

Master's Thesis

Novel ketomimetic agents suppress epileptiform activity in Alzheimer's disease and Dravet syndrome mouse models

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Research conducted in Eric Verdin's laboratory,
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ABSTRACT

The ketogenic diet is a century-old validated therapy against epilepsy whose mechanism remains unknown. It is currently in clinical use mostly for intractable inherited epilepsies such as the Dravet syndrome. Meanwhile, emerging results from mouse models and small clinical studies show that epileptiform activity in Alzheimer's disease, which seems mechanistically-related to the Dravet syndrome, might be directly responsible for certain aspects of the cognitive and behavioural decline in this dementia. In a mouse model of Alzheimer's disease, feeding a ketogenic diet reduced epileptiform activity and improved learning. However, it cannot reasonably be widely implemented in elderly patients with dementia due to its side effects and limitations.

Here, we set out to test in mice a set of novel ketomimetic compounds with the eventual goal of delivering the main benefits of the ketogenic diet to patients with Alzheimer's disease while remaining on a normal diet.

First, compounds were demonstrated to be ketogenic in mice, both via 50 µL intraperitoneal injection and orally as a 10% w/w component of chow. The most promising compounds induced blood β-hydroxybutyrate levels around those normally generated by calorie restriction without any short-term adverse effects. Second, intraperitoneal injection of one of the compounds decreased by 40% epileptiform spikes in electroencephalograms of both Alzheimer's disease and Dravet syndrome mouse models.

This work may help to elucidate the mechanism by which the ketogenic diet acts against epilepsy, and, more importantly, suggests potential new therapeutics for both the Dravet syndrome and Alzheimer's disease that could help decrease abnormal network activity by mimicking the ketogenic diet's effects on epilepsy while on a normal diet.

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Abbreviations

AD	Alzheimer's disease
AED	Anti-epileptic drug
APP	Amyloid precursor protein
A β	Amyloid-beta
BD	1,3-butanediol
BL	Baseline
CI	Confidence interval
CPT1a	Carnitine palmitoyltransferase 1a
CR	Calorie restriction
DS	Dravet syndrome
EEG	Electroencephalogram(graphy)
FA	Food anticipation
FFAR3	Free-fatty acid receptor 3
FST	Fast(ing)
GPCR	G-protein coupled receptor
HCAR2	Hydroxycarboxylic acid receptor 2
HDAC	Histone deacetylase
HRP	Horseradish peroxidase
i.p.	Intraperitoneal(ly)
Kbhb	β -hydroxybutyrylation
KD	Ketogenic diet
MCFA	Medium-chain fatty acid
MCI	Mild cognitive impairment
MCT	Medium-chain triglycerides
NS	Non-significant
PDH	Pyruvate dehydrogenase
PTM	Post-translational modification
PV	Parvalbumin
VGSC	Voltage-gated sodium channel
w/w	Weight/weight

Literature review

Alzheimer's disease

Alzheimer's disease (AD) is a chronic neurodegenerative disorder. It is the most common cause of dementia in the elderly and may account to 60–70% of the cases¹. Today, 47.5 million people live with dementia worldwide².

At the cellular level, AD is primarily characterized by a progressive loss of cortical neurons, which control higher cognitive processes. It also causes synaptic dysfunction early in the disease. The neuronal damage in AD is linked to the deposition of abnormal proteins inside and outside of neurons. More precisely, there are two hallmarks of the disease: plaques and tangles².

“Senile plaques” are extracellular accumulation of insoluble amyloid-beta (Aβ)². Soluble Aβ is secreted by neurons via cleavage of a transmembrane glycoprotein, amyloid precursor protein (APP). Aβ’s normal functions are not well understood but probably include protection against microbial infection³. The regular form is continuously cleared from the brain. AD, on the other hand, involves abnormal cleavage of the APP, which leads to aggregation of Aβ in senile plaques².

“Neurofibrillary tangles” are intracellular aggregates of an abnormally hyperphosphorylated form of the cytoskeleton protein tau². Tau normally stabilizes microtubules. When hyperphosphorylated, it is unable to bind microtubules. As a result, they become unstable and disintegrate, and the unbound tau forms aggregates. Over time, these aggregates form filamentous tangles that may interfere with intracellular function⁴.

Epileptiform activity in AD

AD is associated with an increased incidence of seizures. At first, this was widely interpreted as a consequence of neurodegeneration and advancing age, but recent discoveries in mouse models of AD challenged this notion. Briefly, a strain of transgenic mice expressing human APP (hAPPJ20) was discovered to have epileptiform activity recognizable by the presence of spikes and sharp waves on electroencephalogram recordings (EEG)⁵. Although this mouse model shows behavioural and synaptic deficits consistent with AD, it does not show obvious neurodegeneration, suggesting that the etiology of epileptiform activity may be more specific to AD. This epileptiform activity on

EEG was subsequently confirmed in other transgenic mouse models of AD, including hAPPJ9/FYN (unpublished data), Tg2576⁶ and hAPP/PS1⁷. These results suggest that high levels of A β are sufficient to evoke epileptiform activity, even in the absence of neuronal loss⁵. Surprisingly, epileptiform activity may actually be triggered directly by APP overexpression, and not A β overproduction⁸. In any event, epileptiform activity triggers a variety of compensatory responses in hippocampal circuits to balance the aberrant increase in network activity. However, these interfere with normal neuronal and synaptic functions required for learning and memory. In this way, epileptiform activity in AD, triggered by APP or A β , may actually be a cause of cognitive decline, rather than a simple consequence of the neurodegeneration⁵.

In sporadic AD patients, just as in hAPP mice, convulsive seizures are rather rare, but still significantly more frequent—about 6–10-fold—compared to control individuals⁹. Most of the seizures found by rigorous EEG monitoring of AD patients are actually non-convulsive and difficult to detect in routine clinical practice¹⁰. Among the existing data, a relatively small study found that AD patients with epilepsy developed cognitive decline 5.5 years earlier than those without epilepsy, and more than half (55%) of these epilepsies were non-convulsive¹⁰. More practically, the cognitive functions of AD patients vary from time to time within a same day, which cannot be explained only by progressive neuronal loss. Alternatively, amnestic wandering, disorientation and intermittent inability to retrieve memories may be caused by aberrant neuronal network activity⁵. In a brief report, two AD patients who displayed amnestic wandering and disorientation were shown to have epileptiform activity on EEG. Consequently, treatment with anti-epileptic drugs (AEDs) lead to complete resolution of their episodic behavioural changes. Although not a definitive proof, it strongly suggests that the amnestic episodes were epileptic in origin. This finding, if confirmed on a larger scale, is of particular interest because it is easily treatable with AEDs, with the potential to improve the quality of life of patients¹¹.

Several mechanisms have been proposed to link A β or APP and epilepsy. In one of the most plausible, APP mice have been shown to have a deficit in the expression of the voltage-gated sodium channel Nav 1.1, whose α subunit is encoded by the gene SCN1A¹². Nav 1.1's expression and localization seem to be regulated by β -secretase, which catalyses the first cleavage of APP in the amyloidogenic pathway^{12,13}. More specifically, the deficit in Nav 1.1 is primarily found in a population of fast-spiking GABAergic interneurons expressing parvalbumin, preventing them from correctly firing action potentials¹⁴. In this model, known as the interneuron hypothesis, seizures are explained by a lack of inhibition of the principal cells which are innervated by the parvalbumin-expressing interneurons, which release insufficient GABA¹⁵.

Dravet syndrome

To explain epileptiform activity in AD, the interneuron hypothesis is the model that received the most attention so far. This is because mutation of *SCN1A* also causes two pediatric epilepsy syndromes: generalized epilepsy with febrile seizure plus (GEFS+) and severe myoclonic epilepsy of infancy (SMEI). Incidentally, in APP mice as well as patients with GEFS+ or SMEI, anti-epileptic drugs that block sodium channels make seizures more frequent rather than less, which tends to confirm that deficit in Nav 1.1 is indeed the common cause for these three epileptic conditions¹².

SMEI, also called the Dravet syndrome (DS), is a rare and intractable form of epilepsy caused by a loss-of-function mutation in *SCN1A*, leading to hypoinsufficiency of the voltage-gated sodium channel Nav 1.1¹⁵. Seizures start in the first year of life and continue life-long. Children with DS have poor language development, impaired motor skills, hyperactivity and mental retardation. They have a poor prognosis and a higher incidence of SUDEP (Sudden Unexplained Death in Epilepsy). Seizures are difficult to control but can be decreased by anti-convulsant drugs¹⁶. The ketogenic diet can also be beneficial, leading to a greater than 50% reduction in seizure frequency in around 65% of the patients^{16,17}.

The ketogenic diet

Historically, the first known treatment for epilepsy was fasting. This therapy was first mentioned in the Hippocrates Corpus around the 5th century BC¹⁸. Five hundred years later, fasting was also documented in the Bible, when Matthew relates the story of Jesus curing an epileptic boy¹⁹. Modern use of starvation against epilepsy were first recorded in the early 20th century in France and USA¹⁸.

With a goal of mimicking the metabolic effects of fasting and being able to maintain the treatment for a longer time, Dr. Wilder at the Mayo Clinic invented in 1921 the “ketogenic diet”. In the ketogenic diet (KD), all calories come from fat and proteins, with no or very low quantities of carbohydrates. Numerous variations of Wilder’s first attempt have now been formulated, but the basic principle stayed unchanged. KD is called so because its main consequence is to induce mild ketosis, which is an elevation in the levels of blood ketone bodies. The KD was widely used as a treatment for epilepsy throughout the 1920s and 1930s until the first AED was discovered in 1938. Subsequently, and with the development of new generations of AEDs, the use of the KD dropped and almost completely disappeared. This greatly changed in 1994 when the KD suddenly received media and public attention after a documentary aired on US television telling the true story of a

young child with intractable seizures that were successfully treated with the KD¹⁸. Soon after, the first multicentre prospective study on its efficacy was published²⁰ and really sparked the resurgence of the KD in scientific literature and clinical use (Fig. 1). It is now prescribed by numerous institutions all over the world, generally to children with drug resistant epilepsy¹⁸. Finally, it should be noted that the KD could be helpful in a myriad of conditions other than epilepsy, including obesity; cancer; insulin-resistant states such as type 2 diabetes; Parkinson's disease, autism and other neurological disorders; and in the context of different genetic disorders, such as deficiency in pyruvate dehydrogenase (PDH) and Friedreich's ataxia^{21,22,23}. The KD is also used or could potentially be used in non-pathogenic contexts, such as to lose weight, enhance cognitive or physical performances²⁴, and possibly, slow the effects of aging.

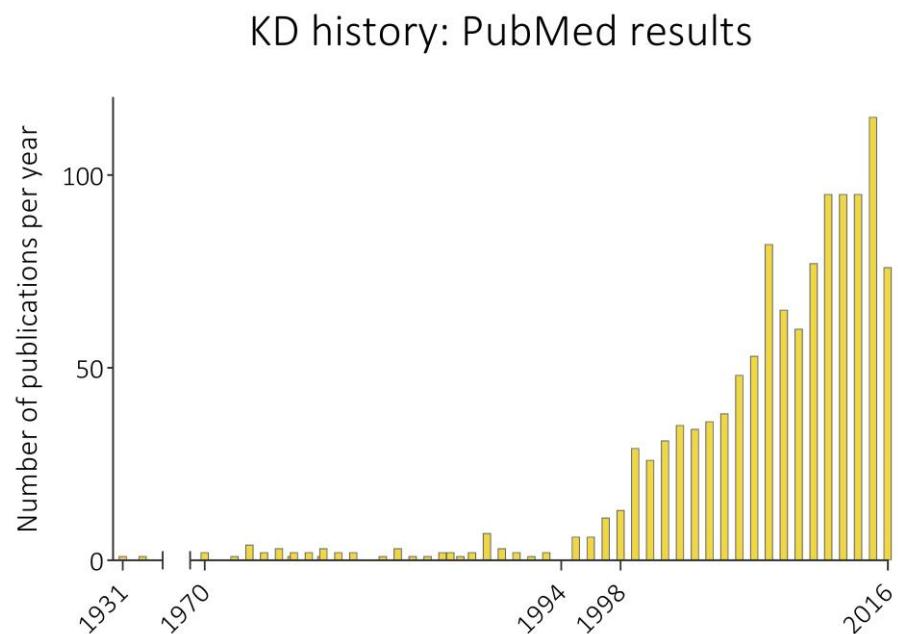


Fig. 1 | History of the KD as told by the number of publications on the topic per year. In 1994 was the diffusion of a documentary on the subject; in 1998 was published the first modern study on its efficacy against epilepsy. Data are from PubMed.gov; search performed was *ketogenic diet epilepsy*.

The efficacy of the KD against epilepsy has been assessed in multiple randomized controlled trials. A systematic review of the clinical trials conducted after 1990 concluded that an average of 15.6% (95% confidence interval [CI] 10.4-20.8%) of the patients became seizure-free 6 months after commencing the diet, and 33.0% (95% CI 24.3-41.8%) achieved greater than 50% reduction in seizure frequency²⁵.

Traditionally, the KD is perceived by physicians as a last resort solution when AEDs do not help in decreasing seizures. In this regard, no clinical factors have been identified that predict which patients may benefit the most. Nonetheless, it is now considered a first-line therapy for a few specific epilepsy syndromes, including epilepsy due to mutation in the blood-brain barrier glucose transporter GLUT1, epilepsy due to pyruvate dehydrogenase (PDH) deficiency, infantile spasms, myoclonic-astatic epilepsy (Doose syndrome), and the Dravet syndrome²³.

To date, no thorough clinical trial of the KD on AD patients has ever been conducted, but some preliminary results exist. For example, medium-chain triglycerides (MCT), which are generally added to the formulation of the KD to make it more ketogenic and in consequence less restrictive, improved cognitive performances of some patients with AD or mild cognitive impairment (MCI)²⁶. Very low carbohydrate diet, which is the main characteristic of the KD, also improved verbal memory performance in MCI patients²⁷. More importantly, both studies found that memory performance was correlated with ketone bodies concentration in plasma. Data from *in vitro* and *in vivo* animal models of AD are more abundant.

In vitro, addition of 4mM BHB on hippocampal cells, whose loss is causing memory deficit in AD patients, protected them from Aβ toxicity²⁸. *In vivo*, three mouse models of AD have been tested on the KD so far: APP/PS1 and APP/V7171, which are two models of amyloid deposition, and Tg4510, which is a model of tau deposition. In APP/PS1 and Tg4510 mice, the KD improved motor function on the rotarod test, but did not significantly affect grip strength and latency to fall in the wire suspension test. Furthermore, it did not improve spatial learning in the radial arm water maze and had no effect on tau and Aβ deposition^{29,30}. In APP/V7171 mice, the KD reduced total brain Aβ by around 25% but did not affect memory performance on an object recognition task³¹. These results may be underestimated because of the age of the mice. Indeed, these studies used young mice, aged 1–5 months. Yet, some metabolic alterations, such as insulin resistance and impaired glucose utilization, are improved by the KD, but mainly appear in the elderly³².

Despite being in use for centuries, the mechanism of action of fasting or the KD against epilepsy is mostly unknown. The KD has many potential effects, and numerous hypotheses have been formulated³³. The fact that the KD is effective in mechanistically different epilepsy syndromes suggests that there may not be a “one mechanism fits all” answer²³.

Overall, the ketogenic diet is a validated, cost-effective therapy that would probably benefit many patients if it was more commonly used. Nevertheless, it is far from flawless.

The KD has various limitations. First, it induces a significant increase in plasma levels of total cholesterol, low-density lipoproteins (LDL), very-low density lipoproteins (VLDL), non-HDL cholesterol, triglycerides and total apolipoprotein B (apoB), all of which are considered atherogenic (i.e., increasing the risk of atherosclerosis), whereas levels of high-density lipoproteins (HDL), considered antiatherogenic, significantly decrease³⁴. Other very common (reported by 25–50% of patients on KD), early-onset complications include dehydration, gastrointestinal disturbances, such as diarrhoea, nausea/vomiting and constipation, and hyperucinemia, which frequently leads to nephrolithiasis, also called kidney stones. Osteopenia is also regularly observed as a late-onset complication³⁵.

More practically, the KD is usually considered unpalatable and restrictive. As a consequence, it is hard to observe for a prolonged time. Yet, any transgression from the diet has the potential to abolish ketosis and resume the seizures³⁶. This issue might be less critical for adults but is especially relevant when the patients are children or elderly with dementia. For AD patients, adherence to a low-carbohydrate diet is also complicated by the other medications that the patient receives, which regularly use dextrose (D-glucose) as a solvent. The KD also induces weight loss. This is favourable as a potential treatment against obesity³⁷, but unintentional weight loss is already a serious concern in the elderly³⁸.

Overall, of all the patients starting the diet, an average of 20.1% (95% CI 12.5–27.7%) will have discontinued it after 3 months, 39.4% (95% CI 29.1–49.7%) after 6 months, and 65% (95% CI 51.6–78.4%) after a year. The most reported reasons are lack of efficacy, lack of compliance, and health complications²⁵.

Ketone bodies

Ketone bodies are small lipid-derived molecules produced by the liver when glucose is not readily available, typically during prolonged strenuous exercise or dietary interventions, such as fasting, calorie restriction (CR), and the ketogenic diet (KD). They are produced from fatty acids stored in

the adipose tissue and serve as a circulating energy source for metabolically active tissues, especially the brain and skeletal muscles^{39,40}. There are three ketone bodies: acetoacetate (AcAc), β -hydroxybutyrate (BHB), and acetone³⁹ (Fig. 2).

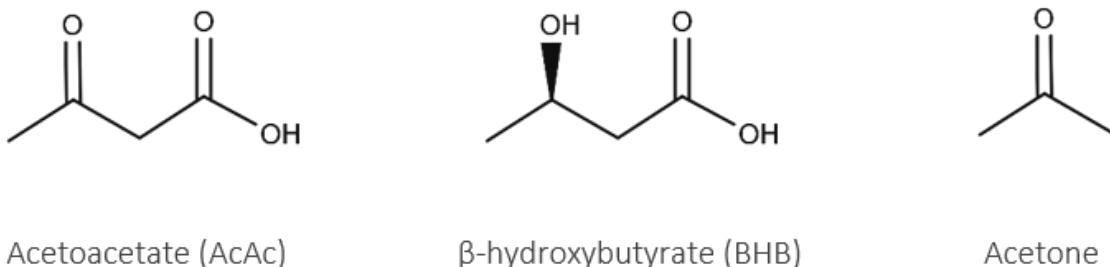


Fig. 2 | Structures of the three endogenous ketone bodies.

BHB is the most abundant ketone body produced during fasting, with plasma levels rising two- to threefold higher than the other ketone bodies⁴¹. It may also have the most interesting range of biological effects.

In humans, BHB plasma concentrations in the fed-state are usually as low as less than 100 μM ⁴². They rise to 400 μM or higher after an overnight (12 hours) fast, then to 2000–3000 μM after 2 days starvation^{41,43}. The plasma level of a 2-day fast is also reached after 90 minutes of strenuous exercise⁴². A prolonged fast of 10 days or more leads to BHB levels of 4000–8000 μM ⁴¹. In children, the KD induces consistent BHB levels of 3000–7000 μM ⁴⁴. CR, usually defined as a reduction of 30–45% calorie intake without malnutrition, should also raise BHB levels above baseline, perhaps as high as 500–1000 μM ⁴⁵. Young children more efficiently produce and utilize BHB; newborns' BHB levels are consistently 2000–3000 μM ⁴¹.

Ketoacidosis, which should be carefully distinguished from the non-pathogenic mild ketosis induced by the KD, is a life-threatening complication of untreated type 1 diabetes mellitus. It is caused by an aberrant, unregulated production of ketone bodies in the absence of any functional insulin⁴⁶. Plasma levels of BHB can reach values in excess of 25,000 μM during ketoacidosis⁴⁷, which can exceed the blood's buffering potential and result in low blood pH. Due to the absence of functional insulin, ketoacidosis usually presents together with life-threatening hyperosmolar hyperglycaemia⁴⁶.

β -hydroxybutyrate

BHB has a broad range of functions and effects, many of which are only starting to be studied now. Fundamentally, they can be clustered into two identities: a fuel and a signalling molecule.

BHB as a fuel

BHB, together with the other ketone bodies, provide a circulating source of energy to the tissues⁴¹. Historically, this was also their first recognized function.

Catabolism of BHB differs in several important ways from that of glucose. Succinctly, BHB produces more ATPs per unit than glucose with less oxygen consumption and the mitochondrial electron transport chain seems to produce fewer ROSs when on ketone bodies^{21,48}. Moreover, BHB do not rely on glucose transporters to be transported into tissues²¹. This is clinically relevant because defects in glucose transporters are frequently associated with pathological conditions. For instance, deficiency in GLUT1 causes a catastrophic neurological disorder in children that includes seizures⁴⁹, and AD patients have reduced GLUT1 and GLUT3 expressions in the cerebral cortex⁵⁰. Furthermore, BHB in the tissues is rapidly converted into acetyl-CoA, which can directly enter the citric acid cycle. By doing so, pyruvate dehydrogenase (PDH), which converts pyruvate into acetyl-CoA when glucose is the energy source, is by-passed. Again, this is relevant in both epilepsy and AD. First, pyruvate dehydrogenase complex deficiency (PDCD) leads to seizures, which may be reduced by the KD⁵¹, and second, Aβ disrupts glucose metabolism in the brain by directly inhibiting several mitochondrial enzymes, including PDH⁵².

BHB as a signalling molecule

While BHB's function as a circulating energy substrate has long been known, several signalling functions were recently discovered and may be equally important for BHB's biological functions.

Cell-surface receptors

BHB is a ligand for at least two GPCRs that also bind short-chain fatty acids: hydroxycarboxylic acid receptor 2 (HCAR2, also known as PUMA-G or GPR109) and the free fatty acid receptor 3 (FFAR3, also known as GPR41).

HCAR2 is a $G_{\alpha i}$ -coupled GPCR that was first identified as a nicotinic acid receptor⁵³. HCAR2 activation by BHB reduces lipolysis in adipocytes by inhibiting the hormone-sensitive triglyceride lipase. This probably constitutes a negative feedback mechanism to reduce the availability of fatty acids, from which ketone bodies are generated. By doing so, BHB negatively regulates its own production, preventing ketoacidosis and ensuring an efficient use of fat reserves⁵⁴.

FFAR3 is also a $G_{\alpha i}$ -coupled GPCR, mainly found in sympathetic ganglia. BHB is an antagonist of FFAR3. In mice, its binding thus suppresses sympathetic activity and, in turn, the overall metabolic rate⁵⁵.

Post-translational modifications and gene expression

HDACs INHIBITION

BHB directly inhibits several histone deacetylases (HDACs)⁵⁶. HDACs are a class of proteins that remove acetyl groups from lysine residues of histone and non-histone proteins. When the substrate is a histone, deacetylation is generally associated with repression of gene expression⁵⁷.

More precisely, *in vitro* BHB inhibits at least HDAC1, HDAC3 (Class I) and HDAC4 (Class IIa) with an IC₅₀ of 2–5 mM⁵⁶. Based on the high level of homology of histones within Class I and Class IIa, it would be safe to extrapolate from these results that BHB inhibits all members of these two classes⁵⁷. BHB also increased in a dose-dependent manner histone acetylation in HEK293 cells and in tissues of mice infused with BHB or fasted for 24 hours, presumably by inhibiting the HDACs⁵⁶.

In other words, BHB, by inhibiting Class I and Class IIa HDACs, promotes histone acetylation, which tends to boost gene expression.

LYSINE β-HYDROXYBUTYRYLATION

BHB is also, by itself, a protein post-translational modification (PTM) of the lysine ε-amino group, called lysine β-hydroxybutyrylation (Kbhb). As expected, starvation increases histone Kbhb in mice. Results also suggest that it is actually a widespread histone PTM, with 38 Kbhb sites in human HEK293 cells and 26 in mouse liver, including H3K9 (histone 3 lysine 9). H3K9bhb was associated with active expression of genes involved in a series of starvation-responsive metabolic pathways, namely the spliceosome, selenoamino acid metabolism, PPAR signalling pathway, fatty acid metabolism, and the proteasome⁵⁸.

Inhibition of the NLRP3 inflammasome

BHB also modulates the innate immune system. Indeed, it specifically inhibits activation of the NLRP3 inflammasome, which might be one mechanism underlying the anti-inflammatory effects of the KD and CR. Notably, activation of the NLRP3 inflammasome was shown to contribute to the pathology of type 2 diabetes, atherosclerosis, multiple sclerosis, AD, age-related functional decline, bone loss, and gout⁵⁹.

Food anticipation and appetite regulation

BHB is necessary for food anticipation (FA). FA is a set of biological changes controlled by the peripheral circadian rhythm and which precede recurring food availability. Namely, FA is characterized by a boost in activity, a rise in body temperature and an increase in corticosteroids 1–3 hours before meal time⁶⁰.

Regarding appetite regulation, direct administration of BHB reduces voluntary food intake in rats, and the KD is often reported by patients to reduce hunger^{61,62}. However, the underlying molecular mechanism is mostly uncertain and probably complex, involving numerous other factors, such as ghrelin, adiponectin, glutamate and GABA. Mechanistically, BHB is both orexigenic (i.e., increasing appetite) and anorexigenic (i.e., suppressing appetite). The net outcome of these conflicting stimuli is supposed to be a perceived reduction in hunger⁶².

Overall, BHB is undoubtedly an essential signalling molecule, having a broad range of effects, sometimes contradictory, on metabolism, epigenetics, innate immune system, and behaviour. This is highly unlikely to be an exhaustive summary of the signalling functions of BHB, and many more will presumably be discovered in the near future.

Exogenous ketones

In summary of the previous sections, the KD has various and appealing therapeutic potentials and is beneficial in several conditions, including epilepsy. Mechanistically, many of the potentials can convincingly be traced back to ketone bodies and BHB in particular. However, as briefly explained above, the KD is far from being the ideal solution to increase blood levels of BHB. As a result, there has been several attempts at safely providing exogenous ketones to patients in the hope of exempting them from relying upon the KD.

Medium-chain triglycerides

Medium-chain triglycerides (MCTs), which are naturally present in coconut oil, are often added to the formulation of the KD to make it more ketogenic²⁶.

During endogenous ketogenesis, the liver mostly utilizes long-chain fatty acids as ketone body precursors. After activation in the cytoplasm (i.e., addition of a CoA), they are transported through the outer mitochondrial membrane to the intermembrane space by the carnitine palmitoyltransferase (CPT) 1a. However, CPT1a gene expression is regulated by the dietary state in such a way that it is repressed when glucose is available. Therefore, supplying long-chain fatty acids to a patient on a normal diet would not work to raise ketosis, unless glucose is also restricted (as in the KD). Short- and medium-chain fatty acids (MCFA), on the other hand, can enter the mitochondria by simple diffusion and, thus, can be more readily utilized by the hepatocytes as ketone bodies precursors even with abundant glucose⁶³.

MCTs alone, even without KD, are sufficient to raise ketosis over baseline and give physiological benefits. Supplementation with MCTs in aged dogs, which are a natural model of amyloid

aggregation, lead to a dramatic improve in mitochondrial function and reductions in APP and A β levels, mostly in the parietal lobe⁶⁴. Several small clinical studies also yielded promising results in patients with AD or MCI. Supplementation with MCTs induced a transient mild ketosis of 200–800 μ M plasma BHB. Cognitive functions were generally improved and correlated with BHB levels^{26,65,66,67}. Results of MCT supplementation alone for epilepsy do not exist or are very scarce⁶⁸, and no data exist about MCT supplementation in the context of epileptiform activity in AD.

1,3-butanediol

BHB is an organic acid. Therefore, if significant quantities of exogenous BHB were fed or infused in pure form, it would necessarily generate a threatening acidosis. In the salt form (Na-BHB) sometimes used in studies, it would generate a similarly dangerous salt load.

A first approach to tackle this problem is to use 1,3-butanediol (BD) instead. BD is an alcohol that is converted into BHB in the liver by enzymes involved in the alcohol metabolism, namely the alcohol and aldehyde dehydrogenases⁶⁹.

BD is generally considered safe, even when fed or infused in large quantities to different animal models and humans. The literature is quite ample on the subject, although not recent⁷⁰. Nevertheless, preliminary experiments in C57BL/6J mice found some long-term liver toxicity when mice were fed BD at the doses required to induce high ketosis. As it was never reported, the effect might be specific to this mouse strain (John Newman, unpublished data).

Esters

Another approach is to combine two ketone body molecules via an ester bond. This neutralizes the acidity of the resulting molecule⁷¹. When administered, the ketone ester is then cleaved into ketone body molecules by the esterases in the blood and the gut⁷².

Ketone esters have already been synthesized between BHB and itself, BHB and BD (BHB-BD), BHB and glycerol, and BD and AcAc, the latter in mono- and diester (BD-AcAc). These compounds were tested in various animal models, and some of them in humans⁷³. BD-BHB was fed to an AD mouse model (3xTgAD) and resulted in a significant decrease in anxiety and increase in performance on learning and memory tests. Brain immunochemistry also revealed reduced levels of plaques and tangles⁷⁴. A case study was also published of an AD patient receiving BHB-BD orally. Results were reported improvements in mood, affect, self-care, cognitive and daily activity performance⁷⁵. Finally, BD-AcAc diester was tested in two models of artificially-induced seizures in rats, with successful results^{76,77}.

Aim of the study

Although a link between AD and epilepsy exists and is well known since a relatively long time, the importance of this phenomenon might have been underestimated. Epileptiform activity may indeed be responsible for aspects of the cognitive deficits and transient behavioural changes observed in AD patients.

The efficacy of the KD against epilepsy has been extensively demonstrated, including in patients with the Dravet syndrome (DS). This specific epileptic condition is particularly interesting because it may share its mechanism with the epileptiform activity in AD.

Nevertheless, the effectiveness of the KD on epileptiform activity in AD had never been assessed. Prior to this study, we thus conducted a preliminary experiment of the KD on the hAPPJ20 mouse model. Mice underwent a 24-hour baseline EEG recording, then were fed a KD for two days before a second 24-hour EEG recording session. The results were a significant reduction in the number of epileptiform spikes (Fig. 3A), which were around 30% less frequent than when the APP mice were fed normal chow. Furthermore, this translated into an amelioration of cognitive functions (Fig. 3B and Fig. 3C) (John Newman, unpublished data).

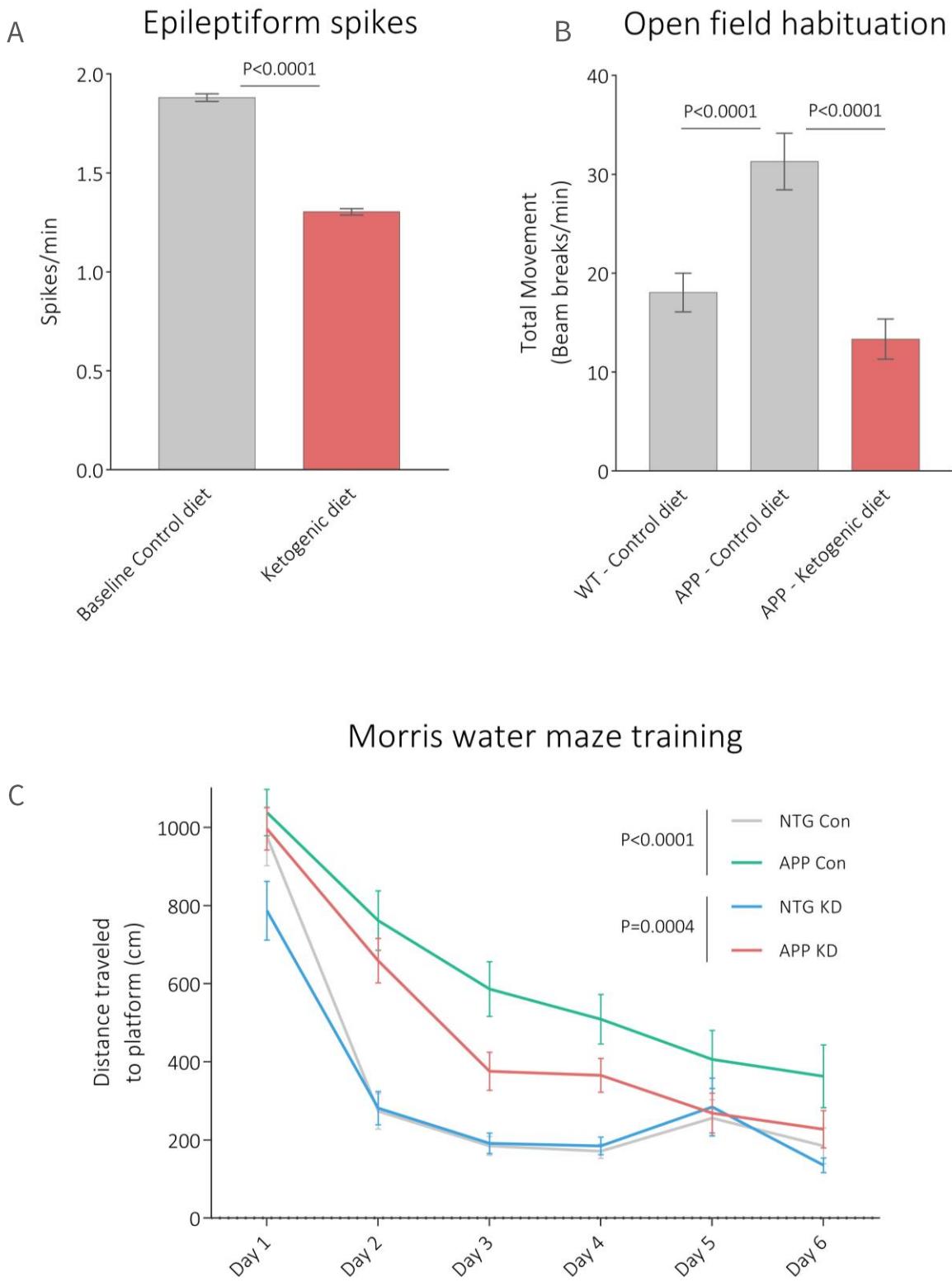


Fig. 3 | In hAPPJ20 mice, the KD significantly reduces epileptiform spikes and improves cognitive functions. **(A)** The KD reduced the number of epileptiform spikes in APP mice by 30%. N = 12–13 mice. **(B)** APP mice on the KD moved as little as WT mice on control diet in the open field. Less movement suggests that the mouse is already habituated to the open field from previous exposures. It thus assesses long-term memory. N = 7–12 mice depending on the group. **(C)** The KD improved performance in the Morris water maze, which evaluates spatial learning and memory. N = 22–56 mice depending on the day and the group. For all three graphs, mice had been on the specified diet for about 3 months prior to testing (John Newman, unpublished data).

We developed a set of novel compounds that have the potential to mimic the effects of KD while on a normal diet. These compounds might be able to deliver some of the therapeutic benefits while avoiding some of the limitations and side effects of the special ketogenic diet. The small-scale but promising results with MCTs or ketone ester supplementation against epilepsy and AD suggest that this approach may have merit, although none of these prior studies tested effects on epileptiform activity in AD models.

These compounds are comprised of a MCFA (C6 or C8), either combined with itself or with a molecule of BD or BHB. These compounds represent a novel solution to the acid or salt problems of delivering BHB in physiologically relevant quantities. It is the first known attempt at combining MCFAs and BHB or BD by an ester bond. Ideally, this should couple the indirect effects of MCFAs on ketogenesis with the direct effects of ketone body molecules on plasma BHB levels to generate both rapid and sustained elevated blood levels of BHB.

We first set out to test the efficacy of these compounds in elevating blood BHB levels when either injected or fed as a component of normal chow. The goal was to identify the compounds that generated the most substantial increases in blood BHB levels without major side effects. We then sought to test whether one of the compounds could, by itself, replicate the suppression of epileptiform activity in hAPPJ20 and DS mouse models that we had seen previously from ketogenic diet. We hope that this work could both help advance the mechanistic understanding of how abnormal epileptiform activity in a mouse model of AD and patients with DS is controlled by the KD; and suggest potential new therapeutics for these two conditions that target the epileptiform activity by mimicking specific aspects of the KD while remaining on a normal diet.

Materials and Methods

Novel ketomimetic agents

Seven novel ketone esters have been conceived and synthesized in collaboration with Scott Ulrich of Ithaca College (Ithaca, New York, USA)

The nomenclature of the compounds is as follows: C6 or C8 (based on the length of the fatty acid unit) with or without BD (1,3-butanediol) or BHB (β -hydroxybutyrate). If the compound is a diester (comprised of two fatty acid units), x2 is added after C6 or C8. For example, C8x2-BD is a diester composed of two C8 fatty acids ester-linked to a molecule of 1,3-butanediol in the middle. All compounds were synthesized using R-enantiomer BHB or BD, and they retain this chirality in the ester form.

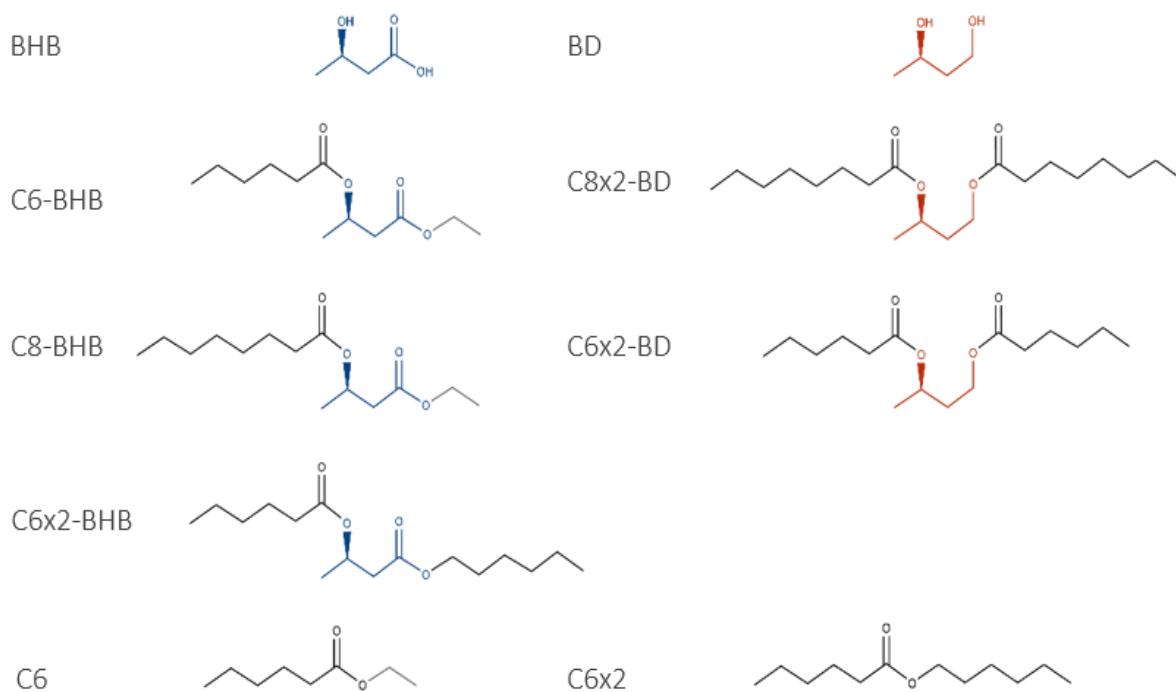


Fig. 4 | Structures of the novel compounds. BHB and BD were added for reference, but are not included in the seven compounds.

Mice

Mice were maintained in a barrier facility on a 7.00 am to 7.00 pm light cycle and had free access to water. Except if stated otherwise, they were housed in littermate groups of up to 5 mice per cage, and fed ad libitum a standard chow diet (5053 PicoLab diet, Ralston Purina Company, St. Louis, MO).

Ethics statement

All mice were maintained according to the National Institutes of Health guidelines, and all experimental protocols were approved by the University of California San Francisco (UCSF) Institutional Animal Care and Use Committee (IACUC). UCSF is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

C57BL/6J mice

Apart from studies on specific mouse models, all experiments used C57BL/6J wild type male mice which were 13–20 months old at the time of the experiment. They were obtained at 11–12 months old from the Aged Rodent Colony maintained by the National Institute on Aging.

Dravet mice

Dravet mice all carry a heterozygous deletion of the *Scn1a* gene, and are thus of *Scn1a* ^{-/+} genotype, on a mixed C3HeB/FeJ X C57BL/6J background⁷⁸. They were originally obtained from M. H. Meisler (Department of Human Genetics, University of Michigan), and this specific line was originally generated by K. Yamakawa (Laboratory for Neurogenetics, RIKEN Brain Science Institute). They were 16–19 months old at the time of the experiment. These mice were bred and maintained by our collaborator Jorge Palop prior to our experiments (Gladstone Institute of Neurological Disease, San Francisco, CA, USA).

hAPPJ20 mice

hAPPJ20 mice carry a transgene containing human APP with the Swedish and Indiana FAD mutations, on a C57BL/6J background⁷⁹. These mice were bred and maintained by our collaborator Jorge Palop prior to our experiments (Gladstone Institute of Neurological Disease, San Francisco, CA, USA).

Experimental procedures

Injections

Injections were always of 50 µL of pure compound injected intraperitoneally (i.p.). Depending on the compound, this represented 132–298 µmol. Saline solution was 150 mM NaCl in water.

Feeding

For the first feeding experiment, which involved all seven compounds and BD, standard chow pellets (5053 PicoLab diet) were ground, and the resulting powder was mixed with pure compound to reach a concentration of 10% weight/weight. BD (R/S-1,3-butanediol) was obtained from Sigma-Aldrich. The control chow used in this experiment was also ground.

For the second feeding experiment, which focused on C6x2-BHB, standard chow pellets were also ground and mixed with 10% w/w of compound. Around 60% w/w water was also added to the powder in order to obtain a dough, which was moulded into pellets and left to dry for a few days. The final pellets contained around 7% w/w water when they were used. The control pellets used in the experiment were prepared identically so that control and compound pellets would be similar in shape, texture and colour.

All feeding experiments, together with the 24-hour fast experiment, were starting at 7.00 pm to synchronize with the normal feeding cycle of the mice.

Blood draws

Blood for plasma glucose and BHB testing was obtained via minimal distal tail snip, with mice placed into a whole-body restrainer (Braintree Scientific) for comfort. Glucose testing requires a single drop of blood, and ~40 µL was drawn for a BHB assay. Blood was collected into microvettes coated with Li-Heparin (Sarstedt), and plasma was collected by centrifugation at 1500 x G for 15 min at 4 °C. Plasma was then kept frozen at – 20 °C until use.

BHB concentrations

BHB concentrations were determined from plasma using a BHB enzymatic detection kit (Stanbio Laboratory, Boerne, TX).

Glucose concentrations

Glycaemia was evaluated using a glucose meter (FreeStyle Freedom Lite) with blood glucose test strips (FreeStyle Lite).

Sedation

Sedation was evaluated qualitatively based on the mouse exploratory behaviour and response when held by the tail. Typically, sedated mice stopped exploring their environment, did not fidget when held by the tail, had closed or semi-closed eyelids, and were sometimes tremulous. In extreme cases, body temperature was also abnormally low. To our observations, no mouse ever became unconscious during the experiments. Thus, attempts at evaluating sedation more quantitatively using published scales of scores created for anaesthetic depth, involving for instance the righting reflex or response to pinching the paw, were unhelpful⁸⁰.

Western blots

Directly after dissection, mouse organs were snap-frozen in liquid nitrogen and then stored at -80 °C. For preparation of whole-cell lysates, a portion of the frozen organ was homogenized with 0.5 mm zirconium oxide beads in a Bullet Blender (Next Advance) while suspended in 1% SDS lysis buffer. Whole-cell lysates were separated on an 8% polyacrylamide gel, then transferred to nitrocellulose membranes. Membranes were blocked with a 5% solution of dry milk in wash buffer prior to antibody blotting with anti-acetyllysine (Cell Signaling 9441L) or anti-betahydroxybutyryllysine (PTM 1201) primary antibodies, followed by HRP-conjugated secondary antibodies. Signal was developed with Supersignal chemiluminescent substrate (ThermoFisher) followed by film exposure. The raw image of the scanned film may have been adjusted in global brightness or contrast for clarity.

Electroencephalograms

Dravet and hAPPJ20 mice were implanted (after anaesthesia) with Teflon-coated silver wire electrodes (0.005-inch diameter) attached to a microminiature connector bilaterally into the subdural space over frontal, central, parietal, and occipital cortices (surgeries were performed by our collaborator, Jorge Palop's laboratory). They were examined by electroencephalography during recording sessions of 50 min each using the Harmonie software from Stellate Systems. Gotman spike detectors from Harmonie were used to automatically detect epileptiform spikes, which is defined by a peak lasting less than 80 ms and reaching an amplitude greater than fivefold the average baseline amplitude measured during the 5 sec before the spike. During the recording, mice are free to move in the round, 50-cm-diameter EEG open field^{78,81}. Raw power in various frequency ranges was quantified from the EEG recordings using ADInstruments LabChart software. Gamma-fraction is defined as the fraction of total EEG power output which is in the 30–90 Hz range, excluding the power 58–62 Hz which is prone to artefacts from the 60-Hz electrical current.

The several data types (epileptiform spikes and frequency power) were time-correlated for analysis using custom software.

Data analysis

Data analysis was performed using GraphPad Prism version 7.0. Difference between two data sets was assessed using unpaired (for most experiments) or paired (for EEG data for which the same mouse is both control and treated at different time), two-tailed Student's t-test. For more than two data sets, differences were assessed by one-way ANOVA with Tukey correction for multiple-hypothesis testing. Non-significance was defined as a P-value over 0.05.

Graphs were all designed with GraphPad Prism version 7.0. Error bars in the figures represent the standard error of the mean (SEM).

Molecular structures were drawn using MarvinSketch 15.12.7.0 from ChemAxon.

Results and Discussion

Novel compounds are ketogenic in mice

The broad aim of this work is to mimic certain therapeutic effects of KD by supplementing with ketogenic compounds while on a regular diet containing normal amounts of carbohydrates.

The main characteristic of the KD is to induce elevated levels of plasma BHB, so the first goal of this work is to assess the ketogenic potential of the new set of compounds. This will allow us to select within this set the most effective compounds that safely induced ketosis and which will be used in subsequent studies.

Compounds induce ketosis via intraperitoneal injection

An efficient way to preliminary assess their ketogenic potential and safety is to directly inject the compound i.p. in mice, followed by blood draws at different time points to assess plasma BHB levels. The i.p. route was chosen because it should subject the compounds, specifically the MCFA component, to first-pass metabolism in the liver, similar to if they had been absorbed from the gut. In addition to plasma BHB levels, we evaluated glycaemia after injection, and we kept track of the daily calorie intakes and weights over a longer time scale. The KD and supplementation with MCT are both reported to affect glucose levels and body weight in mice⁸². Moreover, the KD is also reported to be overall anorexigenic, and administration of BHB in rats reduced voluntary food intake^{61,62}. Yet, low glucose levels or low calorie intake may alone be responsible for inducing ketosis.

A first round of i.p. injections in C57BL/6J mice was performed with the seven compounds and saline as control. All injections were of 50 µL. Depending on the compound, this corresponds to 130–300 µmol or 43–48 µg of compound. For these mice which weigh around 30 g, this represents a 1430–1600 mg/kg dose. Assuming it is totally absorbed and metabolized, this volume of compound would release around 0.4 kcal, or around 1/30–1/40 of a typical daily calorie intake. This volume is also well below the volume limit for an i.p. injection which would be around 300 µL for these mice. Given all this, we expected that the injection would produce measurable effects over a couple of hours. Blood was collected just before injection for baseline BHB levels and at different time points post-injection, until 24 or 30 hours. Glycaemia was evaluated on the same time points than BHB

levels, and weights and daily calorie intake were measured for two of the seven compounds until 29 days after injection.

All compounds efficiently induced a mild ketosis that lasted over 12 hours. For most compounds, BHB levels reached concentrations over those usually generated by calorie restriction. Trends of BHB levels over time after injection were relatively different depending on the compound. Some directly produced a high peak lasting from 30 minutes to 4 hours post-injection, but others resulted in a more long-term ketosis which lasted around 16 hours, starting around the 8th hour post-injection, culminating around the 12th, and concluding around the 24th (Fig. 5A).

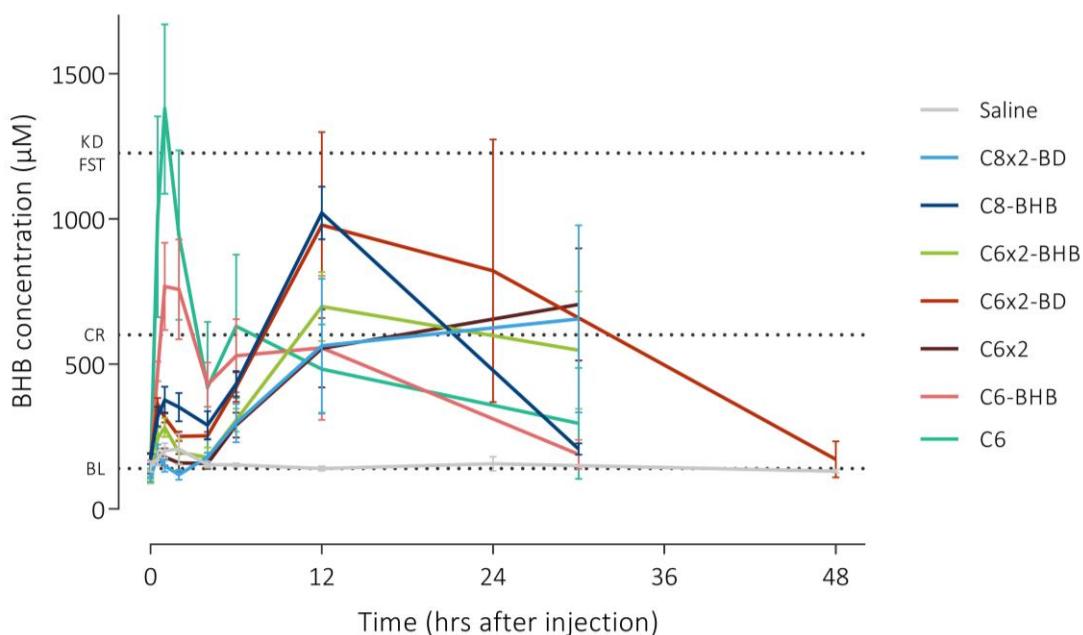
Mice ate considerably less on the first day after injection. Calorie intake was around 30% of the calories they usually eat. This quickly increased the second day, and normal calorie intake was reached back on the third day. The following days, mice had the tendency to slightly overeat, reaching levels of 17–23 kcal/day (Fig. 6A).

The drop in calorie intake coincided with a rapid loss of weight after injection. Weights reached a minimum of 85–90% of the initial weight around the first or second day after injection. From this point, weights slowly but steadily increased, reaching values close to the initial weight around 15–25 days (Fig. 6B).

Concerning short-term safety, we noticed two possible adverse effects. First, some compounds affected glycaemia. Generally, glycaemia remained within the normal range, but a few compounds induced hypo- or hyperglycaemia (Fig. 5B). Second, some mice were identified as sedated a few hours after injection. They recovered in most cases, but a total of two mice, out of 12 marked as sedated, did not and had to be euthanized at the end of the experiment.

A

Injection: BHB concentration



B

Injection: Glycaemia

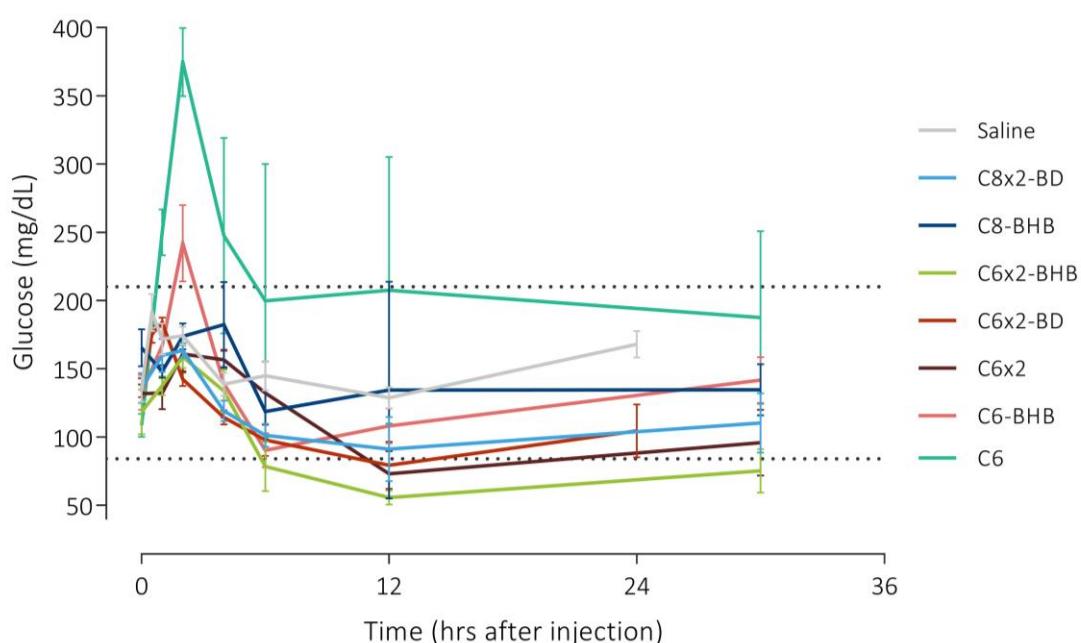
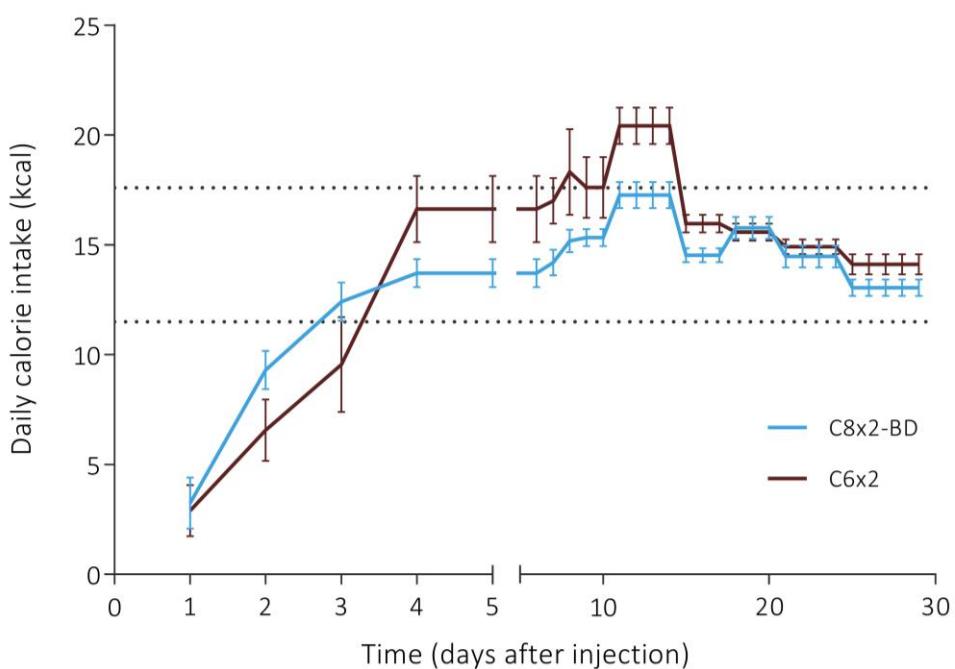


Fig. 5 | Compounds induce ketosis via intraperitoneal (i.p.) injection. **(A)** 50 μ L of compound was injected i.p. Some compounds (C6 and C6-BHB) directly produced a high peak in BHB levels lasting from 30 min to 4 h post-injection. Others resulted in a more long-term ketosis that lasted around 16 h. BL, CR, FST, KD represent the average BHB levels on baseline, on calorie restriction (60% of ad libitum), on a 24-h fast, and on the ketogenic diet, respectively. **(B)** Some compounds affected glycaemia. While it generally remained within the normal range, C6 induced hyperglycaemia and C6x2-BHB induced hypoglycaemia. The two lines represent the normal range of glucose levels in these mice under control situations. N = 4 mice/condition for both graphs.

A

Injection: Calorie intake



B

Injection: Weight

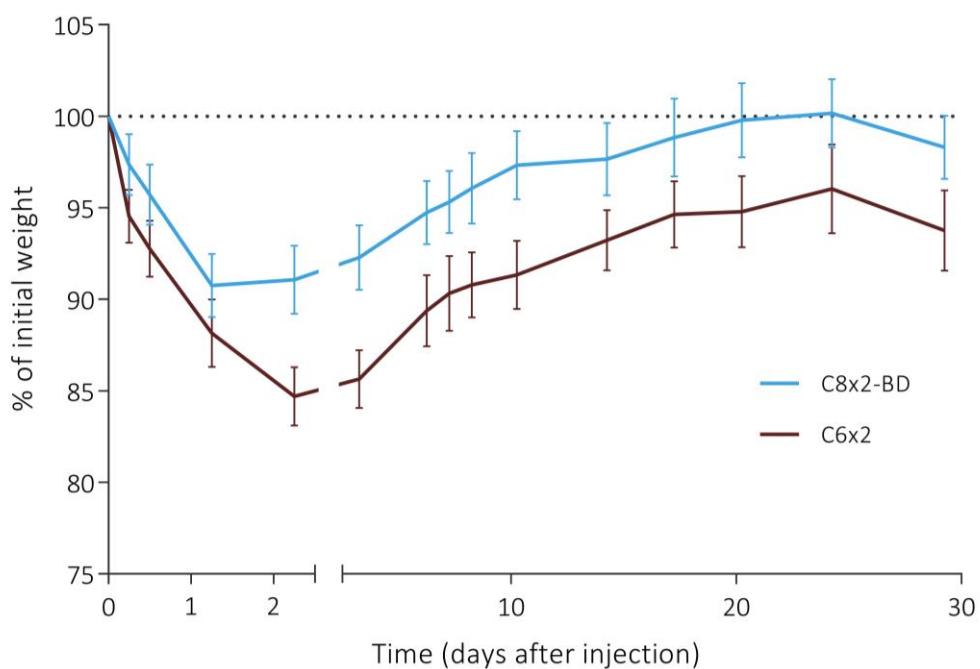


Fig. 6 | Mice eat considerably less and lose weight after injection. **(A)** Calorie intake on the first day after injection was around 30% of the calories they would usually eat. This increased on the second day, and normal calorie intake was reached back on the third day. The following days, mice had the tendency to slightly overeat. The two lines represent the normal range of calorie intake in these mice. **(B)** The mice rapidly lost weight after injection. The minimum was 85–90% of the initial weight on the first or second day post-injection. Initial weight was reached back around 15–25 days. N = 8 mice/condition for both graphs.

Sedation may be explained in part by binding of BHB to FFAR3⁵⁵, even though high BHB levels were not consistently accompanied by sedation. It is in fact a very common side effect of well-established AEDs such as valproate and has also previously been reported, albeit to a lesser degree, with MCFA supplementation⁸³. The explanation remains uncertain but is likely to be linked with their anti-epileptic mechanism, which is poorly understood⁸⁴.

Explaining how some of the compounds had an effect on glycaemia is extremely challenging, and could be the subject of a follow-on study. Briefly, elevated BHB levels have been reported to both increase insulin sensitivity in some studies and increase insulin resistance in others⁸⁵. Some also found that BHB concentrations correlated with increased insulin secretion, but others with decreased insulin secretion⁸⁵. Moreover, direct infusion of BHB in dogs can induce hypoglycaemia or hyperglycaemia, depending on their initial dietary stage and glucose concentrations⁸⁶. The effect is further complicated here by the presence of fatty acids, which have their own effects on glycaemia. Taken alone, free fatty acids concentrations in plasma correlate with gluconeogenesis, and are inversely correlated to glycogenolysis⁸⁷.

The loss of weight can logically be explained by the lower calorie intake, which is close to fasting on the first day after injection. Incidentally, the values coincide with those measured on a 24-hour fast (87.5% of the initial weights at the 24th hour). The fact that calorie intake was lower than usual after injection raises the logical concern that the long-term ketosis we observed might have actually been an artefact due to calorie restriction and not a direct result of the compounds.

BHB and glucose values measured during a 24-hour fast can help us better understand these results. First, the BHB trend we measured during the 24-hour fast (Fig. 12A) allowed us to fully confirm that the first sharp peak observed from 30 minutes to 4 hours after injection of C6 or C6-BHB is directly induced by the compound, as even a strict fast would not generate elevated BHB levels this quickly. Second, the glucose and BHB concentrations measured during the 24-hour fast, together with values measured during other control situations (i.e., not involving any compound) allow us to gain some insight from temporal correlations between glucose and BHB. When gathering all these values (380 data points, each of which is a glucose value and a BHB value measured in the same mouse at the same specific time under control situations), we reached a coefficient of determination (R^2) of 0.357. This is expected: low glycaemia should normally correlate with ketosis. In the exact same manner, when gathering all measures performed during the injection experiment more than 4 hours (included) post-injection of the compound (to focus on

the sustained ketosis and exclude the immediate effect post-injection; 222 data points), we reached an R^2 of 0.004. The fact that glycaemia and BHB levels do not correlate in this situation supports the unexpected result that the compounds could well have a lingering effect on ketosis, lasting much longer than the immediate 2 to 3 hours post-injection. This should be taken with caution though, as BHB and fatty acids have complex and sometimes counterintuitive effects on glycaemia that could confound the usual low glycaemia seen in a fasting state.

For future experiments using i.p. injection as route of administration, we selected C6-BHB as our candidate compound. Indeed, it generated a neat early peak lasting around 3 hours and reaching BHB levels over those induced by CR⁵⁶. Moreover, it was very well tolerated by the mice. It induced mild sedation in only one mouse that perfectly recovered in just a few hours. Glycaemia also remained within normal range.

Compounds also induce ketosis orally via feeding

Intraperitoneal injection is a fast, acute way of testing the ketogenic potential and short-term adverse effects of the compounds in mice. However, injections are not a viable way of administrating the compound over a long term, especially given the fact that patients who may be targeted for such a therapy are young children or elderly with dementia. Oral administration would be far more practical and tolerable. In the few existing studies, MCTs and ketone esters were usually provided orally, often mixed in a drink⁸⁸. We thus needed next to assess if the compounds were also able to induce ketosis when fed to the mice.

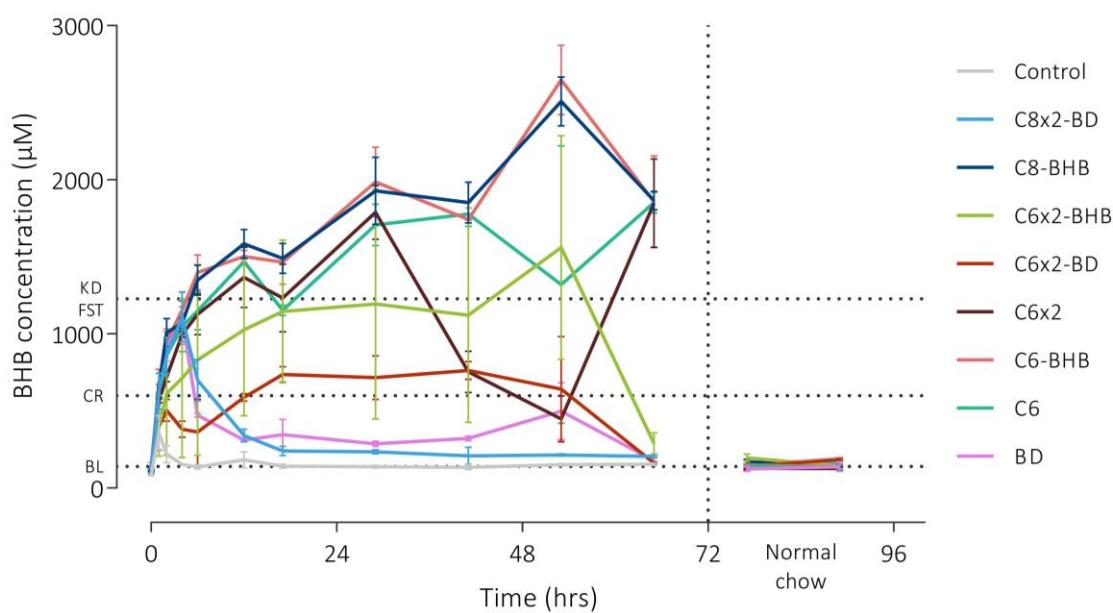
Each of the seven compounds, together with normal chow (control) and R/S-1,3-butanediol (BD) respectively as control and reference for comparison, was tested in feeding. The diet was composed of 10% w/w of pure compound mixed in ground normal chow, and was placed in the cages in lieu of normal chow for 3 days. We regularly measured BHB levels and glycaemia, and kept track of the daily calorie intake. The 10% w/w concentration was chosen based on a pilot experiment in which we tested one of the compound at 2.5% and 5% w/w in chow. The compound was well tolerated by the mice, but did not seem sufficient to generate any meaningful ketosis.

BHB concentrations were notably higher than baseline values for 6 of the 7 compounds. These results varied depending on the compound, but overall were 800–2000 μM BHB (Fig. 7A). No sedation was observed at any time during the experiment, suggesting that the mechanism of sedation may require aspects of the parenteral administration, such as bolus dosing or transiently high peak plasma levels.

Calorie intakes were very different for each compound. For most of them, it was unusually low on the first day, but steadily increased on the second and third days of feeding (Fig. 8A). Mice also lost weight, including those fed control chow and BD. They reached a minimum of 80–95% of the initial weight just before diets were switched back to ground normal chow at the 72nd hour. They directly started to increase afterwards, gaining back around 5% from most compounds in the following 24 hours (Fig. 8B). Finally, four compounds seemed to induce a reduction in glucose levels to approximately 70 mg/dL. These levels are slightly lower than usual but are considered normal and not hypoglycaemic (Fig. 7B).

A

Feeding: BHB



B

Feeding: Glycaemia

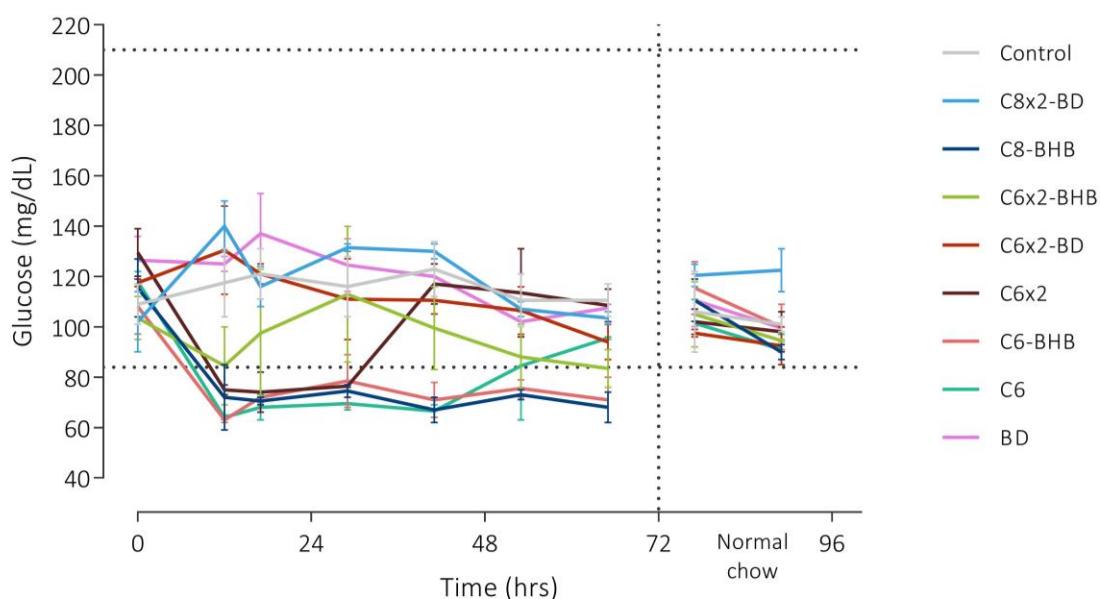
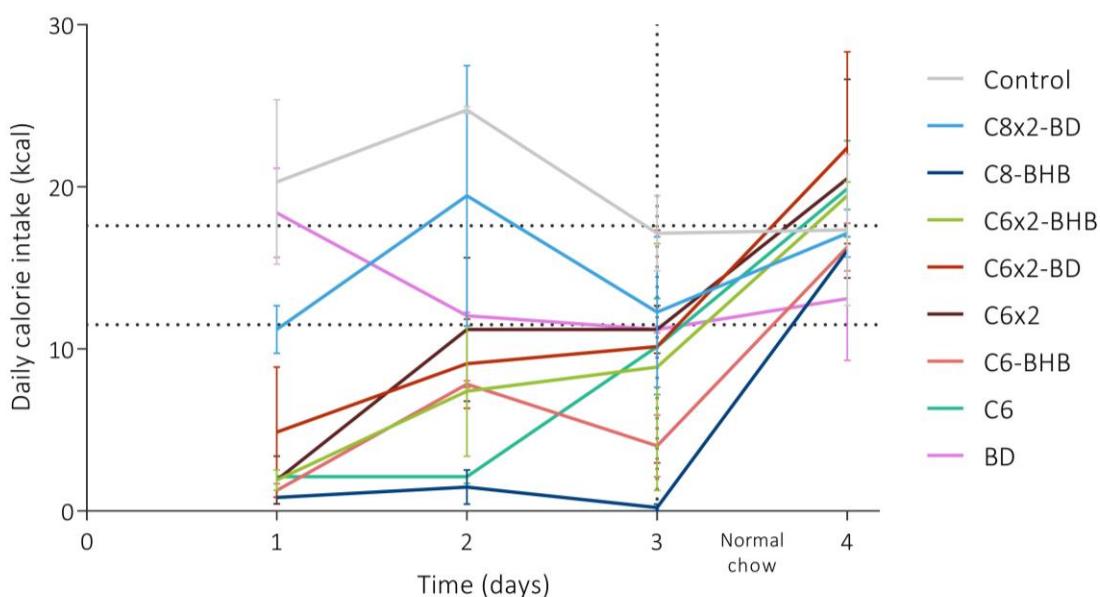


Fig. 7 | Compounds also induce ketosis orally via feeding. **(A)** When feeding 10% w/w compound in ground chow, BHB levels were notably higher (800–2000 μ M) than baseline for 6 of the 7 compounds. Only C8x2-BD did not seem to induce noticeable ketosis. All BHB levels went back to baseline after placing normal chow at 72 h. BL: baseline, CR: calorie restriction, FST: 24-h fast, KD: ketogenic diet (see Fig. 5A). **(B)** Four compounds (C6, C8-BHB, C6-BHB, C6x2) seemed to induce a reduction in glycaemia. Glucose levels were around 70 mg/dL for these compounds, which is slightly lower than normal, but not considered hypoglycaemia. All glucose levels went back in the usual range after placing normal chow at 72h. The two lines represent the normal range of glucose levels. N = 2 mice/condition for both graphs.

A

Feeding: Calorie intake



B

Feeding: Weight

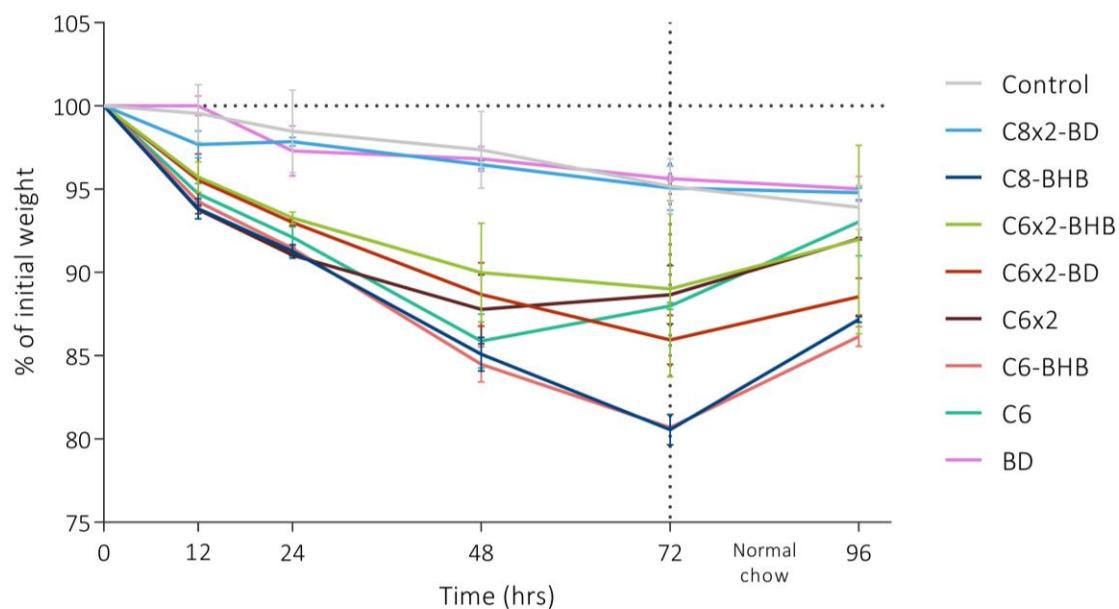


Fig. 8 | Daily calorie intakes and weights throughout three days of feeding ground chow containing 10% w/w compound. **(A)** For most compounds, calorie intake was unusually low on the first day of feeding, then steadily increased on the second and third day. Mice directly ate normal amounts of the chow containing BD, and the calorie intake for control chow was higher than usual. Calorie intakes went directly back to normal, or even above, when normal chow was put in the cage at 72 h. The two lines represent the normal range of calorie intake. **(B)** All the mice lost weight throughout the experiment, including those fed control chow. Minimum was reached at the third day of feeding and was 80–95% of the initial weight depending on the compound. Weights started to increase after normal chow was placed in the cage at 72 h, except for control, BD, and C8x2-BD. Mice were single-housed in this experiment. N = 2 mice/condition for both graphs.

The period of habituation required to reach normal calorie intake could be explained by the distinctive odour of most of the compounds. Like most esters, the compounds have pronounced and distinct odours, which one might describe as similar to apple or whiskey, and probably similarly strong tastes. They might not be palatable at first to the mice, which therefore need an adaption period. This seems to be confirmed by the fact that calorie intake for BD, whose odour is less strong and quite different, is higher and immediately comparable to control chow. Although the length of this experiment was limited by the quantity of compounds available, the steadily increasing trend of calorie intake for most compounds suggests that additional days of feeding would allow the mice to become fully habituated and reach normal calorie intake.

Calorie intake for control chow, on the other hand, was higher than normal. This can be explained by the different localization and form of the food. Indeed, mice usually have to climb or stand to get the food, which is on a grill over them. Also, it is usually in hard pellets, which they have to crumble. Here, the food was ground in a jar on the cage floor, which is much more convenient for the mice and might encourage them to overeat. Furthermore, mice in this experiment were single-housed to monitor individual food intake. As a consequence, they tend to lose more heat, for which they have to compensate by eating more. This did not seem sufficient, however, as weights decreased even for control-fed mice. Weight loss seemed to coincide entirely with calorie intake. Overall, 5% of the weight loss can be explained by higher heat loss, as it happened with control chow, BD, and C8x2-BD, which the mice ate well. The rest of it correlates with differences in calorie intakes.

Comparing BHB and glucose levels with calorie intake can again, as in the injection study, help confirm whether elevated plasma BHB levels were due to the compounds themselves, or a side effect of reduced calorie intake. Reassuringly, we found that some compounds generated elevated plasma BHB even in condition of normal calorie intake.

Most low glucose levels seemed to coincide with the low calorie intake. Indeed, the four compounds that induced unusually low glucose levels were also those with the lowest calorie intake on the first day. Moreover, for two of these compounds, namely C6 and C6x2, low glucose levels resolved when the mice started to eat larger quantities of food. It is thus unlikely that these compounds had a direct effect on glycaemia, which was rather affected by fasting. Incidentally, glucose levels during a 24-hour fast drop to similar values (Fig. 12B). In contrast, some compounds may have contributed to lower glucose levels, such as C6-BHB, for which consistent low glucose levels were not affected at all by changes in calorie intake. Additionally, the changes in calorie

intake during the first 2 days were almost equal for C6-BHB and C6x2-BHB, but glucose levels for the latter stayed within normal range, unlike the first.

Some of the highest BHB levels, especially those encountered with C6 and C8-BHB, cannot be explained by the compound as the mice barely ate any of it. These high values are, without much doubt, generated by fasting. For the other compounds, however, calorie intake after 48 hours should not be sufficiently low to induce BHB levels over 500–600 µM.

An interesting way to summarize this complex data and to select our most efficient compounds at inducing ketosis in feeding is to plot on a scatter graph BHB levels as a function of calorie intake (Fig. 9). The data points were picked after the 48th hour of feeding to avoid, as much as possible, the initial period of habituation to the diets.

Briefly, compounds for which calorie intake was particularly low but BHB levels were high (upper left corner of the graph) were eliminated. Indeed, the high ketosis was most likely induced by prolonged fasting or calorie restriction, and not directly by the compounds. These included C8-BHB and C6-BHB. C6-BHB also induced consistent low glucose levels, despite variations in calorie intake.

Conversely, compounds for which calorie intake was normal or high but BHB levels were close to baseline (lower right corner of the graph) did not sufficiently raise ketosis despite good calorie intake, and were eliminated. These included C8x2-BD and C6x2. Additionally, results with the latter were inconsistent: BHB levels dropped just when the mice started to eat normal amount.

C6 was eliminated because it induced consistent hypoglycaemia throughout the experiment, despite a calorie intake close to normal.

We thus concluded that C6x2-BHB and C6x2-BD were our best compounds for inducing ketosis via feeding. At the third day, calorie intake was close to the usual range for both, and they both induced high BHB levels, reaching levels similar to CR for C6x2-BHB, and similar to fasting for C6x2-BD. We can also add, if there was any doubt, that the high BHB levels encountered with the two compounds cannot be explained by a lingering ketosis remaining from the previous days when calorie intake was abnormally low. Indeed, as seen during refeeding at the end of the 24-hour fast, as little as 1 hour of normal eating is enough to completely suppress ketosis and pull BHB levels back to baseline (Fig. 12A). Finally, glycaemia also stayed within the normal range with C6x2-BHB and C6x2-BD.

Feeding: Calorie intake vs BHB

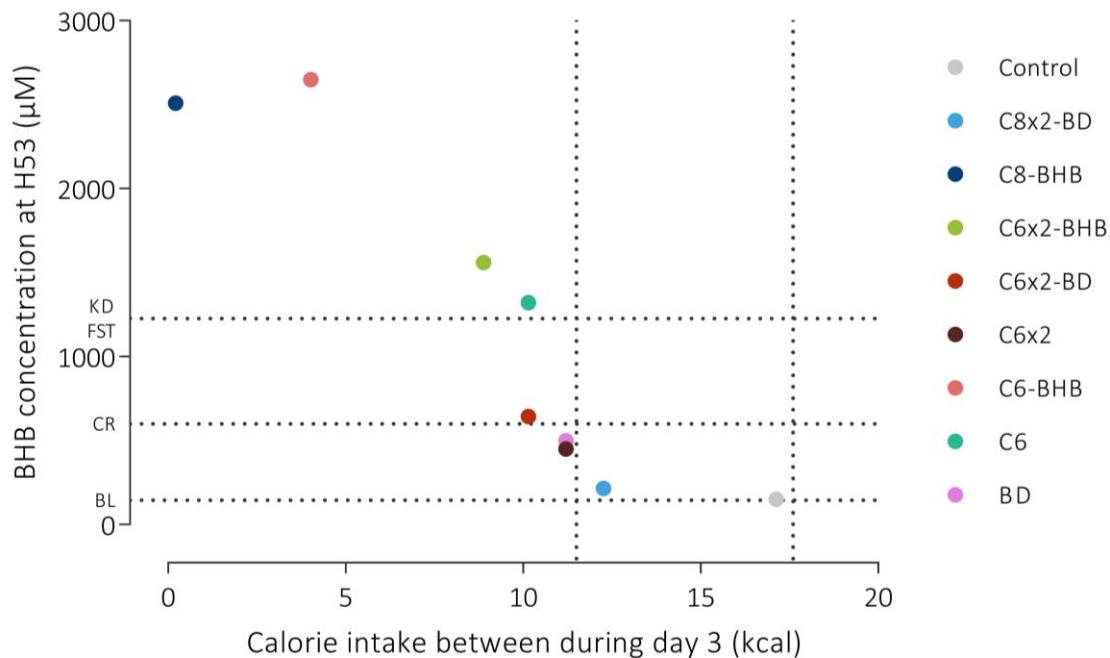


Fig. 9 | BHB levels as a function of calorie intake. Data were picked after the 48th h of feeding in order to avoid as much as possible the initial period of habituation to the diets during when calorie intakes were abnormally low. C6x2-BD and C6x2-BHB were picked as our best compounds in feeding because they generated high BHB levels under conditions of close-to-normal calorie intake. Each data point represents the average of the two mice on the diet. BL: baseline, CR: calorie restriction, FST: 24-h fast, KD: ketogenic diet (see Fig. 5A). The two vertical lines represent the normal range of calorie intake.

C6x2-BHB in food induces protein β-hydroxybutyrylation in the liver

We thus identified compounds that produced substantial ketosis and were well-tolerated in oral feeding. Next, we investigated whether they could also induce biochemical changes associated with fasting and ketosis in tissues. More specifically, fasting induces changes in histone modifications, including lysine acetylation⁵⁶ and, more recently, lysine β-hydroxybutyrylation⁵⁸. β-hydroxybutyrylation is thought to be due to nonenzymatic modification of histones by CoA-activated BHB, while acetylation may be stimulated by increased availability of acetyl-CoA or via inhibition of deacetylases by BHB. Following these findings, we decided to test if lysine β-hydroxybutyrylation (Kbhb) and acetylation (Kac) were also detectable in whole proteins from different tissues, and if feeding a ketogenic compound was sufficient to induce noticeable levels of Kac and Kbhb.

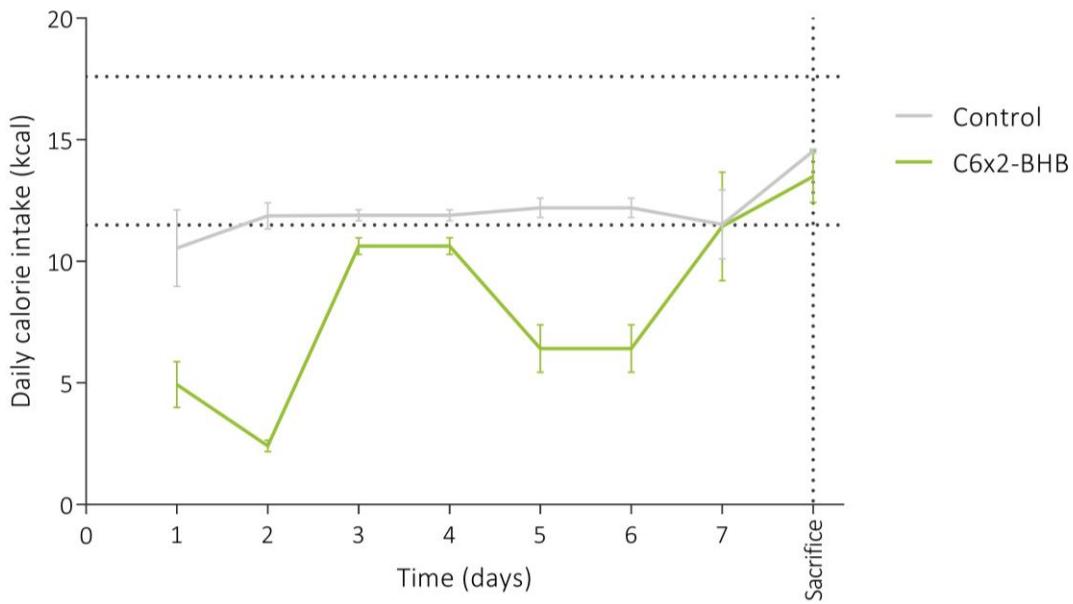
The diet was composed of pellets containing 10% w/w C6x2-BHB. C6x2-BHB was used in this experiment, having been shown in earlier tests to be well tolerated by the mice while inducing noticeable ketosis. Pellets were used instead of ground chow to avoid artefacts due to food

texture, such as overeating of the control-fed mice. Six mice were used in this experiment. Three were fed C6x2-BHB pellets, and the others were fed control pellets. They were all sacrificed on the 8th night, between midnight and 02.00 am. By the 7th day, calorie intakes were comparable in both groups, and it was suspected that BHB levels would be highest during the night-time feeding cycle. Blood was then drawn from cardiac puncture for BHB and glucose levels assessment, and organs were harvested for whole protein immunostaining of Kbhb and acetylation in liver and brain.

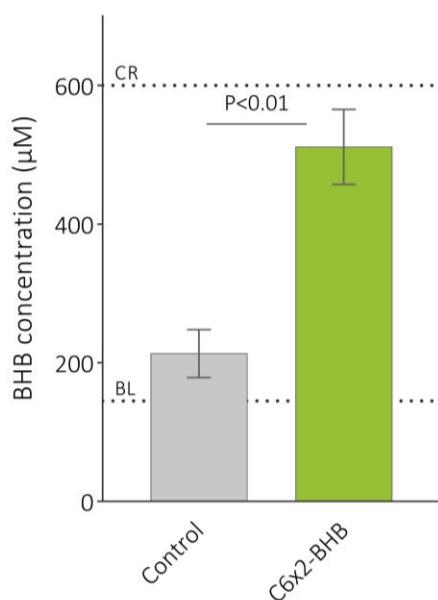
At the time of sacrifice, plasma BHB concentrations of the mice fed the ketomimetic compound were more than twice those fed control pellets (500 µM vs. 200 µM). The more modest BHB elevation compared to the earlier feeding experiment suggests that low calorie intake may have boosted levels in the earlier experiment. Nevertheless, ~500 µM average plasma BHB level achieved here is similar to that generated by calorie restriction. Glycaemia was slightly lower in mice fed compound pellets (69 mg/dL vs. 113 mg/dL), but were close to the normal range and the difference with mice fed control pellets was not significant ($P = 0.16$).

Preliminary western blots of whole proteins from liver revealed promising differences in levels of protein Kbhb in the liver. Several band in the 90–150 kDa range appeared more highly Kbhb in mice fed compound than in control-fed mice. One band slightly above 100 kDa, and one band around 120 kDa looked particularly hyper-Kbhb in livers of mice fed compound. Unexpectedly, one band at 70 kDa seemed more Kbhb in the control-fed mice. Differences in protein Kbhb in the brain, and differences in protein acetylation in both tissues were difficult to detect (data not shown).

A C6x2-BHB feeding: Calorie intake



B C6x2-BHB feeding: BHB



C C6x2-BHB feeding: Glycaemia

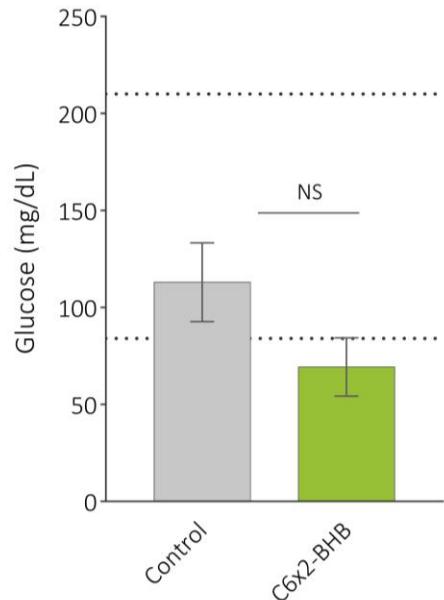


Fig. 10 | After 8 days of feeding, C6x2-BHB in chow (10% w/w) generated significant ketosis while calorie intake was comparable to control-fed mice. **(A)** Calorie intakes of the control-fed group of mice and the compound-fed group of mice were comparable by the 7th day of feeding. All mice were sacrificed during the night of the 8th day for further experiments. **(B)** At the 8th day, blood BHB levels were significantly higher in the mice fed C6x2-BHB compared to the mice fed control (500 μM vs. 200 μM). BL: baseline, CR: calorie restriction (see Fig. 5A). **(C)** Glycaemia of compound-fed mice was slightly lower than glycaemia of control-fed mice at the 8th day of feeding (69 mg/dL vs. 113 mg/dL). However, this difference was not significant, and 69 mg/dL is not considered hypoglycaemia *per se*. NS: not significant. N = 3 mice/condition for all three graphs.

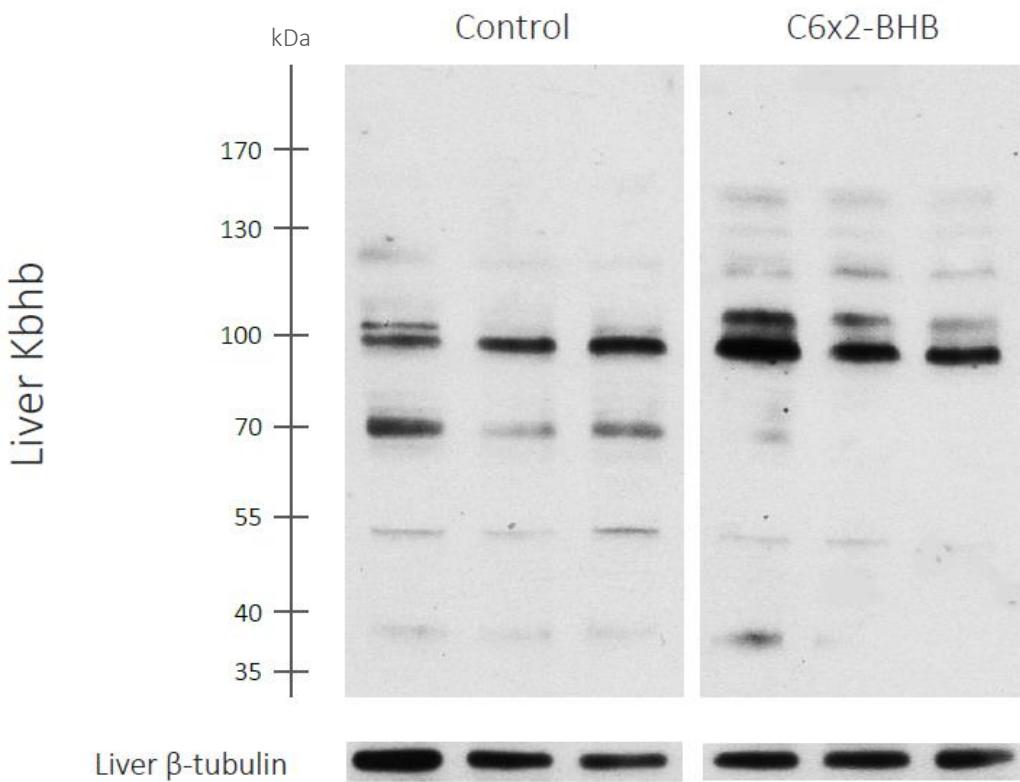
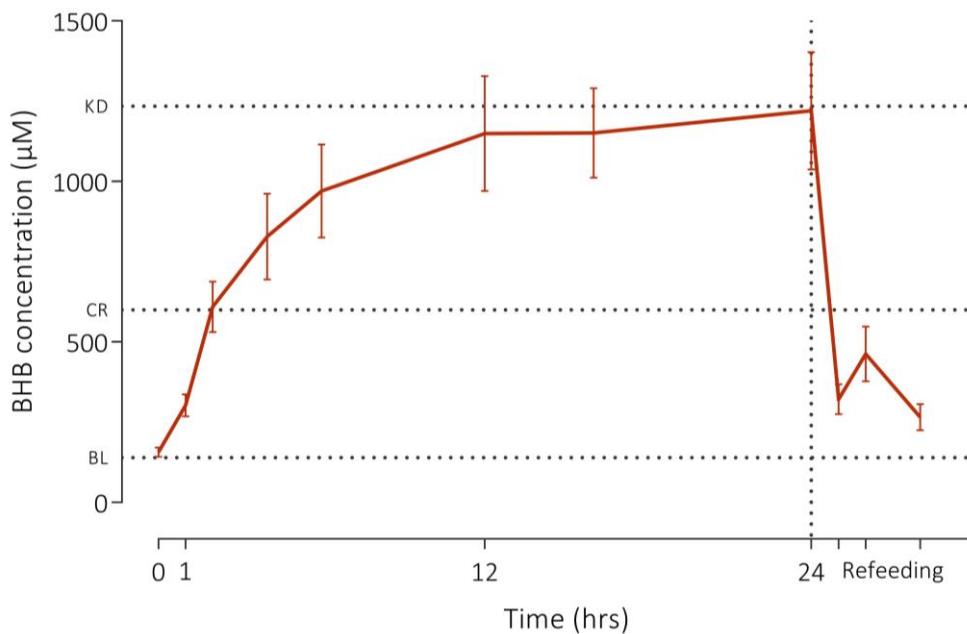


Fig. 11 | C6x2-BHB in food induces protein Kbhb in the liver. Western blot of whole proteins from livers of mice fed C6x2-BHB-pellets (10% w/w) for 8 days using anti-Kbhb antibody. Several bands in the 90–150 kDa range appear more highly modified with C6x2-BHB than on control diet. Western blot using anti-β-tubulin antibody was performed for loading control. Kbhb: lysine β-hydroxybutyrylation. N = 3 mice/condition.

These preliminary results represent a proof of concept that feeding a ketone body precursor compound like C6x2-BHB even in the context of a normal high-carbohydrate diet can both increase blood BHB levels and generate biological effects of BHB in target tissues. Kbhb-modified proteins can be detected in tissue whole-cell lysates. It also appears that Kbhb affects proteins other than histones, with hypothetical consequences on intracellular functions such as signalling and metabolism. Modification of non-histone proteins has not been previously reported; the paper by Xie et al.⁵⁸ focused only on Kbhb as a histone mark. The fact that Kbhb was detected in the liver in our conditions may also be a promising sign that modestly increased BHB levels to around 500 μM may be sufficient to trigger important intracellular biological effects.

A

24-hour fast: BHB



B

24-hour fast: Glycaemia

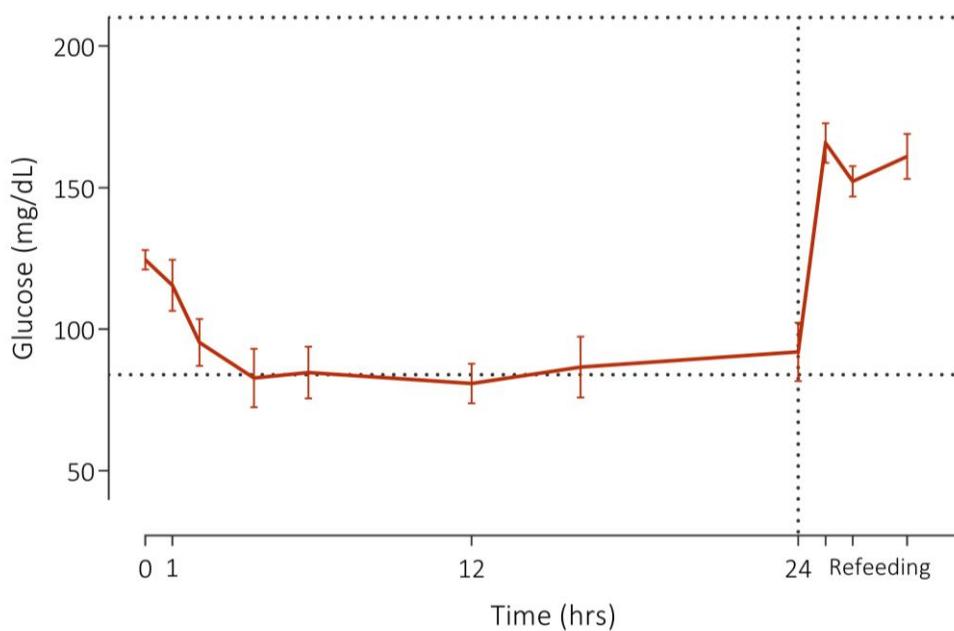


Fig. 12 | Ketosis and glycaemia during a 24-h fast. **(A)** During the 24-h fast, BHB levels reached a peak at $\sim 1220 \mu\text{M}$ at the 24th hour. BHB values dropped surprisingly quickly back to baseline within the first hour of refeeding. **(B)** Glucose values reached a minimum of $\sim 81 \text{ mg/dL}$ around the 12th of fasting. Values rose to $\sim 166 \text{ mg/dL}$ an hour after the end of the fast. N = 8 mice for both graphs.

Intraperitoneal injection of C6-BHB suppresses epileptiform spikes in Dravet and APP mouse models

From the previous experiments, we can conclude that some of the compounds safely generate elevated BHB levels in mice, both via i.p. injection and feeding. They thus mimic the principal physiological effect of the KD, which is to induce mild ketosis. We next sought to determine if this translates into the similar therapeutic effects than the KD on certain pathological conditions. The most documented use of the KD, which is also the first reason why it was invented, is to treat epilepsy. Studying epileptiform activity in AD is particularly interesting in our context for several reasons. First, as mentioned before, there might be an emerging relationship between epileptiform activity and cognitive deficits in AD patients, which may go much further than AD being a simple risk factor for epilepsy. Second, for reasons explained above, although the KD is promising for treating epileptiform activity in AD, it cannot reasonably be implemented in elderly patients with dementia. Therapeutics that mimic elements of the KD without a drastic dietary change might thus be very useful clinically. The DS is an equally interesting model in our context. First, the KD is beneficial in this condition, and is widely used clinically. Second, the mechanism by which seizures are triggered in the DS may be similar to what happens in AD patients, and thus serves as a good reference for comparison. Third, the DS mostly affects very young children, in which long-term implementation of the KD is also difficult.

An efficient, initial method to assess the anti-epileptic activity of the ketogenic compound is to administer it via i.p. injection. As shown previously, i.p. injection of C6-BHB triggers a neat early peak in the BHB levels which lasts a few hours. We thus used this compound in this experiment. We recorded by electroencephalography an AD mouse model (hAPPJ20), and a DS mouse model (*Scn1a*^{-/-}), which both present epileptiform activity on EEG. EEG may also provide clues on the mechanism of action of the compound.

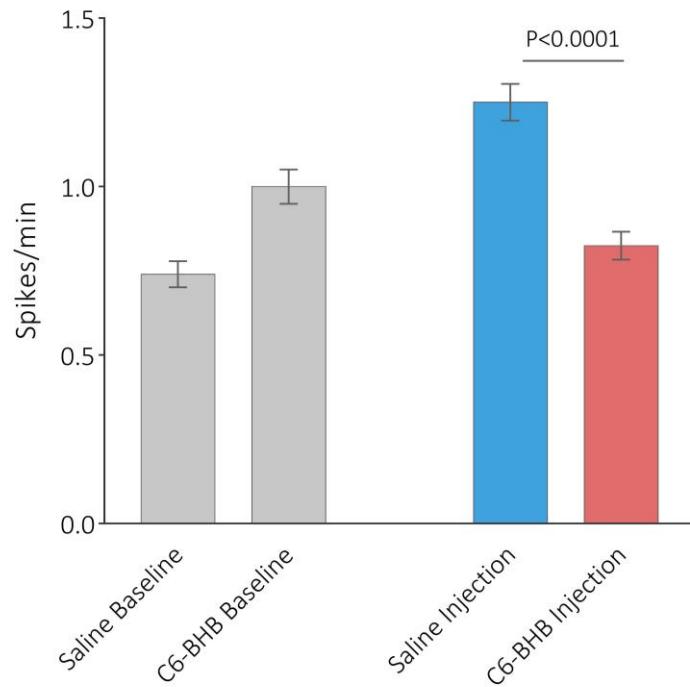
Each mouse underwent four 50-minute EEG recordings: twice on the first day and twice on the consecutive day. On each day, after a baseline EEG recording session of 50 minutes, 50 µL of pure C6-BHB or saline were injected i.p. The mouse was then allowed to rest in its home cage for 20 minutes, before another recording session of 50 minutes. At the end of the second session, blood was drawn from the tail for BHB testing. Whether a specific mouse was receiving saline on the first day then C6-BHB on the second or the opposite was randomized. EEG data included the average number of epileptiform spikes during the recording session, and the fraction of total power output within the gamma range of frequencies.

C6-BHB significantly reduced epileptiform activity in both Dravet and APP mice. Both models responded in a very similar fashion with approximately 40% less epileptiform spikes when the ketogenic compound was injected compared to when saline was injected (Fig. 13A and 14A).

C6-BHB increased plasma BHB levels in both models. While the increase in BHB levels was more than twofold in APP mice (Fig. 15A), the raise observed in Dravet mice was not significant compared to levels measured after saline injection ($P = 0.087$) (Fig. 15B).

A

APP: Spikes



B

APP: Gamma fraction

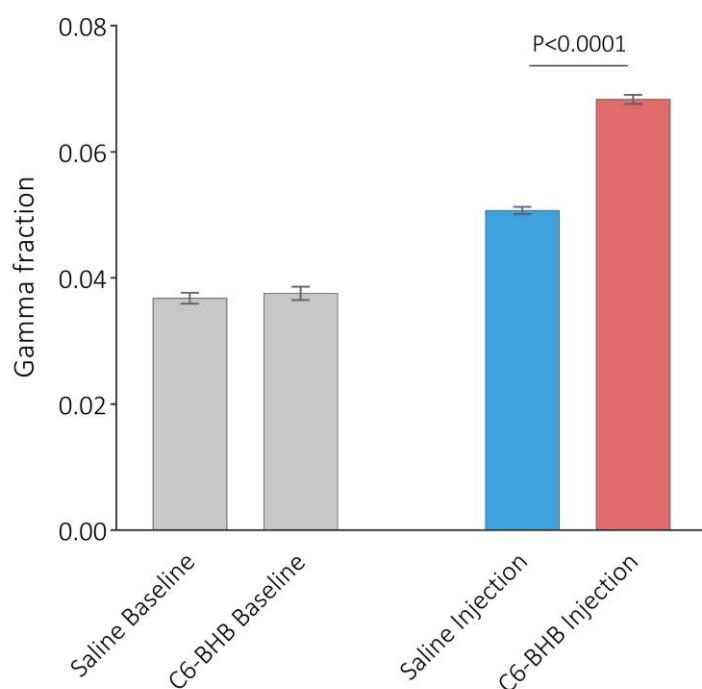
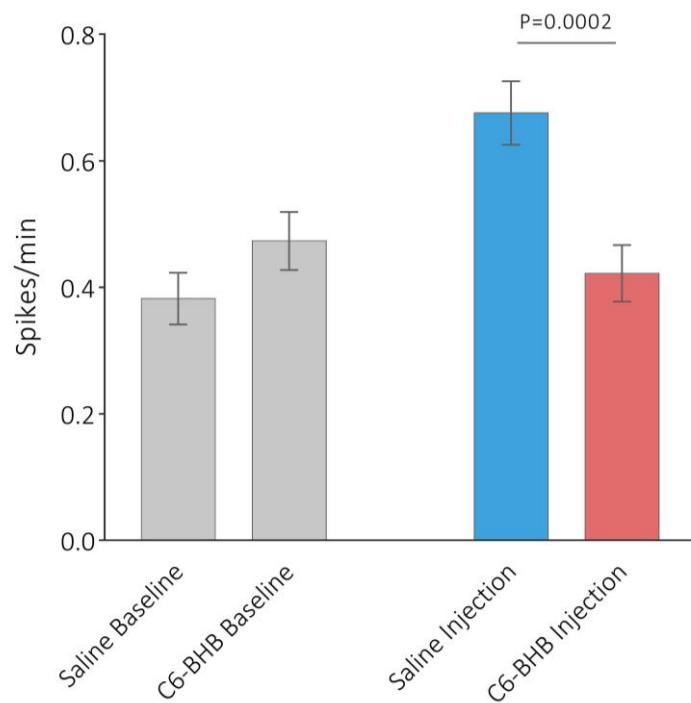


Fig. 13 | C6-BHB suppresses epileptiform spikes in the hAPPJ20 Alzheimer's disease mouse model. **(A)** Intraperitoneal injection of 50 μ L of C6-BHB significantly decreased the frequency of epileptiform spikes in the hAPPJ20 mouse. Baselines (i.e., spikes before injection) are added for comparison, but should not be directly compared to spikes after injection because the injection by itself affects the frequency of spikes. **(B)** Compared to saline injection, C6-BHB significantly increased the gamma fraction in the hAPPJ20 mouse. N = 17 mice for both graphs (John Newman, unpublished data).

A

Dravet: Spikes



B

Dravet: Gamma fraction

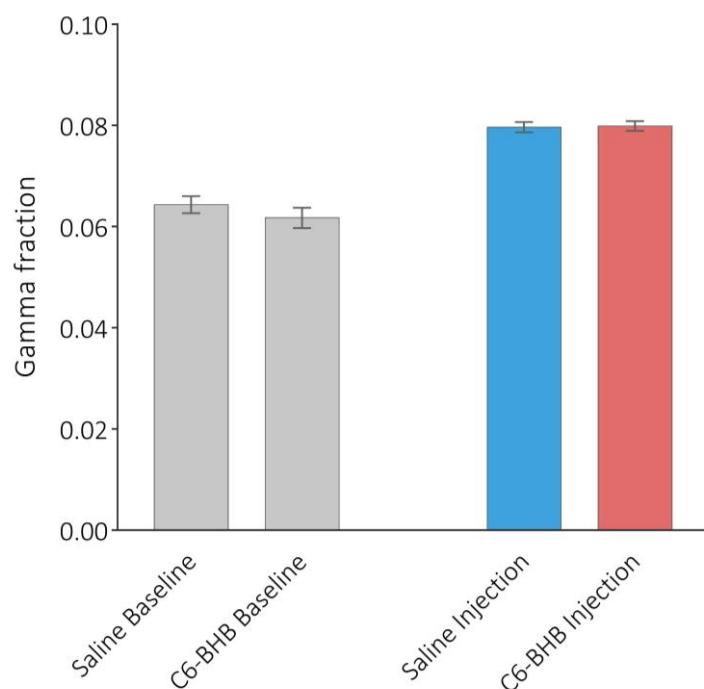


Fig. 14 | C6-BHB suppresses epileptiform spikes in the Dravet syndrome mouse model. **(A)** Intraperitoneal injection of 50 μ L C6-BHB significantly decreased the frequency of epileptiform spikes in the Dravet mouse. Baselines (i.e., spikes before injection) are added for comparison, but should not be directly compared to spikes after injection because the injection by itself affects the frequency of spikes. **(B)** C6-BHB and saline both increased gamma fraction in the Dravet mice. N = 9 mice for both graphs.

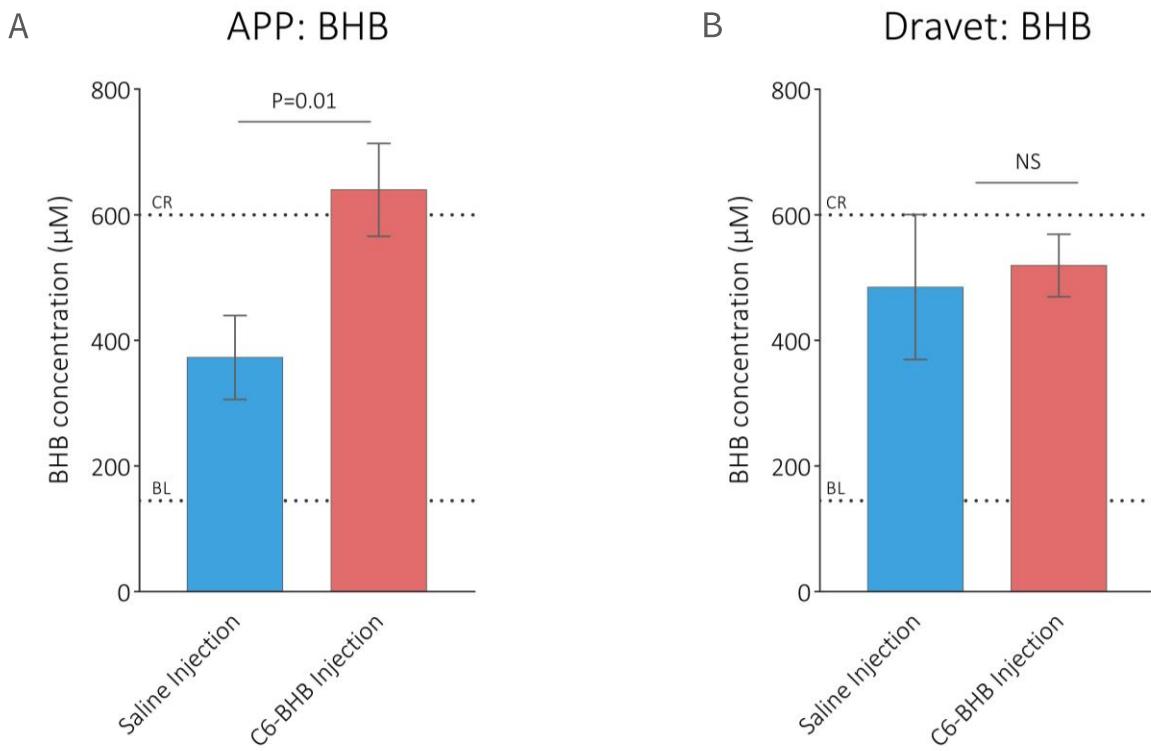


Fig. 15 | C6-BHB induces ketosis in the hAPPJ20 Alzheimer's disease mouse model and the Dravet syndrome mouse model. **(A)** Intraperitoneal injection of 50 μ L of C6-BHB significantly increased BHB levels in the hAPPJ20 mouse (John Newman, unpublished data). N= 18 mice. **(B)** Intraperitoneal injection of 50 μ L of C6-BHB seemed to slightly increase BHB levels in the Dravet mouse. However, this difference was not significant (NS) in part due to unusually high BHB levels in two mice after saline injection, perhaps due to a stress response. N = 8 mice. BL: baseline, CR: calorie restriction (see Fig. 5A). Blood for measures in both graphs were drawn 70 min after injection.

Reasons for the modest difference in plasma BHB levels for Dravet mice (Fig. 15B) may involve a small effect size in this strain but more importantly seem to include high control BHB concentrations, as measured after saline injection. As observed during the first round of i.p. injections, baseline values should normally be around 100–200 μ M. They are around 500 μ M here, which is already considered a relatively high ketosis, and thus seemed to mask the ketosis induced by the compound. This abnormally high baseline may be caused by the stress induced by the EEG recording. For instance, we can notice in the first round of i.p. injections a little bump in BHB levels directly after saline injection, which tends to show that stress does induce a small ketosis. Thus, a more prolonged stress like a 50 minutes EEG recording may induce a more pronounced ketosis in these mice. Epinephrine and norepinephrine induce lipolysis^{89,90}. Under the action of stress hormones, the adipose tissue thus releases glycerol and free fatty acids, of which the latter may be utilized as ketone bodies precursors by the liver. Moreover, this explanation is consistent with the pathophysiology of both mouse models. Dravet mice are reported to display increased levels of anxiety-like disorder and exhibit inflexibility or avoidance of environmental change⁹¹. hAPPJ20

mice, on the meanwhile, are actually less anxious than control mice on the elevated plus-maze⁹². This would coincide with a higher stress response from the Dravet mouse than the APP mouse when placed in the new environment of the EEG open field, thus translating into differences in BHB baselines.

Interestingly, the magnitude of the reduction in spikes is extremely similar to what was measured with hAPPJ20 mice on the KD (Fig. 3A). This is a very good sign that even though C6-BHB induces a milder ketosis than the one generated by the KD, it is sufficient to mimic the effects of the KD on epileptiform activity, hence called a ketomimetic agent.

The mechanism by which C6-BHB reduces epileptiform activity in hAPPJ20 mice involves increased interneurons firing

As previously explained, fast-spiking GABAergic interneurons expressing parvalbumin (PV neurons) are proposed to be involved in epileptogenesis in AD and the DS. Specifically, a deficit in the voltage-gated sodium channel (VGSC) Nav 1.1/SCN1A would cause them to fire fewer action potentials and thus release insufficient GABA. As a result, cortical neurons innervated by these interneurons would lack inhibition, and tend towards spontaneous synchronous firing that manifests as epileptiform activity¹². PV neurons are involved in the generation of gamma oscillations, which are high-frequency “brain waves” in the 30–90 Hz range. Indeed, inhibiting the PV neurons *in vivo* results in loss of gamma oscillations, while stimulating them is sufficient to generate gamma waves⁹³. This model has been confirmed in hAPPJ20 mice. Briefly, inhibiting VGSC with drugs in hAPPJ20 mouse further decreases gamma oscillations, triggers more intense epileptiform activity, and worsens learning and memory impairments. Increasing *Scn1a* expression with a transgene, on the other hand, increases gamma activity, reduces epileptiform spikes, and fully rescues learning and memory deficits, as assessed by the water Morris maze and the open field¹⁴.

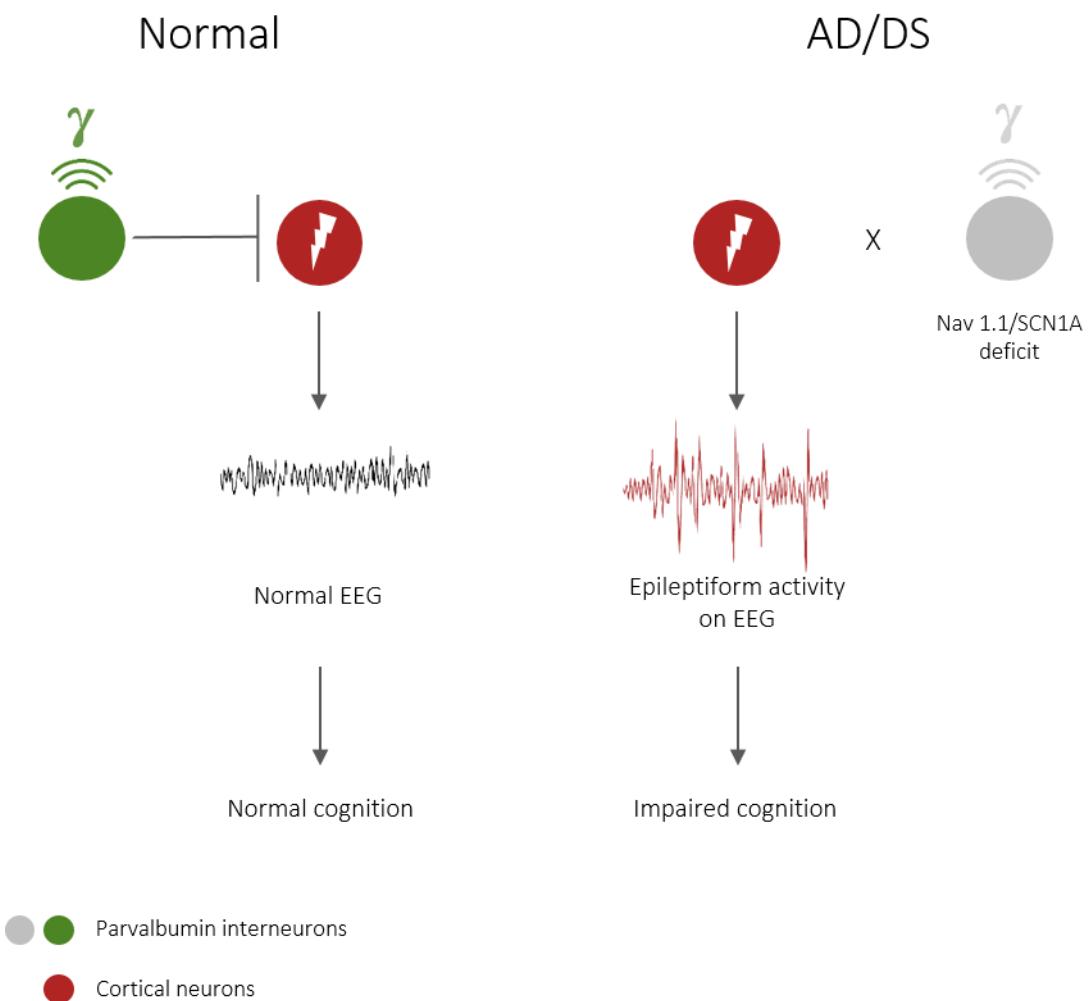


Fig. 16 | Graphical summary of the interneuron hypothesis. In AD and DS (right), the deficiency in Nav 1.1/SCN1A in the parvalbumin interneurons would prevent them from correctly inhibiting the cortical neurons they innervate. As a result, cortical neurons would tend towards spontaneous synchronous firing, which manifests as epileptiform activity on electroencephalogram (EEG). Finally, synchronous firing would disturb normal cognitive function. AD: Alzheimer's disease, DS: Dravet syndrome.

The fraction of total power output within the gamma range of frequencies, hereafter called gamma fraction, was significantly increased in hAPPJ20 mice after administration of the ketomimetic compound (Fig. 13B). In Dravet mice, a similar raise was observed. Saline injection, however, also seemed to increase gamma fraction in Dravet mice (Fig. 14B).

Presumably, the increased gamma fraction in hAPPJ20 mice after C6-BHB injection represents a boosted activity of the PV neurons. The results thus imply that the mechanism by which C6-BHB suppresses spikes in hAPPJ20 mouse involves stimulation of the PV neurons, which might allow them to inhibit neurons responsible for triggering epileptiform activity.

In Dravet mice, the data are lightly more puzzling. The fact that saline and C6-BHB injections both increased gamma fraction in a similar fashion is surprising (Fig. 14B). The data might be complicated here by the fact that the Dravet mouse does not show altered network activity in the first place⁹⁴. This study confirmed the hypoexcitability and decreased firing of PV neurons in isolated brain slices, but shows that it does not translate into decreased gamma signal in EEG. Briefly, two hypotheses may reconcile these seemingly conflicting results. First, *Scn1a*^{-/+} neurons may simply lose their ability to fire at gamma frequencies⁹⁴. However, assuming that epileptiform activity is mechanistically related in the DS and in AD, this solution would not explain why gamma fraction significantly increased after compound injection in hAPPJ20 mice, whose PV neurons should also have similar Nav 1.1 deficit. Second, compensatory mechanisms may mask the dysfunction of the PV neurons⁹⁴. Meanwhile in the hAPPJ20 mice, these compensatory mechanisms could be jeopardized by APP aggregation, thus revealing at the network level the increase in activity of the PV neurons after compound injection.

Succinctly, i.p. injection of a small volume of the ketomimetic compound C6-BHB induces a mild ketosis that is sufficient to suppress around 40% of the epileptiform spikes in mouse models of AD and DS (Fig. 13A and 14A). In the AD mouse model, the mechanism involves stimulation of the PV neurons (Fig. 13B), which would in return suppress firing of cortical neurons responsible for eliciting epileptiform activity (Fig. 16). The mechanism may be the same in the DS mouse model, although more research will be needed to confirm. Finally, results seem to point towards a similar mechanism when the KD is fed to the APP mouse, which gives an interesting lead towards understanding how the KD operates against epilepsy.

Future directions

From what we accomplished with this work, two broad directions take shape and can be pushed forward in the future: moving towards development of therapeutics for clinical trials, and gaining a deeper understanding of the actions of the KD and BHB in AD and epilepsy.

Towards clinical use

A first goal is to move towards clinical trial in patients with AD or other epileptic conditions for which the KD has been proven to be beneficial, such as the DS. Beforehand, several questions need to be answered. It should be first tested in AD and DS mouse models if the reduction in epileptiform spikes is also induced when the compound is fed rather than injected i.p. This would bring us closer to what is the most feasible route of administration in patients. If successful, it will be necessary to assess if the reduction in epileptiform spikes in these mice translates into an improvement in cognitive functions and behaviour. In our experiments, supplying the ketogenic compound in chow induced similar BHB levels than i.p. injection, and the KD was previously shown to reduce the frequency of spikes in hAPPJ20 mouse, which correlated with better performance in cognitive tests (Fig. 3). Given these facts, performing EEGs and cognitive tests on APP and DS mice fed the ketomimetic compound is likely to yield interesting results. Subsequently, it would be useful to test if other AD mouse models give similar results. An interesting model could be 3xTg-AD, which exhibits both A β and tau pathologies⁹⁵. Ablation of tau actually suppresses epileptiform activity in the DS mouse model⁷⁸. Thus, studying the action of tau on epileptiform activity in both the 3xTg-AD mouse model and the DS mouse model could be a fruitful approach. A mouse model which only exhibits tauopathy, such as Tg4510⁹⁶, could be useful as comparison in this context. Another interesting AD model could be the APP/PS1 (prenisilin-1) knock-in mouse model⁹⁷. It has the advantage of being well characterized in our context: it was already confirmed to display epileptiform activity on EEG⁷; and it was already tested for cognitive performance and motor function while on the KD³⁰.

As regard with safety, most preparations of the KD in humans are supplied with MCTs, and different ketone esters, including some carrying a BD unit, have already been tested in small clinical trials which did not conclude in any serious adverse effects (see *Exogenous ketones* section). Therefore, the present compounds, which are esters comprised of MCFAs and ketone

body molecules, are likely to be safe in humans. Nevertheless, the potential concerns we observed in mice have to be better characterized beforehand. They included low glucose levels, loss of weight, and sedation, although the last was not observed when the compound was fed. Low glycaemia occurred in both feeding and i.p. injection, and the compounds that induced it were not picked for further research. Low calorie intake clearly contributed to hypoglycaemia in some compounds, but was not the sole etiology. In this context, an interesting experiment to be performed would be the hyperinsulinemic-euglycaemic clamp⁹⁸. Briefly, two catheters are implanted in a mouse, one in the jugular vein for infusion of insulin and glucose, and one in carotid artery for drawing blood samples. Baseline glucose level is first measured, then insulin is infused at a constant rate to reach physiological hyperinsulinemia. Glucose is then infused to counter the effects of insulin. The goal is to regularly (usually every 10 min) adapt the rate of glucose infusion to maintain euglycaemia, which is defined by the baseline. At the end of the experiment, a low average rate is associated with insulin resistance, while a high average rate is associated with insulin sensitivity. Glucose uptake of specific tissues, such as the brain, can also be assessed with this technique⁹⁸. Yet, AD can be described as brain-specific diabetes mellitus, often called “type 3 diabetes”. Numerous parallels have been discussed in the literature, including insulin deficiency and resistance in the brain of AD patients⁹⁹. Therefore, if the low glycaemia we observed when administrating the ketomimetic compound is induced by increased insulin sensitivity, which we can assess by the hyperinsulinemic-euglycaemic clamp technique, it would in fact be a promising effect in the AD context. The ketogenic diet induces strong hepatic insulin resistance in mice¹⁰⁰, but direct intracerebroventricular infusion of BHB improved serum glucose levels and peripheral insulin sensitivity in diabetic rats through potentiation of leptin and insulin signalling in the hypothalamus¹⁰¹. Given the fact that we usually observed lower glucose levels after administration of the compound and not higher, it is more likely that our situation is reflected by the second study. As leptin activates anorexigenic neurons¹⁰², potentiation of the leptin signalling pathway is also in accord with the low food intake we observed after compound i.p. injection. Finally, models of AD which display insulin signalling impairments as well as neurodegenerative pathologies similar to those seen in AD exist, and could be the ideal subjects for a hyperinsulinemic-euglycaemic clamp experiment¹⁰³.

Weight loss was clearly associated with reduced calorie intake following compound injection or the switch to a compound-containing diet, but it is possible that other factors affected body weight homeostasis as well. Monitoring food intake of individual animals requires that they be single-housed, and the higher heat loss associated with this could contribute to weight loss or slow

weight gain. The compounds might also affect energy usage by changing the basal metabolic rate, or the activity level. A first interesting experiment would be to feed the compound to mice housed in metabolic cages, alongside mice fed the control diet. Metabolic cages continuously monitor body temperature, CO₂ production, O₂ consumption, food intake and activity. Overall, they give data about the metabolic rate of the mice, which could help us understand weight loss, and quantify any changes in activity level.

Incidentally, concerns about the compounds' effects on weights and glycaemia should not necessarily be extrapolated as such to humans. Mice have a very high metabolism – roughly estimated to be around 7 times faster than humans when expressed as mass-specific metabolic rate¹⁰⁴. Consequently, important variations in weights and glycaemia are not unusual in mice. For instance, mice eat the equivalent of 10 to 20% of their weight every night, and we have seen that a mouse will typically lose more than 10% of its weight during a 24-h fast.

Finally, the study we performed here, together with the future ones and better characterization of the potential safety concerns, will give us valuable experience with these compounds that can eventually be useful in the design of small clinical trials. More specifically, better appreciation of the potential safety concerns would help in monitoring safety in stage I clinical trial. This initial trial would presumably consist in feeding small-but-increasing doses of the compound to a small number of healthy volunteers. Timing and duration of ketosis would be measured, while carefully monitoring for side effects, especially effects on glycaemia, appetite and activity. If successful, results in mouse models may also help in designing stage II clinical trial in a way most likely to capture efficacy. This would presumably consist in administrating the compound to some AD patients who display epileptiform activity on EEG. The most important data to be collected would be epileptiform spikes on EEGs, blood BHB levels, and performance in small cognitive tests.

Towards mechanistic understanding

Concomitantly, a second goal would be to advance towards mechanistic understanding of the compounds' action on epilepsy. Although thorough molecular comprehension is not necessary to move towards clinical use, the ketomimetic compounds are a good model to understand the mechanism of the KD against epilepsy in the DS and epileptiform activity in AD. While it is possible that the KD has different modes of action in mechanistically unrelated epilepsies, it is likely that the mode of action is similar in these two conditions. However, while understanding all molecular aspects of the KD is a mammoth task, such compounds can serve as a simplified version which has fewer molecular effects but still suppresses epileptiform activity, as we have shown in AD and DS

mouse models. Eventually, understanding the molecular process by which the KD suppresses seizures in these contexts could potentially lead to the development of a new generation of potent AEDs specifically targeting this mechanism.

This work already gives some leads towards this goal, which also exemplify the pertinence of this approach. The ketomimetic compound C6-BHB, when i.p. injected in mouse models of AD and the DS, suppressed 40% of epileptiform spikes. Assuming that the KD's mechanism is similar or identical, this would imply that some proposed mechanism of the KD could be ruled out. For instance, glucose restriction alone was proposed to be a key mechanism of the KD action. By demonstrating that suppression of epileptiform spikes can be achieved even in presence of normal amounts of carbohydrates in the diet, we show that it may in fact not be essential.

Most probably, the mechanism of the KD involves ketone bodies, and BHB in particular. Indeed, BHB blood levels of patients on the KD are inversely correlated with seizure frequency and duration^{44,105,106}. In the assumption that BHB is directly responsible for seizure inhibition, its mechanism necessarily includes components of one or both of its broad biological functions - BHB as a fuel, and BHB as a signalling molecule (see β -hydroxybutyrate section). Interestingly, it is possible to experimentally assess which one of these broad biological functions is essential to BHB's anti-epileptic effects. First, metabolic cages can help us estimate the importance of BHB as energy substrate in our context. CO₂ production and O₂ consumption which are measured by the metabolic cage are used to calculate the respiratory quotient (RQ), which serves as an estimation to which fuel is being utilized as energy source. RQ is 1 if exclusively carbohydrates are being metabolized, while it is 0.7 if exclusively lipids are being used. In humans on the KD, RQ is around 0.73¹⁰⁷. By telling us if the mouse's metabolism is significantly switched to ketone body utilization when fed compound, it would allow us to have a guess on whether biological effects of the BHB that require its catabolism should be considered relevant as potential KD mechanisms. A more specific experiment would be to test the (S)-enantiomer of BHB. This is impractical with the fatty acid-linked compounds used in this study, as the C6 or C8 fatty acid will be metabolized into R-BHB even if ester-linked to S-BHB. Direct infusion of S-BHB itself has the same acid or salt problem as R-BHB. Instead, a good candidate for this experiment would be the (S)-enantiomer of the BHB-BD ester (see *Exogenous ketones* section). Upon ester lysis, (S)-BHB will be released, and (S)-BD will be metabolized to (S)-BHB by liver dehydrogenases. (S)-BHB cannot enter the TCA cycle⁵⁹, but specific chirality is not necessary to some BHB's signalling functions, including at least binding to HCAR2⁵⁴ and inhibition of the NLRP3 inflammasome⁵⁹. On the one hand, if feeding or injecting (S)-BHB-BD to AD or DS mice is ineffective at suppressing epileptiform spikes, it would point towards

effects of BHB which require its catabolism. Hypotheses of the KD's mechanism are numerous and will not be reviewed here, but such effects include for instance increased synthesis of GABA, increased ATP synthesis, and less production of ROS¹⁰⁸. On the other hand, if (S)-BHB does suppress seizures, we would know for sure that catabolism of BHB is not essential to the KD efficacy. Rather, it would suggest that the signalling effects of BHB are at the core of the KD's anti-epileptic mechanism. These include for instance NLRP3 inflammasome inhibition, whose inhibition suppresses seizures in rats¹⁰⁹. While this approach seems elegant, it would not be surprising to observe that (S)-BHB suppresses epileptiform spikes but to a lesser extent. Indeed, anti-epileptic effects of the KD, and of BHB in particular, are likely to be multiple and synergistic.

Furthermore, in our particular context, the mechanism by which C6-BHB suppresses epileptiform spikes seems to involve stimulation of the PV neurons. We thus seem to have the endpoint of the mechanism. However, we have seen that the data is less clear in the Dravet mouse model. Even though PV neurons from the Dravet mouse are hypoexcitable in isolated brain slices, the deficiency in gamma oscillations cannot be detected during EEG⁹⁴. To clarify this, we could start by measuring BHB concentrations in the brains of mice fed the compound for around a week. Similar enzymatic kits than the one we used for blood measurements allow to perform this on whole cell lysates. If, compared to control, brain BHB concentrations are significantly higher in the mice fed the compound, we could, in a similar approach than De Stasi et al.⁹⁴, record firing rates of Dravet mouse's PV neurons *in vitro* in presence of BHB, perhaps medium-chain fatty acids, and control solution. This approach would mean starting from the probable endpoint of the BHB mechanism, which could be a better strategy than testing each and every possible effect of the KD by trial and error.

Conclusion

To briefly summarize the major points of our results, we showed that a novel set of compounds, consisting of medium-chain fatty acids ester-linked with a ketone body molecule, were ketogenic in mice via intraperitoneal injection and feeding. The safest and most promising compounds, which were selected for further studies, induced a mild ketosis that reached levels comparable to those induced by calorie restriction. The ketosis seemed sufficient to generate in the liver some biochemical effects usually associated with fasting, namely protein β -hydroxybutyrylation. Moreover, we demonstrated that one of these compounds is also able to mimic in mice the primary clinical use of the ketogenic diet, which is treating epilepsy. Indeed, when administered via intraperitoneal injection, it suppresses 40% of epileptiform spikes in mouse models of Alzheimer's disease and Dravet syndrome.

By showing their promising efficacy in mouse models, this work represents an advance towards the use of ketogenic compounds in patients as a new class of therapeutics for Alzheimer's disease, as well as perhaps certain epilepsies. Elderly with dementia could possibly benefit the most from these type of ketomimetic agents. Indeed, while the ketogenic diet is not reasonably implementable in these patients, data from small clinical trials and mouse models point towards appealing effects of a mild ketosis. In a mouse model of AD, these include suppression of epileptiform discharges, which may constitute a direct cause of certain aspects of the cognitive deficits and behavioural changes. While the KD was shown with greater confidence to reduce the frequency of seizures in most patients with the Dravet syndrome, its limitations remain similarly constraining, and allowing patients to circumvent its use would improve the quality of life of these children and their family.

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