SLEEP AND CARDIAC DYSFUNCTION IN Kv1.1 KO MICE, A MODEL OF SUDDEN UNEXPECTED DEATH IN EPILEPSY

By

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A MASTER'S THESIS

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DEDICATION

To my sister Lavanya and my parents, Mr. A.G Hariharan and Mrs. Lalitha Hariharan

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Chapter 1.
Introduction

Epilepsy, incidence and etiology:

Epilepsy is a chronic, broad spectrum neurological disease which is characterized by recurrent and unprovoked seizures (Fisher et al. 2014). It is the fourth most common neurological disease affecting approximately 50 million people globally (Leonardi and Ustun 2002). In fact, it is estimated that about 1 in 26 people in the United States, will develop epilepsy during some point in their lifetime (Hesdorffer et al. 2011). The pathophysiology of epilepsy and seizures involves a chronic hyper excitability of the brain caused by hyperactive and hyper synchronous neuronal firing. This increase in neuronal excitability in epilepsy may be caused by a varied range of factors, channelopathies, and alterations in synaptic transmission, changes in levels of neurosteroids or neuropeptides or metabolic disorders. These abnormal changes may be the resultant of genetic abnormalities and developmental disorders, brain injury or trauma, stress or may be idiopathic in nature (Berg and Scheffer 2011; Shorvon 2011).

Epilepsy and Sudden death:

Studies have shown that people with epilepsy have a higher premature mortality than the general population as indicated by high values of the standardized mortality ratios (or SMR) for epileptic population. SMR is the ratio of observed deaths in a cohort population compared to the expected number of deaths in the general population; a value higher than 1, as seen in the epileptic population, indicates higher mortality in the cohort population (Forsgren et al. 2005; Gaitatzis et al. 2004; Tomson 2000). Sudden unexpected death in epilepsy (SUDEP) has been identified as the most common cause of mortality due to epilepsy with an incidence rate of 1 in 1000 cases of epilepsy every year. In the U.S, amongst the various neurological disorders where potential years of life are lost, SUDEP ranks second to stroke, claiming about 3000 lives every year (Thurman,

Hesdorffer et al. 2014). SUDEP has been defined as the sudden, unexpected, witnessed or unwitnessed, non-traumatic, and non-drowning death of a subject affected by epilepsy, with or without evidence for a seizure (excluding documented status epilepticus) in which autopsy does not reveal a structural or toxicological cause of death (Nashef 1997).

SUDEP: Mechanisms and Risk Factors:

While the precise etiology of SUDEP is unknown, the events immediately preceding SUDEP might provide some perspective into the possible mechanisms involved in causing death. Witnessed cases of SUDEP or near SUDEP in epilepsy monitoring units have shown that 50-90% of the cases show evidence of a seizure immediately preceding death, however this represents a subgroup of the SUDEP cases and needs to be studied further (Kloster and Engelskjøn 1999). Since seizures are an important clinical manifestation of epilepsy, it is logical to assume that factors like the type of seizure, seizure frequency, duration etc. might have some role in the pathological mechanisms involved in SUDEP. In fact, pooled data from numerous case control studies of risk factors for SUDEP conducted in the USA and Europe show that an increased seizure frequency, particularly generalized tonic clonic seizures, a young age of epilepsy onset and increased duration of epilepsy, are some of the factors that increase the risk for SUDEP in the epileptic population (Hesdorffer et al. 2011).

SUDEP: Cardiovascular abnormalities as possible risk factors

For a long time, it has been known that seizures can cause ictal and inter-ictal cardiac abnormalities (Erickson et al. 1939). Studies involving simultaneous electrocardiogram (ECG)-electroencephalogram (EEG) recordings in patients as well as animal models of epilepsies, have described many severe cardiac conduction abnormalities including ictal sinus tachycardia, ictal

and post-ictal bradycardia and asystole, atrioventricular block, QT- abnormalities etc. (Naritoku et al. 2002; Nei and Sperling 2000; Keilson et al. 1989; Howell and Blumhardt 1989). Since a leading cause of sudden death in the general non-epileptic population is often cardiac dysfunction, it makes this a potential candidate mechanism involved in SUDEP. While many case studies have reported arrhythmias and conduction blocks as events preceding SUDEP in patients (Mehvari et al. 2014; Ferlisi et al. 2013; Nei et al. 2004), an extensive retrospective study by the MORTality in Epilepsy Monitoring Unit Study (MORTEMUS) reported that following a seizure, there was a transient period of non terminal asystole, followed by a long bradycardia event which eventually resulted in terminal asystole (Ryvlin et al. 2013). Mouse models of Dravet syndrome and long QT syndromes, which are useful genetic models for SUDEP, have also shown cardiac electrophysiology, myocyte hyper excitability, ventricular fibrillation, bradycardia and asystole preceding death (Glasscock 2014; Auerbach et al. 2013; Goldman et al. 2009).

Kv1.1 KO mice, a model of temporal lobe epilepsy and SUDEP:

The animal model which was used in my study is the Kv1.1 KO mouse, generated on the C3HeB/FeJ background strain, as described by Smart et al. from the University of Washington. The Kv1.1 heterozygous mice are paired to generate Kv1.1 KO mice and their wild type litter mates which have this gene and serve as controls. The Kv1.1 channel which is a delayed-rectifier voltage-gated potassium channel, is widely distributed in the mouse nervous system at sites including the juxtaparanodal regions of myelinated axons and synaptic terminals (Wang et al. 1994; Hao Wang et al. 1993). The broad distribution of this channel suggests that the Kv1.1 channel maybe important in regulating action potential generation, membrane repolarization, neurotransmitter release and deletion of this channel can lead to neuronal hyper excitability

which is seen in the Kv1.1 KO mice (Al-Sabi et al. 2010; Tempel 2003). In fact, studies have shown that functional mutations in the human homologue of the Kv1.1 gene, are associated with episodic ataxia and partial epilepsy (Eunson et al. 2000; Zuberi et al. 1999).

Kv1.1 KO mice predictably develop temporal lobe epilepsy at the third postnatal week and exhibit recurrent, spontaneous limbic seizures for the duration of their life, with seizure severity worsening with age (Wenzel et al. 2007; Smart et al. 1998). Along with the worsening seizure phenotype, one important feature of this model is that the Kv1.1 KO mice show a distinctly shorter life span compared to their wildtype littermates and show mortality by postnatal week 7. Interestingly, studies involving simultaneous EEG-ECG recording in this mouse model have revealed numerous cardiac abnormalities including episodes of post ictal bradycardia and atrioventricular blocks, despite the fact that the Kv1.1 channels have no direct role in the function of mouse heart or vascular tissue (Glasscock et al. 2010). However, there seems to be a relationship between cardiac abnormalities, worsening seizure pathology and the premature mortality seen in these mice, suggesting a role of the central and/or autonomic nervous system in mechanisms of death. This has led to the development of the Kv1.1 KO mice as a robust SUDEP model to study cardiovascular and neuronal mechanisms preceding death (Moore et al. 2014).

Kv1.1 KO mice, sleep and SUDEP:

Our previous EEG studies using the Kv1.1 KO mice have revealed the presence of sleep dysfunction in these mice. Typical manifestations include increased latency to sleep, sleep fragmentations and decreased REM and NREM sleep (Roundtree et al. 2016). This is very similar to studies in humans that have shown a high prevalence of sleep disorders and related co-morbidities in epileptic patients (Yazdi et al. 2013; Manni and Terzaghi 2010). Studies in

animals and humans have also indicated sleep deprivation can result in seizure exacerbation, in terms of both seizure frequency and intensity (Foldvary-Schaefer and Grigg-Damberger 2009; Mattson et al. 1965). Sleep dysregulation can also result in autonomic imbalance and increased susceptibility to potentially lethal arrhythmias (Mullington et al. 2009; Meier-Ewert et al. 2004).

In the light of all these facts, we hypothesize that sleep dysfunction and cardiac abnormalities promote SUDEP in the Kv1.1 KO mice and thus can be used as biomarkers to predict SUDEP in these mice.

Chapter 2.

Cardiac abnormalities and effect of orexin receptor antagonism in Kv.1.1 KO mice

INTRODUCTION

Epilepsy is a chronic neurological disease characterized by recurrent and unprovoked seizures (Fisher et al. 2014). One in 26 people develop epilepsy at some point in their lives (Hesdorffer et al. 2011). People with epilepsy have a higher risk of mortality when compared to the general population. Sudden Unexpected Death in Epilepsy is one the leading causes of mortality in epilepsy and has an incidence of 1:1000 in patients with epilepsy (Thurman et.al 2014). Retrospective studies have indicated that the increased seizure frequency and intensity and resultant cardiovascular abnormalities maybe some of the major risk factors for SUDEP (Ferlisi et al. 2013; Nei et al. 2004). One of the pivotal studies in the epilepsy monitoring units across the world by MORTEMUS, reported the sequence of cardiovascular events preceding SUDEP. All patients had a generalized tonic clonic (GTC) seizure, then a transient cardiac asystole followed by a long bradycardia event which ultimately lead to a terminal cardiac asystole (Ryvlin et al. 2013). Studies in animal models of epilepsy have also reported arrhythmias, ventricular fibrillation, atrioventricular blocks and asystole as the events preceding death (Auerbach et al. 2013; Goldman et al. 2009).

Kv1.1 KO mice and cardiac abnormalities:

The Kv1.1 KO mouse model used in our study, is a model for temporal lobe epilepsy. These mice have a predictable age of epilepsy onset at around P21. They exhibit spontaneous limbic seizures throughout the duration of their life, with the seizure phenotype worsening with age (Wenzel et al. 2007; Smart et al. 1998). Recently, the Kv1.1 KO model has emerged as an important animal model for SUDEP. EEG-ECG studies is this model have revealed that the severe seizure phenotype is accompanied by increased vagal nerve activity accompanied by

several episodes of atrioventricular block and arrhythmia that maybe fatal (Glasscock et al. 2010). Detailed study of the events preceding SUDEP in these mice have shown that following a terminal GTC seizure, these mice have post-ictal bradyarrhythmia that eventually leads to terminal asystole, similar to the events reported in patients who died of SUDEP (Moore et al. 2014; Aiba and Noebels 2015).

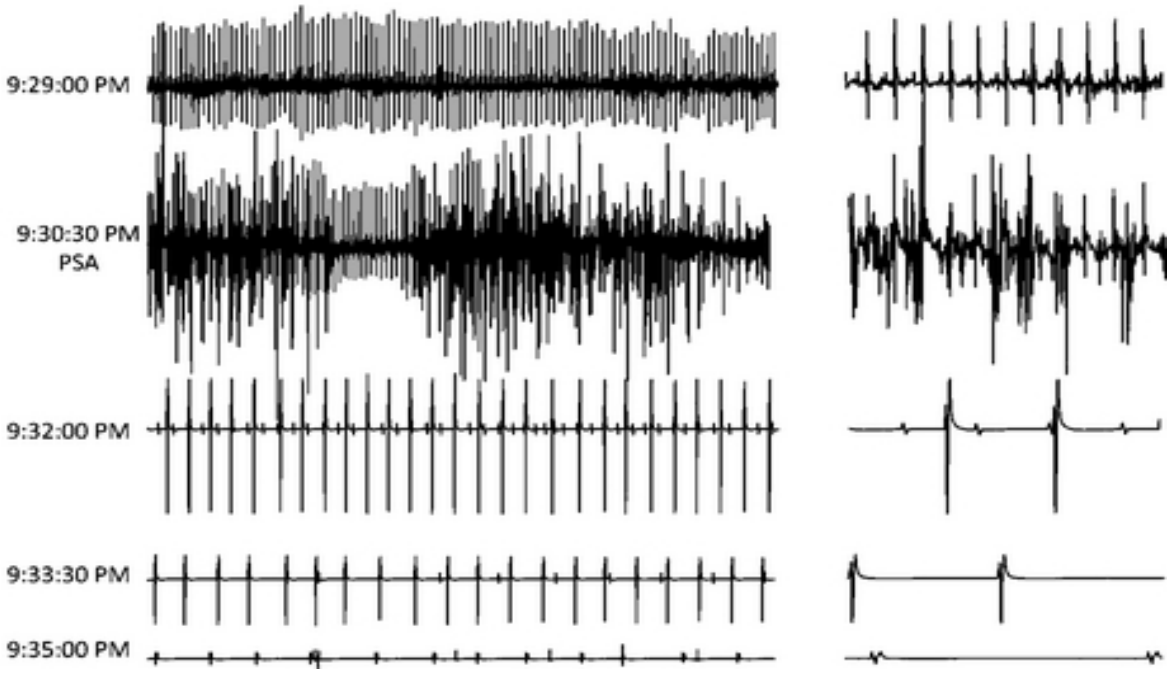
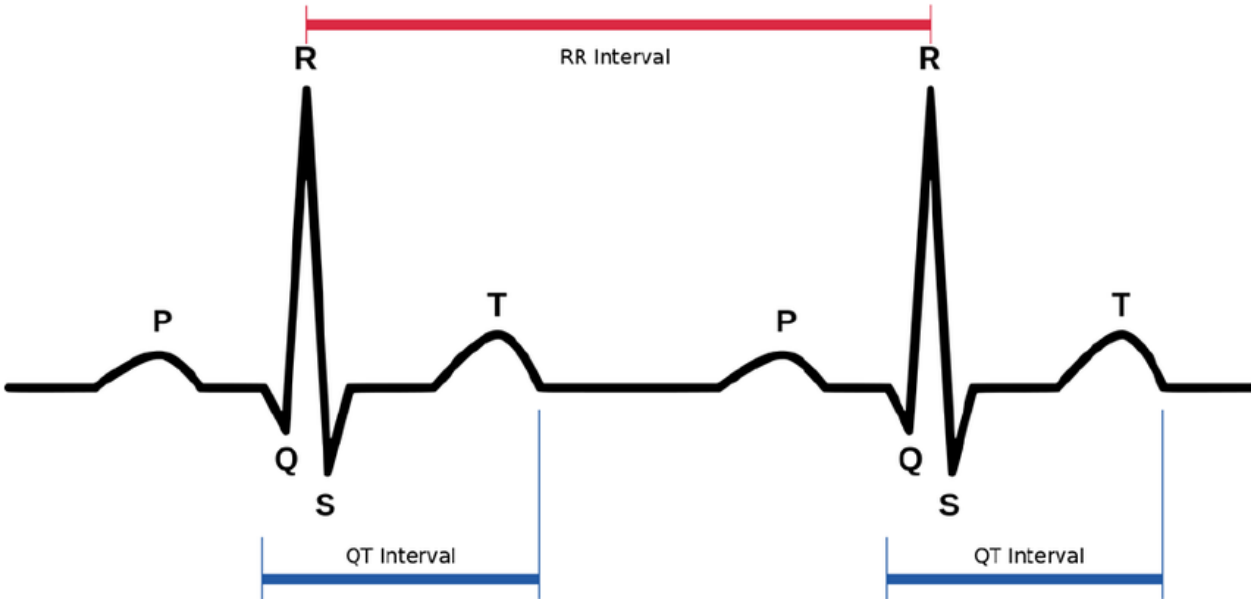


Figure 1: SUDEP in a Kv1.1 KO mouse was preceded by cardiac abnormalities including severe bradycardia and asystole. Simultaneous EEG-ECG recording in Kv1.1 KO mice shows the sequence of events preceding death in Kv1.1 KO mouse. In this figure, only the ECG recordings are indicated. Initially the ECG shows normal rhythm (9:29:00 p.m.), seizure activity (9:30:30 p.m.), and sinus bradycardia with complete heart block (9:32:00 p.m.), progressing to junctional escape rhythm (9:33:30 p.m.) and then an agonal slow ventricular escape rhythm which a slow terminal ventricular rhythm <50 bpm (9:35:00 p.m.) and progressed to asystole and death. (Modified Moore et al. 2014).

The cardiac abnormalities are closely related to the severity of epilepsy (Naritoku et al. 2003). A case study recently found that cardiac abnormalities were negligible several months prior to SUDEP, however nearing SUDEP, there was a dramatic in parasympathetic activity, which corresponds to the temporal increase in seizure severity from epilepsy onset age to SUDEP in the Kv1.1 KO model (Simeone et al. 2016; Jeppesen et al. 2014). Interestingly, all of the cardiac studies in Kv1.1 mice have described the cardiac abnormalities immediately preceding death, however the development of these abnormalities in relation to seizure ontogeny has not yet been reported. In this study, one of our objectives was to determine whether parasympathetic overdrive and related cardiac events change from epilepsy onset age to ages close to SUDEP. We compared various cardiac parameters in the Kv1.1 KO mice and their WT littermates at three different ages, P28-36 (younger age), P37-P44 (age close to SUDEP) and P45-52 (SUDEP age) when majority of the mice die.

Electrocardiography, common terms and definitions:

We evaluated cardiac function in these mice using the electrocardiography method which is the process of recording the electrical activity of the heart over a period of time using electrodes. The ECG so obtained is measurement of the electrical changes associated with depolarization and repolarization of the heart during each heartbeat. The ECG is composed of three main parts, 1) P-wave that represents atrial depolarization; 2) QRS complex that represent ventricular depolarization and the 3) T-wave that represents ventricular repolarization (Becker 2006a). Some of the basics of ECG related cardiac parameters are discussed below.



Heart rate (HR): Heart rate is the speed of the heartbeat measured by the number of contractions or beats of the heart per minute (bpm). Heart rate is regulated by the autonomic nervous system. In simplistic terms, an increase in heart rate (tachycardia) is usually mediated by the sympathetic predominance, while a decrease in heart rate (bradycardia) is mediated by parasympathetic predominance (Purves et al. 2001). Normally, both bradycardia and tachycardia events occur in animals in response to various physiological and external stimuli, but since the autonomic nervous system (ANS) is a very well-regulated system, these events are transient. However, increases or decreases in heart rate outside the normal physiological range, suggests dysregulation of the ANS (Becker 2006b). Interestingly, while the occurrence of seizures itself is associated with tachycardia, the cause of death in SUDEP patients is severe bradycardia and asystole (Mehvari et al. 2014; Almansori, Ijaz, and Ahmed 2006).

R-R interval: The time interval between successive R-waves of the QRS complex is known as the R-R interval. It is inversely related to heart rate, increase in HR results in shortening of R-R

intervals, while a decrease in HR is associated with prolongation of the R-R interval (Becker 2006a).

QT interval: The QT interval is measured from the beginning of the QRS complex to the end of the T wave. It represents the time during which the ventricles depolarize and repolarize and is a measure of ventricular action potential (AP) duration. The corrected QTc interval is calculated using Bazett's formula:

$$QTc = \frac{\text{QT interval}}{\sqrt{\text{R-R interval}}}$$

Prolongation of the QTc interval is an indicator of arrhythmogenic disorders and can result in sudden cardiac death. In fact, long QTc is one of the proven risk factors for Sudden Infant Death Syndrome (SIDS) (Border and Benson 2007; Ackerman 2005). Prolongation of QTc is also seen in epileptic patients and could be a possible cardiac factor involved in SUDEP (Glasscock 2014; Anderson et al. 2012).

Root mean square of standard deviation or rmSSD: This indicates the variability in the R-R intervals and is of one the parameters used to judge short term heart rate variability. Heart rate variability is a normal physiological phenomenon occurring due to change in sino-atrial node rhythmicity in response to stimuli. rmSSD indicates the relative predominance of the parasympathetic and sympathetic nervous system in controlling heart rate. Increased rmSSD values are associated with parasympathetic overdrive (DeGiorgio et al. 2011; Thayer, Yamamoto, and Brosschot 2010).

Kv1.1 KO mice: Orexinergic hyperactivity and cardiac abnormalities

While the autonomic nervous system (ANS) controls the cardiovascular homeostasis in the body, the ANS itself is regulated by a number of nuclei located in the brainstem. Orexinergic neurons located in the lateral hypothalamus are involved in appetite, sleep and autonomic regulation (Tsujino and Sakurai 2013; Sakurai et al. 1998). Orexinergic neurons project to many cardiovascular nuclei, including the nucleus ambiguus, nucleus tractus solitarius and the rostral ventrolateral medulla, and can regulate the predominance of sympathetic or parasympathetic drive depending upon external stimuli (Shahid et al. 2011; Ciriello, McMurray et al. 2003). Excessive orexinergic stimulation can induce a parasympathetic vagal overdrive and cause severe bradyarrhythmia (de Oliveira et al. 2003; Ciriello et al. 2003). Interestingly, our previous studies have shown that when compared to WT controls, there is an increase in the orexinergic neurons in the Kv1.1 KO mice, at the pre-SUDEP age (P40), which is not seen at younger ages (P21 or P30) (Roundtree et al. 2016). In the light of all these facts, we hypothesize that increased orexinergic activity maybe upstream of the excessive parasympathetic drive and resultant cardiac abnormalities preceding SUDEP in Kv1.1 KO mice. To test this hypothesis, we used almorexant, a dual orexin receptor antagonist which is a competitive antagonist at the Orexin-1 and 2 receptors in the brain (Mang et al. 2012). We hypothesized that blocking the activity of the orexinergic system would reduce the cardiac abnormalities normally seen prior to SUDEP and attenuation of the parasympathetic overdrive in Kv1.1 KO mice.

Recent studies have shown that orexin receptors are present in the periphery; it is possible that the influence on cardiac activity might be a result of orexin acting directly on these receptors (Jöhren et al. 2011; Zhang et al. 2005). Studies have also shown an increase in serum orexin levels in epileptic patients (Kaciński et al. 2012). Hence we also evaluated if there is an increase

in the blood orexin levels at the SUDEP age compared to the younger ages in the Kv1.1 KO mice, when compared to their age matched WT controls.

MATERIALS AND METHODS

Animals: C3HeB/FeJ Kv1.1 knockout and wild-type littermates were acquired from The Jackson Laboratory and bred and reared at the Animal Resource Facilities at Creighton University. The mice were subjected to a strict 12 hour light/dark cycle and provided with food and water ad libitum. The genotype of the mice was determined by tail clipping methods using Transnetyx, Inc (Cordova, TN, USA). All experiments were carried out in accordance with the guidelines of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee at Creighton University.

Drugs and reagents: All reagents were purchased from Sigma unless otherwise noted. Almorexant was provided by Acetlion Pharmaceuticals (San Francisco, CA, USA).

ECG: ECGs were recorded non-invasively from Kv1.1 KO and WT mice periodically from P28 until the age where mice die, using the ECGenie recording platform (Mouse Specifics, Inc., Boston, MA, USA). Briefly, mice were placed on the ECGenie recording platform and each mouse was allowed to acclimatize for 10 min prior to ECG recording. ECG signals were recorded and digitized at a sampling rate of 2000 samples/s. Data from continuous recordings of more than 2secs were analyzed using e-MOUSE software (Mouse Specifics, Inc., Boston, MA, USA). The software uses a peak detection algorithm to find the peak of the R-waves and to calculate heart rate. Time domain analysis of the recordings was done and the following endpoints were determined: 1) Heart rate in beats per minute (bpm); 2) R-R interval in ms; 3) Corrected Q-T interval (QTc) and 4) Root mean square of standard deviation (rmSSD). Data

were collapsed across animals and divided into three different age bins; P28-P36 (younger age), P37-P44 (near SUDEP age) and P45-P52 (SUDEP age). There were about 10-12 segments of ECG recording for each mouse. The data was averaged for each age bin and plotted.

Almorexant ECG experimental design: Kv1.1 KO mice were injected with vehicle (50% DMSO in sterile saline), given a day of rest and injected 100 mg/kg almorexant (i.p., 25 mg/mL in vehicle). The vehicle and drug injections were done at three age ranges, P30-33, P38-40 and P44-48. ECG was recorded after vehicle and drug injections using the ECGenie recording platform described above. There were about 10-12 segments of ECG recording for each mouse. The following endpoints were determined: 1) Heart rate in beats per minute (bpm); 2) R-R interval in ms; 3) Corrected Q-T interval (QTc) and 4) Root mean square of standard deviation (rmSSD).

Enzyme Immunoassay (EIA) for quantification of blood orexin-A: WT and KO mice belonging to two age groups, P23-P38 and P40-P52, were anesthetized with isoflurane, quickly decapitated and trunk blood was collected. Serum was separated by centrifuging the samples at 3000 rcf for 10 mins at 4°C and was stored at -80°C. The serum samples were further purified using reverse phase C18 Sep-columns (Phenomenex, Torrance, CA, USA) and washing and elution buffers (Phoenix Pharmaceuticals, Mountain View, CA, USA) as per the instructions provided with the extraction columns. The extracted samples were concentrated using a Savant Speedvac (model, company, city, state) for 40 mins and then lyophilized. Orexin A levels were measured in the reconstituted serum sample using commercially available murine orexin-A chemiluminescent EIA kit (Phoenix Pharmaceuticals, Mountain View, CA, USA). The minimum detectable concentration was 12.2pg/ml. This EIA kit is based on the principle of competitive

enzyme immunoassay. The concentration of sample was calculated using a standard curve with a linear range of 12.2 to 156pg/ml.

RESULTS

Changes in cardiac parameters as a function of age and genotype:

Kv1.1 KO mice show an age dependent decrease in heart rate compared to the WT controls

We first compared the cardiac parameters between the Kv1.1 KO mice and their WT littermates at three different ages P28-P36 (younger age), P37-P44 (near SUDEP age) and P45-P52 (SUDEP age), which have been referred as P28, P37 and P45 respectively, throughout this section. We used the 2-way ANOVA with Sidak's multiple comparison *post hoc* test for analyzing the differences in heart rate. A $p<0.05$ was considered significant.

When compared between the two genotypes, we found that the KO mice have significantly reduced heart rate (bpm) when compared to the WT controls ($F(1, 91) = 54.09$, $p<0.0001$). At the younger age bin of P28, we found no significant difference in heart rate in the KO and WT mice) (Figure 2A-B). However, at the near SUDEP age (P37), the KO mice showed about 10% reduction in heart rate when compared to WT mice (699 ± 17.3 and 789 ± 7.42, respectively. $p<0.001$). At the SUDEP age (P45), there was a 15% reduction in heart rate in KO mice compared to the WT controls (657 ± 10.2 and 778 ± 14.6, respectively, $p<0.001$).

When compared within group across age, the KO mice showed a significant decrease in the heart with age ($F(2, 91) = 14.73$, $p<0.0001$). There was a 10% reduction in heart rate from P28 to P37 ($p<0.001$), and a further 7% decrease from P37 to P45 ($p< 0.05$). The WT heart rate was the same at all three ages.

Overall, our data indicate that there is a decrease in heart rate in Kv1.1 KO mice at ages closer to SUDEP and an increased incidence of bradycardiac events increase prior to death.

The duration of the R-R interval increases with age in Kv1.1 KO mice when compared to the WT controls

On comparison between the two genotypes, we found that the KO mice have a significantly longer duration of the R-R interval (ms) when compared to the WT controls ($F(1, 91) = 52.36$, $p < 0.0001$), the longer R-R interval is consistent with slower heart rate. Like the heart rate data, we found that at the younger age of P28, there are no significant differences in the average R-R interval between the KO and WT mice (Figure 2 C-D). However, the R-R interval becomes significantly longer in duration in the KO mice at both the near SUDEP age P37 and the SUDEP age P45($p<0.001$).

When compared within group across age, the KO mice showed a significant increase in R-R interval with age ($F(2, 91) = 13.44$, $p<0.0001$). When compared to P28 (77.4 ± 1.7) there is a 10% increase in R-R interval at the near SUDEP age P37 (87.4 ± 1.2, $p<0.001$), and a 20% increase at SUDEP age P45 (92.6 ± 1.5, $p< 0.05$). There was no change in the duration of R-R interval in the WT mice across age. Overall our data indicates that there was an increase in the duration of the R-R interval in Kv1.1 KO mice at ages closer to SUDEP, with no difference at younger ages.

QTc interval is prolonged in Kv1.1 KO mice compared to age matched WT controls

On comparing the two genotypes, we found that the KO mice have significantly longer duration of the QTc interval (ms) when compared to the WT controls ($F(1, 77) = 33.92$, $p< 0.0001$) (Figure 2 E-F). Similar to the R-R interval, we found that at the younger age of P28, there are no

significant differences in the duration of QTc interval between the KO and WT mice. However, the R-R interval becomes significantly longer in duration in the KO mice at both the near SUDEP age P37 and the SUDEP age P45 ($p<0.001$).

When compared within group across age, there were no significant differences in QTc interval in the Kv1.1 KO mice across the various ages ($F(2, 77) = 1.682$, $p = 0.1927$). However, when compared to younger P28 age group, there was a significant increase in the duration of QTc intervals at both near SUDEP (P37, $p<0.05$) and SUDEP (P45, $p<0.01$) ages.

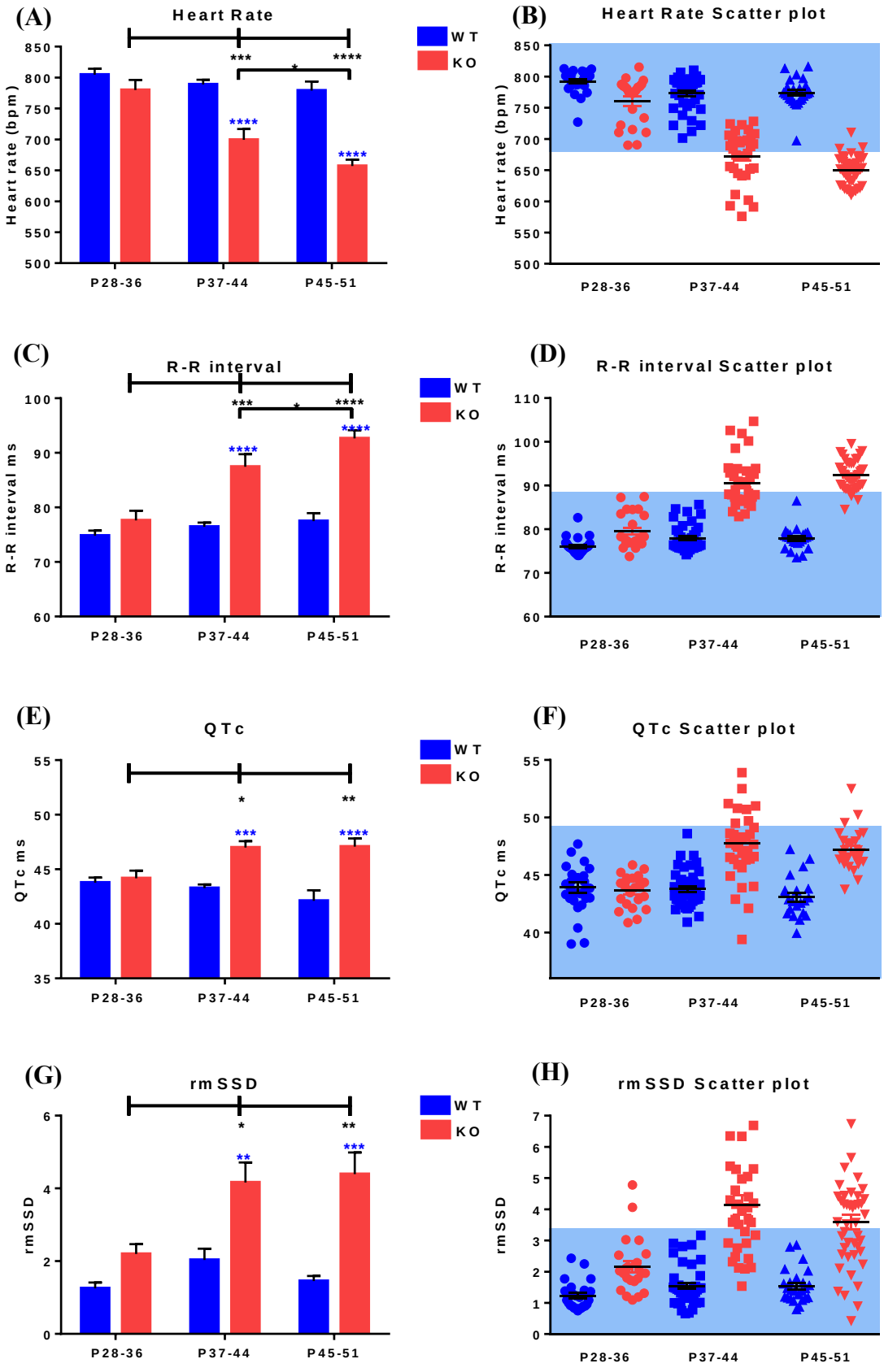
Our data suggests that the prolongation of QTc in the ages closer to SUDEP indicate that long QTc may be an important risk factor for SUDEP similar to SIDS.

There is an increase in the rmSSD values in Kv1.1 KO mice compared to age matched WT controls

We hypothesized that parasympathetic overdrive maybe a mechanism behind the bradycardia and asystole occurring prior to death in the Kv1.1 KO mice. Hence we decided to evaluate the differences in rmSSD between the Kv1.1 KO and WT mice at ages closer to SUDEP, since increased rmSSD indicates parasympathetic predominance.

The KO mice had significantly higher rmSSD value when compared to the WT controls ($F(1, 91) = 27.22$, $p < 0.0001$) (Figure 2 G-H). The rmSSD values of KO and WT mice are similar at P28. However, at P37 (4.164 ± 0.55, $p<0.001$) and at P45 (4.39 ± 0.5, $p<0.001$), the rmSSD of KO mice is twice that of WT controls (2.035 ± 0.3). When compared within groups across age, we found that in Kv1.1 KO mice, the rmSSD values at P37($p<0.05$) and P45($p<0.01$) are significantly higher compared to the young P28 mice.

Our data indicates that the KO mice have increased rmSSD values compared to WT controls at SUDEP ages, supporting our hypothesis that an increase in parasympathetic tone plays a critical role in the cardiac events prior to SUDEP.


(A)
Heart Rate
WT
KO
Heart rate (bpm)
850
800
750
700
650
600
550
500
***
*
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****
****
P28-36
P37-44
P45-51
(B)
Heart Rate Scatter plot
Heart rate (bpm)
P28-36
P37-44
P45-51
(C)
R-R interval
WT
KO
R-R interval ms
100
90
80
70
60
***
*
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P28-36
P37-44
P45-51
(D)
R-R interval Scatter plot
R-R interval ms
110
100
90
80
70
60
P28-36
P37-44
P45-51
(E)
QTc
WT
KO
QTc ms
55
50
45
40
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P28-36
P37-44
P45-51
(F)
QTc Scatter plot
QTc ms
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40
P28-36
P37-44
P45-51
(G)
rmSSD
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KO
rmSSD
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P28-36
P37-44
P45-51
(H)
rmSSD Scatter plot
rmSSD
7
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P28-36
P37-44
P45-51

Figure 2. Kv1.1 KO mice show significant decrease in heart rate (bpm), prolongation of R-R and QTc (ms) intervals and high rmSSD values compared to their age matched WT littermates prior to SUDEP. The cardiac parameters were analyzed using 2-way ANOVA with Sidak's *post hoc* test. **(A)** Kv1.1 KO mice show significantly lower heart rates compared to age matched WT mice at ages approaching SUDEP. KO mice closer to SUDEP age have reduced heart rate compared to younger KO mice. **(B)** Scatter plot shows that KO mice have a large number of bradycardia events at older ages, indicated by a large number of heart rate values lying below the normal WT heart rate threshold or normal range (blue box). **(C)** The Kv1.1 KO mice show prolonged R-R interval compared to age matched WT mice at ages approaching SUDEP with no difference at a younger age. KO mice closer to SUDEP age have increased R-R interval compared to younger KO mice. **(D)** Scatter plot shows at ages closer to SUDEP KO mice have a number of R-R intervals lying above the normal WT threshold. **(E- F)** Plot**s** depict that KO mice have prolonged QTc interval compared to age matched WT mice at ages approaching SUDEP. There is also age-dependent increase in QTc interval. Similarly, at older ages, QTc values are well above the control range, indicating risk of ventricular arrhythmia. **(G-H)** The Kv1.1 KO mice have higher rmSSD values compared to age matched WT mice at ages approaching SUDEP with no difference at younger age. There is also an increase in rmSSD in KO mice with age. Furthermore, scatter plot shows that in the KO mice rmSSD values are greater than WT rmSSD at P37-P51, with younger mice having values within the WT range. (Blue asterisk,*, indicates comparison with age matched WT mice and black asterisk,*, indicates comparison within KO mice across age). Data are expressed as Mean ± SEM; n=5-6 for each group; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Effect of Almorexant on the cardiac parameters in Kv1.1 KO mice:

In this study, we found that there were significant differences in the cardiac parameters between Kv1.1 KO and WT mice. Furthermore, in the Kv1.1 KO mice, these differences are more apparent in the ages approaching SUDEP. We hypothesized that the hyper activation of the orexinergic system might be responsible for these abnormalities in cardiac parameters in the Kv1.1 KO mice. Hence, we evaluated whether pharmacological antagonism of the brain orexin receptors by administration of almorexant, a DORA, restores the cardiac parameters to WT levels. We injected Kv1.1 KO mice with vehicle on day 1 and almorexant at day 3, at three different ages, P32-36(younger age), P38-40(near SUDEP age) and P44-48(SUDEP age). Data was analyzed using both one way and two way ANOVA with Sidak's multiple comparison *post hoc* test.

Almorexant treatment increases heart rate and shortens the R-R interval at ages closer to SUDEP in Kv1.1 KO

As mentioned in the results previously, the Kv1.1 KO have significantly reduced heart rates in the ages approaching SUDEP because of the numerous seizure related bradycardia episodes at this age. Considering that the orexinergic hyper drive may be upstream of this increased parasympathetic activity, we would expect that orexin antagonist will reduce the parasympathetic hyperactivity and reduce the bradycardia episodes in the KO mice. This would be reflected as an increase in the mean heart rate for the mice.

Data analyses using two way ANOVA: Since there were about 8-20 segments of ECG signals for each group, we first took the mean value for each animal and every age. We analyzed the data using a 2 way ANOVA and found that overall, there were no differences in the heart rate

between the vehicle controls and the almorexant treated groups ($F(1, 32) = 0.07018$, $p=0.7928$). Although, overall there was no effect of age on the heart rate in these groups ($F(2, 32) = 0.3392$, $p=0.7149$), we found that, the vehicle treated group at P44-48 which is the general age range for SUDEP had significantly lower heart rate than the vehicle treated group in the age range of P32-36 (724.6 ± 16.93 and 658.4 ± 21.4 respectively, $p< 0.05$).

Data analyses using one way ANOVA: However, considering the biological distribution of our data within animal, simply averaging the heart rate data for animals reduces the sensitivity of this assay and our ability to detect the bradycardia events that occur intermittently in these animals. We determined the heart rate range for WT mice from the cardiac data described previously, this was considered as the control value for the almorexant study. We plotted the raw heart rate values for every animal and looked at the distribution of the heart rate between vehicle and almorexant treated groups using a scatter plot (Figure 3C). Visually, we observed that at the younger age bin the vehicle treated KO mice show heart rate values which are in the WT range (obtained from the previous results), however at older ages, the distribution of the heart rate values is well outside that of WT control range. At older ages, almorexant treated animals show heart rate values distributed closely around the WT control range, suggesting that almorexant may restore the heart rate in the KO mice to the WT levels in ages approaching SUDEP.

We analyzed the raw data using a one way ANOVA, in order to test for the differences between the vehicle and almorexant treated groups while preserving the biological variability (Figure 3B). We found that administration of almorexant caused a significant increase in heart rate at P38-40 and at SUDEP age (P44-48) in the KO mice ($F(5, 188) = 29.76$, $p<0.0001$). At P38-40, there was a 5% increase in heart rate in almorexant treated KO animals compared to vehicle treatment (691.9 ± 3.62 and 654.8 ± 5.082 respectively, $p< 0.0001$). At the SUDEP age, there was an 8%

increase in heart rate in almorexant treated KO animals compared to vehicle treatment (725.1 ± 3.24 and 665.6 ± 5.4 respectively, p< 0.0001). The younger vehicle treated KO mice have heart rates similar to WT mice and do not differ from almorexant treated KO mice.

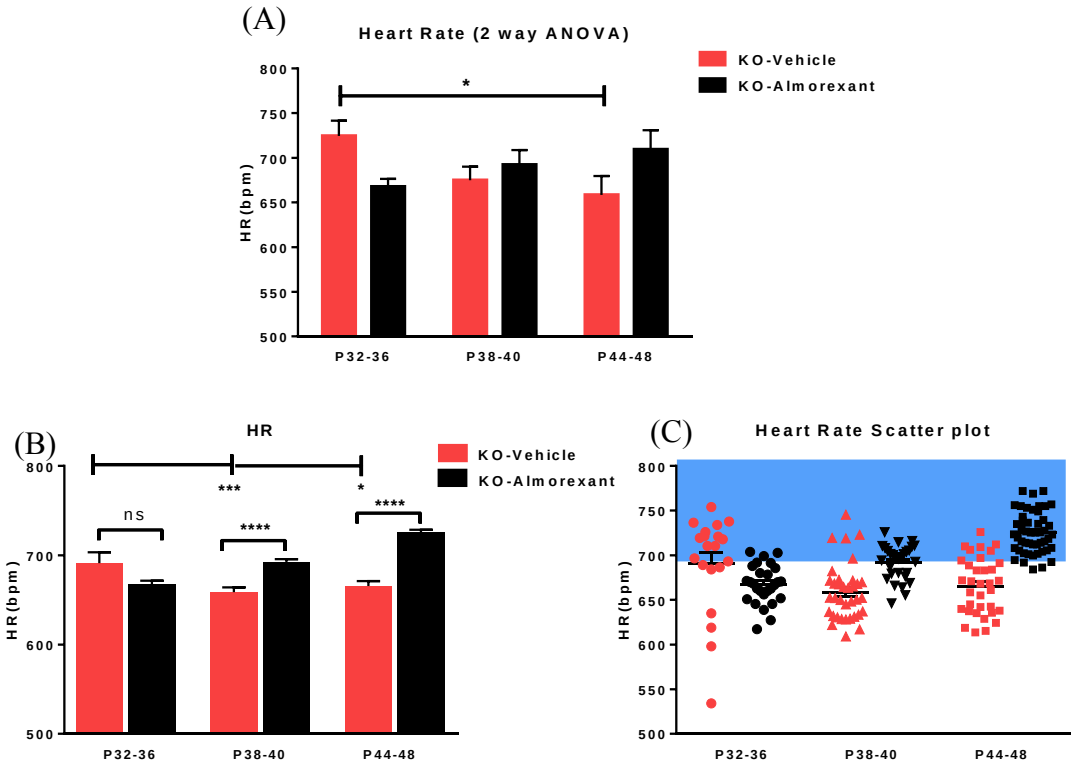


Figure 3. Almorexant restores the heart rate in Kv1.1 KO mice to WT levels at ages closer to SUDEP. **(A)** 2-way ANOVA with Sidak's *post hoc* test indicates that compared to vehicle control, almorexant treatment had no significant effect on heart rate across age in Kv1.1 KO mice. Vehicle treated group at P44 shows lower heart rate when compared to vehicle treated P32 KO mice, similar to the results described earlier in this section. **(B)** One way ANOVA, however, indicates that almorexant treated mice have higher heart rates compared to vehicle controls, at P38 and P44 age bins; with no difference between the groups at younger age. Compared within the vehicle treated groups, there was a decrease in heart rate with age. **(C)** The scatter plot

depicts the vehicle treated KO mice have a large range of heart rate values below the WT control range (blue box) suggesting an increase in bradycardia events. Almorexant treated groups have heart rate values closer to or in the WT range. Thus almorexant may be successful in restoring the heart rate at ages preceding death. Data are expressed as mean ± SEM; n=5-6 for each group; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Since R-R interval has an inverse relationship with heart rate, an increase in heart rate would be reflected as shortening of the R-R interval. Hence, we would expect that, blocking the orexin receptors with almorexant would reduce the R-R interval in the KO mice.

Data analyses using two way ANOVA: Using the two way ANOVA we found that there was no significant difference in the R-R interval between vehicle treated and almorexant treated Kv1.1 KO mice ($F(1, 32) = 0.1128$, $p=0.7391$) (Figure 4A). We also found that there was no change in heart rate across age in both almorexant and vehicle treated groups ($F(2, 32) = 0.4401$, $p=0.6478$).

Data analyses using one way ANOVA and scatter plot: Using the scatter plot, we can see that at the younger age bin the vehicle and almorexant treated KO mice have R-R intervals distributed in the WT range. At older ages the disparity in distribution of the R-R intervals between vehicle and almorexant treated groups appears greater (Figure 4B). At P38-40 and P44-48, a large number of R-R intervals are distributed above the WT range, however almorexant treatment seems to restore the R-R intervals to an extent, which is similar to the WT range.

Using one-way ANOVA, we found that, almorexant causes significant shortening of the R-R interval in heart rate in at P38-40 (92.29 ± 0.67, $p<0.0001$) and P44-48 (91.67 ± 1.06, $p<0.001$) when compared to the vehicle treatment at these ages (85.93 ± 0.83 and 83.31 ± 0.41 respectively) in the KO mice ($F(5, 188) = 21.48$, $p<0.0001$) (Figure 4C). We found no difference in the R-R interval duration between vehicle and almorexant treated KO mice at P30-34.

Overall we found that almorexant increases heart rate and decreases R-R interval in Kv1.1 KO mice at age closer to SUDEP, but not at younger ages.

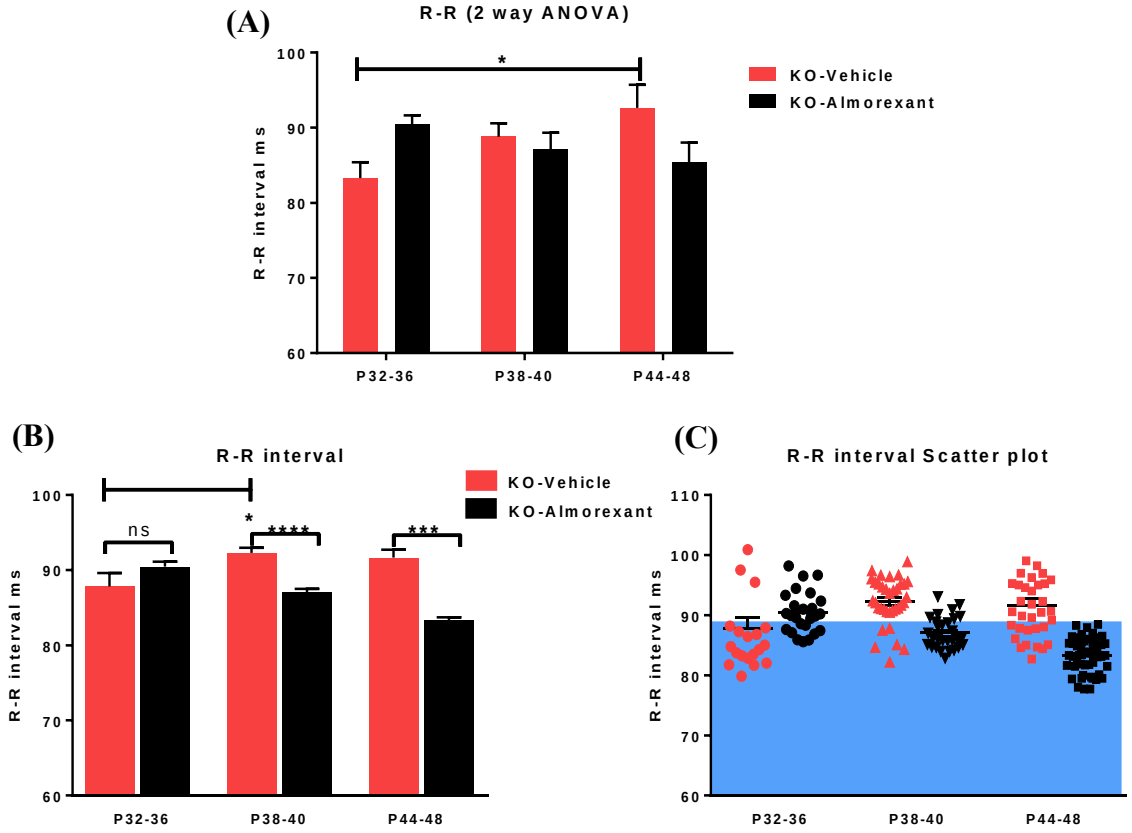


Figure 4. Almorexant shortens the duration of the R-R interval in Kv1.1 KO mice at ages closer to SUDEP. **(A)** 2-way ANOVA with Sidak's *post hoc* test indicates that compared to vehicle control, almorexant treatment had no significant effect on R-R interval across age in Kv1.1 KO mice. Vehicle treated group at P44 shows significantly prolonged R-R interval compared to vehicle treated P32 KO mice. **(B)** One way ANOVA indicates that Almorexant treatment shortens R-R interval duration compared to vehicle controls, at P38 and P44 age bins; with no difference between the groups at younger age. Compared within vehicle treated groups, there is prolongation of R-R interval with age. **(C)** The scatter plot for R-R interval duration in KO mice depicts that at ages closer to death, a large range of values are above the prescribed control range. Almorexant treatment restores the R-R interval duration similar to the WT control

levels at P38-48 in the KO mice. Data are expressed as Mean ± SEM; n=5-6 for each group; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Blocking orexin receptors has mixed effect on the QTc duration in Kv1.1 KO mice

Previously, we found that there is prolongation of the QTc interval in KO mice compared to WT controls at ages closer to SUDEP, with no difference at younger ages.

Data analyses using 2-way ANOVA: We found that overall there is no effect of almorexant treatment on the duration of QTc ($F(1, 32) = 1.863$, $p=0.1818$) **(**Figure 5A). However, at P30-34, we found that almorexant treated KO mice have a longer QTc compared to vehicle controls (50.83 ± 1.76, and 46.64 ± 0.75 respectively. $p<0.05$). We also found that there was no effect of age on the duration of QTc in both vehicle and drug treated groups ($F(1, 32) = 1.863$, $p=0.1818$).

Data analyses using one way ANOVA and scatter plots: The scatter plots show that the distribution patterns for QTc intervals is similar that of R-R intervals described earlier for both almorexant and vehicle treated groups at the different ages **(**Figure 5C). Using a one way ANOVA, we found that at the younger ages of P32-36, almorexant administration to KO mice results in significant QTc prolongation compared to the vehicle control (50.83 ± 0.38, and 48.19 ± 0.62 respectively. $p<0.05$). At P38-40, there is significant reduction in QTc interval in almorexant treated group compared to vehicle control. We however found no significant differences between the almorexant and vehicle treated animals at P44-48 SUDEP age **(**Figure 5B). Our data suggest that prolonged QTc in the KO mice maybe occurring due to reasons other than orexinergic hyperactivity, since blocking the orexin receptors had no effect at the age of SUDEP. We also need to design further experiments to evaluate the reason for prolongation of

QTc at the younger ages by almorexant administration and if it has other targets that might cause this anomaly.

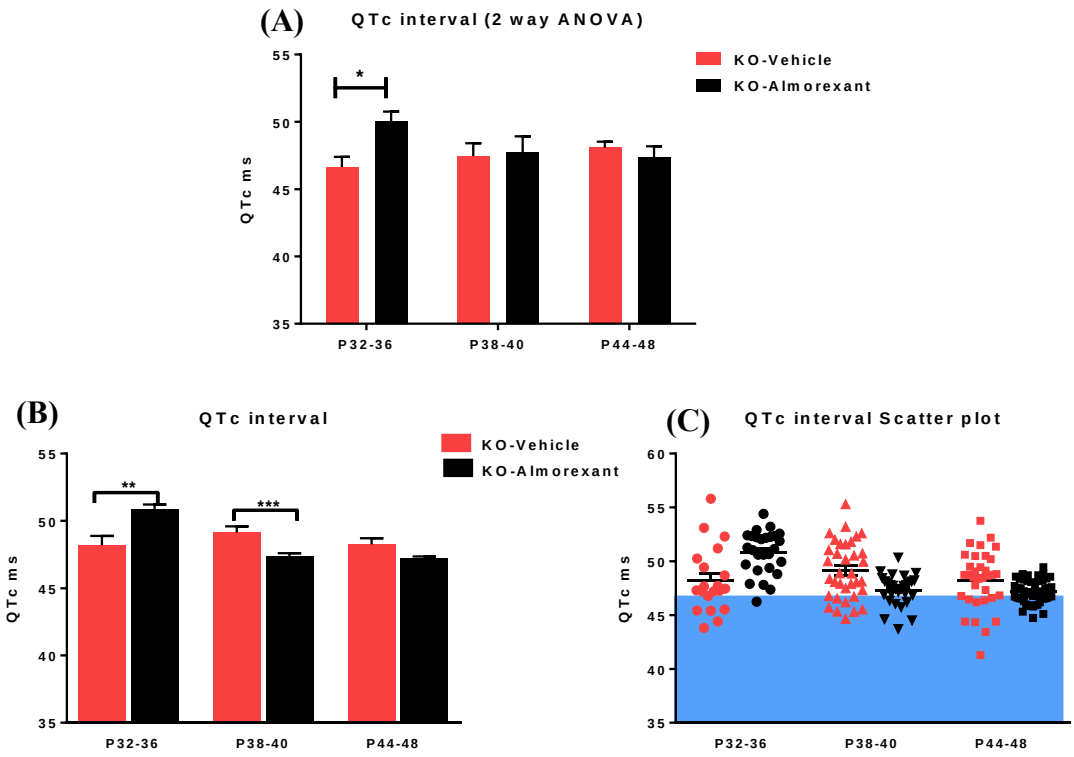


Figure 5. The effect of almorexant on QTc interval Kv1.1 KO mice. (A) 2-way ANOVA with Sidak's *post hoc* test indicates that compared to vehicle control, almorexant treatment had no significant effect on QTc interval, except at younger P32-36 where it actually prolongs the QTc interval even more in Kv1.1 KO mice. **(B)** One way ANOVA suggests that almorexant treatment shortens QTc interval duration compared to vehicle controls, at P38, while at P32 it prolongs it. **(C)** The scatter plot for QTc duration in KO mice depicts that the distribution of QTc interval data for both vehicle and almorexant treated groups is mostly outside the WT control range at all ages. Data are expressed as Mean ± SEM; n=5-6 for each group; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Orexin receptor antagonism by almorexant results in a decrease in rmSSD as SUDEP approaches in Kv1.1 KO mice.

As mentioned previously, we found a significant increase in rmSSD in the Kv1.1 KO mice compared to WT controls at ages nearing SUDEP. Here, we evaluated whether orexin receptor antagonism would normalize the parasympathetic overdrive and reduce the rmSSD values in the KO mice.

Data analyses using 2 way ANOVA: We analyzed the data using a 2 way ANOVA and found that overall, we found that there is a significant effect of almorexant treatment on the rmSSD values across age ($F(2, 32) = 4.763$, $p<0.05$) **(**Figure 6A). We found that, the vehicle treated group at P44-48 which is the general age range SUDEP had significantly higher rmSSD value than the vehicle treated group in the age range of P32-36 (4.3 ± 0.94 and 1.5 ± 0.24 respectively, $p< 0.05$)

Data analyses using one way ANOVA and Scatter plot: The scatter plot shows that at younger age (P30-34), the vehicle and almorexant treated KO mice have rmSSD values distributed in the WT range **(**Figure 6C). However at P38-40 and P44-48, the distribution of rmSSD values in the vehicle groups is above the WT range. Almorexant treatment at these ages seems to restore the rmSSD values to WT levels, indicated by the large number of values that lie in the WT range.

Using a one way ANOVA, we found that almorexant treatment resulted in a significant reduction in rmSSD at the P38-40 age bin **(**Figure 6B), compared to the vehicle treated KO mice (2.054 ± 0.133 and 2.874 ± 0.167 respectively, $p<0.05$). When compared to vehicle controls, the reduction in rmSSD value by almorexant treatment is even more prominent at P44-48 (3.5 ± 0.2 and 1.51 ± 0.11 respectively, $p<0.05$).

Our data indicates that, orexin receptor antagonism causes a decrease in rmSSD value in the Kv1.1 KO mice, and may play a role in reducing the parasympathetic hyperactivity and associated cardiac anomalies seen during SUDEP.

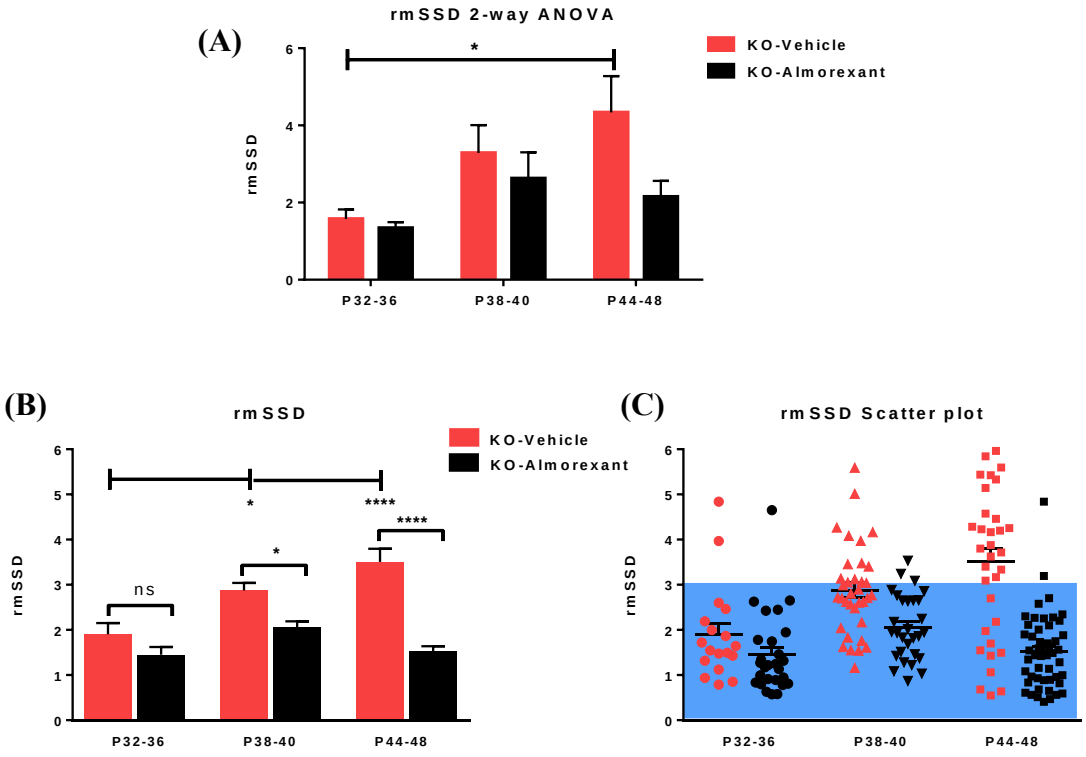


Figure 6. Blocking orexin receptors decreases rmSSD in Kv1.1 KO mice as SUDEP approaches. **(A)** 2-way ANOVA with Sidak's *post hoc* test indicates that Almorexant had no effect on rmSSD across age in Kv1.1 KO mice. Vehicle treated group at P44 shows significantly higher rmSSD compared to vehicle treated P32 KO mice, indicating possible increased parasympathetic activity at older ages. **(B)** One way ANOVA indicates that compared to vehicle controls, almorexant treated KO mice have significantly lower rmSSD values at P38-40 and P44-48 ages (closer to SUDEP), with no difference between the vehicle and drug treated groups at younger age. Compared within vehicle treated groups, there is an increase in rmSSD with age. **(C)** The scatter plot for rmSSD values indicates that at younger age P32-36, vehicle and

almorexant treated KO mice have similar rmSSD values and within the WT range. Between P38-48, a number of rmSSD values for vehicle groups is distributed well above the WT range; to a large extent, almorexant treatment restores the rmSSD in KO mice to the WT levels. Data are expressed as Mean ± SEM; n=5-6 for each group; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Peripheral orexin-A levels in Kv1.1 KO mice:

We found that there is extensive variability in the orexin-A levels in WT and KO mice. We tried to attribute the cause of this variability to a number of factors like time of age, blood collection, gender etc. However, in spite of accounting for these factors, there was still a large disparity in orexin-A concentration values within each group. Using a two way-ANOVA, we found that, there are no differences in the serum orexin A levels (pg/ml) between the Kv1.1 KO mice and their age matched WT controls both at younger and older ages Figure 7. We will also be collecting blood via non-fatal methods, like the submandibular collection method, so that we can analyze serum orexin-levels periodically and analyze the pre-SUDEP data retrospectively.

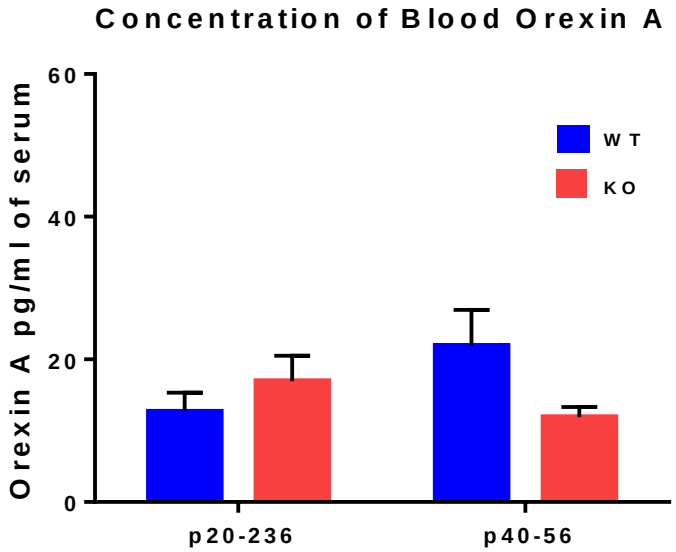


Figure 7. There is no difference in peripheral Orexin-A levels between Kv1.1 KO and WT mice across age. 2-way ANOVA with Sidak's *post hoc* test indicates that there was no difference in serum Orexin A concentration between Kv1.1 KO and WT mice at both younger ages and ages approaching SUDEP. Data are expressed as Mean ± SEM.

DISCUSSION

Kv1.1 KO mice are a model for temporal lobe epilepsy and SUDEP (Moore et al. 2014; Smart et al. 1998). Studies have shown severe cardiac abnormalities associated with this model (Glasscock et al. 2010). We studied the difference in cardiac parameters at epilepsy onset age and ages closer to SUDEP. Additionally we hypothesized that parasympathetic overdrive might be involved in SUDEP related cardiac abnormalities.

Previous studies in Kv1.1 KO mice have indicated the occurrence of severe bradycardiac events which ultimately leads to SUDEP in these mice (Moore et al. 2014). We found that Kv1.1 KO mice have decreased heart rate, increased R-R interval, which corresponds to higher incidence of bradycardia closer to SUDEP (Becker 2006a). Long QTc interval has been associated with sudden infant death syndrome and sudden cardiac death (Border and Benson 2007; Ackerman 2005). We also found an increase in QTc interval which indicates that there is a high risk for ventricular arrhythmias and related fatality. We also found an increase in rmSSD values in the KO mice compared to their WT controls, indicating that parasympathetic overdrive might be upstream of the cardiac abnormalities in these mice (DeGiorgio et al. 2010).

Our previous studies in Kv1.1 KO mice have shown that the seizures progressively worsen from epilepsy onset age reaching a maximum level at pre-SUDEP age (Simeone et al. 2016). Here, we found that differences in cardiac parameters between the Kv1.1 KO and WT mice are only significant at ages approaching SUDEP and not at younger ages. Thus the development of cardiac abnormalities closely correspond to the ontogeny of seizures in these mice. This is also similar to human studies where there were no differences in rmSSD values at seven months prior

to death, however there was a significant increase in rmSSD in patients a few days prior to SUDEP (Jeppesen et al. 2014).

We have previously reported that the Kv1.1 KO mice show disturbed rest-wake cycles and sleep co-morbidities similar to epileptic patients (Fenoglio-Simeone et al. 2009). Orexin is a hypothalamic neuropeptide that promotes wakefulness and can regulate the autonomic nervous system by its effects on the nuclei located in the brain stem (Tsujino and Sakurai 2013). Studies have shown that direct orexin microinjections in the nucleus ambiguous region results in baroreflex-triggered bradycardia, indicating its role in increasing parasympathetic drive (Ciriello, de Oliveira, et al. 2003). We hypothesized that orexin is upstream of parasympathetic drive in SUDEP and orexin receptor antagonism with a DORA, will result in attenuation of the parasympathetic over activity. We found that administration of a DORA (almorexant) results in increase in heart rate and decreases the R-R interval and rmSSD indicating that the parasympathetic drive might be affected by this drug. The effect on the QTc interval seemed to be unclear, we need further pharmacological studies to elicit the role of orexinergic system in ventricular arrhythmias.

Chapter 3. Sleep dysfunction and effect of ketogenic diet in Kv1.1 KO mice

Sleep, seizure and SUDEP

The intimate relationship between epilepsy and sleep has been studied extensively. There is an increased risk for sleep co-morbidities in epilepsy; about 30% of epileptic patients suffer from sleep disorders that result in inadequate sleep. (Manni and Terzaghi 2010; Gilliam et al. 2005) Inadequate sleep can result in other co-morbidities like memory and cognitive impairments and psychological disorders in the epileptic population (Steinsbekk, 2013; Plihal and Born 2008; Drummond and Brown 2001)

Apart from lowering the quality of life in epileptic patients, inadequate sleep can also precipitate further seizures (Mattson et al. 1965). Sleep and seizures share a reciprocal relationship; insufficient sleep can result in worsening seizure frequency and severity, and seizure exacerbation results in further sleep deprivation which forms a vicious cycle (Foldvary-Schaefer and Grigg-Damberger 2009; Matos et al. 2010). Sleep deprivation can also result in a variety of physiological disturbances, autonomic imbalance, cardiac problems and increased susceptibility to potentially lethal arrhythmias (Mullington et al. 2009; Meier-Ewert et al. 2004). In fact sleep deprivation has been identified as a major risk factor in Sudden Infant Death Syndrome (SIDS), which has been closely associated SUDEP (Franco et al. 2003; Simpson 2001; Emery 1959). Considering all these aspects it is reasonable to assume that sleep deprivation maybe a candidate biomarker for SUDEP.

Kv1.1 Mouse Model: Sleep abnormalities

In our studies, we used the Kv1.1 KO mouse which is a model for both temporal lobe epilepsy and SUDEP.(Smart et al. 1998) We have previously shown that the Kv1.1 KO mice show disrupted diurnal rhythmicity; these mice show increased activity during periods of rest and

abnormally low activity during wake periods. In addition there is also seizure periodicity seen in these mice, the seizure frequency is greater during the light phase or rest periods and very few seizures in the active phase (Fenoglio-Simeone et al. 2009). Detailed studies on sleep architecture in these mice have also revealed decreased REM and NREM sleep, increased sleep fragmentation and latency to sleep onset, all of which are very similar to the sleep problems associated with epileptic patients (Roundtree et al. 2016; Kotagal and Yardi 2008; Malow 2005).

The Kv1.1 KO mice develop epilepsy in the third post-natal week and have spontaneous seizures throughout their life. Our recent studies using video analysis of behavioral seizures in these mice show that the age of epilepsy onset in these mice is around P21- where the seizure frequency and severity are relatively low (Simeone et al. 2016) **(**Figure 8). We found that seizures progressively increased in number and severity with age, continuing until postnatal week 7 where these mice die of SUDEP.

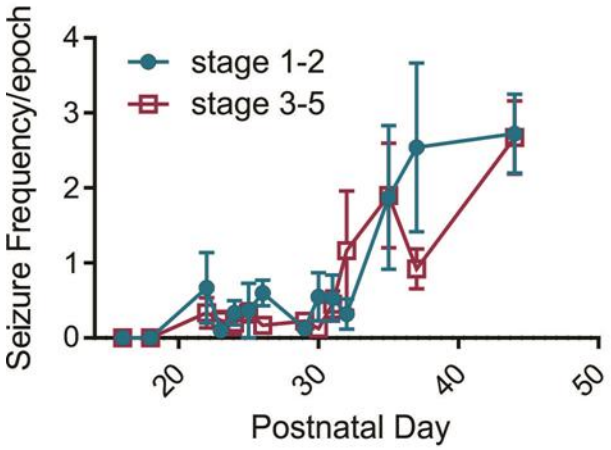


Figure 8. Ontogeny of seizure frequency and intensity in Kv1.1 KO mouse model of SUDEP. Stages 1-2 indicate mild seizure intensity while stages 3-4 indicate severe seizure intensity. Seizure frequency and intensity are both low at epilepsy onset age, around P21-30,

thereafter the seizure burden progressively increases with age until P45-50 where most of the KO mice die of SUDEP.

Considering the close association of seizures and sleep problems in this model, one of the first objectives of our study was to evaluate the ontogeny of sleep in this mouse model; if the sleep disruption also increases with age, particularly as SUDEP age approaches. The second objective was to identify sleep parameters which are most sensitive to the physiological changes that occur as the SUDEP age approaches. These parameters would be expected to exhibit a drastic change in the days preceding death, which could be used as a biomarker to predict SUDEP in these mice.

Ketogenic diet

Despite the availability of a wide variety of medications, 30-40% of the epileptic population have refractory epilepsy, where the seizures cannot be controlled by medication (Felton and Cervenka 2015) . Incidentally, people with poorly controlled seizures are at an increased risk for SUDEP. The high fat, low carbohydrate/protein ketogenic diet (KD) is a highly effective therapeutic option for treating seizures of various types and intensities in a wide variety of epileptic population (Acharya, Hattiangady, and Shetty 2008). Ketogenic diet (KD) has been known to abolish seizures in about 13% of the epileptic patients with more than 50% reduction in seizures in two-thirds of patients with refractory epilepsy (Hallböök et al. 2007). In animal models, KD has been to known to decrease brain cerebral hyper excitability, increase seizure threshold and attenuates epileptogenesis in pentylenetetrazole (PTZ) and pilocarpine kindled mice and rats (Hansen et al. 2009; Hori et al. 1997). In our previous studies, we have shown that KD decreases hippocampal excitability, reduces seizures by about 75% and delays the seizure

progression in the Kv1.1 KO mice (Simeone et al. 2016; Simeone et al. 2014; Fenoglio-Simeone et al. 2009).

KD is also known for improving sleep problems in epileptic patients. KD increases the REM sleep, reduces excessive daytime sleepiness and improves the overall quality of sleep in pediatric patients with refractory epilepsy (Hallböök, Lundgren, and Rosén 2007). We have previously shown that KD induces normalization of the circadian rhythm in the Kv1.1 KO mice and improves their rest-activity cycles (Fenoglio-Simeone et al. 2009). Hence, KD could be a potential therapeutic target for reducing sleep deprivation which maybe a risk factor for SUDEP.

Interestingly KD has also been shown to increase the lifespan by more than 4 fold in animal models of amyotrophic lateral sclerosis and succinic semi aldehyde dehydrogenase deficiency (Ari et al. 2014; Nylen et al. 2008). Recently, we reported than in Kv1.1 KO mouse model of SUDEP, KD increases longevity by about 47% compared to KO mice fed standard animal chow (Simeone et al. 2016).

Considering the broad spectrum activity of KD in attenuating seizure progression, improving sleep deprivation and increasing the lifespan in humans and animal models, we decided to evaluate the efficacy of KD on the ontogeny of sleep deprivation and SUDEP prevention.

There were three main objectives to this study: 1) To study the ontogeny of sleep disruption in the Kv1.1 KO mice and their WT controls from age of epilepsy onset to ages approaching SUDEP; 2) To identify one or more of the rest parameters as a potential biomarker for SUDEP; 3) To study the effect of KD on ontogeny of sleep disruption in the Kv1.1 KO mice particularly in the ages closer to SUDEP.

MATERIALS AND METHODS

Animals: C3HeB/FeJ Kv1.1 KO1-null and wild-type littermates were acquired from The Jackson Laboratory and bred and reared at the Animal Resource Facilities at Creighton University. The mice were subjected to a strict 12 hour light/dark cycle with lights on at Zeitgeber time 0 hr (ZT 0) and provided with food and water ad libitum. The genotype of the mice was determined by tail clipping methods using Transnetyx, Inc (Cordova, TN, USA). All the experiments were carried out in accordance with the guidelines of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee at Creighton University.

Dietary treatments: The Kv1.1 KO mice and their wildtype littermates were weaned onto either a standard diet (SD) or a KD (6.3:1, fat to carbohydrates plus proteins; Bio-Serv F3666, Frenchtown, NJ, U.S.A.) at P21.

Actigraphy: The rest-activity cycles of the both the SD and KD treated mice were acquired using an integrated radio telemetry technology and switch-closure activity monitoring (Vital View data acquisition system, Mini Mitter Company, Inc; Bend, OR). Mice in the age range of P23 to P27 were individually housed in an 8" x 8" x 16" transparent plexiglass cage and allowed to habituate for 12 h. The breaks in the infrared beam caused by activity of the mice were recorded in 3-min epochs and scored on a scale of 0–150. Data were recorded continuously until SUDEP occurred in the KO mice. The WT mice were age matched with the KO mice.

ANALYSES

Ontogeny: The data acquired during the resting phase of the animals between ZT 0 to ZT 10.30 were analyzed using ACTIVIEW Biological Rhythm Analysis software (Mini Mitter Company,

Inc.). Each epoch was defined as either active (with an actigraphy count greater than 3) or resting (with an activity count of 0-3). The data were collapsed across animals. The rest parameters were calculated on a per day basis (1) total number of rest epochs; (2) total activity; (3) total number of transitions from rest to wake. Data were plotted by age in order to study the progressive change in rest parameters with age.

Retrospective Analyses: One of the important objectives of our study was to identify possible changes in rest parameters in the KO mice that can be used as potential biomarkers for SUDEP. However, there is a wide age range of SUDEP in the KO mice (n=5) ranging from P43 to P56. The difference in age of mortality could be a potential source of variability in the rest data. For example, a mouse with mortality age of P43 might have slightly different rest parameters compared to a mouse with mortality age of P56. In order to eliminate this variation, the data were averaged retrospectively in 4-day bins, beginning with the day prior to SUDEP (PTS).

Sleep debt was operationally defined as the average amount of time KO mice spent resting when subtracted from the average time spent resting by the WT controls.

Statistics: The differences between genotypes were assessed using Student's t test. The ontogeny of rest parameters and retrospective data were analyzed using 2 way ANOVA with Dunnett's multiple comparison *post hoc* test. Data is expressed as Mean ± SEM.

RESULTS

A representative actogram obtained from one of the WT and KO mice is depicted in Figure 9. It shows that the KO mouse overall has disrupted rest patterns compared to the WT control.

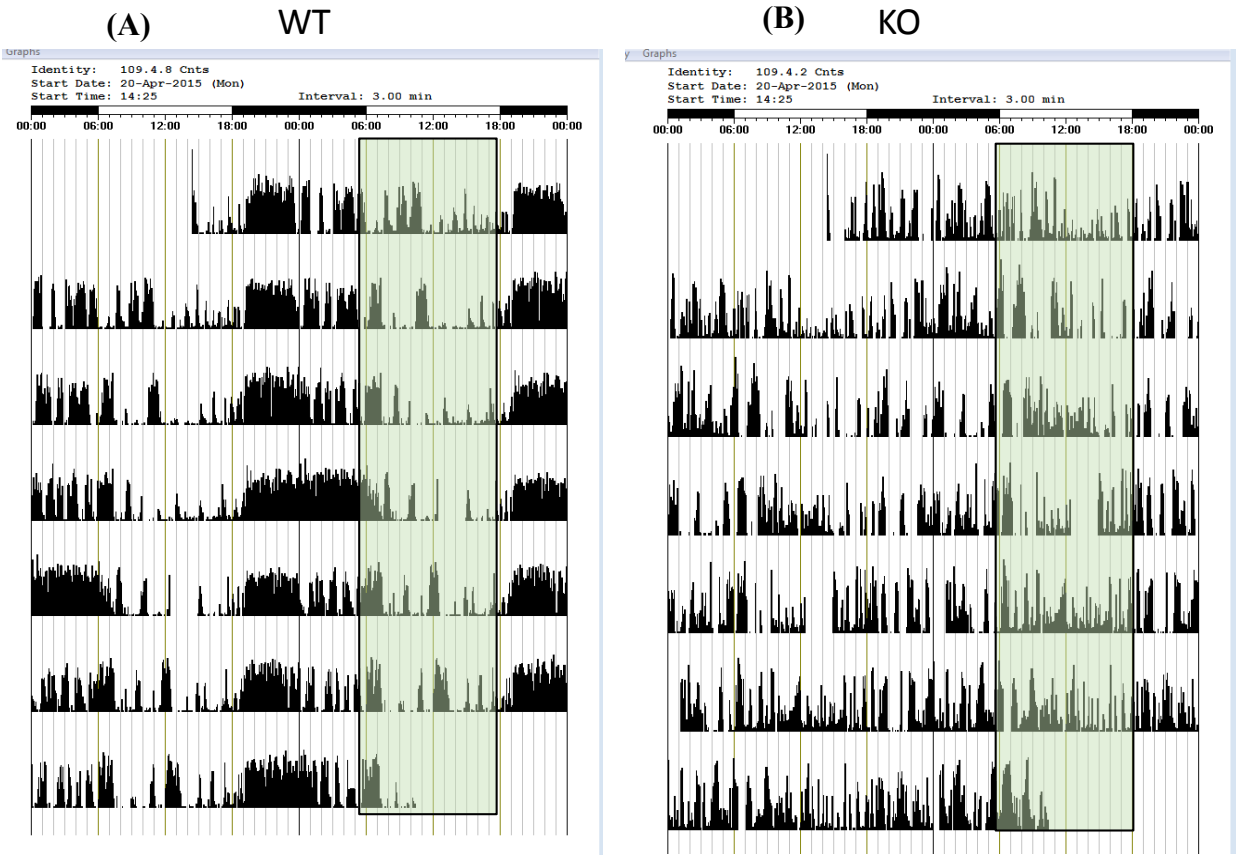


Figure 9. A representative actogram obtained from actigraphy of Kv1.1 KO and WT controls: The figure depicts activity obtained from the WT and KO for over 7 days using actigraphy. The blue box indicates the light phase (resting phase) for the mice. **(A)** The WT mice have clearly defined periods of rest (less black lines) and activity (clusters of black lines). This clear distinction of activity and rest is lost in the KO mouse which has dispersed activity throughout periods of rest. (B) The KO mice rest less during the rest phase compared to WT mice which show minimal activity during that phase.

Kv1.1 KO mice have altered rest parameters compared to their age matched WT littermates.

The data was collapsed across age and averaged. The Student's t-test with two tailed P-value was used to assess rest parameters between the two genotypes. A p-value < 0.05 was considered

statistically significant. The KO mice showed about a 40% decrease in the number of rest epochs per day when compared to the WT control animals (103.2 ± 4.88 and 163.6 ± 2.34 respectively; $p<0.001$) (Figure 10 A). The KO mice were also about two times more active than the WT mice during the rest phase, which was indicated by the significantly higher actigraphy counts per day in the KO mice (1985 ± 123.6 and 916.4 ± 46.64 respectively. $p<0.001$) (Figure 10 B). The transitions from rest to wake epochs were found to be two times higher in the KO mice compared the WT controls (23.14 ± 0.64 and 11.85 ± 0.5734 respectively; $p<0.001$) (Figure 10 C).

Our data indicates that the KO mice have significantly altered rest parameters compared to the WT controls.

KD treatment results in a slight improvement in rest parameters

The aforementioned data from standard diet treated KO and WT mice was re-plotted along with data from ketogenic diet treated WT (KD-WT) and KO (KD-KO) mice (Figure 10). The data from all the four groups were analyzed using a one way ANOVA with Tukey's multiple comparison *post hoc* test. A p-value<0.05 was considered statistically significant.

When comparing the four groups, KO mice, WT mice, KD-KO mice and KD-WT mice, we found significant differences in the average number of rest epochs among the groups ($F(3, 179) = 64.16$, $p<0.0001$) (Figure 10 D). There was no difference in the average number of rest epochs between the KD-KO mice and the KO mice (110.7 ± 4.41 and 103.2 ± 4.88 respectively; $p=0.4945$). Similar to the KO mice, we found a significant reduction in rest epochs in the KD-KO mice (110.7 ± 4.41), when compared to both the KD-WT mice (151.5± 1.94, $p<0.0001$) and the WT mice (163.6 ± 2.34, $p<0.0001$). We found no significant differences in rest epochs

between WT and KD-WT groups (p=0.086). Overall, KD did not affect the rest epochs per day in the KO mice.

We found significant differences in the average activity counts between the groups ($F(3, 179) = 28.81$, $p<0.0001$). There was a 25% reduction in the activity KD-KO mice when compared to the KO mice (1543 ± 86.66 and 1985 ± 123.6 respectively; $p<0.01$). However the KD treated KO mice still had significantly higher activity when compared to both the KD-WT mice (1078 ± 66.05, $p<0.001$) and the WT mice (916.4 ± 46.64, $p<0.0001$). We found no significant differences in activity between WT and KD-WT groups (p=0.5094) (Figure 10 E). Our data indicate that there is some improvement in activity per day due to KD treatment in the KO mice.

There were significant differences in the average number of rest to wake transitions between the groups ($F(3, 179) = 99.72$, $p<0.0001$). There was no significant difference in the rest to wake transitions between the KD-KO mice and the KO mice (22.02 ± 0.49 and 23.15 ± 0.64 respectively; p=0.44). The KD-KO mice had significantly higher rest to wake transitions compared to both the KD-WT mice (15.69 ± 0.38, $p<0.0001$) and the WT mice (11.86 ± 0.57, $p<0.0001$). Interestingly, we found that the KD-WT mice have significantly higher rest to wake transitions compared to WT mice ($p< 0.01$) (Figure 10 F). Overall, KD had no effect on rest to wake transitions in these mice.

Our data indicates that, when data were averaged across ages, KD treated KO mice show reduced activity without improvement in the other rest parameters.

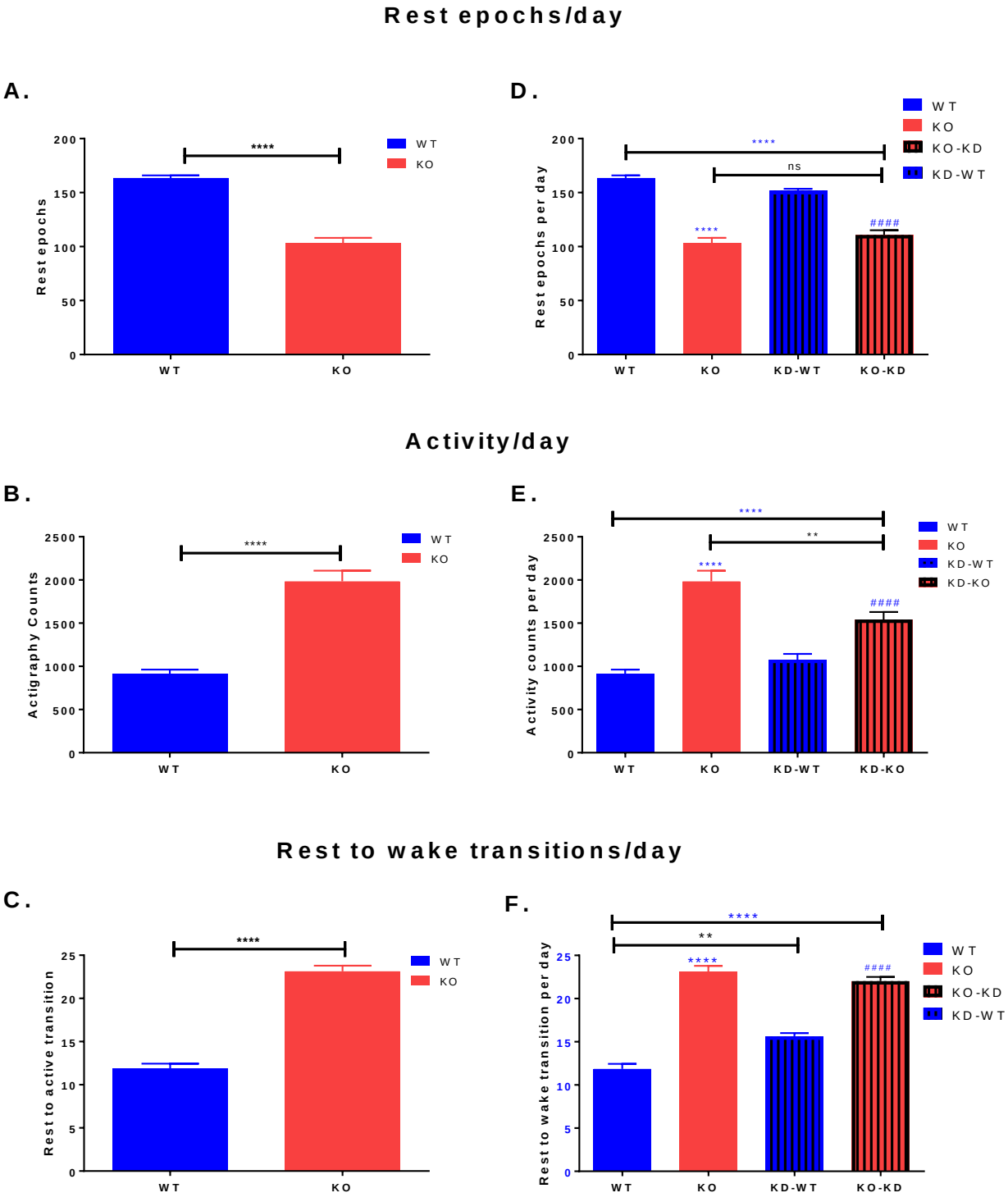


Figure 10. Kv1.1 KO mice have altered rest parameters compared to their age matched WT littermates. (A-B-C) were analyzed using Student's two tailed t test. **A)** Kv1.1 KO mice have significantly reduced rest epochs/day when compared to WT controls (n=5 for each). B)

Kv1.1 KO mice have significantly higher daily activity when compared to WT controls (n=5 for each). **C)** Kv1.1 KO mice have significantly higher daily rest to wake transitions when compared to the WT mice (n=5 for each). (**D-E-F)** were analyzed using one way ANOVA with Tukey's multiple comparison *post hoc* test. **D)** KD-KO (n=6) have reduced rest epochs compared to both KD-WT (n=4) and WT. There were no differences in KD-KO and KO groups. **E)** KD-KO have higher activity compared to both KD-WT and WT. However, the KD-KO show significantly reduced activity when compared to the KO group. **F)** KD-KO have higher rest to wake transitions compared to both KD-WT and WT. There were no significant difference in KD-KO and KO groups. However, the KD-WT have significantly higher transitions when compared to the WT group. (Blue asterisk,*, indicates comparison with age matched WT mice and Blue hash sign,#,, indicates comparison with KD-WT mice. Data are expressed as Mean ± SEM; */# $p<0.05$, **/## $p<0.01$, ***/### $p<0.001$, ****/#### $p<0.0001$.

Ontogeny of sleep and effect of ketogenic diet in Kv1.1 KO mice:

On average, the mortality age in standard diet treated KO mice is around P49.6 ± 2.42 (n=5), while that for KD-KO is P69 ± 3.48 (n=6). Here, we determined whether rest parameters change as SUDEP approaches. Indeed, we found that while there are differences in rest parameters between the two genotypes in the younger mice (P23-P34), these differences become even more significant at the age range approaching SUDEP (42-P56). Similarly, there is an age-dependent effect of ketogenic diet on the rest parameters, it improves the rest parameters at certain ages better than the others.

Here, we first discuss the ontogeny of every rest parameter, followed by effect of KD treatment on the rest parameter in the various age groups.

<u>There is a progressive decrease in rest epochs with increase in age in Kv1.1 KO mice.</u>

To assess the effect of age on the various rest parameters, we divided the data into four-day bins; with P23-26 being the first bin, P27-30 being the second, and so on. As aforementioned, the data were analyzed using 2-way ANOVA with Dunnett's multiple comparison *post hoc* test. When compared within group across age, the KO mice showed a prominent trend of decrease in the number of rest epochs with increasing age ($F(7, 508) = 10.02$, $p<0.0001$) (Figure 11). When compared to the young age bin of P26 (136.2 ± 6.539) there is a 30% decrease in rest epochs at P42 (92.25 ± 7.363, $p<0.05$) and 50% decrease at P50 (71.54 ± 6.315, $p< 0.001$). There is approximately 8-10% decrease in rest epochs between consecutive age bins.

In the between group design, the KO mice had decreased rest epochs when compared to the WT mice across all ages ($F(3, 509) = 111.6$; $p <0.0001$) except the younger age of P26. However, there was no change in the number of rest epochs in the WT mice with age (167.05 ± 2.503).

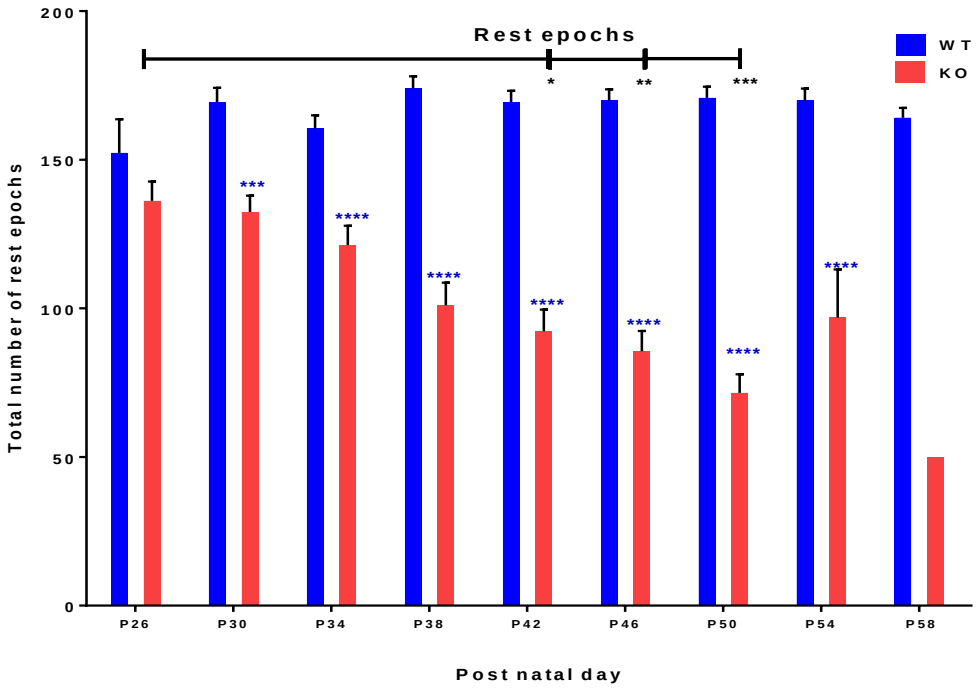


Figure 11. There is progressive decline in the number of rest epochs with age in Kv1.1 KO mice: Data were analyzed using 2-way ANOVA with Dunnett's multiple comparison *post hoc* test. Kv1.1 KO mice have significantly reduced rest epochs when compared to WT controls at all ages except the younger P26 group. At P26, the KO mice have rest epochs similar to WT mice (n=5 for each). In the KO mice the rest epochs progressively decreases from P26 to P54. By P58 (n=2) most of the KO mice in our study died, and this bin was not included for statistical analyses. There is no change in rest epochs for WT mice with age. (Blue asterisk,*, indicates comparison with age matched WT mice. Data are expressed as Mean ± SEM; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Ketogenic diet treatment and rest epochs in Kv1.1 KO mice

When compared between groups across age, there is approximately 10-15% increase in rest epochs in KD-KO when compared to the KO mice, however this increase is not statistically significant. However at P42, there is a 45% increase in rest epochs in the KD-KO group when compared to the KO mice (132.5 ± 9.498 and 92.25 ± 7.363 respectively. $p<0.001$) (Figure 12)

In the younger age bins (P26-P34) there were no significant differences in rest epochs in KD-KO mice when compared to the WT and KD-WT mice. At P38, there is a significant decrease in rest epochs in the KD-KO (109 ± 8.099) compared to KD-WT and WT mice (160.5 ± 4.63 and 174.1 ± 4.05 respectively; $p<0.001$).

When compared within group across age, we found that the KD-KO mice also have reduction in the number of rest epochs with increasing age ($F (13, 396) = 4.356$; $p<0.0001$). However, the decline in rest epochs in KD-KO mice is not as sharp as the age matched KO mice. In KD-KO mice, when compared to the young age bin of P26 (153.6 ± 5.611) there is about 40% decrease in rest epochs at P50 (87.38 ± 10.36, $p< 0.001$) and 50% decrease at P58 (75.47 ± 10.55, $p< 0.001$). Interestingly, after P58, there is no further decline in rest epochs; thereafter the number of rest epochs remain constant, but significantly higher than younger P26 age, till death in these mice.

Overall, KD does not appear to alter the daily rest epochs in Kv1.1 KO mice.

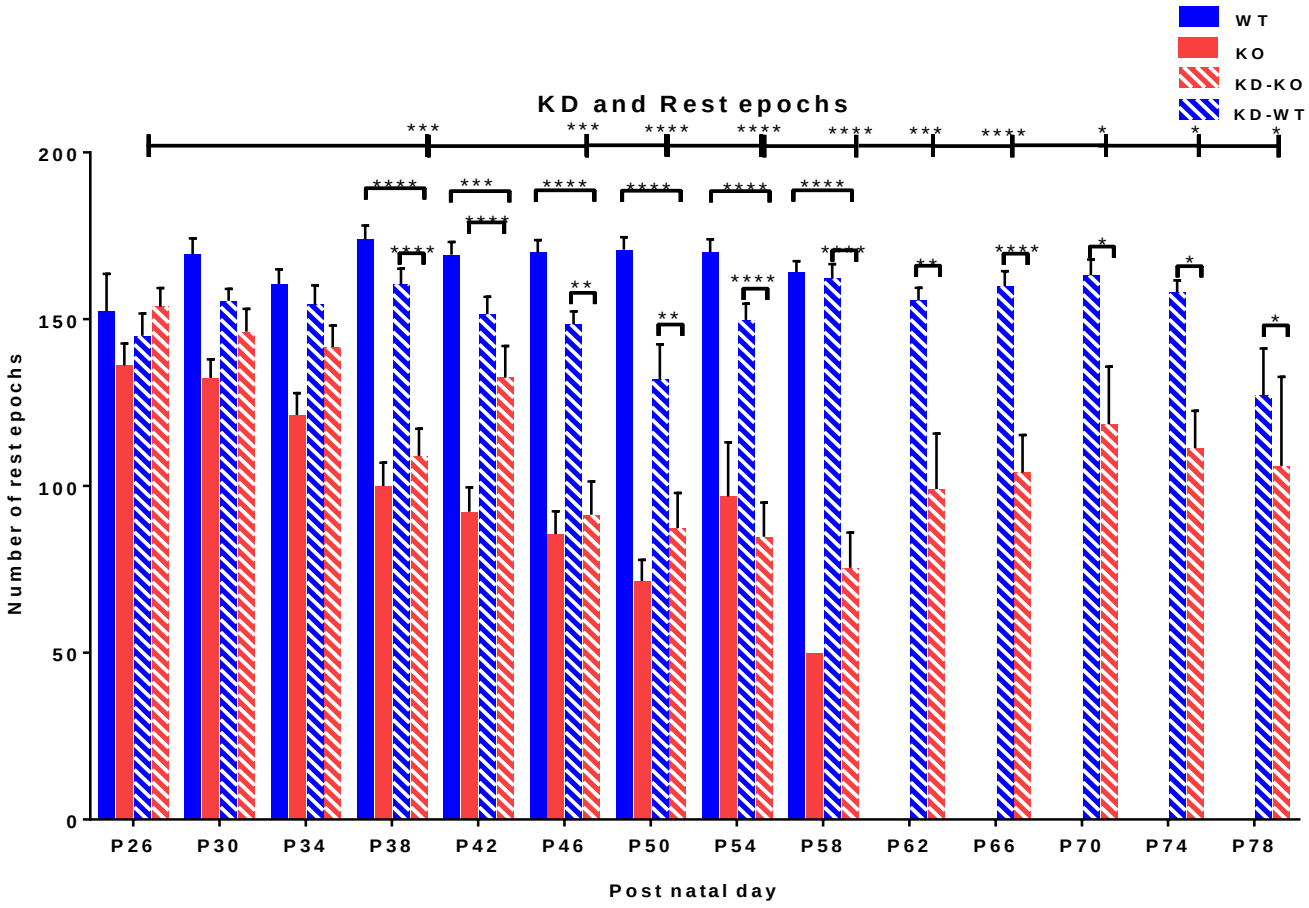


Figure 12. Ketogenic diet treatment and rest epochs in Kv1.1 KO mice: Data were analyzed using 2-way ANOVA with Dunnett's multiple comparison *post hoc* test. Kv1.1 KO on ketogenic diet do not show any significant difference in number of rest epochs compared to KO mice on standard diet. They also show a progressive decline in rest epochs with age until P58 after which there is no further decline (n=6). KD-KO mice have significantly reduced rest epochs when compared to KD-WT and WT controls at all ages except the younger P26-34. There is no difference in rest epochs between WT and KD-WT mice. Data are expressed as Mean ± SEM; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

The Kv1.1 KO mice show increased activity with increase in age.

When compared within group across age, the KO mice became increasingly more active during rest period, with increasing age ($F(7, 509) = 13.76$, $p<0.0001$). There was approximately 15-20% increase in activity between consecutive age bins (Figure 13). When compared to the younger age bin of P30 (1266 ± 97.09), the activity increased by 50% by P42 (1958 ± 127.7, $p<0.01$) which increased dramatically by 130% by P50 (3013 ± 273, $p<0.001$).

In the between group design, the KO mice had increased activity when compared to the WT mice across all age groups ($F(3, 509) = 84.69$, $p<0.0001$).There was no change in activity in the WT mice with age. (840.15 ± 41.265)

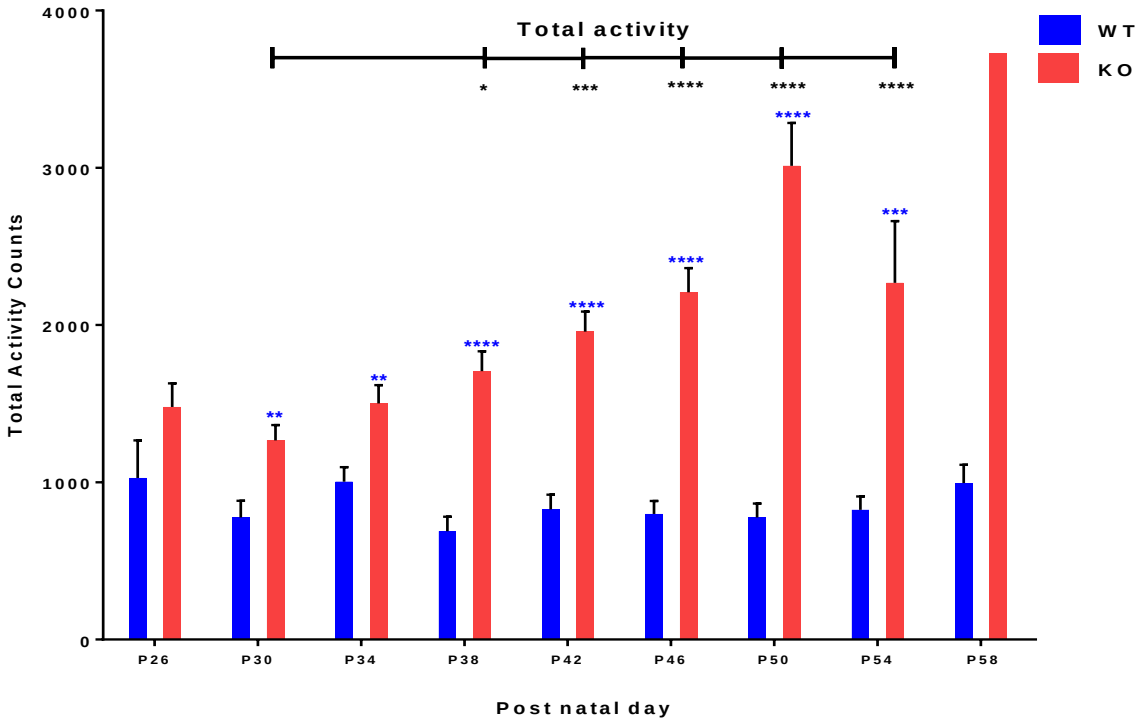


Figure 13. There is an increase in activity with age in Kv1.1 KO mice: Data were analyzed using 2-way ANOVA with Dunnett's multiple comparison *post hoc* test (n=5 each). Kv1.1 KO mice have significantly higher activity when compared to WT controls at all ages except the younger P26, where both groups have similar activity. Within the KO group, the activity counts progressively increased from P30 to P54. P58 (n=2) bin was not included for statistical analyses. There is no change in activity for WT mice with age. (Blue asterisk,*, indicates comparison with age matched WT mice Data are expressed as Mean ± SEM; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

KD significantly reduces activity during the light phase in Kv1.1 KO mice

When compared between groups across age, there is approximately 30-40% decrease in activity in KD-KO when compared to the KO mice at every age bin ($F(3, 509) = 84.69$, $p<0.0001$) (Figure 14). At the younger age bin of P26, the KD-KO mice are only about half as active in the light phase as the age matched KO mice. (886.3 ± 84.14 and 1479 ± 150.6 respectively. $p<0.05$) Similarly, at the older age bin of P46, there is about 30% reduction in activity in KD-KO mice compared to KO mice (1515 ± 139.2 and 2209 ± 153.2 respectively. $p<0.01$).

Up to P34, there is no significant difference in activity of KD-KO mice when compared to KD-WT and WT mice. At P38, KO-KD (1372 ± 117.3) are more active than both WT (688 ± 93.25, $p< 0.001$) and KD-WT groups (802.9 ± 69.61, $p< 0.01$)

When compared within group across age, we found that the activity in KD-KO mice increases with increasing age, however the rise in activity in not as pronounced as seen in KO mice. In fact, dramatic differences in activity were seen only after P50, where the activity became almost twice of that at P26. (886.3 ± 84.14 and 2121 ± 237 respectively. $p<0.001$)

Our data indicates that ketogenic diet reduces the daily activity in the Kv1.1 KO mice without affecting the activity of the WT control group.

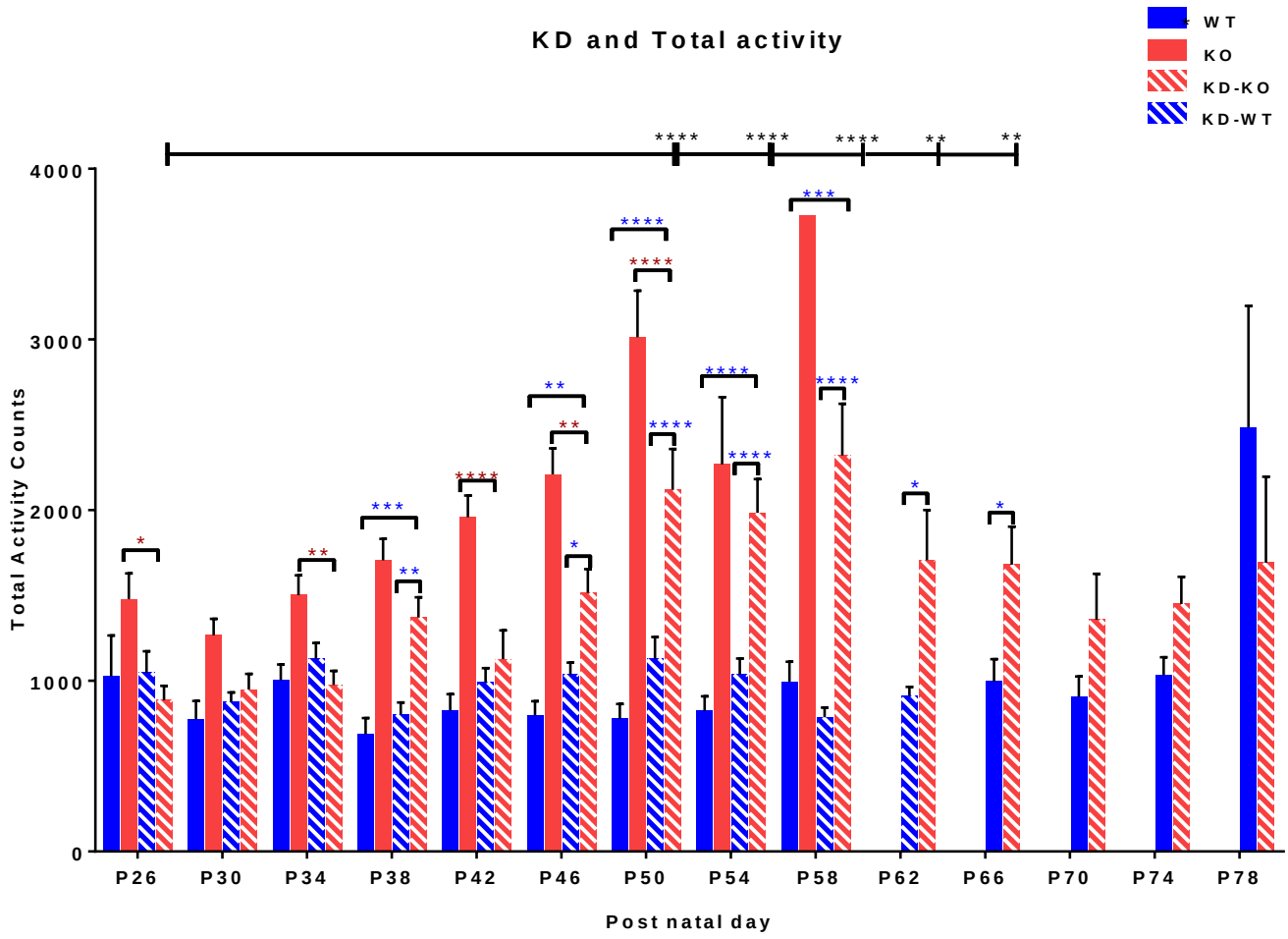


Figure 14. Ketogenic diet treatment significantly reduces activity during light phase in Kv1.1 KO mice: Data was analyzed using 2-way ANOVA with Dunnett's multiple comparison *post hoc* test. Kv1.1 KO on KD have significant reduction in activity at older ages (P42-50) compared to KO mice which pre-SUDEP age for the KO mice on standard diet. KD-KO also show a progressive increase in activity with age but it is not as prominent as it is for KO mice. KD-KO mice have significantly higher activity when compared to KD-WT and WT controls at all ages except the younger P26-34. There is no difference activity between WT and KD-WT mice across age. Data are expressed as Mean ± SEM; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ****

$p<0.0001$. Blue asterisk,*, indicates comparison with age matched WT or KD-WT mice and red * is comparison between KD-KO and KO groups.

There is no change in the rest to wake transitions with progressing age in Kv1.1 KO mice

When compared within group across age, we found that while there was no progressive increase or decrease in rest to wake transitions with age in the KO mice ($F(7, 509) = 1.44$, $p=0.1841$). However, the KO mice at P50 show 40% higher rest to wake transitions when compared younger P26 mice (Figure 15).

When compared to WT mice across age however, the KO mice show significantly higher rest to wake transitions at all ages ($F(3, 509) = 115.9$, $p<0.0001$).

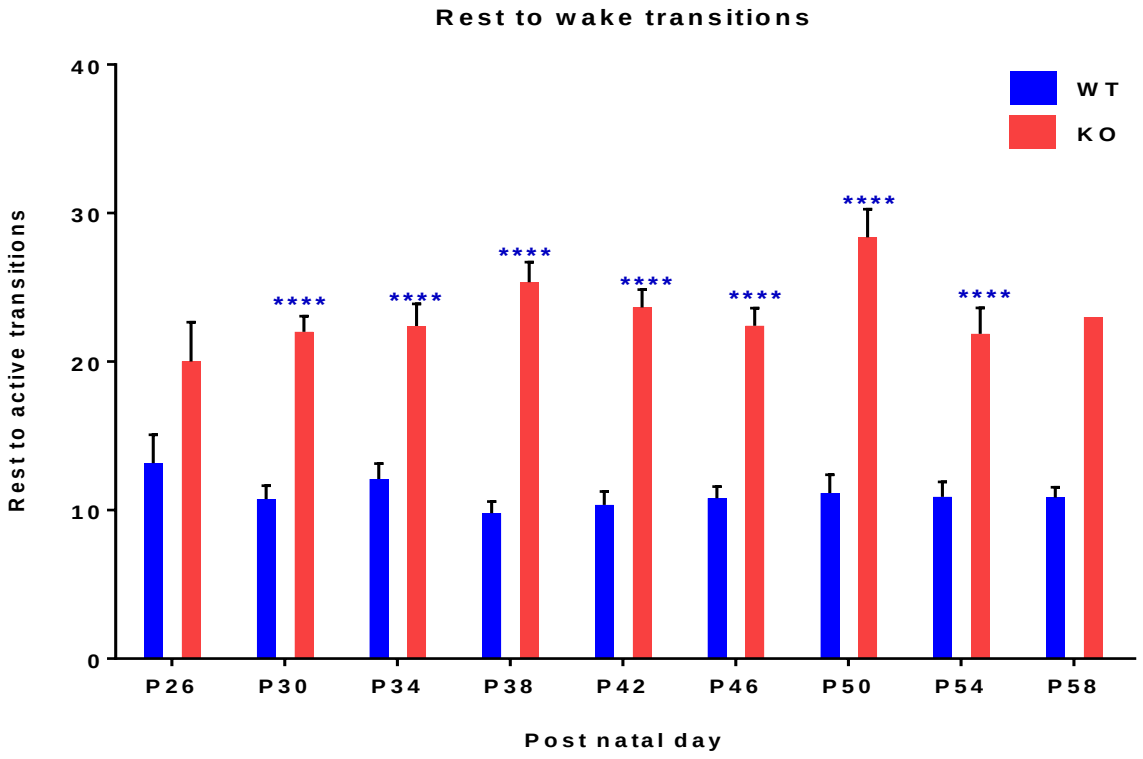


Figure 15. There is no change in rest to wake transitions with age in Kv1.1 KO mice: Data was analyzed using 2-way ANOVA with Dunnett's multiple comparison *post hoc* test (n=5 each). Kv1.1 KO mice have significantly higher rest to wake transitions when compared to WT controls at all ages except the younger P26, where both groups have similar transitions. Within the KO group, there was no change in transitions as a function of age. There is no change in transitions for WT mice with age. (Blue asterisk,*, indicates comparison with age matched WT mice Data are expressed as Mean ± SEM; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

Ketogenic diet treatment does not have any significant effect on rest to wake transitions in Kv1.1 KO mice

When compared between groups across age, (Figure 16), the KD-KO mice have more rest to wake transitions that KO mice at P46 (28.25 ± 2.445 and 22.41 ± 1.48, $p<0.05$). However at P50 it is the exact opposite, the KD-KO have less transitions than KO group (21.38 ± 1.87 and 28.36 ± 1.89, $p<0.01$). Overall, there is no effect of ketogenic diet on rest to wake transitions in the KO mice.

KD-KO mice have significantly higher transitions when compared to WT mice at every age group ($p<0.01$). When compared to KD-WT mice however, the KD-KO mice show higher transitions after P58 (13.44 ± 1.22 and 21 ± 1.37, $p<0.01$). Interestingly the KD-WT mice also show significantly higher transitions when compared to WT mice across age ($p<0.01$).

When compared within group across age, we found that there are a higher number of transitions in KD-KO mice at P38 (25.46 ± 1.431, $p<0.01$) and P46 (28.25 ± 2.445, $p<0.01$) when compared to P26 mice (19.29 ± 1.45).

Collectively our data suggest that ketogenic diet does not have any effect on the rest to wake transitions in the KO mice, however it seems to increase the transitions in the WT mice.

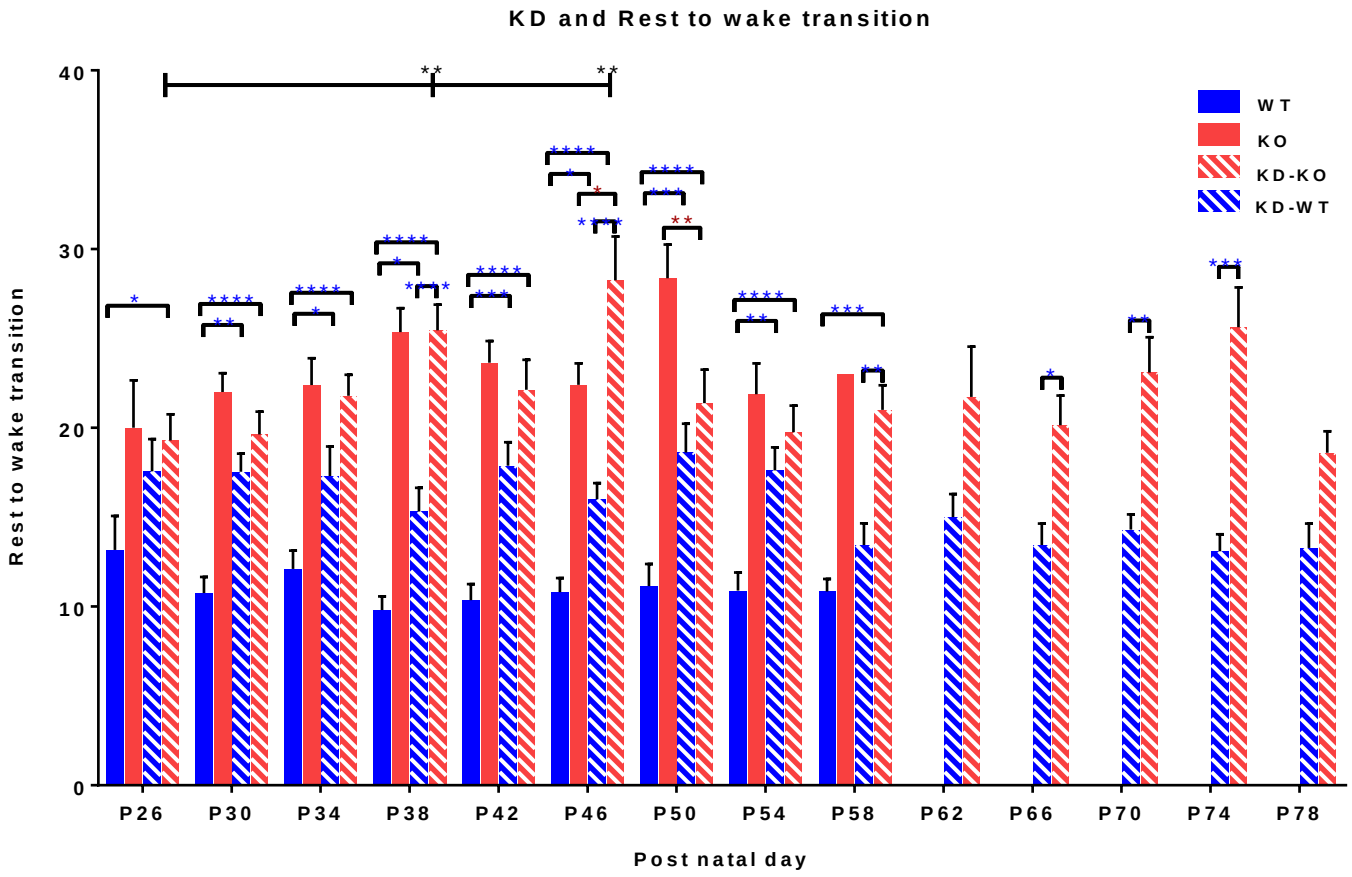


Figure 16. KD does not have any effect on rest to wake transitions in Kv1.1 KO mice: Data was analyzed using 2-way ANOVA with Dunnett's multiple comparison *post hoc* test. Kv1.1 KO on ketogenic diet do not show any significant difference in the rest to wake transitions compared to KO mice on standard diet except at P46 and P50. However, KD-KO mice have significantly higher transitions when compared to both KD-WT and WT controls at all ages except the younger P26-34. KD-WT mice also had significantly higher rest to wake transitions compared to WT mice across age. Data are expressed as Mean ± SEM; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Blue asterisk,*, indicates comparison with age matched WT or KD-WT mice and red * is comparison between KD-KO and KO groups.

Retrospective analyses depict significantly lower rest epochs, higher rest debt and higher activity in the days immediately preceding death in Kv1.1 KO mice

As mentioned earlier, through this study, we intended to detect changes in one or more of the rest parameters that can be used as possible biomarkers for SUDEP in the KO mice. Considering the wide age range of SUDEP in the KO mice (P43 to P56), we decided to eliminate the effect of age induced variability in our data and instead analyze the data retrospectively from day of death. The data were plotted in 4-day bins from day prior to SUDEP (PTS) being Day 1, regardless of the age of the mice.

Retrospective analyses also indicate that in the KO mice, there is a significant decrease in the number of rest epochs at the days immediately prior to SUDEP when compared to several days before SUDEP ($F(7, 260) = 2.499$, $p<0.05$) (Figure 17A). Dunnett's multiple comparison *post hoc* test was used to analyze data further. The retrospective plot depicts almost a step wise decline in the rest epochs in the KO mice. Initially there is a very slow decline in rest epochs till 28-PTS after which we saw a sharp 20% decline from 28-PTS (138.5 ± 8.192) to 24-PTS (113.5 ± 7.574). This was followed by a plateau phase of 10 to 12 days where the rest epochs remain apparently unchanged (112 ± 5.52) followed by a 15% decline in rest epochs at 12-PTS (95.85 ± 9.19, $p<0.05$). After this the rest epochs decreased gradually, reaching a minimum value at 1-PTS (83.5 ± 8.22) which differed significantly from 28-PTS (138.5 ± 8.192, $p<0.01$).

Even though the rest epochs decrease as SUDEP approaches, this endpoint did not appear to be sensitive enough to predict SUDEP accurately. Hence, we looked at another novel rest parameter, sleep debt which was operationally defined as the average amount of time KO mice spent resting when subtracted from the average time spent resting by the WT controls. So,

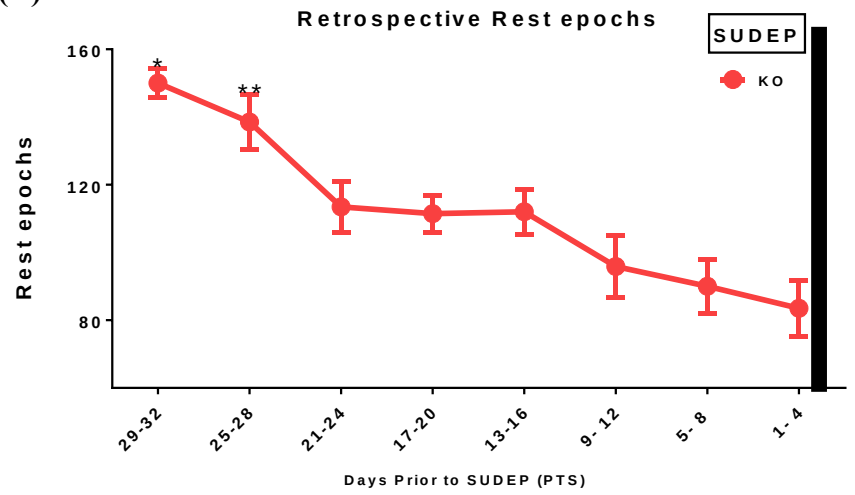
essentially, the sleep debt in WT is considered as zero and the KO values are measured accordingly.

We found that, in the KO mice, there was a two-step rise in the sleep debt; our data indicates that there was a significant increase in the rest debt at the days immediately prior to SUDEP when compared to several days before SUDEP ($F(7, 238) = 3.771$, $p<0.001$). We also found at 1-PTS (274.7 ± 20.45) there was 50% and 150% increase in the rest debt at as compared to 16-PTS (183.8 ± 18.61, $p<0.01$) and 28-PTS(110.6 ± 21.47, $p<0.001$) respectively (Figure 17B).

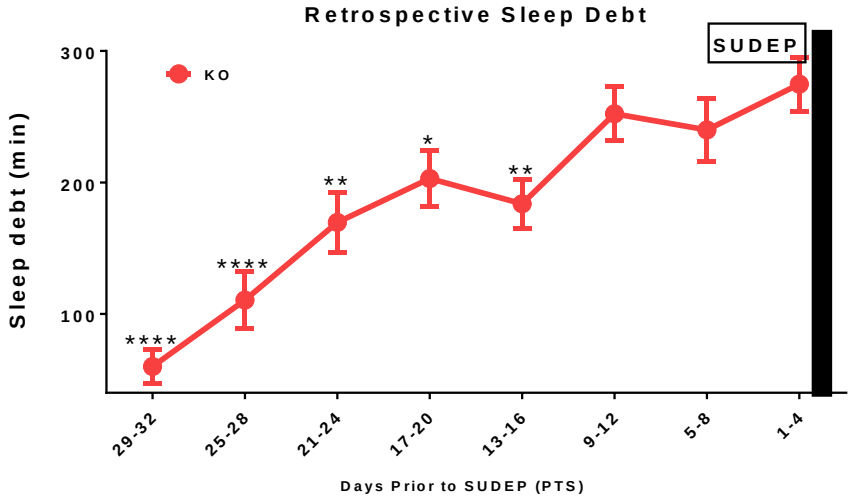
There was a three-step increase in activity from 32-PTS to the pre-SUDEP age. There was approximately 20% rise in activity from 28-PTS (1356 ± 129.1) to 24-PTS (1671 ± 153.8), a plateau phase, and a significant rise in activity from 16-PTS (1543 ± 118.2) to 1-PTS (2436 ± 192.3, $p<0.01$) (Figure 17C). However, overall, the increase in activity does not seem to vary significantly from several days preceding death and day immediately prior to death.

These data collectively indicate that sleep debt and activity counts are both sensitive metrics that can help us predict SUDEP with reasonable accuracy.

(A)


Retrospective Rest epochs
SUDEP
KO
Rest epochs
160
120
80
*
**
29-32
25-28
21-24
17-20
13-16
9- 12
5- 8
1- 4
Days Prior to SUDEP (PTS)

(B)


Retrospective Sleep Debt
SUDEP
KO
Sleep debt (min)
300
200
100
****
****
**
*
**
29-32
25-28
21-24
17-20
13-16
9-12
5-8
1-4
Days Prior to SUDEP (PTS)

(C)

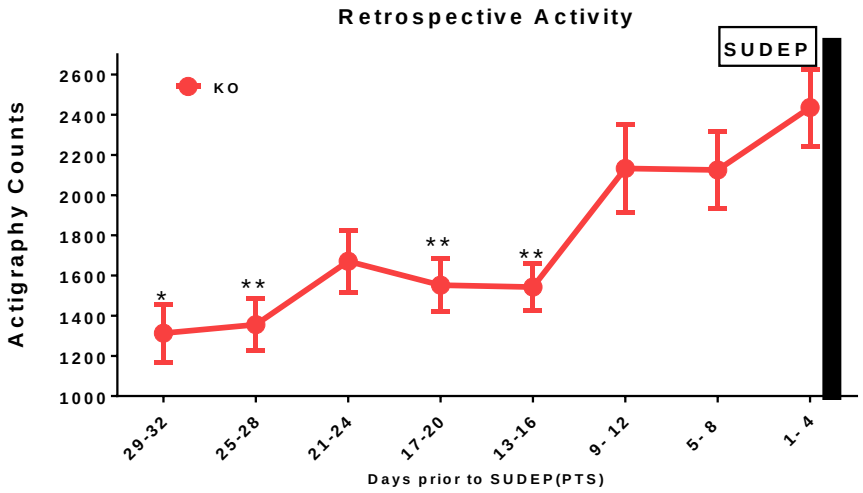

Retrospective Activity
SUDEP
KO
Actigraphy Counts
2600
2400
2200
2000
1800
1600
1400
1200
1000
*
**
**
**
29-32
25-28
21-24
17-20
13-16
9- 12
5- 8
1- 4
Days prior to SUDEP(PTS)

Figure 17. Retrospective Analyses of rest parameters in Kv1.1 KO mice: **(A)** There is significant reduction in number of rest epochs 1-4 days prior to SUDEP compared to several days before death in Kv1.1 KO mice. **(B)** There is a significant increase in rest debt 1-12 days PTS compared to 13-29 days before death in Kv1.1 KO mice. Also the trend of rest debt increase is sharp and indicative of imminent death with a 2-week margin. **(C)** There is a significant increase in activity 1-4 days prior to SUDEP compared to several days before death in Kv1.1 KO mice. Data are expressed as Mean ± SEM; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Blue asterisk,*, indicates comparison with age matched WT or KD-WT mice and red * is comparison between KD-KO and KO groups

Retrospective analyses indicate that KD treated Kv1.1 KO mice show similar pattern in rest parameters as SUDEP approaches

We plotted the rest data from KO and KD-KO mice starting from day of death backwards in 4-day bins. KD-WT data was combined with WT mice to form one WT control group and were plotted to age-match the KD-KO data.

Retrospective analyses test indicates that between 56-PTS and 32-PTS there was no significant difference in number of rest epochs between KD-KO and the WT mice (Figure 18 A). Interestingly, even though KD treated KO mice live 40% longer compared to standard diet treated KO mice, 28 days prior to SUDEP, there is a sudden decline in rest epochs in the KD-KO mice when compared to WT control (91.46 ± 11.02 and 157.3 ± 5.32, respectively. $p<0.001$), which then resembles the step wise pattern seen in the KO mice. We found no significant difference in rest epochs between KD-KO and KO mice from 28 PTS to the day prior to SUDEP.

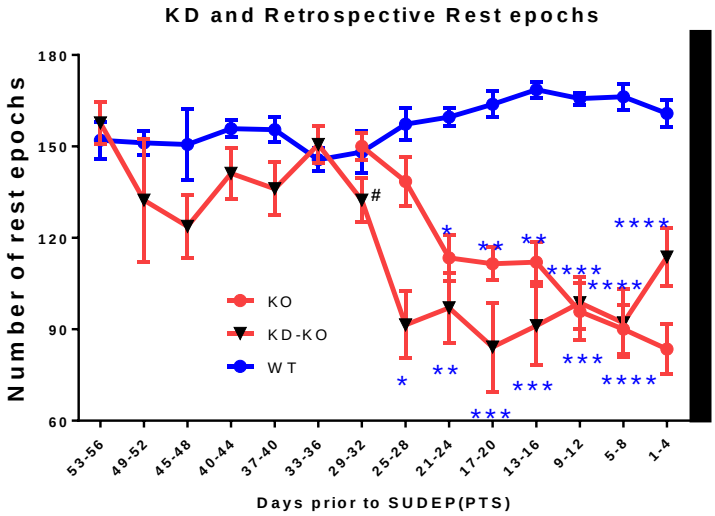
Comparing the KD-KO to the WT group, we found that the KD-KO have significant sleep debt at all days prior to SUDEP (Figure 18 B). However, the sleep debt becomes significantly higher in KD-KO (250.6 ± 28.85, $p<0.001$) at 28-PTS compared to WT group and remains high thereafter. Thereafter the increase in sleep debt in KD-KO resembles the step wise pattern seen in the KO mice. At 28-PTS, the KD-KO have significantly higher sleep debt compared to KO group (250.6 ± 28.85 and 110.6 ± 21.47, respectively. $p<0.05$). Thereafter, we found no significant difference in the sleep debt between the two groups.

Similar to the rest epochs, between 56-PTS until 24-PTS, we found no there is no significant difference in the activity between KD-KO and the WT mice (Figure 18 C). At 20-PTS however,

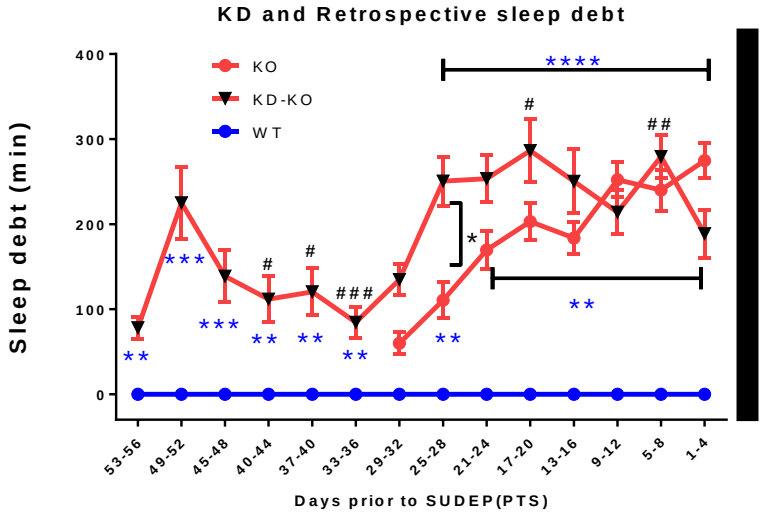
there is about 3 fold increase in activity in the KD-KO mice when compared to WT control (2037 ± 350.2 and 816 ± 69.42, respectively. $p<0.01$). From 28-PTS to 8-PTS there were no significant differences in activity between the KD-KO and KO groups. 4 days prior to SUDEP however, the KD-KO mice have about 40% lower activity compared to KO mice (2436 ± 192.2 and 1426 ± 185.9, respectively. $p<0.05$).

The retrospective analyses suggest that there are significantly lower rest epochs, higher rest debt and higher activity in the days immediately preceding death in Kv1.1 KO mice. Moreover, in the KO mice, KD treatment seems to restore the rest parameters WT levels up to about a month prior to SUDEP. This effect is however lost eventually as the KD treated mice suffer a similar fate to that of the regular KO mice. Sleep debt and activity have again emerged as metrics sensitive to the sleep changes associated with SUDEP. However, they are not yet sensitive enough to be used as a biomarker to indicate imminent SUDEP in the Kv1.1 KO mice.

(A)


KD and Retrospective Rest epochs
Number of rest epochs
180
150
120
90
60
KO
KD-KO
WT
53-56
49-52
45-48
40-44
37-40
33-36
29-32
25-28
21-24
17-20
13-16
9-12
5-8
1-4
Days prior to SUDEP(PTS)

(B)


KD and Retrospective sleep debt
Sleep debt (min)
400
300
200
100
0
KO
KD-KO
WT
53-56
49-52
45-48
40-44
37-40
33-36
29-32
25-28
21-24
17-20
13-16
9-12
5-8
1-4
Days prior to SUDEP(PTS)

(C)

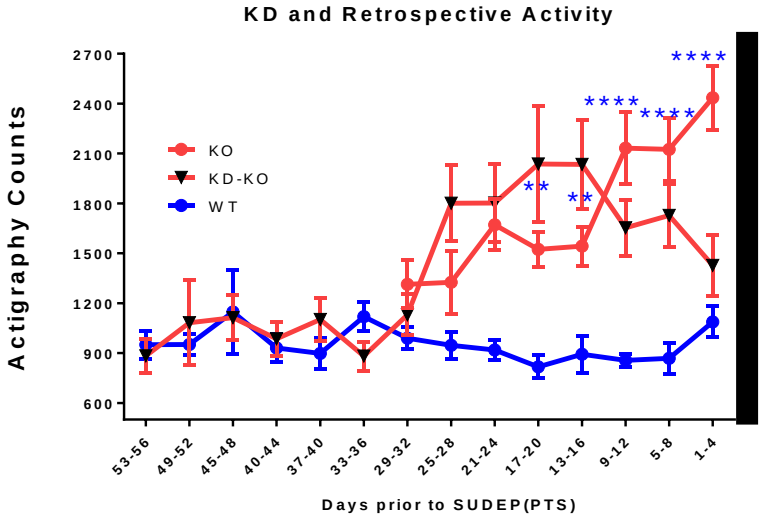

KD and Retrospective Activity
Actigraphy Counts
2700
2400
2100
1800
1500
1200
900
600
KO
KD-KO
WT
53-56
49-52
45-48
40-44
37-40
33-36
29-32
25-28
21-24
17-20
13-16
9-12
5-8
1-4
Days prior to SUDEP(PTS)

Figure 18. Retrospective Analyses depict that KD treated KO mice show similar trends in rest parameters prior to SUDEP as seen in standard diet treated Kv1.1 KO mice: (A) 40-50 days prior to death, the KD treated KO mice have rest epochs similar to that of WT controls. However 4-30 days before SUDEP, the KD-KO show similar trend of decrease in rest epochs compared to KO mice, and vary significantly from WT controls in these days. **(B)** KD treated KO mice have significantly higher rest debt than WT controls at all days prior to SUDEP. 25 days prior to SUDEP the KD KO show higher sleep debt compared to KO on standard diet. However thereafter, the KD-KO show similar trend of increase in sleep debt compared to KO mice, and vary significantly from WT controls in these days. **(C)** 40-50 days prior to death, the KD treated KO mice have similar activity as that of WT controls. However 12-30 days before SUDEP, the KD-KO show similar trend of increase in activity as that of KO mice, and vary significantly from WT controls in these days.. Data are expressed as Mean ± SEM; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Blue asterisk,*, indicates comparison with age matched WT or KD-WT mice and red * is comparison between KD-KO and KO groups

DISCUSSION

Numerous studies have shown that there is a complex relationship between epilepsy and sleep; occurrence of seizures is associated with sleep deprivation which in turn leads to seizure exacerbation forming a vicious cycle (Vaughn and D'Cruz 2004; Matos et al. 2010). Sleep deprivation is often associated with increased nocturnal seizures which incidentally, has been identified as one of the risk factors contributing to SUDEP in humans (Lamberts et al. 2012). Considering all this, we decided to assess the relationship between sleep deprivation and SUDEP, and if sleep deprivation poses an increased risk for SUDEP.

Sleep abnormalities in Kv1.1 KO mice

The Kv1.1 KO mice used in this study are a robust animal model for both temporal lobe epilepsy and SUDEP (Smart et al. 1998; Moore et al. 2014). We previously reported that these epileptic Kv1.1 KO mice altered diurnal rhythmicity and also had deficiencies in sleep architecture (Roundtree et al. 2016; Fenoglio-Simeone et al. 2009). Here we used the non-invasive method of actigraphy for long term monitoring of the rest activity cycles in these mice and found that during the light phase, the Kv1.1 KO mice are more active, have less resting periods and tend to wake up more from rest as compared to their WT controls. These results are quite similar to the sleep disorder symptoms described in people with epilepsy, which include slow onset of sleep, increased wakefulness, and decreased sleep efficiency and NREM sleep (Kotagal and Yardi 2008; Malow, Bowes, and Lin 1997).

SUDEP and progressive worsening of seizure and sleep abnormalities

Further we determined the ontogeny of sleep disruption in these mice starting from the young age of P21-25 until the age of SUDEP. The effect of age on resting patterns is a critical feature of

this study owing to the fact that in Kv1.1 KO model of epilepsy, the postnatal age of the mice is basically an indicator of the epilepsy severity in these mice. Ontogeny of seizure burden in these mice indicates progressive worsening of seizures from epilepsy onset age at around P21-P25 to the age of SUDEP (Simeone et al. 2016) **(**Figure 8). Indeed we found that ontogeny of sleep disruption corresponds to that of seizure worsening and epilepsy progression. The start of sleep disruption corresponds with epilepsy onset indicated by the higher rest to wake transitions in the Kv1.1 KO mice compared to WT mice. The sleep disruption might not be very severe at this age, as the other rest parameters like activity and number of rest epochs do not vary significantly from the WT controls. The sleep disruption eventually becomes more severe in the KO mice, as indicated by the progressive increase in activity and decline in the amount of time spent resting with age and reaches pathologically high value in the age range of P46-54 when SUDEP occurs in these mice. This strengthens our hypothesis that sleep disruption, epilepsy progression and SUDEP are delicately connected events and need to be studied further.

Ketogenic diet: Sleep and SUDEP:

The high-fat, low-carbohydrate ketogenic diet (KD) which is used in the treatment of refractory epilepsy, reduces seizure frequency in humans and increases seizure threshold in flurothyl and amygdala kindled animal models of epilepsy (Felton and Cervenka 2015; Hallböök et al. 2007; Rho et al. 1999; Hori et al. 1997). KD also improves the sleep quality in children with refractory epilepsy. Our previous studies in the Kv1.1 KO model of temporal lobe epilepsy have indicated that KD effectively reduces seizures in these mice and also improves the diurnal rhythmicity in these mice (Fenoglio-Simeone et al. 2009; T. A. Simeone et al. 2014). Recently, we have also shown that KD delays the seizure progression and increases longevity in these mice (Simeone et al. 2016). In this study we evaluated whether KD treatment can improve the progressive nature

of sleep disruption in these mice and help in preventing SUDEP. Indeed, KD treatment seems to delay the progression of sleep disruption and improving the rest parameters the KO mice having little effect on that of the WT controls. Interestingly, we also observed an increase in rest to wake transitions in KD treated non epileptic WT mice. This observation closely corresponds to a recent study in non-epileptic healthy population where high fat-low carbohydrate diet similar in composition to KD was found to cause sleep abnormalities in these subjects (Afaghi, O'Connor, and Chow 2013). However, future studies will look more closely at the significance of this difference between the genotypes on KD treatment.

Retrospective analyses have shown interesting similarities between KD treated KO mice and the standard diet treated KO mice. On an average the KD-treated Kv1.1 KO mice live 60% longer and show better resting patterns compared to those weaned onto standard diet. Interestingly, comparing the rest parameters, in the ages closer to SUDEP the KD-treated KO mice behave similar to the standard diet KO mice. This means that while KD treatment restores the rest parameters in the KO mice to WT levels at very young ages, at some point the protective effect of KD is lost. After this the KD- treated KO mice also have progressively deteriorated sleep which ultimately ends in death. Our future studies will look into the reason for why KD-treatment becomes ineffective at a certain point, and if this can be prevented.

Need for biomarker and prevention of SUDEP:

Studies in humans and animal models have identified several genomic, cardiac and structural parameters which maybe potential biomarkers of SUDEP, however each of these potential biomarkers have been associated with only a certain a sub population of epileptic patients (Wandschneider et al. 2015; Glasscock 2014; DeGiorgio et al. 2011). Hence there is need for a

universal biomarker that can be used to predict SUDEP in most of the epileptic population. To our knowledge, we are the first study to assess if one or more of the rest parameters can be used to predict the age of death in a well-established SUDEP model. Our retrospective analyses have indicated that sleep debt could be a potential biomarker for SUDEP. While this method does allow us to anticipate a SUDEP event at least two weeks before its occurrence, we still need to find a more sensitive method that can allow for more accurate prediction of SUDEP. Our future methods include cumulative retrospective plot of the sleep debt data, where the cumulative sleep debt for each 4-day bin prior to death will be plotted to see the point at which all the mice exceed a particular level of cumulative sleep debt after which their death is imminent.

CONCLUSION AND GLOBAL IMPACT:

Overall, we found that the Kv1.1 KO mice had decreased heart rate, increased R-R and QTc intervals and increased rmSSD in the ages approaching SUDEP when compared to the KO mice of younger age. Thus, the incidence of bradycardia, susceptibility to arrhythmias and the parasympathetic drive increase in the Kv1.1 KO mice just prior to SUDEP. We also found that treatment of the KO mice with orexin receptor antagonist, almorexant, restores these cardiac parameters to the WT control range and also reduces the parasympathetic overdrive in these mice. Further preclinical studies can help us determine if almorexant can ameliorate the cardiac abnormalities in SUDEP and whether it can increase the lifespan of the SUDEP animal models. Thus, orexin receptor antagonism can be a potential therapeutic target for further research in SUDEP.

Our studies on sleep dysfunction in the Kv1.1 KO mice show that the ontogeny of sleep disruption corresponds to the ontogeny of seizure worsening in these mice. Treating these mice with ketogenic diet helps in improving some of the rest parameters and increases the lifespan in this mouse model of SUDEP. Thus, we conclude that progressively worsening cardiac abnormalities and sleep disruption correspond to the seizure ontogeny in Kv1.1 KO mice and individually or together may increase the risk for SUDEP in Kv1.1 KO mice.

Through our studies, we have identified sleep and cardiac abnormalities as possible biomarkers for SUDEP and laid out a general foundation for further research in SUDEP. We also identified ketogenic diet and orexin antagonism as two potential therapeutic targets that can be used to design numerous preclinical studies and maybe useful in preventing SUDEP. This has opened a myriad of possibilities in research pertaining to SUDEP and has brought us a little closer to

understanding the factors involved in SUDEP. Hence, maybe several years along the line, we as a research community, may be actually successful in completely understanding the physiological dynamics prior to SUDEP and benefit the epileptic population as a whole.

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