Construction and Optimization of CRISPR Cas 9 mediated KCNV1 Knock-out Models to Study Its Effects in Post Mitotic Neurons

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ABSTRACT

Construction and Optimization of CRISPR Cas 9 mediated KCNV1 Knock-out models to Study Its Effects in Post Mitotic Neurons

A thesis presented to the Graduate Program in Molecular and Cell Biology

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According to K Brodmann, the cerebral cortex was derived from a six layered structure and continues to illustrate these distinct regions. In layer V of the neocortex, it has been observed that sub-types of pyramidal neurons show a variance in cell morphology, projections and physiology (Hattox and Nelson, 2007). One such property is cell adaptation. Adapting neurons decrease their firing rate during constant current injection while non-adapting neurons do not. This property may be a result of potassium ion currents that build up during continuous firing by the neurons. KCNV1 is an auxiliary potassium channel sub-unit expressed in adapting sub-types, but not in the non-adapting sub-types and so is hypothesized to play a role in determining cell adaptation. This study involves the use of the CRISPR Cas 9 gene editing system to construct knock-out models for KCNV1 in post mitotic neurons of the mouse neocortex to enable investigation of KCNV1 and its importance in determining firing properties.
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1.0 INTRODUCTION AND BACKGROUND

The goal of the project is to enable the investigation of the role played by KCNV1, a potassium ion channel sub-unit in determining physiological properties of pyramidal neurons by constructing CRISPR Cas 9 mediated knock-out models of KCNV1. The different aspects of this study involves three main areas. First, a vector system was designed to deliver CRISPR Cas 9 components. Second, the design was validated in-vivo using mammalian cell culture. Third, the system was implemented on slice culture of mouse neocortex to enable knock-out of endogenous KCNV1 for conducting electrophysiological studies. Before a detailed explanation of the project, a brief background of the different aspects of the system being studied are given below. The different aspects include a summary of neocortical neurons and their firing properties, the role played by ion channels and potassium currents in firing properties and lastly, an explanation of the CRISPR Cas 9 system, which is the gene editing tool being used in this project.

1.1 OVERVIEW OF CORTICAL NEURONS

Neurons are highly specialized cells and form an integral part of the nervous system and their network highly complicated and diverse. Neurons residing in the cortex of the brain are called cortical neurons. The focus of this study is limited to pyramidal neurons of the neocortex in mammalian brains. The neocortex, consists of sub-divisions where specific regions have specific functions. According to K Brodmann, the neurons of neocortex can be grouped into six distinct layers. The layers V and VI consist of pyramidal neurons which have projections that run down to other regions, like the basal ganglia and thalamus thereby forming cortical
connections (Molyneaux et al., 2007). In mouse cortex, layer V pyramidal neurons that have specific target projections have been known to also possess distinct physiological and morphological properties (Hattox and Nelson, 2007). Differentiation or classification of neurons can be done based on their location, morphology and their electrophysiological properties. Some sub-types of pyramidal neurons possess the property of cell adaptation. On receiving continuous current injections, these sub-types reduce the firing rate and hence ‘adapt’ to the stimuli. Firing of impulses or spiking are brought about by the periodic movement of Sodium and Potassium ions through their respective voltage gated ion channels, termed as an action potential. The detailed aspects of an action potential and the roles of these ions is explained in the next section. However, another important point of note is that all spikes are not the same and due to the intricate roles of the ion channels, each unique spike denotes distinct physiological properties to neuron sub-types (Bean, 2007). It is due to this relation that the property of cell adaptation of neurons has been hypothesized to be associated with the potassium channel sub-unit KCNV1.

1.2 ION CHANNELS AND KCNV1

The voltage gated ion channels play a major role in regulating action potentials in neurons. The potential that is always present on the membrane surface of a neuron is termed as resting potential of around -70mV. Due to excitation, there is a rise in the potential due to inward migration of sodium ions into the cell through the sodium ion channel gate. This process is called depolarization. If the rise in potential reaches a certain threshold potential of around -55mV, then an action potential takes place and travels the length of the neuron and fires a signal. At the rise of potential, the potassium channels open and potassium ions leave the cell. At a potential of 50mV the sodium channels close and the only ion movement is potassium ions exiting the cell, bringing
the membrane potential back down and below the resting potential. This is termed as repolarization. After the membrane potential falls below the resting potential the potassium channel closes, excess potassium ions outside diffuse away the the potential rises back to resting potential. This is termed as hyperpolarization. This complete physiological cycle takes place due to the presence of voltage gated ion channels. Out of the several voltage gated ion channels, the potassium ion channels are the most diverse and largest group, consisting of several sub-types and unique properties (Purves D, et al., 2001).

The potassium ion channel is a tetrameric structure with four major identical subunits forming an ion pore in the middle. It was initially named Kv. The first K+ ion channel was successfully cloned into Drosophila, and thus enabled the conquest for it’s molecular mechanism (Kamb et al, 1987). The four major sub-families were known as Kv1 (Shaker), Kv2 (Shab), Kv3 (Shaw) and Kv4 (Shal). Subsequent research over the years led to the discovery of Kv6.1 and Kv7.1 (Drew et al 1992) and Kv5.1 (Zhao et al 1994) and ultimately the Kv8 or KCNV1 subunit was isolated and characterized (Hugnot et al., 1996). KCNV1 was structurally characterized and was also screened for epileptic disease (Ebihara et al, 2004). The sub-units Kv5, Kv6, Kv8 and Kv9 together though are referred to as the KvS since they are thought to be electrically silent, they have been demonstrated to form heterotetrameric Kv2/KvS channels and show unique biophysical properties (Bocksteins, 2016). In this study, the focus is on KCNV1 with the hypothesis being that it may play an important role in determining cell adaptation, since it has been found that KCNV1 is usually present on adapting cells and absent in non-adapting cells. The study engages to construct viable KCNV1 gene knock-out models for conducting electrophysiological studies.
1.3 THE CRISPR CAS 9 SYSTEM AND ITS USE ON NEURONS

The CRISPR (Clustered Interspaced Short Palindromic Repeats) Cas 9 system is a relatively new gene editing tool, and boasts high specificity for gene modification (Ran et al., 2013). The unique nature of this system is that it allows modification at the DNA level by causing double stranded breaks on the target gene, and can be differentially used to either cause a random frameshift mutation or a targeted insertion of a specific sequence, hence inducing mutations. There are a few major components of this system. First, it consists of unique 20bp sequences called CRISPR single guide RNAs. These sequences recognize a “5’ NGG 3’” region adjacent to its own complimentary sequence on the target gene. Hence the CRISPR single guide RNA binds to this complimentary region. Along with the guide, there is also a hairpin sequence adjacent to the guide termed as the Tracr RNA. The final component of the system is the Cas 9 protein mostly isolated from *Streptococcus pyogenes* or *Staphylococcus Aureus* bacteria (Heidenreich and Zhang, 2015). The modes of operation have two major classifications. The first method is called Non-Homologous End Joining (NHEJ). This is a simple method in which Cas 9 results in a frameshift mutation causing either an insertion or a deletion at the target region. Though this method has been error prone, it is still highly efficient for creating knock-out models. The second and more robust method is called Homology Directed Repair (HDR). In this technique a specific sequence can be inserted in the region where the the double stranded break occurs and so specific over-expressions or mutations can be induced into target sequences.

To understand the brain function at the cellular level there have been several advances made by using techniques like RNA interference (RNAi), Homologous Recombination in Embryonic Stem Cells, Zinc Finger Nucleases (ZFNs) and Transcription Activator-like Effector Nucleases
However, the CRISPR Cas 9 system has emerged as the new age tool for conducting gene expression studies, mainly due to its specificity and ease of operation. In this study, the method of Non-Homologous End Joining is utilized to create KCNV1 knock-out models in neurons in slice culture. An elaborate description of how the method is applied is given in the results and discussion section. The unique aspect of this study was the separation of CRISPR Cas 9 components during use. The CRISPR single guide RNA and Tracr RNA components were delivered externally, while Cas 9 was endogenously expressed inside the mouse strains using floxed alleles. While this system and technique is still being perfected, there were definite conclusions drawn from this study about the use of CRISPR Cas 9 on neurons for gene knock-out studies.
2.0 EXPERIMENTAL RESULTS AND DISCUSSION

The project consisted of four major sections in terms of experimental data. The first section includes the validation of the selected tool for knocking out the gene of interest, which is the CRISPR Cas9 system to knock out KCNV1 cDNA in cultured HEK cells. The second section demonstrates the efficiency of CRISPR Cas9 in knocking out endogenous KCNV1 in cortical neurons of mouse in slice culture. The third section consists of a comparative analysis of CRISPR efficiency in different models of study. The fourth section discusses possible reasons for the low mutation efficiency and new approaches to combat the low efficiency. The final section discusses future directions of the study.

2.1 SCREENING OF CRISPR GUIDE RNA ON MAMMALIAN CELL CULTURE

The major CRISPR Cas9 system components include a single guide RNA sequence that is complementary to the target sequence providing specificity for gene knock-out and the Cas9 protein to facilitate the knock-out. These components were delivered into cultured immortalized Human Embryonic Kidney (HEK) cells using a plasmid vector system. To improve the specificity towards KCNV1, a set of eight single guide RNAs, serially numbered from 1-8 were designed as described in the methods section and cloned into the plasmid pL-CRISPR.EFS.tRFP (Heckl et al, 2014) and is referred to as CRRFP. The successfully cloned vectors were CRRFP 4, CRRFP 5 and CRRFP 8. Plasmid MR218015 from NeoGen Biosystems, was used to deliver KCNV1 cDNA. Both plasmid vectors were delivered into cultured HEK cells by co-transfection and incubated for
7 days. The DNA from HEK cells was extracted, amplified and sequenced. As a control set up, an empty plasmid CRRFP not containing a vector was also transfected. The empty CRRFP transfected control sequence and experimental CRRFP 4, CRRFP 5 and CRRFP 8 sequences were aligned. The sequenced data illustrated an evident increase in background noise beyond the binding region of the single guide RNA in all three experimental sequences, which was attributed to the effect of knock-out of that portion of the sequence, as required. Each experimental and control sequence pair was analyzed further using online web tool TIDE (Tracking of Indels by DEcomposition). This tool tracks the difference between control and experimental sequence and displays mutation efficiency based on the probability of insertions or deletions by the CRISPR activity causing a frameshift mutation on the experimental sequence. The percentage mutation efficiency demonstrates the percentage probability of a possible insertion or deletion mutation on the sequence by the single guide RNA. The most efficient vector was found to be CRRFP 8, with a mutation efficiency of 60%, followed closely by CRRFP 4 at an efficiency of 58.7% and CRRFP 5 at an efficiency of 52.2%. The summary of results is provided in Figure 1. This validates the specificity of the designed guides.
Figure 1: Summary of results for screening of CRISPR single guide RNA 8 in HEK cells on KCNV1 cDNA. (a) Illustrates experimental design for procedure. (b) CodonCode Aligner figures from KCNV1 cDNA sequences demonstrating CRISPR activity. Top sequence represents control sequence without gene knock-out. Highlighted region shows the region complimentary to CRISPR single guide RNA. Bottom sequence shows degraded signal in KCNV1 cDNA sequence after complimentary region thus validating CRISPR efficiency. (c) TIDE data demonstrating CRISPR activity by showing level of decomposition and aberration in sequences. Dark green sample represents the control sequence with negligible decomposition and light green represents test sequence approximately after 200bp which from (b) may be observed to be the region after the guide RNA binding area. (d) TIDE data shows the percentage probability of insertion or deletion mutations on test sequence which correlates to mutation efficiency, which is observed to be 60%.
2.2 KCNV1 KNOCK-OUT IN SLICE CULTURE MODEL

With the confirmation of specificity of designed single guide RNAs, the next aim was to validate the use of the slice culture model for gene expression studies by comparing it to acute slices model. A semi-quantitative PCR was implemented on both kinds of slices to amplify KCNV1, the gene of interest and tubulin (a house keeping gene) as control. Both products were run on an agarose gel and compared. Both products illustrated identical bands on gel for gene of interest and control in both slice samples, implying similar amplification of DNA in both models. Hence the use of slice culture to conduct gene expression studies was validated (Humpel, 2015).

For delivery of CRISPR Cas 9 components, Lenti-virus was produced from the viral and CRISPR vector constructs. For Lenti-virus production, the expression plasmid, a packaging plasmid and a coat protein plasmid is expressed in HEK cells and then ultra-centrifuged to obtained a concentrated pellet containing the virus. A viral titer is the minimum dilution of virus that can still be used to cause infection. Hence, higher the viral titer, the better the infection rate of that virus. However, if the size of the expression plasmid is high, the viral titer is lowered. The plasmid size of CRRFP was 12kb, which was deemed to be too high to obtain Lenti-virus of sufficient titer and quality. To reduce the CRISPR expression plasmid size, a new plasmid vector was used which consisted of a similar plasmid but without the Cas9 expressing gene sequence, named pLKO5.sgRNA.EFS.tRFP (Heckl et al 2014) and referred to as the sgRNA vector. sgRNA 8, sgRNA 7 and sgRNA 3 were successfully cloned. The Cas9 protein was expressed endogenously in transgenic mice, obtained from Jackson Labs. Cas 9 mice was bred to achieve a stable Cas 9 mouse line. The Cas 9 expression was also tagged with a GFP sequence, allowing the identification of Cas 9 expression. To facilitate and control the expression of endogenous Cas 9 in the neocortex,
the Cas 9 mice were crossed with Emx1<sup>ires CRE</sup> mice from Jackson Labs (KR Jones et al 2002). The details are provided in the materials and methods section. There were two varieties used for slice culture. Heterozygous Cas 9 mice and Homozygous Cas 9 mice, with varying amounts Cas 9 expression. The Cas 9 heterozygous mice were sliced at Post natal Day 7 (P7) and were infected with Lenti-virus containing sgRNA with CRISPR single guide RNA 8 the following day. Each slice was incubated up to fourteen days before FACS analysis. FACS analysis was carried out on neurons to differentiate between green only cells (Cas 9 only) and green and red together (sgRNA 8 along with Cas 9). DNA was extracted from sorted cells, amplified and the KCNV1 region was sequenced. The detailed protocol is provided in the materials and methods section (Incontro et al, 2014) (Sabatini et al, 2014).

DNA sequencing data from green only cells (control) were aligned to green and red cell sample (experimental) data. A very small increase in background noise was noticed after the CRISPR single guide RNA sequence binding region on the experimental sequence. The sequences were analyzed in TIDE and illustrated a mutation efficiency of 2.9% for sgRNA 8.
Figure 2: Comparison of Slice Culture Model with Acute Slice Model using semi-quantitative PCR.

Illustrates the validation of use of slice culture model. Semi-quantitative PCR was utilized to amplify endogenous KCNV1 gene and housekeeping gene Tubulin in acute cortical slices and in cortical slice culture for comparison of expression of genes in each model. While slice culture sample has slight low intensity, the size of amplified genes in both acute slices and slice culture are identical and so slice culture model was validated for use in this study.
Figure 3: Summary of results for screening of CRISPR single guide RNA 8 on slice culture obtained from mouse strains Heterozygous for Cas 9.

(a) Illustrates experimental design for procedure. (b) CodonCode Aligner sequences of endogenous KCNV1 demonstrating negligible CRISPR activity. Top sequence represents control sequence without gene knock-out. Highlighted region shows the region complimentary to CRISPR single guide RNA. Bottom sequence shows almost identical signal thus demonstrating very low CRISPR efficiency. (c) TIDE data demonstrating CRISPR activity by showing level of decomposition and aberration in sequences. Dark green sample represents the control sequence and light green represents test sequence both illustrating insignificant decomposition. (d) TIDE data shows the percentage probability of insertion or deletion mutations on test sequence which correlates to mutation efficiency, which is observed to be 2.9%.
2.3 DIFFERENCE IN MUTATION EFFICIENCY OF CRISPR IN DIFFERENT MODELS

After obtaining a very low mutation efficiency in heterozygous Cas 9 slice culture model, there were several hypotheses made to attribute for this effect. The first hypotheses to be tested was to investigate whether the level of Cas 9 expression affected the efficiency. The second hypotheses to be tested was to investigate whether reducing the sorting region on the cortex by only sorting neurons from areas that known to express higher levels of KCNV1, which is layer VI of the cortex.

2.3.1 CAS 9 EXPRESSION LEVEL AFFECTS CRISPR ACTIVITY

To test if an increase in endogenous Cas 9 expression will increase the mutation efficiency, homozygous Cas 9 slices were infected with sgRNA 3, sgRNA 7 and sgRNA 8. A similar protocol was followed for this investigation. For each sgRNA, six slices were infected with the Lenti-virus and incubated for fourteen days. Cells were sorted using FACS and DNA was extracted and amplified and sent for sequencing. After aligning the control (green only cells) and experimental (red and green cells) sequences slight background noise was observed in the experimental sequence after the binding region of CRISPR single guide RNA sequence. TIDE analysis of the results showed the mutation efficiency to be 9.8% for sgRNA 8, 8.8% for sgRNA 7 and 8.4% for sgRNA 3. Hence it was concluded that with an increase in the expression of Cas 9, there was more than three-fold increase in mutation efficiency, however, the efficiency obtained still was not significant to conduct knock-out studies. The analysis graphs are provided in figure 4. The striking difference between the mutation efficiency between CRISPR activity on KCNV1 cDNA on HEK cells and CRISPR activity on endogenous KCNV1 may be attributed to several cellular phenomena. In HEK cells, the KCNV1 cDNA is delivered in a plasmid and is hence much easier
to access for the CRISPR Cas 9 components. Also, in HEK cells, the source of Cas 9 and the source of CRISPR single guide RNA sequences are the same plasmid vector. In slice culture, the KCNV1 gene is likely to be deeply embedded in the nuclear assembly and and is not so easily accessible. Also, since the source of Cas 9 and CRISPR single guide RNA have different sources, that may also result in a decrease in the efficiency of the system. Another hypothesis made was that KCNV1 being a potassium channel sub-unit was unlikely to be present throughout the cortex of the slices and may not be very widely expressed and so since cells from the entire cortex was being sorted, that may also be reducing the FACS output for red and green cells in comparison to green only cells.

2.3.2 FACS ANALYSIS OF LAYER VI OF CORTEX

To account for the fact that KCNV1 may not be widely expressed throughout the cortex, layer VI of cortex was micro-dissected from the cortical region and cells were analyzed. Cas9 homozygous slice culture was infected with sgRNA 8 (since it showed the highest mutation efficiency) and incubated for fourteen days. Layer VI was micro-dissected and sorted using FACS. The DNA was extracted and sequenced. The control (green only cells) sequence and experimental (red and green cells) sequence were aligned. A slightly significant background noise was observed on the experimental sequence after the binding region of CRISPR single guide RNA. TIDE analysis of the sequenced data revealed a mutation efficiency of 14.8%. This was an increase by 50% of that of whole cortical sample FACS results. This also confirmed the hypothesis that KCNV1 expression was more concentrated in the layer VI region of the cortex and hence micro-dissection would demonstrate a significant increase in mutation efficiency. If a comparison is drawn between the various mutation efficiencies observed in the different models, significant differences can be
observed in each different model. Figure 5 illustrates the detailed procedure, results and a comparison of different mutation efficiencies of CRISPR single guide RNA 8, in both CRRFP and sgRNA plasmid vectors in the different models utilized.

**FIGURE 4**

![Figure 4](image)

Figure 4: Summary of results for screening of CRISPR single guide RNA 8 on slice culture obtained from mouse strains Homozygous for Cas 9 and a comparison of CRISPR activity of CRISPR single guide RNA 3, 7, 8.

(a) CodonCode Aligner sequences of endogenous KCNV1 demonstrating CRISPR activity. Top sequence represents control sequence without gene knock-out. Highlighted region shows the region complimentary to CRISPR single guide RNA. Bottom sequence shows slight increase in background noise, implying low CRISPR efficiency. (b) TIDE data demonstrating CRISPR activity by showing level of decomposition and aberration in sequences. Dark green sample represents the control sequence showing no decomposition. Light green represents test sequence illustrating slight increase in decomposition (c) TIDE data shows the percentage probability of insertion or deletion mutations on test sequence which correlates to mutation efficiency, which is observed to be 9.8%. (d) A comparison of different CRISPR single guide RNA mutation efficiency in slice culture in Cas 9 homozygous strains. Guide 8 illustrated the highest efficiency at 9.8%, followed by guide 7 at 8.8% and guide 3 at 8.4%. For subsequent experiments, CRISPR single guide RNA was used.
2.4 APPROACHES TO INCREASE MUTATION EFFICIENCY

A wide range of mutation efficiencies were observed in the different slice culture models. However, the efficiency observed was still not adequate for conducting studies. There can be several reasons attributed to this problem as discussed before. One of the conclusions drawn from the previous experiments was that a major player in determining the mutation efficiency is the level of Cas 9 expression in the system. Another conclusion drawn was that focusing on regions of higher expression of the target also increased the mutation efficiency. To further increase the mutation efficiency and increase the knock out level, different approaches were taken.

2.4.1 USE OF MULTIPLE SINGLE GUIDE RNA AND THE MULTIPLEX VECTOR

The first attempt to improve efficiency that was a modification of the CRISPR Cas 9 system. The CRISPR Cas 9 system is designed to cause a double stranded break in the target sequence based on the specificity provided by the CRISPR single guide RNAs. The guide sequence identifies the Proto-spacer Adjacent Motif (PAM) on the target and Cas 9 causes the double stranded break adjacent to binding region of CRISPR single guide RNA. Proceeding with this theory, it was hypothesized that if multiple CRISPR single guide RNA could be delivered simultaneously, then they would simultaneously bind to different regions on the same target and cause multiple double stranded breaks on different regions of the target sequence, thereby increasing the level of knock out of the gene and hence increasing the mutation efficiency. The plasmid vector pX333 has been previously utilized to deliver two CRISPR single guide RNA simultaneously (Maddalo et al 2014) and was utilized for testing this hypothesis and is referred to as the multiplex vector. Since the aim of the project was to increase the level of knock out of KCNV1, it was further hypothesized that
to increase the mutation in the target sequence, a larger region of the sequence should be modified, and hence the CRISPR single guide RNA binding regions on the sequence should be adequately apart from one another. In this regard, the two CRISPR single guide RNA used for constructing the multiplex vector, which were CRISPR single guide RNA 8 and 3, bind to regions on separate exons on the KCNV1 genomic sequence. Guide 8 binds closest to the transcription start site on exon 1 of KCNV1 and guide 3 binds towards the beginning of exon 2. It was hypothesized that this would cause the most efficient knock out of KCNV1. The two CRISPR single guide RNA were successfully cloned into the pX333 vector. The plasmid was also designed such that each guide RNA was driven by a separate U6 promoter, which is a widely used promoter used for siRNA and CRISPR single guide RNA and short RNA sequences (F. Zhang et al. 2013). The schematic diagram of this plasmid is provided in plasmid diagram 4. A drawback of this plasmid was the absence of a fluorescent marker, to enable FACS analysis of the sample. The sequence region consisting of the two cloned CRISPR single guide sequences with their respective U6 promoters and Tracr RNA sequences were to be digested and cloned into a Lenti-viral plasmid containing a red fluorescent marker.

2.4.2 INTRODUCING CELL TYPE SPECIFICITY

The second step taken to increase the mutation efficiency was to introduce a method to infect specifically neurons in the cortex by adding a neuron specific promoter, human synapsin I gene promoter (hSyn). Human Synapsin I gene promoter has been known to provide strong specificity to neurons (Kügler et al, 2003). While conducting FACS analysis, significant red fluorescent cells were observed, however it was also observed that apart from neurons, several glial cells were also
infected with the Lenti-virus and this may be playing a role in reducing the mutation efficiency.
For getting maximum mutation efficiency, the previous hypothesis of using the multiplex vector
was combined with the need for cell type specificity. The plasmid pLV-hSyn-RFP (Nathanson, et
al 2009) was used to construct the combined cell type specific multiplex vector. The sequence
consisting of the U6 promoters, the CRISPR single guide RNAs and the respective Tracr RNA
was cloned into pLV-hSyn-RFP. This plasmid was designed to express RFP under the hSyn
promoter and thus be specific to neurons for infection. The detailed design protocol is provided in
the materials and methods section. The schematic representation of the cloned construct is
provided in Figure 7. The cloned plasmid DNA was used to produce Lenti-virus.
Figure 5: Summary of results for screening of CRISPR single guide RNA 8 on micro-dissected Layer VI of cortical slice culture obtained from mouse strains Homozygous for Cas 9. Also comparison of CRISPR activity of CRISPR single guide RNA 8 in different systems studied.

(a) CodonCode Aligner sequences of endogenous KCNV1 demonstrating CRISPR activity. Top sequence represents control sequence without gene knock-out. Highlighted region shows the region complimentary to CRISPR single guide RNA 8. Bottom sequence shows increase in background noise, implying higher CRISPR efficiency due to micro-dissection. (b) TIDE data demonstrating CRISPR activity by showing level of decomposition and aberration in sequences. Dark green sample represents the control sequence showing no decomposition. Light green represents test sequence illustrating definite increase in decomposition (c) TIDE data shows the percentage probability of insertion or deletion mutations on test sequence which correlates to mutation efficiency, which is observed to be 14.8%. (d) A comparison of mutation efficiency of CRISPR single guide RNA 8 in different models. Guide 8 illustrated the highest efficiency (60%) in HEK cells, followed by 14.8% in micro-dissected Layer VI of cortex, 9.8% in whole cortex of slice culture from homozygous strains of Cas 9 and lastly 2.9% in heterozygous Cas 9 slice culture samples.
3.0 DISCUSSION AND FUTURE DIRECTIONS

The major points that were made from this study was firstly the validation of designed CRISPR single guide RNA. The guides were successfully tested on HEK cells and demonstrated mutation efficiency up to 60%. The secondly the mutation efficiency is greatly reduced when the same CRISPR Cas 9 system is applied on neurons and several theories were formed for this decrement. The first hypothesis tested in this regard illustrated that the level of Cas 9 expression was directly proportional to the level of knock-out and thus the mutation efficiency. The efficiency was almost tripled when the samples were used from Cas 9 homozygous mice strains instead of Cas 9 heterozygous mice strains. The second hypothesis tested illustrated that KCNV1 expression levels also affected the mutation efficiency. It was hypothesized that KCNV1 is expressed in higher levels in the layer VI and this region was micro-dissected and sorted for analysis. This also illustrated a 50% increase in the mutation efficiency.

The future directions of this study firstly involves the application of the constructed novel pX333g8g3-pLV-hSyn-RFP vector to investigate whether the use of multiple CRISPR single guide RNA along with a cell type (neuron) specific promoter would result in an increase in the mutation efficiency. Secondly it may be investigated whether other modes of viral delivery can be implemented such as viral injections or iontoporation of constructs. These methods may also allow the direct insertion of the CRRFP plasmids and hence have a higher expression of Cas 9 and increase the mutation efficiency. Viral injections however require a higher titer for lenti-virus and so high titer lenti-virus would have to be produced for this procedure (Fricano-Kugler et al, 2016).
Another approach that may be taken involves the use of AAV (Adeno Associated Virus) plasmid containing the similar constructs for multiple guide expression as mode of delivery have greater efficiency of vector delivery (J L Nathanson et al, 2009). A mutation efficiency of approximately 30% is expected for knock-out of KCNV1 to enable electro-physiological investigations. A long term goal of this study also involves the use of Homology Directed Repair (HDR) method to over-express KCNV1 gene for conducting electro-physiological studies.
4.0 MATERIALS AND METHODS

The experimental design for the study included a combination of several molecular biology techniques. Each vector used for delivery of the CRISPR Cas9 system components were constructed using molecular cloning. The system used for screening and validating effect of CRISPR Cas9 guide RNAs was in cultured immortalized Human Embryonic Kidney (HEK) Cells. The system used for observing knock out of endogenous KCNV1 was in neurons in cortical slice culture from transgenic mice brain, sliced at Post natal day 7 (P7).

4.1 VECTORS

For effective delivery of the CRISPR Cas9 components, several plasmid vectors were constructed using molecular cloning. There were several types of plasmids used. Each plasmid contained a ampicillin/carbenicillin resistance expressing gene.
4.1.1 pL-CRISPR.EFS.tRFP

The first type of plasmid used was pL-CRISPR.EFS.tRFP and was a gift from Benjamin Ebert (Addgene plasmid # 57819) (Dirk Heckl et al., 2014), obtained from ADDGENE and is referred to as CRRFP with the guide serial number throughout this thesis and on figures. This plasmid encodes for the expression of a CRISPR guide RNA, the Cas 9 protein and a red fluorescent marker (Red Fluorescent Protein, RFP) all expressed under an U6 promoter. Cloned constructs of CRRFP 4, CRRFP 5 and CRRFP 8 were successfully cloned and the sequence was verified using Sanger Sequencing by GENEWIZ.

Plasmid Diagram 3: Illustrates schematic representation of plasmid CRRFP as provided in Addgene. (Heckl et al, 2014)
4.1.2 MR218015 (KCNV1 cDNA)

The second type of plasmid used was obtained from NeoGen Biosystems, Serial ID: MR218015. This was constructed to deliver KCNV1 cDNA to express the KCNV1 protein in mammalian cell culture, to test the specificity of the CRISPR Cas9 guide RNAs. KCNV1 was expressed under a T7 promoter.

Plasmid Diagram 2: Illustrates schematic representation of plasmid KCNV1 as provided in NeoGen Biosystems
4.1.3 pLKO5.sgRNA.EFS.tRFP

The plasmid pLKO5.sgRNA.EFS.tRFP was a gift from Benjamin Ebert (Addgene plasmid # 57823) (D Heckl et al., 2014). This plasmid was used to express the CRISPR single guide RNA without the expression of Cas9 protein in mammalian cell culture and neuronal slice culture through Lenti-viral transduction or Lenti-viral injection. It also expresses a Red Fluorescent Protein (RFP) marker. It is referred to as sgRNA plasmid with serial number of CRISPR single guide RNA.

Plasmid Diagram 3: Illustrates schematic representation of plasmid sgRNA as provided in Addgene. (Heckl et al, 2014)
4.1.4 pX333

The plasmid pX333 was a gift from Andrea Ventura (Addgene plasmid # 64073) (Maddalo et al., 2014). This was used to simultaneously express two CRISPR single guide RNA driven individually by U6 promoters. This plasmid was exclusively used for the cloning of two CRISPR single RNA sequences and then cloned region was digested and ligated into vector plasmid pLV hSyn RFP for expression in HEK cells and in neuronal slice culture.

Plasmid Diagram 4: Illustrates schematic representation of plasmid pX333 as provided in Addgene. (Maddalo et al, 2014)
4.1.5 pLV-hSyn-RFP

The plasmid pLV-hSyn-RFP was a gift from Edward Callaway (Addgene plasmid # 22909) (JL Nathanson et al., 2009). An insert sequence was digested from plasmid pX333 containing two CRISPR single guide RNA sequences with their U6 promoters and was inserted into pLV-hSyn-RFP. This plasmid was used to selectively express multiple guide RNAs from the cloned insert. The presence of the neuron specific human synapsin (hSyn) promoter drives the expression of a Red Fluorescent Protein (RFP) marker DsRedExpress. This increased specificity and limited the expression of the plasmid by Lenti-viral transduction and Lenti-viral injection to only neurons.

Plasmid Diagram 5: Illustrates schematic representation of pLV hSyn RFP plasmid as provided in Addgene. (Nathanson et al, 2009)
4.2 MOLECULAR CLONING

Molecular Cloning is a technique used to edit plasmid vector sequences by digesting it or causing a break in the DNA sequence using a restriction enzyme and then rejoining it or ligating it with newly inserted DNA sequences that were digested with same restriction enzymes. Molecular cloning was used to insert the CRISPR single guide RNA sequences into CRISPR delivery vector plasmids. Molecular cloning was also implemented to construct a multiplex plasmid combining an insert sequence from plasmid pX333 with the vector plasmid pLV-hSyn-RFP. This cloned construct was used to express two CRISPR single guide RNA simultaneously driven with separate U6 promoters. This construct was also designed to selectively transduct cortical neurons due to the presence of a neuron specific promoter human synapsin (hSyn) driving a visible red fluorescent protein marker (RFP), DsRedExpress. When the Lenti-virally transducted cells were sorted, only neurons expressing DsRedExpress were selected.

All molecular cloning was carried out using standard sterile technique. Each vector stock from addgene was obtained as bacteria in agar stab and was stored at 4°C.

Super Broth powder from Teknova Inc was mixed with MilliQ grade water at 66.87grams/litre (weight/volume) and were sterilized by autoclaving for 25 minutes and stored at room temperature. Similarly, Lysogeny Broth (Bertani, 2004) (LB) powder was also mixed with MilliQ grade water and sterilized by autoclaving for 25 minutes and stored at room temperature. Both these media were used for amplification and growth of bacteria containing required plasmids.

Carbenicillin antibiotic stocks were prepared by mixing carbenicillin powder with milliQ grade water and then filtered into 1ml aliquots using sterile filters at a 1000X concentration. Carbenicillin was used a selectable marker to provide specificity to growing bacterial culture containing required plasmids.
Solid growth media on plates were prepared by mixing the following components in ratio. 2.5g of Yeast extract, 5g of Triptome, 5g of Sodium Chloride and 7.5g Agar was mixed in 500ml of MilliQ grade water and sterilized by autoclaved for 25 minutes. Sterilized media was slightly cooled and added with 500µl of Carbenicillin (1000X Carbenicillin diluted 1:1000). Approximately 15ml of mixture was then poured into bacterial culture plates under sterile conditions and solidified. Plates were then stored at 4°C with agar side of plate facing up, to avoid water from condensation to fall on media. These plates were used for amplification and culture of bacteria containing required plasmids.

All restriction enzymes, digestion buffers, ligation enzymes, ligation buffers, Calf Intestine Alkaline Phosphatase (CIP) and T4 Polynucleotide (PNK) were obtained from New England Biolabs (NEB). All digestion and ligation protocols, all reagent volumes, ratios and reaction conditions and online tools such as double digest finder and ligation ratio NEB calculator were obtained from New England Biolabs.

Molecular cloning of CRISPR single guide RNA into CRRFP plasmid was executed using the restriction enzyme BsmBI (Heckl et al., 2013) and was carried out previously at Nelson Lab. The successfully cloned plasmids were transformed into competent bacterial cells and grown overnight. The plates containing bacterial colonies were stored at 4°C, sealed with Parafilm. Glycerol stocks were also made and stored at -80°C.

4.2.1 SEQUENCES FOR CRISPR SINGLE GUIDE RNA

The CRISPR single guide RNA sequences for targeting the KCNV1 gene were designed using the online design tool called CCTop - CRISPR/Cas9 target online predictor (Stemmer. M et al, 2015). The oligonucleotides for the CRISPR single guide RNA sequences were previously obtained from
Integrated DNA Technologies, Inc. Each CRISPR single guide RNA had a forward sequence oligonucleotide and a reverse sequence oligonucleotide, with designed overhang sequences, in accordance with protocol (Heckl et al., 2013), which were stored at -20°C. The list of sequences of the CRISPR single guide RNA is provided in Table 1.

### Table 1

<table>
<thead>
<tr>
<th>SNO</th>
<th>Single Guide RNA Serial Number</th>
<th>CRISPR Single Guide RNA Sequence And Overhang</th>
</tr>
</thead>
</table>
| A   | Single Guide RNA 1             | 5’ caccgGTCTGCACTACTATCGCAC 3’
                                           | 3’ cCAGGACGTGATGATAGCGTGAa 5’ |
| B   | Single Guide RNA 2             | 5’ caccgAAACTTGGGAGGCATTCTAC 3’
                                           | 3’ cTTTGACCCTTCCGTAAGATGcaaa 5’ |
| C   | Single Guide RNA 3             | 5’caccgCAGAGCTTTTGTCGGACAGT 3’
                                           | 3’ cGTCCTGAAACACGCTGTCAa 5’ |
| D   | Single Guide RNA 4             | 5’caccgTCCACAGCAGCGGATCTT 3’
                                           | 3’ cAGGTGTGTCGGGCCTAGAAa 5’ |
| E   | Single Guide RNA 5             | 5’caccgACGACGAGTTCCAGTAGCGGC 3’
                                           | 3’ cTGCTGCTCAGTGATCGCGcaaa 5’ |
| F   | Single Guide RNA 7             | 5’ caccgGACTCGTGCTGCTGGACAG 3’
                                           | 3’ cCTGACAGCGACCGACCTGTGCaa 5’ |
| G   | Single Guide RNA 8             | 5’ caccgACCCCGCAACCGGGCTAC 3’
                                           | 3’ cTGGGCGTTGGCGCGGATGcaaa 5’ |

Table 1: List of sequences for CRISPR Single Guide RNA design for knock-out of KCNV1
4.2.2 AMPLIFICATION OF CRRFP, sgRNA AND KCNV1 cDNA PLASMIDS

Glycerol Stocks for previously cloned CRRFP and sgRNA plasmids containing CRISPR single RNA were used to inoculate 150ml of Sterile Super Broth mixed with 150µl of the antibiotic Carbenicillin in a conical flask. The inoculated mixtures was incubated on a shaker overnight at 37°C. DNA was extracted from this using the Maxiprep Kit from Zymo Research. Extracted plasmid DNA was stored at 4°C for future use. Each sample was previously sequenced by Genewiz and the successfully cloned samples were stored. The stored plasmids for CRRFP included CRRFP 3, CRRFP 4, CRRFP 5, CRRFP 8 and a control CRRFP plasmid without any cloned single guide RNA sequence, referred to as empty CRRFP. Similarly, the stored plasmids for sgRNA included sgRNA 3, sgRNA 7, sgRNA 8.

4.2.3 CLONING OF CRISPR SINGLE GUIDE SEQUENCES INTO PLASMID pX333

Plasmid pX333 was used to hold two adjacent CRISPR single guide RNA sequences. The plasmid was obtained as an agar stab stored at 4°C. The plasmid was used to inoculate 150ml of Sterile Super Broth mixed with 150µl of the antibiotic Carbenicillin in a conical flask and incubated on a shaker overnight at 37°C. DNA was extracted from this using the Maxiprep Kit from Zymo Research and eluted in 400µl of ultra-sterile water at a concentration of 928ng/µl. At the first cloning site, single guide RNA 8 was selected to be cloned in, since it had highest efficiency in both cultured mammalian HEK cells and in neuronal slice culture. For the first restriction cut-site on pX333, the enzyme BbsI was used for digestion. 5µg of pX333 DNA was digested with 2.5µl of BbsI enzyme. The assembled reaction was incubated at 37°C overnight. The complete reaction detail is provided in Table 2. The reaction mixture was added with Calf Intestinal Alkaline
Phosphatase from New England Biolabs (NEB). This results in phosphorylation of the digested ends to prevent ends from re-joining and prevent ligation with the insert sequence. After overnight incubation, the reaction mixture was purified using Zymo Clean and Concentrator kit and was eluted in 25µl of ultra-sterile water.

To clone in CRISPR single guide RNA sequences into pX333, lenti-viral CRISPR/Cas9 and single guide RNA Protocol (Sanjana. NE et al, 2014) from Zhang Lab at MIT was utilized. At the first cloning site, single guide RNA 8 was selected to be cloned in, since it had highest efficiency in both cultured mammalian HEK cells and in neuronal slice culture. The oligonucleotide was thawed and then respective forward and reverse oligonucleotides were phosphorylated and ligated with each other to become double stranded, using T4 PNK enzyme. The complete reaction mixture assembly of reagents and buffers used is provided in Table 3. The reaction was incubated on a thermo-cycler at 37°C for 30 minutes followed by 95°C for 5 minutes. The temperature is then decreased to 25°C at a rate of 5°C/minute. The reaction is then diluted to 1:200 in ultra-sterile water.

For ligation of CRISPR single guide RNA sequence oligonucleotide into pX333 vector, T7 ligase enzyme from NEB was utilized. For ligation, 50ng of digested plasmid pX333 was used. The protocol was also obtained from NEB. The complete reaction mixture components are provided in Table 4. As a control, an additional ligation reaction was setup using only digested plasmid pX333 vector, without the insert sequence, referred to as the vector control. The reaction mixture is incubated for 30 minutes at room temperature. For bacterial transformation, 50µl competent bacterial cell (Mix and Go Escherichia Coli) aliquots were thawed on ice for 15minutes. Pre-made carbenicillin containing bacterial culture plates were warmed to room temperature in incubator. 1.5µl of ligation mixture was added to competent cells and incubated on ice for 5 minutes and
spread on culture plate using a sterilized glass stir rod. Sterile technique was maintained. The procedure was carried out in the presence of a sterilizing flame. The plates were incubated overnight at 37°C. Bacterial colonies were observed the following morning and used to inoculate 4ml of super broth mixed with 4µl of Carbenicillin. Six cultures serially labelled as pX333g8A to pX333g8F were inoculated from six different colonies. The cultures were incubated at 37°C overnight on shaker and DNA was extracted using Miniprep Kit from Zymo Research. To validate successful cloning, each sample was digested using a combination of single and double digests. The complete result of the digest test is provided in Figure 1. From test results, samples Px333g8A, pX333g8C and pX333g8F was validated. 1μg of DNA sample for these three samples were diluted in 10μl ultra-sterile water and mixed with 5μl designed forward primer for pX333 cloning site. This mixture was sequenced by Genewiz and confirmed cloning success.

The second cloning site on pX333 for inserting the second CRISPR single guide RNA sequence oligonucleotide was digested with the restriction enzyme BsaI HF, obtained from NEB. The sample pX333g8A was used as vector plasmid for digestion. The cloning protocol was repeated for single guide RNA 3 exactly as described and resulting sample, serially labelled as pX333g8g3A to pX333g8g3D was validated from sequenced DNA. The sequenced results for pX333g8 and pX333g8g3 are provided in Figures 6 and 7.
FIGURE 6

![DNA trace file observed on CodonCode Aligner. Highlighted region illustrates presence of CRISPR single guide RNA 8 successfully cloned into construct pX333.](image)

Figure 7: Sequenced DNA trace file observed on CodonCode Aligner. Highlighted region illustrates presence of CRISPR single guide RNA 8 successfully cloned into construct pX333.

FIGURE 7

![DNA trace file observed on CodonCode Aligner. Highlighted region illustrates presence of CRISPR single guide RNA 3 successfully cloned into construct pX333.](image)

Figure 8: Sequenced DNA trace file observed on CodonCode Aligner. Highlighted region illustrates presence of CRISPR single guide RNA 3 successfully cloned into construct pX333g8.
### TABLE 2

<table>
<thead>
<tr>
<th>Reagent/Buffer</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>pX333 Plasmid DNA</td>
<td>5.40µl</td>
</tr>
<tr>
<td>NEBuffer 2.1</td>
<td>5.00µl</td>
</tr>
<tr>
<td>NEB enzyme BbsI</td>
<td>2.50µl</td>
</tr>
<tr>
<td>CiP</td>
<td>2.00µl</td>
</tr>
<tr>
<td>H2O</td>
<td>35.1µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>50.0µl</td>
</tr>
</tbody>
</table>

**Table 2:** Assembly of reaction for Digestion of plasmid pX333 for cloning of CRISPR single guide RNA

### TABLE 3

<table>
<thead>
<tr>
<th>Reagent/Buffer</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward Primer</td>
<td>1.0µl</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>1.0µl</td>
</tr>
<tr>
<td>T4 DNA Ligase buffer</td>
<td>1.0µl</td>
</tr>
<tr>
<td>T4 PNK</td>
<td>0.5µl</td>
</tr>
<tr>
<td>H2O</td>
<td>6.5µl</td>
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<tr>
<td><strong>Total</strong></td>
<td>10µl</td>
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</table>

**Table 3:** Assembly of reaction for phosphorylation and Ligation of Forward and Reverse Oligonucleotides for CRISPR Single Guide RNA
**TABLE 4**

<table>
<thead>
<tr>
<th>Reagent/Buffer</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digested Vector DNA</td>
<td>1.5µl</td>
</tr>
<tr>
<td>Diluted (1:200) Insert Oligonucleotide</td>
<td>1.0µl</td>
</tr>
<tr>
<td>T7 DNA Ligase buffer</td>
<td>5.0µl</td>
</tr>
<tr>
<td>T7 DNA Ligase</td>
<td>1.0µl</td>
</tr>
<tr>
<td>H2O</td>
<td>2.5µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>11µl</td>
</tr>
</tbody>
</table>

Table 4: Assembly of reactions for ligation of digested plasmid vector with CRISPR single guide RNA

### 4.2.4 CLONING OF pX333g8g3-pLV hSyn RFP

The cloned construct pX333g8g3 was PCR amplified, digested and inserted into plasmid pLV hSyn RFP. This construct was designed to enable Lenti-viral delivery of the two CRISPR single guide RNA, driven by two separate U6 promoters. The fluorescent marker (DsRedExpress) on this plasmid however is driven by neuron specific promoter human synapsin (hSyn), and so analysis of mutation based on fluorescence is selectively carried out only on neurons. The section of the cloned construct pX333g8g3 that was digested included the two CRISPR single guide RNA sequences, and their respective tracr RNA sequences and U6 promoters. A schematic diagram of the construct is given in the next page.
Plasmid Diagram 7: Illustrates the schematic diagram of cloned construct, pX333g8g3-pLV
hSyn RFP

For PCR amplification of insert region, primers were designed to additionally add specific restriction sites on to the ends of the insert sequence. The restriction site ClaI sequence was added by the Forward Primer. Similarly, the restriction site ApaI was added onto the insert by the reverse primer. The sequences of the forward and reverse primers are provided below:

**Forward Primer:** 5’ **CAGGC**GATCGATTCTCGAGCATGCGCCATT 3’

**Reverse Primer:** 5’ **CTAAT**TGGGCCAACGGGTACCCCATTGTCTG 3’

The underlined section represents the restriction site sequences, ClaI and ApaI respectively. The bold region represents overhang regions on primer sequences that assists in binding primer to the insert sequence during PCR amplification. The reaction details are provided in Table 5. Following PCR amplification, the sample was run on a 0.5% agarose gel. The sample illustrated three different sized DNA samples at 100bp, 500bp and 900bp. The correctly amplified DNA was hypothesized to be at 900bp and this band was cut out from the gel and DNA was extracted using DNA gel extraction kit from Qiagen. The insert DNA was then digested with restriction enzymes ClaI and ApaI from NEB. The complete reaction components are provided in table 6. Before adding ClaI to the reaction mixture, the reaction was incubated at 25°C for two hours. Then after
addition of ClaI, the reaction was incubated at 37°C for two hours. This was carried out to facilitate digestion for both restriction enzymes, as ApaI digestion occurs at 25°C and ClaI digestion occurs at 37°C. The vector plasmid pLV hSyn RFP DNA was also digested using similar ratios and conditions. The digestion protocol was in accordance with the NEB double digest protocol. The reaction mixture assembly is provided in Table 7. After digestion of both plasmids, each reaction mixture was purified using Clean and Concentrator kit from Zymogen and DNA was eluted in 25µl of sterile water. The DNA concentration of each solution was ascertained using UV spectrophotometry by Nanodrop. The ligation reaction component mixture was ascertained from the online tool, Ligation Calculator at NEBioCalculator tools (version 1.6.0) by NEB. The T7 DNA ligation protocol was optimized and followed. The calculator algorithm includes the following.

\[
\text{Required mass insert} = \frac{\text{insert Molar Ratio} \times (\text{mass of vector}) \times \text{insert length}}{\text{vector length}}
\]

The input data includes the following. Insert DNA length = 1 kb, Vector DNA length = 10 kb, mass of vector = 50ng and the (insert/vector) molar ratio was taken at 1:7. The insert mass was calculated at 35ng. The reaction was assembled along with a control reaction without the insert DNA referred to as Vector Control. 1.5µl of ligation reaction mixture was pipetted into 50µl of mix and go competent cell solution and transformed onto bacterial agar plates. These were incubated overnight at 37°C. Colonies were observed the following morning and used to inoculate 150ml super broth media and 150µl Carbenicillin for amplification of cloned sample DNA. The solution was incubated on a shaker at 37°C overnight. Maxiprep kit from Zymo were used extract DNA the following morning. The DNA sample concentration was 927ng/µl and was stored at 4°C.
The sample DNA was then validated using a digest test and also by DNA sequencing. These were major molecular cloning steps that were conducted in this study.

**TABLE 5**

<table>
<thead>
<tr>
<th>SNO</th>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pX333g8g3 DNA template</td>
<td>~ 50ng</td>
</tr>
<tr>
<td>2</td>
<td>HF Buffer</td>
<td>4 µl</td>
</tr>
<tr>
<td>3</td>
<td>dNTP</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>4</td>
<td>Phusion DNA Polymerase</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>5</td>
<td>pX333 Forward 1(10µM)</td>
<td>1 µl</td>
</tr>
<tr>
<td>6</td>
<td>pX333 Reverse (10µM)</td>
<td>1 µl</td>
</tr>
<tr>
<td>7</td>
<td>Water</td>
<td>Up to 20 µl</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

Table 5: Reaction assembly for PCR amplification of insert region on pX333g8g3.
### TABLE 6

<table>
<thead>
<tr>
<th>Reagent/Buffer</th>
<th>Volume</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>pX333g8g3 Insert DNA</td>
<td>40µl</td>
<td></td>
</tr>
<tr>
<td>CutSmart Buffer</td>
<td>5.00µl</td>
<td></td>
</tr>
<tr>
<td>NEB enzyme ApaI</td>
<td>2.50µl</td>
<td></td>
</tr>
<tr>
<td>NEB enzyme ClaI</td>
<td>2.50µl</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>50.0µl</td>
<td></td>
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</tbody>
</table>

**Table 6:** Reaction assembly for Restriction Digestion of pX333g8g3 insert DNA

### TABLE 7

<table>
<thead>
<tr>
<th>Reagent/Buffer</th>
<th>Volume</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>pLV hSyn RFP Vector DNA</td>
<td>5µl</td>
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<tr>
<td>CutSmart Buffer</td>
<td>5.00µl</td>
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<tr>
<td>NEB enzyme ApaI</td>
<td>2.50µl</td>
<td></td>
</tr>
<tr>
<td>NEB enzyme ClaI</td>
<td>2.50µl</td>
<td></td>
</tr>
<tr>
<td>CIP</td>
<td>2.00µl</td>
<td></td>
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<tr>
<td>H20</td>
<td>33µl</td>
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</tbody>
</table>

**Table 7:** Reaction assembly for Restriction Digestion of pLV hSyn RFP Vector DNA
4.3 MAMMALIAN CELL CULTURE

For validating expression of plasmids and for producing lenti-viral constructed, immortalized Human Embryonic Kidney (HEK) cells were cultured and used. HEK cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) from Gibco, supplemented with 10% Nu Serum and 0.1% antibiotic Penicillin-Streptomycin mix. The cell line was used due to its ability to express a wide number of genes due to the presence of a large pool of transcription factors.

For expression of the target genes, the cloned constructs were co-transfected using calcium chloride and HEPES Buffered Saline (HBS) solutions using calcium phosphate transfection method (Kingston RE et al, 2001). For culturing of HEK cells, standard sterile techniques were followed. All techniques were carried out in a biosafety cabinet in a Biosafety level 2.5 facility. For cells being cultured in 10cm dishes, cells were inoculated at a 1:10 or 1:20 concentration in 10ml media. DMEM with mentioned supplements was pipetted into sterile culture dish and then added with inoculum containing HEK cells according to required dilution. Cells after attaining 80-90% confluence were split and re-plated approximately every 3-4 days.

To prepare HEK cells for transfection, six-welled culture dishes were used. The cells were cultured in 1.5ml of media. For transfection of HEK cells using the calcium phosphate transfection protocol, the reagents used include 5% Calcium Chloride and 50% HBS. Approximately 1µg of plasmid DNA was used for co-transfection. The usual ratio for transfection followed included 8.5µl of Calcium Chloride, required volume (1µg) of plasmids to be expressed and then the mix was made up to 85µl with sterile water. This mixture was then added drop-wise to 85µl of HBS solution and the total mixture was bubbled using pipette. The complete 170µl mixture was directly added to HEK cells on the six welled plate. As a control, a well was always transfected without any plasmids to validate that there are no contaminants damaging the cells in the reagents.
For screening of CRISPR single guide RNA, the CRRFP plasmids were co-transfected with KCNV1 expressing plasmid. As a control a well was also transfected with only KCNV1 plasmid and subsequently a well was transfected with an empty CRRFP plasmid as mentioned before. After 6 hours of transfection, the media was drained and fresh media was added without disturbing the cells. Transfection was carried out when cells were at 30% confluence. Successful transfection was validated based on the expression of visible of reporter mCherry or dsRED (Red Fluorescent Proteins) present on the plasmids. Cells were split on day 3 after transfection into new culture dish. Cells were collected for DNA sampling on Day 7. Subsequent splitting of cells after first split demonstrated a loss of fluorescence and transfection efficiency.

For production of lenti-virus for delivery of cloned plasmid into slice culture, a larger 25cm sterile dish containing 25ml of media was co-transfected with the viral components. The first component was plasmid expressing VSV-G envelope. The plasmid used was pMD2.G which was a gift from Didier Trono (Addgene plasmid # 12259). The second component was packaging plasmid, Pax2 (Ulrich Pfisterer et al., 2016). Pax2 was a gift from Malin Parmar (Addgene plasmid # 35002). The third component was the cloned pX335g8g3-pLV-hSyn-RFP plasmid. These components were co-transfected using calcium phosphate method. The media was changed after 6 hours and cells were incubated for 48 hours. After incubation, the supernatant was initially centrifuged at 5000rpm for 5 minutes followed by ultra-centrifugation at 21,000 rpm for 2 hours. The supernatant media was drained without disturbing the pellet and then the lenti-viral construct was re-suspended in 100µl slice culture media (SCM) and stored at -80°C in 20µl aliquots for transduction of neuronal slice culture.
4.4 TRANSGENIC MICE

All transgenic mice were obtained from the Jackson Laboratory. In this study, the varieties of mice used include the use of wild type mice and EMX1\textsuperscript{IRES\textsc{cre}} strain for allowing the use of floxed alleles to express Cas 9 endogenously in mouse cortex (KR Jones et al, 2002). The EMX1\textsuperscript{IRES\textsc{cre}} strain of mice expresses CRE recombinase from the EMX1 locus. A strain of mice containing a target sequence flanked by a LoxP site can crossed with the EMX1 strain can selectively express the target sequence and result in deletion of the flanked LoxP sequence. This expression has been demonstrated to be in around 88% of neurons in neocortex of mice, hippocampus and also glia. Using this method Cas 9 strains of mice were crossed with EMX1\textsuperscript{IRES\textsc{cre}} strains and Cas 9 heterozygous and Cas 9 homozygous strains were obtained. Genotyping of these mice was carried out by extracting DNA from toe samples of Post Natal 6 (P6) pups and gene amplification by PCR. PCR Primers and protocols were designed in accordance with Jackson Laboratory genotyping protocol. The expected amplified gene product lengths were provided in the Jackson Laboratory genotyping protocol (KR Jones et al, 2002). The amplified samples were run on a 1.5% agarose gel with a 1Kb plus ladder. The presence of all genes were verified by analyzing the band sizes of samples on the gel. The target gene of interest for genotyping was amplified using two sets of primers, one for wild type allele and one for mutant allele. A successfully bred strain would show a positive for either only mutant band (homozygous) or both mutant band and wild type band (heterozygous). All mice breeding were set up by Dr Vera Valakh and Lucia Capano.
4.5 SLICE CULTURE, MICRO-DISSECTION AND FACS ANALYSIS

The aim of this study is concerned with producing KCNV1 knock-out models in mouse neurons of neocortex. To achieve this goal, slice culture technique was utilized as the system of study. Brains of successfully genotyped mice were sliced using Compressstome VF-200 on Post-Natal Day 7 (P7). 300µm thick cortical slices were incubated on slice culture membrane in slice culture media at 37°C and 5% CO₂ (C Humpel 2015). The media was changed every three days. Slices were cultured and maintained by Vera Valakh, Lucia Capano and Lorraine Lei.

All cloned plasmid vectors were delivered using transduction by Lenti-viral constructs of target sequences. For enabling of KCNV1 knock-out, the slices were infected with lenti-virus on day 2 of slice culture. The slices after being infected with lenti-virus were incubated at 37°C and 5% CO₂ for two weeks. For FACS analysis, on day 14 of slice culture, the slices first incubated for one hour in oxygenated HEPES-ACSF (artificial cerebrospinal fluid) supplemented with protease at a concentration of 1mg/ml. Then, the slices were incubated in HEPES ACSF supplemented with 1% FBS (fetal bovine serum) and cortical regions were micro-dissected under fluorescent microscope. For subsequent study, layer VI was also micro-dissected for FACS analysis. Micro-dissected regions were triturated in 1% FBS HEPES ACSF supplemented with DNase I with descending order of glass pipette nozzle size, filtered, supplemented with pericol and centrifuged. The pellet obtained was resuspended in 1% FBS HEPES ACSF, re-filtered and analyzed using FACS. Cell sorting was done based on cells consisting of combined fluorescence of Green from Cas 9 and Red from CRISPR single guide RNA vector differentiated from cells with only green fluorescence without the rest of the CRISPR components. FACS Analysis was conducted by Dr Erin Clark. Cells were sorted into DNA extraction buffer. The extracted DNA was PCR amplified using primers designed for KCNV1 genomic sequence and the DNA was sequenced.
4.6 DNA SEQUENCING AND DATA ANALYSIS SOFTWARE

All DNA sequencing was carried out by GENEWIZ. The sample submission guidelines and protocols were obtained from GENEWIZ and was accurately adhered to. For sequencing of plasmid DNA, approximately 1µg of DNA sample was added with sterile water and the total volume was made up to 10µl. Primer concentration was diluted to 5µM and 5µl of primer was added to sample mix. For amplified or purified DNA, 20ng of DNA was used and prepared in a similar manner. All sequencing results passed the quality control guidelines set forth by GENEWIZ. For sequence data analysis the software used was CodonCode Aligner Version 6.0.2. For analysis of CRISPR activity, the web tool TIDE (Tracking of Indels by DEcomposition) from the Netherlands Cancer Institute was utilized. The sequence inputs involved the input of the CRISPR single guide RNA sequence, a control sample sequence without CRISPR activity for reference and the test sequence. The sequences to be analyzed were saved with a ‘.ab1’ extension. All figures illustrating sequence data or CRISPR Analysis data have been obtained from CodonCode Aligner and TIDE.
5.0 REFERENCES


