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Title Page

M-channel activation contributes to the anticonvulsant action of the ketone body β-hydroxybutyrate

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Running title page

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Activation of KCNQ3 by the ketone body β-hydroxybutyrate

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BHB, β-hydroxybutyrate; GABOB, γ-amino-β-hydroxybutyric acid; HBSS, Hank's Balanced Salt Solution; PTZ, pentylene tetrazole;

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Abstract

Ketogenic diets are effective therapies for refractory epilepsy, yet the underlying mechanisms are incompletely understood. The anticonvulsant efficacy of ketogenic diets correlates positively to the serum concentration of β-hydroxybutyrate (BHB), the primary ketone body generated by ketosis. Voltage-gated potassium channels generated by KCNQ2-5 subunits, especially KCNQ2/3 heteromers, generate the M-current, a therapeutic target for synthetic anticonvulsants. Here, we report that BHB directly activates KCNQ2/3 channels (EC_{50}, 0.7 μM), via a highly conserved S5 tryptophan (W265) on KCNQ3. BHB was also acutely effective as an anticonvulsant in the pentylene tetrazole (PTZ) seizure assay in mice. Strikingly, co-administration of γ-amino-β-hydroxybutyric acid (GABOB), a high-affinity KCNQ2/3 partial agonist that also acts via KCNQ3-W265, similarly reduced the efficacy of BHB in KCNQ2/3 channel activation *in vitro* and in the PTZ seizure assay *in vivo*. Our results uncover a novel, unexpected molecular basis for anticonvulsant effects of the major ketone body induced by ketosis.

Significance Statement

Ketogenic diets are used to treat refractory epilepsy but the therapeutic mechanism is not fully understood. Here, we show that clinically relevant concentrations of β-hydroxybutyrate, the primary ketone body generated during ketogenesis, activates KCNQ2/3 potassium channels by binding to a specific site on KCNQ3, an effect known to reduce neuronal excitability. We provide evidence using a mouse chemoconvulsant model that KCNQ2/3 activation contributes to the antiepileptic action of β-hydroxybutyrate.

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Introduction

Epilepsy remains a major, inadequately controlled neurological disorder, affecting around 50 million individuals globally, ~20% of whom are refractory to medications (Shorvon, 1996). A number of inherited and other disorders lie under the umbrella term epilepsy, but a common thread is inadequate control of neuronal excitability, leading to seizures ranging from relatively benign and transient, to life-threatening and severe (Falco-Walter et al., 2018). Aside from medication, one intervention currently attracting renewed interest, especially for treatment of refractory pediatric epilepsy, is the ketogenic diet (Nordli, 2009). The ketogenic diet is a low-carbohydrate, high-fat, adequate-protein diet regime that deprives the body of dietary glucose, which in a normal diet is a particularly important fuel source for energy-hungry neuronal activity. Faced with a low-carbohydrate, high-fat diet, hepatocytes in the liver increasingly convert fats into fatty acids and ketone bodies, which enter the brain via the blood and replace glucose as the predominant neuronal energy substrate (Morris, 2005) (Figure 1A). This metabolic state, referred to as ketosis, has been convincingly demonstrated to reduce incidence of seizures, especially in children with refractory epilepsy (Kinsman et al., 1992, Freeman et al., 1998, Hassan et al., 1999, Freiman et al., 2006, Taub et al., 2014) but also in adults (Kinsman et al., 1992). A meta-analysis of seven studies of the classic ketogenic diet in both adolescents and adults found that the diet was associated with >50% seizure reduction in half of the patients treated, and 13% of the responsive patients were seizure-free (Payne et al., 2011). Fasting produces similar metabolic shifts and its antiepileptic properties have been recognized for millennia (Hartman et al., 2013).

Despite the therapeutic importance of the ketogenic diet, the mechanisms by which it reduces seizures are still unclear. The ketone bodies produced by a ketogenic diet have received most attention in studies investigating the anticonvulsant action of this diet, and in particular, β-hydroxybutyrate (BHB), a ketone body that accumulates during ketosis (Figure 1A). The

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antiepileptic efficacy of ketogenic diets has been positively correlated with BHB reaching an optimal serum concentration of 4 mM (Gilbert et al., 2000). Several potentially therapeutic effects have been attributed to BHB, including replacement of glucose as a neuronal energy source (LaManna et al., 2009), but also less intuitive actions. These include activation of hydroxyl-carbolic acid receptor 2 (HCA2) by BHB, which may be neuroprotective via prostaglandin D2 production (Rahman et al., 2014); BHB also facilitates synaptic vesicle recycling (Hrynevich et al., 2016). Thus, ketone bodies such as BHB may exert a spectrum of effects on neurons to quell aberrant hyperexcitability. A greater understanding of the mechanisms underlying the ketogenic diet may lead to a molecular intervention that mimics the beneficial effects of ketosis without the associated side effects, which can include gastrointestinal disruption, vitamin and mineral deficiencies (Hahn et al., 1979, Tallian et al., 1998, Bergqvist et al., 2003).

K^+ efflux is the primary force behind the cellular repolarization that ends action potentials, and is also instrumental in setting and maintaining resting membrane potential, and preventing neuronal hyperexcitability. In metazoan nervous systems, KCNQ2-5 channels generate the M-current (Biervert et al., 1998, Singh et al., 1998), a muscarinic-inhibited Kv current (Brown and Adams, 1980) that plays an essential part in regulating neuronal excitability (Wang et al., 1998). The M-current is thought to be generated primarily by KCNQ2 and KCNQ3 subunits in the form of heteromeric (KCNQ2/3) channels (Wang et al., 1998, Wang et al., 2009). KCNQ2/3 channels do not inactivate, and open at relatively hyperpolarized membrane potentials. They are therefore uniquely evolved to regulate neuronal excitability and, accordingly, their gating state governs phasic versus tonic firing of neurons (Wang et al., 1998). Furthermore, genetic disruption of KCNQ2 or KCNQ3 function causes epilepsy in humans and mice (Biervert et al., 1998, Schroeder et al., 1998, Singh et al., 1998, Watanabe et al., 2000, Singh et al., 2003, Singh et al., 2008, Soh et al., 2014). We recently discovered that γ-aminobutyric acid (GABA),

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the primary inhibitory neurotransmitter, can directly activate specific members of the KCNQ (Kv7) family of voltage-gated potassium (Kv) channels (Manville et al., 2018). In the same study, we found that two naturally-occurring GABA analogs, γ-amino-β-hydroxybutyrate (GABOB) and the ketone body BHB, can also activate KCNQ2/3 channels. As KCNQ2/3 activation is inhibitory to neuronal activity, and is the mechanism of action for the class of anticonvulsants including retigabine (Rundfeldt, 1999, Main et al., 2000, Wickenden et al., 2000), here we sought to determine the underlying molecular mechanisms and potential anticonvulsant significance of BHB activation of KCNQ2/3. Our results suggest an important role for KCNQ2/3 activation in BHB anticonvulsant activity.

Materials and Methods

PTZ chemoconvulsant assay

Anticonvulsant activities of test compounds were compared in male C57BL/6 mice (Charles River) aged 2-3 months. Mice were housed and used according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publication, 8th edition, 2011). The study was approved by the Institutional Animal Care and Use Committee of University of California, Irvine. Chemicals were sourced from Sigma (St. Louis, MO, USA) unless otherwise indicated. A pentylene tetrazole (PTZ) chemoconvulsant assay was used, as we previously described (Abbott et al., 2014). BHB and GABOB were diluted in Hank's Balanced Salt Solution (HBSS) for injection. Mice were injected intraperitoneally with GABOB and/or BHB, or HBSS vehicle control, and then 30 minutes later were injected intraperitoneally with 80 mg/kg PTZ. Following the PTZ injection, mice were caged individually and an observer (GWA) blinded to the drug used recorded, over 20 minutes, seizure latencies (time to first tail flick or seizure), clonic and tonic incidence, and mortality.

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Channel subunit cRNA preparation and Xenopus laevis oocyte injection

cRNA transcripts encoding human KCNQ2 or KCNQ3 were generated by *in vitro* transcription using the T7 polymerase mMessage mMachine kit (Thermo Fisher Scientific), after vector linearization, from cDNA sub-cloned into plasmids incorporating *Xenopus laevis* β-globin 5' and 3' UTRs flanking the coding region to enhance translation and cRNA stability. cRNA was quantified by spectrophotometry. Mutant channel cDNAs were generated by site-directed mutagenesis using a QuikChange kit according to manufacturer's protocol (Stratagene, San Diego, CA) and then corresponding cRNAs prepared as above. Defolliculated stage V and VI *Xenopus laevis* oocytes (Ecocyte Bioscience, Austin, TX) were injected with Kv channel α subunit cRNAs (5 ng). Oocytes were incubated at 16 ^{o}C in Barth's saline solution (Ecocyte) containing penicillin and streptomycin, with daily washing, for 3-5 days prior to two-electrode voltage-clamp (TEVC) recording.

Two-electrode voltage clamp (TEVC)

TEVC recording was performed at room temperature with an OC-725C amplifier (Warner Instruments, Hamden, CT) and pClamp8 software (Molecular Devices, Sunnyvale, CA) 3-5 days after cRNA injection as described in section above. Oocytes were placed in a small-volume oocyte bath (Warner) and viewed with a dissection microscope. Chemicals were sourced from Sigma. Bath solution was (in mM): 96 NaCl, 4 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES (pH 7.6). BHB was stored at 4 ^{o}C as a 480 mM stock in 100% ethanol and diluted to working concentrations each experimental day. GABOB was stored at -80 ^{o}C as 1 M stocks in molecular grade H_2O and diluted to working concentrations on each experimental day. All compounds were introduced to the recording bath via gravity perfusion at a constant flow of 1 ml per minute for 3 minutes prior to recording. Pipettes were of 1-2 MΩ resistance when filled with 3 M KCl. Currents were recorded in response to pulses between -80 mV and + 40 mV at 20 mV intervals, or a single pulse to +40 mV, from a holding potential of -80 mV, to yield current-voltage

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relationships, current magnitude, and for quantifying activation rate. TEVC data analysis was performed with Clampfit (Molecular Devices) and Graphpad Prism software (GraphPad, San Diego, CA, USA); values are stated as mean ± SEM. Normalized tail currents were plotted versus prepulse voltage and fitted with a single Boltzmann function:

Eq. 1

$$g = \frac{(A_1 - A_2)}{\left\{1 + exp\,[V_{\frac{1}{2}} - V/Vs]\right\} y + A_2}$$

where *g* is the normalized tail conductance, A_1 is the initial value at -∞, A_2 is the final value at +∞, $V_{1/2}$ is the half-maximal voltage of activation and V_s the slope factor. Activation and deactivation kinetics were fitted with single exponential functions.

Chemical structures, *in silico* docking and sequence analyses

Chemical structures and electrostatic surface potentials (range, -0.1 to 0.1) were plotted using Jmol, an open-source Java viewer for chemical structures in 3D: http://jmol.org/. For docking, the *Xenopus laevis* KCNQ1 cryoEM structure (Sun and MacKinnon, 2017) was first altered to incorporate KCNQ3 residues known to be important for retigabine binding, followed by energy minimization using the GROMOS 43B1 force field (van Gunsteren, 1996), in DeepView (Johansson et al., 2012), as we previously described (Manville et al., 2018). Thus, *Xenopus laevis* KCNQ1 amino acid sequence LIT*TL*YIGF was converted to LIT*AW*YIGF, the underlined W being W265 in human KCNQ3. In addition, *Xenopus laevis* KCNQ1 sequence WWGVVTVTTIGYGD was converted to WWG*LIT*L*A*TIGYGD, the underlined L being Leu314 in human KCNQ3. Surrounding non-mutated sequences are shown to illustrate the otherwise high sequence identity in these stretches. Unguided docking of BHB, GABOB and retigabine to predict native binding sites was performed using SwissDock (Grosdidier et al., 2011) with CHARMM forcefields (Grosdidier et al., 2011).

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Statistical analysis

All values are expressed as mean ± SEM. Anticonvulsant actions were compared using two-tailed t-test followed by Bonferroni correction, except for mortality which was compared using chi-squared analysis. Statistical significance was defined as $P<0.05$.

Results

BHB activates KCNQ2/3 channels to hyperpolarize cell membrane potential

Similar to the anticonvulsant and KCNQ2/3 activator retigabine (Kim et al., 2015), GABA and BHB both harbor strongly negative electrostatic surface potential localized close to a carbonyl oxygen; in contrast, glutamate, an excitatory neurotransmitter, does not (Figure 1B), and neither does it activate KCNQ2/3 channels (Manville et al., 2018). Here, we used two-electrode voltage clamp electrophysiology in the *Xenopus laevis* oocyte expression system, which permits long-term recording of ion channel activity (expressed after injection of channel subunit cRNA) and large currents with little baseline drift, to determine the molecular mechanisms underlying BHB enhancement of KCNQ2/3 channels. First, water-injected control oocytes did not respond to bath-applied BHB (100 µM), verifying oocytes as a suitable system for mechanistic studies (Figure 1C, D). In contrast, BHB (100 µM) augmented currents generated by co-injection of cRNA encoding KCNQ2 and KCNQ3, which produces heteromeric KCNQ2/3 channels. This effect was readily apparent in the tail currents, which permit quantification of activation by various prepulse potentials, measured at a single tail potential (in this case -30 mV) to eliminate effects of driving force and give a comparison of the voltage dependence and fraction of channels opened (Figure 1E,F). BHB (100 µM) increased KCNQ2/3 tail currents by ~0.5 µA between membrane potentials of -60 to +20 mV, with lesser absolute increases at -80 and +40 mV (Figure 1F). The relatively uniform absolute increase in current from -60 to +20 mV had the effect of negative-shifting the midpoint voltage dependence of KCNQ2/3 activation by -10 mV

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(from -43.1 ± 1.4 to -53.1 ± 1.7 mV; *n* = 6, p=0.001) (Figure 1G; Supplementary Figure 1; Supplementary Table 1). The strongest augmentation in terms of fold increase versus baseline current occurred at -60 mV, a subthreshold potential at which Kv channel activity is especially influential in governing cellular excitability (Figure 1H). Accordingly, BHB produced a maximal 3.5-fold mean increase in KCNQ2/3 current at -60 mV (EC_{50} = 0.7 µM; Figure 1I), resulting in a resting membrane potential hyperpolarization of -18 mV at optimal BHB doses (Figure 1J).

BHB activates KCNQ3 but not KCNQ2 potassium channels

We next tested KCNQ2 and KCNQ3 isoforms individually to determine whether one or both responded to BHB. Measuring the BHB sensitivity of homomeric KCNQ2 channels using a similar approach to that in Figure 1, we found that they were insensitive to BHB, even up to 1 mM (voltage dependence of activation, $V_{0.5activation}$, was -43.2 ± 0.7 mV in the absence of BHB, versus -44.6 ± 0.7 mV with BHB; *n* = 5, p=0.2) (Figure 2A-D; Supplementary Table 2).

In contrast, BHB produced robust augmentation of currents generated by KCNQ3* (an expression-optimized KCNQ3-A315T mutant that facilitates accurate analysis of homomeric KCNQ3 currents) (Zaika et al., 2008) (Figure 3A,B). $V_{0.5activation}$ (by -11 mV with 100 µM BHB; from -42.2 ± 0.9 to -53.2 ± 1.0 mV; *n* = 6, p=1.0 x 10^{-5}) (Figure 3B,C). This produced a >8.5-fold mean increase in current at -60 mV with 100 µM BHB (EC_{50} = 1.4 ± 0.5 µM BHB) (Figure 3D), and lesser effects at more positive voltages (Figure 3E; Supplementary Figure 2; Supplementary Table 3). Thus, BHB activation of KCNQ channels exhibits isoform specificity, and in KCNQ2/3 channels must be mediated by KCNQ3. The high sensitivity of KCNQ3 for BHB sharply contrasts with canonical $GABA_A$ receptors, which are insensitive to BHB up to at least 5 mM (Yang et al., 2007). Moreover, it places the BHB sensitivity of KCNQ3 channels (and KCNQ2/3 channels) well within the clinical range observed in the ketogenic diet; for example, previous studies found that occipital lobe β-hydroxybutyrate concentrations in children

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rose from ~50 µM (± 50 µM) to ~1 mM after 3 days fasting (Pan et al., 2000).

BHB directly activates KCNQ3 via the S5 tryptophan W265

KCNQ channels are canonical Kv channels, formed from tetramers of α subunits each bearing 6 transmembrane segments (S1-S6). S1-S4 form the voltage-sensing domain (VSD); S5 and S6 form the pore module with the selectivity filter through which K^+ ions pass lying between them and dipping midway into the plasma membrane from the extracellular side (Figure 4A). KCNQ2-5 subunits, but not KCNQ1, each contain a tryptophan in the S5 segment (W265 in KCNQ3; Figure 4A) that mediates channel activation by retigabine and related anticonvulsants. We previously found that activation by GABA requires this residue, and that it is important for direct binding of GABA (Manville et al., 2018). *In silico* binding studies predict that, like retigabine, BHB can bind at or close to KCNQ3-W265 (Figure 4 B,C). Furthermore, in a structural model of KCNQ3 produced by substitution of key residues in the cryo-EM structure of *Xenopus* KCNQ1, W265 lies in a crevice apparently accessible from the extracellular face by BHB and similar-sized molecules (Figure 4D). In support of this, we previously found that extracellularly-applied ^{3}H-GABA, which does not readily cross the plasma membrane, bound to *Xenopus* oocytes expressing KCNQ2-5, but not non-injected oocytes or those expressing KCNQ1, which lacks the W265 equivalent (Manville et al., 2018). To test the hypothesis that W265 mediates BHB activation of KCNQ3, we expressed mutant KCNQ2/3 channels with leucine substituted for the S5 tryptophan, and found that they were largely insensitive even up to 4 mM BHB, except for a small run-up at more positive voltages (Figure 4E). Thus, BHB did not negative-shift the $V_{0.5activation}$ of W236L-KCNQ2/W265L-KCNQ3 channels, even causing a positive shift at 4 mM, from -59.5 ± 0.5 to -55.7 ± 0.7 mV; n = 5, p=0.003 (Figure 4 F), and therefore did not increase current at the physiologically important subthreshold membrane potential of -60 mV (Figure 4G; Supplementary Figure 3; Supplementary Table 4).

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Partial KCNQ2/3 agonist GABOB diminishes BHB activation of KCNQ2/3

To further support the premise that BHB activates KCNQ2/3 channels by binding to KCNQ3-W265, we utilized a competition assay. We previously found that GABOB, a naturally-occurring GABA analog and lesser-known neurotransmitter, activates KCNQ2/3 channels with high affinity (EC_{50} = 0.12 µM at -60 mV) but lower efficacy than GABA or BHB. Thus, GABOB acts as a partial KCNQ2/3 agonist. *In silico* binding studies predict that GABOB also binds to KCNQ3-W265 and that it can access W265 from the extracellular side (Figure 5A, B). Accordingly, we previously found that GABOB competes out GABA and even the synthetic drug retigabine, which also binds to W265, reducing their efficacy for KCNQ2/3 activation (Manville et al., 2018). Here, we employed a similar assay and found that GABOB (100 µM) also greatly reduces the efficacy of BHB (10 µM), presumably by occupying the W265-associated binding site and disallowing BHB binding. Thus, in the presence of GABOB, BHB was unable to shift the KCNQ2/3 $V_{0.5activation}$ (-32.2 ± 1.1, control, versus -34.8 ± 1.0 mV, BHB + GABOB; *n* = 5, p=0.12) (Figure 5 C-G; Supplementary Table 5). In summary, electrophysiological, mutagenesis and GABOB competition assays support the hypothesis that BHB activates KCNQ2/3 channels by binding to KCNQ3-W265.

BHB anticonvulsant activity is diminished by GABOB

The capacity of KCNQ2/3 partial agonist GABOB to competitively inhibit BHB binding to KCNQ3 facilitated a mechanistic analysis of the anticonvulsant effects of BHB. We first tested the efficacy of BHB as an anticonvulsant using the pentylene tetrazole (PTZ) chemoconvulsant assay in mice, intraperitoneally (IP) injecting BHB 30 minutes before IP injection of 80 mg/kg PTZ, and then recording seizures and mortality in a blinded manner for the next 20 minutes. We found that BHB was highly effective at 200 mg/kg, and less so at 40 mg/kg, in increasing latency to first seizure (Figure 6A), and decreasing clonic (Figure 6B) and tonic (Figure 6C) seizures, as well as increasing survival (Figure 6D). Most strikingly, 200 mg/kg BHB

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completely prevented tonic seizures and seizure-related death, which each occurred in >50% of mice pretreated with vehicle instead of BHB (Figure 6C, D). Importantly, 200 mg/kg BHB corresponds to ~2 mM BHB, comparable with the 4 mM serum levels correlated with optimal ketogenic diet anticonvulsant activity in patients (Gilbert et al., 2000).

GABOB (200 mg/kg) was ineffective as an anticonvulsant, but also did not predispose to seizures, resulting in seizure and mortality incidence similar to those observed for vehicle controls (Figure 6A-D). These findings were consistent with previous observations that GABOB is a weak anticonvulsant (Chemello et al., 1980, Garcia-Flores and Farias, 1997). We hypothesized that if GABOB outcompeted BHB for the KCNQ3 binding site, as a partial agonist it would reduce the anticonvulsant efficacy of BHB, and this proved to be the case. Thus, GABOB (200 mg/kg) eliminated the anticonvulsant effects of BHB with respect to seizure latency, incidence, and mortality (Figure 6A-D). These findings, together with the cellular data, support the hypothesis that KCNQ2/3 channel activation is an important component of the anticonvulsant activity of BHB.

Discussion

The ketone bodies produced by fasting or a ketogenic diet are widely considered to be the principal components responsible for the anticonvulsant effects of these diets. In addition, ketone bodies including BHB may mediate other neuroprotective and anti-inflammatory effects. By inhibiting glycolytic flux upstream of pyruvate kinase, BHB competes with glucose as an energy provider in the brain (Valente-Silva et al., 2015, Boison, 2017). By shifting ketones such as BHB into oxidative metabolism, ketogenic diets also increase production of GABA (Zhang et al., 2015), itself an inhibitory neurotransmitter that we recently found activates KCNQ2/3 channels (Manville et al., 2018), in addition to its established role in activating canonical GABA receptors.

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Canonical pentameric $GABA_A$ receptors, which are triggered to open when GABA binds to an extracellularly located, inter-subunit GABA binding site, participate in either phasic or tonic inhibition, depending on their location and subunit composition (Kash et al., 2004, Belelli et al., 2009). Tonic extracellular GABA concentration in mammalian brain is calculated to be 0.16 µM (Santhakumar et al., 2006, Lee et al., 2010), in contrast to the astronomical but transient peak GABA concentrations of several millimolar thought to occur in the synaptic cleft during neurotransmission (Scimemi, 2014).

The GABA EC_{50} of 1 µM we previously found for KCNQ2/3 channels endows them with equivalent sensitivity to that of the most sensitive $\alpha_x\beta_3\gamma_2$ $GABA_A$Rs, which as a family exhibit a spectrum of sensitivities between 1-157 µM (Bohme et al., 2004, Karim et al., 2013). In contrast, while KCNQ3 exhibited a BHB EC_{50} of 1.4 µM in the current study, even at 5-20 mM BHB was ineffective/weakly effective as a $GABA_A$R agonist (Yang et al., 2007). Thus, the complex, intersubunit GABA binding site of $GABA_A$Rs provides much higher selectivity than the more permissive W265-based binding site of KCNQ3, which accepts GABA, BHB and GABOB and likely other GABA analogs and metabolites with low or sub-micromolar affinities, and also binds larger molecules such as the synthetic anticonvulsant, retigabine (Schenzer et al., 2005).

The reported ability of BHB to increase brain GABA levels could also point to a role for increased activity of canonical GABA receptors in the anticonvulsant action of the ketogenic diet due to downstream increases in GABA. However, our cellular electrophysiology and acute PTZ assay results support a hitherto unexpected, prominent and direct role for BHB itself in activating KCNQ3-containing potassium channels, such as KCNQ2/3 heteromers. KCNQ2/3 channels are widely expressed in vertebrate neurons, where they play a prominent role in controlling neuronal excitability. KCNQ2/3 channels are open at subthreshold potentials and do not

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inactivate, but when inhibited by activation of muscarinic acetylcholine receptors, their closure permits neuronal firing. Presynaptic KCNQ2/3 channels are thought to modulate glutamate and GABA release, and are suggested to act pre- and post-synaptically to suppress neuronal excitability (Martire et al., 2004, Peretz et al., 2007). KCNQ2/3 activation by BHB therefore presents an attractive, novel and plausible mechanism for the anticonvulsant effects of BHB.

We previously showed that GABOB is a partial agonist of KCNQ2/3 channels, with an E_{C50} of 120 nM (Manville et al., 2018). This is >5-fold more potent than BHB. Because GABOB has a much lower efficacy than BHB but higher potency, and each binds to the same residue, GABOB is able to prevent BHB binding at Trp265, but cannot activate KCNQ2/3 as effectively. We also previously showed that GABOB can reduce the efficacy of GABA and even retigabine with respect to KCNQ2/3 activation (Manville et al., 2018). In the present study, the lower dose of BHB we used *in vivo* was 40 mg/kg, which is equivalent to 400 µM; this dose did not reduce seizures, whereas the higher dose of 200 mg/kg BHB (2 mM) was effective against seizures (Figure 6). GABOB was not effective against seizures at 200 mg/kg (1.7 mM) (Figure 6). The lack of efficacy in preventing seizures, in the mouse model used herein, of partial agonist GABOB is consistent with our prior findings, which showed that at 1 mM, GABOB is less efficacious with respect to KCNQ2/3 activation than 1 µM BHB (Manville et al., 2018), a dose much lower than the lower dose 400 µM BHB that we found to be ineffective against seizures in the present study.

Vertebrate nervous systems contain many GABA analogs and metabolites, some of which, like GABA, also affect neuronal excitability, but unlike GABA do not modulate canonical GABARs and lack known targets. Our prior (Manville et al., 2018) and current studies suggest that W265 in human KCNQ3 evolved to act as a chemosensor for GABA and its analogs, providing an alternative mechanism for regulating neuronal excitability. We suggest that various neurotransmitters/metabolites including GABA, BHB, GABOB and others compete for binding to

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KCNQ3-W265 and that the resultant activation $V_{1/2}$ of KCNQ3 and KCNQ2/3 channels is set by the specific profile of these molecules that the KCNQ3 subunits expressed at the cell surface are exposed to at any given time. Thus, if GABOB were prevalent, the cell would be more excitable than if BHB were prevalent. As the affinity for KCNQ3 subunits of BHB is ~ten-fold lower than that of GABOB, there would need to be an excess of BHB to outcompete GABOB for binding. Hence, even though the KCNQ3 EC_{50} for BHB is ~1.4 μM and maximal effects are observed at 100 μM, to overcome baseline GABOB levels BHB might need to be at a higher concentration, such as would be achieved during fasting or when on a ketogenic diet.

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Authorship Contributions

Participated in research design: Abbott

Conducted experiments: Manville, Abbott and Papanikolaou

Contributed new reagents or analytical tools: Abbott

Performed data analysis: Abbott and Manville

Wrote or contributed to the writing of the manuscript: Abbott and Manville

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Footnotes

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Legends for Figures

Figure 1. BHB activates KCNQ2/3 potassium channels.

A. Schematic illustrating generation of BHB during ketosis. FFA, free fatty acids; LPL, lipoprotein lipase; *Red*, blood vessel; TCA, tricarboxylic acid cycle; TG, triglycerides.

B. Electrostatic surface potentials (red, electron-dense; blue, electron-poor; green, neutral) and structures calculated and plotted using Jmol.

C. TEVC of water-injected *Xenopus laevis* oocytes. Mean traces showing no effect of BHB (100 µM) on endogenous currents (n = 5). Dashed line here and throughout, zero current level. *Upper inset*, voltage protocol.

D. *Left*, mean I/V relationship from water-injected traces as in C (n = 5). *Right*, mean current fold change in response to BHB (100 µM) in water-injected oocytes (n = 5). Error bars indicate SEM.

E-J. TEVC of KCNQ2/3 expressed in *Xenopus* oocytes in the absence (control) or presence of BHB (n = 5). Dashed line here and throughout, zero current level. Voltage protocol as in C. Error bars indicate SEM. E, mean traces; F, mean tail current versus prepulse voltage (recorded here and throughout at arrow in panel E); G, normalized tail currents (G/Gmax; recorded here and throughout at arrow in panel E) versus prepulse voltage; H, voltage dependence of BHB augmentation of KCNQ2/3 activity; I, dose-dependent fold-increase in current at -60 mV in response to BHB; J, dose-dependent changes in resting membrane potential (E_M) in response to BHB.

Figure 2. BHB does not alter homomeric KCNQ2 activity.

TEVC of KCNQ2 expressed in *Xenopus* oocytes in the absence (control) or presence of BHB (100 µM or 1 mM) (n = 5). Error bars indicate SEM.

A. Mean traces (voltage protocol upper inset).

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B. Mean peak I/V relationships.

C. Mean tail current versus prepulse voltage.

D. Mean normalized tail current (G/Gmax) versus prepulse voltage.

Figure 3. BHB activates homomeric KCNQ3* potassium channels.

TEVC of KCNQ3* expressed in *Xenopus* oocytes in the absence (control) or presence of BHB (10 nM - 10 mM) (*n* = 6). Error bars indicate SEM.

A. Mean traces (voltage protocol upper inset).

B. Mean tail current versus prepulse voltage.

C. Mean normalized tail current (G/Gmax) versus prepulse voltage.

D. Dose response of [BHB] versus KCNQ3* current fold increase at -60 mV.

E. Voltage dependence of BHB effects on KCNQ3* activity.

Figure 4. BHB activation of KCNQ2/3 requires an S5 tryptophan.

A. Upper, chimeric KCNQ1/KCNQ3 structural model (*red pentagon*, KCNQ3-W265); lower, schematic showing membrane topology; domain coloring as above. VSD, voltage sensing domain; pentagon, KCNQ3-W265.

B. Close-up side view of KCNQ structure as in panel A, showing results of SwissDock docking of retigabine in the structural model.

C. Close-up side view of KCNQ structure as in panel A, showing results of SwissDock docking of BHB in the structural model.

D. Close-up view of KCNQ structure from extracellular face, showing results of SwissDock docking of BHB in the structural model.

E-G. TEVC of *Xenopus laevis* oocytes showing effects of BHB (100 µM – 4 mM) on heteromeric KCNQ2-W236L/KCNQ3-W265L channels; *n* = 6; voltage protocol as in Figure 3. Error bars indicate SEM. E, mean tail current versus prepulse voltage; F, mean normalized tail

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current (G/Gmax) versus prepulse voltage; G, Dose response of [BHB] versus KCNQ2-W236L/KCNQ3-W265L current fold increase at -60 mV.

Figure 5. GABOB diminishes BHB activation of KCNQ2/3.

A. GABOB electrostatic surface potential (red, electron-dense; blue, electron-poor; green, neutral) and structure, calculated and plotted using Jmol.

B. *Left*, side view of KCNQ1/3 chimera model structure, showing results of SwissDock docking of GABOB. Right, view of KCNQ1/3 chimera model structure from the extracellular face, showing results of SwissDock docking of GABOB.

C-G. TEVC of *Xenopus laevis* oocytes showing effects of BHB (10 µM) in the presence of GABOB (100 µM) on heteromeric KCNQ2/3 channels; *n* = 5. Error bars indicate SEM. C. Mean traces (voltage protocol upper inset); D, peak current; E, mean tail current versus prepulse voltage; F, fold-change in tail current versus prepulse for 10 µM BHB with versus without 100 µM GABOB; G, mean normalized tail current (G/Gmax) versus prepulse voltage.

Figure 6. GABOB diminishes anticonvulsant effects of BHB.

Comparison of anticonvulsant effects of vehicle versus 40 mg/kg or 200 mg/kg BHB, in the absence (*n* = 8) or presence (*n* = 7-11) of 200 mg/kg GABOB, IP-injected 30 minutes before IP injection of 80 mg/kg PTZ. Seizures were quantified during the first 20 minutes post-PTZ injection: A, latency to first tail flick or seizure; B, clonic seizure episodes; C, tonic seizure episodes; D, seizure-related mortality.

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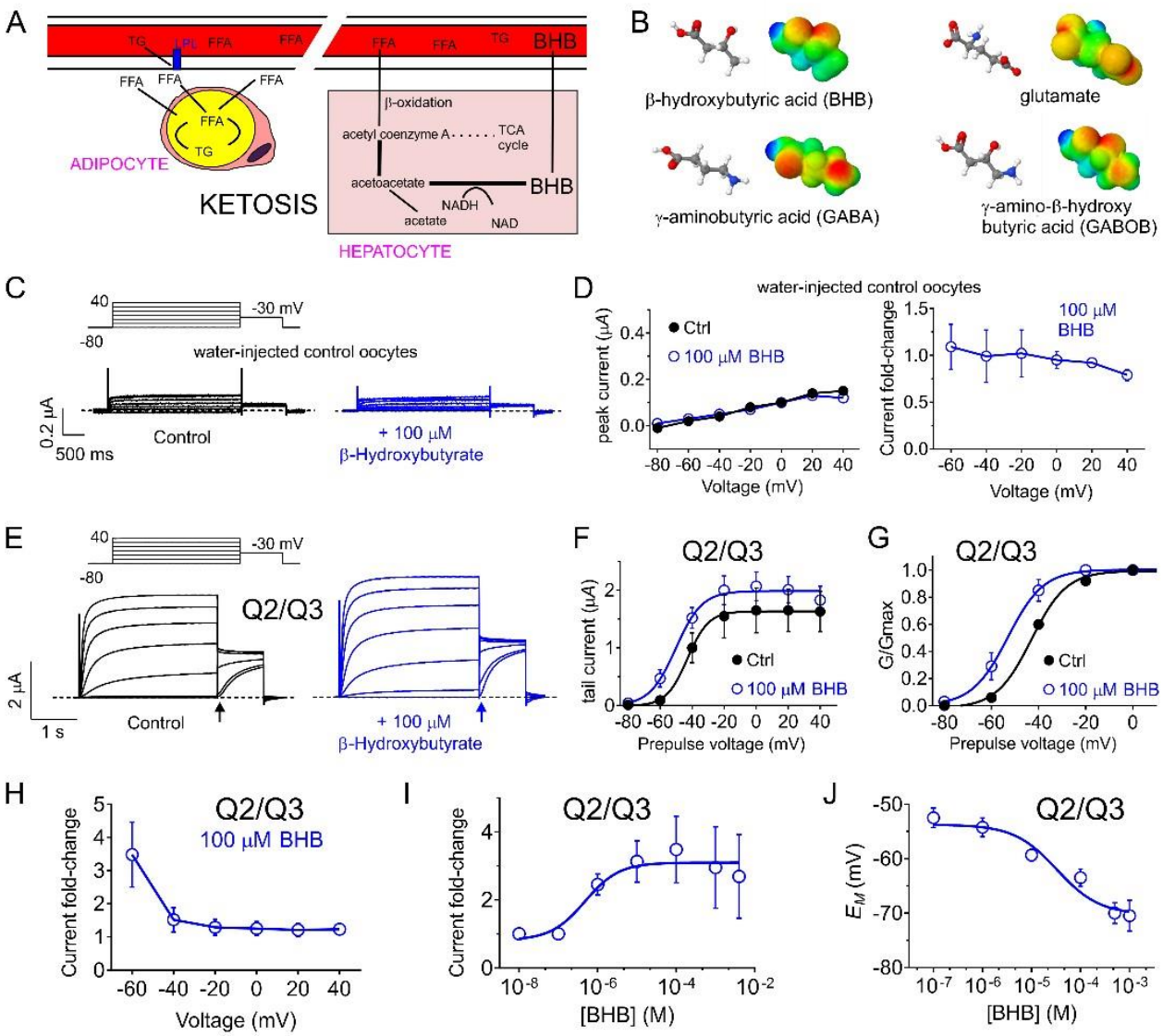
Figures

Figure 1.



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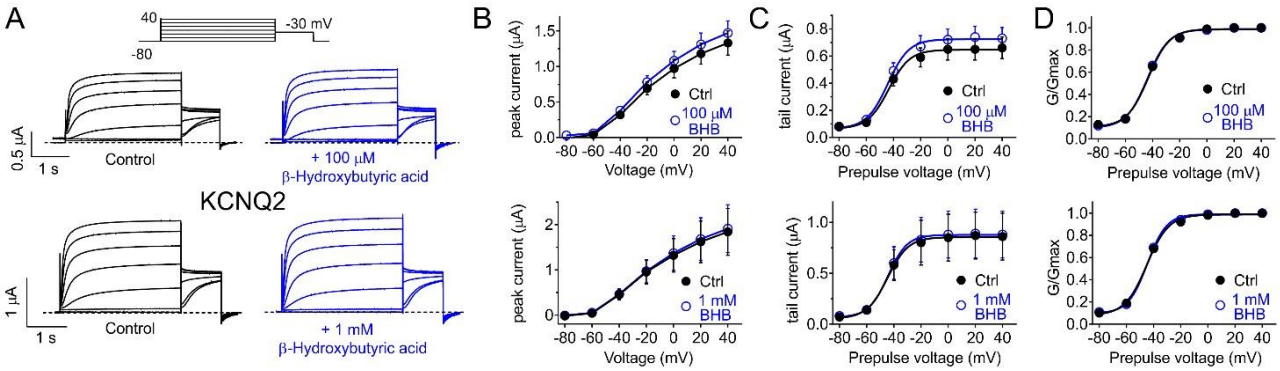

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Figure 3.

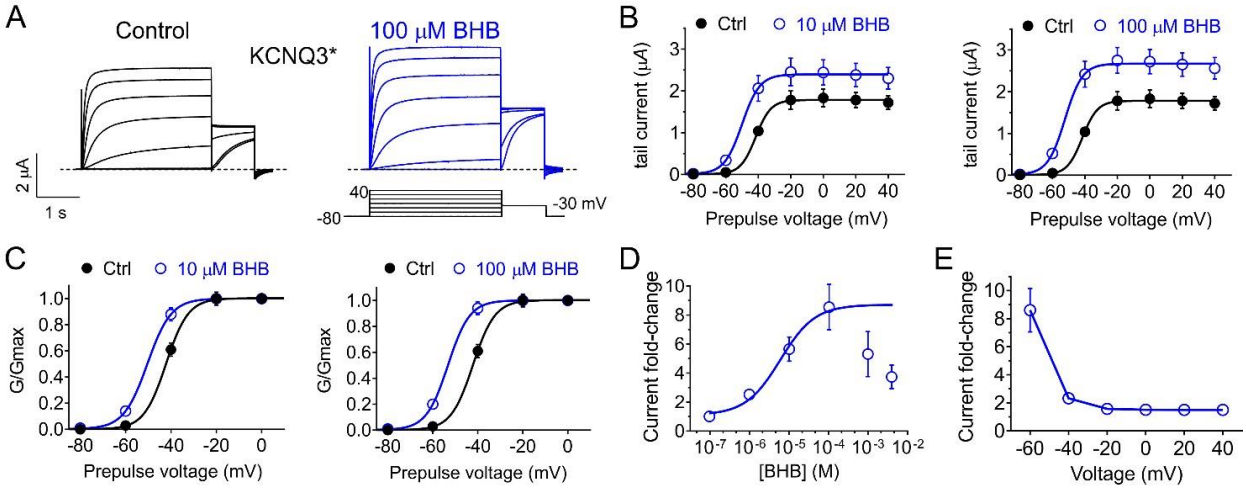

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Figure 4.

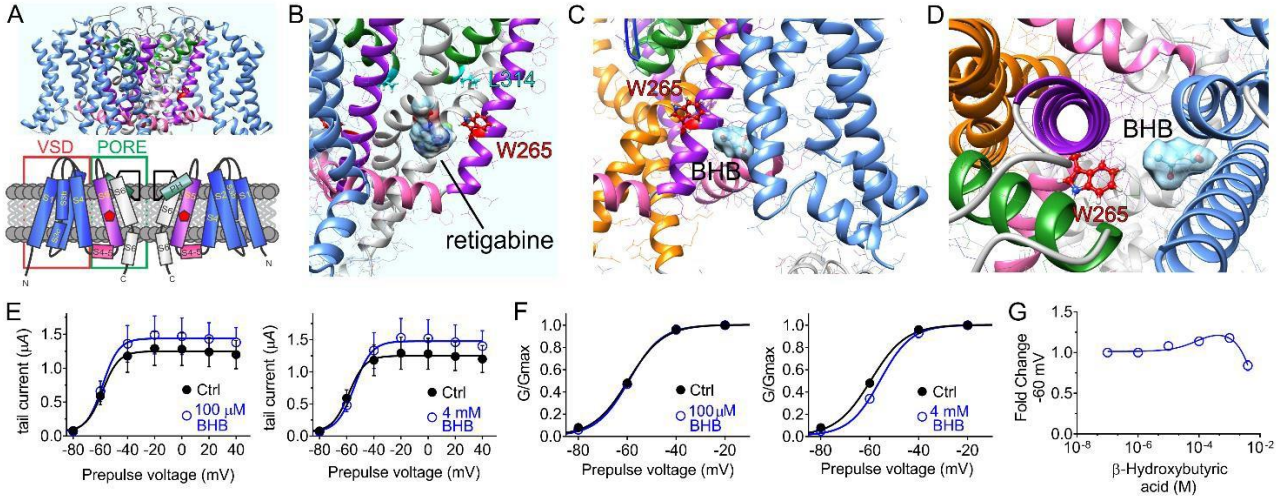

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Figure 5.

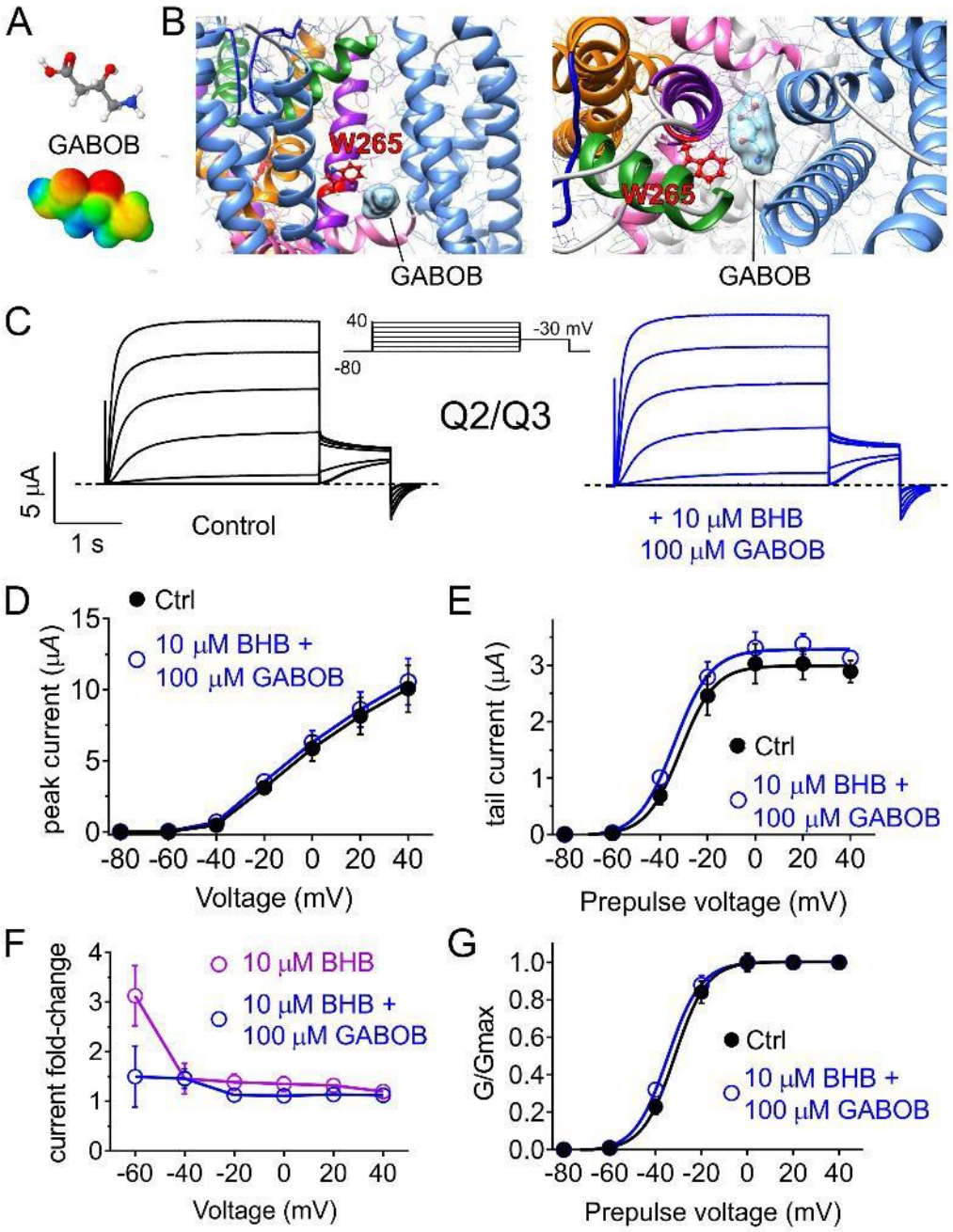

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Figure 6.