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PRINCIPAL INVESTIGATOR: Dr. Geoffrey Murphy

CONTRACTING ORGANIZATION: University of Michigan
Ann Arbor, MI 48109

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Geoffrey Murphy

E-Mail: murphyg@umich.edu

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14. ABSTRACT
The overall objective of this Discovery Award was to explore the hypothesis the ketogenic diet (KD) regulates neuronal excitability by influencing potassium channel activity via the auxiliary potassium channel subunit Kvβ2. To test this hypothesis we have examining the impact of the ketogenic diet on mice in which the gene that encodes Kvβ2 has been deleted (Kvβ2 KO mice) using an in vitro model of seizure induction in which we recorded from three interconnected brain regions: the hippocampus, entorhinal cortex and amygdala. Our main findings are 1) acute treatment (in vitro) with ketones significantly reduces epileptiform activity in the hippocampus from WT mice but not in the Kvβ2 KO mice 2) this effect is not observed in ex vivo slice prepared from mice maintained on the KD 3) the KD appears to increase excitability (as measured by the inter-ictal interval) in the entorhinal cortex and amygdala 4) deletion of Kvβ2 itself has a differential impact on bursting activity in the amygdala in untreated mice and this observation is not reversed by the KD which to some extent supports our initial hypothesis.

15. SUBJECT TERMS
Epilepsy, Ketogenic Diet, Seizure Disorder, Potassium Channels, Neurophysiology.

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1. Introduction:

The overall goal of this Discovery Award was to explore the hypothesis that the ketogenic diet (KD), which is used to treat epilepsy (primarily in children) exerts a positive effect on seizure activity by regulating neuronal excitability via a subclass of potassium auxiliary subunits known as Kvβ2 subunits. Therefore we have been treating genetically engineered mice that lack these subunits (specifically the Kvβ2 subunit) with the KD and examining the interaction of diet and seizure activity in vitro.

2. Keywords:

Epilepsy, Ketogenic Diet, Seizure Disorder, Potassium Channels, Neurophysiology

3. Overall Project Summary:

To determine the impact of KD on epileptiform activity we used an ex-vivo model in which seizure-like activity was induced in acutely prepared horizontal brain slices which contained the hippocampus, entorhinal cortex and amygdala. Simultaneous extracellular field recordings were made (Figure 1) in the CA3 region of the hippocampus (Hp) entorhinal cortex (EC) and lateral nucleus of the amygdala (LA). After a stable baseline was recording, the normal artificial cerebrospinal fluid (aCSF) was replaced with aCSF which contained 0.5 mM magnesium (Low Mg^{2+} in Fig 1A above) and was continuously perfused throughout the remainder of the recording. Typically, after several minutes of low Mg^{2+} spontaneous rhythmic burst events were observed (Fig 1B) which consisted of a biphasic waveform containing multiple spikes of varying amplitude.

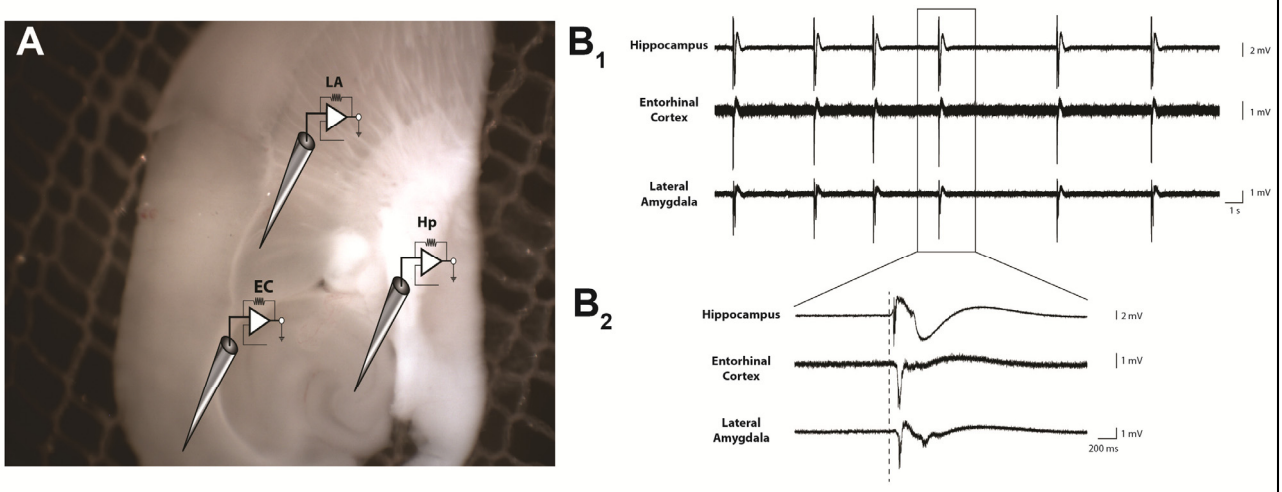

Figure 1. Basic recording method. (A) Photograph of the horizontal slice preparation and electrode placement in the hippocampus (Hp), entorhinal cortex (EC) and lateral amygdala (LA). (B_1) Representative extra cellular recordings exhibiting rhythmic bursting across time. (B_2) Example of synchronized activity in which activity in the Hp precedes the EC which in turn precedes the LA.

Some of our early work was to determine the correct conditioning in which to make the recordings. Initially we reasoned that the ex vivo slices prepared from mice on the ketogenic diet should be maintained in aCSF that contained ketones. Therefore we typically included beta-hydroxybutyrate (BHB; the primary ketone body generated by the liver under ketogenic conditions) at near physiological conditions (0.4 mM). Somewhat surprisingly, treatment of slices with BHB significantly decreased the number of slices that exhibited ictal-like activity but only in mice that were previously maintained on a normal diet (ND; Figure 2). Of significant interest is the fact that this

suppression was not observed in mice in which Kvβ2 was deleted (Kvβ2$^{-/-}$). These results suggest that Kvβ2 may mediate ketone related changes in neuronal excitability. However, it appears that this response is only under acute conditions as we observed no difference between any of the groups when slices were prepared from mice that were maintained on the KD. Furthermore, these alterations appear to be hippocampal specific in that we did not observe these changes in the EC or LA (data not shown). Although we do not yet know the mechanism that underlies the acute effects of BHB treatment these experiment demonstrate that our original design of maintaining slice prepared from mice on the KD in BHB is sound.

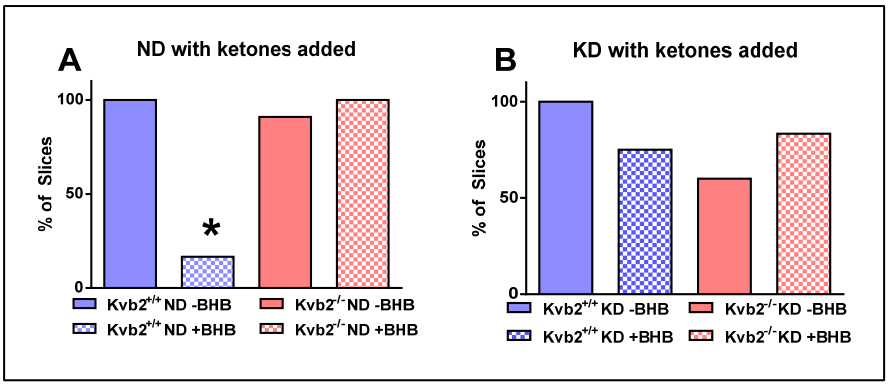

Figure 2. Differential effect of including ketones during ex-vivo recording in the hippocampus. (A) Addition of ketone bodies (BHB) significantly recued the number of wild-type (Kvb2$^{+/+}$) slices that exhibited ictal-like activity. (B) Slices prepared from mice maintained on the KD were unaffected by ex vivo treatment with BHB regardless of genotype. * p<0.05 Chi squared test.

In addition, we discovered that deletion of Kvβ2 itself leads to an increase in the frequency of bursting activity in the presence of low Mg^{2+} (Figure 3). Using the slices that exhibited sustained bursting we analyzed all of the ictal-like events (see Fig $1B_2$) across the entire recording period after the introduction of low Mg^{2+} (typically 1 hr) and calculated each inter-event interval. The inter-event interval data from all of the slices from each treatment group then then plotted as the relative frequency (cumulative distribution). The data acquired from the amygdala is plotted in Figure 3.

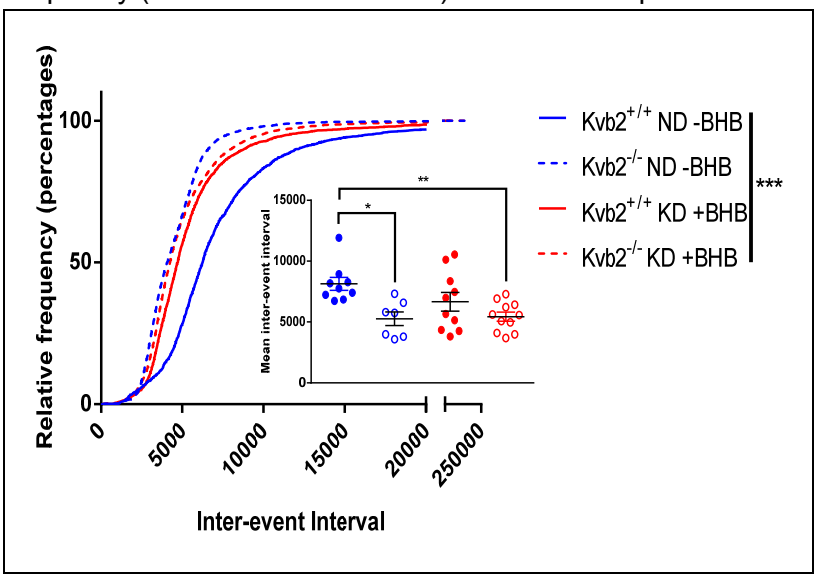

Figure 3. Inter-event interval data from the lateral amygdala. The relative frequency (cumulative probability) of all inter-event intervals is plotted for each group. All groups exhibited a leftward shift when compared to the wild-type mice maintained on the ND (Kruskal-Wallis, ***p<0.001). **Inset**: average inter-event interval (mean ± S.E.M) for slices prepared from WT mice on the ND (closed blue circles) or KD (open blue circles) and Kvβ2 KO mice on the ND (closed red circles) or KD (open red circles). * p < 0.05 ** p < 0.01; ANOVA with post hoc test adjusted for multiple comparisons.

Somewhat surprisingly slices prepared from mice on the KD exhibited a significant leftward shift when compared to ND slices regardless of genotype suggesting a KD induced increase in excitability. This was also true in the entorhinal cortex and to some extent in the hippocampus (data not shown). We also calculated the average inter-event interval for each slice (inset in Figure 3).

When we compared the meaning inter-event interval between the groups we observed that deletion of Kvβ2 resulted in a significant reduction in the mean inter-event interval. This reduction was not reduced in the Kvβ2 KO mice suggesting that Kvβ2 might mediate the effcts of the KD on neuronal excitability. Admittedly this interpretation is somewhat complicated by the observation that the KD appears to reduce the inter-event interval relative frequency in the WT mice although the difference in the mean inter-event interval between WT ND and WT KD was not statistically different. Also we should point out that these differences were confined to the amygdala suggesting that Kvβ2 must play a differential role in this brain structure.

Finally, we have also analyzed the role that Kvβ2 plays in mediating putative changes in functional connectivity mediated by the KD (Figure 4). For this analysis, we made two qualitative assessments: which region was the first to burst and once continuous bursting was established, which region was more likely to "drive" the adjacent region. Referring back to Figure 1B$_2$—this is an example of a slice in which the hippocampus drives the entorhinal cortex which in turn drives the lateral amygdala. In Figure 4, the pie charts represent the proportion of slices in which that specific region was the first to burst. For example Figure 1, panel A presents the data from the WT mice maintained on the ND in which the hippocampus was the region most likely to burst first followed by the entorhinal cortex and amygdala in somewhat equal proportions. The arrows between the pie charts represent the percentage of slices in which repetitive bursts in one region preceded another region. Staying with panel A, we see that in 75% of the slices, bursts in the amygdala preceded burst in the entorhinal cortex and the converse was true in 21% of the slices. Although this analysis is only quantitative, it appears that there are basal differences in functional connectivity between the WT and

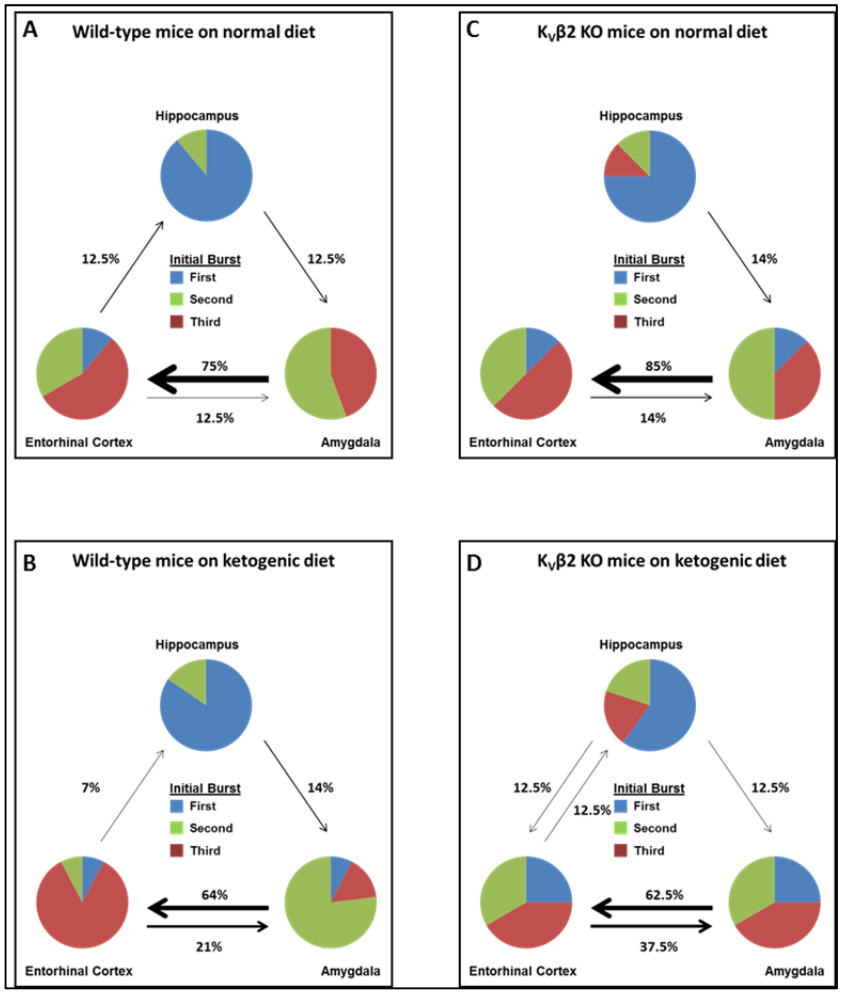

Figure 4. Qualitative analysis of functional connectivity. Pie charts in group represent the proportion of slices in which that region exhibited bursting first, second or third in relation to adjacent and interconnected regions. Arrows and associated percentages indicate to what extent a specific region drove the activity in the adjacent region as assessed by the latency of bursting un the adjacent region(s) which were typically in the millisecond time range.

Kvβ2 KO mice and that the KD alters the functional connectivity such that the relationship between the entorhinal cortex and the amygdala becomes more reciprocal.

4. Key Research Accomplishments:
Using an ex-vivo slice preparation and simultaneous recordings we have discovered :1) acute treatment (in vitro) with ketones significantly reduces epileptiform activity in the hippocampus from WT mice but not in the Kvβ2 KO mice 2) this effect is not observed in ex vivo slice prepared from mice maintained on the KD 3) the KD appears to increase excitability (as measured by the inter-ictal interval) in the entorhinal cortex and amygdala 4) deletion of Kvβ2 itself has a differential impact on bursting activity in the amygdala in untreated mice and this observation is not reversed by the KD which to some extent supports our initial hypothesis.

5. Conclusion:
Although it appears that Kvβ2 may mediate some aspects of the beneficial aspects of the ketogenic diet, it seems unlikely that this protein can account for the entirety of the effects. At a minimum we conclude that Kvβ2 must play different roles in different brain regions. Furthermore it seems likely that the ketogenic diet itself may have different effects on different brain regions. We plan to follow up on this finding in hopes of gaining a better understanding of how this might relate to the efficacy of the ketogenic diet in epilepsies that are refractory to other therapies. Our data also suggest that there are significant experimental differences between the excitability results obtained when ketones are administered acutely in vitro and when they are generated in vivo under the ketogenic diet. We also plan to follow up on this observation because there have been multiple studies using acutely applied ketones and we would like to determine to what extent this experimental approach emulates the conditions in vivo.

6. Publications, Abstracts and Presentations:
This work will be presented at the American Epilepsy Society meeting in Philadelphia (12/4/2015-12/8/206). We also anticipate writing this work up for publication early next year.

7. Inventions, Patents and Licenses:
Nothing to report

8. Reportable outcomes:
Nothing to report

9. Other Achievements:
We anticipate the data generated under this Discovery Award will be used as preliminary data for a larger Federal grant submitted to do the follow up studies.

10. References:
None

11. Appendices:
None