PROJECT PROPOSAL OPEN ACCESS

The Potentiation of Cortical Pyramidal Neurons Due to the Gain of Function Mutation of Kv.4.2 Channels

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SUMMARY

Epilepsy is a disease characterized by recurrent seizures caused by abnormally high levels of activity in certain parts of the brain. High levels of activity in neurons, that is, increased neural firing rates, are commonly caused by specific genetic mutations. Several forms of therapies for epilepsy are available that broadly reduce the firing rates of neurons in the brain. These therapies are effective yet come with several side effects, such as dizziness. A targeted pharmacological intervention will reduce these side effects as it will not influence the brain in a broad manner and will aim to reduce the magnitude of the mutation's effect on the specific neurons involved in epilepsy. In 2014, a new mutation was discovered in a pair of twins suffering from seizures resistant to typical epilepsy medications. The way this mutation leads to seizures is still a mystery. This proposal aims to uncover the mechanism of action of this mutation, potentially leading to targeted pharmacological interventions to treat seizures caused by this mutation.

ABSTRACT

Kv4.2 channels are a type of K^+ channel responsible for the early repolarization phase in an action potential. In 2014, a gain of function (GOF) mutation in these channels was shown to lead to seizures. Since this mutation led to a more sustained K^+ current in the mutant neurons' repolarizing phase, it is unclear as to why it would lead to hyperexcitability rather than hypoexcitability. It has been shown that the transient silencing of glutamatergic neurons can lead to their potentiation, more specifically known as homeostatic potentiation. This proposal aims to test whether homeostatic potentiation of cortical pyramidal neurons of layer 2/3 is the underlying mechanism behind the seizures induced by this mutation. To examine this, three markers of potentiation will be investigated in pyramidal neurons of layer 2/3 in mice that are either wildtype (WT) or mutant for the Kv4.2 GOF mutation. These markers include the excitatory post-synaptic current (EPSC), spine density and the AMPA receptor density on the post-synaptic density (PSD), which should all increase after potentiation. The results of this research will reveal the mechanism behind this mutation's effects, potentially leading to the development of targeted pharmacological interventions for the form epilepsy induced by this mutation.

Keywords: GOF, Kv4.2; homeostatic potentiation, seizure, epilepsy, gain of function of kv4.2

INTRODUCTION

Epilepsy is a chronic condition characterized by recurrent seizures.[1] The majority of epilepsies originate from genetic mutations.[1] The bulk of known epilepsy mutations are monogenic mutations in different ion channels in the central nervous system (CNS).[1] These mutations often lead to neuronal hyperexcitability, leading to seizures.[1] Several unique mutations in K^+ channels have been identified as the cause of different types of epilepsy.[2] Most notably, voltage-gated K^+ channels are responsible for repolarization during the action potential (AP).[3] Hence, a loss of function (LOF) mutation in these channels will disrupt the repolarization event causing sustained depolarization, potentially leading to seizures.[4]

One subtype of K^+ channel is called Kv4.2.[5] These channels carry a transient potassium current, called the A-current.[5] The A-current is thought to be involved in the early phase of the neuron's repolarization.[6] The presence of Kv4.2 channels on the dendrites of pyramidal neurons in the CA1 region of the hippocampus of mice has been demonstrated.[7] These channels have been proven to prevent the axo-somatic backpropagation of action potentials and reduce the chance of the rapid firing of these neurons.[8]

Unexpectedly, in 2014, Lee and colleagues identified a gain of function (GOF) mutation in a Kv4.2 channel in identical twins with intractable seizures and autism.[9] Given that the Kv4.2 channels reduce the chance of rapid firing in these neurons, the discovery that a GOF mutation in the Kv4.2 channels leads to epileptic episodes, which are characterized by the rapid firing of the neurons, is quite counterintuitive.[8] They identified this mutation by performing whole-exome sequencing. They detected a *de novo* mutation in the *KCND2* gene[9] which codes for Kv4.2 channels.[10] The mutation led to the replacement of valine for methionine in the 404th position in the transmembrane S6 helix of the channel.[9] Lee and colleagues further studied this channel's kinetic properties by performing multiple voltage-clamp studies at membrane potentials ranging from -80 mV to +70 mV. These functional analyses revealed that the decay of K^+ current was significantly slower in the mutated channel compared to the wildtype (WT).[9] These results revealed that the Val404Met mutation significantly slows down the K^+ current inactivation leading to a sustained K^+ current.[9] There are currently two competing theories that attempt to explain these unexpected results.[2]

The first explanation relies on the discovery made by Paul and colleagues that the chandelier cells have high levels of expression of Kv4.2 channels.[11] Chandelier cells are interneurons that provide GABAergic input to the axon initial segment (AIS) of pyramidal neurons.[12] A GOF mutation in Kv4.2 channels in chandelier cells can lead to their hyperpolarization and reduce their GABAergic output to pyramidal neurons.[2] This can lead to abnormally high AP firing rates from the excitatory pyramidal neurons.[2] However, the pyramidal neurons also carry the same Kv4.2 GOF mutation as the chandelier cells. Consequently, the hyperpolarizing effects of this GOF mutation in the pyramidal neurons should, to some extent, counteract the reduction in the GABAergic input from the chandelier cells.

An alternative explanation relies on a phenomenon called homeostatic plasticity, which is the response of a neuron or a network of neurons to internal and external stressors to re-establish their baseline activity.[13] Studies show that the transient silencing of glutamatergic neurons can lead to compensatory responses that mimic long term potentiation (LTP).[14] For example, in 2013, Lambo and Turrigiano demonstrated that monocular deprivation of Long-Evans rats leads to the net potentiation of synapses deprived of the signal from the affected eye in area $V1_a$ and $V1_b$ of the primary visual cortex.[15] Interestingly, by blocking the trafficking of new AMPA receptors in these neurons, this effect was significantly reduced and even entirely disappeared in some cases.[15] This finding suggests that the mechanism underlying the homeostatic plasticity of glutamatergic neurons have some similarities to the process of LTP.[15] The cellular mechanism underlying this form of homeostatic plasticity may involve calcium-dependent sensors. These can sense the neuron's firing rate and adjust the trafficking of receptors to the synapse accordingly and change the protein expression profile of the neuron.[14] This is further evidence for the similarities between this form of homeostatic plasticity and LTP.[15]

The excitatory synapses onto the GABAergic neurons can also potentially undergo potentiation because of this mutation. This would reduce the effect of the potentiation of the cortical pyramidal neurons. However, it can be speculated that the potentiation of pyramidal neurons is having a higher influence due to the clinical presentation of this mutation. Since the mutation leads to generalized seizures, the potentiation of the GABAergic interneurons is either absent or outweighed by the potentiation of the cortical pyramidal neurons. This could partially be due to the fact that GABAergic interneurons make a small proportion of all the cortical neurons (10-15% in rodents).[16]

Considering the evidence, it can be expected that homeostatic plasticity is the underlying mechanism of the role of the GOF mutation in the Kv4.2 channels in epilepsy.[2] Since the GOF mutation itself would initially lead to hypoexcitability of the pyramidal neurons, a compensatory homeostatic response with LTP-like characteristics is possible. These synaptic changes could lead to hyperexcitability and uncontrollable firing of the pyramidal neurons, which could clinically present as seizures.

1.1 HYPOTHESIS

If the Kv4.2 channel GOF mutation strengthens the excitatory synapses on cortical pyramidal neurons, then the pyramidal neurons with this mutation should demonstrate significantly higher levels of synaptic potentiation compared to the wildtype pyramidal neurons.

1.2 MODEL ANIMAL

Since the Val404Met GOF mutation in Kv4.2 was recently discovered in humans in 2014, there are currently no model animals present with this specific point mutation. Also, because this mutation is a point mutation, a gene knockout technique cannot produce the desired model animal. Therefore, to perform the following experiments, mice with this specific mutation will be requested from a biotechnology company. Furthermore, Lee and colleagues demonstrated that the patients carrying these mutations were seizure free for the first two months of their lives.[9] Therefore, it can be expected that the effects of the mutations are amplified over early development. To study the role of development on the magnitude of the effects of this

mutation, all the following experiments will be done on 15 and 60-day old mice. It can be expected that the effects of the mutations will be significantly stronger in the 60-day old mice compared to WT and 15-day old mice. This prediction will be investigated by conducting all the following experiments on 15 and 60-day old mice. Therefore, there are four different animal models that will be studied per brain area.

1.3 AREA OF INTEREST

The cerebral cortex is a complex network of neurons with six distinct layers.[17] Layer II of the cortex contains small pyramidal neurons and layer III contains medium-sized pyramidal neurons that are vertically oriented.[17] Even though these two layers have discrete populations of cells, they are not easily distinguished in rodents based on their cytoarchitecture and are referred to as the pyramidal neurons of layer 2/3.[18] Due to these connections, an abnormal rapid firing in layer 2/3 can spread to other. Therefore, layer 2/3 pyramidal neurons are commonly studied for their role in seizures and their effects of monogenic mutations in pyramidal neurons. Consequently, all the following studies will be conducted in layer 2/3 of the cerebral cortex.

Since the specific locations of the start of the epileptic events caused by the Val404Met GOF are not yet identified, multiple areas of the brain must be investigated in this research. Brain slices will be taken from specific locations that are highly associated with epilepsy based on previous research. In 2005, Chabardès and colleagues showed that the anterior part of the cerebral cortex of the temporal lobe, which is also called the temporopolar cortex (Brodmann area 38), is a major site of epileptogenesis.[22] Therefore, temporal tissues for all the following studies will be collected from this area. The slices in the parietal lobe will be taken from the primary somatosensory cortex, since a study performed in 2017 by Niday and colleagues demonstrated that a dysfunction of KCNQ2 K^+ channels leads to hyperexcitability of layer 2/3 pyramidal neurons in this region.[21] The seizures that originate from the supplementary motor area (SMA) of the frontal lobe are common and have been the well documented.[23] Therefore, the frontal lobe slices will be taken from the SMA.

2.1 AIM 1

To measure EPSC amplitude in layer 2/3 pyramidal neurons with the Val404Met GOF mutation in Kv4.2 channels.

2.2.1 RATIONALE

A defining characteristic of the strengthening of synaptic function and potentiation of a neuron is an increase in the amplitude of excitatory postsynaptic current (EPSC).[24] The measurement of EPSCs is often used to evaluate whether a neuron has undergone potentiation.[20] A local pyramidal neuron and its glutamatergic target neurons can be identified by their morphological features by using infrared differential interference contrast microscopy.[21] Then the presynaptic neurons can be stimulated by a stimulating electrode and the response of the postsynaptic neurons can be recorded.

2.2.2 METHOD

A. Extracting the Tissue
   a. Both wild type (WT) and mutant animals will be kept in similar conditions. The experiment will be done on 15- and 60-day old animals.
   b. The animals will be anesthetized with diethyl ether and quickly decapitated. The brain tissue of animals will be removed. The brains will be fixed in a solution of paraformaldehyde in PBS. After fixation, 300 µm coronal sections of the layer 2/3 of the pyramidal neurons in the temporal, parietal and frontal cortex will be cut by using a vibratome. The thickness of these slices maintains the interconnected network of the neurons.[25] After slices are cut, they will be kept at 4°C in artificial cerebrospinal fluid (CSF) until they are needed for electrophysiological analysis.

B. Whole-Cell recording
   a. A local presynaptic pyramidal neuron in layer 2/3 and its postsynaptic targets will be identified in each slice based on their morphology. Since the slices are relatively thick, infrared differential interference contrast will be used to identify the target neurons.[21]
   b. A stimulating electrode will be inserted in the identified presynaptic neuron's membrane. The presynaptic neuron will be stimulated with suprathreshold pulses of current with a duration of 2-10 ms.[25] A whole-cell recording will be performed on the postsynaptic targets of the neuron. This involves positioning a small glass capillary tube onto the membrane of the postsynaptic cell, then applying suction until the membrane is ruptured. The capillary tube (i.e., electrode) will be connected to a signal amplifier, and the currents will be recorded on a computer. The recording solution of the electrode will mimic the intracellular solution of the neuron. Calcium chelators such as 1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid will also be included in the recording solution to prevent the induction of LTP during the protocol.[26] The net-

work of neurons in layer 2/3 is highly interconnected with interneurons between the pyramidal neurons. The action of these interneurons can interfere with postsynaptic recordings because interneurons are indirectly excited by pyramidal cell stimulation, therefore reducing the probability of firing of the pyramidal neurons. This effect can mask the underlying EPSC. To prevent this, GABAergic transmission will be blocked by introducing a $GABA_A$ receptor antagonist called picrotoxin into the artificial CSF solution.[21]

c. To induce an AP in the presynaptic neuron, a depolarizing current will be injected into the presynaptic neuron. The EPSC in the postsynaptic neurons will be measured. This protocol will be repeated 100 times per prepared slice. A resting time of 1 second will be utilized between each recording to allow for neurons to return to their resting membrane potential. The maximum amplitude of each EPSC in all the neurons in each trial will be measured.
d. The maximum amplitude of EPSC in each trial in the WT and mutant animals will be averaged to calculate the mean EPSC. Since the aim of the experiment is to study the changes in mean EPSC as a result of two different variables (age and the allele) a two-way ANOVA will be utilized to determine if a significant difference in mean EPSC exists between the model animals. This analysis will be performed in each of the three brain regions independently.

2.2.3 EXPECTED RESULTS & LIMITATIONS

If the Val404Met GOF mutation in the Kv4.2 channels of pyramidal neurons in layers 2/3 contributes to the potentiation of postsynaptic pyramidal neurons, we would expect to see a larger mean EPSC amplitude in slices from mutant animals versus WT animals. In particular, the mean EPSC amplitude is expected to be highest in the 60-day old mutant mice.

Since slices from three distinct locations of the CNS will be collected, this study can reveal if the potentiation is localized to a specific area in the CNS. It has been shown before that there are subtle electrophysiological differences between different population of neurons.[27] However, the Kv4.2 channel seems to be widely expressed in all cortical pyramidal neurons.[28] Therefore, we do not expect to see a significant difference between the mean EPSC amplitude in different regions of the CNS within the same animal model

There are some potential limitations to this experiment. For instance, it is assumed that the presynaptic pyramidal neuron and all its postsynaptic targets can be identified by their unique morphology. Even though this was done before by Niday and colleagues in 2017, it is a difficult task due to the complexity of the network of neurons in layer 2/3. Moreover, it should be noted that the addition of a $GABA_A$ receptor antagonist removes the influence of the GABAergic input in the measurements taken in this experiment. The reason behind this step is to directly investigate the effect of this mutation on the glutamatergic neurons by eliminating some of the complexities that are present in a cortical network of neurons. Further studies can investigate the effect of this mutation in the inhibitory postsynaptic current (IPSC) induced by the GABAergic neurons to get a clear understanding of the influence of this mutation on the cortical networks of neurons.

3.1 AIM 2

To compare the dendritic spine density in layer 2/3 cortical pyramidal neurons in WT animals versus animals carrying the Val404Met GOF mutation in Kv4.2 channels.

3.2.1 RATIONALE

Dendritic spines are the main site of excitatory input onto a neuron.[29] It is expected that an increase in dendritic spine density will be observed due to potentiation. More dendritic spines will allow the neuron to receive more excitatory input, increasing its chance of firing in response to multiple stimuli.[30] For instance, in 2015, Watson and colleagues demonstrated that the density of dendritic spines significantly increased in hippocampal pyramidal neurons after LTP induction.[30] Therefore, dendritic spine density is a good marker of potentiation, and it will be assessed according to the following procedure.

3.2.2 METHOD

a. Both the WT and mutant animals will be kept in similar conditions. The experiment will be done in 15day old and 60-day old animals. The animals will be anesthetized with diethyl ether and quickly decapitated. The brain tissue of animals will be removed
b. The cerebrum of the animal will then be placed in a light-proof glass jar containing Golgi-Cox solution.[31] This is an ideal stain for visualizing dendritic spines.[31] The tissue will stay in the solution for 48 hours at 37°C in the dark.[31] 150 µm thick coronal brain sections will be taken from the three regions of interest. The tissues will be viewed under the 100x objective light microscope. The pyramidal neurons of layer 2/3 will be identified due to their morphology.
c. In each animal model 16 cortical pyramidal neurons will be randomly selected for further analysis.[32] The

total length of all the dendritic branches including the primary branches arising from the soma and the secondary subsequent branches of the neuron will be measured using the image processing program Image-J.[32]

d. Mushroom type spines (head larger than the neck), thin type spines (head smaller than neck) and stubby spines (no neck) will be counted.[32] This categorization ensures that other protrusions such as filopodia are not counted as stable spines. The number of spines will be counted by two different investigators and the average value of these two counts will be calculated.[33]
e. The mean number of spines per 10 µm will be calculated from each animal and each brain region by dividing the total number of spines per neuron by the total dendritic length. A two-way ANOVA will be performed to determine if there is a significant difference between the WT and mutant animals depending on two variables (age and allele). This analysis will be performed independently for each brain region.

3.3 EXPECTED RESULTS & LIMITATIONS

If the cortical pyramidal neurons in the mutant animal have undergone potentiation, then a higher density of dendritic spines can be expected in the mutant animal compared to the WT. Furthermore, the spine density is expected to be the highest in the 60-day old mutant mice.

The dendritic structures seem to vary between different cortical regions.[34] Therefore, differences between spine densities are expected if different regions of the CNS were compared to each other. That's why in this analysis the two-way ANOVA is performed in each region independently. Since the Kv4.2 channel is present on all cortical pyramidal neurons, it can be expected that all the three regions of interest will demonstrate the highest spine density in the mutant 60-day old mice.[28] A potential issue with this method is mischaracterizing protrusions that are not dendrites with functional, stable synapses. To combat this issue, only the thin, stubby, and mushroom-type protrusions will be counted.

4.1 AIM 3

To compare the AMPA receptor density in the PSD of the pyramidal neurons in layer 2/3 of the cortex of mutant animals vs WT animals.

4.2.1 RATIONALE

A common feature of potentiation of glutamatergic synapses is an increase in AMPA receptor density in the post synaptic density (PSD).[35] A higher density of AMPA receptors makes the postsynaptic neuron more responsive to presynaptic glutamatergic input.[35] This effect is partially responsible for the predicted results mentioned in Aim 1, as an increase in AMPA receptor density can increase the size of EPSC.[35] A method of quantifying the number of receptors in the PSD is called immunoelectron microscopy.[36] In this method, antibodies specific for a receptor can be coupled with secondary antibodies carrying electron-dense particles (such as gold particles) to reveal the targeted antigen's location.[36]

4.2.2 METHOD

A. Extracting the Tissue and Labeling

a. Both the WT and mutant animals will be kept in similar conditions. The experiment will be done in 15-day and 60-day old animals.
b. The animals will be anesthetized and intracranially perfused with paraformaldehyde, phosphate buffer and glutaraldehyde to preserve the tissue.[37] These tissue blocks will be dehydrated by the process of free-substitution with resin.[37]
c. Initially, 0.5 µm semi-thin sections will be cut from each region of interest and stained with toluidine blue (labels for acidic residues such as DNA) to orient the sample and identify the area of interest in each block.[36] Once the pyramidal cells of layer 2/3 are identified, 60 nm ultrathin sections will be cut and mounted on nickel grids.[37] Sections will be emersed in normal goat serum for 30 minutes to block nonspecific antibody binding.[37] The sections will be incubated with primary antibodies against common AMPA receptor subunits such as GluR1, GluR2 and GluR4 overnight.[37] Then the sections will be rinsed and incubated for one hour with secondary antibodies that are attached to gold particles.[37]

B. Electron microscopy

a. Electron microscopy will be started with low magnification to identify the pyramidal cells of the layer 2/3 based on their morphological futures. The synapses will be identified by the presence of presynaptic vesicles and postsynaptic density.[37]
b. The receptors will be quantified by counting the number of the gold particles which will appear as black dots located in the PSD.[37]
c. The area of PSD will be estimated by measuring the length of the PSD and multiplying it by the thickness of each section.[37]
d. By dividing the number of gold particles counted per area of PSD, the density of channels per section can be calculated.
e. A two-way ANOVA will be performed to determine if there is a significant difference between the spine density on neurons from WT and mu-

tant animals depending on age and allele. This analysis will be performed independently for each brain region.

4.3 EXPECTED RESULTS & LIMITATIONS

If the GOF mutation in Kv4.2 K^+ channels induce potentiation in pyramidal cells of layer 2/3 of the cortex, it can be expected to see a significantly higher density of AMPA receptors in the PSD of mutant pyramidal neurons. Due to the ubiquitous expression of Kv4.2 channels on all pyramidal neurons, it can be expected that all brain regions demonstrate the result described above.

This investigation will be difficult due to the high complexity of the neural network in layer 2/3 of the cortex. Since the neurons have several connections, and since GABAergic interneurons are present in this area, it is essential to narrow down the targeted pyramidal cell's dendrite before conducting electron microscopy. To achieve this, the slices will be initially labelled with toluidine blue. This step is included in an immunogold labelling guideline by Zhaong and colleagues (2013). This label allows us to orient the samples appropriately, identify the region of interest based on morphology, and cut the surrounding tissue under the light microscope.

CONCLUSION

There are currently no experiments conducted to reveal why a GOF mutation in Kv4.2 channels can lead to the rapid firing of a neuron. This question can potentially be answered with this research. This understanding can significantly advance the field of neuroscience by providing a deeper insight in the field of homeostatic potentiation. The causes and effects of homeostatic plasticity are not well understood in the cortical pyramidal neurons, especially in the context of epilepsy. The findings of this research can propose the transient silencing of neurons with an increased K^+ current as a potential mechanism that induces homeostatic potentiation on cortical pyramidal neurons. Further research can build upon the findings of this study and investigate the effects of GOF mutations in voltage gated K^+ channels in other populations of neurons, especially the subcortical structures, since they are also often involved in localized seizures.[38] Furthermore, the findings of this research will shed light on the potential impact of homeostatic plasticity on the pathogenesis of epilepsy specifically and more broadly, on the activity rates of cortical networks of neurons.

With all the advancements of modern medicine, epilepsy is a highly manageable disease that responds well to treatments such as benzodiazepines. In fact, over 70 % of patients can become seizure-free with the appropriate treatment protocol.[1] However, some of these treatments do not specifically target the affected channels and neurons. Consequently, they lead to some side effects. For instance, benzodiazepines, which are a common treatment for the management of epilepsies, can also lead to side effects such as sedation, tolerance, and addiction, to name a few.[39] Side effects can be reduced if the specific neurons and channels involved in the pathogenesis of epilepsy are targeted for treatment.

Furthermore, the seizures induced as a result of this mutation were highly resistant to treatments such as benzodiazepines.[9] Hence, this research can provide the scientific community with a deeper understanding of the pathophysiology of the GOF mutation of the Kv4.2 channels. This knowledge can further be used to design specific pharmacological tools that target the Kv4.2 channels and reverse the mutations' effects. An example of a targeted epilepsy treatment is phenytoin. It targets voltage-gated sodium channels mostly in the motor cortex, which has been shown to be a very effective treatment for tonic-clonic seizures with less side effects than benzodiazepines.[40] Designing such targeted therapies is not possible without first understanding the pathology induced by the mutation.

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