# Characterising the transcriptional profile of CACNA1C expression in rodents




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Thesis submitted for the degree of Master in Science (by Research) in Psychiatry

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# ABSTRACT


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**Introduction:** The CACNA1C gene, which codes for the α1-subunit of the voltage-gated calcium channel $Ca_{v1.2}$, has been repeatedly associated with psychiatric disease in genome-wide studies. $Ca_{v1.2}$ can be expressed in a number of isoforms produced through alternative splicing of pre-mRNA. Splice variants in CACNA1C have been shown to be spatially and temporally regulated, many variants being tissue specific or changing expression during development. Moreover, CACNA1C splice variants are known to have differing electrophysiological properties as well as differences in expression. With the advent of long-read sequencing technologies, it has become possible to reliably identify and characterise mRNA splice variants, and recent evidence from human post-mortem brains suggests that the transcriptional profile of CACNA1C may be under-characterised in rodents. In this project, we have set out to fully characterise the full range of mRNA splice variants in mice and rats across neural and cardiovascular tissues.

**Methods:** We extracted whole-tissue RNA from aorta, heart, cerebellum, and frontal cortex of 8 mice and 8 rats. Full-length CACNA1C cDNA was isolated from each tissue using RT and PCR amplification of transcripts starting with exon1a, exon1b, or exon1c. Sequencing libraries were prepared as per the Nanopore 1D Ligation Sequencing protocol, and sequenced on the Nanopore MinION. Transcripts were then translated using the online ExPaSy tool and aligned on UniProt to annotate and predict functional significance of individual splice variants.

**Results:** Principal component analysis (PCA) of sequencing reads reveals a clear segregation of transcript isoforms enriched in the brain compared to isoforms enriched in the heart and isoforms enriched in smooth muscle tissue. Across all samples, reads showed 53 different transcript variants, out of which 45 novel splice variants, among which 37 are predicted to be coding, and 3 which contain novel previously-uncharacterised exons..

**Conclusion:** The transcriptional profile of CACNA1C in rodents has been under-characterised. Although our results need to be validated at the proteomic level, we reveal 45 novel mRNA splice variants. Further study of these may reveal insights to the pathophysiology of psychiatric disorders associated with CACNA1C, and potentially lead to novel therapeutic targets.

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# 1. INTRODUCTION

## 1.1 PSYCHIATRIC DISEASE AND THE ROLE OF CACNA1C

### 1.1.1 THREE SPHERES OF RESEARCH IN BIPOLAR DISORDER AND SCHIZOPHRENIA: GENETICS, NEUROBIOLOGY, AND PHARMACOLOGY

Bipolar disorder (BD) and schizophrenia (SCZ) are psychiatric disorders that manifest in patients with a complex, multifaceted pattern of symptoms affecting emotional, cognitive, and sensory processes (Ruderfer *et al.*, 2018). Individuals with SCZ may suffer from delusions, hallucinations, mood disturbances, and cognitive deficits. While patients with BD may also experience hallucinations and illogical thinking, the disorder is mainly characterized by an episodic nature of extreme highs, or manic states, and depression. Both disorders bear a substantial social and economic burden, with unemployment rates of 60% among people with BD (Bowden, 2005)and up to 8-% among individuals with SCZ (Insel, 2010). In the hopes of developing better treatments, there have been concerted international efforts to characterize the aetiology and pathophysiology of these diseases through the lens of genetics, neurodevelopmental biology, and pharmacology.

Although the two have historically been considered distinct diagnoses, BD and SCZ are closely related and have both shared symptomology and genetics (Eckart *et al.*, 2016). Both disorders are highly heritable, with SCZ estimated to have heritability rates of 65-80% (Harrison, 2015) and BP up to 89% (Craddock and Sklar, 2013). Moreover, taking into consideration the substantial overlap in symptoms and genetic vulnerabilities across the two disorders suggests a shared underlying pathologies leading to common endophenotypes (Meyer-Lindenberg and Weinberger, 2006). The genetic architecture of SCZ and BP has proven to be complex, implicating the involvement of up to hundreds of genomic loci each having a small effect on increasing disease risk (Ruderfer *et al.*, 2018).

One pitfall of genome-wide association studies (GWAS) is that associations are formed to loci, or regions within chromosomes, rather than to genes. As loci often encompass multiple genes in linkage disequilibrium (Harrison, 2015), it is often necessary to validate their involvement in disease pathology through further experiments. The picture is further complicated in complex polygenic traits, including height, weight, and IQ, which have consistently shown a pattern of GWAS hits that are uniformly distributed across the genome (Boyle, Li and Pritchard, 2017). In this model, although traits may be primarily under the control of a number of genes which have convergent downstream effects on phenotype (Harrison, 2015), the interconnected nature of signalling networks in a cell and the involvement of genes in linkage disequilibrium means that most if not nearly all genes in the genome will have a small effect on the final phenotypic outcome (Boyle, Li and Pritchard, 2017).

That said, a recent study by the Psychiatric Genomics Consortium has demonstrated that despite the polygenic nature of these psychiatric disorders it is possible to correlate polygenic risk scores (quantitative measurements of clusters of risk genotypes) with 28 endophenotypes in SCZ and BD – showing that it is possible to predict psychiatric symptomology through genomic data (Ruderfer *et al.*, 2018). Expectedly, genes only weakly associated with psychiatric diagnoses can show a stronger association with specific symptoms, such as altered brain processing of emotional and cognitive information. Moreover, this paves the way for understanding these disorders as multidimensional spectra of symptoms rather than dichotomous incompletely accurate diagnoses, allowing a better way to account for and more accurately treat the heterogenous range of symptoms presented in patients. For example, the study showed that individuals with SCZ with polygenic risk scores highly correlating to BD present with manic symptoms (Ruderfer *et al.*, 2014), but standardized treatment guidelines for SCZ often fail to account for such heterogeneity.

In fact, psychiatry often lacks objective biological measurements through which clinicians can assess patient symptom severity, highlighting the potential for genetics to inform personalised medicines much more than it can in other fields of medicine. Nonetheless, there is still a need to dissect GWAS results in order to properly guide development of improved therapies. Although GWAS can identify which genes are associated with a pathology, it does not inform us whether increased genetic risk is caused by a gain-of-function, loss-of-function, or dysregulation of a gene or signalling pathway. For SNPs identified in SCZ and BP studies the majority of loci are actually found in non-coding sequences, suggesting that disease pathogenesis may involve dysregulation of transcriptional control (Nicolae *et al.*, 2010). As can be expected, many of these genes converge on pathways associated with neurodevelopmental and neuronal function.

Even though symptoms of BD and SCZ generally manifest in late adolescence, both disorders are considered to have a neurodevelopmental component. Longitudinal studies such as the Dunedin birth cohort demonstrated early cognitive deficits associated with increased risk of schizophrenia later in life (Insel, 2010), and a follow up on the Copenhagen birth cohort has confirmed a history of delayed developmental milestones in the first year of life (Sørensen *et al.*, 2010). Although the evidence for motor, social, and cognitive abnormalities in children at risk for BP is weaker than it is for SCZ, a number of studies suggest the pathology may have a neurodevelopmental origin (Sanches *et al.*, 2008). This is replicated in animal models, where disruptions in cortical development *in utero* and in early infancy lead to cognitive abnormalities that do not emerge until early adulthood (Birnbaum *et al.*, 2014).

A prevalent hypothesis is a model in which lesions or trauma early in development are masked by compensatory mechanisms until a later developmental stage, when such mechanisms are no longer sufficient (Insel, 2010). By collecting epigenetic data through methylation microarrays, the Weinberger lab was able to analyse temporal expression patterns of genes associated with schizophrenia and other neurodevelopmental disorders (Birnbaum *et al.*, 2014; Jaffe *et al.*, 2016). Postmortem samples from

hundreds of individuals at different stages of development, ranging from prenatal and fetal tissues to aging brains, reveal that the 108 known schizophrenia risk loci were enriched for changes in methylation (and therefore presumable changes in gene expression) in early life (Jaffe *et al.*, 2016). These changes in gene expression reflect a shifting cell-type composition in the brain in early life, and could disturb the formation of essential brain circuits (Jaffe *et al.*, 2016).

SCZ and BD have a high degree of comorbidity with other factors such as depression, obsessive-compulsive disorder, and addiction (Insel, 2010). Moreover, in both of these illnesses there is an increased prevalence with obesity (Insel, 2010) and autoimmune diseases (Stringer *et al.*, 2014; Cremaschi *et al.*, 2017), suggesting a potentially pleiotropic aetiology involving non-neuronal tissues. However, although there are numerous pharmacological and psychological therapies available to affected patients, they appear to primarily target symptoms of hallucinations, delusions, and mood fluctuations (Insel, 2010). Although antipsychotic medications and mood stabilizers are fairly effective at treating these symptoms, epidemiological studies fail to show significant improvements in functional recovery in socio-economic domains such as unemployment and homelessness (Insel, 2010).

This shortcoming may be due to the fact that although hallucinations and delusions are the most visible symptoms of schizophrenia, they are not as debilitating as the cognitive impairments associated with the disease, which both current pharmacological therapies largely fail to improve (Insel, 2010). Individuals with SCZ exhibit a global neuropsychological deficit, with severe impairments in processing speed, verbal learning, and executive function (Bowie *et al.*, 2018). Although the degree of neurocognitive impairment is lower in euthymic BD patients, there are significant deficits in verbal learning in manic/mixed states, and verbal fluency in depressed states (Bowie *et al.*, 2018). It is increasingly evident that a deeper understanding of the molecular pathophysiology and genetics of the disease is needed for the successful design of improved drugs.

The standard treatments for BD is lithium, which acts as effective mood stabilizers (Flores-Ramos *et al.*, 2017). However, even in undiagnosed individuals lithium appears to have a neuropsychiatric impact. Epidemiological evidence suggests that lithium concentrations in drinking water negatively correlate with suicide rates in the population (Jakobsson *et al.*, 2017), and post-mortem brain hippocampal histological analyses show changes in cell-type and cell density following lithium treatment in rodents (Rajkowska, 2003).

Interestingly, although lithium is an essential nutrient naturally found in the environment, to date no lithium-selective macromolecular binding sites in protein domains or transporters have been identified (Jakobsson *et al.*, 2017). In fact, its mechanism of action in human physiology appears to be through the competition of binding sites that are specific to other cations such as sodium and magnesium (Jakobsson *et al.*, 2017), as well as the GSK3 and BDNF signalling pathways which are

involved in neuronal development (Flores-Ramos *et al.*, 2017). Both of these are also involved in Ca2+ ion signalling, suggesting a possibly convergent pathway leading to lithium action.

However, the current mechanism of action of lithium in the brain is still unclear or disputed, and 50% of BD patients fail respond to it (Licht, 1998). A popular effective alternative is valproate, which has potent antimanic effects but appears to be less effective against depressive episodes than lithium (Flores-Ramos *et al.*, 2017). Both have undesirable side effects, including increased teratogenic potential and menstrual abnormalities, increasing the risk of inducing polycystic ovarian syndrome in women (Flores-Ramos *et al.*, 2017).

Given the above, it is evident that current treatments are insufficiently effective and less than ideal. It is imperative to identify drug targets that have the potential to affect the cognitive symptoms of SCZ and BD in order to improve treatment outcomes for patients, guided by genetics-based approaches. Moreover, there is a need to develop our understanding of these disorders by studying genetic pathways not only for the development of drug targets to manage symptoms, but also to identify the underlying neurodevelopmental mechanisms by which they occur. One target that shows potential is CACNA1C, which encodes for the α subunit of the voltage-gated calcium channel $Cav_{1.2}$.

### 1.1.2 CALCIUM SIGNALLING IN PSYCHIATRIC DISEASE

Genome-wide studies, neuroimaging experiments, and observations in animal models have frequently linked dysregulation of the calcium signalling with endophenotypes in bipolar disorder and schizophrenia. In BD, elevated calcium concentrations have been detected in platelets (Dubovsky *et al.*, 1994), lymphocytes (Emamghoreishi *et al.*, 1997), and serum (El Khoury *et al.*, 2002) of patients. These observations have been replicated across both manic and depressive states (Dubovsky *et al.*, 1994), as well as euthymic states (El Khoury *et al.*, 2002), demonstrating altered calcium homeostasis across all mood states in BD.

Ca2+ is a highly versatile intracellular messenger ubiquitous in almost all biological systems, initiating processes as diverse as muscle contraction, hormone secretion, and gene expression. Due to its diverse functionality it is imperative that the movement of Ca2+ ions in cells is tightly regulated by the combined action of channels, pumps, transporters, and binding proteins. In eukaryotes, Ca2+ signals arise either by influx into the cell through transmembrane protein channels, or release from intracellular compartments where calcium ions are stored. These signals are in general transient, as cytosolic Ca2+ is rapidly sequestered into intracellular stores or extruded through the action of calcium pumps and exchangers (Zamponi *et al.*, 2015).

There are a number of protein families that allow Ca2+ entry from the extracellular space, either in responses to changes in membrane potential, binding of extracellular ligands, or regulation by intracellular messengers. Given the electrochemical gradient present across the membrane, channel activation leads to passive bursts of Ca2+ influx into the cell. Once in the cytosol, Ca2+ is bound to effector proteins or calcium-sensitive enzymes, inducing conformational changes in them that allow the initiation of downstream signalling pathways. As effector proteins have specific intracellular localizations, the route by which Ca2+ transients enter is an important determinant of signal transduction by calcium ions (Bartels *et al.*, 2018).

Gene ontology pathway analyses of genome-wide studies of risk loci suggest that calcium signalling plays a role in the development of endophenotypes in BD and SCZ. In a large case-control study, genes belonging to the family of voltage-gated calcium channels (VGCCs) showed a robust link not only to schizophrenia, but also to working memory performance in healthy adults (Heck *et al.*, 2014). VGCCs are transmembrane proteins activated following membrane depolarization, and are an important route of calcium entry in all excitable cells. They are composed of a pore-forming α subunit, which denotes the channel's identity and may associate with different combinations of auxiliary subunits that endow the channel with different electrophysiological properties and allow its proper expression and localization.

α subunits of VGCCs in mammals are encoded by one of the ten CACNA1- genes, and each has distinct tissue-specific expression profiles and electrophysiological properties. CACNA1C, which codes for the α subunit of the $Cav_{1.2}$ channel, is among the strongest signals in GWAS for BD risk (Keers, Farmer and Aitchison, 2009; Psychiatric GWAS Consortium Bipolar Disorder Working Group, 2011) and consistently appears in genomics studies in schizophrenia (Schizophrenia Psychiatric Genome-Wide Association Study (GWAS) Consortium, 2011). In fact, SNPs in this gene were found to have shared effects across a range of psychiatric conditions, including attention-deficit hyperactivity disorder, autism, and depression (Wessa *et al.*, 2010; Wray *et al.*, 2012; Moon *et al.*, 2018), suggesting a role in a transdiagnostic endophenotype common in all of these psychiatric diseases.

Mutations in CACNA1C have been associated with changes in brain anatomy (Kempton *et al.*, 2009; Franke *et al.*, 2010) and function (Bigos *et al.*, 2010; Erk *et al.*, 2010). As global homozygous knockout rodent models of CACNA1C die *in utero* (Seisenberger *et al.*, 2000), animal models of CACNA1C function often rely on conditional knockout systems or partial, heterozygous, or cell type-specific knockouts. It has been shown that mice with global haploinsufficiency, forebrain-specific knockouts, and prefrontal-cortex-specific knockdowns of CACNA1C exhibited increased anxiety-related behaviours (Lee *et al.*, 2012), sleep disturbances (Dedic *et al.*, 2018), decreased sociability, and impairments in spatial memory (Moon *et al.*, 2018). These mice also appeared to exhibit changes in neuronal development, with increased cell death in immature neurons (Moon *et al.*, 2018).

**Table 1: SNPs associated with increased risk for psychiatric disease**

**The table below has been adapted from (Bhat *et al.*, 2012), and lists SNPs within CACNA1C that associate with psychiatric disease in GWAS studies.**

| **SNP/Region** | **Association with/comments** |
|---|---|
| rs72552065 (WIAF-13171)<br>Exon 44 | BPD (Nominal) |
| Exon 8 & 8a | Timothy syndrome, a multisystem syndrome including autistic features |
| rs1006737 | BPD; GWAS |
| rs1006737 | BPD; GWAS |
| *CACNA1C* | BPD; Gene-wide significance was assessed in genome-wide data rather than at SNP level |
| 4 independent signals<br>(including rs1006737) | BPD; BPD GWAS data analyzed with lower threshold for significance |
| *CACNA1C* | MDD; GWAS; reported the p-value for the entire gene and not for a specific SNP |
| rs2370419; rs2370411 | SNPs in the genomic region of *CACNA1C* analyzed. Significant interaction with |

| SNP/Region | Association with/comments |
|---|---|
| | sex. Increased risk of mood disorder (BPD and MDD) in females only |
| rs1006737 | SCZ |
| rs1006737; rs10848635 | Increased risk of treatment-emergent suicidality in non-psychotic MDD |
| rs1006737 | SCZ |
| rs1006737 | SZ and MDD |
| rs1006737 | Higher psychopathology scores for depression, anxiety, obsessive-compulsive thoughts, interpersonal sensitivity, and neuroticism on Beck Depression Inventory or State Trait Anxiety Inventory |
| rs1006737 | Lower extraversion and higher harm avoidance, trait anxiety, and paranoid ideation scores as assessed via personality questionnaires |
| rs1006737 | Higher depressive and manic symptom scores among all groups |
| rs7297582; rs1006737 | Combined population of BPD and MDD in a meta analysis; GWAS |
| rs4765913 | BPD; GWAS; Combined analysis of BPD dataset and that of SZ dataset also showed significant association with both the disorders combined |

| SNP/Region | Association with/comments |
|---|---|
| rs4765905 | Combined analysis of SZ and BPD; GWAS |
| rs1006737 | Psychotic subgroup of BPD |
| rs4765905 | SZ; GWAS |

In humans, neuroimaging data and voxel-based morphometry have shown that genetic variation at a number of SNPs within the CACNA1C gene has been linked to changes in gray matter volume (Kempton *et al.*, 2009; Radua *et al.*, 2013), particularly in frontotemporal structure including the amygdala (Perrier *et al.*, 2011; Wolf *et al.*, 2014), prefrontal cortex (Bigos *et al.*, 2010), cingulate cortex (Wang *et al.*, 2011), and hypothalamus (Bigos *et al.*, 2010), as well as brain stem structures (Franke *et al.*, 2010). Healthy subjects carrying the risk allele A/G at the rs1006737 SNP have also demonstrated enhanced amygdala responses (Wessa *et al.*, 2010; Tesli *et al.*, 2013), and reduced activation of the hippocampus (Krug *et al.*, 2014), cingulate cortex, and frontotemporal cortices (Erk *et al.*, 2010) in response to emotional stimulation compared to subjects with a non-risk genotype, indicating that genetic variation at this locus induces structural and functional differences even in individuals without psychiatric disease.

These findings have generated interest within the field of psychiatry, as dysfunction of the limbic system – particularly within the amygdala and hypothalamus – has long been observed in patients with bipolar disorder and schizophrenia (Wessa *et al.*, 2010). Follow-up *post-mortem* studies have also revealed that patients with bipolar disorder have revealed altered mRNA levels in different brain regions (Bigos *et al.*, 2010; Gershon *et al.*, 2014; Eckart *et al.*, 2016). In fact, chIP-seq and microarray analyses show that CACNA1C SNPs associated with disease risk correspond to expression quantitative trait loci (eQTLs), and therefore have an impact on total gene expression (Eckart *et al.*, 2016). Changes in mRNA expression of CACNA1C in risk-associated genotypes has been replicated in cultured iPSC neurons (Yoshimizu *et al.*, 2015), though as the direction of effect appears to vary between brain regions (Bigos *et al.*, 2010; Gershon *et al.*, 2014; Eckart *et al.*, 2016) it is likely that the gene is subject to cell type-specific transcriptional regulation. Moreover, CACNA1C expression also appears to be activity

dependent, as subjecting rats to chronic stress has been shown to lead to increased mRNA expression in dentate gyrus, amygdala, and hippocampus (Klaus *et al.*, 2018)..

## 1.2 CACNA1C ACROSS THE HEART AND BRAIN

### 1.2.1 PHARMACOLOGY OF CAV1.2 IN PSYCHIATRIC DISEASE

Like all LTCCs, $Cav_{1.2}$ channels are characterized by their susceptibility to antagonism by dihydropyridines such as nimodipine, nifedipine, isradipine, verapamil (Striessnig, Ortner and Pinggera, 2015). Dihydropyridines are widely used in the treatment of angina and hypertension (Zamponi *et al.*, 2015), and therefore are well characterized in terms of their pharmacological profile, side effects, and dosage toxicity. However, case reports over the years as well as a recent meta-analysis of patient data suggest their potential in the treatment of psychiatric disorders. In Swedish patients with serious mental illness, individuals that were also prescribed LTCC antagonists exhibited lower rates of self-harm as well as reduced rates of psychiatric hospitalisation (Hayes *et al.*, 2019). Combined with GWAS data implicating CACNA1C in the aetiology of SCZ and BD, $Cav_{1.2}$ appears as an attractive target for the development of future psychiatric drugs.

The data surrounding the LTCC antagonist nifedipine is controversial, and appears to have heterogeneous effects on patients. In a subset of patients without any history of psychiatric illness, prescription of nifedipine correlated with the onset of depressive symptoms (Kabir, Martínez-Rivera and Rajadhyaksha, 2017). However, the same drug appears to have antidepressant effects in rodent models of depression (Mogilnicka, Czyrak and Maj, 1988). It is however possible that the effects of nimodipine are dependent on mood state and symptom severity or on other physiological factors. When trialled in combination with a mood stabiliser on patients with BD, nifedipine appeared to alleviate depressive symptoms, which recurred once the drug treatment stopped (De Beaurepaire, 1992). However, in two of the patients in the trial, nifedipine had no effect on manic symptoms of the disease, suggesting that it may only work against depressive symptoms.

Similar results were obtained in trials with nimodipine (Keers, Farmer and Aitchison, 2009) and isradipine (Levy and Janicak, 2000), which have both shown efficacy in the treatment of BD in conjunction with lithium. In the case of verapamil, a double blind RCT showed that in some cases of mania it can be even more successful than lithium (Garza-Treviño ES, Overall JE, 1992). Verapamil has also shown efficacy in patients who were insensitive to lithium , and has been known to attenuate psychotic symptoms in schizophrenia (Kabir, Martínez-Rivera and Rajadhyaksha, 2017)

Given the evidence implicating a causative role of CACNA1C in the development of psychopathology, thoroughly dissecting the role of CACNA1C and its protein product $Cav_{1.2}$ within the human body can lead to insights the aetiology of SCZ and BD. The transcriptional profile of CACNA1C is complex, as it is expressed not only throughout the brain but also the cardiovascular system. As such,

consideration of this gene as a potential target for development of future pharmacological therapies for psychiatric disorders must take into account the potential for systemic side effects. The following sections will briefly review the literature on the role of $Cav_{1.2}$ in cellular biology across neural and cardiovascular tissues, and delineate the molecular mechanisms that regulate its expression and functionality.

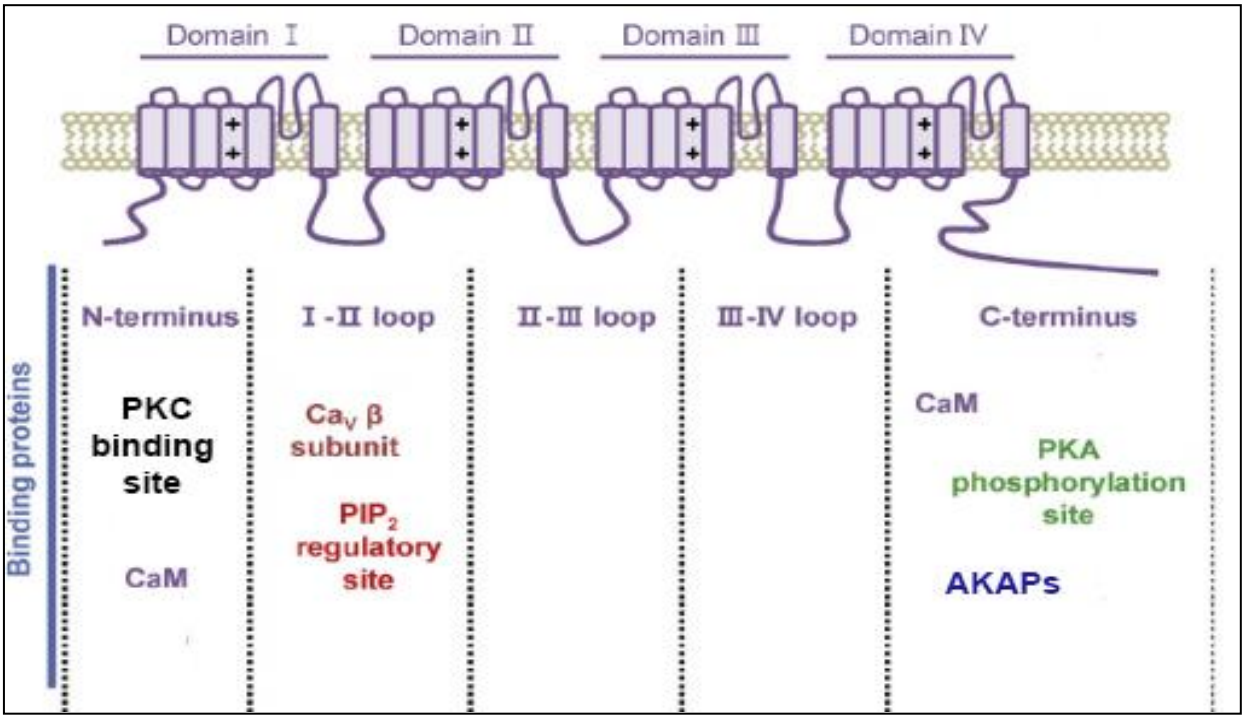
### 1.2.2 STRUCTURE AND FUNCTION OF VOLTAGE-GATED CALCIUM CHANNELS

Voltage-gated calcium channels are transmembrane proteins that open in response to membrane depolarization, allow an influx of Ca2+ from extracellular spaces or intracellular compartments. Present in almost all excitable cell types, VGCCs are key mediators of physiological processes as diverse as neurotransmitter secretion, maintaining intracellular calcium homeostasis, and muscle contraction (Jurkat-Rott and Lehmann-Horn, 2004). They are composed of an α subunit that makes up the pore-forming unit, which can be bound to β and/or α2delta auxiliary subunits. Although the α subunit is sufficient to induce VGCC currents, auxiliary subunits are needed in order to modulate electrophysiological channel kinetics and ensure its appropriate localization and stability in cells.

B subunits can substantially shift channel kinetic and voltage sensitivity of the channels, in addition to increasing channel surface expression by preventing ubiquitination and channel internalization. Klugbauer and colleagues have described genes encoding for four α2delta subunits (Klugbauer, Marais and Hofmann, 2003). Although the functional effect they exert on channel electrophysiological properties appears to be modest, they are involved in promoting channel surface localization. In addition to the auxiliary subunits, VGCCs also associate with a number of intracellular molecules, containing binding sites to calmodulin, PIP2, PKA, and AKAPS. VGCC currents have been shown to respond to modulation by an extensive range of hormones and neurotransmitters in different tissues.

VGCCs α subunits are believed to have evolved through multiple gene duplication of an ancestral one-domain potassium channel (Strong, Chandy and Gutman, 1993). As shown in **Figure 1**, VGCCs have four domains that are homologous to one another, each containing six transmembrane α-helical segments labelled S1-S6 (Jurkat-Rott and Lehmann-Horn, 2004). The channel obtains its pore-forming structure by tetrameric association of its four domains (Jurkat-Rott and Lehmann-Horn, 2004). The intra-domain linker regions between each transmembrane domain lie in the intracellular space, and can associate with various second messenger molecules and regulatory proteins.

**Figure 1: Voltage-gated Calcium Channels and Associated Intracellular Molecules**




**The figure above outlines the structure of Voltage-gated Calcium Channels, denoting four homologous Domains I – IV, each composed of six transmembrane segments S1-S6. The intracellular linker regions between each domain contain motifs that allow binding to intracellular second messengers and regulatory molecules, listed below as binding proteins. The figure above has been adapted from (Park and Suh, 2018)**

Structurally, the coding regions of the transmembrane segments S1-S5 are well conserved across each of the four domains between all of the VGCCs. The S4 segments are rich in positively charged amino acid residues and act as the voltage-sensors of the channel, moving outwards in response to depolarization causing the channel to open (Jurkat-Rott and Lehmann-Horn, 2004). The S6 segments and intracellular N- and C-terminal domains on the other hand show more heterogeneity suggesting their importance channel-specific functional diversity (Jurkat-Rott and Lehmann-Horn, 2004). The C-terminus in fact contributes to a third of the total channel protein, and contains binding elements to scaffolding proteins such as AKAPs and regulatory proteins including calmodulin and G-proteins (Jurkat-Rott and Lehmann-Horn, 2004; Catterall, 2015)

Channel identity is determined by α subunit, which are encoded by one of ten CACNA1* genes in mammals. These are subdivided into L-type, P/Q-type, N-type, R-type, and T-type depending on their electrophysiological properties and tissue-specific expression pattern. A summary of the CACNA1* genes, the VGCCs they encode, and their nomenclature can be found in Table 2 below. L-type Calcium Channels (LTCCs) have longer-lasting currents and have a in important role in neuronal and muscular physiology. Of the four LTCCs, $Cav_{1.1}$ and $Cav_{1.4}$ have highly tissue-specific expression patterns: $Cav_{1.1}$ is

mainly restricted to skeletal muscles, and $Cav_{1.4}$ is primarily found in retinal cells (Zamponi *et al.*, 2015). On the other hand, $Cav_{1.2}$ and $Cav_{1.3}$ are found throughout the nervous system in both neurons and astrocytes, as well as cardiac muscle cells and in smooth muscle tissues.

**Table 2: Nomenclature of Voltage-Gated Calcium Channels**

**The table below denotes the ten voltage-gated calcium channel genes and the channels associated with their protein products. The three naming systems are often used interchangeably in the literature, though it must be specified that names in the first column refer to genes, second column refers to proteins, and third column refers to more generalized subcategories of VGCCs.**

| Gene | Associated VGCC | Properties |
|---|---|---|
| CACNA1S | Cav1.1 | L-type channels |
| CACNA1C | Cav1.2 | |
| CACNA1D | Cav1.3 | |
| CACNA1F | Cav1.4 | |
| CACNA1A | Cav2.1 | P/Q-type channels |
| CACNA1B | Cav2.2 | N-type channels |
| CACNA1E | Cav2.3 | R-type channels |
| CACNA1G | Cav3.1 | T-type channels |
| CACNA1H | Cav3.2 | |
| CACNA1I | Cav3.3 | |

In neurons, $Cav_{1.2}$ is expressed post-synaptically on dendrites and cell bodies (Biase *et al.*, 2008). It is the most widely expressed LTCC in the brain, account for 89% of L-type currents in neurons. $Cav_{1.3}$ channels, which account for most of the remaining 11%, are often co-expressed within the same neurons (Berger and Bartsch, 2014). In the cardiovascular system, $Cav_{1.2}$ is the major LTCC involved in cardiac excitation-contraction coupling, and its activity in vascular smooth muscle is required for homeostatic maintenance of blood pressure (Moosmang *et al.*, 2003).

### 1.2.3 CACNA1C IN EXCITATION-TRANSCRIPTION COUPLING

Synaptic activity and neuronal membrane depolarization leads to activation of gene expression progammes in the nucleus (Deisseroth, Heist and Tsien, 1998; West, Griffith and Greenberg, 2002). Up to 300 different genes show synaptic-activity dependent regulation (West, Griffith and Greenberg, 2002), and this process is believed to underlie mechanisms leading to neurogenesis, long-term potentiation, and synaptic pruning.

Calcium is a crucial second messenger in biochemical transduction of synaptic activity into gene transcription. As mentioned in previous sections, as effector proteins are highly localized in cells, the route by which Ca2+ enters a cell is an important determinant of signal transduction. Intracellular Ca2+ transients in neurons are known to regulate gene expression at several steps, including transcription initiation, elongation, splicing, stability, and translation (West, Griffith and Greenberg, 2002). Preferentially expressed at the basal dendrites and cell soma of neurons, $Cav_{1.2}$ is well-placed in coupling Ca2 influx with phosphorylation of transcription factors, including cAMP response element-binding protein (CREB), NFAT, and BCL2, implicated in synaptic plasticity and neurogenesis (West *et al.*, 2001).

Although Ca2+ can enter cells through multiple other routes, such as NMDA receptors and other VGCCs, activation of CREB is tightly coupled to $Cav_{1.2}$. In fact, although pharmacological inhibitors of LTCCs only block a fraction of the calcium influx induced by spontaneous synaptic activity, they almost completely block the transcription of CREB-induced genes (West *et al.*, 2001). Conversely, even under conditions in which global cytoplasmic rise in Ca2+ is blocked (such as through artificial sequestration of Ca2+ ions), localized elevation of Ca2+ transients through $Cav_{1.2}$ is sufficient to trigger CREB phosphorylation, suggesting that signalling pathways are activated close to or at the channel (West, Griffith and Greenberg, 2002).

This is likely due to the fact that signalling molecules are physically associated with the channel itself, and only dissociate once bound to Ca2+. Several signalling molecules are known to bind to LTCCs, including AKAP, Src kinases, and calmodulin. By generating a series of mutated isoforms of $Cav_{1.2}$, Dolmetsch and colleagues showed that calcium-dependent activation of CREB requires the binding of calmodulin to an IQ domain of the c-terminal of $Cav_{1.2}$. Once released, calmodulin may translocate to the nucleus and activate CaMKII and CamKIV, initiating CREB phosphorylation (Deisseroth, Heist and Tsien, 1998).

However, the interaction of LTCCs with calmodulin alone cannot explain their selective ability to activate CREB, as calmodulin also binds to homologous domains in N, P/Q, and R type calcium channels that may be found in the same cells (West, Griffith and Greenberg, 2002). Moreover, although in neurons, phosphorylation of CREB is mediated by the activation of CaMKIV by calmodulin, but prolonged-activity- dependent phosphorylation is mediated by the Ras-MAPK pathway (West, Griffith and Greenberg, 2002). Ca2+ influx leads to dephosphorylation of NFAT by calcineurin and its translocation to the nucleus. CNS targets of NFAT aren't clear, but most calcium activated gene expression in lymphocytes is dependent on calcineurin, suggesting that NFAT might have a general role in calcium induced gene expression (West, Griffith and Greenberg, 2002).

### 1.2.4 CACNA1C IN NEUROGENESIS AND BRAIN FUNCTION

Due to the post-synaptic localization of $Cav_{1.2}$ channels, CREB-mediated gene expression can be induced in neurons in response to neurotransmitters and neurotrophic factors, and lead to long-term changes in the developing brain through the modulation of neuronal differentiation, survival, and morphogenesis (Deisseroth *et al.*, 2004; D'Ascenzo *et al.*, 2006). Not only does immunocytochemistry show a clear correlation between $Cav_{1.2}$ expression and neuronal-lineage differentiation in cultured progenitor cells (D'Ascenzo *et al.*, 2006), but pharmacological manipulation of $Cav_{1.2}$ using antagonists blocks this effect. In an *in vivo* mouse model with a conditional hippocampal deletion of CACAN1C, although the size of the stem cell pool was not affected, there was an increase in differentiation in the astrocytic lineage and a reduction in the neuronal lineage (Völkening *et al.*, 2017). Moreover, $Cav_{1.2}$-deficient mice display an increase in neuron death in the hippocampus, showing that LTCC activation is necessary for neuronal survival (Lee *et al.*, 2016).

The effects of CREB in neurogenesis are likely due to its target BDNF, which is highly expressed throughout the hippocampus and cortex, and plays an important role in neuronal survival and differentiation (Tao *et al.*, 1998). Expectantly, expression of BDNF is tightly linked to neuronal activation, increasing within 30 min of membrane depolarization (Tao *et al.*, 1998). As a trophic factor BDNF is required for the survival of neurons in development, particularly in the hippocampus and cortex, and in its absence neurons die at an accelerated rate of 50% (Tao *et al.*, 1998). Moreover, post-natal deletion of CREB leads to rapid neurodegeneration in the striatum and hippocampus, reinforcing the importance of CREB-induced gene activation in the brain (West, Griffith and Greenberg, 2002).

The effects of knockdown and inhibition of CREB/BDNF in rodent models are reminiscent of cognitive symptoms implicated in BD and SCZ, suggesting a common molecular pathway in which dysfunction or dysregulation of Ca2+ influx through $Cav_{1.2}$ affects downstream activation of CREB/BDNF signalling (Moon *et al.*, 2018). This involves downstream generation of long-lasting forms of synaptic plasticity, necessary for hippocampal spatial memory formation (Lee *et al.*, 2012)

### 1.2.5 CACNA1C IN CARDIOVASCULAR PHYSIOLOGY

$Cav_{1.2}$ channels are the primary LTCC in the heart, having essential roles in both cardiomyocytes and the sino-atrial node (SAN) (Harvey and Hell, 2013). In cardiomyocytes, $Cav_{1.2}$ channels is located along the T-tubules, which are invaginations of the plasma membrane that facilitate and coordinate depolarization with muscle contraction. Ca2+ entering through $Cav_{1.2}$ activates intracellular ryanodine receptors, which in turn trigger release of Ca2+ stores from the sarcoplasmic reticulum (Kushnir and Marks, 2010) and activate troponin C and enable interaction of myosin with actin filaments. As such, $Cav_{1.2}$ is essential for the excitation phase of the cardiac cycle, contributing to the first step in the contraction.

Moreover, $Cav_{1.2}$ channels tightly associate with signalling molecules such as PKA and second messengers such as cAMP (Balijepalli *et al.*, 2006), coupling muscle contractility with hormonal activation (Striessnig *et al.*, 2014). In the fight-or-flight response, in which the sympathetic nervous system triggers the release of catecholamine hormones, β-adrenergic receptors on the surface of cardiomyocytes are stimulated by epinephrine and norepinephrine (Weiss *et al.*, 2013). The β-adrenergic receptor is a G protein that increases intracellular cAMP, and in turn cAMP-dependent protein kinases such as PKA (Balijepalli *et al.*, 2006). PKA in turn phosphorylates sites on the C-terminal domain of $Cav_{1.2}$, and in so doing attenuates channel inactivation and therefore increases channel activity (Lei *et al.*, 2018). In physiological terms, β-adrenergic stimulation therefore leads to a signalling cascade culminating in prolonged $Cav_{1.2}$ activation and therefore increased force of muscle contraction and increased blood pressure.

The mechanism of action of $Cav_{1.2}$ in arterial smooth muscle is similar, in which influx of Ca2+ by $Cav_{1.2}$ allows muscle contraction. Intravascular pressure stimulates the depolarisation of myocytes in the artery, leading to activation of $Cav_{1.2}$ channels and therefore Ca2+ influx and vasoconstriction (Fan *et al.*, 2018).

As delineated in the sections above, CACNA1C has an important and complex role both in signal transduction in myocytes and activation of gene expression programmes in neural cells. It is of no surprise that mutations affecting its protein sequence may have significant physiological effects, either by causing variation in channel gating kinetics or its regulation by intracellular molecules. However, it appears that the vast majority of CACNA1C SNPs associated with psychiatric disease are in fact located in intronic, or non-coding, regions of the gene (Keers, Farmer and Aitchison, 2009). Although these sequences are typically excluded from mRNA prior to translation, they can affect $Cav_{1.2}$ function by influencing its transcription and expression. Splicing is an important regulatory mechanism of gene expression and function, particularly in eukaryotic genomes.

## 1.3 ALTERNATIVE SPLICING AS A GENETIC REGULATORY MECHANISM

### 1.3.1 EUKARYOTIC GENE ARCHITECTURE AND ALTERNATIVE SPLICING

Eukaryotic genes are typically transcribed as long precursor mRNAs, containing short protein-coding regions called “exons” that are interrupted by long non-coding stretches known as “introns” (Staley and Guthrie, 1998). Introns are removed during or shortly after transcription in a process known as splicing, and flanking exons are ligated together to create a final mRNA product that can direct protein synthesis (Fairbrother *et al.*, 2002).

The existence of introns in a genome places a substantial burden on organisms, slowing down transcription and increasing energy consumption required by cells (Chorev and Carmel, 2012). Moreover, introns vary greatly in length and show rapid sequence divergence between species (Staley

and Guthrie, 1998). Derisively labelled as “junk DNA” (Pennisi, 2012) when they were first discovered, introns were thought to have arisen as remnants of viral insertions in the genome or as selfish genetic elements that have persisted because of genetic bottlenecks and subsequent gain of function mutations within their sequences (Lynch, 2002).

Although it is theorized that introns in early eukaryotes were non-functional, it is now clear that they serve as essential repositories of regulatory elements and participate in tissue-specific and spatiotemporal transcriptional regulation (Chorev and Carmel, 2012). Introns are known to positively regulate gene expression levels (Buchman and Berg, 1988; Chorev and Carmel, 2012), mRNA degradation (and therefore the duration of gene expression (Kalyna *et al.*, 2012), and subcellular mRNA localization (Chorev and Carmel, 2012).

However, the most versatile mechanism by which introns modulate cellular function is through alternative splicing. In this process, the inclusion or exclusion of individual exons or introns within a gene in the final mRNA transcript can vary based on sequence-specific or cell-specific signals. Proteins translated from alternatively spliced transcripts of the same gene may therefore have differences in how they function or interact with other intracellular or extracellular molecules.

Over 95% of eukaryotic genes are known to undergo alternative splicing, displaying developmental stage-specific, age-specific, and tissue-specific expression diversity (Pan *et al.*, 2008). The sites at which introns are excised and exons are ligated are known as splice junctions, and are dependent on an interaction between splicing motifs within the genetic sequence and the spliceosome, a dynamic molecular complex assembled on nascent pre-mRNA molecules (Staley and Guthrie, 1998).

### 1.3.2 MECHANISMS OF ALTERNATIVE SPLICING

The spliceosome is a ribonucleoprotein complex composed of five small nuclear RNAs and between 50 to 150 proteins (Will and Lührmann, 2011). The structure interacts with the nascent transcript at three locations in the sequence: the 5’ splice site, the branch point sequence, and the 3’ splice site. After the first subunit of the spliceosome binds to a 5’ splice site sequence, it cleaves the transcript and guides it through a series of conformational changes involving two consecutive phosphoryl transfer reactions. First, the intron is coerced into a lariat structure with the 5’ splice site associating with an adenosine in the branch point sequence; the 3’ splice site is then cleaved and the exons flanking either side of the intron are ligated together (Staley and Guthrie, 1998).

The spliceosome is assembled anew for each nascent transcript, interacting with RNA polymerase II in mammals to ensure that mRNAs produced are ready for translation and protein synthesis. The interaction of the spliceosome with RNA Pol II is bidirectional; not only is spliceosome assembly dependent on the activation of RNA Pol II, its activity is also known to upregulate and facilitate future rounds of transcription. In fact, intron-mediated enhancement of transcription is well-

documented, and insertion of certain intron sequences into a vector can increase its expression by several orders of magnitude (Chorev and Carmel, 2012).

In order to identify exon-intron boundaries, the spliceosome typically searches for a pair of closely spaced splice sites in the gene being transcribed, and exon boundaries are then defined by the binding of U1 and U2 snRNPs with associated splicing factors (Berget, 1995). However, although this process is strongly dependent on the attachment of spliceosome components to complementary sequences, there is some heterogeneity in the sequences that will be accepted by the spliceosome depending on its composition and on the splicing factors available (Chorev and Carmel, 2012).

Although some splice sites will be recognized and cleaved by the spliceosome every time, weaker splice sites will be missed, at least in certain tissues or under certain conditions (Nilsen and Graveley, 2010). As a result, exons may be excluded in certain tissues, leading to alternative splice variants that are translated into proteins with differences in functionality. Following this notion, it is useful for a cell to be able to regulate the expression, inclusion, or exclusion of exons to generate functional diversity using the same gene. In fact, introns flanking exons exhibiting tissue-specific inclusion often contain signals that inhibit splicing (Zavolan *et al.*, 2003). Nonetheless, these sequence-specific signals are only consensus sequences and are not able to provide the specificity and degree of control needed for fine regulation of transcript expression, and the recognition of exons by the spliceosome requires a series of interactions between trans-acting factors such as proteins and riboproteins (Douglas and Wood, 2011).

Exonic splicing enhancers (ESEs) are responsible for the recognition of weak exons (Fairbrother *et al.*, 2002). ESE's are often purine-rich and are found in proximity to splice sites within introns and exons, and bind to splicing factors known as SR proteins (Berget, 1995). These SR proteins include protein families such as Nova, RBFOX, nPTB, and SF2 (Lipscombe, Andrade and Allen, 2013), and express tissue specificity. For example, Nova-1 is primarily expressed in inhibitory neurons, and binds to intronic motifs encoding YCAY (Y denoting either cytosines or thymines) (Ule *et al.*, 2005). On the other hand, Fox1 is expressed in heart and skeletal muscles and binds to intronic motifs encoding GCAUG (Jin *et al.*, 2003). ESEs often function additively, with multiple ESEs found in proximity to the same splice site (Han *et al.*, 2005).

Expectedly, tissue-specific alternative exons are typically flanked by longer introns that are able to contain more of such regulatory sequences and serve as scaffold elements to ensure the correct assembly of nucleosomes (Vinogradov, 2006). However, intron length is extremely heterogeneous, and in fact genes with longer first introns may be less transcriptionally active on average (Bieberstein *et al.*, 2012), due to the fact that the expression of corresponding genes is entirely silenced in certain tissues. Conversely, genes with shorter first exons have a robust transcriptional profile that is relatively uniform across tissues, and these are characteristic of many house-keeping genes (Zavolan *et al.*, 2003).

Besides regulating exclusion and inclusion of exons, many genes also contain mutually exclusive exons. Alternative splicing of mutually exclusive exons essentially results from competition between pairs of splice sites for splicing factors and for recognition by the spliceosome (Stamm *et al.*, 2000). In a model using predicted splice sites across the genome, it was found that for exons that are tissue-specific or developmentally regulated, splice sites have a tendency to be weaker and are less well defined (Fairbrother *et al.*, 2002). These findings were validated using transcriptomic data of tissue-specific exons expressed in neurons, blood, liver, muscle, testis, and thymus tissues (Stamm *et al.*, 2000). Splice site strength is therefore a key determinant in the expression profile of a given transcript, and in fact large human introns have been found containing sequences that resemble splice sites and are flanked by "pseudoexons", which resemble exons in terms of length but are not known to be ever spliced (Wang and Burge, 2008), suggestive of alternative exons that have been eliminated as a result of purifying selection.

Splicing patterns may also be unique to developmental stages as well as tissues-specific, for example changes in intron inclusion or exclusion appear to shift during neuronal development (Luisier *et al.*, 2018). Studies of iPSC neurons show that while intron retention is common during early stages of differentiation, at later stages the majority of alternative splicing events shifts to intron skipping or alternate exon usage(Luisier *et al.*, 2018). Intron retention typically results in decreased gene expression, as intronic sequences may contain premature stop codons or may lead to mRNA degradation through nonsense-mediated decay (Liu *et al.*, 2017). The majority of genes with higher rates of intron retention in adult neurons appear to correspond to genes with effector functions relevant to non-neuronal tissues, such as pluripotency, DNA replication, and cell cycle, and genes with lower intron retention rates appear to be enriched in those relevant to neurotransmitter transport and synapse function (Braunschweig *et al.*, 2014). It is possible that intron retention is a mechanism by which genes irrelevant to cell type-specific functions are downregulated. In fact, throughout differentiation there may be an increase in the availability of splicing factors required to induce intron skipping in genes relevant to neuronal function, progressively increasing cell type specific functions while preventing excessive expression of un-needed genes until their function is required by more mature cells.

Introns may also include functional elements such as microRNAs, small nuclear RNAs, and piwi-interacting RNAs, and small-interfering RNAs. These are embedded within introns and only expressed upon intron removal (Chorev and Carmel, 2012), and are often involved not only in cis-acting gene regulation, but in silencing or modulating expression of other genes. Moreover, introns are often sites of antisense genes, which are activated during transcription rather than as a result of intron excision (Reis *et al.*, 2005).

Lastly, splicing may modulate gene expression by its interaction with transcriptional machinery in the cell as well as with epigenetic markers. For example, the U1 snRNP is known to bind to RNA

Polymerase II (Bieberstein *et al.*, 2012), and enhancing formation of the pre-initiation complex stimulating transcription elongation (Damgaard *et al.*, 2008). Chromatin-associated elements have also emerged as significant in the regulation of splicing (Oesterreich, Bieberstein and Neugebauer, 2011); removal of either intronic sequences from the first intron of a gene (and therefore eliminating splicing) reduces the levels of methylation marker H3K4me3 and acetylation marker H3K9ac in chIP-seq data (Bieberstein *et al.*, 2012), and therefore transcription. Moreover, H3K3me3 also has been shown to interact with the U2 snRNP and promote splicing and transcription (Sims *et al.*, 2007).

### 1.3.3 IMPACT OF ALTERNATIVE SPLICING ON PROTEOME DIVERSITY

It has been proposed that the availability of alternative protein isoforms resulting from splicing is the key to eukaryotic complexity (Tress, Abascal and Valencia, 2017). By creating multiple mRNA variants from the same gene, alternative splicing has the potential to greatly diversify the proteome beyond the number of genes present in the genome (Chorev and Carmel, 2012). In fact, alternatively spliced exons in the humans were shown to predominantly map to surface regions of protein, increasing the likelihood they may affect protein function or protein interaction with second messengers and enzymes (Douglas and Wood, 2011).

Several large-scale computational analyses of transcriptomic data demonstrated the impact of tissue-specific alternatively spliced exons on intracellular signalling cascades. Tissue-specific exons typically map to disordered domains in proteins that lack a well-defined structure (Buljan *et al.*, 2012; Ellis *et al.*, 2012). Moreover, these domains are enriched in protein-interaction motifs (Ellis *et al.*, 2012). As such, tissue-specific exons are predicted to be key factors in shaping the protein-interaction network within a cell, and the inclusion or exclusion of an exon can in turn modify the activity of entire signalling pathways (Buljan *et al.*, 2012).

### 1.3.4 TECHNIQUES IN THE STUDY OF ALTERNATIVE SPLICING

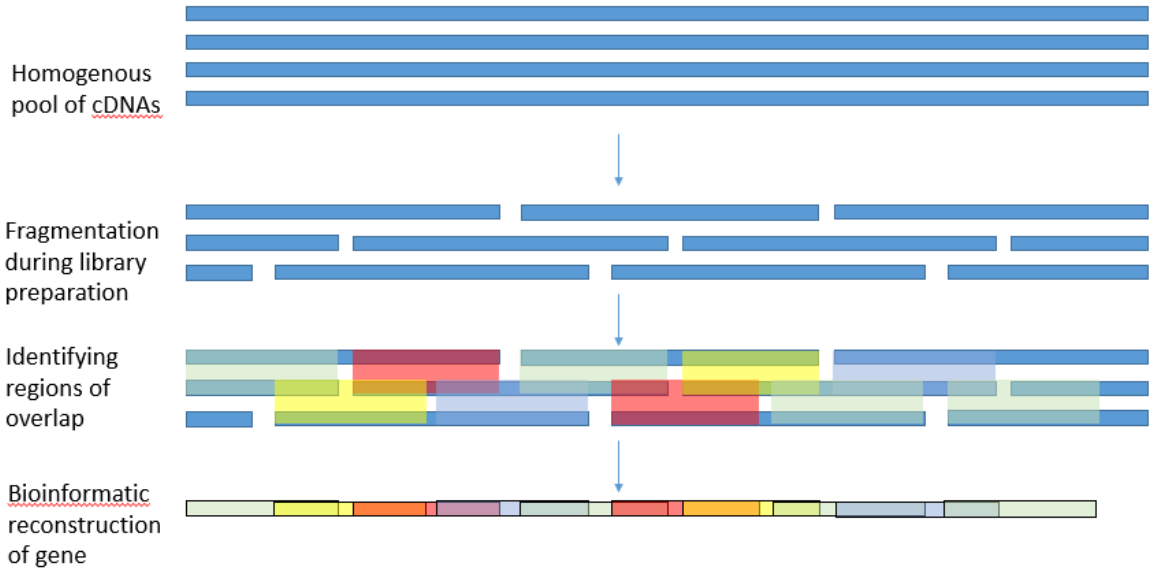
Until recently, the identification and characterisation of alternatively spliced transcripts was wrought with challenges. Unlike proteins or DNA, which can be easily manipulated and handled once extracted from tissues of interest, RNA is highly unstable and can rapidly degrade at room temperatures in the presence of ubiquitous RNAses present in the environment. Moreover, the stability of RNA prepared from post-mortem samples is often dependent on post-mortem interval, age of death, and the pH of the tissue being handled (Preece and Cairns, 2003).

Most studies on alternative splicing take advantage of microarrays and short-read sequencing techniques. High throughput sequencing of genetic information has grown exponentially more affordable following the development of Next-Generation Sequencing (NGS) technologies. Vastly superior in speed to traditional Sanger sequencing, NGS techniques are based on being able to sequence short fragments of nucleic acids in parallel. This is made possible during the preparation of sequencing

libraries, in which genomic DNA or cDNA is fragmented. Assuming homogeneity in cDNAs extracted from the same sample, bioinformatic analysis of sequenced fragments can be used to reconstruct the full-length DNA or cDNA sequence by accounting for overlapping regions between each fragment.

However, although this works incredibly well for sequencing of genomic DNA, this approach does not work for studying alternative splice variants. First of all, alternative splicing by definition adds heterogeneity to the pool of cDNAs in a prepared sample, meaning that reordering fragments based on regions of overlap in their sequence cannot perfectly reconstruct a full-length cDNA sequence. Although such discrepancies in overlapping regions may allow researchers to infer that alternative splicing is occurring, and may even identify alternative splicing occurring for individual exons or exon-exon junctions located in the middle of a sequencing fragment, it is not possible to reliably match combinations of alternative exon usage in transcripts that are longer than the sequencing library's fragment length.

**Figure 2: Analysis of genetic information with NGS**




**The figure above outlines the process by which bioinformatic analysis must be used to reconstruct full-length sequences based on libraries of fragmented cDNAs, based on the assumption that the pool of nucleic acids in the library is homogeneous.**

Taking a different approach, Effengerger and colleagues attempted to generate a high throughput screening assay that uses small molecule inhibitors of the spliceosome in order to identify the contribution of different components of the splicing machinery in a cell to alternatively spliced transcripts (Effenberger, Urabe and Jurica, 2017). A separate study using a similar approach found thousands of genes whose expression was altered by the depletion of a single splicing factor, PRPF8 (Liu

*et al.*, 2017). However, although these studies demonstrate the extent of alternative splicing in the transcriptome, they are still focused on splicing at an exon level rather than a transcript level.

There have been attempts to study splicing by analysing data on expressed sequence tags (ESTs), which are short partial cDNA sequences (Boguski, 1995), but these have run into the same issue as the full length transcript must be inferred from reconstructed sequences combining multiple ESTs. It is evident that transcriptomic annotations are vastly incomplete and do not reflect the whole range of transcript variation present even in well-studied model organisms such as mice (Mortazavi *et al.*, 2008). As reviewed in the literature, the number of alternative splice variants of a given gene appears to correlates with the depth of the sampling in any technique, and is unlikely to ever be complete using short-read sequencing approaches (Zavolan *et al.*, 2003).

Recent technological advancements have however made the sequencing of full-length cDNA possible. The Oxford Nanopore MinION platform is based on using biological pores embedded in an electrically charged membrane. These pores are permeable to single molecules of nucleic acids, which will disrupt the flow of ions passing across the pore at any given time. However, the disruption of current across a pore by a given hexanucleotide sequence has a unique pattern, which can be detected by a sensor and recorded. Using basecalling algorithms, it is therefore possible to convert recorded patterns of current fluctuations across a current into nucleotide sequences of cDNAs.

As full-length molecules can be read, the bioinformatics burden of the MinION platform is much lower than NGS techniques, as there is no need for reassembly of fragment cDNA. Moreover, once reads are produced they can be directly aligned to the genome and alternative splice variants can be identified and characterised.

## 1.4 AIMS AND OBJECTIVES

### 1.4.1 IDENTIFICATION OF CACNA1C SPLICE VARIANTS

$Cav_{1.2}$ is known to have multiple functionally diverse isoforms produced through alternative splicing of pre-mRNA. Splice variants in CACNA1C have been shown to be spatially and temporally regulated, many variants being tissue-specific or changing expression levels during development.The CACNA1C gene spans over 700kb and is known to code for 50 exons, at least 20 of which have been identified to be alternatively spliced. Splice variation in CACNA1C has been associated with differences in the $Ca_{v1.2}$ channel's inactivation kinetics (Soldatov *et al.*, 1998), voltage sensitivity (Tang *et al.*, 2004), and susceptibility to calcium channel antagonists (Welling *et al.*, 1997). Calcium ion flow through $Ca_{v1.2}$ channels is known to affect neuronal gene expression through interaction with multiple intracellular pathways, including CAMKIV signalling and MAPK signalling (Bhat *et al.*, 2012), the phosphoinositide cycle (Levy and Janicak, 2000), and the activation of the CREB/CBP transcription factor complex, and

inactivation of histone deacetylases (Berridge, 2014). As such, functional changes in $Ca_{v1.2}$ channels may have implications for downstream gene expression.

The last attempt to comprehensively characterize transcriptional variants of CACNA1C used a transcript-scanning approach. Using a series of primers covering overlapping regions of the CACNA1C gene, Tang et al were able to identify the presence of 20 possible sites of alternative splice variation (Tang *et al.*, 2004). However, this experiment was done on a single human tissue sample and was limited in that it could only identify sites of alternative splicing, but was not able to characterize the different combinations in which exons assemble into full-length transcripts, nor identify their distribution across tissues.

Using a novel approach combining RT-PCR amplification with cDNA sequencing through the Oxford Nanopore MinION plaform, this project aims to comprehensively characterize the full range of possible splice variants of CACNA1C in order to allow further studies on their functional relevance. This will be the first time that full-length CACNA1C transcripts will be sequenced from rodent tissues.

### 1.4.2 CHARACTERIZATION OF TISSUE-SPECIFIC EXPRESSION OF CACNA1C SPLICE VARIANTS

CACNA1C is expressed in a range of tissues, including brain, cardiomyocytes and arterial smooth muscle (Uhlen *et al.*, 2015). The literature reveals that there are splice variants known to be differentially expressed in each of these tissues (Hofmann *et al.*, 2014), and the expression of two different isoforms of CACNA1C have been observed to shift during the development of cardiomyocytes in rats (Wang *et al.*, 2006).

Disease-associated SNPs across the genome have been demonstrated to be enriched in genes expressed in cell types relevant to disease (Boyle, Li and Pritchard, 2017). For example, SNPs associated with schizophrenia in GWAS are enriched in neuronal expression, while genes expressed only in peripheral tissues contribute little to disease risk (Boyle, Li and Pritchard, 2017). As such, it is important to not only study genes in their broader function, but also take a closer look at cell type-specific contexts of gene expression and regulation.

More importantly, tissue-specificity in splice variants may be a clinically relevant target for the development of pharmacological therapies. Given both the effectiveness of calcium channels antagonists in the treatment of manic symptoms in bipolar disorder, as well as their lower teratogenic potential compared to lithium, $Cav_{1.2}$ is an attractive target for drug development. However, to date the expression of this channel in peripheral tissues has rendered it unsuitable, as currently available calcium channel antagonists would give patients undesired systemic cardiovascular side-effects. Comparing sequencing reads from cDNAs from brain and cardiovascular tissues, this project aims to identify splice

variants enriched in brain tissues that may be specifically targeted for future drug development in psychiatric disease.

### 1.4.3 ANIMAL MODELS IN PSYCHIATRIC DISEASE

Currently, a characterization of the full transcriptional profile of CACNA1C and its splice variants is believed to be incomplete, even in rodents. Given the need for animal models in order to conduct future research on the role of CACNA1C in psychiatric disease, it would be valuable to gain a full understanding of transcriptional regulation in a system that is amenable to genetic manipulation and behavioural testing. Given the already existing literature using mice and rats as models in psychiatric disease, and the homology of CACNA1C to both species, this project aims to elucidate the validity of using rodent models in psychiatric disease.

## 2. METHODS AND OPTIMISATION

### 2.1 INTRODUCTION

Our approach to capturing the full range of alternatively spliced transcripts of CACNA1C involved an combining RT-PCR to with our adaptation of the existing Oxford Nanopore Technologies protocol SQK-LSK108 for sequencing genomic DNA. In summary, following RNA extraction from our tissues of interest, we used a reverse transcription reaction and subsequently performed long-range PCR to isolate and amplify cDNAs of transcripts from our gene of interest. Following multiplexing and library preparation, samples were sequenced using a Nanopore MinION and analysed through a customized bioinformatics pipeline to identify novel splice isoforms and their distribution across tissues.

### 2.2 SAMPLE PREPARATION

Snap-frozen brains, hearts, and aortas were obtained from eight C57BL/6 mice (4 male, 4 female) and 8 Sprague-Dawley rats (4 male, 4 female). Upon arrival, brains were further dissected to isolate samples from frontal cortex and cerebellum samples, and hearts were cut to facilitate subsequent tissue homogenization.

Brain-derived tissues were homogenised using the Qiagen TissueLyser system. However, heart and aorta tissues were homogenised using the Qiagen TissueRuptor system instead as they would not homogenise completely otherwise. Bulk-RNA was extracted using the Qiagen RNeasy Kit as per manufacturer's protocol. All samples were diluted to 100ng/ul stocks and stored at -80C until needed.

Concentrations were measured using a Qubit fluorometer calibrated to Broad Range RNA, and an Agilent TapeStation was then used to assign RNA-Integrity Number (RIN) values as a measure of RNA quality to each sample. Only samples with RIN values higher than 7.0 were included in our study.

**Table 3: Summary of RIN values obtained for each sample**

**The table below shows each sample and corresponding RINs obtained following RNA extractions**

| Mouse # | Gender | Frontal Cortex | Cerebellum | Heart | Aorta |
|---|---|---|---|---|---|
| 1 | M | 8.5 | 9.3 | 8.3 | 9.2 |
| 2 | M | 8.9 | 8.6 | 7.9 | 5.6 |
| 3 | M | 8.8 | 8.4 | 8.5 | 8.4 |
| 4 | M | 8.2 | 8.5 | 8.2 | 8.5 |
| 5 | F | 8.5 | 8.0 | 8.3 | 8.1 |
| 6 | F | 8.3 | 7.9 | 7.8 | 8.5 |
| 7 | F | 8.3 | 8.5 | 8.3 | 9.0 |
| 8 | F | 8.4 | 8.7 | 8.2 | 7.8 |

| Rat # | Gender | Frontal Cortex | Cerebellum | Heart | Aorta |
|---|---|---|---|---|---|
| 1 | M | 7.5 | 8.6 | 8.1 | 8.2 |
| 2 | M | 7.5 | 9.3 | 8.4 | 8.4 |
| 3 | M | 7.6 | 8.6 | 8.4 | 8.3 |
| 4 | M | 8.3 | 8.3 | 8.5 | 8.4 |
| 5 | F | 8.4 | 7.9 | 8.2 | 8.5 |
| 6 | F | 7.5 | 7.7 | 8.5 | 8.2 |
| 7 | F | 8.4 | 6.1 | 8.8 | 8.5 |
| 8 | F | 7.7 | 9.1 | 6.4 | 8.3 |

A limitation of our data is that as the tissues were obtained already snap-frozen, it was not possible to perform more careful dissections and isolate more specific brain regions, nor account for possible differences in splice variation between different heart regions.

## 2.3 REVERSE TRANSCRIPTION

We converted our RNA to cDNA using the Promega GoScript Reverse Transcription System as per manufacturer's protocol. This kit used OligoDT primers to capture denatured, full-length mRNAs. Each RT reaction used 400ng of input RNA and produced 20ul of product, which was aliquoted into four stocks of 5ul each that were stored at -20C.

## 2.4 LONG-READ PCR

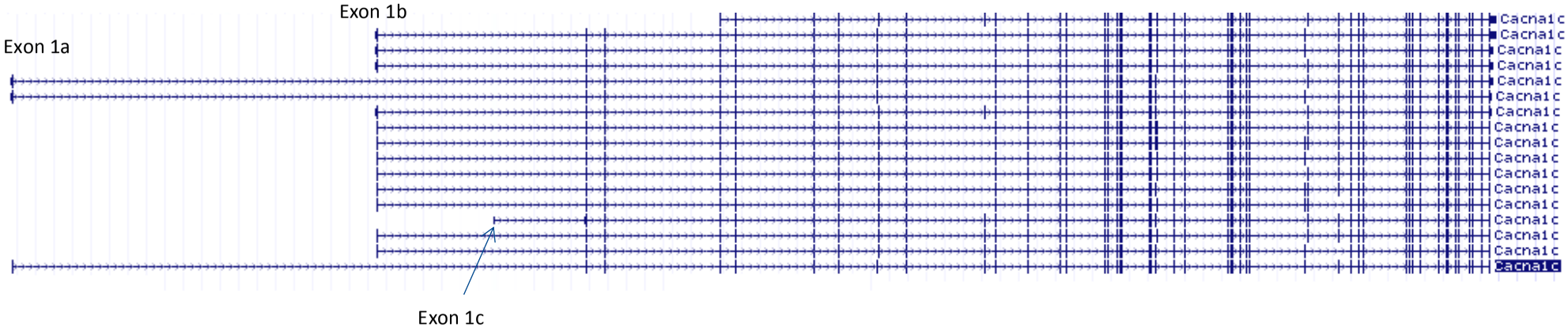
Our long-read PCR experiments used TaKaRa PrimeSTAR GXL polymerase, a high-fidelity polymerase shown to have better capabilities for amplifying long PCR products than other commercially available enzymes(Jia *et al.*, 2015). In order to isolate CACNA1C transcripts, we used primers targeting the beginning and end of the coding sequence of the gene, as shown in **Figure 3**. This would ensure that all amplicons with 5' and 3'-end sequences aligning to CACNA1C would be amplified, regardless of whether there was alternative exon usage or exon inclusion or exclusions within the amplicon itself.

Forward primers were designed by using 5' sequence complementing the sense strand and reverse primers were designed matching the 3' sequence of the same strand for CACNA1C on the UCSC Genome Browser. AnnHyb software was used to determine whether primer sequences were likely to form unwanted dimers or hairpin structures and to check whether annealing temperatures for forward and reverse primers were 1°C apart or less. All of the primer sequences were also checked for target specificity using UCSC BLAT.

As CACNA1C has three known alternative start exons, forward primers were designed to complement the 5' ends of each of the exons. Primer sequences and their locations on the gene are mapped in Figure 3. For the purpose of multiplexing our samples, we appended a nongenomic sequence to the end of our forward and reverse primers, which is compatible with a Nanopore barcoding kit that would allow the attachment of unique barcode identifiers to distinguish transcripts from each of our different samples.

**Figure 3: Genetic architecture of CACNA1C**

**A**




**B**

| Primer location | Primer sequence (A) | Primer sequence (B) |
|---|---|---|
| Mouse Exon 1a (forward) | ACAATGATTCGGGCCTTTGTTCAG | tttctgttggtgctgatattgcACAATGATTCGGGCCTTTGTTCAG |
| Mouse Exon1b (forward) | CCAATGGTCAATGAAAACACGAGGAT | tttctgttggtgctgatattgcCCAATGGTCAATGAAAACACGAGGAT |
| Mouse Exon1c (forward) | GAGATGCTCTGCTGTGCTTTGG | tttctgttggtgctgatattgcGAGATGCTCTGCTGTGCTTTGG |
| Mouse 3'UTR (reverse) | GGACTACAGGTTGCTGACGTAGG | acttgcctgtcgctctatcttcGGACTACAGGTTGCTGACGTAGG |
| Rat Exon 1a (forward) | GTCACAATGATTCGGGCCTTCG | tttctgttggtgctgatattgcGTCACAATGATTCGGGCCTTCG |
| Rat Exon1b (forward) | CAATGGTCAATGAAAACACGAGGATGTAC | tttctgttggtgctgatattgcCAATGGTCAATGAAAACACGAGGATGTAC |
| Rat Exon1c (forward) | GCAGAGATGCTCTGCTGTGC | tttctgttggtgctgatattgc GCAGAGATGCTCTGCTGTGC |
| Rat 3'UTR (reverse) | GGACTACAGGTTGCTGACATAGGAC | acttgcctgtcgctctatcttc GGACTACAGGTTGCTGACATAGGAC |

**A) The figure above diagrams the genetic architecture of CACNA1C and its known alternative splice variants characterized on the UCSC genome browser. Three alternative start sites are known: exon 1a, exon 1b, and exon 1c.**

**B) The table on the left denotes the primers used for amplification of CACNA1C cDNAs from each sample**

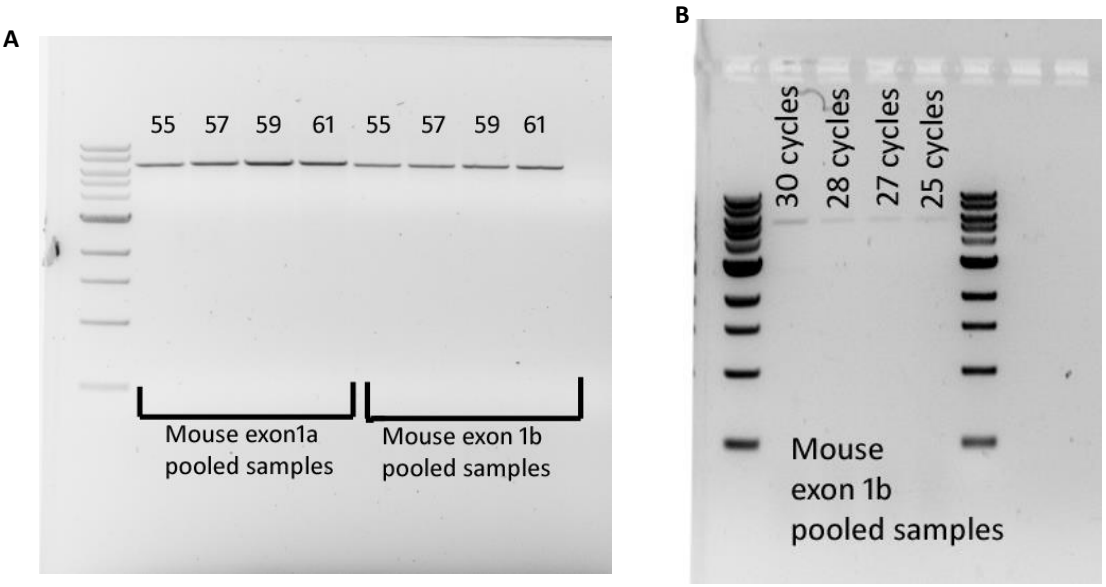
A limitation of this approach is that by targeting the beginning and end of our target gene, we are excluding any transcript variants that have either not yet identified alternative start sites, or transcripts that are truncated. Truncated variants of CACNA1C have been previously reported (Jurkat-Rott and Lehmann-Horn, 2004), but it is unclear whether they have a physiological function or if they are a result of an experimental artefact.

Prior to performing our final sequencing experiment, we first optimised PCR settings by testing different annealing temperatures. These optimisation steps were initially done using primers from column A of Figure 3B, which lacked the nongenomic sequence for barcodes. For each pair of primers we tested annealing temperatures of 55°, 57°, 59°, and 61°C (representative gel image shown in **Figure 4**). cDNA samples from each tissue were pooled and PCR was performed at each temperature, and products were run on a 0.5% agarose/TAE gel stained with Ethidium Bromide and imaged under UV.

In both Mice and Rats, 59°C was determined to be the optimal annealing temperature for both forward primers for Exon1a and Exon1b paired with the same reverse primer that aligned to the beginning of the 3' UTR of the gene. Exon 1c was found to have an optimal annealing temperature of 55°C in mice, but we were not able to amplify it at all in our rat samples.

We further optimised our PCR reactions to find the minimum number of cycles required to get a band. The reason for this was to minimize the possibility of PCR artefacts as much as possible. As full-length CACNA1C has previously been successfully amplified using 30 cycles of PCR, our experiment tried 20, 25, and 30 cycles of PCR. As it was still possible to amplify CACNA1C with 25 cycles, we chose to run our final experiment with the following settings: denaturing temperature of 98°C for 10s, annealing temperature of 59°C for 15s, and elongation temperature of 68°C for 7 minutes (1min/kb) for 25 cycles, followed by a single elongation step at 68°C for 10 minutes and a holding temperature of 4°C.

**Figure 4: Optimisation of PCR Reaction**




**The gel images above are representative images of optimisation steps taken to find the most efficient annealing temperature for PCR (A) and the lowest number of cycles that can be used and still obtain a visible band (B). Optimisation steps were done on pooled cDNA samples.**

### 2.1.3 GEL EXTRACTION AND PCR PURIFICATION

Following PCR amplification, each sample was run on a 0.5% agarose/TAE electrophoresis gel with an NEB BioLabs 6X Blue Loading dye containing glycerol and SDS, stained with GelGreen and imaged under an LED light. Bands corresponding to 6.5-7kb were excised and extracted. This allowed us to exclude any samples shorter than 6kb, which would have been generated as a result of PCR artefact. Due to technical constraints, we were not able to perform all of our gel extractions at the same time, as the electrophoresis equipment available meant we could only run 8 samples at a time. To avoid potential batch effects, samples were randomised using an online list randomiser (www.random.com)

We originally attempted to perform our gel extractions as per manufacturer's protocol using the Qiagen QIAquick Gel Extraction kit. However, measuring cDNA concentration using a Qubit fluorometer after using this kit consistently revealed excessively low yields (too low to measure using the Qubit).

We subsequently switched to the NEB Monarch Gel Extraction Kit. Although we were never able to resolve what the problem was with the Qiagen Kit, we no longer had any problems after our switch to the new NEB Monarch Kit.

## 2.5 MYSTERIOUS HIGH-VISCOSITY CONTAMINANT

An unresolved difficulty we consistently faced following PCR amplification using Takara PrimeSTAR GXL polymerase has been the presence of a tick, highly viscous gelatinous substance in our PCR products, which for the purposes of this discussion will be henceforth referred to as “goo.”

This goo gets stuck in pipette tips, rendering it difficult to handle samples following PCR amplification. It also gets stuck in the wells of agarose gels, appearing to fluoresce brightly when imaged. It is uncertain if the goo contains DNA, though as it does not fluoresce under UV in the absence of Ethidium Bromide or GelGreen in the gel we assume that it does, and that by therefore ignoring it we are losing part of our yield from our amplifications.

This goo consistently appears when amplifying CACNA1C. It has been observed that increasing the number of cycles of PCR also increases the viscosity of the goo, reaffirming the hypothesis that it contains or sequesters DNA in our PCR product. However, besides subjective measures of “gooeyness” based on the difficulty of pipetting the goo, it was impossible for us to objectively quantify the gooeyness of a sample.

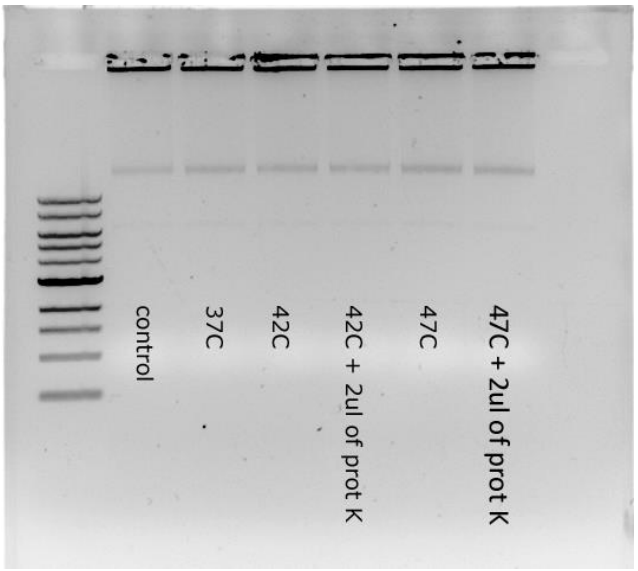
We tried four different approaches to deal with this problem and eliminate the goo from our samples. Our first method consisted of performing a PCR purification using the Qiagen PCR Purification kit. Like with the QIAquick Gel Extraction kit described in the previous section, we had an issue with loss of yield following purification. The goo was effectively eliminated, but that is possibly because we were losing our entire product. We did not continue with this approach at the time, but as since then we have had success in preventing loss of yield in our gel extractions by switching to a commercial kit by a different company, it would be attempting the same thing when replicating this experiment in the future.

The second approach consisted of diluting our samples with $ddH_2O$. By performing a 1:2 dilution, the goo dissolves and sample is easier to handle. However, this meant that the sample had to be split across multiple wells in order to obtain the same amount of cDNA, and that we therefore had to pool multiple bands after excising them to perform gel extraction. As there were still issues with loss of yield following gel extraction at the time, we discontinued this method. However, it would be worthwhile revisiting this approach the future now that we have resolved the issue of loss of yield using a different commercial kit.

Thirdly, we attempted to remove the goo using a Proteinase K digest, in case the goo was in fact proteinaceous in nature. We attempted adding Proteinase K at 2ul as per the manufacturer’s recommendation, and at 4ul, at room temperature and at 42°C and at 47°C. As seen in the gels below, the effect of Proteinase K was minimal. While it does appear to reduce the brightness of the material

stuck in the wells, it does not substantially improve DNA yield nor reduce sample "gooeyness." As a result, we chose to discontinue this approach as well.

**Figure 5: Removal of gooeyness through incubation with Proteinase K**




**The gel image above is a representative image of attempted removal of high viscosity contaminants through proteinase K. There was no significant improvement after 1 hour incubation with proteinase K.**

The last approach, which is what we chose to adopt going forward, was to ignore the goo and pipette around it. We observed that when using a loading dye containing SDS (NEB Gel Loading Dye Blue 6X), the goo within the sample remains unstained and is therefore visible by eye, and easier to avoid.

In conclusion, we were not able to resolve this problem but due to time constraints and lack of progress we chose to ignore it.

## 2.6 MULTIPLEXING SAMPLES

After determining the optimal annealing temperatures for our PCR reactions, we switched to using primers in column B of Figure 3B which had non-genomic sequences appended. Unfortunately, using these primers caused bands in agarose gel electrophoresis to appear fainter. This may be due to secondary structures, hairpins, or dimers forming in our primers resulting in reduced PCR efficiency.

While bands for amplicons starting with Exon 1a and Exon 1b in both mice and rats were still visible, we were no longer able to visualise bands for amplicons with Exon 1c in mice using these primers. Due to time constraints and lack of progress, we decided to not proceed with amplification of Exon 1c amplicons at this time and instead only focus on amplicons containing Exon1a and Exon1b.

Following gel extraction, each amplicons sample had a unique barcode attached to them from the Oxford Nanopore Technologies PCR Barcoding Kit EXP-PCB096. This process consisted of running each sample through a 15 cycle PCR reaction, using the kit's barcodes in the place of primers, with the same settings as the first-round PCR.

After barcoding, samples were quantified on a Qubit Fluorometer.

## 2.7 MYSTERIOUS HIGHER-MOLECULAR WEIGHT BAND

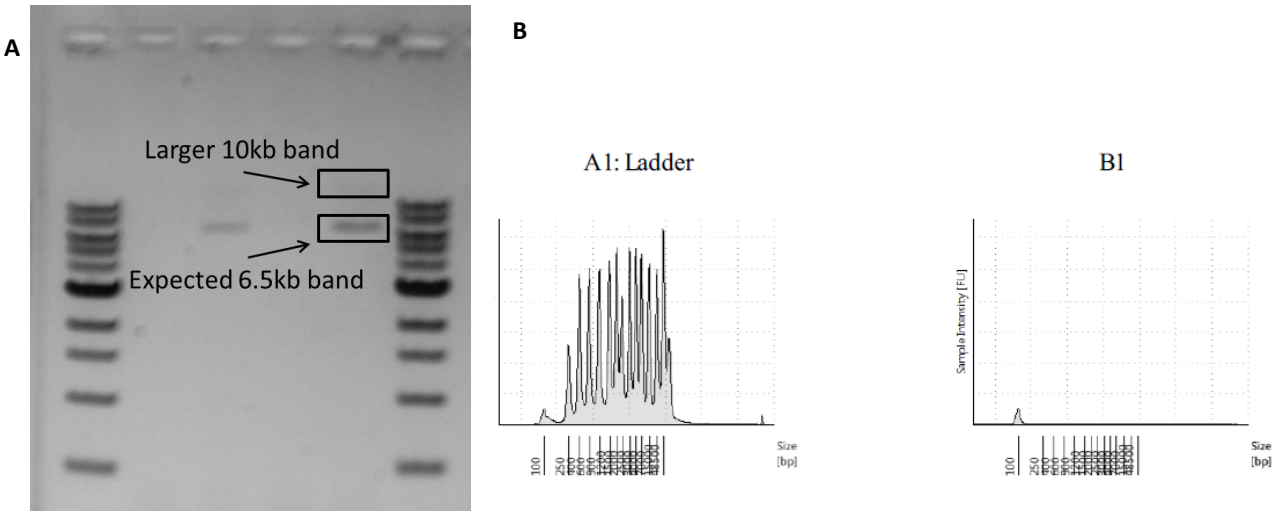
In many of our samples run on agarose gels prior to gel extraction, we observed what appeared to be a faint higher molecular weight band in addition to the 6.5kb band expected of CACNA1C amplicons, in the region of 10kb. We optimistically believed this band may consist of amplicons containing a previously uncharacterized splice variant possibly containing a retained intron.

After attempting to optimise PCR conditions to allow better amplification of this band by increasing elongation time to 10minutes, we failed to observe any increase in band brightness. Furthermore, performing a gel extraction on the band failed to produce sufficient yield to be quantified by a Qubit Fluorometer.

Despite these negative results, we tried to analyse our extracted band using the Agilent DNA TapeStation, which would reveal length of DNA fragments even at low concentrations. Unfortunately the TapeStation failed to reveal any DNA product in our extracted band larger than the standard 6.5kb size expected of CACNA1C.

In the end, we were unfortunately unable to obtain enough of this product to sequence it. Moreover, our TapeStation data suggests that this band was an artefact in our agarose gels; as our amplified product is several kilobases long, it is possible that it may form supercoiled or otherwise non-linear structures that affect its gel migration kinetics.

**Figure 6: High molecular weight band**




**The figure above shows the high molecular weight band found in some of our gels (A). However, when the band was extracted and analysed by TapeStation, no products above 100bp were found.**

## 2.8 LIBRARY PREPARATION

Prior to library preparation, we pooled our barcoded cDNAs into separate libraries for mouse samples and rat samples. An equal amount of cDNA from each sample was obtained from each sample to reach a total of 1ug of cDNA per library. These libraries were then dried using a SpeedVac and resuspended in 45ul of nuclease-free water.

The sequencing libraries for both the mouse and rat samples was prepared using the Oxford Nanopore Ligation Sequencing 1D (SQK-LSK108) kit. First, DNA end-repair and dA-tailing was performed using NEBNext Ultra II End-repair/dA-tailing Module for 5min at 20°C and 5min at 60°C along with a DNA calibration strand (DNA CS) to use as a control. The end-prepped library was then purified with 1x Omega MagBind TotalPure NGS magnetic beads, washed in 70% ethanol, and eluted in 31ul of nuclease-free water, before being once again quantified on the Qubit Fluorometer to confirm at least 85% retention of the original library.

The remaining 30ul library was then subjected to a ligation reaction to attach 20ul of AMX1D sequencing adaptors by co-incubating with 50ul of NEB Blunt/TA Ligase Master Mix for 10min. The adapted library was then purified again with MagBind TotalPure NGS magnetic beads and eluted in 15ul of elution buffer.

## 2.9 MINION SEQUENCING

Flowcells were quality controlled on the MinKNOW software to ensure sufficient pore availability for good-quality reads (at least >1000 pores). Prior to loading, flowcells were primed with a

mix of 480 uL Running Buffer with Fuel Mix (RBF) and 520ul nuclease-free water. We prepared a loading mix of 35ul RBF, 25.5ul library loading beads (LLB), and 14.5ul cDNA library, which was then loaded onto the primed flowcell.

We allowed sequencing to run for 48 hours, after which basecalling and demultiplexing was performed using Albacore software as per recommendation by Oxford Nanopore Technologies.

### 2.10 READ-MAPPING ALIGNMENT PIPELINE

Read mapping was performed by our collaborators Wilfred Haerty and Tomasz Wrzesinki at the Earlham Insitute using a customized bioinformatics alignment pipeline. The pipeline works by using LAST to align our reads to species-specific annotated transcriptomes available on ENSEMBL databases, and identifying any reads that contain mismatches with known transcripts. Mismatches in these novel transcripts are then aligned to the genome to determine whether their sequence corresponds to unannotated regions of the gene found between those exons. Positive results are classified as novel exons.

By aligning reads to both the transcriptome and the genome, the pipeline is able to determine novel isoforms resulting from novel combinations of previously annotated exons as well as identifying unannotated exons.

Following this process, the data from sequencing reads was filtered to only include reads that appeared in at least 100 reads per sample. Although this threshold was arbitrary, the intention was to exclude “novel” splice variants that may have been generated as a result of sequencing errors and could therefore have been artefacts of our data analysis pipeline.

### 2.11 PREDICTING IMPACT ON AMINO ACID SEQUENCE

Nucleotide sequences from our finalised reads were translated to amino acid sequences using the online translation tool ExPaSy. Amino acid sequences were then aligned to known isoforms of CACNA1C on UniProt, where protein domains have been annotated. This allowed us to directly observe the impact of transcriptional variation through deletions, insertions, and alternative exon usage at the protein level.

## 3. RESULTS

### 3.1 AMPLIFICATION OF TRANSCRIPTS WITH ALTERNATIVE START SITES

It was not possible to cover the full range of transcripts of CACNA1C using our methodology at this time. Although three alternative start exons are known (exon 1a, 1b, and 1c), it was not possible to

amplify amplicons with all three start exons across all tissues. Transcripts containing exon 1a were in high abundance in cDNA isolated from heart tissue, but bands were not bright enough to be extracted from agarose gel electrophoresis following PCR amplification in samples from any of the other tissues. It is possible that its expression was too low, and it may be useful to repeat this part of the experiment in the future using a nested-PCR.

It was however possible to successfully amplify transcripts containing exon1b in both brain regions, as well as aorta and heart samples.

**Table XX: Quantification of Amplicons Following Barcoding**

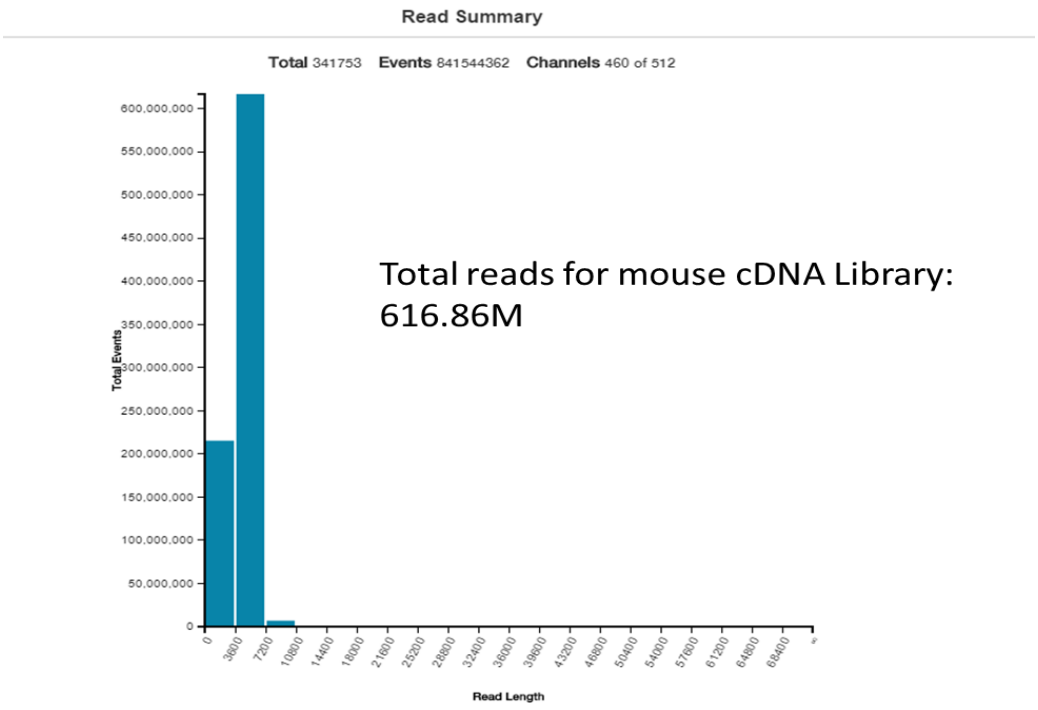
The table above shows the concentration (ng/ul) of each amplicon following our barcoding PCR reaction. At this stage, the samples were ready to be pooled and processed using our library preparation method.

## 3.2 SEQUENCING OF MOUSE AND RAT SAMPLES

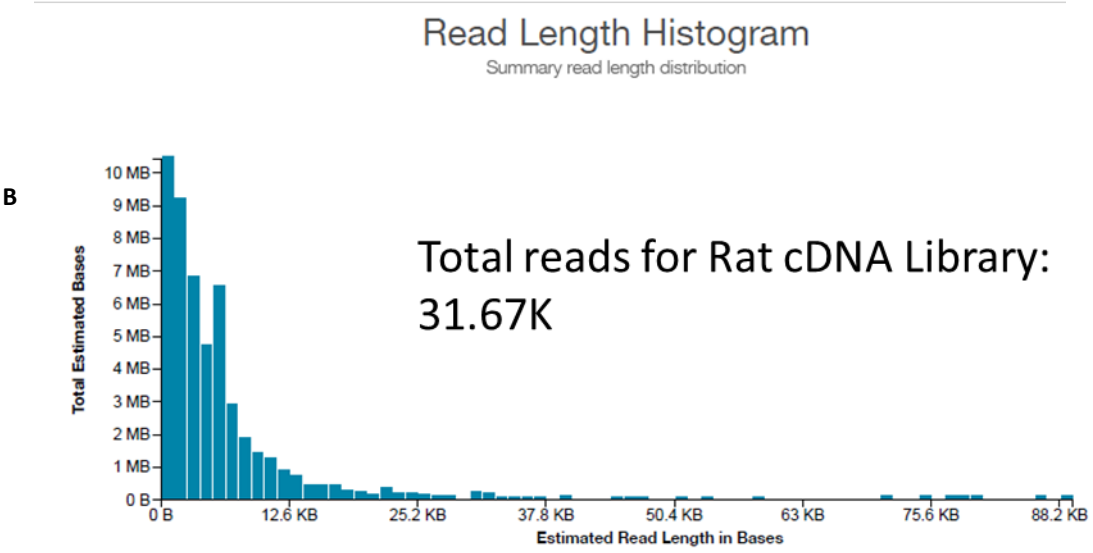
The two sequencing libraries (corresponding to cDNAs from mice and rats, respectively) were sequenced on separate flow cells, and a summary of each sequencing run can be seen in **Figure 7.**

**Figure 7: Read counts and read length distribution histograms**

A




B




**The figure above shows the MinKNOW readout with the number of reads from each experiment and a histogram of read-length distribution. Read length for the vast majority of mouse cDNAs (A) was between 3.6 and 7.2kb, which corresponds to the expected size of CACNA1C transcripts. As the sequencing run for the rat library (B) yielded an incredibly low number of reads, it was discounted and not included in any further analyses. Differences in histogram formatting are due to a software update of MinKNOW between our first and second sequencing run.**

## 3.3 RELATIVE ABUNDANCE OF SPLICE VARIANTS

Our dataset of reads from mouse transcripts found 53 splice variants across the four tissues, 45 of which have not been previously annotated on the ENSEMBL database. Eight of these variants are expected to be noncoding as they encode either a frameshift or a premature stop codon. Unsurprisingly, some of the variants with the highest inferred expression have been previously annotated.

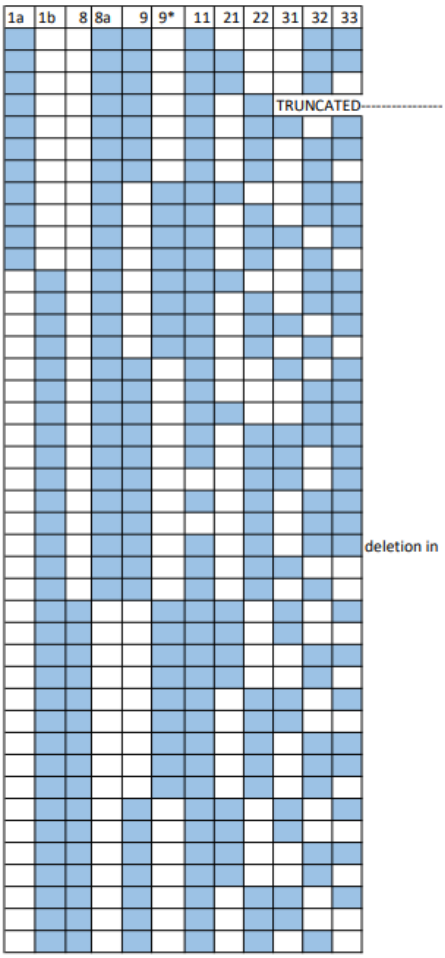
Because the methodology used includes two PCR amplification steps, for a total of 40 cycles of PCR, it is inherently biased towards smaller cDNA amplicons that are more efficiently amplified. As such, it is not directly quantitative. However, it is still possible to infer that transcripts with a higher number of reads are more commonly expressed than reads with low relative abundance. Based on this, as seen in **Figure 8** it was possible to map the relative abundance of transcripts across each of our tissues on a histogram.

**Figure 8: Relative abundance of transcripts**

A

| Transcript_ID | FCX | CER | AOR | HEA B | HEA A |
|---|---|---|---|---|---|
| Cacna1c_novel-2008 | 3.827817458 | 2.287364304 | 0.179605658 | 2.261599736 | 2.250869374 |
| ENSMUST00000187940 | 0.056170675 | 0.560885358 | 0.393012277 | 7.227754234 | 7.942537071 |
| Cacna1c_novel-2634 | 0.461809044 | 0.052475633 | 0.369385556 | 1.342698033 | 2.590941769 |
| Cacna1c_novel-2194 | 5.170938336 | 4.647864436 | 0.01964623 | 0.784202265 | 1.910517385 |
| ENSMUST00000186889 | 5.643400731 | 12.1981597 | 0.178875884 | 2.053499047 | 6.649654135 |
| Cacna1c_novel-894 | 18.23822544 | 14.70513006 | 0.637223483 | 6.020663834 | 22.05886903 |
| Cacna1c_novel-2569 | 7.387287197 | 5.444479219 | 0.240073581 | 3.051980365 | 5.654769672 |
| Cacna1c_novel-248 | 0.398171867 | 0.062831206 | 0.127884948 | 4.00446173 | 6.252536834 |
| Cacna1c_novel-260 | 0.910083512 | 2.260102761 | 0.27684994 | 5.214333079 | 16.01129802 |
| Cacna1c_novel-277 | 0.091525942 | 1.183489716 | 0.041450584 | 3.535453675 | 5.121601645 |
| Cacna1c_novel-1089 | 0.071726524 | 0.462525961 | 0.239915582 | 2.605386053 | 4.354341393 |
| Cacna1c_novel-164 | 0.07656791 | 0.111256932 | 1.169401203 | 46.17916523 | 0.174148518 |
| Cacna1c_novel-212 | 7.815374786 | 26.56658007 | 1.85300926 | 90.0800882 | 0.422323444 |
| Cacna1c_novel-231 | 3.186847869 | 25.2302983 | 0.724122176 | 45.13020769 | 0.099329896 |
| Cacna1c_novel-781 | 2.257363518 | 7.923935512 | 2.273820487 | 29.58463072 | 0.163105508 |
| Cacna1c_novel-1179 | 8.236133718 | 19.42749828 | 0.326443071 | 3.404463099 | 0.039785943 |
| Cacna1c_novel-1240 | 37.6073815 | 27.23161294 | 1.016154825 | 13.76444128 | 0.063403802 |
| Cacna1c_novel-443 | 1.434228894 | 1.593294021 | 1.52447313 | 56.41159301 | 0.216462198 |
| Cacna1c_novel-114 | 15.45841163 | 25.41419536 | 0.014731903 | 10.50543589 | 0.03290386 |
| ENSMUST00000075591 | 127.8920866 | 245.2243183 | 2.525102375 | 48.62936638 | 0.260601058 |
| Cacna1c_novel-1487 | 11.38989772 | 24.18504772 | 0.115956394 | 2.623163244 | 0.025134173 |
| ENSMUST00000112790 | 548.2578187 | 389.9588295 | 5.353942022 | 127.9927152 | 0.536863325 |
| Cacna1c_novel-1513 | 51.16996215 | 37.76411568 | 0.345604639 | 11.49164169 | 0.07443121 |
| Cacna1c_novel-1944 | 15.1964738 | 14.86014816 | 0.013390127 | 7.735209738 | 0.067330227 |
| Cacna1c_novel-2090 | 31.39307621 | 56.896221 | 0.741460511 | 14.76347518 | 0.034779549 |
| Cacna1c_novel-2051 | 138.2995847 | 92.60454589 | 4.878294146 | 37.56043137 | 0.136562914 |
| Cacna1c_novel-218 | 0.368387335 | 1.458849777 | 14.60622677 | 74.1665807 | 0.008243701 |
| ENSMUST00000188106 | 0.927684298 | 1.776897828 | 7.938249023 | 89.37581071 | 0.005318229 |
| ENSMUST00000188522 | 0.717358725 | 0.660560873 | 18.95336552 | 34.0544736 | 0.053060435 |
| Cacna1c_novel-771 | 0.547676585 | 2.1926627 | 33.30154387 | 131.3232514 | 0.043275064 |
| Cacna1c_novel-180 | 0.739621951 | 0.141932647 | 15.27200594 | 24.06054896 | 0.008717775 |
| Cacna1c_novel-852 | 0.211611216 | 0.452186684 | 8.540220826 | 23.08781018 | 0.006817063 |
| Cacna1c_novel-205 | 0.905980433 | 2.134585191 | 21.31678386 | 44.51314981 | 0.013814881 |
| ENSMUST00000188865 | 1.455447894 | 1.054877433 | 15.77875651 | 8.277752792 | 0.012913502 |
| Cacna1c_novel-676 | 0.373058731 | 1.169842555 | 27.37514432 | 57.276541 | 0.008531206 |
| Cacna1c_novel-522 | 0.410415995 | 0.769767912 | 10.26154437 | 27.90269902 | 0.008243701 |
| Cacna1c_novel-1928 | 0.407415661 | 0.917251572 | 9.544757248 | 65.77249308 | 0.005318229 |
| Cacna1c_novel-594 | 0.065159576 | 0.649963263 | 14.30504772 | 17.70969174 | 0.073390289 |
| Cacna1c_novel-2065 | 0.296787041 | 1.721384747 | 25.28294602 | 54.44120756 | 0.007050949 |
| ENSMUST00000188078 | 0.673128896 | 0.493864372 | 14.32694317 | 7.811757752 | 0.010481446 |
| Cacna1c_novel-1638 | 0.303542696 | 0.427107268 | 9.994975369 | 23.49446288 | 0.007276848 |
| Cacna1c_novel-2218 | 1.303328079 | 0.711343271 | 16.00083526 | 24.02911933 | 0.008046567 |

B



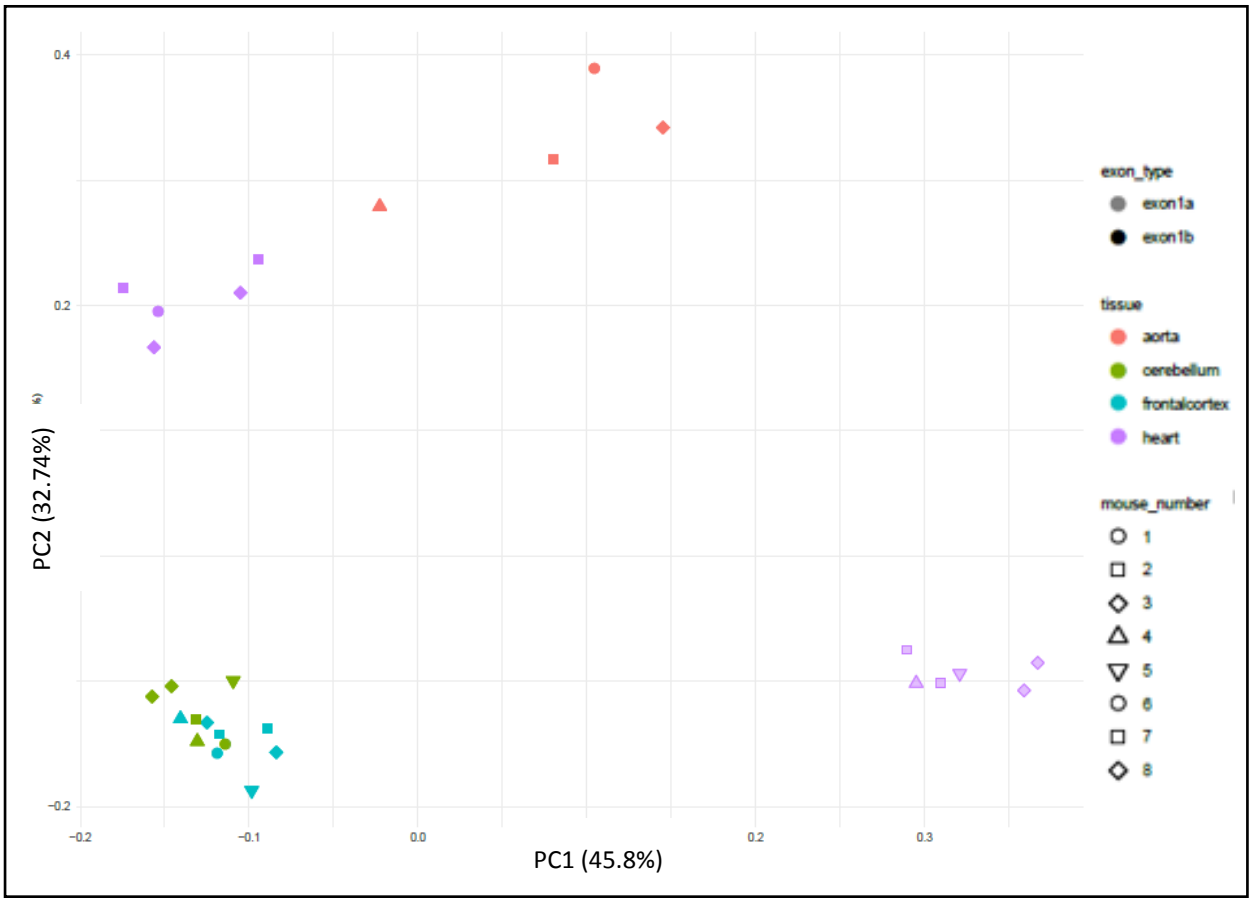

**The figure above uses read counts of each identified transcripts as an indirect measure of relative abundance. (A) displays histograms showing relative transcript abundance for each tissue (A) [FCX =**

**frontal cotex, CER = cerebellum, AOR = aorta, HEA B = heart cDNAs amplified with exon 1b primers, HEA A = heart cDNAs amplified with exon 1a primers]. (B) shows a map of alternatively spliced exons and their presence in each transcript.**

## 3.4 TISSUE-SPECIFIC ENRICHMENT OF TRANSCRIPTS

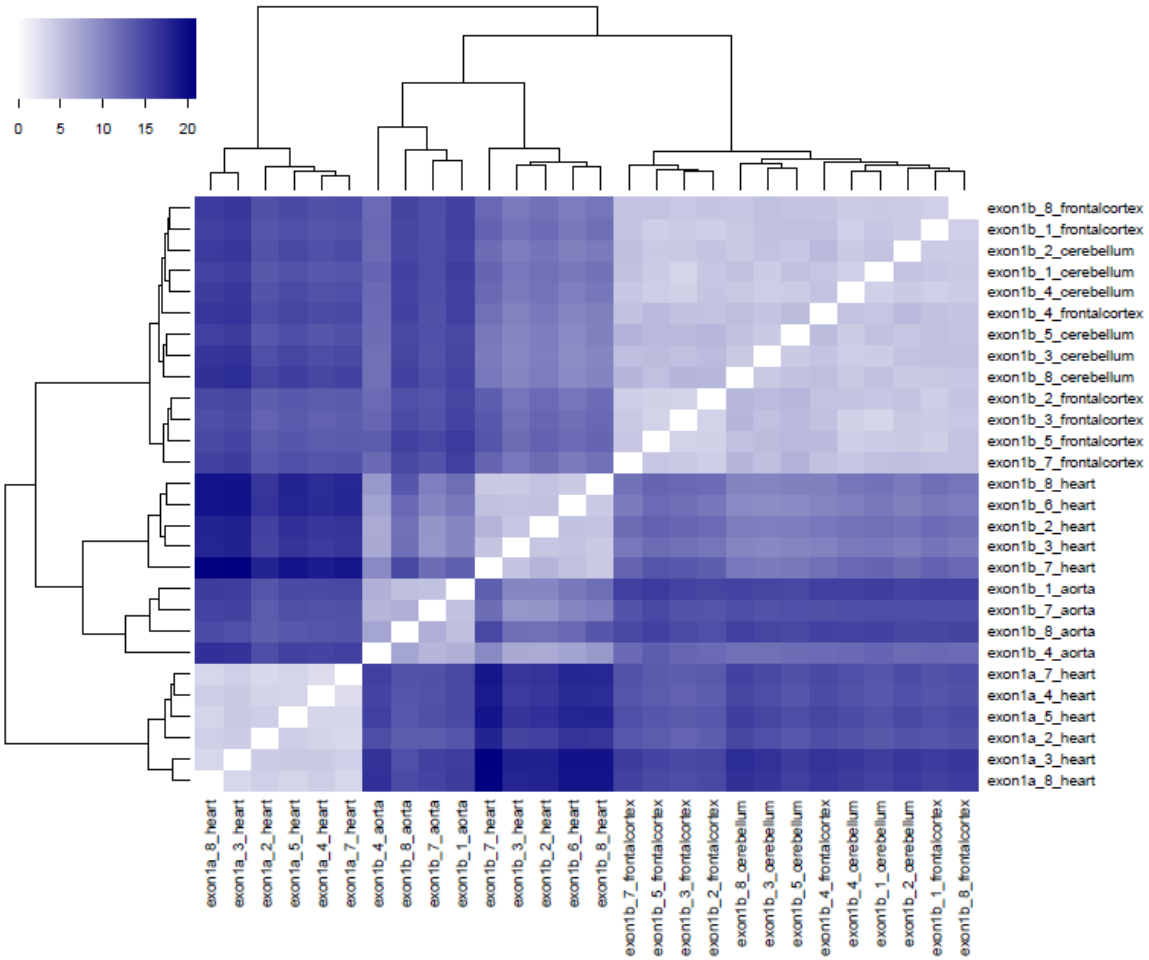
By using principal component analysis of transcript isoforms and their read counts, it appears there is substantial variation among the splice variants expressed in the cardiovascular system and in the brain.

**Figure 9: Principal component analysis of sequencing reads**




**The principal component analysis takes into account the number of reads per transcript per sample. Clustering of transcript isoforms shows that the variation in transcriptional profile between tissues is greater than the variation between individuals. The read count distribution within each sample was therefore less varied for samples from brain regions than it was for samples from cardiovascular tissues.**

**Figure 10: Relationship of transcriptional profiles between samples**

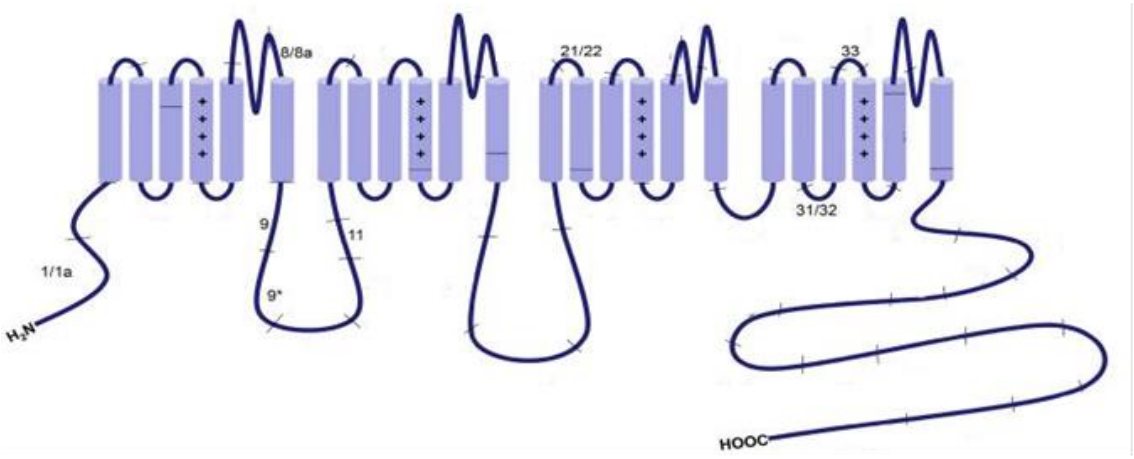



**The heatmap above displays samples in terms of similarity based on read count distribution of each identified transcripts, with light blue denoting less variation between corresponding samples and dark blue denoting greater variation. As can be seen above, read count distribution of samples was less variation between samples from similar tissues and greater variation across tissues.**

## 3.5 MAPPING ALTERNATIVE EXONS ACROSS THE PROTEIN PRODUCT

Each transcript variant was translated and manually aligned to the $Cav_{1.2}$ amino acid sequence on UniProt. It was therefore possible to map sites of alternative splicing to protein domains on the channel. This approach allowed for two things: identification of tissue-specific distribution of exons (shown in **Figure 8)**, as well as identification of which sites on the protein are most susceptible to alternative splicing and therefore tissue-specific functionality (shown in **Figure 11)**.

**Figure 11: Cav1.2 and the location of identified alternative splice sites**




**The figure above displays the protein structure of Cav1.2 and marks the sites of alternative splicing that have been identified in our dataset. The study found alternative splicing occurring in exon 1, exon 8, exon 9, exon 11, exon 21/22, exon 31/32, and exon 33.**

# 4. DISCUSSION

## 4.1 FUNCTIONAL SIGNIFICANCE OF ALTERNATE EXON USAGE

There were nine sites of alternative exon usage found in our study: exon 1, exon 8, exon 9, exon 11, exon 21/22, exon 31/32, and exon 33, as well as the C-terminus. All of these were known in the literature to undergo tissue-specific alternative splicing, but it had not been previously possible to conclusively determine the different combinations in which they were used.

Alternative usage of exon 1a and exon 1b is known to differentiate how $Cav_{1.2}$ responds to PKC stimulation. Exon 1a contains two threonine residues at positions 27 and 31, allowing the channel's N-terminal region to be phosphorylated by PKC and inducing channel inactivation (McHugh *et al.*, 2000). As these PKC binding sites are not present in exon 1b, non-cardiac isoforms of $Cav_{1.2}$ are not subjected to the same regulatory mechanism and remain active despite the presence of PKC. As PKCs are highly expressed in the brain and are involved in several processes overlapping with $Cav_{1.2}$ stimulation, such as long-term potentiation and long-term depression (Saito and Shirai, 2002), neural-specificity in expression of exon 1b over 1a may be useful for preventing erroneous inactivation of $Cav_{1.2}$ channels.

Alternative splicing of exon 1 also contributes to specific changes in subcellular localization and therefore channel activity. Exon 1b transcripts exhibit a two-fold higher surface expression of $Cav_{1.2}$ (Bartels *et al.*, 2018), leading to bigger current densities and enhanced channel activity. Although the mechanism behind this is unclear, the shorter N terminal generated by exon 1b may have increased affinity for β-subunits, which prevent the channel from being ubiquitinated and degraded by the proteasome (Zamponi *et al.*, 2015).

Alternative splicing of Exon 8/8a has an effect on modifying voltage-dependent inactivation of the channels. As they encompass the S5-S6 extracellular linker region of domain I of the channel, they form part of the channel pore. Whole-cell patch clamp of cell lines transfected with alternatively spliced constructs of CACNA1C revealed that transcripts containing exon 8a had longer inactivation kinetics following excitation (Bartels *et al.*, 2018).

Splicing at exons 8/8a, and exons 21/22 also has implications for sensitivity to pharmacological antagonism by dihydropyridine drugs (Wang *et al.*, 2006), with exon 8 (Welling *et al.*, 1997) and exon 22 (Soldatov, Bouron and Reuter, 1995) exhibiting increased sensitivity to dihydropyridines. Our data shows these exons to be enriched in aorta tissue in comparison to brain and heart tissues, which is in line with the existing body of literature showing that LTCCs in smooth muscle are more sensitive to calcium channel antagonists than cardiomyocytes (Wang *et al.*, 2006).

Exons 9 and 9* correspond to an intracellular region linking domains I and II. This region is a primary target for association with β-subunit, and is required for channel inactivation as well as cell

surface expression of the channel (Kobrinsky *et al.*, 2005). Previously, expression of exon 9* has only been reported in scar tissue following ischaemic injury (Liao *et al.*, 2009), and was characterized as exhibiting increased sensitivity to membrane depolarization and inactivation. In our study, exon 9* was only expressed in cardiovascular tissues and was not present in any of the amplicons isolated from the brain.

Alternative splicing at both exon 21/22 and exon 31/32 is known to be subjected to developmental regulation. In ageing human aortic smooth muscle, a switch from expression of exon 22 to 21 is correlated to an increased risk of atherosclerosis (Jurkat-Rott and Lehmann-Horn, 2004). Splicing of exon 31/32 is also known to shift with ageing, though functional differences in their role are yet unclear.

Our data also revealed a transcript isoform with a deletion in the C-terminus, as well as an isoform that was truncated at exon 31. Both of these splice variants were found in cardiac tissue, but at low relative abundance. It is unclear what the functional role of these variants may be, but the C-terminus of $Cav_{1.2}$ is known to contain a number of intracellular protein interaction domains and is also responsible for channel inactivation. In neuronal VGCCs, it has been shown that shorter C-termini correlate to greater current amplitudes and greater calcium-dependent inactivation kinetics (Soldatov *et al.*, 1997). Truncation of the protein at exon 31 on the other hand may not actually form a functional channel, as it would be lacking the majority of the final transmembrane domain. However, it is difficult to dissect based on our data whether this splice variant actually exists or is merely a result of coincidental failed sequencing reads culminating at the same place.

Clearly, although the dataset of full-length transcripts reveals the existence of previously uncharacterised combinations of exons, it is not in itself able to explain their functional significance. Further study is required to dissect the role of individual splice variants, and individual transcripts must be cloned in cell culture to determine electrophysiological properties or differences in intracellular protein interactions. However, the clear tissue-specific enrichment of sets of transcripts holds promise for the future development of tissue-specific pharmacological agents.

## 4.2 EXTENT OF SPLICE VARIATION

As VGCCs are large genes composed of four homologous domains, likely arisen through gene duplication of an ancestral single-domain protein, it is to be expected that they have a large number of splice variants – particularly as splice sites are likely to be repeated in homologous locations across domains. However, it is yet unclear why a single gene requires such a high number of splice variants. Splicing sites are typically conserved among the CACNA1* genes, suggesting that they hold biological significance and confer a selective physiological advantage. Moreover, recent studies using long-read

sequencing on other genes have revealed similarly extensive splice variation (Byrne *et al.*, 2017), which was previously likely simply missed due to the insensitivity of NGS techniques.

There is some debate as to what fraction of the repertoire of alternative splice variants of a gene is functional (Nilsen and Graveley, 2010). In a large mass-spectrometry study, it was shown that although genes may have a number of different transcript variants, proteins are usually found expressed as one main isoform, and other isoforms are likely to be expressed at an extremely low level and may therefore not have a serious impact on cellular function (Gonzàlez-Porta *et al.*, 2013). However, this study adapted stringent filters to reduce the risk of false-positives in their detection of peptide sequences, and it is likely that the lack of sensitivity of the technology used may have caused low expression isoforms to be missed.

It is possible that some of the splice variants arise through stochastic noise in spliceosome machinery. Any biochemical reaction in a cell is subject to a degree of stochasticity, and transcription itself is frequently observed to be an extremely noisy process (Chubb, 2016). As splicing sites within introns must compete with one another for recognition by the spliceosome, it is to be expected that a gene transcribed enough times will be able to express all possible permutations of alternatively spliced exons, merely as a result of probability. Despite this, the number of splice variants found in our study is lower than the possible number of permutations, and appears to occur with high fidelity, displaying clear tissue-specificity for certain transcripts. We attempted to account for potential noise by excluding any reads that appeared less than 100 times in a sample, but admittedly this number was arbitrarily chosen and not a defined cut-off that ensures exclusion of variation due to noise.

# 5. CONCLUSIONS

Despite the success of antipsychotics in reducing delusional and hallucinatory symptoms in patients with BD and SCZ, they do not appear to target cognitive symptoms of the disease. Moreover, they are accompanied by side effects that decrease compliance with treatment regimens and fail to significantly improve living standards using metrics such as unemployment and homelessness (Insel, 2010). As such, there is a high demand for more effective pharmacological therapies.

Calcium channels have shown promise as drug targets in the field of psychiatry. Not only are calcium channel antagonists well-characterized and known to have safe therapeutic profiles, they been demonstrated as effective in the treatment of BD and SCZ. The issue with these drugs, however, is that even at low doses they appear to have cardiovascular side-effects.

Our study shows that the variability between alternatively splice transcripts is much higher than previously characterized. More importantly, a significant proportion of transcript isoforms show tissue-specific expression enrichment. While these results need to be validated at the protein level before they can be applied in drug development, they suggest that brain-specific pharmacological targets of $Cav_{1.2}$ are possible.

As all of our data was derived from mouse cDNAs, the next logical step would be to replicate the study using human tissues in order to validate their relevance to the clinic. Moreover, although the CACNA1C genetic sequence is fairly homologous across mammalian species, it would be interesting to note whether this similarity extends to its splicing profile. If a given splice variant and its tissue-specific expression pattern is evolutionarily conserved, that would signify an important functional physiological role of that splice variant. One future experiment that could be done is a bioinformatics comparison of exon usage across tissues across different species. Such an experiment could be done using available transcriptomic databases, and would be illuminating even if the available data is from short-read sequences rather than full-length transcripts.

Secondly, it will be important to validate the functional role of the full-length transcripts that have been identified in the study. Depending on the region of the gene and splice site of interest, functional analyses can be done in different ways. If the regions subjected to alternative splicing are located in domains relevant to voltage-sensitivity or to channel inactivation, transcripts can be cloned into a neutral cell line in order to perform electrophysiology experiments. If instead, the regions of interest are located in intracellular inter-domain loops, or N- or C-terminal domains, it would be beneficial to use techniques such as co-immunoprecipitation in order to see how alternative splicing affects intracellular interaction networks. If alternative splicing is occurring in domains exposed to the extracellular surface of the cell, it will be beneficial to perform pharmacological sensitivity assays to see if they can be manipulated to modulate channel function and activity.

Lastly, a severe limitation of our protocol is that it does not allow us to directly quantify expression of individual transcript variants. Although this can be inferred based on read counts, the data is inherently biased towards amplicons that have more efficient amplification in PCR. Before any further studies can be done, it will be important to validate the relative abundance of splice variants in a given tissue by qPCR.

In conclusion, the above study has set the groundwork for future studies on the role of CACNA1C in the aetiology and pathophysiology of BD and SCZ, as well as the basis for development of tissue-specific targeted pharmacological agents. The work will have to be replicated and validated, but it is an encouraging start to furthering the field of psychiatric genetics and clinical therapies.

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