. 2007, Loscher 2007)(Loscher W. et al., 2007). Surprisingly, for individuals who are drug-resistant, like those suffering from temporal lobe epilepsy (TLE), half of them achieve seizure control through the “ketogenic diet”; a diet that substitutes fats and protein to obtain an equivalent calorie intake from foods containing carbohydrates. Due to the response of patients to dietary manipulation, Garriga-Canut, M. et al. consider metabolic pathways as targets for future pharmacological intervention in the treatment of epilepsy.

In particular, investigators have studied the effects of the glycolytic inhibitor 2-deoxy-D-glucose (2DG) in a rat model of TLE, where they have shown that 2DG blocks
seizure-induced increases in the expression of brain-derived neurotrophic factor and its receptor, TrkB. 2DG displays anticonvulsant and antiepileptic properties, suggesting that antiglycolytic compounds may represent a new class of drugs for treating epilepsy (Garriga-Canut, Schoenike et al. 2006). 2DG differs from glucose in that a hydroxyl group at the C2 position has been replaced by a hydrogen atom. The successful use of 2DG in this model is associated with its increase in the antiepileptic threshold and decrease in the progression of kindling, respectively, suggesting that targeting pathways of energy metabolism can control seizures. By targeting metabolism, remodeling chromatin structure, and altering gene expression, the authors suggest 2DG may represent the founding member of a new class of antiepileptic drugs.

Similarly, another group has found that ketone bodies reduce the firing rate of neurons in the rodent substantia nigra pars reticulata, a region in the brain that may control the spread of seizures. Results of the study indicate that the ketogenic diet may work by activating neuronal $K_{ATP}$ channels, thereby reducing the electrical activity of neurons (Ma, Berg et al. 2007). Consistent with a role for potassium channels as a novel target for future epilepsy, retigabine is a first-in-class potassium-channel opener that has been developed. Retigabine is thought to reduce susceptibility for epileptic seizures primarily by opening neuronal voltage-gated potassium channels that allow potassium ions to flow out of the cell, increasing the threshold for excitation, and thereby decreasing cellular excitability. Specifically, retigabine activates neuronal KCNQ-type $K^+$ channels, a gene product involved in idiopathic epilepsy (Steinlein 2004).

Finally, there is a drug named gabapentin, which was synthesized to be a GABA
agonist but was later discovered that it did not act at the GABA receptor but rather as a modulator of voltage-sensitive Ca\(^{2+}\) channels that indirectly increase GABA levels in the brain (Taylor, Gee et al. 1998). Still, the exact mechanism of action is unknown. Nonetheless, gabapentin is effective for partial seizures as an alternative therapy, with and without secondary generalization, when used in addition to other antiseizure medications (Goodman, Gilman et al. 2006). Related to gabapentin in structure is the drug pregabalin that is in clinical trials. It was identified through investigations of alkylated analogues of GABA. In preclinical models of epilepsy and neuropathic pain, pregabalin was found more potent than gabapentin (Dworkin and Kirkpatrick 2005).

3. Temporal lobe epilepsy (TLE) and animal models

One form of epilepsy prevalent in adults, known as temporal lobe epilepsy (TLE), consists of seizures that originate focally in the temporal lobe. These seizures frequently lead to impaired consciousness and cognitive impairment such as memory disruption (Bell, Lin et al. 2011). Although approximately 65-70% of patients suffering from epilepsy can manage or minimize seizure occurrences with a single therapeutic agent, the remaining patients either require multi-drug treatments or are entirely refractory to current ACDs. Remarkably, for those who are drug-resistant, a common struggle confronted by TLE patients, seizure control can be achieve through a dietary regimen known as the “ketogenic diet”; a diet that substitutes fats and protein to obtain an equivalent caloric intake from a typical carbohydrate-rich diet, as described earlier in this chapter. The success of the ketogenic diet suggests an energetic and metabolic component to the mechanism of epileptogenesis.
The initial injuries leading to chronic acquired epilepsies converge upon many cellular and molecular changes such as those that increase production of reactive oxygen species, oxidative stress and mitochondrial dysfunction. Potential sequelae to these changes are dysregulated energy and cellular metabolism. Epilepsy has been associated with oxidative stress, mitochondrial dysfunction, as well as neuronal death depending on the region of the brain (Waldbaum and Patel 2010). These interconnected cellular processes can culminate in decreasing ATP supply, altering cellular energy and metabolism. One cellular response that may mitigate these changes is mitochondrial biogenesis. Neuroprotection via mitochondrial biogenesis may be conferred, in part, by restoring levels of ATP (Maalouf, Rho et al. 2009), as well as local regulation of the second messenger calcium ion, which play roles in neurite outgrowth and synaptic plasticity (Mattson 2007).

3.1 Pilocarpine model of TLE

An important criterion for an in vivo model of epilepsy is its ability to recapitulate the human condition in the formation of spontaneous recurring seizures. One such model in rats uses pilocarpine, a cholinergic agonist, at a convulsant dose to assault the brain and induce a prolonged seizure known as status epilepticus (SE). Following SE, the animals undergo a latent period of no seizure development. Despite the apparent quiescent phase, it is believed that there are ongoing biochemical and cellular changes occurring in the brain that underlie the development of chronic spontaneous seizures, the hallmark of epileptogenesis. For instance, brain lesions can be found in the pilocarpine model that mimic the observations in human patients with mesial temporal sclerosis.
Other morphological features associated with epilepsy include the loss of GABAergic interneurons in the dentate hilus, proliferation of astrocytes, activation of microglia indicative of an inflammatory response, and death of pyramidal neurons within the CA3 and CA1 strata of hippocampus (McNamara, Huang et al. 2006). Although the granule cells of the dentate gyrus are resistant to cell death (Grooms, Opitz et al. 2000), the loss of their normal target cells leads to altered mossy fiber (axons of granule cells) sprouting into the outer molecular layer of the gyrus and reinnervating the dendrites of granule cells (Sutula, Cascino et al. 1989, Sarkisian 2001). These observations suggest a reorganization of neural networks and a contributor of hyperexcitability in epileptic brains. In addition to these changes in cellular connectivity, animal models suggest that there are key molecular responses in neurons driven by increased levels of BDNF.

3.2 BDNF signaling and epilepsy

In response to seizures, BDNF expression is increased markedly (Ernfors, Bengzon et al. 1991, Isackson, Huntsman et al. 1991, Springer, Gwag et al. 1994) at both the levels of mRNA (Murray, Isackson et al. 2000) and protein (Takahashi, Hayashi et al. 1999) in the hippocampus, particularly in the dentate gyrus and CA1-CA3 pyramidal cells (Binder, Croll et al. 2001). As a member of the neurotrophin family, BDNF is essential to neuronal survival, differentiation and long-term potentiation during development of the CNS. These processes are regulated by the binding of BDNF to its higher and lower affinity receptors, TrkB and p75NTR, respectively. Although its physiological function may suggest a neural protective role, genetic models seem to provide evidence for the role of BDNF signaling in epileptogenesis. An additional animal
model to pilocarpine used to study epileptogenesis is kindling, which enhances a neuron’s sensitivity to electrical stimulations and to the subsequent stimulation of seizure activity. This model allows researchers to induce seizures using electrical stimuli for the animal’s lifespan. Comparing BDNF\(^{+/+}\) wild type and heterozygous BDNF\(^{+/−}\) knockout animals undergoing kindling, BDNF\(^{+/−}\) knockout animals exhibited reduced rate of kindling development, meaning more stimulations were required to reach the same level of seizure severity as the wild type animals (Kokaia, Ernfors et al. 1995). The suppressed epileptogenesis in the kindling model is also supported by another study where fusion antibodies composed of the TrkB receptor ligand-binding domain and Fc portion of human immunoglobulin IgG1 were introduced to compete against the actions of endogenous neurotropins in the hippocampus (Binder, Routbort et al. 1999). CNS specific elimination of either BDNF or TrkB shows that while homozygous knockout of BDNF had little effect on epileptogenesis, epileptogenesis was completely eliminated in the TrkB receptor knockout when employing the kindling model (He, Kotloski et al. 2004). This finding implies a major role for the TrkB receptor in epileptogenesis and a potential role for additional TrkB ligands such as Neurotrophin 3 (NT3). In addition, substitution of phenylalanine for tyrosine at residue 816 in TrkB has been shown to disrupt the activation of phospholipase C\(γ1\) (PLC\(γ1\)) which inhibits limbic epileptogenesis (He, Pan et al. 2010).

The molecular basis of epileptogenesis involving BDNF signaling continues to be an active area of research. Our laboratories (Russek and Brooks-Kayal) have identified BDNF signaling as a mediator of GABAR \(α1\) subunit downregulation and GABAR \(α4\)
subunit upregulation that follows SE in the pilocarpine model of TLE (Brooks-Kayal, Shumate et al. 1998, Roberts, Hu et al. 2006, Lund, Hu et al. 2008). We reported how BDNF can alter the number of GABARs at the synapse and how one receptor subtype can be replaced with another that desensitizes rapidly (Lund, Hu et al. 2008). A decrease in α1 subunit levels has also been observed in human TLE hippocampal tissues (Loup, Wieser et al. 2000) suggesting that altered GABAR subunit gene (GABR) transcription is a hallmark of the human condition. Ultimately, changes in BDNF signaling may chronically alter the expression of inhibitory systems, especially its function via the altered expression of certain GABAR subunits in target neurons, leaving the brain susceptible to the development of spontaneous seizures and to a subsequent decline in both cognitive function and emotional stability in patients.

3.3 GABARs and epilepsy

There are nineteen different subunit genes, grouped into eight classes (i.e. α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3), that contribute to the diversity of GABA_A receptors, and in turn, to the differential assembly of these subunits into receptors at the cell surface. The majority of the GABA_A receptors are composed of two α, two β subunits and either γ2 or δ. The γ2 subunit is required for the clustering of GABA_A receptors at the synapse (Essrich, Lorez et al. 1998). GABARs containing the γ2 subunit exhibit low affinity for GABA, but are positioned at the synapse where there is the highest concentration of GABA. In contrast, GABARs containing the δ subunit are commonly outside of the synapse (e.g. perisynaptic or extrasynaptic) and exhibit a high affinity for GABA. This is important as GABA diffuses away from the synapse and is much lower in concentration.
The GABAR is a ligand-gated Cl⁻ ion channel that mediates inhibitory neurotransmission in the adult mammalian CNS. For each receptor, two molecules of GABA binds, one at each α and β subunit interface (Connolly and Wafford 2004, Olsen and Sieghart 2009). In the adult brain, GABA activation leads to anion influx, resulting in hyperpolarization of the cell membrane. While in the developing brain, due to the expression of the Na-K-Cl cotransporter (NKCC1), Cl⁻ ions are taken up into the cell. This is complemented by a low to modest expression of the K-Cl co-transporter (KCC2) that is responsible for Cl⁻ efflux from neurons (Rivera, Voipio et al. 1999). Ultimately, the net effect is the maintenance of a high intracellular Cl⁻ concentration in the immature neuron.

The subunit composition of GABARs and their cell surface localization provide a molecular basis for both phasic and tonic inhibition in the nervous system. In addition to the δ subunit, the α4 subunit plays a major role in mediating tonic inhibition. In the dentate granule (DG) cells of α4 knock-out mice, tonic inhibition is markedly reduced (Chandra, Jia et al. 2006). Pharmacologically, recombinant GABAₐRs containing α4 and α6 subunits are insensitive to modulation by benzodiazepines (Wisden, Herb et al. 1991) and more sensitive to zinc inhibition than those containing α1 (Knoflach, Benke et al. 1996, Fisher and Macdonald 1998). Our laboratory discovered that α4 subunit gene expression is specifically under the control of BDNF via the Trk B receptor and MAPK signaling (Roberts, Hu et al. 2006). In animal models of TLE, α4 subunit expression is increased in DG after prolonged seizures (Brooks-Kayal, Shumate et al. 1998, Roberts, Raol et al. 2005) and SE increases the abundance of α4 subunits in γ2 containing
receptors in this region (Lund, Hu et al. 2008). Such subunit changes like the up-regulation of α4 in the hippocampal formation may underlie ACD resistance as demonstrated in phenobarbital-resistant epileptic rats (Bethmann, Fritschy et al. 2008). Along with decreased α1 subunit expression, these changes in GABRA4 transcription may contribute greatly to alterations in neural excitability and the process of epileptogenesis.

3.4 β1 subunit-containing GABARs

While the transmembrane domains of each GABAR subunit are highly conserved, the intracellular domains are genetically divergent, giving rise to residues for differential receptor regulation. These include sites for ubiquitination, palmitoylation, and phosphorylation (Kittler and Moss 2003, Jacob, Moss et al. 2008) that affect receptor cell surface expression, trafficking, and localization. These post-translational modifications of GABARs ultimately determine neuronal excitability. In particular, the intracellular domains of the β subunits have emerged as a key component of GABAR synaptic expression. For cell surface expression, GABAR subunits are assembled in the ER and directed through the secretory pathway. Part of this process is mediated by the ubiquitin-like protein Plic-1, a protein that links integrin-associated protein with the cytoskeleton-1, thereby stabilizing the receptor in the ER and inhibiting ubiquitin-mediated degradation (Walters, Kleijnen et al. 2002). Plic-1 interacts with both α and β GABAR subunits (Bedford, Kittler et al. 2001). NSF (ethylmaleimide sensitive factor) in association with GABARAP (GABAR-associated protein) and PRIP1/2 (phospholipase C-related catalytically inactive proteins 1 and 2) also bind with β subunits, as well as γ subunits, and control the trafficking of GABARs to the cell surface (Uji, Matsuda et al. 2002,
β subunits of GABARs show differential post-translational modification. While β1 and β3 can be phosphorylated by cAMP-dependent protein kinase A (PKA), the β2 subunit is resistant (McDonald, Amato et al. 1998). The phosphorylation state of GABARs determines its cell surface expression and synapse localization. The dephosphorylated β subunits can interact with the μ2 subunit of the AP2 protein (Clathrin adaptor protein 2) that internalizes GABARs as part of clathrin-mediated endocytosis, leading to either degradation or receptor recycling (Kittler, Chen et al. 2005, Kittler, Chen et al. 2008). The β subunit also participates in the receptor’s endocytic recycling via interacting with BIG2 (brefeldin A-inhibited GDP/GTP exchange factor 2) (Charych, Yu et al. 2004). Interestingly, huntingtin-associated protein 1 (HAP1) can interact with the cytoplasmic domains of β subunits to facilitate GABAR vesicular transport back to the cellular membrane (Kittler, Thomas et al. 2004).

The human β1 subunit gene (GABRB1) is located on chromosome 4, as part of a GABAR gene cluster that contains α2, α4, and γ1 (McLean, Farb et al. 1995). Recently, it was reported that specific mutations in Gabrb1 of mice produce increased alcohol consumption associated with increased tonic inhibition (Anstee, Knapp et al. 2013). These findings suggest a role for GABRB1 in alcoholism, consistent with data from association studies (Parsian and Zhang 1999, Sun, Cheng et al. 1999, Zinn-Justin and Abel 1999, Song, Koller et al. 2003).

Disrupted GABAR-mediated neurotransmission is implicated in various nervous
system disorders (Hines, Davies et al. 2012). Expression of GABRβ1 is reduced in the lateral cerebella of subjects with bipolar disorder, major depression, and schizophrenia compared to healthy controls (Fatemi, Folsom et al. 2013). Levels of β1 subunits are also reduced in the brains of subjects with Autism (Fatemi, Reutiman et al. 2010). In the Fragile X mental retardation mouse model, where the gene Fragile X mental retardation 1 (fmr1) is removed, both β1 and β2 subunit mRNAs are reduced (D’Hulst, De Geest et al. 2006).

3.5 Transcription of the β1 subunit gene

The TATA-less GABRB1/Gabrb1 promoter (GABRB1-p/Gabrb1-p) is known to contain multiple transcriptional start sites within a CpG island (Ma, Song et al. 2004, Roberts, Raol et al. 2005) and exhibit neuronal-selective expression (Chandra, Jia et al. 2006). In unraveling the molecular determinants of GABAR β1 subunit gene regulation, our laboratory has demonstrated that the minimal GABRB1-p lies within the first 500 bp of the 5’ flanking region. Within this region of the promoter, we have found a conserved initiator element (Inr) that mediates down-regulation of Gabrb1 transcription by GABA (Russek, Bandyopadhyay et al. 2000). Recently, we identified a role for the polycomb-like protein, PHF1b, as a transcriptional regulator that mediates GABA-induced downregulation (Saha, Hu et al. 2013). In my thesis project, I identified a new regulator for GABRB1; and, for the first time, link its transcription to potential programs of cellular respiration and energy consumption. Although previously identified as important for the coupling of neuronal excitation to increased mitochondrial biogenesis via NRF-1,
my findings are the first to link the mitochondrial and nuclear genomes to the potential regulation of GABAergic signaling.

C. CREB and activity-dependent gene expression

1. Post-translational modification

In response to either electrical or chemical stimuli, a wave of biochemical changes occur in neurons that ultimately lead to the alteration of synaptic strength and neural excitability. A key intracellular component that these extracellular signals converge on is the messenger molecule cyclic adenosine monophosphate (cAMP). cAMP is formed from ATP by the enzyme adenylate cyclase, which is activated by specific G proteins that are coupled to various receptors at the membrane surface. Binding of cAMP liberates the catalytic subunit of the cyclic AMP-dependent protein kinase or Protein Kinase A (PKA), which mediates the transfer of a phosphate group from ATP to serine or threonine residues of its substrates. Depending on the nature of the target protein, the phosphorylation event can either lead to activation or inactivation of the protein, a form of molecular “on/off” switch. A set of PKA targeted proteins are known as transcription factors, which can enter the cell nucleus, bind to gene promoters, and alter transcription and gene expression. One such factor important to the function of the nervous system is the cAMP response element-binding protein (CREB).

CREB is essential in the formation of long-term memory as mediated by the hippocampus and the amygdala (Montminy and Bilezikjian 1987). The relative level of CREB activity has also been associated with the selection of neurons for inclusion in a circuit that is believed to (Han, Kushner et al. 2007). As a dimer, CREB acts as a
transcriptional regulator by binding to the promoter region of a gene that contains the eight-bp canonical cAMP Response Element (CRE), 5’-TGACGTCA-3’. The activated CREB recruits cofactors and components of the transcriptional machinery necessary for RNA synthesis. An important residue in CREB responsible for its activation is serine 133 (S133), one of several serine residues within the regulatory domain known as kinase-inducible domain (KID) that spans residues 123-151 (Johannessen, Delghandi et al. 2004, Johannessen, Delghandi et al. 2004). Once S133 is phosphorylated, CREB associates with one of its co-activator, the CREB-binding protein (CBP) or its paralog p300, at the kinase inducible interacting (KIX) domain (Chrivia, Kwok et al. 1993). In turn, CBP recruits the RNA polymerase II complex to the promoter for transcription initiation.

However, the regulation of CREB is more complex. This regulatory KID domain allows for the convergence of a variety of kinases, including protein kinase A (PKA), calcium-calmodulin-dependent kinases (CaMKs), and Ras/MAPK, to regulate CREB’s function via post-translational modification.

In addition to cAMP and PKA signaling, CREB activation can be induced in response to increased intracellular Ca^{2+} (West, Griffith et al. 2002). Typically, intracellular homeostatic Ca^{2+} concentration is kept at low levels. Ca^{2+} influx can occur through the opening of ligand-gated ionotropic glutamate receptors, such as NMDA receptors during glutaminergic transmission, and voltage-gated Ca^{2+} channels (VDCCs), particularly the L-type Ca^{2+} channel. Ca^{2+} interacts with calmodulin (CaM), a calcium sensor, which acts to activate Ca^{2+}-CaM kinases (e.g. CaMKI, CaMKII, and CaMKIV). In vitro, these CaMKs can phosphorylate CREB (Dash, Karl et al. 1991, Sheng,
Thompson et al. 1991, Enslen, Sun et al. 1994, Matthews, Guthrie et al. 1994, Sun, Enslen et al. 1994, Bito, Deisseroth et al. 1996, Kornhauser, Cowan et al. 2002, West, Griffith et al. 2002). The most notable and well-characterized of these is CaMKIV, which phosphorylates CREB S133 in vivo (Ho, Liauw et al. 2000, Ribar, Rodriguez et al. 2000, Kang, Sun et al. 2001, Anderson and Means 2002, Wei, Qiu et al. 2002, Wei, Qiu et al. 2002). Evidence for CaMKIV regulation of CREB is supported by over-expression studies where constitutively active CaMKIV drives CREB-dependent gene expression (Enslen, Sun et al. 1994, Matthews, Guthrie et al. 1994, Sun, Enslen et al. 1994). These results are complemented by additional evidence from animal models where CaMKIV inhibition and CaMKIV deficiency display a loss in CREB phosphorylation and decreased CREB-targeted gene expression (Bito, Deisseroth et al. 1996, Ho, Liauw et al. 2000). In addition, it has been shown that Ca\(^{2+}\)-CaM kinase signaling, in part, accounts for the induction of CREB S133 phosphorylation in neurons as a response to stimulation by the neurotrophin brain derived factor (BDNF) in hippocampal slices. In fact, it has been suggested that CREB plays a key role in mediating gene expression in responses to neurotrophins (Finkbeiner, Tavazoie et al. 1997).

CaMK phosphorylation does not always lead to CREB activation. For instance, CaMKII can also phosphorylate CREB on residue Serine 142 (S142) in vitro, which disrupts CREB dimerization and CREB-CBP interactions without affecting DNA binding capacity (Radhakrishnan, Perez-Alvarado et al. 1997, Parker, Jhala et al. 1998, Wu and McMurray 2001). Thereby, inactivating transcription of CRE-dependent genes. Despite these findings, S142 phosphorylation cannot be generalized as a marker of CREB
inactivation because S142 phosphorylation has also been linked to active CREB mediated transcription (Gau, Lemberger et al. 2002, Kornhauser, Cowan et al. 2002). Together, these observations point to the possibility that CREB-dependent gene expression is more complicated than originally described and may even take place irrespective of CBP interactions (Lonze and Ginty 2002).

In addition to BDNF, activation of growth factor signaling and receptor tyrosine kinase (RTK) results in the phosphorylation of CREB at S133. First, stimulation by the neurotrophin, nerve growth factor (NGF), activates its Trk A receptor, an RTK that activates Ras and stimulates the serine/threonine kinase, Raf, that then activates MEK as part of a sequence of kinase cascades (Raf-MEK-ERK-RSK). The activated ERKs and RSKs then phosphorylate CREB at S133 (Xing, Ginty et al. 1996) (Finkbeiner, Tavazoie et al. 1997). In contrast to the Ca\(^{2+}\)-CaM-CaMKIV pathway, this Ras-ERK-RSK2 pathway results in slow phosphorylation kinetics instead of the rapid phosphorylation/dephosphorylation that is a function of S133 regulation. Therefore, the effect of phosphorylation on CREB may be achieved by diverse mechanisms, in a pathway and cell-type dependent manner. The complexity of CREB signaling and its importance to processes of cognition argues that it will be critical to develop molecular tools in the future that can be used to dissect out the distinct molecular signatures associated with different aspects of CREB-mediated brain function, an area of focus in the research of my thesis.

2. Models for CREB-mediated gene regulation

The prevailing model for CREB-dependent gene regulation is that CREB binds
constitutively to the cAMP-response element (CRE) within promoters in its inactive form (i.e. not phosphorylated at S133). Upon stimulation of intracellular signaling, CREB is phosphorylated on the DNA, facilitating transcriptional activation, with gene expression increasing several fold (Barco, Lopez de Armentia et al. 2008). However, through chromatin immunoprecipitation (ChIP) analysis using genomic extracts from unstimulated cultured cortical neurons, the Ginty group found little to no binding of CREB in promoters with known functional CREs. Their findings, taken together with our own (Lau, Saha et al. 2004), suggest that CREB may be recruited to the DNA in an activity-dependent manner.

CREB is part of a larger family of transcription factors that possess a highly similar basic region/leucine zipper (bZIP) domain, which recognize CREs. This family includes a subcategory of related factors CREM (cAMP response element modulator) and ATF-1 (activating transcription factor 1). The basic region alpha helices contact the major groove of DNA, interacting with the DNA backbone at phosphates. Results of mutagenesis studies show that leucine residues are required for dimerization. Homology zipper DNA-binding domain in the C-terminus of transcription factors classifies them as a member of the CREB/Activating Transcription Factor (ATF) family. Two glutamine-rich regions Q1 and Q2 flank the KID domain. These regions help to anchor CREB to CRE-containing promoters and facilitate its interactions with the transcriptional machinery. CREB can homodimerize or heterodimerize with its family-members that contain leucine zippers, ATF-1 or cAMP responsive element modulator (CREM).
3. Potential mechanisms in the attenuation of CREB signaling

The inducible cAMP early repressor (ICER) is a gene product transcribed from the intronic region of the CREM gene via promoter P2 (Molina, Foulkes et al. 1993). Unlike CREM, ICER contains both, or the second DNA binding domain of CREM, but lacks the transactivating domain that is responsible for CREM’s function as a transcriptional activator. ICER can form homodimer and heterodimers with all other CREM and CREB proteins, but homodimer formation is more favorable (Molina, Foulkes et al. 1993, Stehle, Foulkes et al. 1993). As a homodimer, ICER can bind to the CRE, but since it lacks a transactivation domain, it represses the transcription of CRE containing genes by competing for interaction with the coactivator proteins CBP or TFIID, or both (Sun and Maurer 1995, Nakajima, Uchida et al. 1997). Support for ICER as a repressor of CREM mediated transcription comes from studies of ICER IIγ overexpression where forskolin-induced CRE-driven transcription is blocked (Burkart, Mukherjee et al. 2006, Misund, Steigedal et al. 2007, Steigedal, Bruland et al. 2007). ICER splice variants have also been shown to exert cell-type specific effects on cell growth (Razavi, Ramos et al. 1998, Memin, Yehia et al. 2002, Misund, Steigedal et al. 2007). In summary, ICER, like CREB, is critical for diverse activity-dependent processes; and, in fact, there may be a critical balance between CREB and ICER genomic programs that underlies brain plasticity and its associated functional disorders.

As a goal of my thesis work, I developed a novel reporter system to study the effects of ICER expression on CREB/CBP interactions that can be used in the future to...
monitor the presence of ICER in response to chronic exposure to neurotrophins, like BDNF whose levels rise dramatically upon status epilepticus (See Chapter 4).
CHAPTER II: MATERIALS AND METHODS

A. Cell culture

_Human embryonic kidney (HEK) cell culture_

HEK cells were grown in Dulbecco’s Modified Eagle Medium containing 4.5g/L D-Glucose and 4mM L-Glutamine, supplemented with 0.1mM non-essential medium and 10% fetal bovine serum. Cells were cultured in an incubator that maintained the cells at 37°C and 5% CO₂. Cells were detached with 0.25% (w/v) Trypsin-0.53mM EDTA solution and subcultivated at ratio of 1:20 every 3-4 days in 75 cm² flask.

_Partial neuronal cell culture_

Primary hippocampal and neocortical neurons are isolated from embryonic day 18 Sprague-Dawley rat embryos (Charles River Laboratories). Isolated embryonic brains and the subsequently dissected hippocampi cortices were maintained in ice-cold modified calcium-magnesium free Hank’s Balanced Salt Solution (HBSS) (4.2mM sodium bicarbonate, 1 mM sodium pyruvate, and 20 mM HEPES, 3mg/ml BSA) buffering between pH range 7.25 – 7.3. Tissues are then separated from HBSS dissection solution and trypsinized (0.05% trypsin-EDTA) for 10 minutes in 37°C and 5% CO₂. The trypsin reaction is stopped with serum inactivation using Neural Basal Medium (NBM) containing plating medium (10% FBS, 10 U/ml penicillin/streptomycin, 2mM L-glutamine). Tissues are titrated with 1000 mL micropipette and diluted to a concentration of 0.5x10⁶ cells/mL in plating media for plating. Cells are allowed to adhere onto Poly-L-lysine coated culturing surface for 1 h prior to changing to serum-free feeding medium.
(2% B-27, 2mM glutamine, 10 U/ml penicillin/streptomycin supplemented NBM).

Neuronal cultures are maintained in 37°C and 5% CO₂ incubator.

**B. Chromatin immunoprecipitation (ChIP)**

ChIP is performed according to Magna ChIP A protocol (Millipore). Briefly, primary neurons in 100 mm dishes are fixed with final concentration of 1% formaldehyde in culturing medium. The remaining unreacted formaldehyde is quenched with Glycine. Genomic DNA and protein complex are extracted from cells using nuclear lysis buffers supplemented with protease and phosphatase inhibitors. The lysates containing DNA-protein complexes are sonicated (nine times, 5 minutes each at a 30s on/off interval) in an ice-cold water bath with Bioruptor (Diagenode) in order to generate fragments predominantly in the range of 200 – 1000 bp in size. The sheared chromatin is immunoprecipitated with either anti-NRF-1 antibody (Abcam ab34682 ChIP grade antibody) or normal rabbit IgG overnight at 4°C with constant rotation. The antibody/transcription factor bounded chromatin is separated from unbound chromatin using Protein A conjugated magnetic beads and magnetic pull-down. The isolated complexes are washed with a series of salt buffer solutions prior to eluting and reverse cross-linking DNA from complexes using NaCl, EDTA, Proteinase K and heating. DNA fragments are recovered through column purification. The immunoprecipitated DNA fragments are identified by quantitative PCR (qPCR) using specific primer and TaqMan probe that flank putative responsive elements in gene promoters. qPCR was performed using the ABI7900HT real-time PCR system and FastStart Universal Probe Master mix (Roche) in 20 μl reactions.
C. Electrophoretic mobility shift (EMSA) and supershift assay.

Briefly, ~30 bp DNA probes containing putative NRF-1 binding sequence were incubated with 25µg of neocortical nuclear extracts for electrophoresis under non-denaturing conditions. Following electrophoresis, the protein-DNA complexes were detected by exposing the gel to x-ray film (Kodak) as part of autoradiography. The DNA probes were created from annealing synthesized oligonucleotides (www.idtdna.com) and 5’ end labeling using [γ-³²P] ATP (PerkinElmer) in a T4 polynucleotide kinase (NEB) reaction. Nuclear extracts were prepared from DIV 7-9 primary neocortical neurons grown on 10-cm plates. Protein-DNA binding specificity were determined by adding poly (dI-dC) (Roche) or/and 100-fold excess unlabeled DNA probe prior to the addition of labeled probe during the binding reaction. To generate a supershift complex, NRF-1 antibody (AbCam) was added to the reaction mixture for 15 min to loading onto gel. The binding reactions were loaded onto 5% polyacrylamide gel in 0.5X TBE buffer and ran at 200V for 2 h at 4ºC. The positive control probe consisted of sequence from the Rat cytochrome c with NRF-1 binding site previously described (Evans and Scarpulla, 1990).

Table 1. EMSA probes and competitor oligonucleotide sequences

| GABRB1 NRF1 | 5’-agccgcgcTCTCGCATGCAGcattc-3’ | 5’-gaatggaccTGCGCATGCAGcgcgtcgt-3’ |
| GABRB1 NRF1 mutant | 5’-agccgcgcTCTGccCATGgGCAgttccattc-3’ | 5’-gaatggaccTGccCATGgGCAGcgcgtcgt-3’ |
| rCysc NRF1 | 5’-ctgctatGCGCATGCGCgcgcacctta-3’ | 5’-taaggggtgcGCGCATGCGGCTagcag-3’ |

D. Preparation of nuclear protein extracts.

Cortical nuclear extracts were isolated from 4-5 100 mmm DIV 7-9 primary E18 neocortical neurons. Lysis ‘Buffer A’ (salts and a cocktail of protease inhibitors which
includes DDT, PMSF, aprotinin, leupeptin and pepstatin) was used to disrupt the plasma membrane and protect proteins (e.g. transcription factors) from enzymatic degradation. The lysis reaction was incubated on ice for 15 minutes. Following incubation, 25μl of 10% NP40 was added to each extract sample, and briefly vortexed. The resulting nuclei were pelleted by centrifugation at 12,000 rpm for 2 minutes at 4ºC. Once the supernatant is removed, the nuclei pellet was resuspended in 100-120μl of hypotonic solution ‘Buffer C’, breaking down the nuclear envelope, while protecting nuclear proteins from degradation. Mixing of buffer C and cellular extract should be done with care, only use pipette tip to swirl pellet in buffer. Incubate samples on a rocking platform for 1 hr at 4ºC. Nuclear debris is separated from nuclear proteins by centrifugation (12,000 rpm for 5 minutes at 4ºC). The subsequent supernatant, containing nuclear proteins, was collected and stored as 20% glycerol mixture in -80ºC freezer.

E. Molecular cloning

Subcloning promoter reporter constructs

The minimal GABRA4p-Luc (pGL2-GABRA4) containing the first 500 bp of the 5’ flanking region have been previously described (Roberts et al., PNAS 2006). A 367 bp fragment extending from +62 to -307 with respect to the transcription start site of the rat cytochrome c gene (Cycs) was amplified from total rat genomic DNA (Clontech Laboratories, Mountain View, CA) using Phusion High-Fidelity polymerase (NEB, Ipswich, MA) and directionally cloned into pGL2-basic vector (Promega, Madison, WI) using restriction sites Kpn I and Xho I. The GABRB1p-Luc (pGL2-GABRB1) containing the 5’ flanking region had been previously described (Russek SJ et al., PNAS 2000).
promoter containing a mutated NRF-1 element within the *GABRB1p-Luc* was created by PCR-driven overlap extension. Using wild type *GABRB1p-Luc* as PCR template, two PCR fragments were amplified using GL1 primer (Promega) and the antisense mutant NRF-1 oligonucleotide from EMSA, and sense mutant NRF-1 oligonucleotide and GL2 primer (Promega), resulting in fragments with 30 bp overlapping sequences that contain the mutant NRF-1 element. A second PCR step using GL1, GL2 primers and both initial PCR products produced the mutant *GABRB1* promoter insert.

**Construction of dominant-negative (DN) NRF-1.**

The pcDNA3.0-NRF-1 DN expresses amino acid residues 1-304 of NRF-1, which encodes the DNA-binding, dimerization and nuclear localization domains of NRF-1, but not the transactivation domain (amino acids 305-503). The construct was created using PCR with the forward primer 5’-CGGGGTTACCCATGGAGGAACACGGAGTGA CCCAAAC-3’, containing the underlined Kpn I restriction site and the kozak sequence (ACC) on the 5’ end, and the reverse primer 5’GCTCTAGATCCTGTGATGGTGACTACA AGATGAGCTAATATGTGCTGAGTGGC-3’, containing stop codon (TGA) and Xba I restriction site. PCR products were digested with restriction enzymes Kpn I and Xba I, and ligated into pcDNA3.0 vector (Invitrogen) with T4 DNA Ligase (NEB, Ipswich, MA). Constructs were amplified by transforming ligation products into subcloning efficiency DH5α Competent cells (Life Technologies, CA).

**Split-complement luciferase constructs**

The design of the split luciferase proteins, KID-LucC and KIX-LucC were previously described and tested in HEK293 cells (Ishimoto T. et al., 2012). The plasmids that encode
these recombinant proteins were constructed (generated by Zhuting Li and Sabita Bandyopadhyay) using In-Fusion HD Cloning Kit (Clontech) in accordance with manufacturer’s directions. For each construct, five DNA fragments (i.e. two PCR-amplified, two annealed oligonucleotides, and EcoRI and NotI linearized pCAG-Cre vector) were engineered to contain a 15 bp overlap that directionally fuses the fragments at their ends. The synthetic amino-terminal fragment contains an overlapping sequence with vector, the Kozak consensus sequence, and nucleotides encoding the start codon and nuclear localization signal (NLS) DPKKKRKVDPKKKRKV. The internal fragment, containing either KID of CREB (gift of ME Greenberg) or N-terminus of Luciferase (LucN) from the pGL2-basic vector (Promega), was PCR-amplified with 5’-end primers overlapping with the NLS DNA sequence. The polyGGGS linker was synthesized with an overlap with either the 3’ end of KID or LucN at the 5’ end. The carboxyl-terminal fragment, containing either the C-terminus of Luciferase (LucC) from the pGL2-basic vector or KIX (total rat cDNA), were PCR-amplified with primers overlapping the sequence with the polyGGGS linker at the 5’ end and linearized pCAG-Cre vector at the 3’ end. S133A-KID-LucC was created by PCR site-directed mutagenesis by changing a single nucleotide from TCC to GCC.
Table 2. Primers for PCR amplification of split-complement luciferase fragments

<table>
<thead>
<tr>
<th>pCAG KID-LucC</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>KID Fragment</td>
<td>F: 5’-AAG AAG AGG AAG GTC CAG ATT TCA ACT ATT GCA GAA AGT G-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-AGT CTC CTC TTC TGA CTT TTC-3’</td>
</tr>
<tr>
<td>LucC Fragment</td>
<td>F: 5’-GGT GGA GGT GGA AGT GGA TGG CTA CAT TCT GGA GAC ATA GC-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-CT GAG GAG TGC GGC CGC CAA TTT GGA CTT TCC GCC CTT C-3’</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pCAG KIX-LucN</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>KIX Fragment</td>
<td>F: 5’-GGT GGC GGC GGA TCT GGT GTT CGA AAA GGC TGG-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-CT GAG GAG TGC GGC CGC TCA TTT TCC TAG TTC TTT TGT TAT TTT ATA GAT TTT-3’</td>
</tr>
<tr>
<td>LucN Fragment</td>
<td>F: 5’-AAG AAG AGG AAG GTC ATG GAA GAC GCC AAA AAC-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-ATC CTT GTC AAT CAA GGC GT -3’</td>
</tr>
</tbody>
</table>

Table 3. DNA sequence of split-complement luciferase fragments

<table>
<thead>
<tr>
<th></th>
<th>DNA Sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kozak Sequence +</td>
<td>T TTT GGC AAA GAA TTC GAC CCG AAG AAG AGG AAG GTC</td>
</tr>
<tr>
<td>Start Codon + NLS</td>
<td>AAG GTC GAC CCG AAG AAG AGG AAG AGG AAG GTC</td>
</tr>
<tr>
<td>KID overlapping</td>
<td>TCA GAA GAG GAG ACT GGT GGC GGT GGC TCG GGC GGT GGT GAT GTC</td>
</tr>
<tr>
<td>polyGGGS linker</td>
<td>GGC TCG GGC GGC GGA TCT</td>
</tr>
<tr>
<td>LucN overlapping</td>
<td>TTG ATT GAC AAG GAT GGT GGC GGT GGC TCG GGC GGT GGT GAT GTC</td>
</tr>
<tr>
<td>polyGGGS linker</td>
<td>GGC TCG GGC GGC GGA TCT</td>
</tr>
</tbody>
</table>

F. DNA Sequencing

All recombinant DNA constructs were verified with sequencing performed by GENEWIZ, Inc. (South Plainfield, NJ) using specific forward and reverse primers (Table 4).
Table 4. Vector sequencing primers

<table>
<thead>
<tr>
<th>Vectors</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3.0/pcDNA3.1</td>
<td>T7: 5’-TAA TAC GAC TCA CTA TAG GG-3’</td>
</tr>
<tr>
<td></td>
<td>SP6: 5’-GAT TTA GGT GAC ACT ATA G-3’</td>
</tr>
<tr>
<td>pGL2</td>
<td>GLprimer1: 5’- ATG TAT CTT ATG GTA CTG TAA CTG</td>
</tr>
<tr>
<td></td>
<td>GLprimer2: 5’- CTT TAT GTT TTT GGC GTC TTC CAT -3’</td>
</tr>
<tr>
<td>pCAG</td>
<td>F69: 5’-GTG TGA CCG GCG GCT CTA -3’</td>
</tr>
<tr>
<td></td>
<td>R1879: 5’- AGA TCT CAG TGG TAT TTG TGA GCC -3’</td>
</tr>
</tbody>
</table>

G. Transfection for transient mammalian cell expression.

Calcium phosphate-mediated transfection

Calcium phosphate mediated transfection of DNA into primary neuron cultures was previously described by Dudek H et al., 2001. For each well of a 6-well plate, 4-8 µg of DNA was transfected in 80 µl volume. In one sterile tube, DNA and one-tenth volume of CaCl₂ were diluted in nuclease-free water to 40 µl volume. In the other sterile tube, 40 µl of 2X Heps Buffer Saline (HBS) was allocated. While gently vortexing 2X HBS, the diluted DNA and CaCl₂ solution was slowly added to the 2X HBS in a dropwise manner. The DNA precipitates were allowed to form at room temperature in the dark for 20 minutes prior to transfection, primary neurons were washed with pre-warmed DMEM (minus glutamine) and subsequently incubated in DMEM. The formed DNA precipitates were added to neurons in DMEM and allowed to incubate on cells for 30-45 min at 37°C and 5% CO₂. Neurons were returned to feeding media after washed twice with DMEM. 24 h after transfection, neurons were typically assayed for luciferase activity.

Magnetofection™ with NeuroMag reagent.

The day prior to transfection, half of the culture media was replaced with fresh feeding media on cells to be transfected. For each well of a 6-well plate, 2-4 µg of DNA was
transfected with NeuroMag reagent at a 1:1 DNA to NeuroMag reagent ratio. First, DNA was diluted in 200 μl of NBM in a microcentrifuge tube, and the NeuroMag reagent was set aside. The diluted DNA was then gently mixed into the tube of NeuroMag reagent, and DNA-NeuroMag complexes were allowed to form for 20 minutes at room temperature. The newly formed complexes were distributed onto neurons and allowed to incubate for 20 minutes on a magnetic plate at 37°C and 5% CO₂. After 24 h, neurons were evaluated for transgene expression in downstream assays.

*Lipofection-mediated transfection.*

The day before transfection, HEK 293 cells were seeded at 6.25x10⁶ cells per well. Prior to transfection, all reagents were allowed to warm to room temperature. For the screening of active NRF-1 targeting shRNAs, each well was transfected with 0.5μg of pcDNA3 NRF-1 and 1.0 μg of pSilencer NRF-1 shRNA, or non-targeting control. 4.5μl of XtremeGene 9 DNA transfection reagent (Roche) was diluted in 150 μl of Opti-MEM serum-free medium for 3:1 ratio using a sterile tube. After gentle mixing, DNA was added to the 150 μl of diluted transfection reagent and allowed to incubate for 30 min at room temperature. The formed reagent:DNA complex was then added to the cells in a dropwise fashion. After 48 h incubation, cell extracts were harvested for protein expression.

**H. Promoter luciferase reporter assays**

E18 primary cortical neurons (1 X 10⁶ cells) were plated into each well of a 6-well plate (NUNC). On DIV7, neurons were transfected with 1μg of expression construct, 2μg of promoter reporter construct, and 3μl of NeuroMag transfection reagent (1:1 DNA to
reagent ratio). Twenty-four hours after transfection, neurons were actively lysed by scraping. Cell lysates were cleared of precipitates by centrifugation and then assayed for luciferase activity by adding Luciferase Assay Substrate (Promega). Luciferase reporter activity was measured by using the GloMax Multi-Detection system luminometer (Promega). Luciferase activity was normalized to total protein as determined by Coomassie protein assay (Thermo Scientific Pierce). All transfections were performed in duplicates and using three or more platings of neurons, each from an individual animal.

I. Assays for protein determination

Bradford protein assay

A total protein quantification method was employed that uses a dye-based bio-rad protein assay (Bio-Rad #500-0006) and an albumin based standard curve to quantify protein extracts. Standard curve proteins were prepared using a 1mg/ml stock of Bovine Serum Albumin (BSA) to create standards with known amounts 0, 1, 2, 4, 8, and 16 μg of BSA. The background/baseline solution contained 4 μl of lysis buffer (e.g. 1X RIPA) and the remaining volume was made up of water for a total volume of 800μl. Four μl of unknown protein sample was diluted with 796 μl of water for a 1:250 dilution. To all samples and standards, 200 μl of Bio-Rad Protein Assay Dye was added and samples were vortexed. The mixture was then incubated in room temperature for about 20 minutes (or until there was an appropriate color change) prior to reading samples using a spectrophotometer at absorbance at 600 nm GloMax (Promega). Protein concentrations and absorbance readings were positively correlated and linear within the given concentration range.

Coomassie protein assay
A total protein quantification method that uses a Coomassie assay dye (Thermo Scientific) and an albumin based standard curve was used to quantify protein extracts in Passive Lysis Buffer (Promega). The known standards (2000, 1500, 1000, 750, 500, 250, 125, 25 μg/ml of BSA) were prepared using a 2mg/ml BSA stock. The background/baseline solution contained lysis buffer (e.g. 1X Passive Lysis Buffer) and the remaining volume was made up of water for a total volume of 800μl. Four μl of an unknown protein sample was diluted with 796 μl of water for a 1:250 dilution. To all samples and standards, add 300 μl of Coomaissie Protein Assay Dye and vortex samples. Allow the mixture to incubate in room temperature for about 20 minutes prior to reading samples using a spectrophotometer absorbance at 600 nm GloMax (Promega). Protein concentrations and absorbance values (595 nm) were positively correlated and related by second-order polynomial.

J. Quantitative reverse-transcription PCR (qRT-PCR)

Total RNA was isolated from cultured primary neocortical or hippocampal neurons using RNeasy Micro Kit (Qiagen) according to manufacturer’s protocol. RNA concentration was determined by Nanodrop (Thermo Scientific) spectrophotometer at 260 nm absorbance. For each qRT-PCR, 20-100 ng of total RNA was reverse-transcribed to cDNA, and PCR amplified in a single reaction mixture using TaqMan® One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems), and target specific TaqMan probe and primer set. Incubation and thermal cycling conditions were performed using the ABI7900HT in the 384-well PCR plate format (Applied Biosystems). RT reaction was held at 48ºC for 30 min, followed by 95ºC for 10 min for polymerase activation. PCR
was then performed with 15 sec denaturation at 95°C, annealing and extension for 1 min at 60°C for 40 cycles. Rat cyclophilin A gene expression served as internal control. Relative gene expression was quantified using \( \Delta \Delta C_T \) and standard curve generated based on amplification of total RNA extracted from untreated cultured neurons.

K. RNA interference

The pSilencer™ siRNA expression vector system was used to express NRF-1 specific hairpin siRNAs under control of the human U6 RNA pol III promoter (pSilencer 2.0-U6, Life Technologies). Four hairpin siRNAs were constructed with a common hairpin loop sequence (TTCAAGAGA), RNA Polymerase III terminator sequence (TTTTTTT), and 19 complementary nucleotide sequences targeting the coding region of NRF-1. siRNA sequences were selected and validated as previously described (Dhar S.S. and Won-Riley, M.T.T., 2009). Directional cloning of the siRNA templates into the pSilencer vector was facilitated using BamH1 (G^GATC_C) and HindIII (A^AGCT_T) restriction sites. A negative control vector expressing a non-targeting siRNA (5’-ACTACCGTTGTTATAGGTG-3’) that lacks significant homology to the human and murine genomes, as validated by the manufacturer, was used as a control in our silencing assays.
Table 5. Sequences encoding NRF-1-targeting shRNAs

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRF-1 shRNA #6</td>
<td>GATCCGAAAGCTGCAAGCCTATCTTTCATTTCAAGAGAAGATAG GCTTGCAGCTTTTTTGGAAGA</td>
</tr>
<tr>
<td>NRF-1 shRNA #7</td>
<td>GATCCGACACAGGGAGGAATTTATTTTTGGAAGAAGATGAATT AACCTCTCTGTGCTTTTGGAAGA</td>
</tr>
<tr>
<td>NRF-1 shRNA #8</td>
<td>GATCCGCATTACGGACCATTATTCAAGAGAGAATGAAGTT ACTACTGTTGCTTTTTGGAAGA</td>
</tr>
<tr>
<td>NRF-1 shRNA #9</td>
<td>GATCCAGAGCATGGAGCTATTTAATTTCAAGAGAGAATCTTCC AGGATCATGCTCTTTTTTGGAAGA</td>
</tr>
<tr>
<td>NRF-1 shRNA #10</td>
<td>GATCCGGCCACAGGAGGAAGTTTATTTTTGGAAGAAGATGAATT AACCTCTCTGTGCTTTTGGAAGA</td>
</tr>
</tbody>
</table>

L. Western blot analysis

Total proteins were extracted from cells using RIPA lysis buffer (Tris, pH 7.4, 10 mM; Nonidet P-40 1%; NaCl 150 mM; SDS 0.1%) supplemented with protease and phosphatase inhibitors (Roche Applied Science), EDTA 1mM; sodium orthovanadate 1 mM; sodium deoxycholate 0.1%; phenylmethylsulfonyl fluoride 1 mM. 20-30 μg of whole cell extracts were separated by SDS-PAGE under reducing conditions on either 10% or 4-20% Tris-glycine gel according to mass/size. The electrophoresed samples were transferred to nitrocellulose membrane for detection. The membrane was probed for proteins of interest using primarily rabbit polyclonal antibodies and peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000 dilution in 1X TBS-T). Proteins were detected with enhanced chemiluminescence reagent (ECL) (GE Healthcare Life Sciences) and visualized on x-ray film (Thermo Scientific). Immunoblot films were scanned and protein band intensities were quantified using ImageQuant™ TL software (GE). Levels of β-actin (mouse monoclonal Anti-β-actin Ab) were used to normalize whole cell/cytoplasmic protein extracts.
M. Bioluminescence Imaging

In vivo imaging system

Primary cortical neurons (5 x 10^4 per well of 96-well black-walled plate) were transiently transfected with plasmids using the NeuroMag (Oz Biosciences) protocol at DIV 8. To image live cells, the D-luciferin substrate (20 µl of 1.5 mg/ml in Calcium and Magnesium free 1X PBS) was added to wells containing 200 µl of media. Nine to 10 min were given for cellular uptake of the membrane permeant D-luciferin prior to imaging. Photon flux for each well was measured with an IVIS charge-coupled device camera (1 or 4 min exposure).

Bioluminescence microscope imaging system

Primary cortical neurons (1 x 10^6 per 35mm confocal dish) were transiently transfected with plasmids (NeuroMag (Oz Biosciences)) described above at DIV 8. To image individual live neurons, D-luciferin (20 µl of 1.5 mg/ml in Calcium and Magnesium free 1X PBS) was added to wells containing 200 µl of media. Cells were given 9-10 min for D-luciferin uptake prior to imaging. Live cell images were acquired using the Olympus LV200 Bioluminescence Imaging System, where neurons were maintained in an incubation chamber kept at 37°C and 5% CO₂.
CHAPTER III. A role for nuclear respiratory factor 1 (NRF-1) in the control of β1-containing GABARs

A. Abstract

The physiological role of β1 subunit-containing type A GABA receptors (GABARs) has not been well characterized, although altered expression of β1 is associated with neurological and neuropsychiatric disorders. In particular, down-regulation of β1 subunit levels is observed in the brains of patients with epilepsy, autism, bipolar disorder, and schizophrenia. A pathophysiological feature of these disease states is an imbalance in energy metabolism and mitochondrial dysfunction. The transcription factor, nuclear respiratory factor 1 (NRF-1), has been shown to be a key mediator of genes involved in oxidative phosphorylation, mitochondrial biogenesis and synaptic transmission. Using a variety of molecular approaches (including mobility shift, promoter/reporter luciferase assays, and RNA silencing), we now report that NRF-1 regulates the transcription of the GABAR β1 subunit gene (GABRB1) in neurons and that its core promoter region contains a conserved canonical NRF-1 element responsible for sequence specific binding and transcriptional control over expression. Our identification of GABRB1 as a new target for NRF-1 suggests that the genes coding for inhibitory neurotransmission, in addition to excitatory, may be coupled to cellular metabolism.

B. Introduction

The type A γ-aminobutyric acid receptor (GABAR) is a ligand-gated Cl⁻ ion channel that mediates inhibitory neurotransmission in the adult mammalian central nervous system. The majority of GABARs are composed of two α and two β subunits, and either a γ2 or δ subunit (Farrar, Whiting et al. 1999, Barrera, Betts et al. 2008, Patel,
Mortensen et al. 2014). For each receptor, there is the binding of two molecules of GABA, one molecule at each α and β subunit interface (Rabow, Russek et al. 1995, Connolly and Wafford 2004, Olsen and Sieghart 2009). In the mature neuron, activation of GABARs leads to hyperpolarization. Depending on its subunit composition, GABARs may contain binding sites for barbiturates, benzodiazepines, ethanol, and/or neuroactive steroids. There are nineteen different subunit genes to date, grouped into eight classes (i.e. α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3) that contribute to the diversity and differential assembly of receptor subtypes. The β subunits, which contain the domains that interact with mediators of receptor trafficking and endocytosis (for reviews see (Jacob, Moss et al. 2008, Vithlani, Terunuma et al. 2011)), play an important role in the expression of GABARs at the cell surface.

The human GABRB1 gene, located on chromosome 4, is part of a GABAR gene cluster that contains the genes that encode the α2, α4, and γ1 subunits. A dysregulation of GABAR-mediated neurotransmission has been implicated in various neurological disorders (Hines, Davies et al. 2012) that show altered levels of GABAR subunits, including β1. Through linkage studies, GABRB1 has been associated with alcohol dependence (Parsian and Zhang 1999, Sun, Cheng et al. 1999, Zinn-Justin and Abel 1999, Song, Koller et al. 2003); and recently, it was reported that specific mutations in mouse Gabrb1 produce increased alcohol consumption that is linked to increased tonic inhibition (Anstee, Knapp et al. 2013).

GABRB1 expression is also reduced in the lateral cerebella of subjects with bipolar disorder, major depression, and schizophrenia compared to healthy subjects
Fatemi, Folsom et al. 2013). Particularly in schizophrenia, a significant association of 
GABRB1 has been identified by genome-wide association studies that were coupled to a 
protein-interaction-network-based analysis (Yu, Bi et al. 2014). As GABRB1 and 
GABRA4 lie within the same GABAR gene cluster, it is interesting to note that GABRA4 
association with the risk of developing autism increases with a GABRB1 interaction (Ma, 
Whitehead et al. 2005, Collins, Ma et al. 2006), suggesting that these genes may be 
coordinately regulated. Further evidence for a GABRB1 association with autism is seen in 
the decreased levels of β1 subunit protein within the brains of autistic subjects (Fatemi, 
Reutiman et al. 2009, Fatemi, Reutiman et al. 2010). In addition, the levels of both β1 
and β2 subunit mRNAs are reduced in a Fragile X mental retardation mouse model, 
where the gene Fragile X mental retardation 1 (fmr1) was removed (D'Hulst, De Geest et 
al. 2006). Finally, down-regulation of β1 subunit mRNAs and protein are observed in the 
at rat pilocarpine-model of epilepsy (Brooks-Kayal, Shumate et al. 1998). Yet, despite its 
prevalent association with neurological disorders, there is still little known about the 
function and/or regulation of β1 in neurons.

The TATA-less GABRB1/Gabrb1 promoter (GABRB1-p (human)/Gabrb1-p 
(rodent)) contains multiple transcriptional start sites that lie within a CpG island (Russek, 
Bandyopadhyay et al. 2000, Saha, Hu et al. 2013). In unraveling the molecular 
determinants of GABAR β1 subunit gene regulation, our laboratory demonstrated that the 
minimal GABRB1-p lies within the first 500 bp of the 5’ flanking region. Within this 
region there is a conserved initiator element (Inr) that mediates down-regulation in

Nuclear respiratory factor 1 (NRF-1) is a transcription factor that functions primarily as a positive regulator of nuclear genes involved in mitochondrial biogenesis and oxidative phosphorylation (Scarpulla 2006, Scarpulla 2008). Physiologically, changes in neuronal activity result in parallel changes in cellular metabolism, as well as the expression of NRF-1 and its associated control over mitochondrial biogenesis. In the rat pilocarpine-model of epilepsy, increased levels of NRF-1, and its upstream regulator PGC-1α, are observed in the face of mitochondrial impairment and/or dysfunction (Han, Lin et al. 2011). Furthermore, it has been reported that NRF-1 is a transcriptional activator of glutamate receptor subunit genes under conditions of depolarizing stimulation in neurons (Dhar and Wong-Riley 2009). So therefore, in addition to its role in cellular metabolism, via the regulation of the mitochondrial genome, evidence suggests that NRF-1 coordinates activities in the nucleus to couple neuronal excitability with energy demands of synaptic neurotransmission.

Here, we ask whether NRF-1 may also affect synaptic transmission through control over the transcription of GABAR subunit genes, and in particular the human β1 subunit gene, GABRB1, a gene that has been associated with neuronal developmental disorders, the pathophysiology of epilepsy, and alcohol dependence. In this study, we have uncovered a functional regulatory element within GABRB1 that demonstrates sequence specificity and is responsible for the majority of GABRB1 promoter-reporter activity in transfected primary cortical neurons.
C. Results

1. Identification of a Conserved NRF1 Element in the GABRB1 Promoter

Previously, we mapped the 5’-regulatory region of GABRB1, identifying transcriptional start sites (TSSs) within a 10 bp functional initiator element (Inr) that mediates the response of the gene to chronic GABA (Russek, Bandyopadhyay et al. 2000, Saha, Hu et al. 2013). Now we report that directly upstream of this Inr is a canonical NRF-1 element spanning -11/+1 relative to the major TSS found for Gabrb1 expression in neocortical neurons. As shown in Figure 1, the location of the NRF-1 element within the promoter region is conserved across multiple species. Given the ubiquitous expression of NRF-1, its conservation across species, and its established role in metabolism and mitochondrial biogenesis, our sequence comparison strongly suggests that the NRF-1 element is functionally relevant to β1 subunit expression in the mammalian brain.
Figure 1. Sequence alignment of the 5’ region of β1 subunit gene promoters in reference to the ATG translational start site in multiple mammalian species. The β1 subunit promoters contain a conserved NRF-1 element, which is indicated in bold type upstream of the major initiator element (Inr) that is underlined. Sequences were aligned using ClustalW, where conserved nucleotides are as indicated “*”.

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**Species**

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2. NRF-1 Recognizes a Cis-Element in the GABRB1 Promoter

To determine the specific binding site within GABRB1-p that binds to NRF-1, we performed the electrophoretic mobility shift assay (EMSA) using a $^{32}$P-labeled probe that contains the NRF-1 binding element (Fig. 1) in reaction with the nuclear protein extracts from E18 primary cortical neurons. To validate the specificity of NRF-1 antibody for EMSA analysis, nuclear extracts were incubated with a positive control probe (Dhar, Ongwijitwat et al. 2008) containing the NRF-1 binding site of the rat cytochrome c promoter. As shown in lane 2 of Figure 2, the control radiolabeled probe (rat Cyt C) displays specific DNA recognition of binding proteins in nuclear extracts from cortical neurons that is confirmed by supershift with the addition of NRF-1 specific antibody (Fig. 2, lane 4). Next, specific binding to the putative NRF-1 in GABRB1 was confirmed using the same nuclear extracts, with sequence specificity defined by competition with an unlabeled double stranded oligonucleotide that was identical to the probe sequence (competitor) (Fig. 2, lanes 6 and 7). Addition of an unlabeled competitor mutant probe, containing substitutions within the GC core, failed to compete for complex formation (Fig. 2, lane 8). Presence of endogenous NRF-1 at its site in GABRB1 was further confirmed by supershift analysis using the NRF-1 specific antibody (Fig. 2, lane 9). Finally, a radiolabeled probe containing the sequence of the mutant NRF-1 site in GABRB1 shows little or no complex formation using the nuclear extracts under study (Fig. 2, lanes 10-12).
In vitro binding of NRF-1 to the putative NRF-1 site in GABRB1. $^{32}$P-labeled probes encompassing the NRF-1 binding site were incubated with 20 μg of DIV7 primary rat cortical nuclear extracts. 100-fold excess of unlabeled probe was added to the binding reaction to assess specificity. NRF-1 Abs were pre-incubated with nuclear extracts and radiolabeled probe to test for “supershift” and protein identification. (Left Panel) The NRF-1 element in the rat cytochrome c (Cyt C) promoter displays NRF-1 specific binding (lane 2) and “supershift” (lane 4). (Right Panel) The proposed NRF-1 element in the human GABRB1 promoter displays a probe specific shift (lane 6) (note excess probe was run off of the gel), competition of complex formation with cold competitor (lane 7), lack of competition with mutant cold competitor (lane 8), and supershift upon addition of NRF-1 specific Ab (lane 9). In contrast, binding to radiolabeled probe for NRF-1 mutant GABRB1 shows markedly reduced signal (lanes 11 and 12). ** indicates specific interaction between labeled probe and nuclear extract, ← indicates location of supershift.

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Figure 2. In vitro binding of NRF-1 to the putative NRF-1 site in GABRB1. $^{32}$P-labeled probes encompassing the NRF-1 binding site were incubated with 20 μg of DIV7 primary rat cortical nuclear extracts. 100-fold excess of unlabeled probe was added to the binding reaction to assess specificity. NRF-1 Abs were pre-incubated with nuclear extracts and radiolabeled probe to test for “supershift” and protein identification. (Left Panel) The NRF-1 element in the rat cytochrome c (Cyt C) promoter displays NRF-1 specific binding (lane 2) and “supershift” (lane 4). (Right Panel) The proposed NRF-1 element in the human GABRB1 promoter displays a probe specific shift (lane 6) (note excess probe was run off of the gel), competition of complex formation with cold competitor (lane 7), lack of competition with mutant cold competitor (lane 8), and supershift upon addition of NRF-1 specific Ab (lane 9). In contrast, binding to radiolabeled probe for NRF-1 mutant GABRB1 shows markedly reduced signal (lanes 11 and 12). ** indicates specific interaction between labeled probe and nuclear extract, ← indicates location of supershift.
3. Overexpression of NRF1 Induces GABRB1 Promoter Activity in Transfected Primary Cortical Neurons

To evaluate whether there is a functional consequence to NRF-1 binding to its consensus site in GABRB1-p, primary cortical neurons were transfected with the GABRB1p-luciferase construct containing the 541 bp 5’ flanking region upstream of the human β1 subunit gene (Russek, Bandyopadhyay et al. 2000). We chose this approach to study functional relevance of the NRF-1 site to GABRB1 transcription because NRF-1’s influence on the genome is difficult to detect due to its robust expression at baseline and stability (Baar, Song et al. 2003, Scarpulla 2006, Ramachandran, Yu et al. 2008).

As the expression of NRF1:VP16 fusion proteins has been shown to induce the promoter activity of NRF-1 responsive genes in cell lines (Ramachandran, Yu et al. 2008, Gonen and Assaraf 2010), we transfected primary cortical neurons with NRF1:VP16 expression constructs along with the GABRB1p-luciferase reporter and observed a marked increase (~70%) above baseline (when compared to co-transfection with empty vector control) (Fig. 3). Mutations were introduced into GABRB1-p using site-directed mutagenesis (based on the loss of specific binding of NRF-1 as identified in EMSA (see Fig. 2, lane 8)). As can be seen in Figure 3 for mGABRB1-p, with and without NRF1:VP16 overexpression, mutation of the NRF-1 regulatory element in GABRB1-p reduces basal activity to around 30% of wild type. Overexpression of NRF-1:VP16 has no effect on mGABRB1-p, showing that increased GABRB1 promoter activity directed by NRF-1 is sequence specific; and, moreover, that NRF-1 may be an important positive regulator of β1 subunit expression in developing neurons, especially interesting because
β1 is found in the germinal zones and associated with pre-migrating neurons (Ma and Barker, 1995) and increased mitochondrial biogenesis has also been associated with neuronal differentiation (Vayssiere, Cordeau-Lossouarn et al. 1992, Cheng, Hou et al. 2010).
Figure 3. Overexpression of NRF-1:VP16 increases GABRB1 promoter activity in a sequence specific manner. Primary cortical neurons were co-transfected with 2 µg of wild type GABRB1p (wtGABRB1p) or the NRF-1 binding site mutant (mGABRB1p) and 1 µg of empty vector pcDNA3 or the NRF-1:VP16 fusion construct. Cells were assayed for luciferase activity 24 hours after transfection. Data represent the average ± SEM (n = 5 independent transfections) of luciferase activity relative to wild type GABRB1p in the absence of NRF-1:VP16. “*” and “ns” represent significance based on p < 0.05 and not significant, respectively, according to Student’s t-test.
4. Inhibition of NRF-1 Function in Neurons

To evaluate the direct contribution of endogenous NRF-1 to the regulation of
GABRB1-p, NRF-1 expression constructs were co-transfected with vectors containing
putative NRF-1 targeted shRNAs, or scrambled negative control shRNAs, in HEK cells
to identify shRNAs that could be used subsequently for NRF-1 silencing in primary
neurons. NRF-1 shRNA8 exhibited ~50% knockdown of NRF-1 protein levels (Fig. 4).
Therefore, GABRB1 promoter activity was assayed in the presence of either NRF-1
shRNA8 or NRF-1 shRNA9 in transfected primary cortical neurons. As can been seen in
the upper panel of Figure 5, co-transfection of the promoter construct with shRNA8 led
to a small but significant ~20% reduction in GABRB1-p as compared to the scrambled
shRNA control (Fig. 5A).

To further evaluate the specific effect of NRF-1 on GABRB1 transcription, we
utilized a dominant negative form of NRF-1 that contains the DNA binding domain but
lacks the trans-activation domain (Gugneja, Virbasius et al. 1996). Co-expression of
dominant negative NRF-1 represses GABRB1 promoter activity by 45% (Fig. 5B). When
taken together with the fact that the NRF-1 element in the β1 subunit gene is completely
conserved across species, and that there is a mutational loss of binding and function as
assayed in transfected primary neurons, our results strongly suggest that NRF-1 is an
essential feature of β1 subunit expression in neurons.
Figure 4. Silencing efficiency of NRF-1 shRNAs in HEK293 cells. (Top Panel) HEK cells in 6-well plates were co-transfected with NRF-1 expression constructs (0.5 µg) and vectors containing NRF-1 targeted (1 µg) or random sequence (RS) shRNAs. Forty-eight hours after transfection, total proteins were extracted and monitored for NRF-1 and β-actin expression. A representative western blot is shown. (Bottom Panel) NRF-1 levels were quantified by densitometry and normalized to levels of β-actin. NRF-1 abundance is expressed relative to RS control. Data represent the average ± SEM of n = 3 independent transfections. “*”, p < 0.05, Student’s t-test. NRF-1 shRNAs 8 and 9 were chosen for NRF-1 silencing in transfected primary cortical neurons.
Figure 5. Overexpression of dominant negative NRF-1 and application of NRF-1 directed shRNAs reduce GABRB1 promoter activity in transfected primary cortical neurons. (A) Primary cortical neurons were co-transfected with GABRB1p and NRF-1 shRNA constructs. Forty-eight hours after transfection, cells were assayed for luciferase activity. Data represent the average ± SEM of n = 3 independent transfections, normalized to wild type GABRB1p-luciferase and random sequence (RS) shRNA. **, p < 0.01, Student’s t-test. (B) Primary cortical neurons were co-transfected with either pcDNA3 or a dominant negative variant of NRF-1 (NRF-1 DN) and the GABRB1p reporter (2 µg). Twenty-four hours after transfection, cells were assayed for luciferase activity. Data represent the average ± SEM of n = 6 independent transfections, normalized to wild type GABRB1p and the pcDNA3 control. *, p < 0.05, Student’s t-test.
D. Discussion

The results of these studies in primary cortical neurons strongly suggest that the GABAR β1 subunit gene is regulated by NRF-1, a crucial transcription factor involved in oxidative phosphorylation and mitochondrial biogenesis. Here, we expatiate the role of NRF-1 in coordinating synaptic activity and energy metabolism beyond that of glutamatergic neurotransmission (Dhar, Liang et al. 2009, Dhar and Wong-Riley 2009, Dhar and Wong-Riley 2011), extending it to selective inhibitory GABAergic neurotransmission that may be relevant to brain disorders where β1 is implicated.

Previously, our laboratory mapped the 5’ flanking region of the human β1 subunit promoter. Within this TATA-less promoter, we identified the major transcriptional start site (TSS) and described an initiator element (Inr) that senses the presence of prolonged GABA to mediate the autologous downregulation of β1 subunit expression (Russek, Bandyopadhyay et al. 2000). Our recent studies have discovered that such decreases in β1 subunit RNA levels may reflect a change in the chromatin state as mediated by PhF1b, a polycomb-like protein (Saha, Hu et al. 2013). In our present work, we have found a conserved canonical NRF-1 binding element (Fig. 1) that interacts with NRF-1 in vitro as verified by mobility shift assays (Fig. 2). Interestingly, our results are consistent with a peak of NRF-1 binding in embryonic stem cells H1-hESC as archived in the ENCODE database (Gerstein, Kundaje et al. 2012, Wang, Zhuang et al. 2012, Wang, Zhuang et al. 2013) (http://genome.ucsc.edu/). This significant peak for NRF-1 is located immediately upstream of the β1 Inr element and associated major TTS.
It is thought that NRF-1 binds as a homodimer to the consensus binding sequence (T/C)GCGCA(C/T)GCGC(A/G), making contact with DNA at the guanine nucleotides (Virbasius, Virbasius et al. 1993). This model is supported by the results of our mutational studies that show that a single mutation of G->C eliminates that ability of a cold double stranded oligonucleotide to compete for complex formation as assayed by mobility shift. The location of the NRF-1 element in GABRB1 centers at -12 relative to the major TTS in neocortical neurons. The GC-rich NRF-1 binding motif is often associated with TATA-less promoters and found within 100 bp DNA regions around transcriptional start sites in the human genome (Virbasius, Virbasius et al. 1993, Xi, Yu et al. 2007). The proximity of the NRF-1 element in GABRB1 to the Inr that binds PCL proteins associated with chromatin remodeling and DNA methylation (Vire, Brenner et al. 2006) may underlie its major role in controlling basal levels of β1 subunits in neurons. Whether GABRB1 is epigenetically regulated remains to be determined and could be a feature of why its transcription decreases in disease, especially since NRF-1 binding is blocked by DNA methylation (Gebhard, Benner et al. 2010).

Using the sensitivity of the luciferase reporter system, we have exploited the knockdown of endogenous NRF-1 in primary neurons to confirm the results of promoter mutational studies both with overexpressed NRF-1:VP16 in living neurons and in in vitro binding assays where mutations disrupt NRF-1 specific recognition. Our results are consistent with previous studies from our laboratory, using the same wild type GABRB1p-luciferase reporter construct, where promoter truncation and/or deletion that removes the Inr and disrupts the element for NRF-1 results in a 75-90% decrease in
luciferase gene transcription (Russek, Bandyopadhyay et al. 2000) (Fig. 3). Given that GABAR blockade by bicuculine has also been shown to drive NRF-1 dependent transcription (Delgado and Owens 2012) and that bicuculine reverses GABA-induced downregulation of β1 mRNA levels (Russek, Bandyopadhyay et al. 2000), presumably through PhF1b binding to the Inr, our new results suggest that the NRF-1 responsive element and Inr may act synergistically to regulate β1 subunit levels as neurons adapt to changes in their activity state.

Although differential expression of α subunits in relationship to brain disorders has clearly been associated with their region specific changes in tonic and phasic inhibition, it is only recently that the importance of differential β subunit containing GABARs has been noted. This selective property of GABARs controlled by β subunit expression, however, has been limited to β2 and β3, with β1 present in only a limited population of receptors in the brain. However of all β subunits, β1 has been mostly associated with both neurological and neuropsychiatric disorders. The reason for this functional relationship remains to be described and is an active area of investigation in our laboratory.

Recent identification of fragrant dioxane derivatives that show a 6-fold preference for β1-containing GABARs (Sergeeva, Kletke et al. 2010) suggest that the β1 subunit is required for the modulation of wakefulness that is mediated by the histaminergic neurons of the posterior hypothalamus (tubermamillary nucleus-TMN) (Yanovsky, Schubring et al. 2012). Given that energy metabolism is sensitive to restoration during the sleep cycle and that NRF-1 levels rise with sleep deprivation (Nikonova, Naidoo et al. 2010), it is
interesting that β1-containing receptors are the major source of inhibitory control over sleep.
CHAPTER IV. A Biosensor for CREB Activation in vivo Responds to BDNF and PKA Signaling

A. Abstract

Determining the individual contribution of neurons to the output of vast multicellular brain networks is paramount in neuroscience. We have employed a protein-protein interaction reporter system, based on the firefly luciferase protein, to track the activation of the memory molecule cAMP-response element binding protein (CREB) in living primary cortical neurons, as a first step to integrating principles of molecular dynamics into systems neuroscience. The luciferase enzyme is split into N- and C-terminal fragments, which are fused to the kinase interacting domain (KIX) of the CREB-binding protein (CBP) and the kinase-inducible domain of the cAMP-response element binding protein (CREB), respectively. Luciferase is reconstituted and its activity is restored upon the phosphorylation of KID and its subsequent binding with KIX. Using this biosensor, we observe CREB activation and recruitment of CBP (in response to forskolin-induced cAMP production) that is attenuated with alanine mutation at Serine 133 (S133A). Activation of CREB and recruitment of CBP via BDNF is also detected and while attenuated by the S133A mutation in KID, there is still a persistent emission of light suggesting a contribution of additional regulatory features to the program of CREB regulation in living neurons. In addition, our studies using this novel biosensor have revealed that the inducible cAMP early repressor (ICER), a known repressor of CREB target genes, can exert its repressor effects by disrupting the ability of phosphorylated CREB to recruit CBP rather than acting solely at the level of DNA. This technology, therefore, has the promise of providing a platform for researchers to track CREB-
activated transcription within a neural network, as well as to characterize regulators of CREB-CBP interactions and CREB function \textit{in vivo}.

B. Introduction

The function of cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) is essential in the formation of long-term memory as mediated by the hippocampus and the amygdala (Montminy and Bilezikjian 1987). Moreover, the relative level of activated CREB has also been associated with the selection of neurons for inclusion in a circuit that is believed to underlie a memory trace (Han, Kushner et al. 2007). When phosphorylated at a key residue in the protein, CREB acts as a transcriptional regulator binding to the promoter region of particular genes that contain the canonical cAMP Response Element (CRE). Upon binding and activation, a diversity of cofactors and components of the transcriptional machinery are recruited to promote the stabilization of RNA Polymerase II to the initiator sequence and subsequent RNA synthesis.

Activation of CREB occurs in response to multiple signaling pathways that are activated by synaptic transmission. An important residue on the protein that is responsible for the majority of CREB activation is serine 133 (S133). After phosphorylation at this residue, CREB associates with its co-activator, the CREB-binding protein (CBP) via the CBP kinase inducible interacting (KIX) domain, which helps to stabilize the pre-initiation complex on the open promoter to initiate transcription. Serine 133 is located within the kinase-inducible domain (KID) of CREB, where the activity of a variety of kinases, including protein kinase A (PKA), calcium-calmodulin-dependent
protein kinase (CaMK), and mitogen-activated protein kinase kinase (MEK), converge to regulate the protein’s function via post-translational modifications that can either lead to activation or repression. Most detailed studies of CREB function in neurons have been done in vitro using immunohistochemistry or Western blot analysis. Little is known about the dynamic changes that occur in its activation and inactivation in living cells and whether there is specificity to such regulation dependent upon extracellular signals and the intracellular pathways they control. Therefore, we reasoned that by monitoring the interaction of KID-KIX in living neurons and subsequently in a living organism, we might be able to better study the underlying properties of its tight control over synaptic plasticity and the neural circuits that are associated with its role in memory formation.

Here, I report implementation of a genetic biosensor that will enable us to visualize dynamic KID-KIX interactions in primary cultured neurons as a first step towards developing a molecular tool for future use in animal models. We have utilized the firefly luciferase complementation bioluminescence (BLI) system as our strategy to detect KID-KIX interactions in living neurons. This system has the advantages of maintaining good cellular viability and an outstanding signal-to-noise detection ratio. In brief, the activity of the reporter enzyme luciferase is disrupted by separating it into two components, where each is fused to a domain of interest on CREB and CBP. Under conditions of CREB phosphorylation, such as neural stimulation or kinase activation, KID and KIX will associate and the enzymatic activity of the luciferase reporter will be reconstituted so that application of substrate into the media will lead to the emission of light.
C. Results

1. Forskolin and BDNF Stimulate the KID-KIX Interaction in Cultured Cortical Neurons.

Forskolin (FSK) is a known activator of adenylyl cyclase, an enzyme that converts ATP into the second messenger cAMP that activates the cAMP-dependent Protein Kinase A (PKA), which phosphorylates CREB at S133. Serine 133 phosphorylation then stimulates the recruitment of the CBP/p300 co-activator. To validate this established protein-protein interaction at the KID and KIX domains in neurons, we treated cultured primary cortical neurons, transiently expressing KID-LucC and KIX-LucN, with either DMSO (vehicle control) or 20 µM FSK for 2 h. Under these conditions, FSK stimulates the KID-LucC/KIX-LucN interaction (as measured by light emission) in cultured primary cortical neurons by 4-fold, as compared to the vehicle control. This stimulation is significantly reduced (~65%) when the KID-LucC construct contains the S133A mutation (Fig. 6).

Brain-derived neurotrophic factor (BDNF) is a major signaling molecule in the nervous system that is intimately connected to cell survival, the formation of synapses, the development of neurites and their maintenance (Kwon, Fernandez et al. 2011), as well as spatial learning (Mizuno, Yamada et al. 2000). Endogenous expression of BDNF is activity-dependent and induces CREB activation at S133 in vivo (Ying, Futter et al. 2002). Individual expression of either KID-LucC or KIX-LucN does not elicit luciferase activity (light emission) in the presence of BDNF (Fig. 7A). However, when they are co-expressed to generate the KID-KIX interaction there is a dose dependent increase in light
at ~5-fold over baseline at 10 ng/ml (20 nM) BDNF and 12-fold over baseline at 50 ng/ml (100 nM) BDNF (Fig. 7B). The 100 nM BDNF-stimulated KID-KIX interaction is attenuated ~50% upon mutation of S133 to A133 in KID-LucC compared to wild type. As shown in Figure 8, the emission of light can also be detected in individual transfected neurons using bioluminescence microscopy (LV200 Olympus BLI microscope) suggesting that in the future this biosensor may prove important for the specific detection of individual neurons within a circuit whose gene expression program is being modified by synaptic activity.
Figure 6. Forskolin induces a KID-CLuc/KIX-NLuc interaction in cultured primary cortical neurons. Neurons in a 96-well format were transfected with either wild type or S133A mutant KID-CLuc and KIX-NLuc pairs. Wells were treated with either vehicle or forskolin (20 μM) for 2 h before Xenogen IVIS imaging. A representative IVIS bioluminescence image of live neurons in pseudocolor for one N is shown on left. ** p < 0.01; Error bars represent mean ± SEM of at least 3 independent cultures where each N contained duplicate or triplicate wells.
Figure 7. BDNF induces a KID-CLuc/KIX-NLuc interaction in cultured primary cortical neurons that is partially reversed with S133A mutation. (A) Neurons grown in a 96-well format were transfected with either KID-CLuc or KIX-NLuc and treated with BDNF (50 ng/ml). (B) Neurons were transfected with KID-CLuc and KIX-NLuc and then treated with vehicle, low concentration (10 ng/ml), or high concentration (50 ng/ml) of BDNF. (C) Neurons were transfected with either wild type or S133A mutant KID-CLuc and KIX-NLuc pairs. Wells were treated with either vehicle or BDNF (50 ng/ml) 2 h prior to IVIS imaging. IVIS bioluminescence images of live neurons in pseudocolor are shown. * p < 0.05, “n.s.” = not significant; Error bars represent mean ± SEM of at least 3 independent cultures where each N is from duplicate or triplicate wells.
Figure 8. Bioluminescence detection induced by BDNF in individual transfected neurons containing KID-CLuc and KIX-NLuc expression constructs. Primary cortical neurons were transfected with KID-CLuc and KIX-NLuc and then treated with vehicle (top panel) or BDNF (50 ng/ml) (bottom panel) for 2 h. Merged bright field and bioluminescence images represent sampling of different fields within a single dish. Bioluminescent signal is indicated by cyan blue and was visualized using the Olympus LV200 bioluminescence imaging system.
2. ICER IIγ may inhibit the association between KID and KIX

Similar to CREB, CREM (the cAMP response element modulator) is a cAMP-responsive nuclear factor that binds to CRE containing promoters. Unlike the ubiquitously expressed CREB, the expression of the CREM gene product is cell-type and developmentally specific (Misund, Steigedal et al. 2007). A splice variant of CREM gives rise to the gene product ICER IIγ (Inducible cAMP Early Repressor); a nuclear factor that contains a CRE binding domain but lacks the transcriptional activation domain of CREM. Our lab has previously shown that induction of ICER IIγ expression reduces CREB activation of the promoter for the type A GABA receptor α1 subunit gene (Lund, Hu et al. 2008). Preliminary data now shows that there may be an interesting relationship between CREB and ICER at the level of CREB/CBP interactions that is independent of the CRE site (Fig. 4), as overexpression of ICER IIγ is trending towards a decrease in the BDNF induced KID-KIX association from 5-fold to 2.5-fold.
Figure 9. ICER IIγ over-expression may attenuate the BDNF-induced KID-KIX association in primary cultured neurons. Cells were triple-transfected with either pcDNA3 (empty vector) or ICER IIγ expression plasmids, and the KID-CLuc and KIX-NLuc pair. Both groups were treated with vehicle or BDNF (100 nM) for 2 h before IVIS imaging. An IVIS bioluminescence image of live neurons in pseudocolor is shown. * p < 0.05: Error bars represent mean ± SEM of at least 3 independent cultures where each N contained duplicate or triplicate wells.
Discussion

In this report we demonstrate that induced KID-KIX interactions can be visualized in primary cultured neurons using the Xenogen IVIS imaging system and the LV200 Olympus BLI microscope to detect single neuron light emission. Results from the BDNF-mediated KID-KIX interaction suggests that there may be other regulatory residues responsible for the CREB-CBP association in living neurons as S133A does not completely block association between the protein domains. The trending loss of light emission after over-expression of ICER IIγ suggests that ICER may associate with KID or KIX to repress CREB-mediated gene expression. This is a new observation and given the role of ICER in processes of learning and memory, especially long term depression (LTD) (Mioduszewska, Jaworski et al. 2003, Kojima, Borlikova et al. 2008) it may indicate a new area for future research and the development of target therapeutic molecules as cognitive enhancers.

Future studies will be aimed at the use of this novel biosensor in vivo to map the specific neurons in a circuit whose genome is being altered in response to synaptic activity over time. In order for this to happen one must employ a sensitive means to detect light emission in the living animal, an area currently being developed in the laboratory of Tim Gardner at Boston University that will be used to test the principles of this particular protein reconstitution approach in collaboration with our laboratory. These biosensors can also be used to signal the activation of particular intracellular pathways when coupled to the mutation of certain residues that are pathway specific; for instance, to distinguish between PKA signaling vs that of mitogen activated protein kinase.
(MAPK). Finally, these approaches can also be applied to other important interacting molecules that are of great significance to the field of molecular neuroscience so that their dynamics can be studied in vivo to determine the implication of such interactions to systems neuroscience.
APPENDIX

Altered expression of GABAA receptors (GABAR) containing α4 subunit has been implicated in both physiologic and pathologic processes, such as learning and memory, alcohol withdrawal, and temporal lobe epilepsy (TLE). In particular, up-regulation of α4 subunit expression has been observed in both TLE patients and animal models of TLE. One aspect of α4 containing GABAARs is that they control the majority of tonic inhibition in the brain, an important feature of synaptic plasticity and neuronal excitability. Moreover, in the face of α1 subunit down regulation 24 hours after status epilepticus (SE), α4/β2/γ2 containing GABARs appear at the synapse. Due to the critical importance of α4 subunits in the normal brain and the pathophysiology of epilepsy, a detailed understanding of the molecular mechanisms regulating its expression becomes increasingly significant.

In this pursuit, we used bioinformatic tools to identify novel regulatory elements in GABAR subunit promoters. We have identified putative binding sites within the GABRA4 promoter for NRF-1. Our preliminary results in primary cortical cultures show that NRF-1 is up-regulated by BDNF stimulation, NRF-1 binds to the human GABRA4 promoter (GABRA4-p) in vitro, and that activation of NRF-1 gene expression through depolarizing stimulation also resulted in a significant increase in the expression of rat Gabra4. Taken together, these observations have led us to hypothesize that NRF-1 directly regulates GABAAR α4 subunit expression at the transcriptional level. The results of these studies should further our understanding of the role that activity plays in
regulating α4 subunit levels, expand on the role of NRF-1 in synaptic transmission, and explore lines of inquiry into mitochondrial biogenesis in epilepsy.
Figure A1. In vitro interactions between NRF-1 and human GABRA4 subunit promoter. $^{32}$P-labeled probes corresponding to NRF-1 putative binding sites were incubated with 20μg of nuclear extract. 100-fold excess unlabeled probes pre-incubated with nuclear extracts serve as specific competitors. NRF-1 antibodies were pre-incubated with nuclear extracts to form specific binding complexes that result in a supershift. The positive control Rat cytochrome c probe has specific shift (lane 3) and supershift (lane 4). Human GABRA4 Probe, containing the putative binding sequence 5’ GCAGCGCAAGCGCGCGG 3’, has specific shift (lane 7) and supershift (lane 8).
Figure A2. Induction of NRF-1 and Gabra4 mRNA expression by KCl depolarization of primary cortical neurons. Primary cortical cultures were treated with either control or 20 mM KCl for 6 hours. Following treatment, cells were collected for analysis by qRT-PCR with primers and probes designed to NRF-1 and Gabra4. Samples were standardized to cyclophilin. (n=5) **p<0.01
Figure A3. Induction of NRF-1 protein expression by KCl depolarization of primary cortical neurons. Primary cortical cultures were treated with either control or 20 mM KCl for 6 and 12 hours. Following treatment, cell extracts were collected for western blot analysis and probed for NRF-1 levels. Samples were standardized to β-actin levels. (n=4) *p<0.05
Figure A4: Induction of NRF-1 and Gabra4 mRNA expression by BDNF stimulation of primary cortical neurons. Primary cortical cultures were treated with either vehicle or 50 ng/ml BDNF for 6 hours. Following treatment, cells were collected for analysis by qRT-PCR with primers and probes designed to NRF-1 and Gabra4. Samples were standardized to cyclophilin. (n=4) *p<0.05
Figure A5. Levels of NRF-1 increases in rat hippocampus 24 h after status epilepticus (SE) in the pilocarpine rat model of TLE. (Control n=3, SE n=4) * p<0.05.
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