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CALCIUM CHANNEL-COUPLED TRANSCRIPTION FACTORS FACILITATE DIRECT CALCIUM SIGNALING

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Technical Abstract:

Voltage gated calcium channels (VGCCs) are known to play crucial roles in maintaining neuronal excitability, activity-dependent changes in gene expression, and long-term learning and memory changes within the central nervous system. The main pore-forming alpha subunits (α1 subunits) of these calcium channels are encoded by 10 distinct genes corresponding to the 10 members of this family (Catterall, 2010). Our lab has previously shown that the VGCC gene *CACNA1A* is a bicistronic cellular gene, producing two functionally distinct proteins from a single mRNA. *CACNA1A* produces the P/Q-type CaV2.1 VGCC via canonical, cap-dependent translation, and the transcription factor α1ACT through a cryptic Internal ribosome entry site (IRES) within the primary open reading frame (Du et al., 2013; Du et al., 2019).

To determine if the L-type VGCC gene *CACNA1C* (encoding the CaV1.2 α1C subunit) and the T-type VGCC gene *CACNA1H* (encoding the CaV3.2 α1H subunit), representing the two additional major subtypes of VGCC, are bicistronic cellular genes similar to *CACNA1A*, I employed a series of molecular biology experiments to manipulate the coding sequences of each VGCC gene in cDNA constructs. Using mRNA transfections, I was able to convincingly demonstrate that the second cistrons of both *CACNA1C* and *CACNA1H* produce secondary proteins independently of their full-length VGCC α1 subunits via a cap-independent, IRES-like mechanism. Additionally, using molecular manipulations to the *CACNA1C* and *CACNA1H* mRNAs, I excluded the possibility that these secondary proteins, termed α1CCT and α1HCT respectively, are produced via other methods of alternative transcription and translation, including alternative splicing of the mRNA, an underlying cryptic promoter in the DNA sequence, or ribosomal skipping/read-through/shunting.

As our lab previously showed that α1ACT functions as a transcription factor, I performed next-generation sequencing studies to investigate the gene regulation capabilities of α1CCT and α1HCT. RNA-seq and ChIP-seq revealed complex networks of genes regulated by each VGCC C-terminal protein (CTP) that promoted neuronal differentiation and synaptic function programs in human neural progenitor cells. Finally, as these novel secondary CTPs are embedded within parent calcium channel genes, I hypothesized that their subcellular localization and possible transcriptional regulation capabilities were regulated in part by neuronal activity. To test this, I employed both neuronal imaging techniques in tandem with depolarizing stimuli to induce calcium influx. In living neurons, calcium spikes induced via glutamate uncaging caused α1CCT and α1ACT to translocate to and from the nucleus, respectively, indicating that these transcription factors are indeed activity dependent. Additionally, pharmacological manipulations showed that calcium influx through specific channels, namely L-type VGCCs and NMDA channels, regulated the intracellular translocation of α1CCT and α1ACT, respectively. These results reveal a conserved method of coordinated protein expression within the VGCC family, as parent VGCC channels are transcriptionally and functionally coupled with their secondary C-terminal transcription factors.

Mutations in these VGCC genes lead to debilitating neurological, muscle, sensory, and cardiac disorders. Loss- or gain-of-function mutations in the *CACNA1C* gene, encoding the α1C calcium channel subunit of the CaV1.2 L-type VGCC, lead to several pleiotropic disorders including the autism spectrum disorder (ASD) Timothy's Syndrome, bipolar disorder, schizophrenia, and major depressive disorder (MDD), as well as severe cardiac arrhythmias (Splawski et al., 2004; Green et al., 2009; Dedic et al., 2017; Boczek et al., 2015). Loss- or gain-of-function mutations in the α1H subunit, comprising the CaV3.2 T-type VGCC, are associated

with chronic neuropathic pain disorders, ASDs, several types of mental illness, and amyotrophic lateral sclerosis (ALS) (Carter et al., 2019; Becker et al., 2017; Rzhepetskyy et al., 2016;Splawski et al., 2006; Souza et al., 2015). Attempts to recapitulate such phenotypically complex disorders in models has often fallen short, in large part due to the genetic complexity of these disorders and the failure of single-mutation models to capture that complexity. While these results push forward our understanding of the complex pathological mechanisms underlying VGC-mediated disorders, future experiments identifying necessary secondary signaling molecules, as well as the underlying activity-dependent changes to gene expression induced by α1CCT and α1ACT, will lead to a better understanding of this complex transcriptional network in function and dysfunction.

Chapter 1: General Introduction

Introduction to neuronal calcium signaling:

Calcium (Ca^{2+}) is a ubiquitous second messenger that regulates myriad biological properties and activity throughout eukaryotic cells. Although Ca^{2+} regulates processes common to all cell types, such as cellular metabolism, enzyme activation, cellular motility, or exocytosis, it also serves important cell-type specific functions. Ca^{2+} signaling is especially important throughout the nervous system, where it signals both intra- and intercellularly to convey critical information necessary for proper nervous system development and function. As a result, extensive mechanisms exist within the nervous system to maintain precise Ca^{2+} homeostasis and signal processing (Carafoli and Crompton, 1978; Brini et al., 2012).

Ca^{2+} signaling has the unique property amongst second messengers of autoregulation, meaning that the cellular controls of proper Ca^{2+} signaling and homeostasis are themselves regulated by Ca^{2+}, both at the transcriptional and post-transcriptional levels. This property is especially important in neurons, where Ca^{2+} signaling is crucial for cellular depolarization leading to action potentials, neurotransmitter vesicle exocytosis from pre-synaptic terminals, and modification of synaptic strength through long-term potentiation (LTP) or long-term depression (LTD). Due to the extensive role of Ca^{2+} within the nervous system, robust mechanisms exist within neurons to maintain precise temporal and spatial regulation of Ca^{2+} signaling (Mellstrom et al., 2008; Carafoli, 2007)

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Voltage-gated calcium channels:

One tool within the neuronal Ca^{2+} signaling toolkit is the diverse family of neuronal calcium-permeable channels present across neuron subtypes, each one having unique gating, kinetic, and downstream signaling properties. Voltage-gated calcium channels (VGCCs) are perhaps the most important type of neuronal calcium channel, and as the name suggests, allow Ca^{2+} ion flux across the plasma membrane in response to local intracellular depolarization. In neurons, the intracellular Ca^{2+} concentration is in the tens of nanomolar range while the extracellular concentration is in the millimolar range, representing more than a thousand-fold difference. Thus, when neuronal VGCCs open, Ca^{2+} ions flow intracellularly according to their steep gradient, and the magnitude of this gradient ensures rapid and robust signal transduction (Clapham, 2007).

VGCCs are comprised of five distinct subunits (α1, α2, β, γ, δ), with the α1 subunit forming the main pore of each channel. Neuronal VGCCs are divided into three families – CaV1, CaV2, and CaV3 – and further subdivided into six subtypes (L-, N-, P-, Q-, R-, and T-) based on their common pharmacological and physiological properties (Figure 1) (Brini et al., 2014). Among these, the L-, P/Q-, N-, and T-type calcium channels play especially important and unique roles within the central nervous system.

L-type calcium channels (LTCCs), often thought of as "post-synaptic" VGCCs, are high-voltage activated (HVA) calcium channels that play a substantial role in coupling neuronal activity to changes in gene expression, a property known as excitation–transcription coupling (Flavell and Greenberg 2008). While there are four identified L-type VGCCs (CaV1.1, CaV1.2, CaV1.3, CaV1.4), only CaV1.2 and CaV1.3 are highly expressed in the brain, with CaV1.2 also highly expressed in the heart. CaV1.2 and CaV1.3 share significant sequence homology yet differ in their neuronal distribution and biophysical properties. CaV1.2 appears to be more

predominant across the hippocampus and cortex than CaV1.3, and homozygous CaV1.2 knockout in mice leads to prenatal death while CaV1.3 homozygous knockout results in congenital deafness and cardiac abnormality (Seisenberger et al., 2000; Platzer et al., 2000).

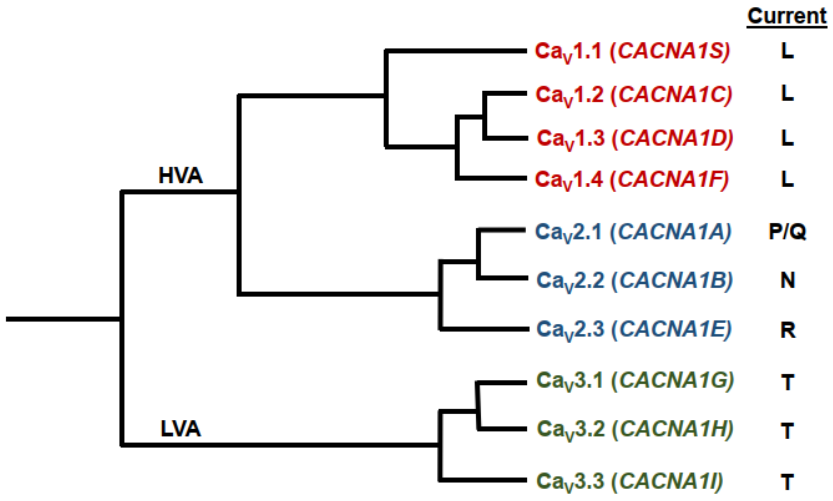

Figure 1. Schematic phylogenetic tree illustrating the various VGCC subfamilies and their respective current type. Adapted from Bidaud et al., 2006.

Ca^{2+} currents conducted by L-type calcium channels have a unique role in the regulation of neuronal gene transcription compared to Ca^{2+} currents of similar magnitude conducted through other neuronal calcium channels (Flavell and Greenberg 2008). Several cellular and physiological properties of LTCCs working in tandem are thought to give rise to this property. For one, LTCCs are primarily located in the cell bodies and proximal dendrites of neurons, compared to P/Q- and T-type VGCCs which are more prevalent at pre-synaptic terminals and dendrites, respectively (Westenbroek et al., 1990; Hell et al., 1993). This localization of LTCCs could make them more suited to interaction with cellular calcium sensors or calcium-dependent transcriptional regulators within the soma, or simply direct calcium signaling to the nucleus.

Studies have shown that local increases in Ca^{2+} are sufficient for neuronal gene expression changes, suggesting that Ca^{2+} current through LTCCs may interact with LTCC-associated calcium-sensor proteins to affect transcription, such as calmodulin and calcineurin, most likely through their intracellular C-terminal region (Wheeler at al. 2008).

The P/Q-type calcium channel CaV2.1, also an HVA channel and perhaps the most familiar neuronal calcium channel to neuroscientists, is often thought of as the "pre-synaptic" VGCC due to its important role of coupling calcium influx to fast neurotransmitter release at pre-synaptic terminals (excitation – secretion coupling) (Jouvenceau et al., 2001). Although CaV2.1 is widely expressed across the CNS, it is most abundant in Purkinje and granule cells in the cerebellum, a localization from which it receives its name ("P" from Purkinje cell and "Q" from granule cell). As with L-type calcium channels, P/Q-type calcium channels have long intracellular C-terminal regions that have been shown to interact with many different proteins, especially those involved with pre-synaptic neurotransmitter release mechanisms, including SNARE protein family members, synaptotagmin, and SNAP25, and the calcium binding protein calmodulin (CaM) (Sheng et al., 1994; Rettig et al., 1996; Charvin et al., 1997; Wiser et al., 1997; Bezprozvanny et al., 1995).

T-type calcium channels (TTCCs), in contrast to L-type and P/Q- VGCCs, are low-voltage activated (LVA), meaning that they require smaller intracellular depolarizations to enter the open state, activate at comparatively negative membrane potentials (in the same range as Na^{+} currents in neurons) and exhibit fast voltage-dependent inactivation compared to other VGCCs (Nowycky et al. 1985). These unique electrophysical properties of T-type calcium channels make them particularly well suited for repetitive, oscillatory firing. As such, they play important roles in the generation of rhythmic action potential bursts within thalamic relay neurons, as well as

helping to drive the pacemaker current in the heart responsible for the heartbeat (Kim et al., 2001; Mangoni et al., 2006). TTCCs have also been implicated in neurotransmitter release in the retina and olfactory bulb (Egger et al., 2003; Pan et al., 2001), as well as synaptic transmission in hippocampal interneurons (Tang et al., 2011). Finally, TTCCs have been implicated in spontaneous release at certain neuronal synapses, a function well-suited to TTCCs due to their low voltage activation threshold and subsequent fast inactivation.

Activity-dependent gene expression:

All cells regulate their gene expression in response to salient external stimuli in their environments. One common mechanism through which they can achieve this dynamic regulation involves the expression of inducible transcription factors (ITFs). These ITFs are often tailored to recognize a particular stimulus in the cells internal or external environment and subsequently alter gene expression in a predictable manner. From insects to primates, ITFs have been shown to respond to a wide array of stimuli, such as sensory input, behavior, or learning (Burmeister et al., 2005; Sommerlandt et al., 2019). In neurons, perhaps the most salient stimulus is membrane depolarization. Yet, membrane depolarization in neurons, especially Ca^{2+}-mediated depolarization, is not spatially uniform, and depending on the source of the depolarization, the temporal pattern, or the magnitude of the activity, the downstream affects may vary greatly. Understanding not only how global depolarization induces ITFs to alter gene expression but also if distinct inputs and outputs leading to spatially and temporally varied depolarizations is critically important for understanding both normal and pathogenic nervous system function.

CaV1 Channels:

LTCCs have long been known to regulate a wide array of gene expression networks, primarily through indirect mechanisms involving secondary effectors and downstream ITFs. Early studies showed that LTCC agonist or antagonist application to cortical neurons either promoted or inhibited the transcription of several early intermediate genes, including *c-fos*, now understood to be a faithful marker of neuronal activity (Murphy et al., 1991). Shortly thereafter, Ca^{2+} influx through LTCCs was shown to activate the calcium-binding protein calmodulin (CaM), which in turn promotes CaM kinase activation, CaM nuclear translocation, and the subsequent phosphorylation and activation of the transcription factor CREB (Deisseroth et al., 1998). An additional layer of complexity is added at the temporal scale, as brief hippocampal depolarizations (1-10 minutes) have been shown to activate a CaM/CaM kinase-dependent pathway of CREB activation, while extended depolarizations (>30 minutes) activate CREB through an additional Ras/MAPK pathway (Wu et al., 2001).

More recently, the calcium-binding protein (CBP) calcineurin has been shown to associate with LTCCs and regulate the intracellular localization and transcriptional regulation activity of several downstream transcription factors following Ca^{2+} influx through LTCCs. Graef et al. demonstrated that Ca^{2+} entry through LTCCs induces calcineurin-mediated activation of the transcription factor NFAT, which in turn results in rapid NFAT nuclear translocation and gene regulation (Graef et al., 1999). Mao and Weidmann (1999) further demonstrated that LTCC Ca^{2+} - mediated activation of calcineurin maintains the transcription factor MEF2A in its transcriptionally active hypophosphorylated state.

Ca^{2+} influx through LTCCs can regulate a diverse set of activity-dependent gene expression programs, ultimately resulting in varied or redundant downstream effects. While

LTCCs have arguably been shown to be one of the most important neuronal calcium channels in terms of activity-dependent gene expression, recent reports have shown that CaV2 channels may also play a role in activity-dependent gene expression.

CaV2 Channels:

Like CaV1 channels, CaV2 channels have been shown to promote NFAT- and CREB-mediated transcription, although the exact molecular mechanisms underlying these transcriptional changes are not well understood. Unlike CaV1 channels, CaV2 channels seem to require a global rise in intracellular Ca^{2+} concentration to affect transcription, while CaV1 channels only require local microdomain $[Ca^{2+}]_I$ elevations. While CaV2-mediated transcriptional regulation requires much stronger membrane depolarizations than CaV1-mediated transcriptional regulation, both appear to share common downstream mechanisms, such as Ca^{2+}/calmodulin-dependent kinase II (CaMKII) activation independent of protein kinase A (PKA), protein kinase C (PKC), or mitogen-activated protein kinase (MAPK) activity (Wheeler et al., 2012). As both CaV1 and CaV2 channels are known to interact directly with CaMKII at their C-terminal domains (Hudmon et al., 2005, Wheeler et al., 2008, Jiang et al., 2008), the differences in CaV1- and CaV2- appear to arise from diffusible populations of CaMKII that preferentially localize near CaV1 channels (Wheeler et al., 2012). Although CaV2-mediated gene transcription is still relatively poorly understood, it appears that global, robust Ca^{2+} elevation is required for CaV2-mediated CREB activation, at least partially resulting from the differential intracellular localization of CaMKII.

Voltage-gated calcium channels and neurological disorders

Although VGCCs play diverse, important, and distinct roles in the developing and mature nervous system, it is striking that mutations in VGCC genes often present as physiologically and phenotypically complex disorders. Mutations in VGCC genes have been associated with a wide variety of developmental and neuropsychiatric disorders, including but not limited to autism spectrum disorder (ASD), bipolar disorder (BD), major depressive disorder (MDD), schizophrenia, and epilepsy (Andrade et al., 2019; Heyes et al., 2015; Zamponi 2015). While these mutations sometimes result in altered VGCC function through gain- or loss-of-function activity, there is often is no discernable change in channel function, gating, or kinetics (Berger and Bartsch, 2014; Craddock and Sklar, 2013; Schmunk and Gargus, 2013; Splawski et al., 2004; Liao and Soong, 2010). Furthermore, single mutation models often fail to recapitulate disease phenotypes, suggesting that disorders related to single nucleotide polymorphisms (SNPs) within VGCC genes may be more than simple channelopathies. Nevertheless, animal models have provided significant insight into the underlying mechanisms related to several VGCC – related disorders.

CACNA1C – mediated neuronal dysfunction:

Due to the crucial role LTCCs play in several biological processes necessary to sustain life, such as initiating excitation-contraction coupling in the heart to produce a heartbeat or facilitating changes in activity-dependent learning the memory, the fact that homozygous knockout (KO) mice for CaV1.1 or CaV1.2 die prenatally is not surprising (Tanabe et al., 1988, Seisenburger et al., 2000). These severe phenotypes underscore the unique function of VGCCs

even within family subtypes, and that the role that each channel plays cannot be compensated by another.

In line with these findings, mutations in human *CACNA1C* have been linked with the complex ASD and developmental disorder Timothy syndrome (TS). TS is a debilitating condition that presents with mental retardation, autism, lethal cardiac arrhythmias, and various other developmental abnormalities (Splawski et al., 2004, 2005). TS is caused by one of two identified single amino-acid substitutions in the CaV1.2 protein, which result in decreased voltage – dependent inactivation of the channel and subsequent aberrant excitability (Bader et al., 2011). The ASD symptoms associated with TS have successfully been recapitulated in a mouse model, and studies using pluripotent stem cells derived from patients with TS have demonstrated activity – dependent retraction of neuronal processes resulting from mutant channel activity (Bader et al., 2011, Krey et al., 2013, Pasca et al., 2011).

The role of LTCCs, specifically CaV1.2, in mental health is further supported by recent sequencing studies of patients with mental illness. Several SNPs have been identified within *CACNA1C* that have been linked with disorders such as bipolar disorder, schizophrenia, and major depression (Dao et al., 2010, Green et al., 2009, He et al., 2014, Strohmaier et al., 2012). Additionally, fMRI studies of patients with a specific noncoding *CACNA1C* risk allele show altered amygdala function, consistent with the altered fear learning phenotype observed in the CaV1.2 cKO mice (Tesli et al., 2013).

To better understand the role CaV1.2 plays in the brain, a conditional knockout (cKO) mouse that lacks the LTCC gene in the hippocampus and cortex was developed. These mice display an anxiety phenotype, as well as impairment of spatial memory and fear learning/extinction (White et al., 2008, Lee et al., 2012). Additionally, I performed two

behavioral tests, the marble burying test and the nestlet shredding test, to assay these 1.2 cKO mice for repetitive, autism-like behavior (Figure 12). While marble burying differences between the cKO mice and control B57 mice were not significant, the cKO mice shredded significantly more nestlet than control mice, indicating that in addition to the anxiety phenotype, the 1.2 cKO mice may display a mild ASD-like phenotype as well.

CACNA1A Mediated Neuronal Dysfunction:

Considering the P/Q-type calcium channel CaV2.1's distribution within the cerebellum and its established role in maintaining proper cerebellar physiology, it follows that mouse models recapitulating human mutations in the *CACNA1A* gene (encoding the CaV2.1 α1 subunit) display an ataxic phenotype. To this end, homozygous *CACNA1A* knockout mice display severe ataxia as well as absence seizures and die before postnatal week four (Jun et al. 1999). Unlike CaV1.2 knockout mice however, there appears to be a functional compensation by CaV2.2 and CaV2.3 within the hippocampus and motor neuron endplates of these mice but not within other brain regions highly expressing the channel, suggesting that CaV2.1's role within the cerebellum is unique, (Jun et al., 1999, Urbano et al., 2003). Several additional mouse models have been developed with mutations in the *CACNA1A* gene, all of which display partially overlapping ataxia and seizure phenotypes (Pietrobon, 2002). Finally, Ca2.1's critical role in cerebellar development was better understood by studies conducted on a conditional CaV2.1 knockout (postnatal) line which only developed neurological dysfunction in late adulthood (Mark et al., 2011).

In humans, both loss-of-function (LOF) and gain-of-function (GOF) mutations have been observed in the *CACNA1A* gene, and these varied mutations present with a wide range of phenotypes. Classically, GOF CaV2.1 mutations appear to present with familial hemiplegic migraine while LOF mutations result in episodic ataxia and in some cases seizures (Pietrobon, 2010, Jouvenceau et al., 2001), although this relationship has been controversial.

A polyglutamine (polyQ) expansion in the CaV2.1 C-terminal region has also been identified and associated with the human disorder spinocerebellar ataxia type 6 (SCA6) (Jodice et al., 1997). SCA6 was originally thought to be a channelopathy, however studies looking at CaV2.1 channels containing a pathogenic polyQ in Purkinje cells revealed normal channel function (Saegusa et al., 2007). In apparent confliction with this result, another study revealed progressive neuronal dysfunction in a mouse model with a polyQ superexpandion CaV2.1 (Watase et al., 2008). Our lab has subsequently revealed that while the pathogenic polyQ repeat observed in patients with SCA6 does not alter CaV2.1 channel function, it does affect the proper transcription factor function of the *CACNA1A* CTP α1ACT. Additionally, in a dose-dependent *CACNA1A* gene deficiency mouse model, we discovered that α1ACT drives a dynamic gene expression network that is critical for proper perinatal Purkinje cell and cerebellar development. Furthermore, re-introduction of wild-type α1ACT during this period rescues the ataxic phenotype observed in this *CACNA1A* deficient mouse model (Du et al., 2013, Du et al., 2019).

<u>*CACNA1H* mediated neuronal dysfunction:</u>

Disorders related to mutations or dysregulation in the *CACNA1H* gene are less well understood than those related to *CACNA1C* or *CACNA1H*. CaV3.2 knockout mice, unlike

CaV1.2 or CaV3.2 knockout mice, are viable and do not die prematurely. These mice show a reduced sensitivity to noxious pain but not chronic pain (Choi et al., 2007). Interestingly, several mouse models of chronic pain show increased CaV3.2 conductance, suggesting that dysregulation of channel expression, not channel function, can lead to neurologic disorders (Orestes et al., 2013; Jagodic et al., 2008; Jagodic et al., 2007). In humans, several mutations in the *CACNA1H* gene have been linked to various forms of generalized epilepsy (Heron et al., 2007). One such mutation, when introduced into a recombinant expression system, has been shown to increase neuronal excitability by reducing the rebound bursting threshold, and this increased firing subsequently leads to enhanced neurite outgrowth and dendritic arborization (Eckle et al., 2014; Singh et al., 2007). Yet, as seen with the *CACAN1A* polyQ repeat and SCA6 pathology, many of the clinical mutations observed in the *CACNA1H* gene, when introduced into recombinant channels, appear to only have a minimal effect on channel localization, biophysical properties, and overall function (Khosravani et al., 2004; Vitko et al., 2005). Finally, mutations that appear to reduce CaV3.2 – mediated current have been linked to autism spectrum disorders (ASDs) (Lu et al., 2012; Splawski et al., 2006). How these mutations contribute to disease pathophysiology, and if interaction with other ASD risk genes is necessary, requires further study.

Introduction to cap-dependent and cap-independent translation:

George Beadle and Edward Tatum first proposed a model of gene expression in which individual genes encode single polypeptides, which later became known as the "one-gene one-polypeptide" hypothesis. While Beadle and Tatum's work laid the foundation for modern genetics, numerous discoveries of alternative translation mechanisms since then, in both

eukaryotes and prokaryotes, have led to the revision of this model. Many genes have been recognized to encode for multiple polypeptides through a wide variety of non-canonical regulatory mechanisms, including alternative splicing, proteolytic cleavage, the presence of cryptic promoters in DNA sequences, and others. Such non-canonical translation mechanisms may lead to a "hidden" proteome, i.e. one serving to expand the proteomic diversity of an organism while coordinating the expression of distinct but functionally interconnected proteins (Han and Zhang, 2002; Hecht et al., 2002; Merrick 2004).

Cap-dependent translation:

Traditional protein synthesis in both eukaryotes and prokaryotes is divided into three stages – initiation, elongation, and termination. The predominant form of translation in eukaryotes relies on the recognition of the m7GpppN (m7G) "cap" structure at the 5' end of all mRNAs by a series of initiation factors that together form the eIF4F complex. These factors, which include eIF4A (a DEAD-box RNA helicase), eIF4E (the cap binding protein), and eIF4G (a scaffold protein), recognize the m7G cap, and subsequently recruit the 40S ribosomal subunit, along with what is called the ternary complex, to form the 48S initiation complex. The 48S initiation complex scans along the mRNA from the cap until it reaches the start codon sequence (AUG), at which point the eIF4F complex is reorganized, the 60S ribosomal subunit joins the smaller subunit, and the resulting 80S ribosome begins the elongation phase of translation. The elongation phase proceeds until the ribosome reaches a termination codon. This type of translation, termed "cap-dependent" translation, accounts for most protein synthesis in eukaryotes (Sonenberg and Hinnebusch, 2009).

Cap-independent translation:

While cap-dependent translation is the predominant form of translation in eukaryotes, several forms of cap-independent translation have been identified in eukaryotic cells, giving credence to the notion that a single gene may encode two or more functionally distinct polypeptides. One such mechanism involves the presence of multiple open reading frames (ORFs) within one mRNA, giving rise to two or more independently regulated proteins. These polycistronic genes allow for translation initiation not only at the m7g 5'-cap structure, but also at downstream ORFs either tandem to or overlapping with the primary ORF, often without recognition of the m7G cap (i.e. cap-independent). Such internal translation initiation is often achieved via utilization and recognition of a highly structured stem-loop RNA element called an internal ribosome entry site (IRES) (Kneller et al., 2006; Stern-Ginossar et al., 2018).

IRESs have long been recognized as a genetic tool utilized by prokaryotes and viruses to maximize proteomic diversity from a limited amount of genetic template. Viral IRES-based translation mechanisms involve the recognition of the RNA stem-loop structure by both canonical elongation initiation factors (eIFs) and specialized RNA binding proteins, termed IRES trans acting factors (ITAFs). Originally discovered in the *Picornaviridae* family of viruses, IRESs have since been found in several pathogenic viruses, including human immunodeficiency virus, hepatitis C virus, and foot and mouth disease (Jang et al., 1998; Pelletier and Sonenberg, 1988; Belsham and Brangwyn, 1990; Buck et al., 2001; Tsukiyama-Kohara et al., 1992). Although these IRESs have somewhat similar secondary structures, they often differ in the specific ITAFs and eIFs required to recruit ribosomes and initiate translation and can be grouped into four main categories based on RNA-structure and IRAF similarities (Kieft 2008).

Once thought to be a purely viral mechanism, IRES-mediated translation has since been identified in eukaryotic cells, and recent studies have indicated that ~10% of mammalian mRNAs may contain IRES-like elements (Spriggs et al., 2008; Weingarten-Gabbay et al., 2016). Unlike viral IRESs, cellular IRESs tend to have more limited and diverse RNA stem-loop structures and lack any significant sequence conservation, making categorization of cellular IRESs challenging. Cellular IRESs were originally identified within the 5' untranslated regions (UTRs) of eukaryotic genes related to cellular stress response. Based on the functions of these genes, these cellular IRESs appear to provide a means of translation and protein synthesis under conditions that may limit or prevent canonical cap-dependent translation, such as during apoptosis, hypoxia, or nutrient scarcity (Komar and Hatzoglou, 2011; King et al., 2010; Baird et al., 2006). Additionally, based on the functions of some genes with identified 5'-UTR IRES elements, such as *myc*-family genes, cyclin-dependent kinase 11, and vascular endothelial growth factor (VEGF) this form of alternative translation may also occur during cell proliferation events. Like viral IRESs, cellular IRESs differ in the eIFs and ITAFs required to initiate internal translation, however the specific factors required have only been elucidated for a small number of identified cellular IRESs (Komar and Hatzoglou, 2011).

Increasing evidence has suggested that cellular IRESs may not be limited to the 5'-UTRs of genes but may also be present in downstream regions overlapping with or following the primary ORF (Karginov et al., 2016). For 5'-UTR IRESs, IRES translation activity appears to be dependent on the expression and localization of specific ITAFs, suggesting strict control and regulation of cellular IRES function (King et al., 2010). While 5'-UTR IRESs have typically been recognized to produce crucial proteins during times of cell stress, cell cycle progression, or

certain developmental stages when cap-dependent translation may be inhibited or reduced, the function and purpose of downstream cellular IRES function and purpose is less understood.

Our lab has demonstrated that the voltage-gated calcium channel (VGCC) gene *CACNA1A*, encoding the P/Q-type calcium channel CaV2.1, is one such bicistronic cellular gene, encoding the well-characterized CaV2.1 α1A pore-forming subunit through canonical cap-dependent translation and a secondary C-terminal protein (CTP), the transcription factor α1ACT, through the use of a cryptic IRES within the *CACNA1A* open reading frame (Du et al., 2013, Du et al., 2019). The presence of an IRES within the ORF as seen with *CACNA1A* enables the production of multiple polypeptides from a single mRNA under distinct but interconnected control. I hypothesized that this bicistronic expression mechanism is conserved throughout the entire VGCC family as a means of coordinated gene expression and regulation. In this dissertation, I demonstrate that two additional VGCC genes, *CACNA1C* (encoding the L-type calcium channel α1C subunit) and *CACNA1H* (encoding the T-type calcium channel α1H subunit) both produce distinct C-terminal proteins, termed a1CCT and α1HCT respectively, through an internal, cap-independent translation mechanism.

VGCC genes and their C-terminal proteins:

Our lab has previously demonstrated that the VGCC gene *CACNA1A* is a bicistronic cellular gene, meaning that it encodes two functionally distinct proteins from a single mRNA. In this dissertation, I explored two additional members of the VGCC gene family that appear to encode two distinct proteins. I report that the neuronal VGCC genes *CACNA1C* and *CACNA1H* are also bicistronic cellular genes, producing an α1 calcium channel subunit (α1C and α1H

respectively) through canonical, cap-dependent translation, as well as secondary, C-terminal transcriptional regulatory proteins, termed α1CCT and α1HCT respectively, through a non-canonical, cap-independent mechanism. These novel VGCC C-terminal proteins (CTPs) bind to DNA to regulate the expression of several genes crucial for proper neuronal development and differentiation and promote neurite outgrowth in human neural progenitor cells. Additionally, we show that two of these CTPs undergo activity-dependent translocation, and that their subcellular localization is modulated in part by calcium influx through specific sources. These findings reveal a conserved strategy of gene expression amongst these neuronal VGCC genes, possibly helping to better explain the widespread, pleiotropic phenotypes observed in disorders related to VGCC gene mutations.

The finding that VGCC genes of all families (L-type, P/Q-type, T-type) utilize a bicistronic gene expression mechanism may help to explain the pleiotropic effects of some human disease-associated mutations. Many such mutations affect putative bicistronic genes from the three main VGCC families, potentially dysregulating two distinct gene products to different degrees.

This project has wide ranging implications for the understanding and potential treatment of a variety of neurological disorders. Viral IRESs have been targeted to suppress viral protein synthesis to treat infection, and our lab has recently demonstrated that expression of α1ACT in hypomorphic mice that have normal CaV2.1 channel function but lack endogenous α1ACT expression rescues the observed disease phenotype. Therefore, if a disease can be attributed to dysfunction or dysregulation of the second ORF protein, as appears to be the case with SCA6, then the second cistron protein becomes an attractive target for therapy development. Knowing that a given mutation affects one cistron or the other could direct focus to the proper pathogenic

target and mechanism. This project seeks to better characterize and understand the interplay of these regulatory expression systems and potentially provide novel targets to treat complex neurological genetic disease.

Chapter 2: Voltage-gated calcium channel genes *CACNA1C, CACNA1A,* and *CACNA1H* produce two distinct proteins from overlapping cistrons

Introduction:

Bicistronic genes are able to generate two distinct proteins from separate open reading frames (ORFs) in a single mRNA. In the case of VGCC genes, my studies indicate that these genes utilize a cryptic internal ribosome entry site (IRES) within the primary open reading frame (ORF) of the gene. IRESs are defined as highly structured RNA elements that can recruit ribosomes and other translational machinery to an internal region of mRNA and subsequently initiate translation. IRESs were once thought to be a virus-specific genetic mechanism that served to produce a diverse set of proteins from a limited amount of viral genetic material. Numerous IRESs have been discovered in pathogenic viruses, including human immunodeficiency virus (HIV), hepatitis C virus (HCV), and foot and mouth disease (Belsham and Brangwyn, 1990; Tsukiyama-Kohara et al., 1992, Buck et al., 2001). Although viral IRESs contain diverse sequences, many of them appear to share conserved secondary structures, as well as utilizing similar sets of canonical translation initiation factors and IRES *trans*-acting factors (ITAFs) (Kieft, 2008). As such, viral IRESs have been classified into four groups based on secondary structure, required eIFs and ITAFs, and translation start codons (Kieft, 2008).

Following the identification of viral IRESs, IRESs and IRES-like structures were subsequently discovered in eukaryotic genomes, however these features were originally thought to be limited to the 5' untranslated regions (5'-UTRs) of eukaryotic genes (Spriggs et al., 2008). Due to the types and functions of the endogenous genes with identified 5' IRESs, cellular IRESs were initially thought to primarily provide a means of expressing key proteins under conditions of cell stress and proliferation, when canonical, cap-dependent protein translation may be

inhibited (Komar and Hatzoglou, 2011). A recent screen indicated that ~10% of mammalian mRNAs may contain elements that function as IRESs (Weingarten-Gabbay et al., 2016).

However, several key challenges hamper the identification and discovery of *bona fide* IRES structures in eukaryotes. For one, unlike viral IRESs, no conserved structure, sequence, or IRES *trans*-acting factors (ITAFs) have been identified among the known cellular IRESs, making categorization of known cellular IRESs as well as the prediction of novel endogenous IRESs difficult. While this apparent diversity in cellular IRESs makes prediction and identification of them difficult, it also may point to a more complicated translational mechanism through which they drive translation in eukaryotic cells. Furthermore, while several ITAFs have been identified that facilitate and promote viral IRES-mediated translation, few have been identified in cellular systems, and the ones that have appear to be highly cell-type specific (King et al., 2010; Mokrejs et al., 2010). The mechanisms through which the identified cellular ITAFs function are also largely unknown.

Finally, as eukaryotic cells can utilize several mechanisms of alternative gene expression and translation, many stringent criteria must be satisfied to conclusively identify cellular IRESs. For one, cryptic promoters in either the DNA sequence of the candidate gene or the reporter plasmid, as well as potential alternative splicing events that may be occurring, must be ruled out. Additionally, mechanisms of secondary protein translation, such as ribosomal scanning or shunting, re-initiation, stop codon read-through, or translational frameshifting, must also be excluded (Van eden et al., 2004; Baranick et al., 2008).

In this chapter, I demonstrate that the VGCC genes *CACNA1C* and *CACNA1H* produce distinct secondary C-terminal proteins (CTPs) in addition to their well-characterized α1 subunits, and that these CTPs persist following the insertion of premature termination codons or truncation

mutations in the primary ORF upstream of the putative CTP start codon. Using several molecular biology techniques, including *in vitro* transcription, dual-luciferase assay, and truncating mutations in the VGCC genes, I show that these CTPs are produced via a cap-independent translation mechanism, and that the segments of mRNA directly upstream of the putative CTP start codons are able to significantly activate downstream translation.

Materials and Methods:

Cell Culture:

HEK293T: HEK293 cells were maintained in Dulbecco's Modified Essential Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO, Grand Island, NY, USA) in a 5% CO_2, 37^{o}C incubator. HEK293 cells were authenticated by ATCC prior to purchase and use. Media was changed every third day. To split, media was aspirated, and cells were washed once with 1xPBS before 3 mL of 0.25% trypsin was added to the plate for one minute. Cells were collected, spun down at 500g for 3 minutes, then plated at a confluency of ~40%.

Transfections:

mRNA: Poly-A tailed mRNA was transfected using *trans*-IT mRNA transfection reagent (Mirus Bio, Madison, WI, USA) as per manufacturer's instruction when cells were approximately 95% confluent. In brief, 2.5ug of mRNA was resuspended in 250ul of Optimem (GIBCO, Waltham, MA, USA) and mixed well. To this, 5ul of mRNA Boost reagent and 5ul of TransIT mRNA transfection reagent were added and mixed well. The reaction was incubated at room temperature for 5 minutes before being added dropwise to cells. Cells were collected for protein 16 hours

post-transfection in RIPA buffer (Sigma, St. Louis, MO, USA) supplemented with Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA, USA) and PMSF (Sigma, St. Louis, MO, USA).

cDNA: Cells were transiently transfected with pcDNA3 vectors expression full-length cDNA of human *CACNA1C* (CaV1.2, NM_199460.3) and *CACNA1H* (CaV3.2, NM_021098.2), a series of 5' truncation cDNA constructs close to the N-terminus of α1CCT or α1HCT, and empty pcDNA3 vector when cells were ~60% confluent using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

Western Blot:

Nuclear and cytoplasmic proteins were extracted from either cell lines or mouse cerebellum/cortex using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA). 50μg of total protein or fraction was subjected to SDS-PAGE (6%, 8%, or 10% Tris-Glycine gel, Invitrogen, Grand Island, NY, USA) and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The transblotted membrane was blocked with TBST containing 5% nonfat milk for 60 minutes and then incubated with primary antibody overnight at 4^{o}C. The next day, the membrane was probed with HRP-conjugated secondary antibodies for 1 hour at room temperature then washed three times with TBST. Immunoblots were detected using chemiluminescent substrate (Thermo Scientific Pierce Protein Biology Products, Rockford, IL, USA) and visualized via autoradiography.

In vitro Transcription and RNA purification:

cDNA constructs were linearized via single enzyme restriction enzyme cutting overnight at 37^{o}C (Xba1) and purified using DNA Clean-Up kit (Zymo Research, Irvine, CA, USA). The resulting

linearized cDNA was used as the template for *in vitro* transcription using the MEGAscript T7 in-vitro transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). For capped constructs, GTP was diluted 1:5 and 6mM of either functional m7G(5')ppp(5')G RNA Cap Structure Analogue (NEB-S1404) or nonfunctional G(5')ppp(5')A RNA Cap Structure Analog (NEB-S1406) was supplemented. T7 enzyme mix was added in 1x and the reaction was incubated at 37^{o}C for 4 hours. The reaction was then DNase treated for 15 minutes at 37^{o}C. To polyadenylate the transcripts, the reaction was then supplemented with E-PAP buffer, 25 mM $MnCl_2$, 10 mM ATP and poly(A)polymerase (PAP) before being incubated at 37^{o}C for 1 hour. The resulting RNA was column purified (QIAGEN RNA Cleanup Kit, QIAGEN, Valencia, CA, USA) and stored at -80^{o}C until required.

Dual Luciferase Assay:

The 1000bp regions directly upstream of the internally initiated methionines of α1CCT, α1ACT, or α1HCT were cloned into dual luciferase reporter plasmid between an upstream *Renilla* and a downstream *Firefly* ORF. These plasmids were transfected into HEK293 cells for 24 hours and *Renilla* and *Firefly* activity were measured using the Dual Luciferase Reporter System (Biorad - E1980). Internal translation was reported as *Firefly* activity normalized to *Renilla* activity.

RNA – collection and qPCR:

Total cellular RNA was isolated using the RNeasy mini kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. cDNA was generated with the Superscript Vilo reverse transcriptase (Invitrogen, Grand Island, NY, USA). qPCR primers were ordered from IDT (Coralville, IA, USA) and Sybr Green reagent from Bio-Rad (Hercules, CA, USA). Real-time PCR was performed as triplicates on an AB7900 HT Fast -Real Time PCR System (Applied

Biosystems, Foster City, CA, USA) and each experiment was repeated in triplicate (n=3). Each 20µL reaction contained 10µL Sybr Green, 1.2µL 5µM Forward + Reverse primer, 3.8µL ddH_2O, and 5µL diluted cDNA. Each reaction was run under the following conditions: 95°C 2 min., (94°C 15s, 60°C 1 min.) x40 cycles, followed by a melt curve. As a relative quantification, fold changes were measured using the ΔΔCt method, using GAPDH as an internal control.

Statistical Analysis:

Statistical analyses were conducted using GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA), RStudio (Boston, MA), and SPSS (IBM, Armonk, NY). For single comparisons, Student's t test was used to establish statistical significance. For multiple sample comparisons, One-Way ANOVA was performed, followed by Tukey post hoc tests. Data were expressed as the mean ± SEM and written with the identification of n under appropriate figure legends, with $*p < 0.05$, $**p < 0.01$; $***p < 0.001$.

Results:

CACNA1C and *CACNA1H* genes produce a secondary C-terminal protein in cultured cells and endogenous mouse neuronal tissue

A few reports have suggested that other members of the VGCC gene family produce secondary C-terminal fragments, however the mechanism of expression and function of these proteins remains unknown (Bannister et al., 2013; Gomez-Ospina et al., 2006; Lu et al., 2015). To determine if other prominent neuronal VGCC genes produced CTPs similarly to *CACNA1A*, I chose two additional VGCC genes representing the two other distinct functional classes of VGCCs in the brain, *CACNA1C* and *CACNA1H*, and transiently transfected C-terminally

3xFLAG-tagged cDNAs of each into HEK293 cells. Protein lysates were collected 48 hours post-transfection, then fractionated into a cytosolic and nuclear fraction. Using anti-sera specific to N-terminal or C-terminal (3xFLAG) epitopes of each VGCC, western blot analysis of transfected HEK293 lysates showed distinct 65- to 70- kDA polypeptides that were enriched in the nuclear fractions when probed with the C-terminal antibody in addition to the full-length 250-kDa calcium channel α1 subunit. Notably, these C-terminal proteins were not detected when probed using an N-terminally directed antibody (Figure 2A and Figure 2B). Additionally, these 65- to 70-kDa secondary gene products were detected using C-terminal specific α1C (CaV1.2) or α1H (CaV3.2) anti-sera endogenously in mouse brain tissue, but not in control tissues (Figure 2C).

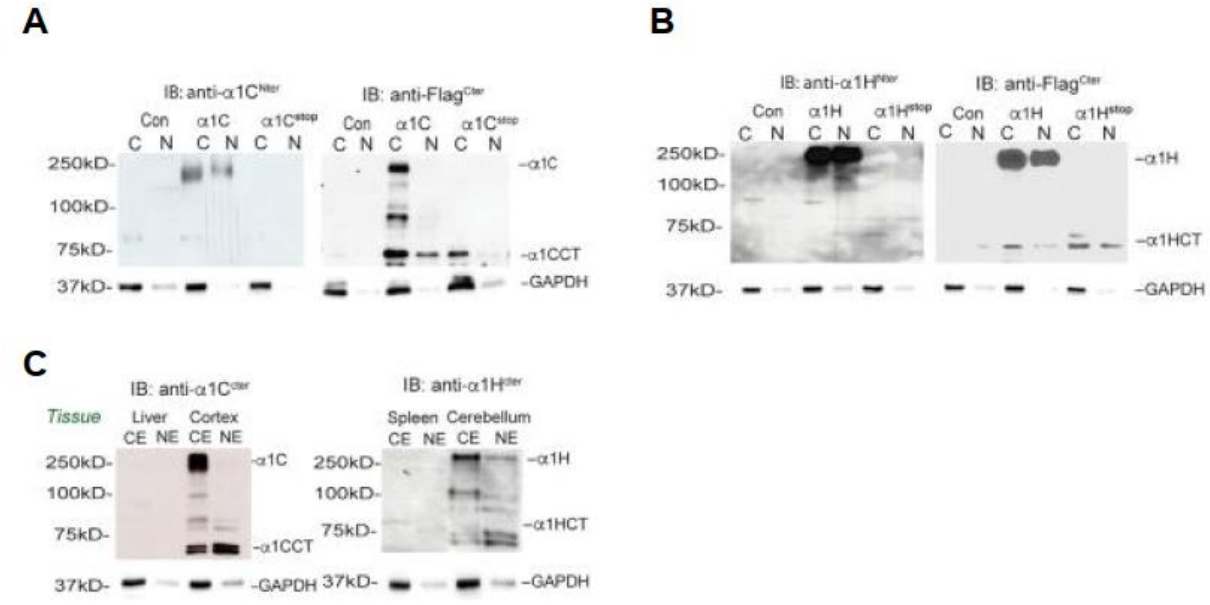

Figure 2. The VGCC genes *CACNA1C* and *CACNA1H* produce secondary, C-terminal proteins in cultured cells and mouse tissue. (A) Western blot analysis of C-terminally 3xFLAG-tagged α1CCT and α1CCT^{STOP} showing a secondary protein product detected by a FLAG antibody (right panel), but not an antibody directed against the N-terminal region of CaV1.2 (left panel). (B) Western blot analysis of C-terminally 3xFLAG-tagged α1HCT and α1HCT^{STOP} showing a secondary protein product detected by a FLAG antibody (right panel), but

Figure 2, continued. not an antibody directed against the N-terminal region of CaV3.2 (left panel). (C) Western blot analysis detecting C-terminal proteins for α1C (left panel) or α1H (right panel) in protein lysate collected from mouse forebrain or cerebellum, respectively. Liver and spleen were used as negative controls for α1C or α1H expression, respectively.

Next, I explored the origin of the *CACNA1C* and *CACNA1H* secondary proteins by introducing a series of premature termination codons (STOP) within the coding regions of the primary α1C and α1H subunits (schematic for STOP and truncation mutations shown in Figure 3A and Figure 3B). The full-length α1 subunit expression was abolished, however C-terminal protein persisted until stop codons were inserted directly downstream of the internal start sites at 1,869 amino acids (aa) and 1,953 aa (Figure 3C and Figure 3D). To estimate the N-terminus of the VGCC CTPs, I generated a series of stepwise 5' truncation cDNA constructs with the final truncation construct initiating around 1,869 aa and 1,953 aa (Figure 3C and Figure 3D). While expression of the VGCC CTPs persisted following the insertion of upstream stop codons within the α1 coding region, deletion of the cytomegalovirus (CMV) – derived eukaryotic promoter from the expression vector resulted in abolished full-length and secondary protein expression in HEK293 cells (Figure 4). Finally, I transfected HEK293 stable cell lines expressing either full-length CaV1.2 or CaV3.2 with siRNAs directed towards the 5' ends of the *CACNA1C* or *CACNA1H* genes. These siRNAs abolished expression of both the full-length α1 subunits as well as the secondary proteins, suggesting that both are expressed from the same mRNA (Figure 5). Together, these results suggest that both *CACNA1C* and *CACNA1H* are bicistronic genes that express secondary, C-terminal proteins that are independent of the full-length α1 subunits.

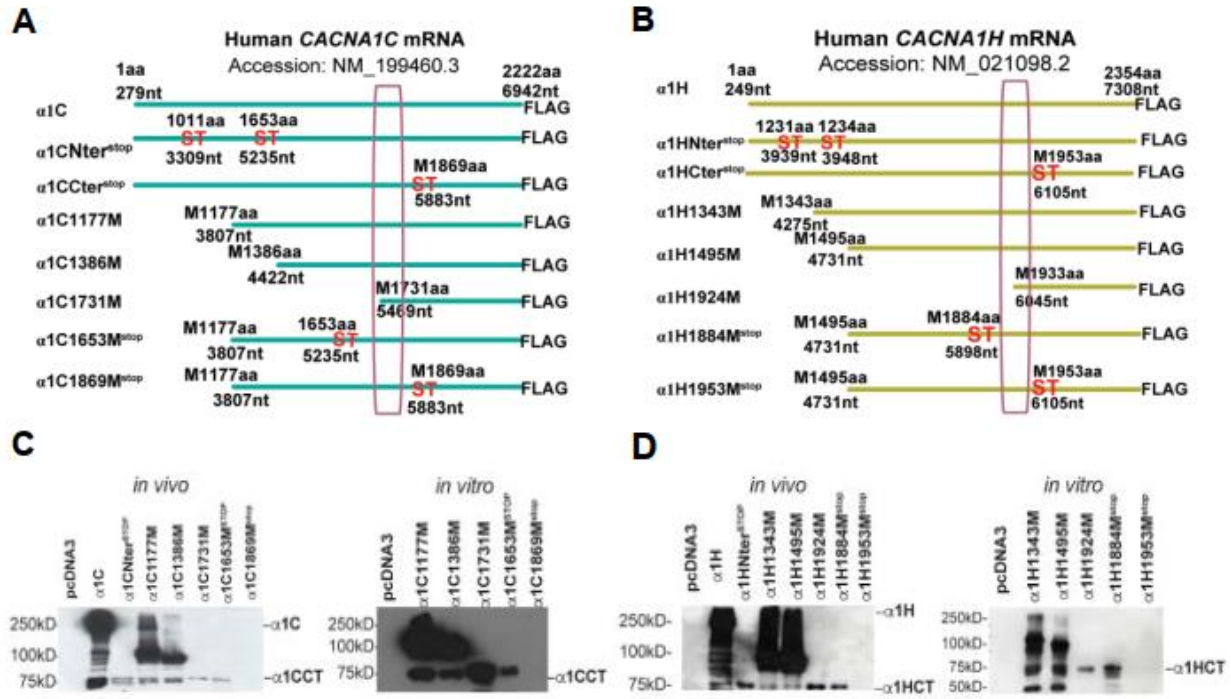

Figure 3. *CACNA1C* and *CACNA1H* gene truncations produce persistent secondary CTPs of consistent size. (A,B) Schematic representation of the cDNA constructs made for *CACNA1C* (NM_199460.3; A) and *CACNA1H* (NM_021098.2;B) 5' deletion truncations and premature termination codon mutations. (C,D) *In vivo* (left), western blot analysis of HEK293T cells transiently transfected with truncated C-terminally 3xFLAG-tagged α1C (C) or α1H (D) to identify the N-terminal methionine of α1CCT and α1HCT. *In vitro* (right) transcription and translation of truncated C-terminally 3xFLAG-tagged α1C (C) or α1H (D), both of which generate the C-terminal proteins α1CCT and α1HCT.

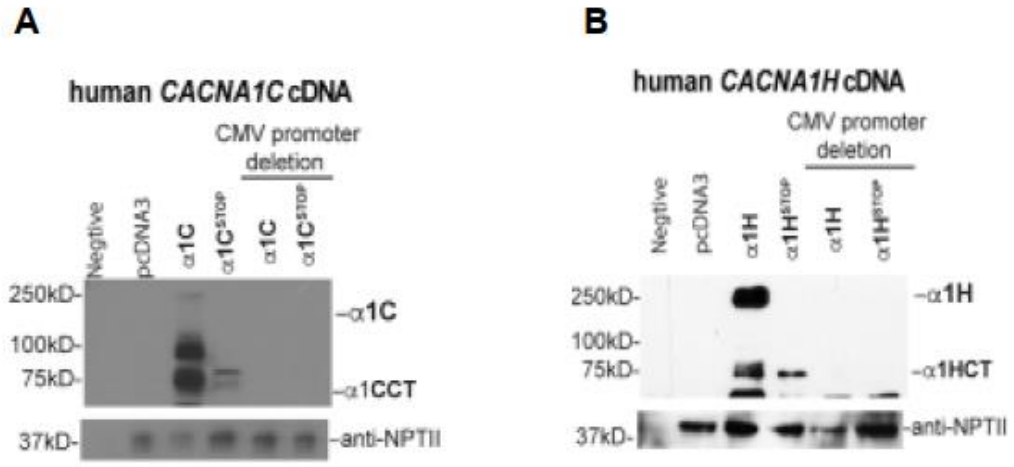

Figure 4. Full-length *CACNA1C* and *CACNA1H* genes produce secondary CTPs in a promoter-less construct. Western blot analysis of HEK293 lysates transiently transfected with promoterless constructs of full-length *CACNA1C* (left) or *CACNA1H* (right) cDNA. Lysates were separated into cytosolic (C) or nuclear (N) fractions. NPTII expression was used as a loading control.

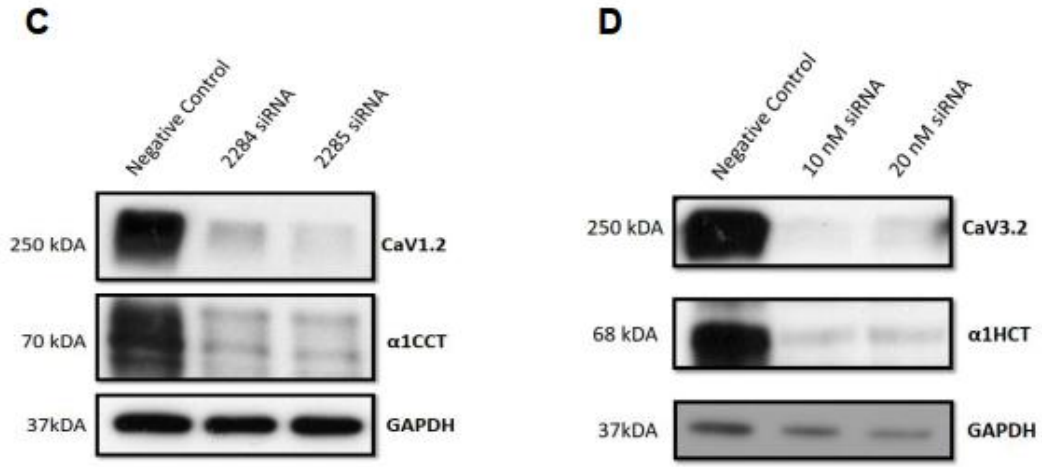

Figure 5. siRNAs targeting the 5' region of the *CACNA1C* and *CACNA1H* genes abolish expression of both full – length and secondary proteins. siRNAs targeting the 5' regions of the *CACNA1C* (left) or *CACNA1H* (right) coding regions were transiently transfected into stable HEK293 lines expressing either *CACNA1C* or *CACNA1H*.

***CACNA1C, CACNA1A, and CACNA1H* mRNAs encode two distinct proteins from overlapping cistrons**

To confirm the bicistronic nature of three neuronal VGCC genes, I transfected HEK293T cells with mature, *in vitro*-transcribed, poly-A-tailed RNAs, expressing the three VGCC alpha subunits extended at the C-terminus end with a 3xFLAG epitope tag. The mRNAs were either capped on the 5' end with a canonical m7G cap, allowing for the initiation of translation, or with a modified 5' cap consisting of m7G(5')ppp(5') A cap. The modified A-cap stabilizes the mRNA within the cell but does not allow for binding of the initiation complex and the initiation of cap-dependent translation. *CACNA1C, CACNA1A,* and *CACACA1H* G-capped mRNAs all expressed the full length α1 VGCC subunits, as well as smaller CTPs that we termed α1CCT (~70 kDa), α1ACT (75 kDa), and α1HCT (~68 kDA) respectively, detected in Western blots using an anti-FLAG antibody. Interestingly, in cells transfected with A-capped mRNAs, the full-length α1 subunit expression was abolished while the CTPs persisted (Figure 6A-C). This finding suggests that while the full-length α1 subunits are generated by canonical cap-dependent translation, the CTPs are generated in the absence of cap-dependent translation. Additionally, using FLAG-specific qPCR primers, I demonstrated that the reduction in CTP expression observed in the A-cap mRNA conditions was not due to an intrinsic mechanistic reason, but because the transfected A-cap mRNAs are significantly more degraded intracellularly than those with the G-cap (Figure 6D-E). Next, we transcribed mRNAs from the cDNA templates for transfection into cells after either of two modifications designed to interfere with translation of the full-length protein: 1) insertion of sequences that generate large RNA hairpins at the very 5' end of the sequence, designed to impede ribosomal elongation, or 2) insertion of premature termination codons between the initiation codon of the full-length α1 subunit and the presumed start site of the putative CTPs. Full-length α1 subunits were not generated from mRNAs bearing either the inserted hairpins or termination codons, but FLAG-tagged CTPs were readily detected. These

results indicate that the CTPs are generated in the absence of a process such as ribosomal slipping/shunting. Furthermore, these findings indicate that the CTPs are not the product of proteolytic cleavage of the full-length subunit.

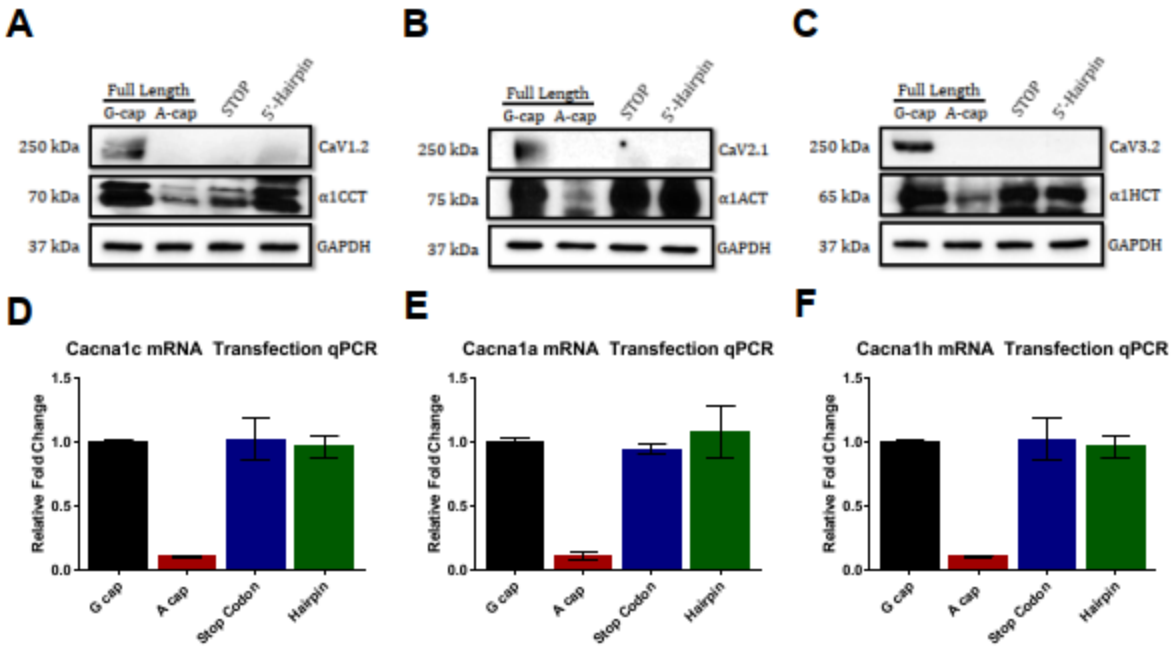

Figure 6. *CACNA1C, CACNA1A,* and *CACNA1H* mRNAs encode two distinct proteins from overlapping cistrons through a cap-independent mechanism. (A,B,C) Western blot analysis of HEK293 protein lysates transiently transfected with *CACNA1C* (A), *CACNA1A* (B), or *CACNA1H* (C) *in vitro* transcribed mRNA. Full-length mRNAs were capped with either an m7G or an m7A cap. STOP construct had two premature termination codons inserted upstream of C-terminal protein start sites. 5'-hairpin constructs had a large hairpin structure inserted directly downstream of the initiating methionine. (D,E,F) qPCR analysis of RNA collected from HEK293 cells transiently transfected with *CACNA1C* (D), *CACNA1A* (E), or *CACNA1H* (F) mRNA. (n=3 for each condition).

mRNA segments within *CACNA1C, CACNA1A,* and *CACNA1H* drive internal, cap-independent translation of CTPs

Our lab previously showed that the expression of α1ACT is mediated by an IRES-like sequence present in the *CACNA1A* coding sequence (Du et al., 2013). I hypothesized that the

α1CCT and α1HCT CTPs are generated from the *CACNA1C* and *CACNA1H* coding sequences by a similar mechanism. To test for IRES-like activity within these regions, we inserted segments of DNA from the regions directly upstream of the putative α1CCT and α1HCT start sites into the bicistronic (*Renilla* luciferase, R-Luc, and firefly luciferase, F-luc) reporter vector, pRF, and transfected these reporters into HEK293 cells. The coding region for R-luc is followed by a termination codon, and an increase in F-luc activity in cell lysates indicates that the inserted RNA segment can enable re-initiation of translation in a cap-independent manner. Insertion of a 1000-base pair region of DNA from directly upstream of the start sites of α1CCT and α1HCT led to significant F-luc activity compared to empty pRF vector control (Figure 7). These results suggest that the expression of α1CCT and α1HCT is driven by the presence of a cellular IRES-like structure within the α1C and α1H coding regions, respectively.

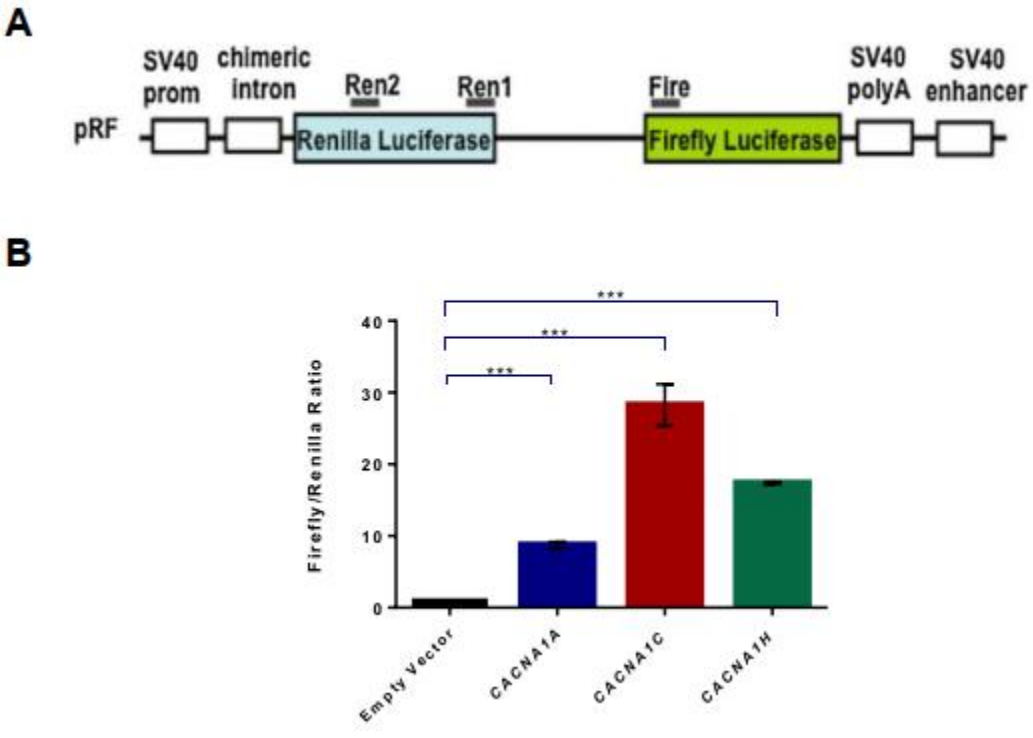

Figure 7. The mRNA segments directly upstream of *CACNA1C, CACNA1A, and CACNA1H* secondary CTP start sites drive cap-independent translation. (A) Schematic representation of

Figure 7, continued. the pRF construct. (B) The luciferase activity as measured by Renilla/Firefly ratio for the bicistronic vector pRF with 1000-bp insertions directly upstream from α1CCT, α1ACT, or α1HCT initiating methionines, compared to empty vector. (n = 3 for each condition, *p<0.05, **p<0.01, ***p<0.001).

Discussion:

Our lab previously demonstrated that the VGCC gene *CACNA1A* was a bicistronic cellular gene, producing two distinct proteins from one mRNA. In this chapter, I show that two additional VGCC genes, *CACNA1C* (encoding CaV1.2) and *CACNA1H* (encoding CaV3.2), representing the two other distinct functional classes of VGCCs, are also bicistronic cellular genes, as each encodes a primary α1 calcium-channel subunit as well as a secondary, C-terminal protein, termed α1CCT or α1HCT respectively. These findings expand our knowledge of the poorly characterized class of cellular bicistronic genes, demonstrating a conserved mechanism of gene expression in eukaryotic cells.

Identifying bona fide cellular IRES-like structures requires stringent exclusionary criteria, as several other methods of alternative gene expression and translation, both cap-dependent and cap-independent, must be excluded from possibility. For one, cryptic promoters in coding regions and reporter plasmids as well as alternative splicing events need to be ruled out, as both can lead to distinct transcripts and secondary protein products (Van Eden et al., 2004; Baranick et al., 2008). Additionally, mechanisms of secondary protein translation, including ribosomal scanning, ribosomal re-initiation, translational frameshifting, or stop codon read-through, have been identified to occur in eukaryotic cells and need to be excluded. To this end, I performed several experiments using cDNA and mRNA expression techniques that yielded converging evidence. The expression of two distinct proteins from single mRNA transcripts directly transfected into cells excludes the possibility that the CTPs are the products of alternative

splicing, as these mRNAs are unable to undergo such a process. This finding also excludes the presence of a cryptic promoter in the underlying DNA sequence, as *in vitro*-transcribed mRNA was transfected directly into cells. To exclude the possibility that the VGCC CTPs were produced via ribosomal read through or shunting mechanisms, the expression patterns of VGCC mRNA transcripts bearing either a 5' hairpin structure or several premature stop codons were assessed. In both conditions, the full-length α1 subunit expression is abolished, while secondary protein expression persists. The 5' hairpin structure is designed to "knock off" cap-dependently initiated ribosomes, excluding any possible read through or shunting mechanism. The premature stop codons exclude ribosomal read through as well as proteolytic cleavage mechanisms. These results convincingly demonstrate that the VGCC CTPs α1CCT, α1ACT, and α1HCT are produced in a cap-independent manner via a cryptic IRES-like structure within the α1 subunits' primary open reading frame.

Unlike viral IRESs, cellular IRESs exhibit no clear conserved sequence, tertiary structure, or required IRES *trans*-acting factors (ITAFs) (Baird, 2006). Therefore, unbiased screens for IRES-like structures within the eukaryotic genome are difficult. Two-dimensional structure analyses of the complete α1C and α1H mRNA sequence using an M-fold-based algorithm did not identify any canonical type I or type II IRES structures in this region (Palmenberg and Sgro, 1997; Zuker, 2003; Baird, 2006). However, the regions directly upstream of the putative start sites for α1CCT and α1HCT within the *CACNA1C* and *CACNA1H* mRNAs are predicted to form a highly stable series of stem loop structures that could represent regions of functional significance for the initiation of translation and interaction with canonical translation initiation factors as well as novel IRES *trans*-activating factors (ITAFs). Indeed, this seems to be the case, as these regions were able to robustly initiate cap-independent translation when inserted into the

dual-luciferase pRF vector (Figure 7). While these results suggest cap-independent translational activity within these mRNA regions, further studies are needed to address the limitations of the pRF vector. For one, the inserted DNA sequence may function as a cryptic promoter instead of an IRES, transcribing a second transcript harboring the F-luc coding sequence. Another non-trivial possibility is that the inserted DNA sequence may harbor cryptic alternative splicing sites when transcribed, leading to potential in-frame fusion R-luc and F-luc ORFs. Finally, ribosomes translating R-luc may be able to restart translation, either by improper termination resulting in read through, translation re-initiation downstream, or shunting to the downstream F-luc start site. Therefore, interpretation of pRF results needs to be done in the context of additional results demonstrating cap-independent translational activity, in this case mRNA transfection experiments discussed above.

The finding that three VGCCs representing all three major subtypes are bicistronic has far-reaching implications for our understanding of normal cellular translation and dysregulation in disease. This coordinated form of gene expression can spatially and functionally couple two distinct protein products, as they do with the VGCC CaV2.1 and its secondary transcription factor α1ACT, ensuring the proper functioning of complex biological programs. However, this coupling also has the potential to manifest as debilitating disorders when dysregulated through mutations in one or both gene products. Nonetheless, the discovery and characterization of novel eukaryotic IRESs may help explain the complex and varied phenotypes observed in many neurological disorders resulting from single polymorphisms, as it did for α1ACT and the pathology spinocerebellar ataxia type 6 (Du et al., 2019).

Chapter 3: VGCC C-terminal proteins bind to DNA to promote gene expression changes in human Neural Progenitor Cells

Introduction:

While every cell in a single eukaryotic organism contains the same genetic material, the expression of this genetic material is not uniform across all cells. Cells expressing different subsets of genes develop into vastly different subtypes, and proper temporal and spatial regulation of this expression is critical for normal development. All organisms achieve this precise regulation at least partially through the function of transcription factors (TFs), proteins that bind to specific DNA sequences and promote or repress transcription to regulate the expression of target genes. Regulation of TF expression by endogenous and exogenous stimuli, as well as regulation of necessary co-factors such as coactivators or corepressors, ensures proper terminal cell localization and differentiation (Bakken et al., 2016; Hong et al., 2005; Tasic et al., 2018).

In the developing and mature nervous system, vast networks of TFs have been identified that give nervous system cells their unique morphologies and functions. Various families of TFs control neuronal cell development at each stage of their development, maintaining neuronal cells in various developmental states and promoting proliferation, differentiation, and maturation. TFs drive the differentiation of neural stem cells to all types of neurons and glial cells, and even a small disruption in proper TF activity, depending on the temporal window and localization of dysregulation, can have wide-ranging downstream consequences (Heavner et al., 2020; Santiago and Bashaw, 2014). Therefore, understanding proper TF function is crucial for our understanding and subsequent treatment of disorders related to TF dysfunction.

Our lab previously demonstrated that the VGCC gene *CACNA1A* produces two proteins from one mRNA transcript, the α1A calcium channel subunit and a newly discovered transcription factor termed α1ACT (Du et al., 2019). α1ACT is critical for proper perinatal cerebellar development, helping to regulate a suite of genes that promote proper Purkinje cell maturation and function. Furthermore, our lab has demonstrated that α1ACT, which contains a polyQ tract, partially loses its function when this tract is expanded to pathogenic repeat numbers, which leads to spinocerebellar ataxia part 6 in human (Du et al., 2019). The discovery that SCA6 is caused by the polyQ-expanded α1ACT protein, and not disrupted channel function of the polyQ-expanded CaV2.1 calcium channel, has wide ranging implications for the understanding and potential treatment of disorders related to IRES-translated proteins.

Disruption of proper TF function during neuronal development and maturation has been linked to the pathogenesis of many debilitating neurological disorders. Specifically, dysregulation of complex gene expression networks, with multiple risk genes contributing, has been implicated in the pathophysiology of several neuropsychiatric illnesses. However, identification of any one TF that contributes significantly to mental illness is still challenging, and no single gene has been identified that contributes significantly to the risk of neuropsychiatric illness. Instead, these disorders are characterized by complex genetics, with many genes, as well as environmental factors that interact with predisposed genetic variations, influencing risk. Additionally, the unique role TF dysregulation may play in the pathophysiology of mental illnesses is highlighted by the fact that most common risk variants for neuropsychiatric disorders do not appear to change the coding sequence of genes. Instead, they are likely to disrupt the regulatory regions of these genes, including potential binding sites of TFs (Bray and O'Donovan, 2018).

In this chapter, I demonstrate that the C-terminal proteins (CTPs) produced by the *CACNA1C* and *CACNA1H* genes, termed α1CCT and α1HCT respectively, function as TFs similarly to α1ACT. These CTPs promote neurite outgrowth in human neural progenitor cells and all bind to DNA to alter the expression of an array of genes related to neuronal development, cell adhesion, and synapse formation. Additionally, I demonstrate that in a conditional CaV1.2 forebrain knockout mouse model, which I and others have shown has an anxiety and ASD-like phenotype, several of the genes α1CCT was shown to regulate *in vitro* have significantly altered expression, partially illustrating the distinct roles the parent VGCC CaV1.2 and the CTP α1CCT play in modulating neuronal gene expression.

Materials and Methods:

Lentivirus Cloning/Packaging:

The pLVX-Puro transfer vector was purchased from Takara, and the α1CCT, α1ACT, and α1HCT coding sequences were cloned into the multiple cloning site. Packaged virus was created in HEK293T cells using the Lenti-X Single Shot Lentivirus packaging system (Takara, Kusatsu, Shiga, Japan). 48 hours after the Lenti-X single shot vector mix was transfected into cells, the media was collected and filtered through a sterile 0.45 µm syringe filter. To purify and concentrate the packaged virus, one volume of concentrator solution (40% w/v polyethylene glycol 8000 (PEG-8000) in PBS solution with 1.2M NaCl) was added to three volumes of filtered viral media, mixed well, and gently rotated overnight at 4^{o}C. The following day, the concentrator-viral media solution was spun down at 1600xg for 60 minutes at 4^{o}C, and the

supernatant was carefully removed. The resulting viral pellet was resuspended in DMEM/F12 media without serum and frozen in aliquots at -80^{o}C until use.

Cell Culture:

Human neural progenitor cells (hNPCs) were obtained from Stem Cell Technologies (Vancouver, BC, CA) and grown in Stem Cell Technologies Complete Neural Progenitor Cell Medium on Matrigel (Corning Life Sciences, Corning, NY, USA), with complete media changes made every day. Stable cell lines expressing either α1CCT-3xFLAG, α1ACT-3xFLAG, or α1HCT-3xFLAG were created by infecting NPCs at a multiplicity of infection (MOI) of 20 with packaged lentivirus expressing each of the VGCC CTPs, along with a pLVX-Puro empty vector control line. Cells were switched to complete NPC medium containing 0.5 µg/mL puromycin for 7 days to select for stably infected cells, then maintained thereafter in NPC medium supplemented with 0.1 µg/mL puromycin.

Morphology Imaging:

hNPC empty pLVX-Puro, α1CCT-3xFLAG, α1ACT-3xFLAG, or α1HCT-3xFLAG stable cell lines were plated on Matrigel covered glass coverslips. 72-hours post plating, cells were fixed with 4% (vol/vol) paraformaldehyde (PFA) + 4% sucrose (wt/vol) in PBS for 10 minutes at room temperature. The PFA/sucrose fixing solution was aspirated and hNPCs were incubated with 0.1% Triton-100 in PBS for 10 minutes. hNPCs were then washed once with PBS and blocked for 1 hour at room temperature in 5% bovine serum albumin (BSA) in PBS, then incubated overnight at 4^{o}C in primary antibody diluted in 1% BSA in PBS. Primary antibodies used were Anti-Nestin (Sigma, St. Louis, MO, USA), Anti-FLAG 1804 (Sigma, St. Louis, MO, USA), all at a 1:500 dilution. The next day, hNPCs were washed with PBS then incubated in the dark for 1

hour in secondary antibody (AlexaFluor594 Anti-Mouse and AlexaFluor488 Anti-Rabbit; Thermo Fisher, Waltham, MA, USA) at room temperature. Coverslips were washed again with PBS then mounted on glass slides with DAPI mounting media (Vector Labs, San Francisco, CA, USA).

RNA-seq:

RNA was collected from hNPC stable cell lines using the RNeasy Micro Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. Total mRNA was sequenced by Novogene Corporation at a depth of 40 million reads. Separate hNPC RNA was collected for downstream quantitative real-time PCR to confirm CTP-mediated fold changes. RNA-seq analysis was done with FastQC v0.11.2, RSeQC, BWA v0.7.5a. Picard tools v1.117, Tophat v2.0.10, edgeR, samtool v0.1.19. All reported genes had a minimum significance of FDR $p < 0.05$.

ChIP-seq:

The hNPC stable cell lines expressing either pLVX empty vector, a1CCT-FLAG, a1ACT-FLAG, or a1HCT-FLAG were grown to 90% confluency in 10 cm dishes. Cells were collected and ChIP DNA was prepared using the Magna ChIP A/G Chromatin Immunoprecipitation Kit (Millipore Sigma, Burlington, MA, USA) according to the manufacturer's instructions, using Anti-FLAG 1804 antibody for immunoprecipitation (Sigma, St. Louis, MO, USA). ChIP DNA libraries were prepared and sequenced at a depth of 20 million reads by Novogene Corporation. Template DNAs included input (5% of starting chromatin as positive control), CTP-ChIP DNA, and immunoglobulin G (IgG)-ChIP as a negative control. For each condition, enriched DNA sequences were identified first by comparing CTP-ChIP DNA to each respective input, then

comparing that DNA pool to the similar pool identified for IgG-ChIP DNA.ChIP DNA was collected in tandem triplicate experiments for downstream quantitative real-time PCR (ChIP-qPCR) to confirm selected α1CCT, α1ACT, and α1HCT target genes. ChIP-seq analysis was done with skewer v0.1.126, FastQC v0.11.2, BWA v0.7.1, MACS2 v2.1.0, meme v4.10.2, PeakAnnotator_Cpp v1.4, diffbind, Goseq/topGO Bioconductor v2.13. All reported genes had a minimum significance of FDR $p < 0.05$.

Statistical Analysis:

Statistical analyses were conducted using GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA), RStudio (Boston, MA), and SPSS (IBM, Armonk, NY). For single comparisons, Student's t test was used to establish statistical significance. For multiple sample comparisons, One-Way ANOVA was performed, followed by Tukey post hoc tests. Data were expressed as the mean ± SEM and written with the identification of n under appropriate figure legends, with $*p < 0.05$, $**p < 0.01$; $***p < 0.001$.

Results:

α1CCT, α1ACT, and α1HCT proteins promote a neuronal differentiation program

α1ACT mediates neurite outgrowth and activates a gene expression program favoring neuronal differentiation in PC12 cells and in Purkinje neurons (PCs) *in vivo* (Du et al., 2013; Du et al., 2018). I hypothesized that α1CCT and α1HCT would elicit specialized differentiation pathways in native human neural progenitor cells (hNPCs). To test this, I generated stable hNPC cells lines expressing either α1CCT, α1ACT, α1HCT, or an empty pLVX transfer vector control line. hNPCs stably expressing any one of the three CTPs grew significantly more neurites than

the empty vector control stable line four days after plating (Figure 8). Additionally, these stable cells lines had significantly longer average neurite length as well as significantly greater longest neurite length, when adjusted for the increased average number of neurites, compared to the control stable line (not shown).

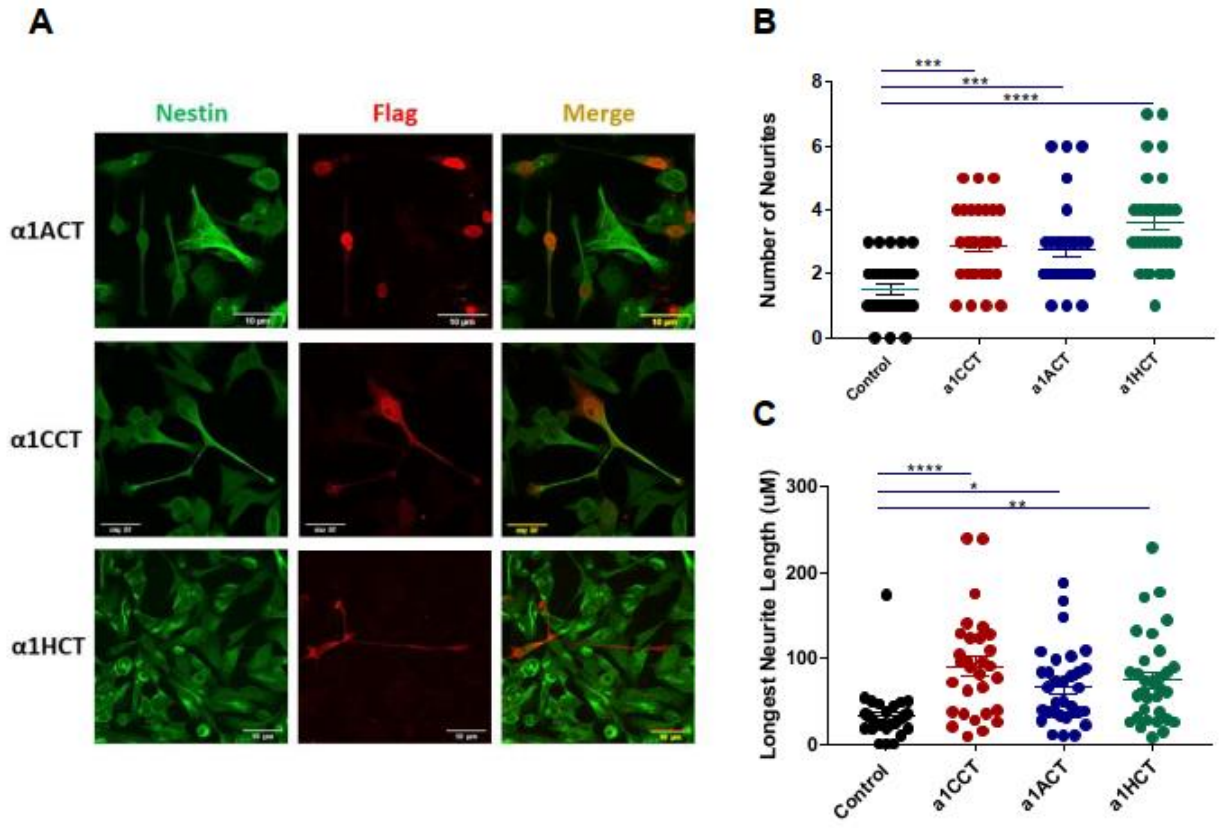

Figure 8. α1CCT, α1ACT, and α1HCT promote neurite outgrowth in human neural progenitor cells. (A) Representative images of human neural progenitor cells (hNPCs) stably expressing C-terminally 3xFLAG tag-labeled α1CCT, α1ACT, or α1HCT. Co-stained with antibodies directed against the hNPC marker Nestin (green) or FLAG (red). (B,C) Quantification of number of neurites per cell and longest neurite per cell (n > 30 cells, *p<0.05, **p<0.01, ***p<0.001).

To explore whether the phenotypic changes induced in hNPCs by α1CCT, α1ACT, or α1HCT were associated with underlying unique or overlapping changes in gene activation, we

conducted an mRNA-sequencing (RNA-seq) and chromatin immunoprecipitation with sequencing (ChIP-seq) analyses of hNPC stable cell lines expressing VGCC CTPs. Consistent with previous studies, we observed that α1ACT was associated with expression of several key genes associated with neurogenesis, nervous system development, and synapse formation (Figure 9B) (Du et al., 2013; Du et al., 2019). Similarly, we observed that α1CCT and α1HCT altered expression of several genes associated with neuronal differentiation and maturation in hNPCs (Figure 9A and Figure 9C). In agreement with the morphological result described above, all three CTPs had several significant associated GO terms related to neuronal projection growth and guidance, cell adhesion, and cell-cell communication (Figure 9A-C). Although there is overlap between the GO terms for each CTP, there are numerous unique terms specific for each CTP, possibly indicating their selective temporal or spatial roles in the developing and mature CNS. α1CCT upregulates genes more associated with synaptic formation, synaptic signaling, and neurotransmitter packaging and secretion, while downregulating genes associated with neurogenesis and generation of neurons. Interestingly, in hNPCs, α1CCT seemed to regulate several genes relating to cardiac system development, consistent with the established role of *CACNA1C* in cardiac electrophysical development, maturation, and function (Li et al., 2016). Conversely, α1HCT upregulates an ensemble of genes more associated with neurogenesis, generation of neurons, and nervous system development while downregulating genes associated with cell migration, locomotion, and localization of cell (Figure 9).

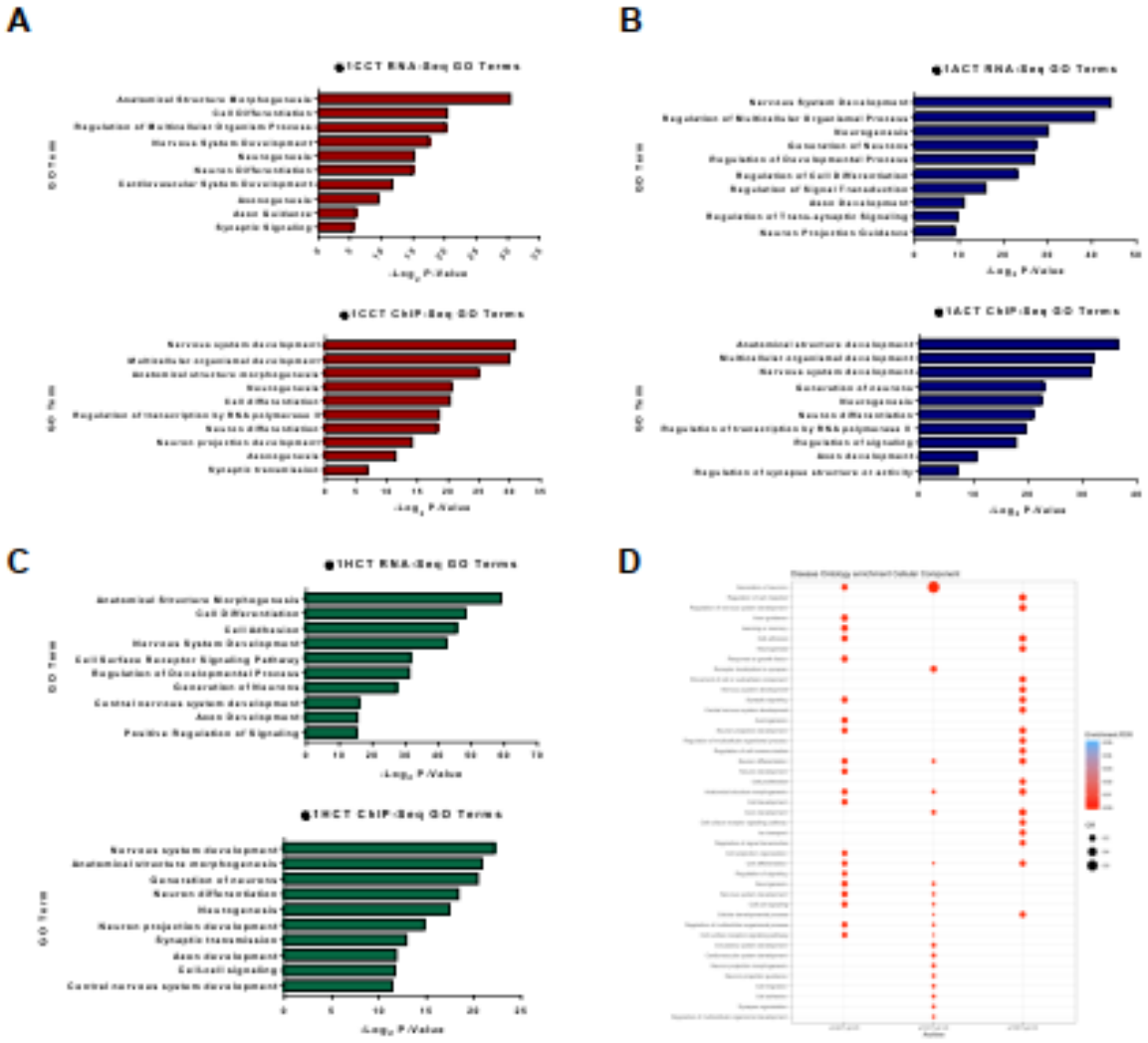

Figure 9. α1CCT, α1ACT, and α1HCT promote a genetic neural differentiation program in hNPCs. (A-C) Selected gene ontology (GO) terms that are significantly altered in hNPCs stably expressing either α1CCT (A), α1ACT (B), or α1HCT (C)identified by RNA-seq (upper panels) or ChIP-seq (lower panels). (D) Top selected enriched biological GO terms identified by RNA-seq related to neuronal development and synaptic signaling for α1CCT, α1ACT, or α1HCT compared to empty vector control. Significantly altered DEGs identified at FDR<0.05.

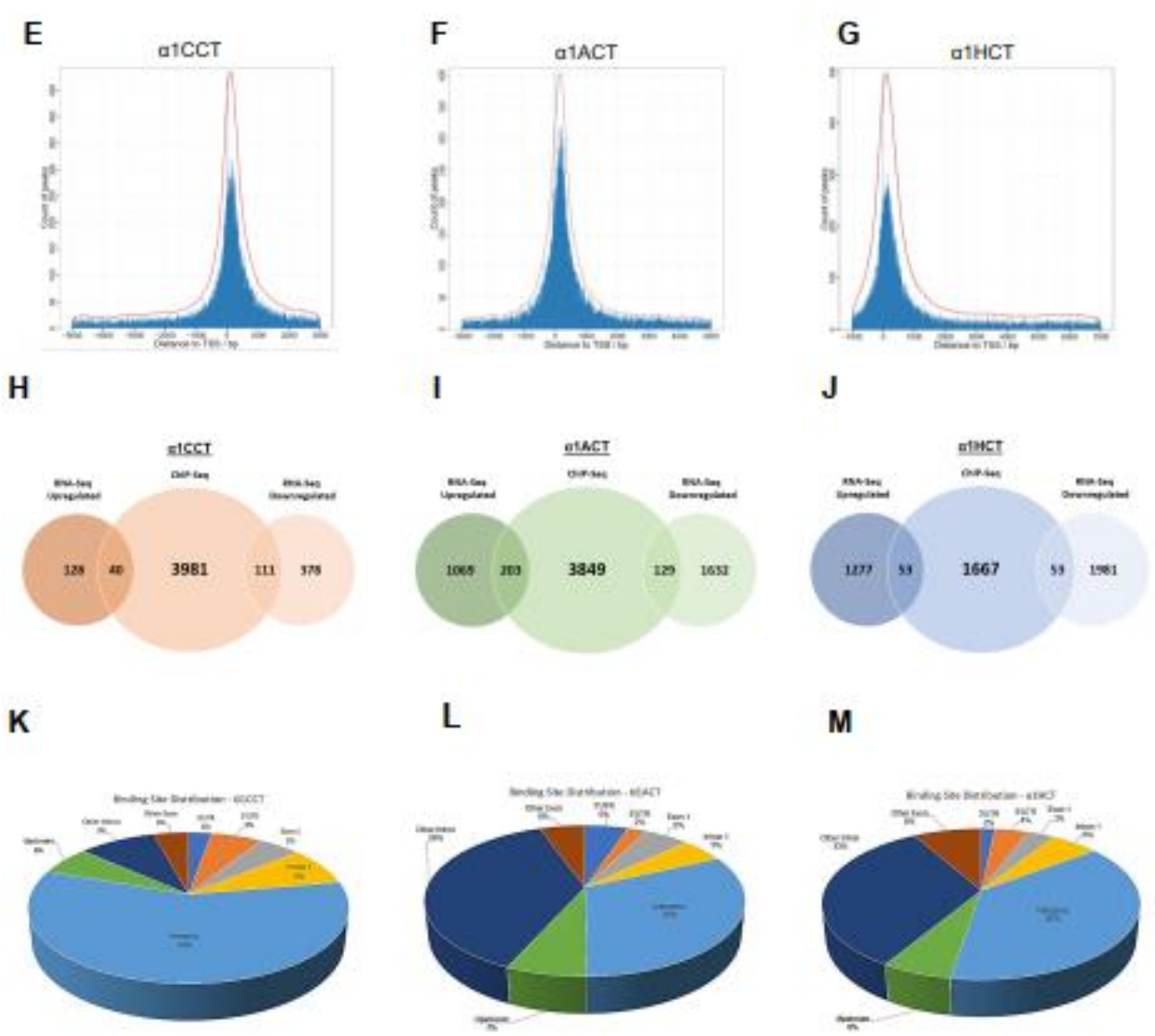

Figure 9, continued. (E-G) Venn diagrams showing the number of significantly upregulated or downregulated DEGs for the three VGCC CTPs identified through RNA-seq, ChIP-seq, and their overlap. (H-J) Pie chart showing the distribution of binding sites for α1CCT (H), α1ACT (I), or α1HCT (J) based on *Homo sapiens* (University of California Santa Cruz Genome Browser [UCSC] version GRCh38/hg38 assembly) gene annotation.

These findings suggest that VGCC CTPs α1CCT, α1ACT, and α1HCT help regulate critical and distinct developmental programs driving the differentiation of hNPCs to mature neurons, as well contributing to proper synaptic maintenance in mature neurons.

α1CCT, α1ACT, and α1HCT bind to DNA to promote gene expression changes in human NPCs

Our lab has shown previously that α1ACT binds to DNA in PC12 cells and directly regulates a suite of genes crucial for proper cerebellar development in Purkinje cell neurons (PCs) (Du et al., 2013). To determine the physiological DNA-binding and direct gene regulatory properties of α1CCT and α1HCT, we performed chromatin immunoprecipitation with next-generation sequencing (ChIP-seq) on hNPC stable cell lines expressing either α1CCT-3xFLAG or α1HCT-3xFLAG, as well as α1ACT-3xFLAG or empty pLVX vector to serve as the positive and negative control, respectively.

α1CCT and α1HCT both bind directly to DNA, like α1ACT, and the majority of α1CCT- or α1HCT- occupied genomic regions occurred within ±1000 bp of annotated transcriptional start sites (TSSs) (Figure 9E-8G). For α1CCT, 42% of binding sites were located within transcribed regions, and 6% were located within regions directly upstream of annotated TSSs. For α1HCT, 62% of binding sites were located within transcribed regions, and 6% were located within regions directly upstream of annotated TSSs. Using the DREME software package to detect significant motif sequences within peaks and the Tomtom package to map motifs to an annotated motif database, I identified several DNA-binding motifs for α1CCT, α1ACT, and α1HCT in hNPCs (Figure 11). Furthermore, for all three CTPs, many key neuronal target genes identified through RNA-sequencing were directly bound by the CTPs, indicating that these genes are bona fide gene targets of the CTPs (Figure 10). Additionally, key selected GO terms identified through ChIP-seq matched those that were identified as significantly modulated in CTP RNA-sequencing (Figure 9A-8C). Finally, to validate select genes identified as

significantly altered by α1CCT, I performed qPCR analysis on cortical tissue collected from a CaV1.2 conditional forebrain knockout mouse model (Lee et al., 2012). This mouse model has been shown to exhibit an anxiety-like phenotype, which I further characterized through the elevated plus maze (EPM) test, open field task, nestlet shredding task, light-dark conflict task, and marble burying task. These behavioral assays test for both an anxiety phenotype as well as an autism-like phenotype (Seibenhener and Wooten, 2015; Komada et al., 2008; Arrant et al., 2013; Angoa-Perez et al., 2013). The CaV1.2 cKO mice exhibited several characteristics of an anxiety-autism-like phenotype, including decreased open arm entrances in the EPM, decreased distanced traveled in the open field task, decreased time spent in light in the light./dark task, and increased nestlet shredding (Figure 12A-10E). Additionally, several of the differentially expressed genes (DEGs) identified by RNA-seq were significantly upregulated or downregulated in the CaV1.2 cKO mice compared to wild-type controls (Figure 12F). Together, these results indicate that the novel VGCC CTPs α1CCT, α1ACT, and α1HCT function as direct DNA-binding proteins to mediate widespread changes in gene expression, promoting the development or maturation of a neuronal phenotype.

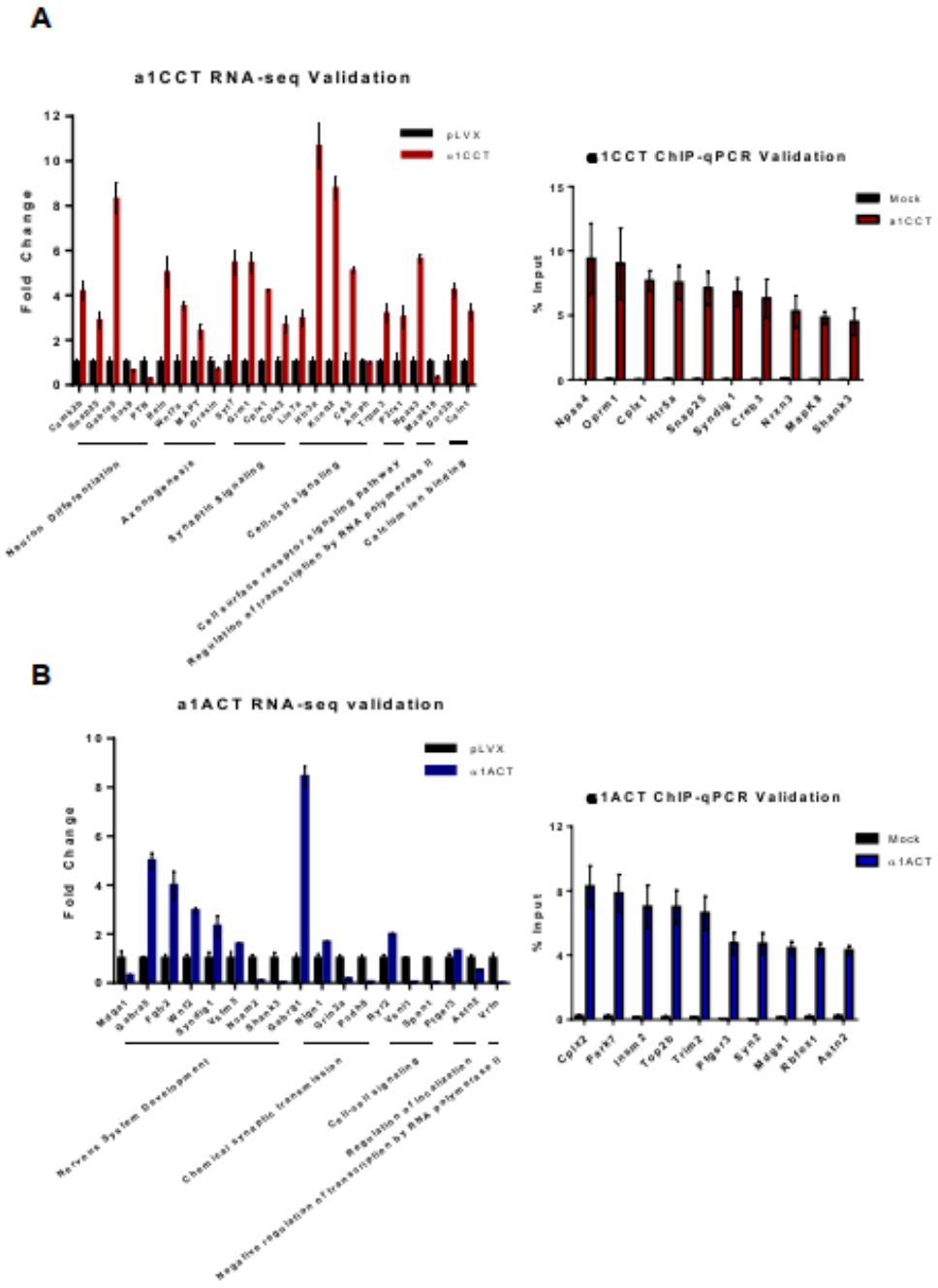

Figure 10. α1CCT, α1ACT, and α1HCT bind to DNA to promote gene expression changes in hNPCs. (A-C) qPCR validation of selected upregulated and downregulated genes (RNA-seq; left panels) or peaks (ChIP-seq; right panels) for α1CCT (A), α1ACT (B), or α1HCT (C). FDR<0.05.

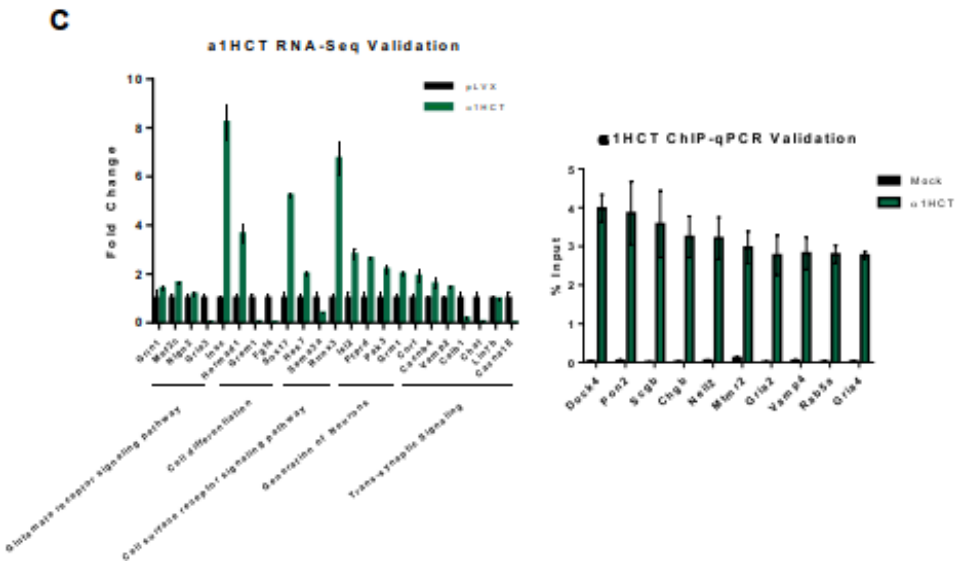

Figure 10, continued. For RNA-seq validation, genes are clustered based on their representative GO terms. For RNA-seq DEGs, all CTPs were compared to empty pLVX lentiviral expression vector (N > 3 for each condition).

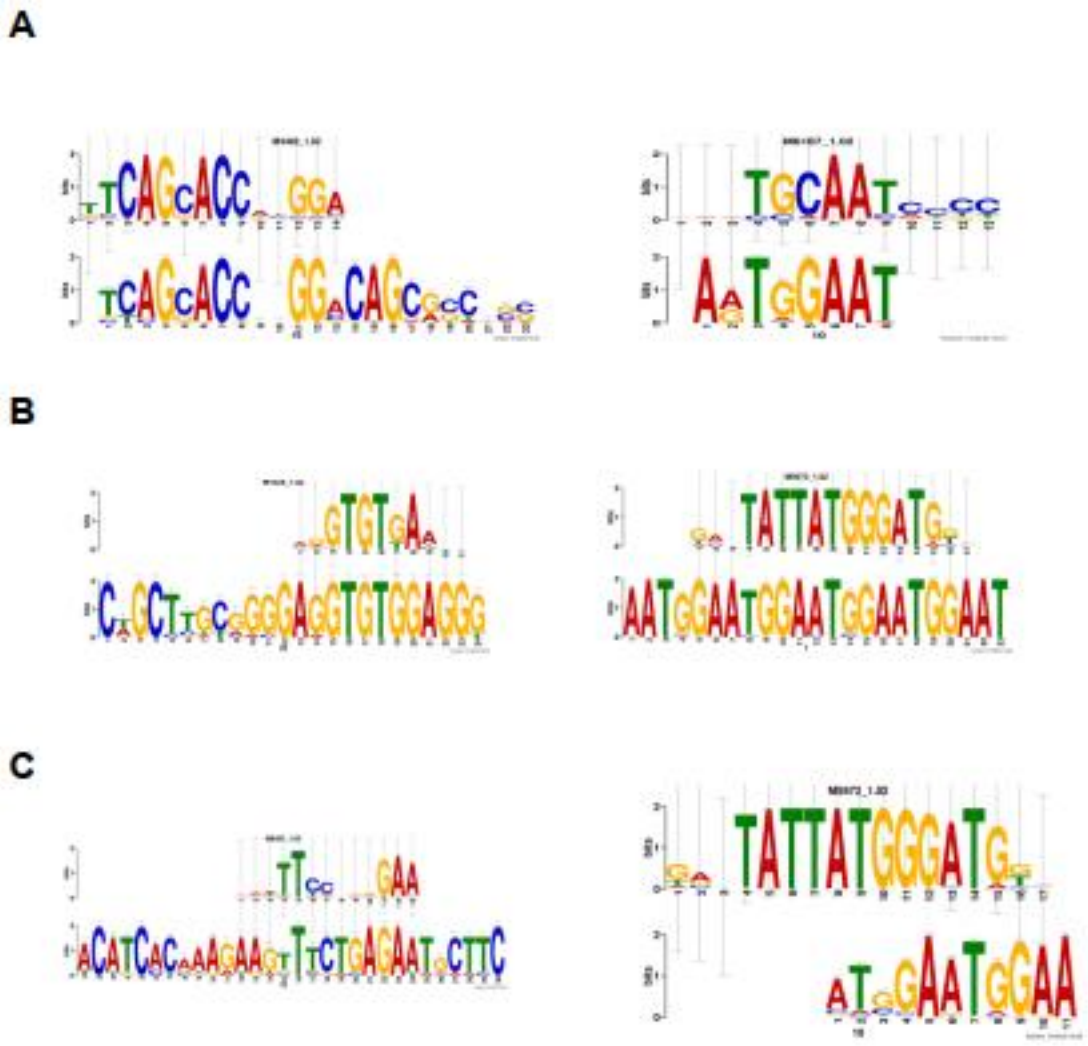

Figure 11. α1CCT, α1ACT, and α1HCT have unique binding motifs as identified by DREME/TOMTOM analysis. (A-C) Enriched consensus binding motifs for α1CCT (A), α1ACT (B), and α1HCT (C). Motifs were identified using Discriminative Regular Expression Motif Elicitation (DREME) software followed by annotation and mapping using Tomtom.

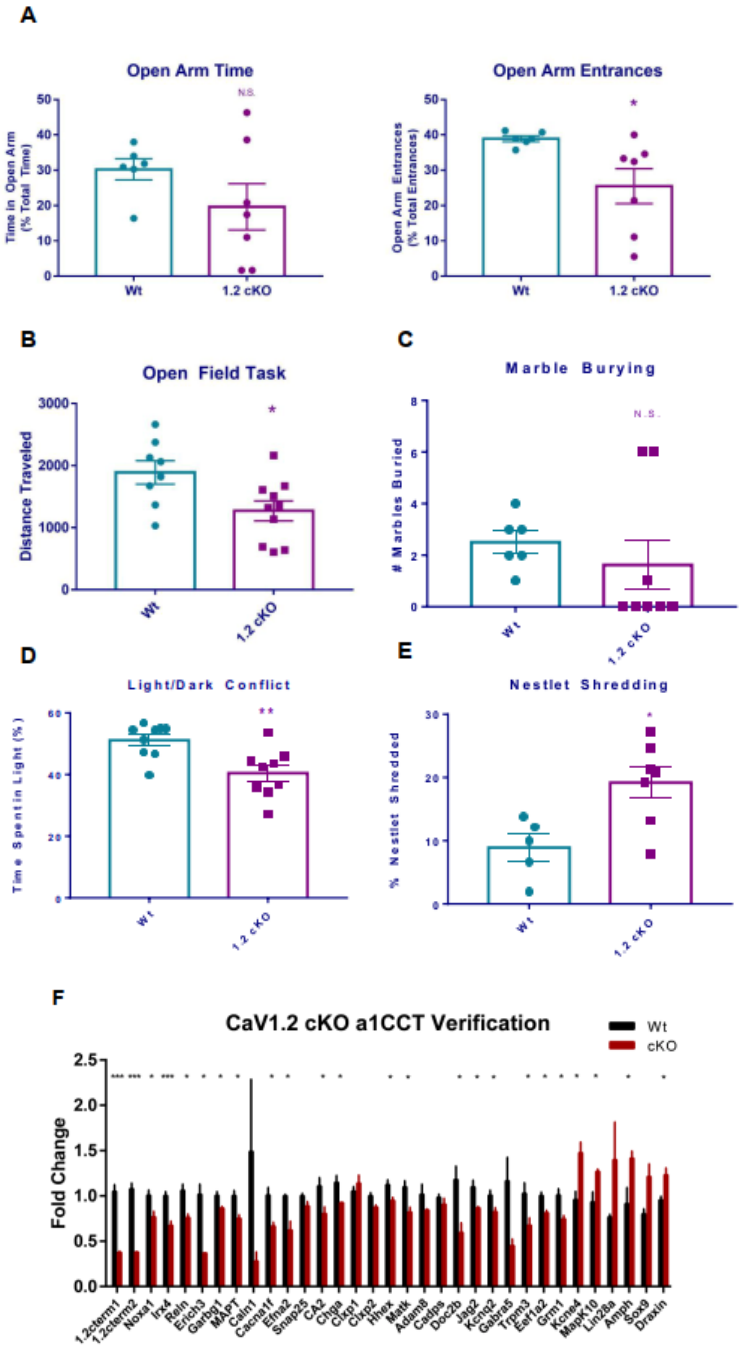

Figure 12. CaV1.2 cKO mice exhibit an anxiety/autism-like phenotype and expression of selected α1CCT – regulated genes is significantly altered in cortex of this cKO mouse line. (A-E) Behavioral assays conducted on CaV1.2 cKO mice for elevated plus maze (A) (WT n = 6, cKO n = 7), open field task (B) (WT n = 8, cKO n = 10), marble burying task (C) (WT n = 6, cKO n = 8), light dark conflict task (D) (WT n = 9, cKO n = 9), and nestlet shredding task (E) (WT n = 5, cKO n = 7) (*p<0.05). (F) qPCR analysis of select α1CCT DEGs identified through RNA-seq in cortical tissue of a conditional forebrain CaV1.2 knockout mouse line, compared to wild-type cortical tissue. (n = 3, *p<0.05, **p<0.01, ***p<0.001)

Discussion:

The proper development and maintenance of the nervous system is orchestrated by a wide array of transcription factors that are precisely regulated in both a spatial and temporal manner. Transcription factors are especially important during neuronal development, where discrete groups of them can drive differentiation of a common neural progenitor cell pool to downstream neuronal subtypes with distinct features. The expression and function of these transcription factors are controlled by a variety of intrinsic and extrinsic neuronal stimuli, and dysregulation of even a single component of these complex signaling cascades can result in debilitating neurological disease ((Tasic et al., 2018; Rosenberg et al., 2018; Bakken et al., 2016). Therefore, understanding the function, regulation, and expression patterns of established transcription factors, as well as identifying novel ones, is of critical importance to understanding normal nervous system development as well as complex genetic disorders.

Previously, our lab demonstrated that the *CACNA1A* CTP, α1ACT, functions as a transcription factor, driving the expression of key genes related to proper Purkinje cell development and function within the cerebellum (Du et al., 2019). In this chapter, I demonstrate that two additional VGCC CTPs, α1CCT and α1HCT, also function as DNA-binding transcription factors in hNPCs, driving distinct neuronal differentiation and synaptic development programs. Additionally, I demonstrate that in a conditional forebrain CaV1.2 knockout mouse line, several of the α1CCT DEGs identified by RNA-seq had significantly altered expression. This finding is especially intriguing as it begins to differentiate the genetic contributions of the parent VGCC CaV1.2 from the newly identified TF α1CCT. Additionally, this cross-species validation of α1CCT DEGs in human cells and mouse brain tissue indicates a conserved evolutionary function of this TF. Finally, while all three CTPs had overlap in the

genes they regulated, they also regulated distinct sets of genes, possibly indicating a cell-type specific or developmental-stage specific role for each. These differences would make sense as each VGCC gene has a unique expression profile within the brain. While these data convincingly demonstrate TF activity of all three CTPs, an important limitation of this study is that this function was assayed in cells over-expressing each CTP, hence endogenous DNA-binding and gene regulatory function may differ from that observed here.

CaV1.2 is well established as being a powerful indirect modulator of activity-dependent gene expression in neurons (Yap and Greenberg, 2018). However, the discovery of the *CACNA1C* CTP, α1CCT, and its transcriptional regulatory function provides new insight into a direct role of the CACNA1C gene in neuronal gene expression. Further studies are needed not just for α1CCT, but for α1HCT and α1ACT as well, to fully understand the unique contributions of the parent VGCC channels and their associated secondary transcription factors in modulating gene expression patterns in neurons. Additionally, studies elucidating the extent of dysfunction of each protein and their respective contributions to disease pathology may help identify pathophysiological mechanisms of complex neurological disorders seen in patients with VGCC mutations as well as inform novel therapeutic targets.

CHAPTER 4: Nuclear translocation of VGCC CTPs is coupled to neuronal calcium signaling

Introduction:

Changes in gene expression allow cells to interact dynamically with salient external stimuli and subsequently store information about past experiences. Neurons face a unique challenge regarding this information processing, as they are post-mitotic and therefore must be highly adaptable while also being able to reliably encode short- and long-term memories related to an organism's experiences. Neurons achieve this dynamic reactivity and regulation through activity-dependent gene expression mechanisms, where the precise temporal patterns of neuronal activity lead to consistent changes in protein expression, dendritic growth, and synapse strength.

Intracellular calcium signaling through voltage gated calcium channels, as well as NMDA receptors, is a key neuronal signal used to drive gene expression changes (West et al., 2001; Moosmang, 2005). Neurons are capable of reacting to changes in intracellular calcium concentration and calcium influx, and subsequently translating these signals into long-lasting changes to DNA accessibility, expression of key genes, or both. These changes often result in the addition or subtraction of dendritic spines, number of receptors at these spines, or global changes to the neuron itself (Hernandez and Abel, 2008).

The discovery that calcium influx through VGCCs or NMDA receptors into neurons resulted in reliable, fast, and predictable changes in gene expression in the 1980s led to the search for the secondary messengers responsible for transducing these calcium signals from synapses to the nucleus (Greenberg and Ziff, 1984). Several key calcium sensor proteins have since been identified in neurons, such as calmodulin (CaM), calcineurin, and Ca^{2+}/calmodulin

dependent protein kinase II (CaMKII), and Ras-mitogen-associted protein kinase (MAPK). In addition to regulating local changes in synapses, such as changes to glutamate receptor localization or concentration, synaptic mRNA translation, or modification of downstream proteins, these proteins also serve to activate signaling cascades that induce or repress the transcription of certain genes within the nucleus (Bito et al., 1996; Hardingham et al., 1997; Sheng et al., 1991; Xing et al., 1996; Holt and Schuman, 2013; Thomas and Huganir, 2004; Martin and Zukin 2006; Wayman et al., 2008).

The intermediate signaling effectors responsible for activity-dependent gene expression changes have been widely studied, and several constitutively expressed proteins that contribute to these changes, including cyclic adenosine monophosphate (cAMP)-responsive element binding protein (CREB), serum response factor (SRF), and myocyte enhancer factor 2 (MEF2), have been identified to play prominent roles (Aizawa et al., 2004, Chawla et al., 1998, Deisseroth et al., 1996, McKinsey et al., 2002, Shalizi et al., 2006, Rivera et al., 1993). Additionally, mutations in components of these signaling cascades often lead to debilitating neurological disorders (Hong et al., 2005, Mullins et al., 2016, Ebert and Greenberg, 2013). Therefore, the identification and further characterization of additional activity-dependent signaling intermediates could lead to better understanding of complex neurological disease and potential therapeutic development.

Previous studies into activity-dependent gene expression in neurons have been hampered by several experimental constraints, such as precisely measuring calcium influx over time or stimulating neurons in a physiologically relevant manner. However, several methodological advances have significantly furthered the field in recent decades. The development of fluorescent calcium sensors has allowed researchers to study protein dynamics and subsequent gene

expression changes in response to calcium signaling with high spatial and temporal precision. Additionally, more sophisticated methods of inducing neuronal activity, such as neurotransmitter uncaging or optogenetic stimulation, have revealed distinct activity-dependent gene expression profiles in the numerous neuronal cell types (Spiegel et al., 2014).

Considering the well documented involvement of the co-expressed "parent" calcium channels in activity-dependent gene expression, as well as the fact that the VGCC CTPs α1CCT and α1ACT are predicted to each have many Ca^{2+}-dependent calmodulin-binding domains within their coding sequences (Figure 13; Mruk et al., 2014), I hypothesized that α1CCT and α1ACT interacted with the calcium-sensor protein calmodulin, or others, and translocated to or from the nucleus in response to changes in intracellular calcium concentration, possibly through interaction with the calcium-binding protein calmodulin. In this chapter, I demonstrate that these two CTPs are indeed activity-dependent signal transducers, traversing to or from the nucleus, for α1CCT or α1ACT respectively, in response to calcium influx derived from extracellular glutamate uncaging in cortical neurons. Additionally, I demonstrate that this activity-dependent translocation is dependent on calcium influx through specific sources.

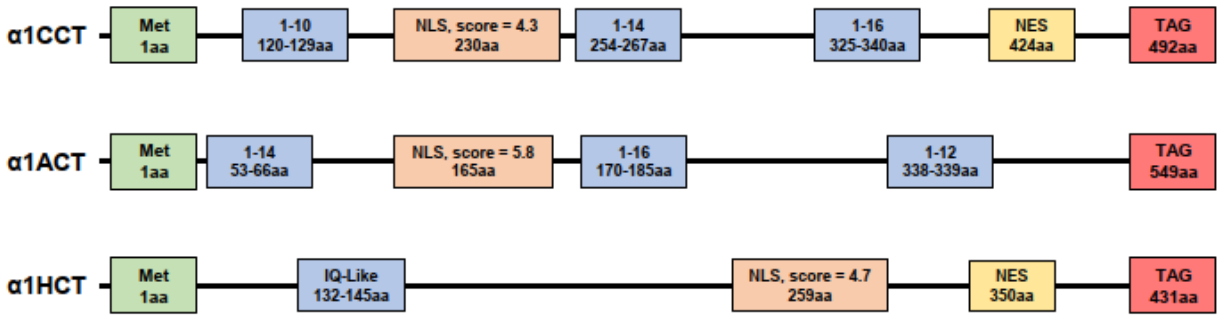

Figure 13. VGCC CTP calmodulin (CaM) binding domains, nuclear localization signals (NLSs), and nuclear export signals (NESs) as predicted by published motif predictor algorithms. NLS (orange), NES (yellow), or CaM binding domains (blue; Ca^{2+}-dependent: IQ-like; Ca^{2+}-independent: 1-10, 1-12, 1-14, 1-16) predicted within the α1CCT, α1ACT, and α1HCT

Figure 13, continued. amino-acid (aa) sequences (Kosugi et al., 2008; Kosugi et al., 2009; Mruk et al., 2014; La Cour et al., 2004). Predicted start sites of each CTP were determined through mass spectrometry analysis and cDNA mutagenesis confirmation.

Materials and Methods:

Fixed Cell Imaging:

Primary cortical neurons were harvested from E18 rat embryos and plated on Poly-D-Lysine (PDL) coated coverslips (50 ug/mL) at a density of $5x10^4$ cells/cm^2 in Neurobasal medium + B27 supplement (GIBCO, Waltham, MA, USA). Neurons were allowed to mature for 15 days. At days in vitro (DIV) 15, neurons were transfected with α1ACT, α1CCT, or α1HCT (1 ug/well) using Lipofectamine Messenger Max (Thermo Fisher Scientific, Waltham, MA, USA). Approximately 12-16 hours following the mRNA transfection, neurons were either left untreated or treated with 20 mM K^+,100 μM glutamate, or 10 μM A23187 (Sigma, St. Louis, MO, USA) for 5 minutes, with or without BAPTA-AM (5 μM) (Thermo Fisher Scientific, Waltham, MA, USA). The treatments were then washed out and neurons were incubated for an additional 10 minutes. Neurons were then fixed in 4% (vol/vol) paraformaldehyde + 4% sucrose (wt/vol) in PBS for 10 minutes at room temperature (RT). The paraformaldehyde/sucrose solution was aspirated and neurons were incubated in 0.1% Triton-100 in PBS for 10 minutes. Neurons were then washed once with PBS and blocked for 1 hour at RT in 5% BSA in PBS. Neurons were then incubated overnight at 4^o in primary antibody diluted in 1% BSA in PBS. Primary antibodies used were Anti-MAP2 (Sigma, St. Louis, MO, USA) or Anti-vGlut1 (Sigma, St. Louis, MO, USA), and Anti-FLAG 1804 (Sigma, St. Louis, MO, USA), all at a 1:500 dilution. The following day, the primary antibody was aspirated and the neurons were washed three times with PBS. Neurons were then incubated in the dark for one hour in secondary antibody (AlexaFluor594

Anti-Mouse and AlexaFluor488 Anti-Rabbit; Thermo Fisher, Waltham, MA, USA) at RT. The coverslips were washed three more times with PBS then mounted on slides with mounting media containing DAPI.

Live Cell Imaging:

Primary cortical neurons were harvested from E18 rat embryos and plated on Poly-D-Lysine (PDL) coated 35 mm live cell imaging dishes. Neurons were matured for 15 days in vitro before being imaged. The day prior to imaging, neurons were infected with Emerald GFP-tagged α1ACT, α1CCT, or α1HCT-expressing AAV9 virus (Vectorbuilder, Chicago, IL, USA). On the day of imaging, the glutamate cage MNI-L-glutamate (20 uM) (Tocris Bioscience, Bristol, UK), the calcium indicator Rhod-2 am (5 uM) (Cayman Chemical, Ann Arbor, MI, USA), and the live-cell nuclear stain Draq5 (5 uM) (Thermo Fisher Scientific, Waltham, MA, USA) were added to the neurons 15 minutes prior to imaging. Neurons were imaged in NB+B27 media with 0.001 mM TTX added, to minimize spontaneous firing and action potentials. Neurons were imaged on a 3i Marianas spinning disk confocal microscope. Neurons were visualized for ten minutes following uncaging with 405 nm UV light (two pulses of 10 ms each), and the Emerald GFP fluorescent change within the nucleus of neurons was quantified using the 3i Slidebook software.

For FRAP experiments, neurons were infected with AAV9 virus as above then loaded with Draq5. A 100 ms pulse of blue light was used to bleach a ROI within the nucleus of observed neurons. Emerald GFP recovery was quantified using 3i Slidebook software.

Immunoprecipitation:

HEK293 cells were transiently transfected with α1CCT-3xFLAG, α1ACT-3xFLAG, α1HCT-3xFLAG, or empty pcDNA3 cDNA vectors using Lipofectamine 2000. Cells were lysed in

passive lysis buffer (Cell Signaling, Danvers, MA, USA) then incubated with Anti-Calmodulin antibody (Santa Cruz Biotechnology, Dallas, TX, USA) and Protein A/G beads (Pierce Protein Thermo Scientific, Waltham, MA, USA) overnight at 4^{o}C with gentle rotation. The next day, the beads were washed and bound protein was eluted per manufacturer's instructions. Eluted protein was analyzed via Western Blot probing with Anti-FLAG 1804 primary antibody (Sigma, St. Louis, MO, USA).

Statistical Analysis:

Statistical analyses were conducted using GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA), RStudio (Boston, MA), and SPSS (IBM, Armonk, NY). For single comparisons, Student's t test was used to establish statistical significance. For multiple sample comparisons, One-Way ANOVA was performed, followed by Tukey post hoc tests. Data were expressed as the mean ± SEM and written with the identification of n under appropriate figure legends, with *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$.

Results:

α1CCT, α1ACT, and α1HCT have significantly lower mobile fractions than EmGFP as measured by FRAP

Fluorescence recovery after photobleaching (FRAP) analysis a powerful microscopy tool that can measure the mobility and DNA binding activity of transcription factors (Govindaraj et al., 2019). As α1CCT, α1ACT, and α1HCT were shown to function as DNA-binding transcription factors, I hypothesized that they would have a significantly lower mobile pool available for active nuclear transport compared to EmGFP control, or that their nuclear

fluorescence would recover more slowly than that of the EmGFP control following photobleaching. Primary neurons were infected with Em-GFP, α1CCT-EmGFP, α1ACT-EmGFP, or α1HCT-EmGFP expressing AAV9 virus driven by the EF1α promoter at 10 days *in vitro* (DIV) and imaged at DIV 15-17. I used a brief (100 m), high intensity pulse of light to bleach the nucleus of imaged cells, and measured recovery of fluorescence signal was measured for ten minutes post-bleach. As expected, α1CCT-EmGFP, α1ACT-EmGFP, and α1HCT-EmGFP all had significantly slower recovery times than EmGFP alone (Figure 14A). Additionally, quantification of the mobile fraction (MF) for each condition revealed significantly lower MFs for α1CCT-EmGFP, α1ACT-EmGFP, and α1HCT-EmGFP compared to EmGFP.

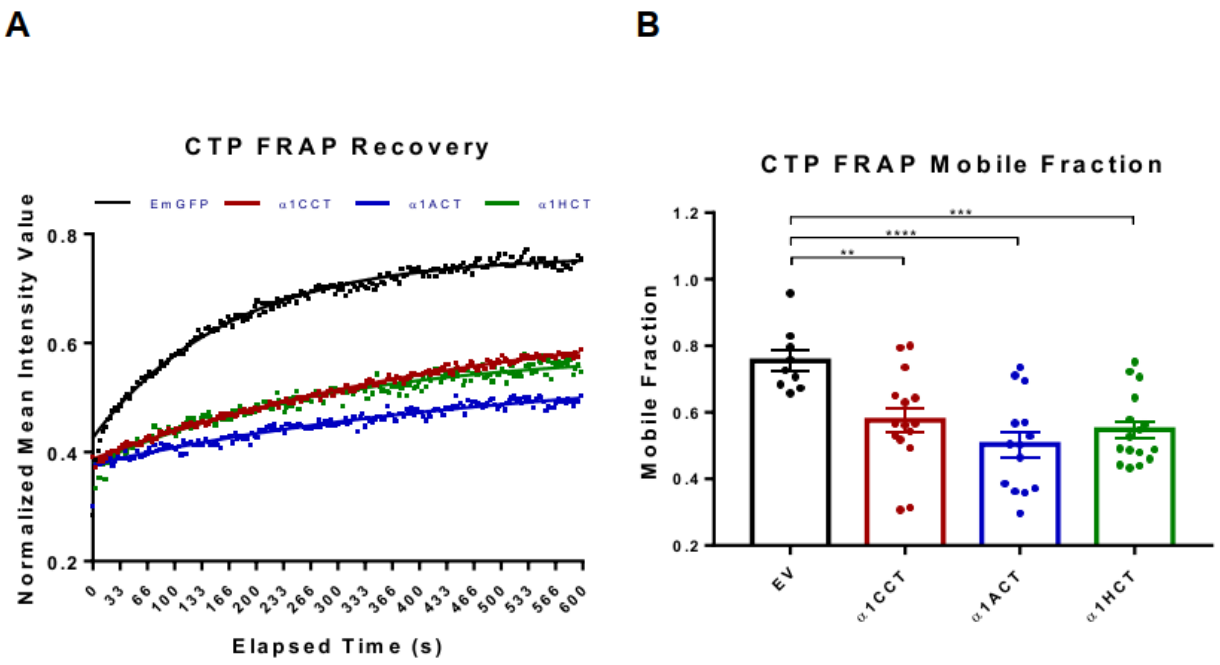

Figure 14. FRAP analysis reveals that α1CCT, α1ACT, and α1HCT have significantly lower mobile fractions than EmGFP. (A) FRAP analysis was performed on cultured rat cortical neurons expressing EmGFP, α1CCT-EmGFP, α1ACT-EmGFP, or α1HCT-EmGFP. The recovery profiles represent the nonlinear fit to average of individually photobleached cells. (N > 10 cells for each condition). (B) Mobile fractions for EmGFP, α1CCT-EmGFP, α1ACT-EmGFP, or α1HCT-EmGFP. (N > 10 cells for each condition, *p<0.05, **p<0.01, ***p<0.001).

Nuclear translocation of α1CCT and α1ACT is coupled to neuronal calcium signaling in cultured cortical neurons

Because α1CCT, α1ACT, and α1HCT are co-expressed with full-length VGCC subunit proteins, we hypothesized that calcium signaling would regulate the subcellular localization of these CTPs in an activity-dependent manner. To test this hypothesis, I first used fixed-cell immunocytochemistry to observe the localization of VGCC CTPs in mature rat cortical neurons. I transfected primary neurons with FLAG-tagged VGCC CTP mRNAs in culture and manipulated intracellular calcium concentrations by standard treatments. Distinct cultures of transfected neurons were treated with 20 mM potassium, 100 μM glutamate, or 5 μM of the calcium ionophore A23187 in the presence and absence of the calcium chelator, BAPTA-AM (40 μM), for five minutes followed by fixation and immunolabeling with an anti-FLAG antibody.

In neurons treated with high potassium, glutamate, or A23187, the nuclear-to-cytoplasmic (N-C) ratio of 3xFLAG tagged α1CCT was significantly higher when compared to the NTC ratio of the untreated neuronal control (Figure 15B,D,F). Additionally, neurons co-treated with either K^+/glutamate/A23187 and BAPTA-AM exhibited similar N-C ratios to the untreated control, indicating that this subcellular translocation in response to activity-mimicking stimuli is calcium dependent. For α1ACT, activity-mimicking stimuli had the opposite effect on subcellular localization, as α1ACT-transfected neurons treated with K^+/glutamate/A23187 all exhibited significantly lower N-C ratios compared with the untreated control. As with α1CCT, this effect was reversed when neurons were co-treated with BAPTA-AM. Interestingly, none of these stimuli had any significant effect on the subcellular localization of α1HCT, indicating that

the localization and translocation of the *CACNA1H*-derived CTP may be independent of calcium signaling.

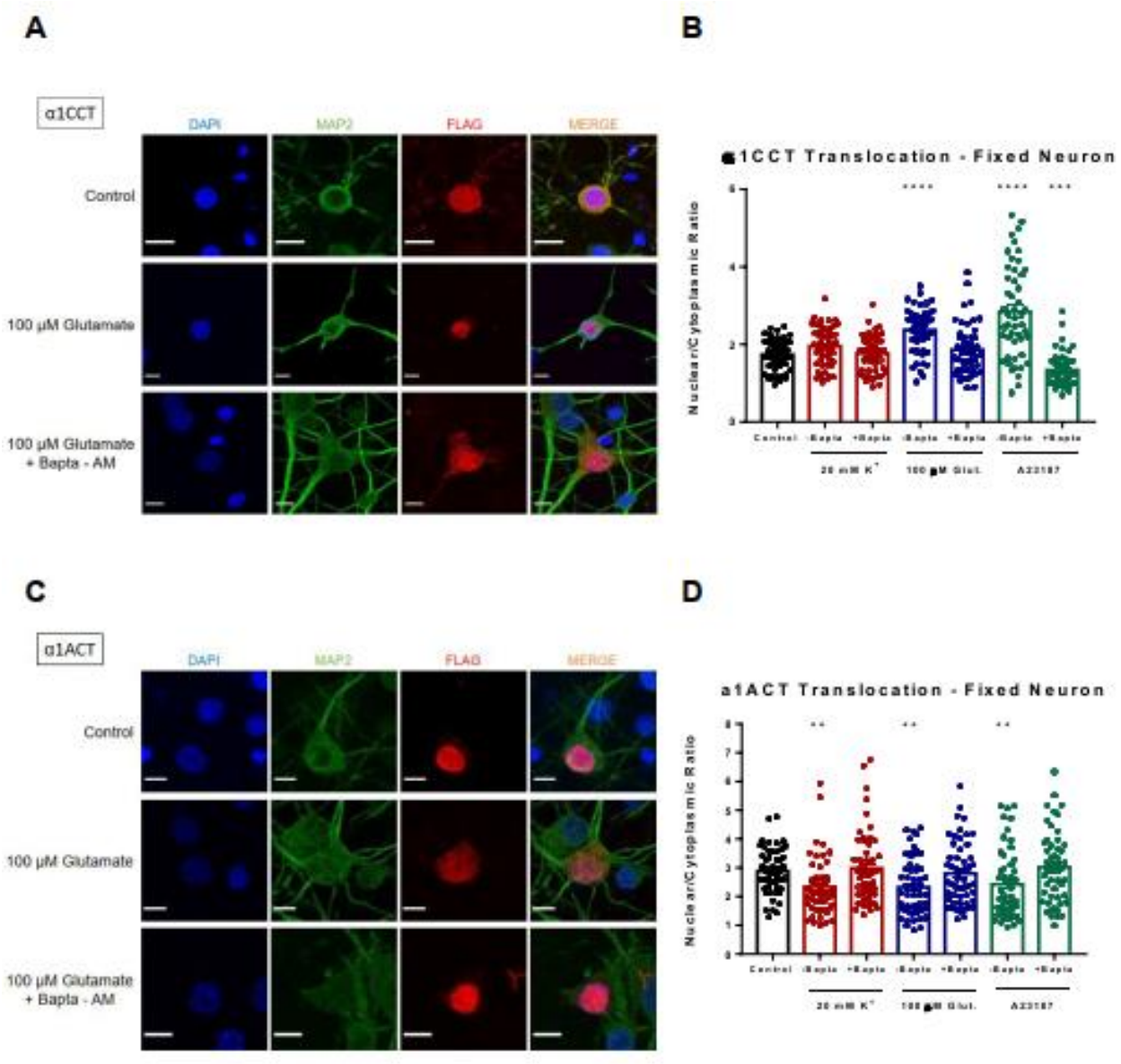

Figure 15. α1CCT and α1ACT translocate to or from the nucleus in fixed rat cortical neurons following global depolarizing stimuli, while α1HCT does not. (A,C,E) Representative images of fixed rat cortical neurons transfected with α1CCT, α1ACT, or α1HCT mRNA. Fixed neurons were stained with DAPI (blue), MAP2 (green), and Flag (red) for control, 100 μM glutamate without BAPTA-AM, and 100 μM glutamate with BAPTA-AM conditions.

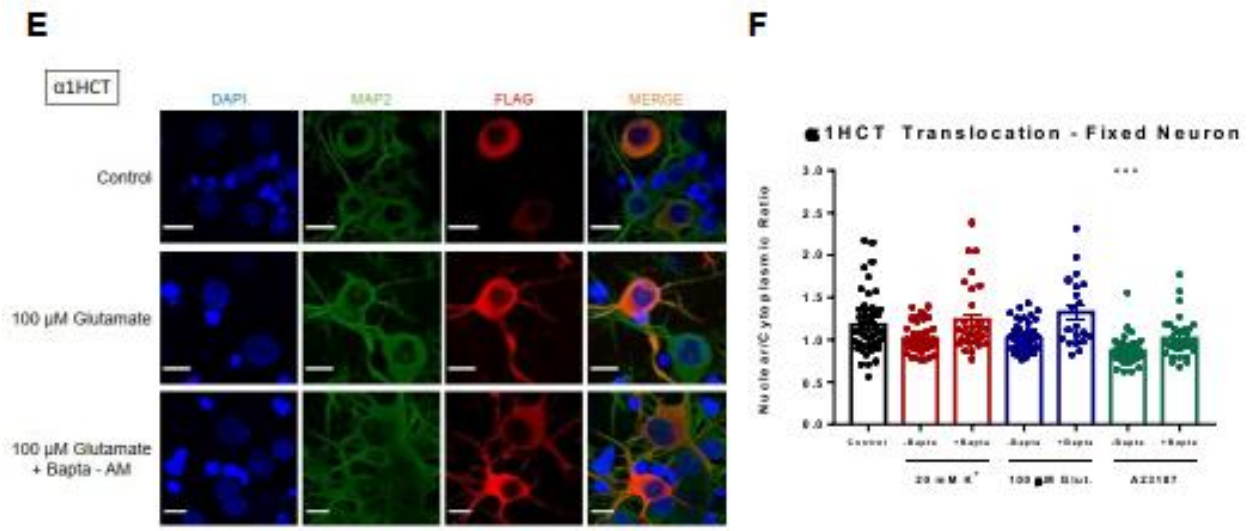

Figure 15, continued. Scale bars = 10 microns. (B,D,F) Quantification of nuclear/cytosolic fluorescence signal of rat cortical neurons transfected with α1CCT (B), α1ACT (D), or α1HCT (F) mRNA. Neurons were treated with either 20 mM K^+, 100 μM glutamate, or the calcium ionophone A23187 with or without a s5-minute pretreatment of BAPTA-AM. (N > 50 cells for each condition, *p<0.05, **p<0.01, ***p<0.001).

To observe the kinetics of α1CCT and α1ACT translocation in real-time, we replaced the C-terminal 3xFLAG tag with Emerald GFP (EmGFP) and followed the localization using live-cell confocal microscopy. Primary neurons were infected with α1CCT-EmGFP, α1ACT-EmGFP, or α1HCT-EmGFP expressing AAV9 virus driven by the EF1α promoter at 10 days *in vitro* (DIV). Five days post infection, I loaded neurons with MNI-L-glutamate, a form of caged glutamate, along with the calcium indicator Rhod2-AM and the live-cell nuclear stain DRAQ5. Uncaging of MNI-L-glutamate extracellularly resulted in reliable calcium spikes within a subset of neurons, which also propagated to the nucleus. In agreement with the fixed-cell imaging results, glutamate-induced calcium spikes induced rapid α1CCT nuclear entry and α1ACT nuclear exit (Figure 16B-D). Additionally, while α1HCT showed no significant movement, it tended to enter the nucleus at a slow rate following intracellular calcium spikes. (Figure 16C).

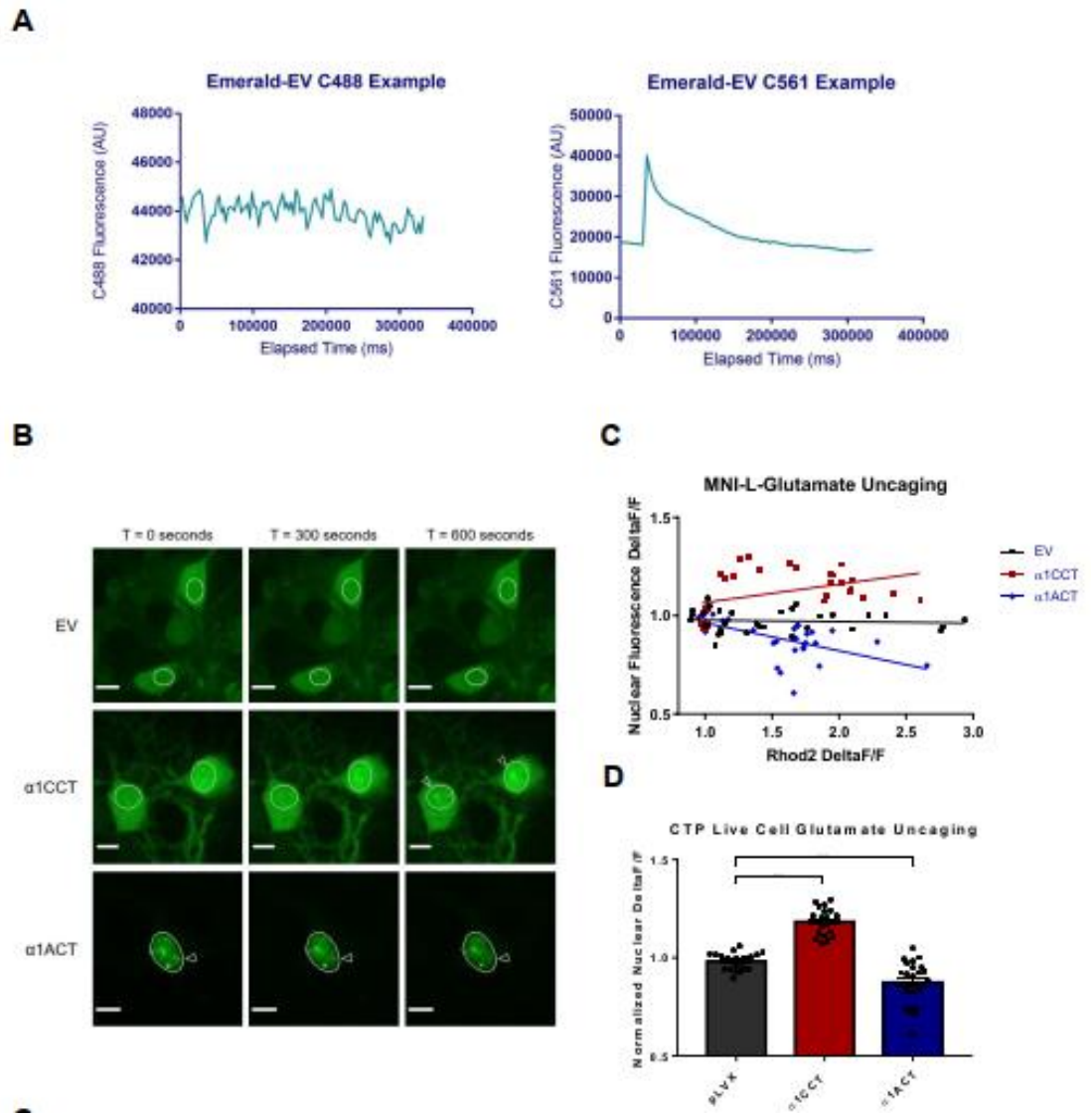

Figure 16. Nuclear translocation of α1CCT and α1ACT is coupled to neuronal calcium signaling in cultured cortical neurons. (A) Example GFP (488 nm – left panel) and Rhod2-AM (561 nm – right panel) live cell traces. Glutamate uncaging occurred at T = 15s with two 10ms pulses of 405 nm light. Images were collected every 3s for 10 minutes total. (B) Representative live-cell images of rat cortical neurons infected with AAV9-EmGFP, AAV9- α1CCT-EmGFP, or AAV9- α1ACT-EmGFP virus at T = 0 seconds, T = 300 seconds, or T = 600 seconds post-glutamate uncaging. Circles denote the nucleus; arrows denote nuclear speckle formation (α1CCT) or dissipation (α1ACT). Scale bars = 10 microns. (C) Graph depicting intracellular

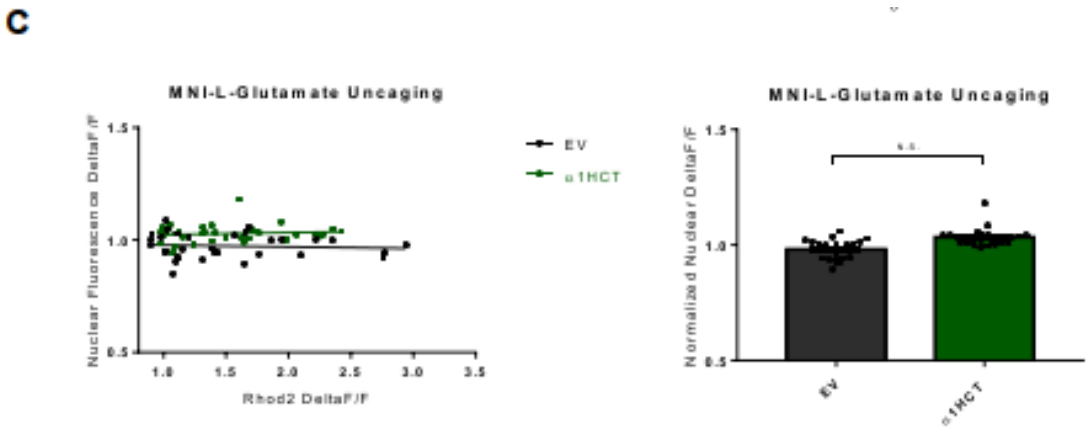

Figure 16, continued. calcium spike (as measured by Rhod2 DeltaF/F) versus neuronal nuclear fluorescence change at T = 600s (DeltaF/F) for EmGFP, α1CCT, and α1ACT infected neurons (N > 30 cells for each condition). (D) Normalized nuclear fluorescence DeltaF/F for EmGFP, α1CCT, and α1ACT infected neurons at T=600s post-glutamate uncaging (N > 30 cells for each condition, *p<0.05, **p<0.01, ***p<0.001).

Calcium signaling through L-type VGCCs and NMDA receptors is necessary for proper α1CCT and α1ACT nuclear translocation

Calcium influx through VGCCs is crucial for several cellular mechanisms, including proper nuclear signaling. To test whether the source of the elevated internal calcium was important for proper CTP translocation, we replicated glutamate uncaging in the presence of various antagonists to sources of extracellular and intracellular calcium release. To determine if calcium influx through CaV1.2, CaV2.1, or CaV3.2 was necessary for subsequent translocation of α1CCT or α1ACT, we loaded neurons infected with α1CCT-EmGFP or α1ACT-EmGFP with blockers specific for each VGCC subtype and subsequently stimulated these neurons through MNI-L-glutamate uncaging. α1ACT nuclear exit was unperturbed in the presence of either nifedipine (CaV1.2 antagonist), ω-agatoxin (CaV2.1 antagonist), or TTA-A2 (CaV3.2 antagonist) (not shown). α1CCT nuclear import was unaffected following CaV2.1 or CaV3.2 blockade, however, when cells were loaded with the CaV1.2 blocker nifedipine, glutamate-

induced calcium spikes elicited no α1CCT translocation, indicating that calcium signaling through L-type VGCCs is crucial for proper CTP nuclear translocation (Figure 17B-C).

To identify the source of calcium responsible for α1ACT nuclear export, I used several additional antagonists in tandem with glutamate uncaging. To inhibit store-operated calcium entry mechanisms, I loaded α1ACT-EmGFP transfected neurons with both the IP3 antagonist 2-APB (Tocris) and ryanodine (Tocris), and glutamate-induced action potentials were elicited. Additionally, I loaded α1ACT-EmGFP transfected neurons with the NMDA blocker AP5. α1ACT nuclear export was unperturbed following antagonism of intracellular calcium store release via 2-APB or ryanodine (not shown), however was significantly lessened following AP5-mediated NMDA antagonism (Figure 17D-E).

Calmodulin is a crucial neuronal calcium binding protein (CBP), facilitating the nuclear translocation and activation of target proteins following calcium influx through L-type VGCCs (Dolmetsch et al., 2001; Sakagami et al., 2005; Deisseroth et al., 1998). As such, we hypothesized that calmodulin could be the downstream calcium sensor responsible for proper α1CCT or α1ACT nuclear transport. To test this idea, I transfected neurons with CTP-EmGFP AAV9 virus and loaded them with the membrane-permeable calmodulin blocker W7 (Tocris). Blockade of calmodulin followed by glutamate uncaging in living neurons resulted in significantly decreased nuclear translocation of α1CCT and α1ACT (Figure 17B-16E). However, this effect is less than that seen in the nifedipine or AP5 treatment conditions, possibly indicating incomplete antagonism of calmodulin or redundant pathways of translocation.

A

Treatment	Concentration	Target	Type
Nifedipine	10 µM	L-Type Calcium Channels	Antagonist
ω-Agatoxin	500 nM	CaV2.1 Calcium Channel	Antagonist
TTA-A2	100 µM	T-Type Calcium Channels	Antagonist
2-APB	50 µM	Ip3 Receptors	Antagonist
Ryanodine	100 µM	Ryanodine Receptors	Antagonist
AP5	100 µM	NMDA Channels	Antagonist
w7 Hydrochloride	100 µM	Calmodulin	Antagonist

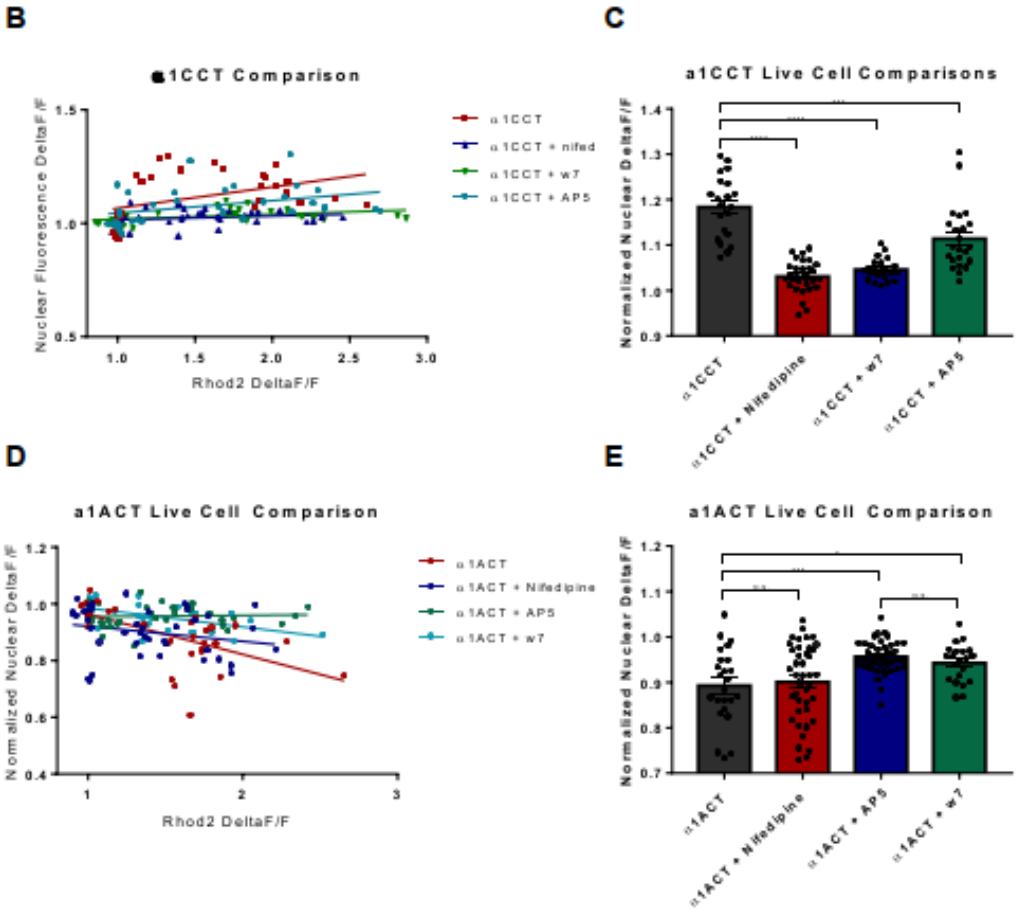

Figure 17. Nuclear translocation of α1CCT and α1ACT is coupled to calcium signaling through L-type VGCCs or NMDA receptors respectively. (A) Table listing the calcium-channel antagonists used in conjunction with live-neuron glutamate uncaging. (B,D) Graphs showing the comparison of α1CCT (B) or α1ACCT (D) nuclear translocation following glutamate uncaging in the presence of L-type calcium channel antagonist (nifedipine), NMDA receptor antagonist (AP5), or calmodulin antagonist (w7 hydrochloride). (N > 30 cells for each condition) (C,E) Quantification of normalized nuclear DeltaF/F at T = 600 seconds for α1CCT treatment conditions (C) and α1ACT treatment conditions (E). (N > 30 cells for each condition, *p<0.05, **p<0.01, ***p<0.001).

α1CCT and α1ACT bind to calmodulin while α1HCT does not

CaV1.2 and CaV2.1 have been shown previously to contain calmodulin-binding domains in their intracellular C-terminal regions (Figure 13; Erickson et al., 2001; Zuhlke et al., 2000; Pitt et al., 2001). To examine whether the *CACNA1C* and *CACAN1A* CTPs α1CCT and α1ACT bound to calmodulin as well, we transfected HEK293T cells with either α1CCT-3xFLAG, α1ACT-3xFLAG, or α1HCT-3xFLAG. Cell lysates, including a mock transfected negative control, were collected and subjected to overnight immunoprecipitation (IP) using an anti-calmodulin antibody. IP'd samples were run on a western blot and probed using an anti-3xFLAG primary antibody. α1CCT and α1ACT bind to calmodulin as predicted, while α1HCT did not (Figure 18).

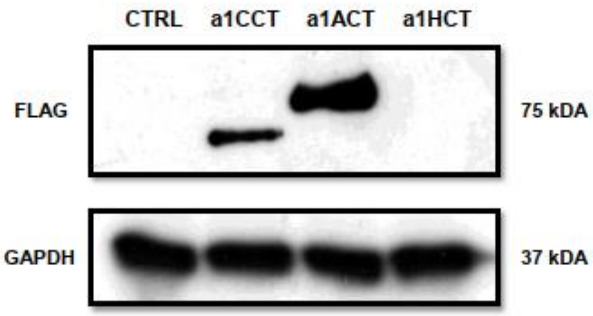

Figure 18. α1CCT and α1ACT bind to calmodulin while α1HCT does not. Western blot analysis of lysates collected from HEK293 cells transfected with either α1CCT-3xFLAG, α1ACT-3xFLAG, α1HCT-3xFLAG, or pcDNA3.1 empty vector, pulled down with anti-calmodulin primary antibody and probed with anti-FLAG antibody.

Discussion:

Activity-dependent gene expression is an important mechanism of gene regulation that is critical for proper neuronal response to salient external and internal stimuli. Among the crucial neuronal processes governed by activity-dependent gene regulation are nervous system plasticity,

mechanism of learning and memory, and proper pain signaling (Fields, 2020). Numerous neurological disorders, especially those affecting cognitive function, arise from altered activity-dependent gene expression, however our understanding of the underlying mechanistic changes mediating such dysfunction, as well as many of the molecular substrates involved, is still evolving (Flavell and Greenberg, 2008). While activity-dependent modulation of key neuronal genes was discovered more than four decades ago, we are just beginning to understand how discrete modulation of activity-dependent genes instruct changes at the behavioral level.

Our lab previously demonstrated that the *CACNA1A* secondary protein product α1ACT functions as a critical transcription factor during cerebellar development. Additionally, a pathogenic polyglutamine expansion within the α1ACT coding region results in altered transcription factor activity and causes spinocerebellar ataxia type 6 (SCA6) (Du et al., 2013; Du et al., 2019). In this chapter, I demonstrate that both α1ACT and the *CACNA1C* secondary protein α1CCT exhibit activity-dependent nuclear translocation, traveling out or into the nucleus following intracellular calcium spikes. Furthermore, calcium influx through L-type calcium channels or NMDA receptors is crucial for proper α1CCT or α1ACT translocation, respectively, as bath application of the L-type calcium channel antagonist or the NMDA receptor antagonist AP5 was able to abolished CTP nuclear import or export. These results reveal a coordinated form of gene regulation utilized by the VGCC genes, as co-translated secondary transcription factors and their subcellular localization are modulated by calcium influx through the parent VGCC channel for α1CCT or NMDA channels for α1ACT.

While these results expand our knowledge of the complex mechanisms employed by neurons to modify gene expression in response to external stimuli, several future studies are needed to truly identify the functional significance of them. A key future direction is directly

demonstrating that α1CCT and α1ACT nuclear translocation has downstream effects on modulating gene expression and behavior in conditional knockout mice. Furthermore, while cytosolic increases in calcium concentration are often accompanied by nuclear increases, studies have demonstrated that cytosolic and nuclear calcium concentration increases are able to activate distinct signaling pathways, and as such determining the role, if any, subcellular calcium concentration plays in α1CCT and α1ACT nuclear translocation is important (Hardingham et al., 1997; Segal and Manor, 1992; Birch et al., 1992; Al-Mohanna et al., 1994). Additionally, the secondary effectors mediating CTP translocation need to be elucidated. To this end, one promising candidate is calmodulin, which has been shown to mediate activity-dependent translocation mechanisms for a wide variety of neuronal transcription factors and binds to α1CCT and α1ACT, but not α1HCT (Figure 18) (Cohen et al., 2018, Bading et al., 1993, Murray et al., 2003). Finally, recent advances in next-generation single-cell sequencing as well as gene editing will allow for thorough characterization of how different physiological states and neuronal sub-types contribute to the fine-tuning of gene expression via activity-dependent mechanisms.

General Discussion:

Neurons are a remarkably diverse set of cells that have myriad specialized functions. Among these, the process of reacting to and encoding environmental stimuli through short- and long-term changes in gene expression is vitally important, and neurons have developed astonishingly precise spatial and temporal mechanisms to do so. One such mechanism harnesses the temporal patterns of membrane depolarization, induced by EPSPs or action potentials, and converts them into discrete modulatory signals. To achieve this dynamic regulation, neurons employ a vast array of activity-dependent calcium channels, signaling proteins, and cascades. While several of these signaling intermediates and downstream transcriptional regulators have been identified in neurons, such as those relying on calmodulin and CaM kinases, CREB, and Npas4, vast gaps in our knowledge of these networks remain (Lin et al., 2014, Bito et al., 1996, Sheng et al., 1991). Additionally, numerous neurological disorders, especially neuropsychiatric disorders, are characterized by mutations in these signaling proteins or an overall excitatory – inhibitory signaling imbalance in populations of neurons (Sohal and Rubenstein, 2019). Understanding the direct effects these mutations may have on gene expression, as well as the normal and pathological functions of the signaling proteins they may affect, is crucial for the development of effective therapies.

This dissertation seeks to carry forward previous work done in our lab that identified the protein α1ACT and its function in Purkinje cell development. We demonstrated that α1ACT is a transcription factor (TF) produced through cap-independent, non-canonical translation via a cryptic internal ribosome entry site (IRES) within the primary open reading frame (ORF) of the voltage-gated calcium channel (VGCC) gene *CACNA1A* (Du et al., 2013, Du et al., 2019). Following up on those findings, I demonstrated that two additional VGCC genes, *CACNA1C* and

CACNA1H, produce similar C-terminal proteins, α1CCT and α1HCT respectively, that function as TFs.

The VGCC genes CACNA1C and CACNA1H produce secondary proteins through a bicistronic, cap-independent mechanism

Chapter in Review: In Chapter 2, I demonstrated that the VGCC genes *CACNA1C* and *CACNA1H*, in addition to *CACNA1A*, produce secondary, C-terminal proteins via a cap-independent mechanism. Using cDNA mutagenesis experiments, I was able to show that these CTPs, α1CCT and α1HCT respectively, were produced independently of the primary α1 calcium channel subunits of each gene, and that this CTP expression persisted following the insertion of upstream termination codons that abolished α1 subunit expression or upstream frameshift mutations that rendered the C-terminal 3xFLAG tags on the α1 subunits unrecognizable to antibody detection via Western Blot analysis. Additionally, through *in vitro* transcription experiments, I excluded the possibility that these secondary CTPs were produced via alternative splicing, a cryptic promoter in the underlying DNA sequence, or ribosomal skipping.

Significance and Future Directions: While viral IRESs have long been recognized to cap-independently translate proteins from internal regions of mRNA, more recently identified cellular IRESs are less characterized and less understood. In fact, several prominent geneticists of the last century, including Marilyn Kozak (discovered the Kozak sequence of translation initiation), have cast doubt on the existence of cellular IRESs altogether (Kozak 2001, Kozak 2005). As such, stringent and rigorous criteria must be met to confidently identify IRES

structures in cellular genomes. Such criteria include the exclusion of other alternative translation mechanisms, as well as reliable assays testing for IRES-like translational initiation within mRNA segments.

The growing number of annotated cellular IRESs and the acceptance that the products of IRES-mediated translation in eukaryotic cells may play more diverse roles than simply mediating cellular stress response opens several potential therapeutic avenues for complex neurological disorders. As shown with the *CACNA1A* CTP α1ACT by our lab, disorders previously thought to be attributed to the primary protein of a gene may in fact be partially or fully mediated by dysfunction of potential secondary, IRES-mediated protein products instead (Du et al., 2019). Further, our lab has demonstrated that the *CACNA1A* cellular IRES can be selectively silenced by the micro RNA miR-3191, and that miRNA silencing of the *CACNA1A* IRES translating a pathogenic α1ACT ($\alpha1ACT_{SCA6}$) fragment that leads to SCA6 results in decreased Purkinje cell degeneration and subsequent ataxia and motor deficits caused by $\alpha1ACT_{SCA6}$. Importantly, miRNA silencing of the α1ACT IRES does not lead to decreased *CACNA1A* mRNA or α1A VGCC subunit (Miyazaki et al., 2016).

Several *CACNA1A*-, *CACNA1C*-, and *CACNA1H*-related disorders have been shown to be associated with mutations within the C-terminal region of each gene, and it is an intriguing possibility to hypothesize that some of these mutations may convey a pathological phenotype at least partially through dysfunction of the secondary proteins α1CCT or α1HCT respectively (Zamponi, 2015). As more cellular IRESs discovered and the functions of their secondary proteins elucidated, such IRESs could become viable therapeutic targets for selective silencing treatments such as miRNA-3191. Already, putative IRESs have been discovered in genes with important neuronal roles such as TCP-BP, NOTCH2 – ICD (intracellular domain), and FGF2

(Hui et al., 2009, Lauring and Overbaugh, 2000, Creancier et al., 2000). One common and complicating feature of cellular IRESs is the cell-type specific expression of ITAFs, and the requirement of specific ITAFs for robust IRES-mediated translation. While some viral ITAFs have been shown to play prominent roles in cellular IRES-mediated translation, such as PTB, several others have shown to play little to no role (Romanelli et al., 2013). Therefore, identifying not only potential cellular IRES structures within mRNAs, but also the necessary ITAFs needed to regulate IRES expression and their cell-type specific expression patterns, is crucial for development of potential therapies targeting those IRESs.

α1CCT and α1HCT bind to DNA and alter expression of key genes related to neuronal development and synapse formation in hNPCs

Chapter in Review: As the *CACNA1A* CTP α1ACT was previously shown by our lab to induce neurite outgrowth and function as a transcription factor in rat phaeochromocytoma (PC12) cells, I hypothesized that the *CACNA1C* and *CACNA1H* CTPs α1CCT and α1HCT, respectively, had similar transcriptional regulation capabilities. In this chapter, I show that α1CCT and α1HCT are able to induce neurite outgrowth in hNPCs, similar to α1ACT. Additionally, I performed next-generation RNA-sequencing (RNA-seq) and chromatin immunoprecipitation with next-generation sequencing (ChIP-seq) to demonstrate that α1CCT and α1HCT both bind to DNA in hNPCs to influence the expression of a diverse set of genes related to neuronal differentiation, neuronal maintenance, and synapse formation. Additionally, I validated the sequencing findings looking at a subset of the genes regulated by α1CCT, α1ACT, and α1HCT in hNPCs, as well as some of the α1CCT regulated genes in CaV1.2 cKO mouse neuronal tissue.

Significance and Future Directions: Terminal neuronal fate is controlled by a vast network of transcription factors acting during precisely stereotyped temporal and spatial windows. The discovery that two prominent neuronal VGCC genes, *CACNA1C* and *CACNA1H,* produce secondary proteins that function as TFs in a neuronal context to promote neural differentiation and maturation is a crucial first step in potentially elucidating the mechanisms through which complex genetic disorders related to these genes exert their pathologic effects. The C-terminal proteins (CTPs) produced by *CACNA1C* and *CACNA1H*, α1CCT and α1HCT respectively, are DNA-binding transcriptional regulators that have significant roles within the developing and mature nervous system, helping to regulate neurogenesis, neuron differentiation, neuronal maturation, proper axonal guidance, and synapse formation (Figure 9). Therefore, logically it follows that mutations in either these TFs, regulatory regions they bind to, cofactors, chromatin regulators, and/or noncoding RNAs that interact with these regions could have wide ranging phenotypic effects across the CNS.

Identifying specifically when α1CCT, α1ACT, and α1HCT are expressed in the developing and mature CNS, as well as the specific sub-types of neurons they are present in, is a critical future direction of this project. As this project was carried out in human neural progenitor cells, which are a type of pluripotent stem cell that can differentiate into any neuron or glial sub-type, probing the role each CTP plays in downstream cell types is important. For example, many mental illnesses are characterized by an excitatory-inhibitory activity imbalance and understanding how each of these CTPs may function differently in excitatory and inhibitory neurons could lead to a better understanding of such disorders. Additionally, several VGCC genes have been shown to be expressed in various glial cell types, including astrocytes and

oligodendrocytes, and the potential role of the VGCC CTPs in these non-excitable cell types cannot be discounted (Akopian et al., 1996; Haberlandt et al., 2011). One immediate experiment that could be performed is to differentiate hNPC cell lines stably expressing each CTP into various types of neurons or glia, as the differentiation protocols for each cell type have been thoroughly characterized and streamlined.

α1CCT and α1ACT are activity-dependent transcription factors regulated by calcium influx through specific sources

Chapter in Review: In this chapter, I used fixed-cell and live-cell glutamate uncaging experiments in cultured rat cortical neurons to reveal the activity-dependent nuclear translocation of α1CCT and α1ACT. Further pharmacological modifications to this experiment demonstrated that nuclear import and export of α1CCT and α1ACT are regulated by calcium influx through L-type calcium channels and NMDA receptors, respectively. Finally, I provide a future avenue of study by showing that both α1CCT and α1ACT bind to the calcium-sensor protein calmodulin, while α1HCT does not.

Significance and Future Directions: Since the first reports of activity-dependent transcriptional regulation in neurons in the 1980s, significant progress has been made in uncovering both the specific effectors responsible for transducing neuronal signals into gene expression changes and the downstream effects these effectors exert (Greenberg et al., 1986; Morgan and Curran, 1986). Nonetheless, as our understanding of the complex networks neurons use to post-mitotically modulate gene expression profiles in response to external environmental stimuli expands, it is

clear that many gaps remain in our knowledge. The results presented in this dissertation, namely that several VGCC genes produce secondary proteins that function as activity-dependent transcription factors, lead to several intriguing possibilities, especially in relation to disease pathology.

The proper functioning of activity-dependent transcription factors requires precise temporal and spatial signals across a vast number of intra- and intercellular networks, and perturbations in even a small fraction of such a network can have wide ranging ripple effects leading to pathology. One of the first genes identified to be under neuronal activity-dependent transcriptional control was Brain-derived neurotrophic factor (BDNF) (Lu, 2003). Studies into BDNF transcription revealed a complicated regulation scheme, as transcription of BDNF was shown to be controlled in a developmental, tissue-specific, and activity-dependent manner (Aid et al., 2007). Several activity-dependent transcription factors, including CREB, USF, CaRF, and MEF2, were identified to transduce calcium signals into altered BDNF transcription profiles (Greer and Greenberg 2008). Further studies into BDNF transcription revealed differential function in excitatory and inhibitory neurons, suggesting that the activity-dependent regulation of BDNF could play a larger role in modulating the excitatory-inhibitory balance in cortical networks (Kohara et al., 2007; Genoud et al., 2004).

Research into BDNF transcriptional regulation and function has illustrated the complexity of neuronal activity-dependent gene expression. The discovery that α1CCT and α1ACT translocate to and from the nucleus in an activity-dependent manner is an important first step, however future research must be done to determine if this translocation has any significant effect on downstream neuronal gene expression. To address this question, ChIP-seq and/or ATAC-seq experiments performed after bulk depolarizing stimuli, such as bath application of K^+

or glutamate, could be particularly helpful. Additionally, further studies on the exact mechanism of α1CCT and α1ACT translocation are necessary. While I identified calcium influx through L-type calcium channels or NMDA receptors as crucial for proper α1CCT or α1ACT translocation respectively, the immediate downstream mechanisms and signal transducers remain unknown. A promising avenue of further research could be looking at calmodulin's role in this translocation, as I show through immunoprecipitation followed by western blotting (Figure 18) that both α1CCT and α1ACT bind to calmodulin, while α1HCT does not. Furthermore, calmodulin and CaM kinases have shown to play dynamic roles in both LTCC- and NMDA-receptor mediated gene expression regulation, therefore examining their role in α1CCT and α1ACT nuclear translocation would be a logical next step (Cohen et al., 2018; Bading et al., 1993; Murray et al., 2003).

Activity-dependent gene expression and neurological disease: CACNA1C has been implicated as a risk gene for several mental illnesses, including autism spectrum disorders (ASDs), bipolar disorder (BD), and major depressive disorder (MDD) (Moon et al., 2018). However, single mutation models often fail to recapitulate disease phenotypes, suggesting that disorders related to *CACNA1C* mutations may be more than simple channelopathies. Our results indicate that α1CCT function and localization may help to explain the complex phenotypes and pleiotropic nature of disorders arising from *CACNA1C* mutations. ASD, BP, and MDD are all characterized at least partially by altered synaptic strengths, and by an overall imbalance in the excitatory-inhibitory balance within key brain regions, such are the cerebral cortex, midbrain, or cerebellum (Sohal and Rubenstein, 2019). An excess of excitatory or inhibitory activity as seen in these disorders could significantly alter the normal localization and translocation properties of α1CCT, resulting

in widespread, downstream gene expression dysregulation. For example, the complex ASD Timothy Syndrome (TS), caused by a single gain-of-function amino acid mutation in exon 8 of *CACNA1C* (either G406R or G402S), results in delayed channel closing and subsequent enhanced cellular excitability (Yarotskyy et al., 2009). TS is characterized by multi-system dysfunction, including cardia arrhythmias, dysmorphic facial features, syndactyly, immunodeficiency, developmental delay, and autism (Krause et al., 2011). Considering the findings reported in this paper, one could imagine a pathophysiological mechanism of TS whereby an excess of excitatory signals could function as a positive feedback loop, causing an abundance of α1CCT to translocate to the nucleus of affected neurons. This would theoretically result in more α1CCT target DNA-binding and upregulation of gene targets, including the suite of synaptic genes we show α1CCT directly affects, ultimately leading to an overall increase in synaptic strength within that neuron/neuronal circuit. These underlying gene expression changes mediated by dysregulated α1CCT could help to explain the complex pleiotropic characterization of TS.

Like α1CCT and α1ACT, α1HCT binds to a suite of genes that promote neurogenesis and nervous system development. While there is overlap between the genes regulated by α1HCT and those regulated by α1CCT and α1ACT, there are many unique genes that could help to elucidate α1HCT's distinctive role in central and peripheral nervous system. Although more studies need to be conducted to identify the regional expression of α1HCT within the nervous system, its parent VGCC gene, *CACNA1H*, is expressed widely throughout the CNS and PNS, particularly in oscillatory circuits where CaV3.2 is crucial in modulating neuronal excitability (Catterall, 2010). We found that α1HCT does not significantly traffic to or from the nucleus following intracellular calcium spikes, however our experiments focused on transient, single spikes and not

the oscillatory, rhythmic activity patterns often observed in neurons that highly express *CACNA1H*. Further studies are needed to systemically examine whether these more complicated calcium signals play a role in α1HCT translocation.

The results presented in this dissertation introduce a novel method of coordinated gene expression, as the VGCC CTPs are translated via cap-independent, IRES-mediated translation. Furthermore, α1CCT and α1ACT are activity-dependent transcription factors, translocating to or from the nucleus in response to elevated internal calcium concentration. These findings have vast implications for not only our understanding of proper nervous system function, but also for characterization of the mechanisms that lead to the complex clinical phenotypes observed in disorders related to the VGCC genes. Often, single mutations in VGCC genes lead to pleiotropic, varied phenotypes, poorly explained by simple disturbances in channel gating or kinetics (Berger and Bartsch, 2014; Craddock and Sklar, 2013; Fernandes-Rosa et al., 2017; Liao and Soong; 2010; Schmunk and Gargus, 2013; Splawski et al., 2004; Yalcin, 2012). As shown with *CACNA1A* and its CTP α1ACT, these disorders may not be pure channelopathies at all, but instead pathology may be conveyed via altered secondary protein function. The fact that such novel proteins in the CNS function in an activity-dependent manner further complicates the picture. Mutations within known activity-dependent related genes, or dynamic targets of those genes, have been shown to correlate positively with various degrees of intellectual disability (Vaillend et al., 2008). For example, a loss-of-function mutation in the FMR1 gene resulting in Fragile X syndrome, the most common inherited form of both mental retardation and ASDs, prevents FMR1, an mRNA-binding protein that regulates translation, from properly modulating gene expression in an activity-dependent manner (Huber et al., 2002; Bhakar et al., 2012). This

dysregulation subsequently leads to synaptic defects caused by altered synaptic pruning and subsequent synaptic overelaboration (Gatto and Broadie, 2008).

Perturbations to activity-dependent gene expression programs are especially relevant to the pathology of mood disorders. While environment has been known to play a non-trivial role in the etiology and progression of mood disorders in those with underlying genetic risk factors, research conducted in recent decades has started to uncover the mechanisms through which environmental stimuli coupled with genetic mutations in key genes may significantly alter proper activity-dependent gene expression leading to disease. For example, several studies have demonstrated that stress can directly lead to atrophy and death in pyramidal neurons in the hippocampus (McEwen, 1999; Sapolsky, 1996). Additionally, in rodent models of depression, stress has been shown to lead to decreased neuronal dendritic branching, neurogenesis, and LTP induction (Kim and Diamond, 2002; Xu et al., 1997; Son et al., 2012). Furthermore, mutations in genes related to activity-dependent gene expression are associated with debilitating disorders of human cognitive function. Mutations in proteins such as RSK2, CBP, MECP2, or UBE3A have been shown to lead to Coffin-Lowry syndrome, Rubinstein-Taybi syndrome, Rett syndrome, and Angelman syndrome, respectively (Flavell and Greenberg, 2008). Therefore, a reasonable hypothesis posits that several of the cognitive and mental illnesses identified to be related to mutations in *CACNA1C, CACNA1A*, and/or *CACNA1H* could result from dysfunction of the novel activity-dependent TFs α1CCT, α1ACT, or α1HCT. Identifying the unique and overlapping roles of each CTP in contrast to their parent VGCC subunits could provide valuable insight into the pathophysiology of mental illness related to those genes.

The proper development of the nervous system relies heavily on the molecular relationship between neuronal activity and changes in gene expression. Proteins such as CREB

and NFAT have been shown to translocate to the nucleus in response to synaptic or Ca^{2+}-dependent stimuli, and dysregulation of this activity-dependent translocation leads to a wide variety of neuronal pathologies (Wang et al., 2018; Saura and Valero, 2011; Lee et al., 2018; Rao, 2019). Similarly, aberrant patterns of neuronal activity or perturbations of normal calcium homeostasis could lead to the improper cellular translocation of α1CCT or α1ACT, significantly altering downstream gene expression networks tremendously. Our findings represent a novel mechanism of coordinated gene expression and could help to explain the complex and varied phenotypes associated with disorders related to VGCCs, which are often poorly explained through disturbances in simple channel gating. Further investigation of the distinct roles of these three VGCC CTPs and the regulation of their subcellular localization could therefore provide valuable insights into the complex genotype-phenotype relationships disorders related to VGCCs, as well as inform the development of future therapies.

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