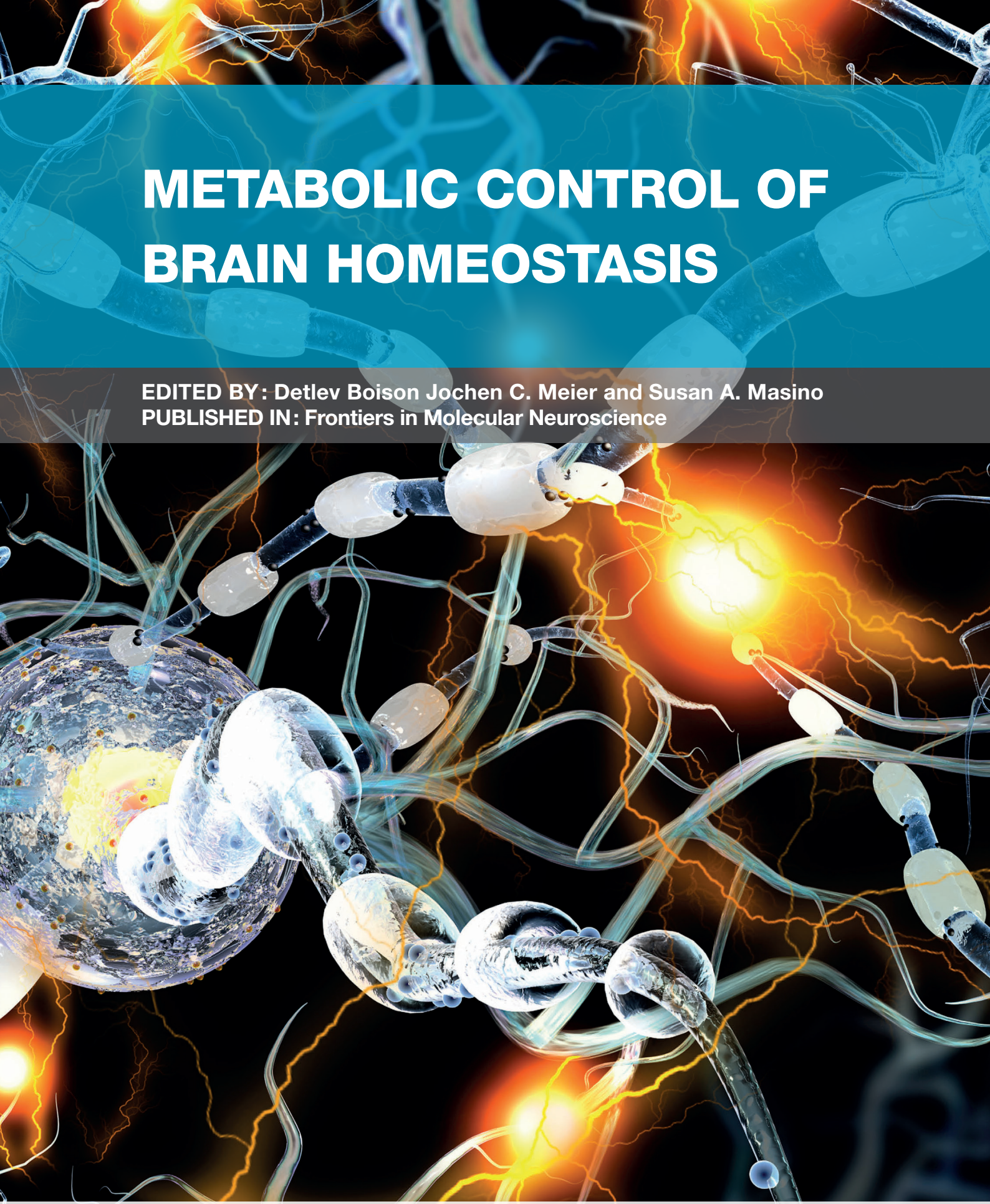
METABOLIC CONTROL OF BRAIN HOMEOSTASIS

EDITED BY: Detlev Boison Jochen C. Meier and Susan A. Masino
PUBLISHED IN: Frontiers in Molecular Neuroscience



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88945-286-6
DOI 10.3389/978-2-88945-286-6

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

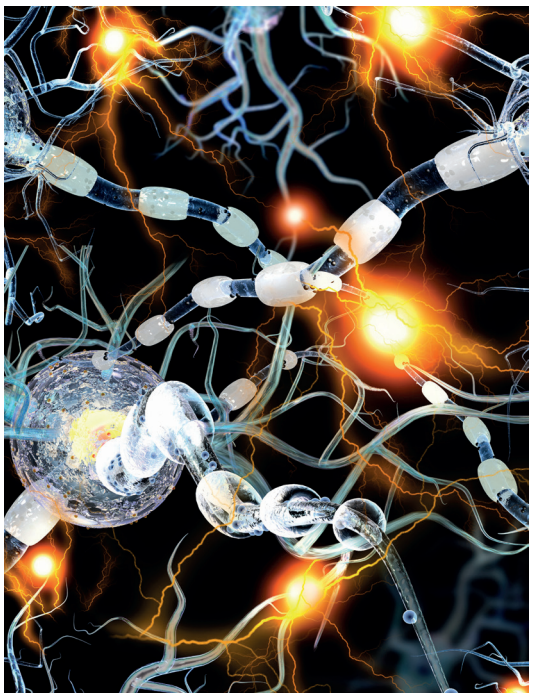
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: **researchtopics@frontiersin.org**

METABOLIC CONTROL OF BRAIN HOMEOSTASIS

Topic Editors:
Detlev Boison, Legacy Research Institute, United States
Jochen C. Meier, TU Braunschweig, Germany
Susan A. Masino, Trinity College, United States



Cover image: Ralwel/Shutterstock.com

Brain function is under metabolic control, which in turn determines the equilibrium of homeostatic systems that affect neuronal and glial networks on the molecular, cellular, and systems levels. The collection of articles ranges from molecules and mechanisms involved in regulating homeostasis and neuronal excitability to therapeutic mechanisms tailored to restore homeostatic function. It also features neurological diseases and novel treatment approaches that are based on metabolic and homeostatic interventions. Together, the collection of articles outlines novel strategies to restore brain function in neurology and highlights limitations of conventional pharmacological approaches. We suggest that restoration of molecular and biochemical networks could lead to a new era of therapeutic opportunities.

Citation: Boison, D., Meier, J. C., Masino, S. A., eds. (2017). Metabolic Control of Brain Homeostasis. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-286-6

Table of Contents

frontiers in Molecular Neuroscience

EDITORIAL
published: 09 June 2017
doi: 10.3389/fnmol.2017.00184

Editorial: Metabolic Control of Brain Homeostasis

Detlev Boison[1], *Jochen C. Meier*[2] *and Susan A. Masino*[3]*

[1] Robert Stone Dow Chair and Director of Neurobiology Research Legacy Research Institute, Portland, OR, United States, [2] TU Braunschweig, Cell Physiology, Braunschweig, Germany, [3] Life Sciences Center, Neuroscience and Psychology, Trinity College, Hartford, CT, United States

Keywords: ketogenic diet, metabolism, epilepsy, brain cancer, Alzheimer's disease, autism, radiation, RNA editing

Editorial on the Research Topic

Metabolic Control of Brain Homeostasis

Fundamental metabolic processes determine homeostasis of neuronal and glial networks at the molecular, cellular, and systems levels. Metabolic resilience promotes brain health and has the potential to prevent or reverse brain disease. A renewed and increasing interest in the relationship between metabolism and homeostasis is evident across multiple disciplines and has the potential to spawn new insights and therapeutic targets. The striking lack of effective treatments in disorders such as brain cancer, Alzheimer's disease, epilepsy, and autism spectrum disorder (ASD) highlights the limitations of conventional, discipline-specific pharmacological approaches.

For this Research Topic authors were encouraged to submit basic research on homeostasis and excitability as well as metabolic mechanisms associated with neurological diseases and novel treatment approaches based on metabolic and homeostatic interventions. This special issue features several papers examining molecular mechanisms involved in neural network homeostasis and higher brain function. From an evolutionary perspective, "early" metabolites such as adenosine (a structural component of ATP and RNA) and glycine (the simplest amino acid) assumed multiple functions as structural building blocks of cellular systems, as biochemical metabolites involved in energy and carbon homeostasis, as epigenetic modulators, and finally (i.e., an evolutionarily "late" function) as receptor ligands and signaling molecules. Not surprisingly, dysregulation of those key metabolites is implicated in virtually every neurological condition, whereas therapeutic reconstruction of biochemical homeostasis has potential for disease prevention and cure (Boison). A molecular link to basic biochemical mechanisms is further provided in an original research article that ascribes a specific role of the methionine cycle-associated enzyme enolase phosphatase 1 in apoptotic response mechanisms triggered by oxidative stress (Zhang et al.). The link between metabolic homeostasis and neuronal activity is evident in the relationship between ion homeostasis and energy demand of fast neuronal network oscillations associated with higher brain functions—including sensory perception, attentional selection, and memory formation and requiring timed synaptic excitation and inhibition with glutamate and GABA, respectively (Kann et al.). Fast neuronal network oscillations are characterized by high oxygen consumption and significant changes in the cellular redox state, indicating rapid adaptations in glycolysis and oxidative phosphorylation. A second review article highlights the complex role of glycogen and its role in brain energy and particularly synaptic plasticity (Waitt et al.). Exquisite sensitivity to metabolic stress is essential for adaptation and plasticity and confers vulnerability of higher brain functions to injury and disease.

Advances in sequencing technologies led to the discovery of amino acid-recoding RNA editing in many gene transcripts. In this context, recent advances in A-to-I and C-to-U RNA editing are reviewed. This mechanism points to the systemic relevance of the neurotransmitter receptor for glycine (GlyR) and possible clues to disease mechanisms. C-to-U RNA editing of GlyR-coding transcripts is increased in the hippocampus of patients with intractable temporal lobe epilepsy

OPEN ACCESS

Edited and reviewed by:
Jean-Marc Taymans, Institut National de la Santé et de la Recherche Médicale (INSERM), France

***Correspondence:**
Susan A. Masino
susan.masino@trincoll.edu

Received: *17 May 2017*
Accepted: *26 May 2017*
Published: *09 June 2017*

Citation:
Boison D, Meier JC and Masino SA (2017) Editorial: Metabolic Control of Brain Homeostasis. Front. Mol. Neurosci. 10:184. doi: 10.3389/fnmol.2017.00184

and can provoke completely different symptoms depending on the neuron type that is affected (Winkelmann et al., 2014; Çalişkan et al., 2016). These recent discoveries provide further evidence for the concept of excitatory/inhibitory "imbalance" in current psychiatric neuroscience (Eichler and Meier, 2008), but we ultimately need to identify susceptible neuron types and, in particular, differentiate among "inhibitory" GABAergic neuron types (Lovett-Barron et al., 2014; Çalişkan et al., 2016; Meier et al.). An excitatory/inhibitory "imbalance" and excess excitability can have many causes: Westmark et al. delineate the homeostatic role of amyloid-β protein precursor (APP) and its metabolites in fragile X syndrome in humans and in the Fmr1KO mouse model. APP seems to serve as a rheostat where too much or too little causes hyperexcitability—suggesting that normalizing APP levels can address aspects of this pathophysiology.

Regarding translational and clinical research, a study of the healthy human brain indicates the existence of specific metabolomic profiles in different brain regions (Cabré et al.). An analysis of post-traumatic brain injury cerebrospinal fluid finds that mortality at 6 months can be predicted by levels of cortisol and BDNF, particularly in younger people, and suggests a regulatory role for cortisol (Munoz et al.). Several articles look at changes mobilized by a metabolic therapy used to treat epileptic seizures for nearly 100 years: the ketogenic diet (Masino, 2017). These studies include strategies to mimic the diet *in vitro* and in transitional *in vitro*/*in vivo* models (Kawamura et al.). In an original research paper, anxiolytic behavioral effects of ketone-based metabolism—mobilized by administering a ketone ester to induce nutritional ketosis—are reported in two rat strains (Ari et al.). A second original paper compared ketosis induced by exogenous ketones vs. a ketogenic diet and found similar biochemical changes but more robust behavioral effects of the diet (Brownlow et al.). Laboratory and clinical evidence is reviewed regarding the prevalence of dysfunctional mitochondria and altered metabolism in ASD alongside current limited but positive data on the role of ketogenic diets and metabolic therapy in reducing ASD symptoms and common comorbidities—potentially via adenosine or other mechanisms (Cheng et al.). An original research article demonstrates that enhancing adenosine in brain through pharmacological blockade of the enzyme adenosine kinase prevents radiation-induced cognitive impairment in rats (Acharya et al.).

Finally, the role of metabolic factors and the promise of metabolic therapy in brain cancer and Alzheimer's disease are implicated directly in several articles: an original research article reveals a critical role of the proneural basic helix-loop-helix transcription factor human achaete-scute homolog 1 (hASH1) in neuroblastoma. This study shows that hASH1 suppresses neuronal differentiation by inhibiting transcription at the retinoic acid receptor element, highlighting hASH1 as a key determinant of neuroblastoma resistance to differentiation therapy (Kasim et al.). Metabolic therapy with a ketogenic diet is highlighted as a multifaceted therapy for glioma, particularly glioblastoma, and clinical and basic research is reviewed indicating that a metabolic approach may both limit tumor growth and augment the efficacy of chemotherapy and radiation (Woolf et al.). Finally, hypometabolism is a hallmark of Alzheimer's disease, and regional glucose hypometabolism is also found in patients at risk for Alzheimer's disease and may be a presymptomatic marker. However, hypometabolism is reversed by administering ketones as an alternative fuel—suggesting that a selective problem with glucose metabolism may underlie the pathophysiology of early onset Alzheimer's disease, and that a metabolic therapy may prevent or delay the disorder (Cunnane et al.).

Taken together, this research topic provides a cross-cutting perspective of the relationship between metabolism and homeostasis and showcases novel metabolic therapies to restore molecular and biochemical networks. The direct and potentially imminent clinical implications of several of these articles will be exciting to follow as the field moves forward. While a range of topics and mechanisms is featured herein, there is room for much more.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

Funding by NIH (NS084920, NS088024, MH083973 to DB; NS065957 to DB and SM; NS066392 to SM) and Deutsche Forschungsgemeinschaft DFG (Priority Programme SPP1784 grant No. ME2075/7-1 to JM) is acknowledged.

REFERENCES

Çalişkan, G., Müller, I., Semtner, M., Winkelmann, A., Raza, A. S., Hollnagel, J. O., et al. (2016). Identification of parvalbumin interneurons as cellular substrate of fear memory persistence. *Cereb. Cortex* 26, 2325–2340. doi: 10.1093/cercor/bhw001

Eichler, S. A., and Meier, J. C. (2008). E-I balance and human diseases-from molecules to networking. *Front. Mol. Neurosci.* 1, 2. doi: 10.3389/neuro.02.002.2008

Lovett-Barron, M., Kaifosh, P., Kheirbek, M. A., Danielson, N., Zaremba, J. D., Reardon, T. R., et al. (2014) Dendritic inhibition in the hippocampus supports fear learning. *Science* 343, 857–863. doi: 10.1126/science.1247485

Masino, S. A. (ed.). (2017) *Ketogenic Diet and Metabolic Therapies: Expanded Roles in Health and Disease.* New York, NY: Oxford University Press USA.

Winkelmann, A., Maggio, N., Eller, J., Caliskan, G., Semtner, M., Häussler, U., et al. (2014). Changes in neural network homeostasis trigger neuropsychiatric symptoms. *J. Clin. Invest.* 124, 696–711. doi: 10.1172/JCI71472

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Molecular Neuroscience

MINI REVIEW
published: 13 April 2016
doi: 10.3389/fnmol.2016.00026

The Biochemistry and Epigenetics of Epilepsy: Focus on Adenosine and Glycine

Detlev Boison *

Robert Stone Dow Neurobiology Laboratories, Legacy Research Institute, Portland, OR, USA

Epilepsy, one of the most prevalent neurological conditions, presents as a complex disorder of network homeostasis characterized by spontaneous non-provoked seizures and associated comorbidities. Currently used antiepileptic drugs have been designed to suppress neuronal hyperexcitability and thereby to suppress epileptic seizures. However, the current armamentarium of antiepileptic drugs is not effective in over 30% of patients, does not affect the comorbidities of epilepsy, and does not prevent the development and progression of epilepsy (epileptogenesis). Prevention of epilepsy and its progression remains the Holy Grail for epilepsy research and therapy development, requiring novel conceptual advances to find a solution to this urgent medical need. The methylation hypothesis of epileptogenesis suggests that changes in DNA methylation are implicated in the progression of the disease. In particular, global DNA hypermethylation appears to be associated with chronic epilepsy. Clinical as well as experimental evidence demonstrates that epilepsy and its progression can be prevented by biochemical manipulations and those that target previously unrecognized epigenetic functions contributing to epilepsy development and maintenance of the epileptic state. This mini-review will discuss, epigenetic mechanisms implicated in epileptogenesis and biochemical interactions between adenosine and glycine as a conceptual advance to understand the contribution of maladaptive changes in biochemistry as a major contributing factor to the development of epilepsy. New findings based on biochemical manipulation of the DNA methylome suggest that: (i) epigenetic mechanisms play a functional role in epileptogenesis; and (ii) therapeutic reconstruction of the epigenome is an effective antiepileptogenic therapy.

Keywords: epilepsy, epileptogenesis, adenosine, adenosine kinase, glycine, glycine transporter 1, epigenetics, DNA methylation

OPEN ACCESS

Edited by:
Jean-Marc Taymans, UMR1172, Jean-Pierre Aubert Research Center, France

Reviewed by:
Massimo Mantegazza, CNRS UMR7275 and University of Nice Sophia Antipolis, France
Rafal Kaminski, UCB Pharma, Belgium

***Correspondence:**
Detlev Boison
dboison@downeurobiology.org

Received: *04 January 2016*
Accepted: *31 March 2016*
Published: *13 April 2016*

Citation:
Boison D (2016) The Biochemistry and Epigenetics of Epilepsy: Focus on Adenosine and Glycine. Front. Mol. Neurosci. 9:26. doi: 10.3389/fnmol.2016.00026

INTRODUCTION

Biological evolution is thought to have started with relatively simple, versatile, and multifunctional metabolites (Miller and Urey, 1959a,b). Adenosine was likely part of the "primordial soup" at the origin of life on Earth (Oro, 1961). Therefore it is not surprising that adenosine is an integral component of ATP, RNA (including poly-A tails), NAD, and other compounds essential for basic biochemistry and mitochondrial bioenergetics. Glycine in turn is the most primitive amino acid, which has additional biochemical functions in carbon metabolism. It is highly likely that the biochemical and epigenetic

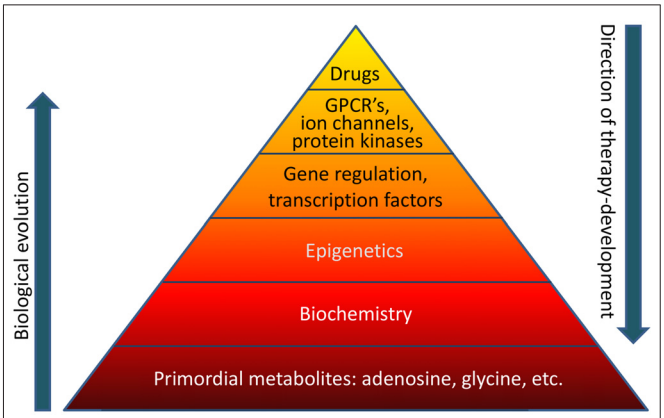


FIGURE 1 | The "pyramid of life". Evolutionary complexity started with key metabolites and biochemical mechanisms, which form the basis of all forms of life. In contrast, conventional drug development follows a top-down approach. GPCR's, G protein coupled receptors.

control of genes through global biochemical regulation preceded the "invention" of transcription factors, which later assumed the role to fine-tune a preexisting primordial biochemistry-based regulatory system. For example, an energy crisis would lower ATP needed for RNA synthesis and increase adenosine, thereby promoting increased DNA methylation as will be discussed in this mini-review in more detail. Both processes would reduce gene transcription globally and conserve energy. Therefore, primordial regulatory networks acting on a global homeostatic level likely preceded the "invention" of gene specific mechanisms that require sophisticated control through transcription factors, which in turn are regulated by G protein coupled receptors (GPCRs) and protein kinase pathways. In contrast, therapy development conventionally starts with the pharmacology of drugs. For example, benzodiazepines were almost discovered by chance in 1957 leading to the subsequent characterization of the "benzodiazepine receptor" in the CNS in 1977. It turned out that the benzodiazepine binding site was in fact an integral part of the $GABA_A$ receptor complex (Möhler and Okada, 1977). Drug-driven therapy development led to a major focus on "druggable" GPCRs, ion channels, and protein kinases, which still form the main-stay of CNS therapeutics today. Methods to exploit gene regulation therapeutically are still in its infancy and the therapeutic potential of epigenetic, biochemical, and metabolomic approaches constitutes a new frontier in therapy development. If the basis of the pyramid depicted in **Figure 1** is overlooked, it becomes obvious that a traditional pharmacological top-down treatment approach has limitations.

THE BIOCHEMISTRY OF EPILEPSY

In the following, I will focus on temporal lobe epilepsy (TLE), the most common form of epilepsy in adult patients, and the most thoroughly studied form of epilepsy in animal models, as well as on two key metabolites, adenosine and glycine, whose homeostasis is known to be affected in the epileptic brain. Adenosine, primarily through activation of adenosine A_1 receptors, is an endogenous anticonvulsant and seizure terminator of the brain (Dragunow, 1991; During and Spencer, 1992) that also controls a wide range of cognitive and psychiatric phenotypes (Boison et al., 2012). In human surgical specimens, as well as in rodent models of TLE, overexpression of adenosine kinase (ADK) and resulting adenosine deficiency associate with astrogliosis and constitute a pathological hallmark of TLE (Riban et al., 2002; Gouder et al., 2004; Fedele et al., 2005; Boison, 2008; Li et al., 2008, 2012; Aronica et al., 2011, 2013). Consequently, therapeutic adenosine augmentation effectively suppresses seizures in a wide range of rodent models of epilepsy (Huber et al., 2001; Zuchora et al., 2001; Gouder et al., 2003; Anschel et al., 2004; Vianna et al., 2005; Li et al., 2007b, 2008; Wilz et al., 2008; Boison, 2009, 2012a; Boison and Stewart, 2009; Van Dycke et al., 2010). A focus of this mini-review however is the underappreciated biochemical adenosine receptor (AR) independent effects of adenosine that are tightly linked to the control of DNA methylation and that are under the control of ADK, an enzyme which also has a specific isoform expressed in the nucleus of cells (Boison, 2013). The cytoplasmic isoform of the enzyme is thought to regulate the homeostatic pool of adenosine thereby determining the level of AR activation (Boison and Aronica, 2015), whereas the nuclear isoform of the enzyme strongly affects DNA methylation status (Williams-Karnesky et al., 2013). Interestingly, ADK undergoes biphasic expression changes during epileptogenesis in modeled TLE (Gouder et al., 2004; Li et al., 2008; Boison, 2013), which form the basis of the ADK hypothesis of epileptogenesis: Acute insults to the brain such as traumatic brain injury (Clark et al., 1997), seizures (During and Spencer, 1992; Gouder et al., 2004), or a stroke (Pignataro et al., 2008) lead to an acute surge in adenosine associated with transient downregulation of ADK within the first 2 to 3 h after the injury (Gouder et al., 2004; Pignataro et al., 2008). This acute phase is followed by a "latent period" of epileptogenesis, which occurs during the first few days or weeks after an insult in rodent models, or weeks and months in humans. During this latent period, inflammatory processes are activated that lead to microglial and astroglial activation (Nabbout et al., 2011; Vezzani et al., 2011; Devinsky et al., 2013). Astrogliosis is associated with increases in ADK expression and consequential development of adenosine deficiency. We have previously shown that seizures originate in areas of astrogliosis with overexpression of ADK (Li et al., 2008, 2012), that seizure onset during epileptogenesis temporally coincides with the emergence of astrogliosis and overexpressed ADK (Li et al., 2007a), that overexpression of ADK as such is sufficient to generate partial seizures (Li et al., 2007a, 2008), and that overexpression of ADK triggers hypermethylation of DNA (Williams-Karnesky et al., 2013). Since therapeutic adenosine augmentation restores normal DNA methylation levels and prevents epilepsy progression long-term (Williams-Karnesky et al., 2013) increased ADK and increased DNA methylation status might form a vicious cycle implicated in the progression and maintenance of epilepsy. Therefore, dysregulation of ADK appears to play a significant role in the processes that turn a normal brain into an epileptic brain.

In the hippocampal formation, glycine can exert opposing effects that depend on the activation of presynaptic (Kubota et al., 2010; Winkelmann et al., 2014) vs. postsynaptic glycine receptors (GlyRs; Aroeira et al., 2011). It has recently been demonstrated (Chen et al., 2014) that low concentrations of glycine (10 μM) exert pro-convulsive effects, whereas higher glycine concentrations (100 μM) attenuate recurrent epileptiform discharges. The pro-convulsive actions of presynaptic GlyRs expressed on glutamatergic synapses (Winkelmann et al., 2014) is further supported by findings showing that the expression of edited GlyR encoding mRNAs are increased in the human epileptic hippocampus (Eichler et al., 2008) and that GlyR RNA editing regulates glycine affinity (Meier et al., 2005). These findings suggest that glycine homeostasis plays a crucial role in maintaining the balance between increased and decreased neuronal excitability. Hippocampal glycine is largely regulated by its reuptake transporter, glycine transporter 1 (GlyT1), found in both excitatory neurons and astrocytes (Tsai et al., 2004; Aragón and López-Corcuera, 2005; Cubelos et al., 2005; Eulenburg et al., 2005; Martina et al., 2005; Betz et al., 2006). Consequently, the genetic deletion of GlyT1 increased synaptic glycine availability (Gomeza et al., 2003). Engineered mice with a genetic deletion of GlyT1 in forebrain were characterized by a decrease in hippocampal glycine uptake, an increase in hippocampal NMDAR function, and a wide spectrum of pro-cognitive effects (Yee et al., 2006; Möhler et al., 2008, 2011). Therefore, GlyT1 is considered a promising target for the treatment of cognitive symptoms in schizophrenia and several compounds have been tested in phase II and III clinical trials (Black et al., 2009; Singer et al., 2009; Möhler et al., 2011). A recent study (Shen et al., 2015) provided the first comprehensive analysis of GlyT1 dysregulation in chronic TLE. GlyT1 expression during epileptogenesis was characterized as a biphasic response with initial downregulation of GlyT1 after epileptogenesis-precipitating seizures followed by sustained pathological overexpression of GlyT1 in chronic epilepsy as demonstrated in two mechanistically different models of TLE in mice and rats (Shen et al., 2015). It was further demonstrated that human TLE is likewise associated with increased levels of GlyT1. Conversely, the pharmacological suppression of GlyT1 or the genetic ablation of GlyT1 in the hippocampus provided robust reduction of both acute as well as chronic seizure activity in three different model systems (Shen et al., 2015). Therefore, glycinergic regulation of network excitability is altered in epilepsy and GlyT1 presents a rational therapeutic target for the treatment of epilepsy. Pathological overexpression of GlyT1 in progressive epilepsy also implies altered interactions of GlyT1 with the transmethylation pathway (**Figure 2**), a novel hypothesis further discussed below.

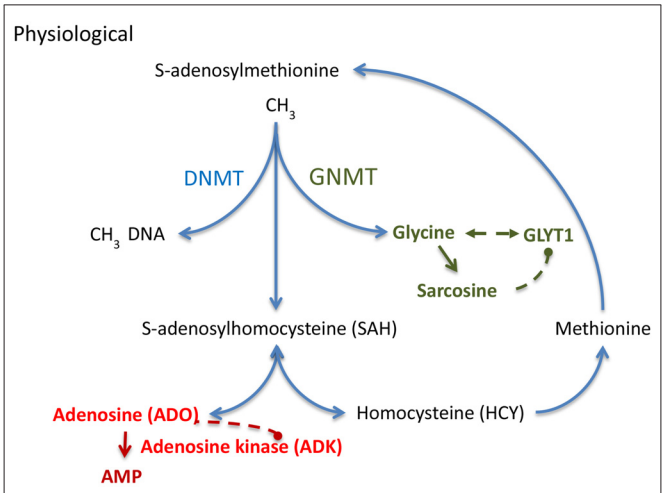


FIGURE 2 | S-adenosylmethionine (SAM) dependent transmethylation pathway, which is under the control of adenosine and glycine. Adenosine and glycine are regulated by adenosine kinase (ADK) and glycine transporter 1 (GlyT1), respectively. DNMT, DNA methyltransferase; GNMT, glycine N-methyltransferase.

THE EPIGENETICS OF EPILEPSY

The role of epigenetics in epilepsy development is a new and emerging research area (Garriga-Canut et al., 2006; Qureshi and Mehler, 2010; Kobow and Blümcke, 2012; Lubin, 2012; Henshall and Kobow, 2015). The fact that epigenetic changes might play a significant role at least in TLE is important because in contrast to genetic mutations, epigenetic changes are potentially reversible. The knowledge of epigenetic mechanisms implicated in the development of epilepsy provides a conceptual and mechanistic framework for the future development of epigenetic therapies tailored to prevent epilepsy (antiepileptogenic) or its progression (disease modifying). Currently used antiepileptic therapies fail to address the underlying causes of epilepsy and do not halt epileptogenesis (Löscher and Brandt, 2010). Epileptogenesis is characterized by a progressive increase in frequency and severity of spontaneous recurrent seizures (SRS). Several mechanisms are implicated in the epileptogenic cascade including neuro-inflammatory responses, neuronal cell loss, mossy fiber sprouting, aberrant connectivity, and gliosis coupled with adenosine dysfunction (Dudek et al., 2002; Seifert et al., 2010; Vezzani et al., 2011; Aronica et al., 2012; Boison, 2012b). One potential unifying factor behind many of the pathological changes in epileptogenesis may be epigenetic modifications, which are likely further potentiated by epileptogenesis itself (Qureshi and Mehler, 2010; Kobow and Blümcke, 2011). Epigenetic modifications, which alter gene transcription without modifying the underlying DNA sequence, are plastic and can respond rapidly in response to environmental cues, an important endogenous mechanism for the control of gene expression. Changes in histone acetylation and methylation, as well as changes in DNA methylation have been shown to occur in mature cells in the central nervous system (CNS; Ma et al., 2009; Feng et al., 2010). Importantly, these changes occur regularly and rapidly. Even a single episode of neural synchronization exceeding 30 s in the hippocampus induces DNA methylation-dependent alterations in transcription of immediate-early genes and initiates a cascade of transcription factors contributing to long-term circuit alterations (Nelson et al., 2008). Therefore,

epigenetic modifications offer new therapeutic alternatives to interfere with epileptogenesis.

THE METHYLATION HYPOTHESIS OF EPILEPTOGENESIS

Although several epigenetic mechanisms such as histone modifications that involve the addition or removal of methyl or acetyl groups might be implicated in epileptogenesis (Henshall and Kobow, 2015), recent evidence points to a critical role of DNA methylation changes for the development and progression of epilepsy, which will be discussed in the following. Altered DNA methylation in the brain has been implicated in psychiatric, neurodegenerative, and neurological conditions, including epilepsy (Kobow et al., 2009; Ma et al., 2009; Feng et al., 2010; Martin and Wong, 2013; Masliah et al., 2013; Coppieters et al., 2014; Tremolizzo et al., 2014). The methylation hypothesis of epileptogenesis suggests that seizures by themselves can induce epigenetic modifications and thereby aggravate the epileptogenic condition (Kobow and Blümcke, 2011). Specifically, increased activity of DNA methylating enzymes as well as hypermethylation of DNA has been associated with the development of human and experimental epilepsy (Kobow et al., 2009, 2013; Zhu et al., 2012; Williams-Karnesky et al., 2013; Miller-Delaney et al., 2015). Thus, interference with DNA methylation offers novel conceptual opportunities to prevent epilepsy.

BRAIN HOMEOSTASIS AND THE CONTROL OF DNA METHYLATION

DNA methylation status depends on the equilibrium of biochemical enzyme reactions that add methyl groups to cytidine groups in the DNA (5-methylcytidine, 5mC) catalyzed by DNA methyltransferases (DNMTs) and those that convert methyl groups to hydroxymethyl groups catalyzed by Ten-eleven translocation (TET) enzymes in preparation for active DNA demethylation. Here I will focus on those mechanisms that add methyl groups to DNA; those mechanisms are linked to the S-adenosylmethionine (SAM) dependent transmethylation pathway (**Figure 2**), which is under the control of adenosine and glycine regulated by ADK (Boison et al., 2002; Boison, 2013) and GlyT1 (Gomeza et al., 2003; Yee et al., 2006), respectively. DNA methylation requires the donation of a methyl group from SAM, a process that is facilitated by DNMTs. The resulting product, S-adenosylhomocysteine (SAH) is then further converted into adenosine and homocysteine (HCY) by SAH hydrolase (SAHH). Critically, the equilibrium constant of the SAHH enzyme lies in the direction of SAH formation (Kredich and Martin, 1977); therefore, the reaction will only proceed when adenosine and HCY are constantly removed (Kredich and Martin, 1977; Boison et al., 2002). If metabolic clearance of adenosine through ADK is impaired, SAH levels rise (Boison et al., 2002). SAH in turn inhibits DNMTs through product inhibition (James et al., 2002). Based on adenosine's role as obligatory end product of DNA methylation, we conclude that an increase in ADK and the resulting decrease in adenosine, as seen in chronic epilepsy (Li et al., 2008; Masino et al., 2011), would drive increased global DNA methylation in the brain. This process may be amplified, because adenosine is an inhibitor of ADK (Boison, 2013). Therapeutic adenosine augmentation may thus effectively reverse pathological DNA hypermethylation and thereby prevent epilepsy progression. The recent discovery of glycine-N-methyltransferase (GNMT) in the hippocampus (Carrasco et al., 2014) suggests that the availability of hippocampal glycine also controls the SAM-dependent transmethylation pathway by competing for methyl-groups. Increased GlyT1 as seen in chronic TLE (Shen et al., 2015) is expected to affect DNA methylation through interference with glycine homeostasis. Interestingly, the methylation of glycine leads to the formation of sarcosine, which is an endogenous inhibitor of GlyT1 (Javitt, 2012). Therapeutic glycine augmentation (e.g., via diet) may thus effectively divert methyl groups to the formation of sarcosine and thereby reduce: (i) pathological DNA hypermethylation; (ii) ameliorate the effects of pathologically overexpressed GlyT1; and (iii) prevent epilepsy progression.

TOWARDS EPIGENETIC THERAPIES FOR EPILEPSY PREVENTION

The antiepileptogenic potential of transient focal adenosine-delivery was tested in a rat model of systemic kainic acid (KA)-induced progressive TLE (Williams-Karnesky et al., 2013). Young male rats (130–150 g) received an acute dose of KA (12 mg/kg i.p.) to trigger status epilepticus (SE). Only rats that exhibited at least 3 h of acute convulsive SE were used further. Rats were subjected to continuous long-term monitoring to quantify seizure activity. Once rats had reached a stable rate of 3–4 SRS per week at 9 weeks post KA, the animals were randomized and each rat received bilateral intraventricular adenosine-releasing silk-implants, silk-only implants, or a corresponding sham treatment. Adenosine releasing implants were designed to transiently deliver a stable dose of 250 ng adenosine per brain ventricle per day restricted to 10 days of drug delivery (Szybala et al., 2009). Twenty four hours after the surgery, continuous video monitoring was initiated, maintained for 4 weeks, and resumed after a 4 week hiatus for an additional 4 weeks. In the control groups seizures continued to increase both in number and severity. In contrast, in recipients of adenosine-releasing implants, seizures were almost completely suppressed after polymer implantation. Remarkably, reduced seizure activity was maintained far beyond expiration of adenosine-release from the polymer for at least 12 weeks following implantation. Even at 12 weeks after implantation, seizure incidence was reduced by more than 70%. Importantly, and in line with prolonged reduction of seizures, mossy fiber sprouting at 21 weeks following KA was significantly attenuated in adenosine-treated rats compared to controls. In line with those profound antiepileptogenic effects, the transient delivery of adenosine restored normal DNA methylation status long-term. These data demonstrate that the transient delivery of adenosine is sufficient

to restore normal DNA methylation status and to prevent epilepsy progression long-term (Williams-Karnesky et al., 2013).

CONCLUSIONS AND OUTLOOK

The realization that a transient local dose of adenosine can have long-lasting antiepileptogenic effects based on shifting the transmethylation equilibrium through mass action may offer new therapeutic opportunities for small molecule ADK inhibitors. ADK inhibitors had been in pre-clinical development in the early 2000's for the indications of seizure management in chronic epilepsy, the control of chronic pain and robust anti-inflammatory effects in chronic conditions (McGaraughty et al., 2005). Although highly efficient in preclinical models, further drug development was halted due to unacceptable side effects related to chronic drug dosing (McGaraughty et al., 2005). The first generation of ADK inhibitors was designed to augment beneficial AR dependent effects of adenosine by raising extracellular levels of adenosine, which are at the same time responsible for wide-spread systemic adverse effects of those agents. Of note, patients with inborn global ADK deficiency develop hepatic encephalopathy and a wide range of neurological symptoms (Bjursell et al., 2011), however it is currently not known whether those symptoms are a primary cause of ADK deficiency in the brain, or secondary to hepatic encephalopathy. Early drug development efforts created a bias for the identification of agents with preferential action on the cytoplasmic isoform of ADK. ADK inhibitors with a higher selectivity for the nuclear isoform of ADK might capitalize on the epigenetic effects of adenosine while minimizing AR-mediated adverse effects. In addition, the short term use of ADK inhibitors, over days as opposed to chronic sustained drug dosing, might be acceptable if long-lasting epigenetically based therapeutic benefits can be achieved. Challenges for drug development remain. It needs to be determined whether new therapeutic agents can enter the brain and whether a higher level of selectivity for specific isoforms of ADK can be achieved. Due to the different distribution of nucleoside transporters within the brain there might be opportunities for the development of cell-type or isoform selective therapies. Glycine modifying therapies might constitute an alternative avenue to affect DNA methylation and potentially epileptogenesis. However, it needs to be determined how the adenosine and glycine systems interact on the epigenetic level. As discussed in this mini-review, understanding the biochemistry of epileptogenesis might light to the development of novel forms of therapeutic intervention.

AUTHOR CONTRIBUTIONS

DB conceptualized and wrote the manuscript.

ACKNOWLEDGMENTS

The author's work is supported through grants from the National Institutes of Health (R01 NS084920, R21 NS088024, R01 MH083973) and through the Legacy Good Samaritan Hospital Foundations.

REFERENCES

Anschel, D. J., Ortega, E. L., Kraus, A. C., and Fisher, R. S. (2004). Focally injected adenosine prevents seizures in the rat. *Exp. Neurol.* 190, 544–547. doi: 10.1016/j.expneurol.2004.07.017

Aragón, C., and López-Corcuera, B. (2005). Glycine transporters: crucial roles of pharmacological interest revealed by gene deletion. *Trends Pharmacol. Sci.* 26, 283–286. doi: 10.1016/j.tips.2005.04.007

Aroeira, R. I., Ribeiro, J. A., Sebastião, A. M., and Valente, C. A. (2011). Age-related changes of glycine receptor at the rat hippocampus: from the embryo to the adult. *J. Neurochem.* 118, 339–353. doi: 10.1111/j.1471-4159.2011.07197.x

Aronica, E., Ravizza, T., Zurolo, E., and Vezzani, A. (2012). Astrocyte immune responses and epilepsy. *Glia* 60, 1258–1268. doi: 10.1002/glia.22312

Aronica, E., Sandau, U. S., Iyer, A., and Boison, D. (2013). Glial adenosine kinase—A neuropathological marker of the epileptic brain. *Neurochem. Int.* 63, 688–695. doi: 10.1016/j.neuint.2013.01.028

Aronica, E., Zurolo, E., Iyer, A., de Groot, M., Anink, J., Carbonell, C., et al. (2011). Upregulation of adenosine kinase in astrocytes in experimental and human temporal lobe epilepsy. *Epilepsia* 52, 1645–1655. doi: 10.1111/j.1528-1167.2011.03115.x

Betz, H., Gomeza, J., Armsen, W., Scholze, P., and Eulenburg, V. (2006). Glycine transporters: essential regulators of synaptic transmission. *Biochem. Soc. Trans.* 34, 55–58. doi: 10.1042/bst0340055

Bjursell, M. K., Blom, H. J., Cayuela, J. A., Engvall, M. L., Lesko, N., Balasubramaniam, S., et al. (2011). Adenosine kinase deficiency disrupts the methionine cycle and causes hypermethioninemia, encephalopathy and abnormal liver function. *Am. J. Hum. Genet.* 89, 507–515. doi: 10.1016/j.ajhg.2011.09.004

Black, M. D., Varty, G. B., Arad, M., Barak, S., De Levie, A., Boulay, D., et al. (2009). Procognitive and antipsychotic efficacy of glycine transport 1 inhibitors (GlyT1) in acute and neurodevelopmental models of schizophrenia: latent inhibition studies in the rat. *Psychopharmacology (Berl)* 202, 385–396. doi: 10.1007/s00213-008-1289-2

Boison, D. (2008). The adenosine kinase hypothesis of epileptogenesis. *Prog. Neurobiol.* 84, 249–262. doi: 10.1016/j.pneurobio.2007.12.002

Boison, D. (2009). Adenosine augmentation therapies (AATs) for epilepsy: prospect of cell and gene therapies. *Epilepsy Res.* 85, 131–141. doi: 10.1016/j.eplepsyres.2009.03.019

Boison, D. (2012a). "Adenosine augmentation therapy for epilepsy," in *Jasper's Basic Mechanisms of the Epilepsies*, 4th Edn. eds J. L. Noebels, M. Avoli, M. A. Rogawski, R. W. Olsen and A. V. Delgado-Escueta (Oxford, NY: Oxford University Press), 1150–1160.

Boison, D. (2012b). Adenosine dysfunction in epilepsy. *Glia* 60, 1234–1243. doi: 10.1002/glia.22285

Boison, D. (2013). Adenosine kinase: exploitation for therapeutic gain. *Pharmacol. Rev.* 65, 906–943. doi: 10.1124/pr.112.006361

Boison, D., and Aronica, E. (2015). Comorbidities in neurology: is adenosine the common link? *Neuropharmacology* 97, 18–34. doi: 10.1016/j.neuropharm.2015.04.031

Boison, D., and Stewart, K.-A. (2009). Therapeutic epilepsy research: from pharmacological rationale to focal adenosine augmentation. *Biochem. Pharmacol.* 78, 1428–1437. doi: 10.1016/j.bcp.2009.08.005

Boison, D., Scheurer, L., Zumsteg, V., Rülicke, T., Litynski, P., Fowler, B., et al. (2002). Neonatal hepatic steatosis by disruption of the adenosine kinase gene. *Proc. Natl. Acad. Sci. U S A* 99, 6985–6990. doi: 10.1073/pnas.092642899

Boison, D., Singer, P., Shen, H. Y., Feldon, J., and Yee, B. K. (2012). Adenosine hypothesis of schizophrenia—opportunities for pharmacotherapy. *Neuropharmacology* 62, 1527–1543. doi: 10.1016/j.neuropharm.2011.01.048

Carrasco, M., Rabaneda, L. G., Murillo-Carretero, M., Ortega-Martínez, S., Martínez-Chantar, M. L., Woodhoo, A., et al. (2014). Glycine N-methyltransferase expression in the hippocampus and its role in

neurogenesis and cognitive performance. *Hippocampus* 24, 840–852. doi: 10.1002/hipo.22274

Chen, R., Okabe, A., Sun, H., Sharopov, S., Hanganu-Opatz, I. L., Kolbaev, S. N., et al. (2014). Activation of glycine receptors modulates spontaneous epileptiform activity in the immature rat hippocampus. *J. Physiol.* 592, 2153–2168. doi: 10.1113/jphysiol.2014.271700

Clark, R. S., Carcillo, J. A., Kochanek, P. M., Obrist, W. D., Jackson, E. K., Mi, Z., et al. (1997). Cerebrospinal fluid adenosine concentration and uncoupling of cerebral blood flow and oxidative metabolism after severe head injury in humans. *Neurosurgery* 41, 1284–1292; discussion 1292–1293. doi: 10.1097/00006123-199712000-00010

Coppieters, N., Dieriks, B. V., Lill, C., Faull, R. L., Curtis, M. A., and Dragunow, M. (2014). Global changes in DNA methylation and hydroxymethylation in Alzheimer's disease human brain. *Neurobiol. Aging* 35, 1334–1344. doi: 10.1016/j.neurobiolaging.2013.11.031

Cubelos, B., Giménez, C., and Zafra, F. (2005). Localization of the GLYT1 glycine transporter at glutamatergic synapses in the rat brain. *Cereb Cortex* 15, 448–459. doi: 10.1093/cercor/bhh147

Devinsky, O., Vezzani, A., Najjar, S., De Lanerolle, N. C., and Rogawski, M. A. (2013). Glia and epilepsy: excitability and inflammation. *Trends Neurosci.* 36, 174–184. doi: 10.1016/j.tins.2012.11.008

Dragunow, M. (1991). Adenosine and seizure termination. *Ann. Neurol.* 29:575. doi: 10.1002/ana.410290524

Dudek, F. E., Hellier, J. L., Williams, P. A., Ferraro, D. J., and Staley, K. J. (2002). The course of cellular alterations associated with the development of spontaneous seizures after status epilepticus. *Prog. Brain Res.* 135, 53–65. doi: 10.1016/s0079-6123(02)35007-6

During, M. J., and Spencer, D. D. (1992). Adenosine: a potential mediator of seizure arrest and postictal refractoriness. *Ann. Neurol.* 32, 618–624. doi: 10.1002/ana.410320504

Eichler, S. A., Kirischuk, S., Jüttner, R., Schaefermeier, P. K., Legendre, P., Lehmann, T. N., et al. (2008). Glycinergic tonic inhibition of hippocampal neurons with depolarizing GABAergic transmission elicits histopathological signs of temporal lobe epilepsy. *J. Cell. Mol. Med.* 12, 2848–2866. doi: 10.1111/j.1582-4934.2008.00357.x

Eulenburg, V., Armsen, W., Betz, H., and Gomeza, J. (2005). Glycine transporters: essential regulators of neurotransmission. *Trends Biochem. Sci.* 30, 325–333. doi: 10.1016/j.tibs.2005.04.004

Fedele, D. E., Gouder, N., Güttinger, M., Gabernet, L., Scheurer, L., Rülicke, T., et al. (2005). Astrogliosis in epilepsy leads to overexpression of adenosine kinase resulting in seizure aggravation. *Brain* 128, 2383–2395. doi: 10.1093/brain/awh555

Feng, J., Zhou, Y., Campbell, S. L., Le, T., Li, E., Sweatt, J. D., et al. (2010). Dnmt1 and Dnmt3a maintain DNA methylation and regulate synaptic function in adult forebrain neurons. *Nat. Neurosci.* 13, 423–430. doi: 10.1038/nn.2514

Garriga-Canut, M., Schoenike, B., Qazi, R., Bergendahl, K., Daley, T. J., Pfender, R. M., et al. (2006). 2-Deoxy-D-glucose reduces epilepsy progression by NRSF-CtBP-dependent metabolic regulation of chromatin structure. *Nat. Neurosci.* 9, 1382–1387. doi: 10.1038/nn1791

Gomeza, J., Hülsmann, S., Ohno, K., Eulenburg, V., Szöke, K., Richter, D., et al. (2003). Inactivation of the glycine transporter 1 gene discloses vital role of glial glycine uptake in glycinergic inhibition. *Neuron* 40, 785–796. doi: 10.1016/s0896-6273(03)00672-x

Gouder, N., Fritschy, J. M., and Boison, D. (2003). Seizure suppression by adenosine A_1 receptor activation in a mouse model of pharmacoresistant epilepsy. *Epilepsia* 44, 877–885. doi: 10.1046/j.1528-1157.2003.03603.x

Gouder, N., Scheurer, L., Fritschy, J.-M., and Boison, D. (2004). Overexpression of adenosine kinase in epileptic hippocampus contributes to epileptogenesis. *J. Neurosci.* 24, 692–701. doi: 10.1523/jneurosci.4781-03.2004

Henshall, D. C., and Kobow, K. (2015). Epigenetics and epilepsy. *Cold Spring Harb. Perspect. Med.* 5:a022731. doi: 10.1101/cshperspect.a022731

Huber, A., Padrun, V., Déglon, N., Aebischer, P., Möhler, H., and Boison, D. (2001). Grafts of adenosine-releasing cells suppress seizures in kindling epilepsy. *Proc. Natl. Acad. Sci. U S A* 98, 7611–7616. doi: 10.1073/pnas.131102898

James, S. J., Melnyk, S., Pogribna, M., Pogribny, I. P., and Caudill, M. A. (2002). Elevation in S-adenosylhomocysteine and DNA hypomethylation: potential epigenetic mechanism for homocysteine-related pathology. *J. Nutr.* 132, 2361S–2366S.

Javitt, D. C. (2012). Glycine transport inhibitors in the treatment of schizophrenia. *Handb. Exp. Pharmacol.* 213, 367–399. doi: 10.1007/978-3-642-25758-2_12

Kobow, K., and Blümcke, I. (2011). The methylation hypothesis: do epigenetic chromatin modifications play a role in epileptogenesis? *Epilepsia* 52, 15–19. doi: 10.1111/j.1528-1167.2011.03145.x

Kobow, K., and Blümcke, I. (2012). The emerging role of DNA methylation in epileptogenesis. *Epilepsia* 53, 11–20. doi: 10.1111/epi.12031

Kobow, K., Jeske, I., Hildebrandt, M., Hauke, J., Hahnen, E., Buslei, R., et al. (2009). Increased reelin promoter methylation is associated with granule cell dispersion in human temporal lobe epilepsy. *J. Neuropathol. Exp. Neurol.* 68, 356–364. doi: 10.1097/NEN.0b013e31819ba737

Kobow, K., Kaspi, A., Harikrishnan, K. N., Kiese, K., Ziemann, M., Khurana, I., et al. (2013). Deep sequencing reveals increased DNA methylation in chronic rat epilepsy. *Acta Neuropathol.* 126, 741–756. doi: 10.1007/s00401-013-1168-8

Kredich, N. M., and Martin, D. V. Jr. (1977). Role of S-adenosylhomocysteine in adenosinemediated toxicity in cultured mouse T lymphoma cells. *Cell* 12, 931–938. doi: 10.1016/0092-8674(77)90157-x

Kubota, H., Alle, H., Betz, H., and Geiger, J. R. (2010). Presynaptic glycine receptors on hippocampal mossy fibers. *Biochem. Biophys. Res. Commun.* 393, 587–591. doi: 10.1016/j.bbrc.2010.02.019

Li, T., Lytle, N., Lan, J.-Q., Sandau, U. S., and Boison, D. (2012). Local disruption of glial adenosine homeostasis in mice associates with focal electrographic seizures: a first step in epileptogenesis? *Glia* 60, 83–95. doi: 10.1002/glia.21250

Li, T., Quan Lan, J., Fredholm, B. B., Simon, R. P., and Boison, D. (2007a). Adenosine dysfunction in astrogliosis: cause for seizure generation? *Neuron Glia Biol.* 3, 353–366. doi: 10.1017/s1740925x0800015x

Li, T., Steinbeck, J. A., Lusardi, T., Koch, P., Lan, J. Q., Wilz, A., et al. (2007b). Suppression of kindling epileptogenesis by adenosine releasing stem cell-derived brain implants. *Brain* 130, 1276–1288. doi: 10.1093/brain/awm057

Li, T., Ren, G., Lusardi, T., Wilz, A., Lan, J. Q., Iwasato, T., et al. (2008). Adenosine kinase is a target for the prediction and prevention of epileptogenesis in mice. *J. Clin. Inv.* 118, 571–582. doi: 10.1172/JCI33737

Löscher, W., and Brandt, C. (2010). Prevention or modification of epileptogenesis after brain insults: experimental approaches and translational research. *Pharmacol. Rev.* 62, 668–700. doi: 10.1124/pr.110.003046

Lubin, F. D. (2012). Epileptogenesis: can the science of epigenetics give us answers? *Epilepsy Curr.* 12, 105–110. doi: 10.5698/1535-7511-12.3.105

Ma, D. K., Jang, M. H., Guo, J. U., Kitabatake, Y., Chang, M. L., Pow-Anpongkul, N., et al. (2009). Neuronal activity-induced Gadd45b promotes epigenetic DNA demethylation and adult neurogenesis. *Science* 323, 1074–1077. doi: 10.1126/science.1166859

Martin, L. J., and Wong, M. (2013). Aberrant regulation of DNA methylation in amyotrophic lateral sclerosis: a new target of disease mechanisms. *Neurotherapeutics* 10, 722–733. doi: 10.1007/s13311-013-0205-6

Martina, M., B-Turcotte, M. E., Halman, S., Tsai, G., Tiberi, M., Coyle, J. T., et al. (2005). Reduced glycine transporter type 1 expression leads to major changes in glutamatergic neurotransmission of CA1 hippocampal neurones in mice. *J. Physiol.* 563, 777–793. doi: 10.1113/jphysiol.2004.080655

Masino, S. A., Li, T., Theofilas, P., Sandau, U. S., Ruskin, D. N., Fredholm, B. B., et al. (2011). A ketogenic diet suppresses seizures in mice through adenosine A1 receptors. *J. Clin. Inv.* 121, 2679–2683. doi: 10.1172/JCI57813

Masliah, E., Dumaop, W., Galasko, D., and Desplats, P. (2013). Distinctive patterns of DNA methylation associated with Parkinson disease: identification of concordant epigenetic changes in brain and peripheral blood leukocytes. *Epigenetics* 8, 1030–1038. doi: 10.4161/epi.25865

McGaraughty, S., Cowart, M., Jarvis, M. F., and Berman, R. F. (2005). Anticonvulsant and antinociceptive actions of novel adenosine kinase inhibitors. *Curr. Top. Med. Chem.* 5, 43–58. doi: 10.2174/1568026053386845

Meier, J. C., Henneberger, C., Melnick, I., Racca, C., Harvey, R. J., Heinemann, U., et al. (2005). RNA editing produces glycine receptor $\alpha 3^{P185L}$, resulting in high agonist potency. *Nat. Neurosci.* 8, 736–744. doi: 10.1038/nn1467

Miller, S. L., and Urey, H. C. (1959a). Organic compound synthesis on the primitive earth. *Science* 130, 245–251. doi: 10.1126/science.130.3370.245

Miller, S. L., and Urey, H. C. (1959b). Origin of life. *Science* 130, 1622–1624. doi: 10.1126/science.130.3389.1622-a

Miller-Delaney, S. F., Bryan, K., Das, S., McKiernan, R. C., Bray, I. M., Reynolds, J. P., et al. (2015). Differential DNA methylation profiles of coding and non-coding genes define hippocampal sclerosis in human temporal lobe epilepsy. *Brain* 138, 616–631. doi: 10.1093/brain/awu373

Möhler, H., Boison, D., Singer, P., Feldon, J., Pauly-Evers, M., and Yee, B. K. (2011). Glycine transporter 1 as a potential therapeutic target for schizophrenia-related symptoms: evidence from genetically modified mouse models and pharmacological inhibition. *Biochem. Pharmacol.* 81, 1065–1077. doi: 10.1016/j.bcp.2011.02.003

Möhler, H., and Okada, T. (1977). Benzodiazepine receptor: demonstration in the central nervous system. *Science* 198, 849–851. doi: 10.1126/science.918669

Möhler, H., Rudolph, U., Boison, D., Singer, P., Feldon, J., and Yee, B. K. (2008). Regulation of cognition and symptoms of psychosis: focus on $GABA_A$ receptors and glycine transporter 1. *Pharmacol. Biochem. Behav.* 90, 58–64. doi: 10.1016/j.pbb.2008.03.003

Nabbout, R., Vezzani, A., Dulac, O., and Chiron, C. (2011). Acute encephalopathy with inflammation-mediated status epilepticus. *Lancet Neurol.* 10, 99–108. doi: 10.1016/S1474-4422(10)70214-3

Nelson, E. D., Kavalali, E. T., and Monteggia, L. M. (2008). Activity-dependent suppression of miniature neurotransmission through the regulation of DNA methylation. *J. Neurosci.* 28, 395–406. doi: 10.1523/JNEUROSCI.3796-07.2008

Oro, J. (1961). Mechanism of synthesis of adenine from hydrogen cyanide under possible primitive earth conditions. *Nature* 191, 1193–1194. doi: 10.1038/1911193a0

Pignataro, G., Maysami, S., Studer, F. E., Wilz, A., Simon, R. P., and Boison, D. (2008). Downregulation of hippocampal adenosine kinase after focal ischemia as potential endogenous neuroprotective mechanism. *J. Cereb. Blood Flow Metab.* 28, 17–23. doi: 10.1038/sj.jcbfm.9600499

Qureshi, I. A., and Mehler, M. F. (2010). Epigenetic mechanisms underlying human epileptic disorders and the process of epileptogenesis. *Neurobiol. Dis.* 39, 53–60. doi: 10.1016/j.nbd.2010.02.005

Riban, V., Bouilleret, V., Pham-Lê, B. T., Fritschy, J. M., Marescaux, C., and Depaulis, A. (2002). Evolution of hippocampal epileptic activity during the development of hippocampal sclerosis in a mouse model of temporal lobe epilepsy. *Neuroscience* 112, 101–111. doi: 10.1016/s0306-4522(02)00064-7

Seifert, G., Carmignoto, G., and Steinhäuser, C. (2010). Astrocyte dysfunction in epilepsy. *Brain Res. Rev.* 63, 212–221. doi: 10.1016/j.brainresrev.2009.10.004

Shen, H. Y., van Vliet, E. A., Bright, K. A., Hanthorn, M., Lytle, N. K., Gorter, J., et al. (2015). Glycine transporter 1 is a target for the treatment of epilepsy. *Neuropharmacology* 99, 554–565. doi: 10.1016/j.neuropharm.2015.08.031

Singer, P., Feldon, J., and Yee, B. K. (2009). The glycine transporter 1 inhibitor SSR504734 enhances working memory performance in a continuous delayed alternation task in C57BL/6 mice. *Psychopharmacology (Berl)* 202, 371–384. doi: 10.1007/s00213-008-1286-5

Szybala, C., Pritchard, E. M., Wilz, A., Kaplan, D. L., and Boison, D. (2009). Antiepileptic effects of silk-polymer based adenosine release in kindled rats. *Exp. Neurol.* 219, 126–135. doi: 10.1016/j.expneurol.2009.05.018

Tremolizzo, L., Messina, P., Conti, E., Sala, G., Cecchi, M., Airoldi, L., et al. (2014). Whole-blood global DNA methylation is increased in amyotrophic lateral sclerosis independently of age of onset. *Amyotroph. Lateral Scler. Frontotemporal Degener.* 15, 98–105. doi: 10.3109/21678421.2013.851247

Tsai, G., Ralph-Williams, R. J., Martina, M., Bergeron, R., Berger-Sweeney, J., Dunham, K. S., et al. (2004). Gene knockout of glycine transporter 1: characterization of the behavioral phenotype. *Proc. Natl. Acad. Sci. U S A* 101, 8485–8490. doi: 10.1073/pnas.0402662101

Van Dycke, A., Raedt, R., Dauwe, I., Sante, T., Wyckhuys, T., Meurs, A., et al. (2010). Continuous local intrahippocampal delivery of adenosine reduces seizure frequency in rats with spontaneous seizures. *Epilepsia* 51, 1721–1728. doi: 10.1111/j.1528-1167.2010.02700.x

Vezzani, A., French, J., Bartfai, T., and Baram, T. Z. (2011). The role of inflammation in epilepsy. *Nat. Rev. Neurol.* 7, 31–40. doi: 10.1038/nrneurol.2010.178

Vianna, E. P., Ferreira, A. T., Doná, F., Cavalheiro, E. A., and da Silva Fernandes, M. J. (2005). Modulation of seizures and synaptic plasticity by adenosinergic receptors in an experimental model of temporal lobe epilepsy induced by pilocarpine in rats. *Epilepsia* 46, 166–173. doi: 10.1111/j.1528-1167.2005.01027.x

Williams-Karnesky, R. L., Sandau, U. S., Lusardi, T. A., Lytle, N. K., Farrell, J. M., Pritchard, E. M., et al. (2013). Epigenetic changes induced by adenosine augmentation therapy prevent epileptogenesis. *J. Clin. Inv.* 123, 3552–3563. doi: 10.1172/JCI65636

Wilz, A., Pritchard, E. M., Li, T., Lan, J. Q., Kaplan, D. L., and Boison, D. (2008). Silk polymer-based adenosine release: therapeutic potential for epilepsy. *Biomaterials* 29, 3609–3616. doi: 10.1016/j.biomaterials.2008.05.010

Winkelmann, A., Maggio, N., Eller, J., Caliskan, G., Semtner, M., Häussler, U., et al. (2014). Changes in neural network homeostasis trigger neuropsychiatric symptoms. *J. Clin. Invest.* 124, 696–711. doi: 10.1172/JCI71472

Yee, B. K., Balic, E., Singer, P., Schwerdel, C., Grampp, T., Gabernet, L., et al. (2006). Disruption of glycine transporter 1 restricted to forebrain neurons is associated with a pro-cognitive and anti-psychotic phenotypic profile. *J. Neurosci.* 26, 3169–3181. doi: 10.1523/JNEUROSCI.5120-05.2006

Zhu, Q., Wang, L., Zhang, Y., Zhao, F. H., Luo, J., Xiao, Z., et al. (2012). Increased expression of DNA methyltransferase 1 and 3a in human temporal lobe epilepsy. *J. Mol. Neurosci.* 46, 420–426. doi: 10.1007/s12031-011-9602-7

Zuchora, B., Turski, W. A., Wielosz, M., and Urbanska, E. M. (2001). Protective effect of adenosine receptor agonists in a new model of epilepsy–seizures evoked by mitochondrial toxin, 3-nitropropionic acid, in mice. *Neurosci. Lett.* 305, 91–94. doi: 10.1016/s0304-3940(01)01816-x

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Molecular Neuroscience

ORIGINAL RESEARCH
published: 31 August 2016
doi: 10.3389/fnmol.2016.00079

Cerebral Microvascular Endothelial Cell Apoptosis after Ischemia: Role of Enolase-Phosphatase 1 Activation and Aci-Reductone Dioxygenase 1 Translocation

Yuan Zhang[1,2,3†], Ting Wang[1,2†], Ke Yang[1,2], Ji Xu[1,2], Lijie Ren[4], Weiping Li[2,5] and Wenlan Liu[1,2,5*]*

[1] The Central Laboratory, Shenzhen Second People's Hospital, Graduate School of Guangzhou Medical University, Shenzhen, China, [2] Shenzhen Key Laboratory of Neurosurgery, Shenzhen Second People's Hospital, Graduate School of Guangzhou Medical University, Shenzhen, China, [3] Department of Pathophysiology, Baotou Medical College, Baotou, China, [4] Department of Neurology, Shenzhen Second People's Hospital, The First Affiliated Hospital of Shenzhen University, Shenzhen, China, [5] Department of Neurosurgery, Shenzhen Second People's Hospital, The First Affiliated Hospital of Shenzhen University, Shenzhen, China

OPEN ACCESS

Edited by:
Detlev Boison,
Legacy Health, USA

Reviewed by:
Jeffrey Iliff,
Oregon Health and Science University, USA
Lei Pei,
Huazhong University of Science and Technology, China

***Correspondence:**
Weiping Li
wpli@szu.edu.cn
Wenlan Liu
wlliu@szu.edu.cn

[†]These authors have contributed equally to this work.

Received: 16 June 2016
Accepted: 19 August 2016
Published: 31 August 2016

Citation:
Zhang Y, Wang T, Yang K, Xu J, Ren L, Li W and Liu W (2016) Cerebral Microvascular Endothelial Cell Apoptosis after Ischemia: Role of Enolase-Phosphatase 1 Activation and Aci-Reductone Dioxygenase 1 Translocation. Front. Mol. Neurosci. 9:79. doi: 10.3389/fnmol.2016.00079

Enolase-phosphatase 1 (ENOPH1), a newly discovered enzyme of the methionine salvage pathway, is emerging as an important molecule regulating stress responses. In this study, we investigated the role of ENOPH1 in blood brain barrier (BBB) injury under ischemic conditions. Focal cerebral ischemia induced ENOPH1 mRNA and protein expression in ischemic hemispheric microvessels in rats. Exposure of cultured brain microvascular endothelial cells (bEND3 cells) to oxygen-glucose deprivation (OGD) also induced ENOPH1 upregulation, which was accompanied by increased cell death and apoptosis reflected by increased 3-(4, 5-Dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide formation, lactate dehydrogenase release and TUNEL staining. Knockdown of ENOPH1 expression with siRNA or overexpressing ENOPH1 with CRISPR-activated plasmids attenuated or potentiated OGD-induced endothelial cell death, respectively. Moreover, ENOPH1 knockdown or overexpression resulted in a significant reduction or augmentation of reactive oxygen species (ROS) generation, apoptosis-associated proteins (caspase-3, PARP, Bcl-2 and Bax) and Endoplasmic reticulum (ER) stress proteins (Ire-1, Calnexin, GRP78 and PERK) in OGD-treated endothelial cells. OGD upregulated the expression of ENOPH1's downstream protein aci-reductone dioxygenase 1 (ADI1) and enhanced its interaction with ENOPH1. Interestingly, knockdown of ENOPH1 had no effect on OGD-induced ADI1 upregulation, while it potentiated OGD-induced ADI1 translocation from the nucleus to the cytoplasm. Lastly, knockdown of ENOPH1 significantly reduced OGD-induced endothelial monolayer permeability increase. In conclusion, our data demonstrate that ENOPH1 activation may

Abbreviations: BSA, Bovine albumin; DAPI, 4′,6-diamidino-2-phenylindole; DCF-DA, 2,7-dichlorodihydrofluorescein diacetate; EDTA, Eathylene diamine tetraacetic acid; FACS, Fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; PBS, Phosphate Buffered Saline; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.

contribute to OGD-induced endothelial cell death and BBB disruption through promoting ROS generation and the activation of apoptosis associated proteins, thus representing a new therapeutic target for ischemic stroke.

Keywords: ENOPH1, oxygen-glucose deprivation, cerebral ischemia, blood brain barrier, apoptosis

INTRODUCTION

Ischemic stroke is a leading cause of disability and mortality in humans. Cerebral ischemia initiates a cascade of cytotoxic molecules responsible for the death of neural cells as well as the damage of the blood brain barrier (BBB) at the injury site. The polyamines, such as putrescine, spermindine and spermine, are elevated in the ischemic parenchyma and contribute to ischemic brain damage via enhancing N-methy-D-aspartate receptor-mediated excitotoxicity, generating toxic aldehydes and reactive oxygen species (ROS), and disrupting oxidative metabolism and mitochondrial function (Takano et al., 2005; Kim et al., 2009). The administration of polyamine antagonists prevents the development of ischemic brain damage (Takano et al., 2005; Li et al., 2007).

Enolase-phosphatase 1 (ENOPH1) is a newly identified enzyme of the methionine salvage pathway, a ubiquitous pathway for the reconstitution of methionine, which is required for the synthesis of polyamine (Sauter et al., 2013). A recent study shows that ENOPH1 is widely expressed in the brain and stress exposure increases ENOPH1 protein levels in brain tissue of C57BL/6J mice (Barth et al., 2014). Since ENOPH1 participates in the synthesis of polyamine indirectly via S-adenosyl methionine (SAM; Takano et al., 2005; Li et al., 2007; Kim et al., 2009; Duan et al., 2011), it is logical to speculate a role of ENOPH1 in ischemic brain injury.

BBB disruption is a common event in ischemic stroke, which leads to vasogenic brain edema and hemorrhagic transformation (Sandoval and Witt, 2008; Hawkins et al., 2014). Brain capillary endothelial cells form the backbone of the BBB structure, their death or apoptosis can result in catastrophic failure of BBB's integrity (ElAli et al., 2011; Wang et al., 2011). We have obtained preliminary data showing that cerebral ischemia induces ENOPH1 mRNA expression in ischemic cerebral microvessels in a rat model of middle cerebral artery occlusion (MCAO; **Figure 1**). However, the exact role of ENOPH1 in BBB damage remains to be determined.

In this study, we applied *in vitro* cultured brain microvascular endothelial monolayer and oxygen-glucose deprivation (OGD) to mimic the BBB and ischemia, respectively, and explored the role of ENOPH1 in ischemic BBB damage. Our data showed that OGD induced a significant increase in the protein levels of ENOPH1 and its downstream molecule aci-reductone dioxygenase 1 (ADI1) in cultured brain endothelial cells, which led to increased ROS generation, endothelial cell apoptosis and increased permeability of endothelial monolayer.

MATERIALS AND METHODS

Rat Model of MCAO

The Laboratory Animal Care and Use Committee of Shenzhen University approved all experimental protocols. Male Sprague Dawley rats (Southern Medical University, Guangzhou, Guangdong, China) weighing 290–320 g were anesthetized with isoflurane (4% for induction, 1.75% for maintenance) in N_2O:O_2 (70%:30%) during surgical procedures and the body temperature was maintained at 37.5 ± 0.5°C using a heating pad. The rats were subjected to 3 h MCAO using the intraluminal filament technique as previously described (Liu et al., 2009). Briefly, a 4–0 silicone-coated monofilament nylon suture was inserted into the internal carotid artery and advanced along the internal carotid artery to approximately 17–18 mm from the bifurcation, thereby blocking the ostium of the MCA. At the end of 3 h MCAO, rats were sacrificed and the brains were removed for microvessel isolation as described below. For a total number of 12 rats included in this study, successful MCAO was confirmed by 2,3,5-triphenyltetrazolium chloride (TTC) staining of the 1 mm-thick brain coronal section 6 mm away from the tip of the front lobe as we described previously (Liu et al., 2008).

Isolation of Cerebral Microvessels

Isolation of cerebral microvessels was carried out as we described previously (Liu et al., 2009). Briefly, the hemispheric brain tissue was dissected and homogenized in ice cold Phosphate Buffered Saline (PBS). The homogenate was filtered through a 41 μm nylon mesh (Spectrum), and the nylon mesh was washed three times with PBS. Microvessels retained on the mesh were then washed off and pelleted by centrifugation at 4000 g for 10 min at 4°C. The pellets were resuspended in 15% dextran T-500 and then added onto 20% dextran T-500, followed by centrifugation at 25,000 g for 10 min at 4°C. The pellets were collected at the cerebral microvessels and used for measuring ENOPH1 mRNA and protein expression by real time RT-PCR and western blot, respectively.

Cell Cultures

Mouse brain microvascular endothelial cells (bEND3 cells; American Type Culture Collection, VA, USA) were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified incubator gassed with 5% CO_2 and 95% room air. The cells were grown to confluence on type I collagen-coated 60 mm dishes before exposure to OGD. For the assays, cell cultures were initiated at a density of 5×10^5 cells/ml to have cells

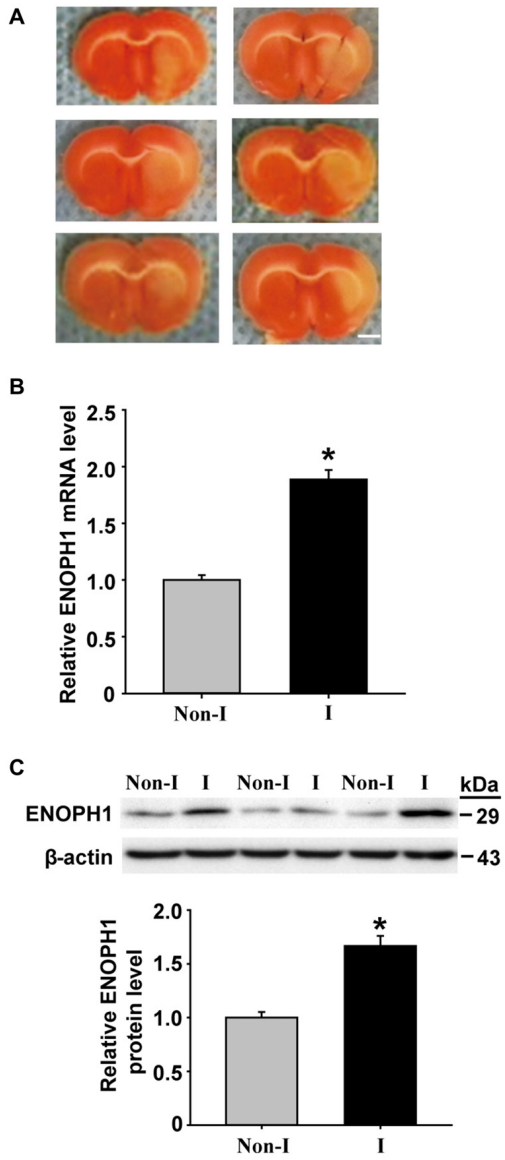


FIGURE 1 | Middle cerebral artery occlusion (MCAO) induces Enolase-phosphatase 1 (ENOPH1) upregulation in ischemic cerebral microvessels. Rats were subjected to 3 h MCAO before isolating hemispheric cerebral microvessels. The mRNA and protein levels of ENOPH1 in cerebral microvessels from nonischemic (Non-I) and ischemic (I) hemispheric tissue were analyzed by real-time RT-PCR and western blot. **(A)** Representative photographs of triphenyltetrazolium chloride (TTC) stained 1 mm-thick brain sections showing tissue infarction (pale white region) in the ischemic hemispheres (right). **(B)** Real-time RT-PCR analysis showed that ENOPH1 mRNA expression was significantly increased in ischemic hemispheric microvessels. $^*P < 0.05$ vs. Non-I; $n = 6$. **(C)** Western blot analysis revealed increased levels of ENOPH1 protein in ischemic hemispheric microvessels. Upper panel: representative immunoblots of ENOPH1 and the loading control β-actin; bottom panel: quantitative data of protein band intensity after normalization to β-actin. $^*P < 0.05$ vs. Non-I; $n = 6$.

in the exponential growth phase. The number of cells was determined with a hemocytometer (Adam MC, Digital bio, Korea).

OGD Treatment

To mimic ischemic condition *in vitro*, bEND3 cells were exposed to OGD as described previously (Liu et al., 2012). In brief, confluent bEND3 cells were subjected to OGD by replacing the normal growth medium with glucose free medium (DMEM without glucose) pre-equilibrated with 95% N_2 and 5% CO_2. The cells were then incubated in a humidified airtight chamber (Biospherix Ltd., Lacona, NY, USA) for 1, 3 or 6 h. Control cultures were incubated with normal DMEM medium without FBS at 37°C in 5% CO_2/95% air. The cells were collected for further analyses immediately after OGD treatment.

Lactate Dehydrogenase Release Assay

After OGD treatment, cell viability was measured using a CytoTox 96®Non-Radioactive Cytotoxicity Assay Kit (Promega) according to manufacturer's instruction. Briefly, 50 μl of each sample medium (e.g., pure culture medium for measuring background LDH release, culture media collected from control or OGD-treated cells for measuring experimental LDH release and lysis buffer-treated cells for measuring maximum LDH release) was collected to assay LDH release. The samples were incubated with the reduced form of nicotinamide-adenine dinucleotide and pyruvate for 30 min at room temperature and the reaction was terminated by adding Stop Solution. LDH release was assessed by measuring the absorbance of supernatants at 490 nm. The cell death rate was calculated as follows: cell death rate = (experimental LDH release − background LDH release)/(maximum LDH release-background LDH release) × 100%. The results were presented as fold increase of the control cells.

Knockdown of ENOPH1 with siRNA

bEND3 cells at 60–70% confluence on 6- and 24-well plates were transfected with 100 nM ENOPH1 siRNA (si-ENOPH1, sc-144654, Santa Cruz, CA, USA) or scrambled control siRNA (si-control, sc-37007, Santa Cruz, CA, USA) using siRNA transfection reagent (sc-29528) according to the manufacturer's instruction. Forty-eight hours after transfection, cells were subjected to OGD treatment. Specific silencing was confirmed by western blot.

ENOPH1 CRISPR Activation Plasmid Transfection

bEND3 cells grown to 60–80% confluence were transfected with the ENOPH1 overexpression plasmid (ENOPH1 CRISPR activation plasmid, Santa Cruz, CA, USA) or control plasmid (control CRISPR activation plasmid, Santa Cruz, CA, USA) using UltraCruz® Transfection Reagent (Santa Cruz Biotech) according to the manufacturer's instruction. In brief, for each transfection, a 300 μl mixture of 1 μg of plasmid DNA with 10 μl of UltraCruz® transfection reagent in plasmid transfection medium was added to each well. Forty-eight hours after transfection, cells were subjected to OGD treatment and subjected to various assays.

In vitro BBB Model of Endothelial Monolayer

An endothelial monolayer grown on a cell culture insert is widely used for *in vitro* BBB models. Nunc cell culture inserts (for 24-well plates) with 0.02 μm Anapore membranes (Nunc Inc., Naperville, IL, USA) were coated by incubation with 70 μg/ml type I collagen (Sigma, St. Louis, CA, USA) in 20 mM acetic acid for 1 h at 25°C. The inserts were then washed with serum free medium to remove excess protein before adding complete medium to equilibrate the membrane for 3 h at 37°C in a cell culture incubator. Then, the endothelial cells were trypsinized from the tissue culture flasks, washed three times with complete medium, and seeded on the inserts at 20,000 cells/cm^2. The cells seeded on inserts were allowed to grow for 3–4 days at 37°C in 5% CO_2/95% air to achieve full confluence, which was confirmed under a phase contrast microscopy.

Endothelial Cell Monolayer Permeability Assay

Endothelial monolayer permeability after OGD was measured as we described previously (Liu et al., 2012). Briefly, bEND3 cells were placed on the upper side of the insert and allowed to grow to confluence. Then, 3.5 μM fluorescein isothiocyanate (FITC)-dextran was added to the endothelial monolayer (luminal compartment) before expose to OGD for 6 h. After OGD treatment, the contents of FITC-dextran in both luminal and abluminal compartments were determined and endothelial monolayer permeability was assessed by calculating the apparent permeability coefficient (Papp) as previously described (Grabovac and Bernkop-Schnürch, 2006): Papp [cm/s] = dQ/(dt*A*Co), where dQ was the amount of FITC-dextran getting into the abluminal compartment, dt was duration of OGD treatment, dQ/dt was the rate of transfer (ng/s), A was surface area (cm^2), and Co was the initial concentration in the luminal chamber (ng/cm^3). To test whether ENOPH1 were implicated in OGD-induced endothelial barrier disruption, cells were pretreated with ENOPH1 siRNA for 48 h before OGD treatment.

Detection of Intracellular Reactive Oxygen Species (ROS) Generation

Intracellular ROS generation was assessed by quantifying fluorescence intensity under a fluorescent microscope or by flow cytometry analysis after staining with 2,7-dichlorodihydrofluorescein diacetate (DCF-DA). The bEND3 cells were pre-incubated in a 24- or 6-well culture plate for 24 h. Subsequently, the cells were transfected with control or ENOPH1 siRNA for 48 h at 37°C. After transfection, the bEND3 cells were treated with OGD for 6 h before incubating with 10 μM DCF-DA (Sigma, St. Louis, MO, USA) for 30 min at 37°C. The cells were then photographed under a fluorescent microscope (Leica, Germany) or resuspended and incubated for another 10 min with propidium iodide (10 μg/ml) before analyzing ROS with a Fluorescence-activated cell sorting (FACS) calibur flow cytometer (Becton Dickinson, excitation: 488 nm). ROS fluorescence was quantified and expressed as fold increase of the control cells.

Western Blot Assay

After indicated treatment, the cells grown on 6 or 10 cm culture dish were harvested and lysed in a cold lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM Ethylene diamine tetraacetic acid (EDTA), 10% glycerol, 1% TritonX-100, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2% protease inhibitor mixture (Sigma, St. Louis, MO, USA)]. The cell lysates were centrifuged for 15 min at 12,000 g at 4°C and protein concentrations were determined using Pierce^{TM} BCA Protein Assay Kit (Thermo Fisher Scientific Inc, Waltham, MA, USA). The cell lysates (30 μg protein/lane) were subjected to electrophoresis on 10–12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to polyvinylidene difluoride membrane. The membranes were blocked in Trisbuffered saline with 0.05% Tween-20 (TBS-T) containing 5% nonfat milk and blotted overnight with primary antibodies (dilution: 1:1000). The membranes were washed and incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibodies (dilution: 1:1000, Jackson ImmunoResearch, West Grove, PA, USA). The membranes were washed and developed using a chemiluminescence kit (Fisher Scientific). The primary antibodies were cleaved-caspase-3, caspase-3, cleaved-PARP, Bcl-2, Bax, PARP, ADI1, PERK, Calnexin, Ire-1α, ENOPH1 and β-actin. ENOPH1 antibody was a product of Proteintech (Cat. Log: 11763-1-AP), and the rest primary antibodies were purchased from Santa Cruz Biotech. Relative protein levels were normalized to β-actin. For subcellular fraction samples, we noticed that the actin levels were comparable for each sample among different fractions, so we only used one normalizing loading control (CF actin) for all three subcellular fractions.

Coimmunoprecipitation of ENOPH1 with ADI1

Coimmunoprecipitation was performed as described previously (Wen et al., 2010). Briefly, bEND3 cells were subjected to OGD treatment for 6 h and then lysed on ice in 1 ml RIPA buffer. After pre-clearing with normal IgG, cell lysates (0.5 mg of protein) were incubated overnight at 4°C with 2 μg of anti-ENOPH1 (Proteintech, Chicago, IL, USA), followed by precipitation with 20 μl of protein A/G Plus-Agarose (Pierce Biotechnology, Rockford, IL, USA) for 1 h at 4°C. The precipitated complexes were separated on SDS-PAGE gels and immunoblotted with anti-ADI1 (Santa Cruz Biotech., Dallas, TX, USA) to detect the presence of this protein in the complex, as described above.

Immunocytochemistry

bEND3 cells grown on type I collagen-coated coverslips were exposed to OGD for 6 h before fixation with 4% paraformaldehyde and permeabilization with 0.1% Triton X-100. After blocking non-specific binding with a blocking solution (3% BSA, 0.1% Tween 20 and 5% goat serum in PBS), the cells were incubated overnight at 4°C with anti-ENOPH1 (Proteintech, 1:200) primary antibodies, then followed by FITC anti-mouse secondary antibodies (Invitrogen, 1:200) for 1 h at room temperature. Coverslips were mounted on glass slides using anti-fade sealed solution (Beyotime

Biotechnology, Jiangsu, China) and immunostaining was visualized under a DMI6000B fluorescence microscope (Leica, Germany). The average fluorescence intensity of single nuclei (Iav) and the area (A) that they occupied in the image were determined and the integrated optical density (IOD), which is proportional to the amount of incorporated ENOPH1 antibody, was calculated by the formula: IOD = Iav *A.

Real-Time RT-PCR

Total RNA was isolated from endothelial cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA samples (2 μg) were reverse-transcribed to generate first-strand cDNA. After reverse transcription using TaqMan® Reverse Transcription Kits (Applied Biosystems), 0.5 μl reverse-transcribed products were amplified with the Vii7 real-time PCR System (Applied Biosystems) in a 10 μl final reaction volume using SYBR® Green PCR Master Mix (Applied Biosystems) under the following conditions: 30 s at 95°C, followed by a total of 40 cycles of two temperature cycles (15 s at 95°C and 1 min at 60°C). Primer sequences were as follows: rat ENOPH1 forward: 5′-ACCACAACCCCGATTGCTTT-3′ and reverse: 5′-TTCTTCAGCCTGCTTCCTCA-3′; mouse ENOPH1 forward: 5′-ACCACAACCCCGATTGCTTT-3′ and reverse: 5′-TTCCTCGGCCTGTTTCCTCA-3′; mouse ADI1 forward: 5′-CCGAATGGAAAGTTGCTC-3′ and reverse: 5′-TAAGTCTTGACAGTTAGGGA-3′; GAPDH served as endogenous control, and the primers were forward: 5′-CAATGTGTCCGTCGTGGAT CT-3′; reverse:5′-GTCCTCAGTGTAGCCCAAGATG-3′. The Ct value was calculated by the comparative $\Delta\Delta C_t$ method using the SDS Enterprise Database software (Applied Biosystems).

TUNEL Assay

Apoptosis was analyzed by TUNEL assay using Click-iT® Plus TUNEL Assay (Life Technologies, Inc., Carlsbad, CA, USA) according to manufacturer's instruction. In brief, at the end of the indicated treatments, bEND3 cells grown on coverslips were incubated with TdT reaction mixture for 2 h at 37°C, followed by 30 min incubation with the Alexa Fluor® 594 dye (red fluorescence). Then, the cells were counterstained with DAPI (Sigma-Aldrich) for 20 min and observed under a fluorescence microscope (magnification, ×200; Leica, Germany). The TUNEL-positive nuclei of six non-overlapping fields per coverslip were counted by a researcher blinded to treatment, and these counts were converted to percentages by comparing the TUNEL-positive counts to the total number of cell nuclei as determined by DAPI counterstaining, that is TUNEL-positive ratio = (number of red nuclei/number of blue nuclei) × 100%.

Statistical Analysis

All data were expressed as means ± SEM. Differences between groups were evaluated by either an unpaired Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

ENOPH1 is Upregulated in Ischemic Cerebral Microvessels

ENOPH1 is found to be widely expressed in the brain and is implicated in stress response (Barth et al., 2014). To determine whether ENOPH1 plays a role in ischemic BBB injury, we first examined the change of ENOPH1 expression in the BBB, i.e., the cerebral microvessels or capillaries, isolated from the rats that were subjected to 3 h MCAO without reperfusion. TTC staining of the 1 mm-thick brain sections collected from the six rats included in this study showed visible tissue infarction (no TTC staining, white color) in the MCA supplied area of the right hemisphere after 3 h MCAO (**Figure 1A**). ENOPH1 mRNA expression was analyzed in isolated hemispheric microvessels by real time RT-PCR and found that 3 h MCAO induced a significant increase (∼1-fold) of ENOPH1 mRNA expression in ischemic hemispheric microvessels compared to nonischemic microvessels tissue (**Figure 1B**). Consistent with its mRNA change, ENOPH1 protein levels were also significantly increased in ischemic cerebral microvessels (**Figure 1C**). These results demonstrate that ENOPH1 is upregulated in the ischemic brain microvessels. To further demonstrate the role of ENOPH1 in ischemic BBB injury and the underlying mechanisms involved, we chose the widely used *in vitro* model of BBB (i.e., brain microvascular endothelial cell monolayer) and the *in vitro* model of ischemia (i.e., OGD) for the rest of this study.

OGD Induces ENOPH1 Expression in Brain Endothelial Cells

As mentioned earlier, ENOPH1 is a newly identified protein and little is known about its biological function. So far, there are no specific pharmacological inhibitors or genetics-manipulated animals available, therefore we chose the widely used *in vitro* model of BBB (i.e., cultured endothelial monolayer) and the *in vitro* model of ischemia (i.e., OGD) to investigate whether ENOPH1 plays a role in ischemic BBB injury. Brain endothelial cells bEND3 were exposed to OGD for 1, 3, or 6 h before analyzing ENOPH1 mRNA and protein levels. Real time RT-PCR analysis showed that ENOPH1 mRNA expression was increased in bEND3 cells after exposing to OGD for 1 h and was further increased at 6 h OGD, while no significant difference was seen between 1 h and 3 h OGD (**Figure 2A**). Western blot analysis showed that ENOPH1 protein levels were significantly increased in bEND3 cells after exposing to OGD for 3 h, but not for 1 h (**Figure 2B**). Different from the mRNA result, 6 h OGD did not further increase ENOPH1 protein levels in bEND3 cells compared to 3 h OGD (**Figure 2B**). To further verify the immunoblot findings and to reveal ENOPH1's intracellular localization, we performed immunocytochemical staining after exposing bEND3 cells to OGD for 6 h. As shown in **Figure 2C**, immunostaining revealed that ENOPH1 protein was located in the cytosol of bEND3 cells and OGD treatment significantly increased ENOPH1's fluorescence intensity (∼1.5-fold increase) compared to control (normoxic) cells. These data demonstrate

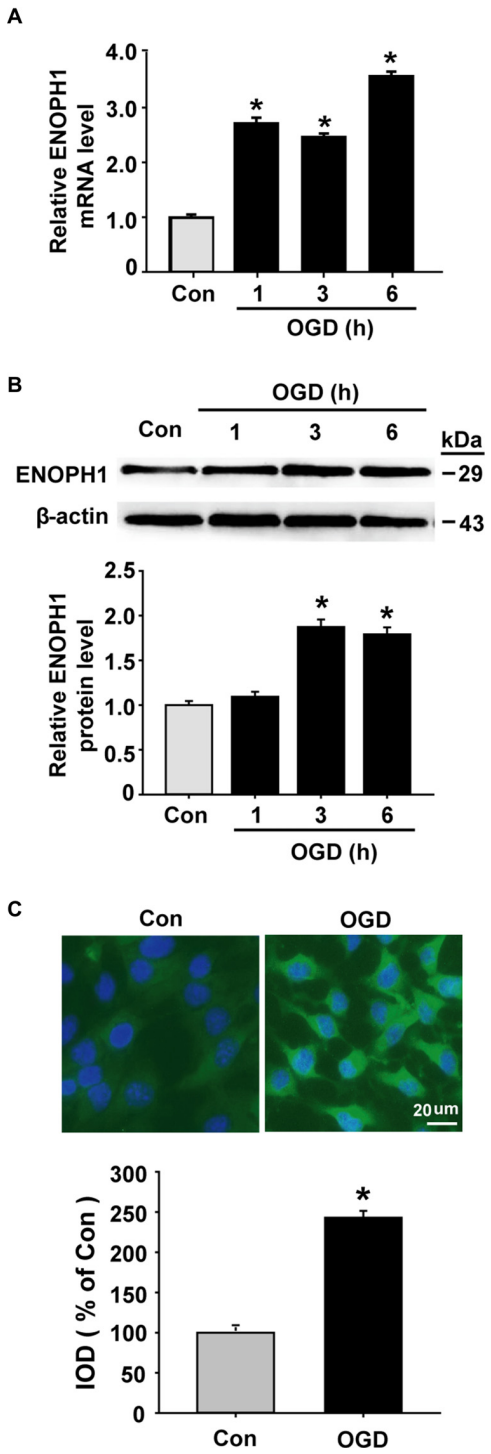


FIGURE 2 | Oxygen-glucose deprivation (OGD) induces upregulation of ENOPH1 in brain microvascular endothelial cells (bEND3 cells). bEND3 cells were subjected to OGD treatment or normoxia (Control, Con) for 1, 3, or 6 h before analyzing ENOPH1 mRNA and protein expression using real-time RT-PCR, western blot and immunostaining. **(A)** Real time RT-PCR analysis showed that ENOPH1 mRNA expression was significantly increased in bEND3 cells at 1 h after OGD treatment and was further increased when OGD was prolonged to 6 h. $^*P < 0.05$ vs. Con; $n = 4$. **(B)** Western blot analysis

(*Continued*)

FIGURE 2 | continued
showed that ENOPH1 protein levels were increased in 3 h OGD and 6 h OGD treated cells, but not in 1 h OGD treated cells. Upper panel: representative immunoblots of ENOPH1 and the loading control β-actin; bottom panel: quantitative data of protein band intensity after normalization to β-actin., $^*P < 0.05$ vs. Con; $n = 4$. **(C)** bEND3 cells were exposed to OGD for 6 h before immunostaining with ENOPH1 (green), and nuclei were counterstained with DAPI (blue). Upper panel: representative immunocytochemical micrographs showed that ENOPH1 was located in the cytosol and OGD significantly enhanced ENOPH1's fluorescence intensity (bar = 20 μm); bottom panel: the intensities of ENOPH1 immunofluorescence were quantitated and expressed as IOD parameter. $^*P < 0.05$ vs. Con; $n = 4$. IOD, integrated optical density.

that OGD significantly induces ENOPH1 upregulation in brain endothelial cells.

Knockdown of ENOPH1 Inhibits OGD-Induced Endothelial Cell Apoptosis

To determine whether ENOPH1 contributes to ischemia-induced BBB injury, we applied siRNA approach to knock down ENOPH1 expression in bEND3 cells and assessed its effect on 6 h OGD-induced endothelial apoptosis. The efficacy of ENOPH1 siRNA in knocking down ENOPH1 expression was shown in **Figure 3A**, in which a ∼90% reduction in ENOPH1 protein level was seen on immunoblots at 48 h after ENOPH1 siRNA transfection. The effect of ENOPH1 siRNA on OGD-induced endothelial cell death was assessed by measuring LDH release (indicating late apoptosis and necrosis) and TUNEL staining (indicating apoptosis). As shown in **Figure 3B**, 6 h OGD significantly induced endothelial cell death, with a death rate of 46.74% ± 2.15 vs. 16.19% ± 1.24 of the control cells. Knockdown of ENOPH1 with siRNA had no effect on LDH release from control bEND3 cells, while it significantly reduced OGD-induced cell death. TUNEL staining showed a similar protective effect of ENOPH1 siRNA on OGD-induced apoptosis, with 19.98% ± 2.03 TUNEL-positive cells for OGD alone vs. 7.14% ± 0.95 for OGD plus ENOPH1 siRNA (**Figure 3C**).

To further verify a role of ENOPH1 in OGD-induced bEND3 cell apoptosis, we assessed the effect of ENOPH1 siRNA on several key apoptosis-associated signal proteins including caspase-3, PARP and Bax/Bcl-2. As shown in **Figures 4A,B**, 6 h OGD induced increased levels of cleaved caspase-3 (caspase-3 activation), cleaved PARP-1 and higher ratio of Bax/Bcl-2 in bEND3 cells, and transfection with ENOPH1 siRNA, but not control siRNA, abolished these changes. As expected, ENOPH1 siRNA alone did not affect these apoptosis-associated signal proteins under control conditions (**Figures 4A,B**). Taken together, these results clearly indicate that ENOPH1 is involved in OGD-induced apoptosis in brain endothelial cells.

Knockdown of ENOPH1 Abolishes OGD-Induced Oxidative Stress in bEND3 Cells

We next asked how ENOPH1 promoted microvascular endothelial cell injury after ischemic stimulation. Endoplasmic reticulum (ER) stress and oxidative stress are well-known

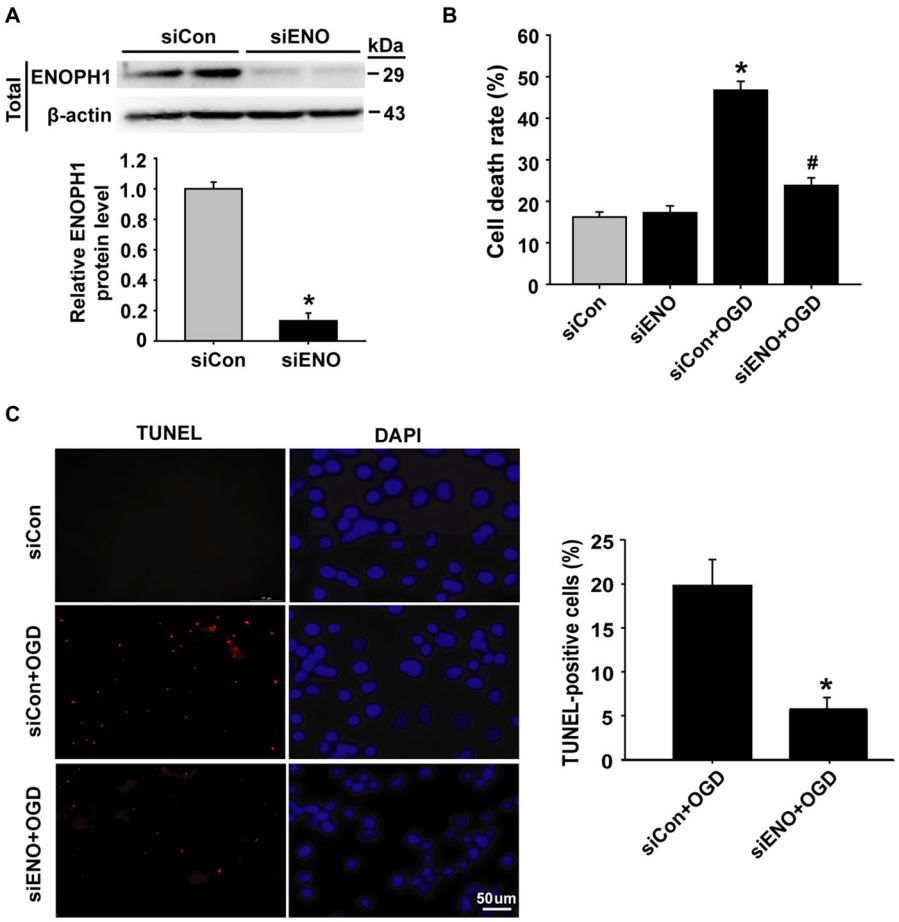


FIGURE 3 | Knockdown of ENOPH1 attenuates OGD-induced apoptosis in bEND3 cells. (A) ENOPH1 siRNA effectively knocked down ENOPH1 protein expression in bEND3 cells. Western blot analysis showed that incubation bEND3 cells with ENOPH1 siRNA (siENO) for 48 h significantly (~90%) reduced ENOPH1 protein levels. Upper panel: representative immunoblots of ENOPH1 and the loading control β-actin; bottom panel: quantitative data of protein band intensity after normalization to β-actin. $^{*}P < 0.05$ vs. Control siRNA (siCon); $n = 4$. **(B)** Knockdown of ENOPH1 significantly reduced 6 h OGD-induced cell death assessed by LDH release. $^{*}P < 0.05$ vs. siCon; $^{\#}P < 0.05$ vs. siCon + OGD; $n = 4$. **(C)** TUNEL assay showed that 6 h OGD significantly increased the number of TUNEL-positive apoptotic endothelial nuclei (red fluorescence) and knockdown of ENOPH1 significantly decreased this increase. Left panel: representative micrographs of double staining of TUNEL and DAPI (blue, counter staining), bar = 50 μm; right panel: quantitative data of TUNEL-positive cells. $^{*}P < 0.05$ vs. siCon + OGD; Experiments were repeated four times ($n = 4$).

mediators for BBB damage in ischemic stroke (Kaur and Ling, 2008; Yang et al., 2014). We speculated that ENOPH1 might contribute to ischemic bEND3 cell death/apoptosis through potentiating ER stress and oxidative stress under OGD conditions. To test this possibility, bEND3 cells were transfected with ENOPH1 siRNA before exposing the cells to OGD for 6 h. ER chaperone proteins (calnexin, PERK, Ire-1a and GRP78) were analyzed by western blot. As shown in **Figure 5A**, 6 h OGD induced a significant reduction in chaperone proteins calnexin, PERK, GRP78 and Ire-1a, and this reduction was significantly inhibited by knockdown of ENOPH1 with siRNA. ROS generation was assessed by DCFH staining and flow cytometry. As shown in **Figures 5B,C**, 6 h OGD induced a significant increase in DCFH fluorescence (green) intensity in bEND3 cells, indicative of increased ROS generation, which was inhibited by ENOPH1 siRNA, but not by control siRNA. Similar results were obtained when using flow cytometry to quantitate ROS generation in OGD-induced bEND3 cells transfected with ENOPH1 siRNA or control siRNA (**Figure 5D**). These results suggest that ENOPH1 may retard ER stress protective effect and potentiate ROS generation to promote endothelial cell death/apoptosis under OGD conditions.

ENOPH1 Overexpression Enhances OGD-Induced Endothelial Injury and Oxidative Stress

To further verify an important role of ENOPH1 in potentiating endothelial cell death/apoptosis under OGD conditions, we transfected bEND3 cells with ENOPH1 CRISPR activation plasmid to elevate the ENOPH1 expression

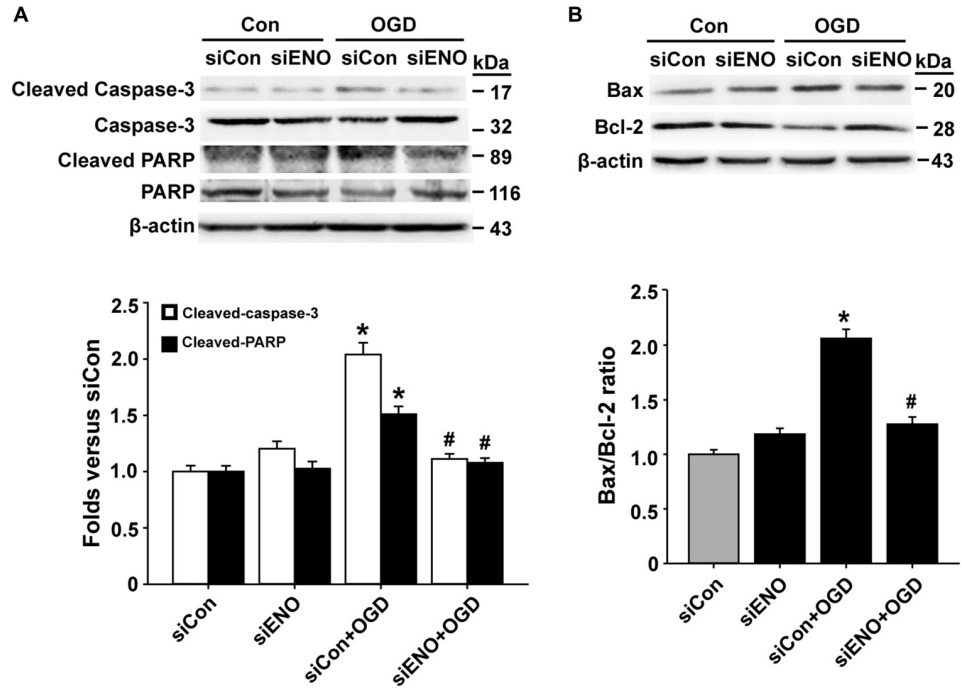


FIGURE 4 | Knockdown of ENOPH1 attenuates OGD-induced apoptosis-related protein expression in bEND3 cells. bEND3 cells transfected with siENOPH1 (siENO) or control siRNA (siCon) before exposing to OGD for 6 h. Apoptosis-related proteins were analyzed by western blot. **(A)** Upper panel: representative immunoblots showing the changes of cleaved and full length caspase-3 and PARP protein bands in bEND3 cells. β-actin was used as a loading control; bottom panel: quantitative data showed that OGD significantly increased the cleavage of caspase-3 and PARP and knockdown of ENOPH1 inhibited this change. $^{*}P < 0.05$ vs. siCon; $^{\#}P < 0.05$ vs. siCon + OGD; $n = 4$. **(B)** Upper panel: representative immunoblots of Bax and Bcl-2 proteins; bottom panel: quantitative data showed that OGD significantly increased the ratio of Bax/Bcl-2 and knockdown of ENOPH1 inhibited this increase. $^{*}P < 0.05$ vs. siCon; $^{\#}P < 0.05$ vs. siCon + OGD; $n = 4$.

before exposing the cells to OGD treatment for 6 h and assessed its impact on cell death/apoptosis associated proteins. As shown in **Figure 6A**, western blot analysis showed that bEND3 cells transfected with ENOPH1 plasmid led to increase in ENOPH1 protein level at 48 h after transfection compared to control plasmid. Of note, ENOPH1 plasmid had no effect on cell death assessed by measuring 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide formation under control condition, but it significantly potentiated OGD-induced increase in cell death (**Figure 6B**). It also significantly enhanced OGD-induced activation of apoptosis-associated signal molecules, reflected by greater ratios of cleaved caspase-3/caspase-3 and Bax/Bcl-2 for OGD plus ENOPH1 plasmid-treated cells than OGD alone treated cells (**Figures 6C,D**). These data indicate that overexpression of ENOPH1 potentiates endothelial cell death/apoptosis under OGD conditions.

ENOPH1 Regulates ADI1 Redistribution in Endothelial Cells Under OGD Condition

ADI1 is a downstream protein of ENOPH1 and has been shown to play an important role in cell apoptosis, oxidoreductase reaction and virus infection (Hirano et al., 2005; Oram et al., 2007; Cheng et al., 2009). Therefore, we speculated that ADI1 might mediate the effect of ENOPH1 on OGD-induced endothelial cell death. To investigate this possibility, we studied the interaction between these two proteins. First, we examined the changes of ADI1 expression in OGD-treated bEND3 cells. As shown in **Figure 7A**, ADI1 mRNA expression was increased at 1 h after OGD treatment, which, unlike ENOPH1, was not further increased when OGD duration was prolonged to 3 h or 6 h. The change of ADI1 protein seemed to lag behind its mRNA, as bEND3 cells did not show increased ADI1 protein levels at 1 h OGD treatment and the increase in ADI1 protein was only seen after 3 or 6 h OGD (**Figure 7B**). To our surprise, knockdown of ENOPH1 with siRNA did not affect ADI1 protein expression under both normoxic and OGD conditions (**Figure 7C**), indicating that OGD-induced ADI1 upregulation was not ENOPH1-dependent. Interestingly, ENOPH1 knockdown significantly stimulated the translocation of ADI1 from the nuclei to the cytosol (**Figure 7D**). To demonstrate a direct interaction of ENOPH1 with ADI1, we conducted coimmunoprecipitation experiments. As shown in **Figure 7E**, ADI1 coimmunoprecipitated with ENOPH1 and OGD treatment enhanced the interaction of ENOPH1 with ADI1 by approximately one-fold over the control group (**Figure 7E**). Collectively, these data demonstrate that ENOPH1 might interact with ADI1 to restrict ADI1 in

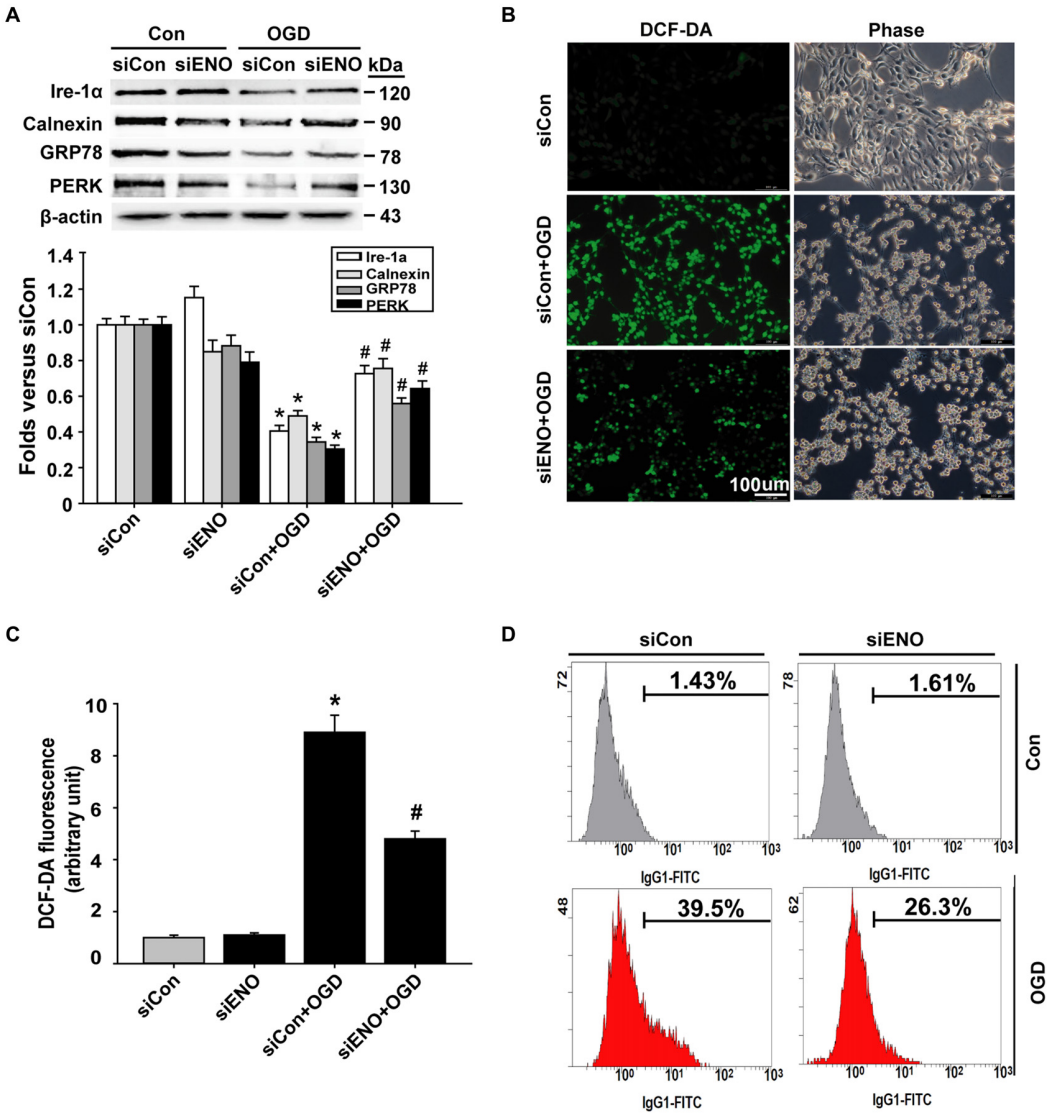


FIGURE 5 | Knockdown of ENOPH1 inhibits OGD-induced oxidative stress in bEND3 cells. Down-regulation of ENOPH1 on OGD induced Endoplasmic reticulum (ER) stress signaling were determined by western blot. **(A)** Upper panel: representative immunoblots showing the changes of Ire-1a, calnexin, GRP78 and PERK protein bands in bEND3 cells after 6 h OGD treatment, with or without siENOPH1 transfection; bottom panel: quantitative data showed that OGD significantly decreased the mentioned ER stress chaperon protein level and knockdown of ENOPH1 inhibited this change. β-actin was used as a loading control. $^{*}P < 0.05$ vs. siCon; $^{\#}P < 0.05$ vs. siCon + OGD; $n = 4$. **(B)** bEND3 cells transfected with siENOPH1 or control were loaded with DCF-DA (10 μM) to detect intracellular reactive oxygen species (ROS) production. Representative microscope pictures were shown (bar = 100 μm). **(C)** Quantification data showed that OGD significantly increased ROS production and knockdown of ENOPH1 inhibited ROS increase. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. OGD; $n = 4$. **(D)** Representative flow cytometry of cells transfected with or without siENOPH1 after 6 h OGD treatment. Cells were stained with DCF-DA solution and then the levels of intracellular ROS were analyzed by flow cytometry; $n = 4$.

the nucleus and this interaction was enhanced under OGD conditions.

Knockdown of ENOPH1 Reduces OGD-Induced Increase in the Permeability of bEND3 Cell Monolayer

The above data have demonstrated a role of ENOPH1 in contributing to brain endothelial cell death under ischemic conditions, therefore we speculated that ENOPH1 was involved in OGD-induced endothelial barrier disruption. To test this possibility, we assessed the impact of ENOPH1 siRNA on the permeability of endothelial cell monolayer to FITC-dextran under control or OGD conditions. bEND3 cells were pre-treated with ENOPH1 siRNA before exposing to OGD for 6 h. As expected, 6 h OGD significantly increased the permeability of bEND3 cell monolayer to FITC-dextran (**Figure 8**). ENOPH1 siRNA, but not scrambled control siRNA, significantly reduced

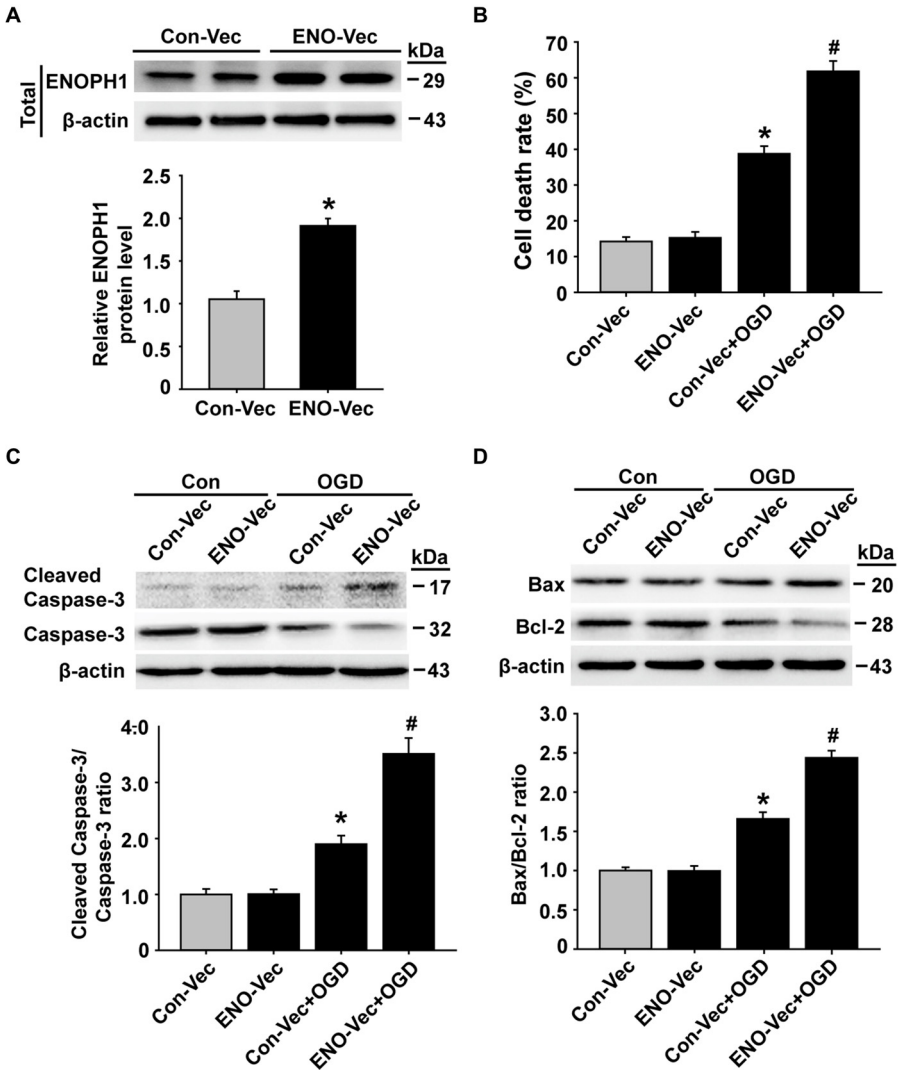


FIGURE 6 | Overexpression of ENOPH1 aggravates OGD-induced apoptosis in bEND3 cells. (A) The ENOPH1 CRISPR activated plasmid effectively increased ENOPH1 protein expression in bEND3 cells. Upper panel: representative immunoblots showing the changes of ENOPH1 protein bands in bEND3 cells after 6 h OGD treatment, with or without ENOPH1 CRISPR activated plasmid (ENO-Vec) transfection; bottom panel: quantitative data showed that transfecting cells with ENO-Vec for 48 h significantly enhanced ENOPH1 protein levels $^{*}P < 0.05$ vs. control activated plasmid (Con-Vec); $n = 4$. **(B)** ENOPH1 overexpression significantly increased 6 h OGD induced cell death assessed by lactate dehydrogenase release. $^{*}P < 0.05$ vs. Con-Vec; $^{\#}P < 0.05$ vs. Con-Vec + OGD; $n = 4$. **(C)** Upper panel: representative immunoblots showing the changes of cleaved and full length caspase-3 protein bands in bEND3 cells. β-actin was used as a loading control; bottom panel: quantitative data showed that OGD significantly increased the cleavage of caspase-3 and PARP and overexpression of ENOPH1 aggregated this change. $^{*}P < 0.05$ vs. Con-Vec; $^{\#}P < 0.05$ vs. Con-Vec + OGD; $n = 4$. **(D)** Upper panel: representative immunoblots of Bax and Bcl-2 proteins; bottom panel: quantitative data showed that OGD significantly increased the ratio of Bax/Bcl-2 and overexpression of ENOPH1 promoted this increase. $^{*}P < 0.05$ vs. Con-Vec; $^{\#}P < 0.05$ vs. Con-Vec + OGD; $n = 4$.

the permeability of OGD-treated endothelial monolayer to FITC-dextran (**Figure 8**). These results indicate that ENOPH1 is involved in OGD-induced loss of endothelial barrier function under ischemic conditions.

DISCUSSION

ENOPH1 is a newly identified enzyme of the methionine salvage pathway, and has been found to play a role in stress reactivity (Barth et al., 2014). In this study, we demonstrated a new role of ENOPH1 in mediating BBB injury under OGD conditions, which is a model of cerebral ischemia. The major findings include: (1) MCAO and OGD significantly increase ENOPH1 expression in cerebral microvessels and cultured brain endothelial cells (bEND3 cells), respectively; (2) knockdown of ENOPH1 with siRNA significantly attenuates OGD-induced bEND3 cell death and the activation of apoptosis-associated signal molecules, and accordingly overexpression

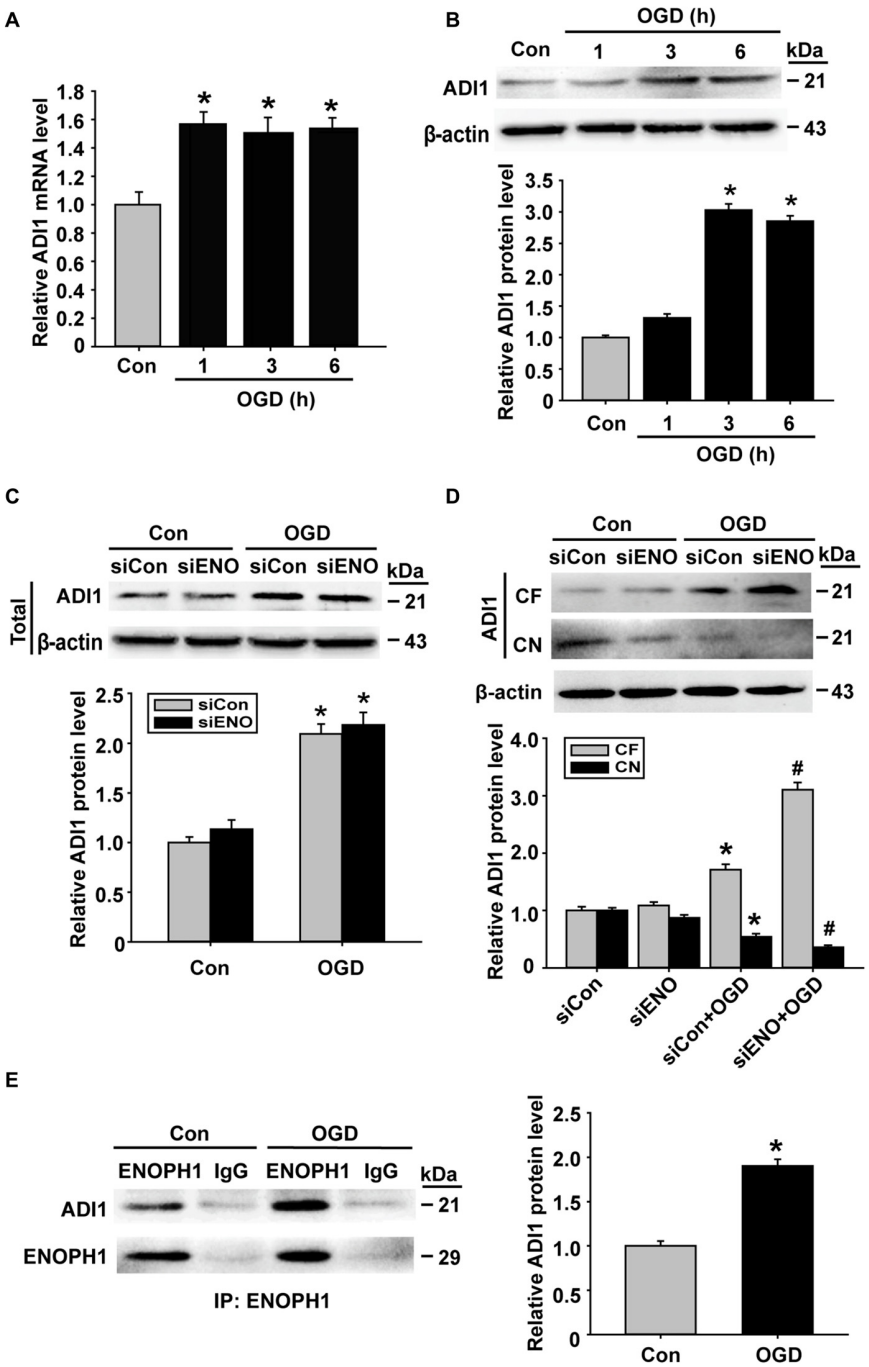


FIGURE 7 | ENOPH1 mediates OGD-induced ADI-1 relocation in endothelial cells. (A) Real time RT-PCR analysis showed that aci-reductone dioxygenase 1 (ADI1) mRNA expression was significantly increased in bEND3 cells at 1 h after OGD treatment and was further increased when OGD was prolonged to 6 h. $^{*}P < 0.05$ vs. Con; $n = 4$. **(B)** Western blot analysis showed that ADI1 protein levels were increased in 3 h OGD and 6 h OGD treated cells. Upper panel: representative immunoblots of ADI1 and the loading control β-actin; bottom panel: quantitative data of protein band intensity after normalization to β-actin., $^{*}P < 0.05$ vs. Con; $n = 4$. **(C)** Upper panel: representative immunoblots of ADI1 and the loading control β-actin; bottom panel: quantitative data showed that transfected with ENOPH1 siRNA had not prevented OGD induced upregulation of total protein level of ADI in bEND3 cells. $^{*}P < 0.05$ vs. siCon; $n = 4$. **(D)** Upper panel: representative immunoblots of ADI1 and the loading control β-actin; bottom panel: quantitative data showed that following OGD treatment, ADI1 levels in the CF was increased, while its level in CN was markedly reduced, which was enhanced by ENOPH1 siRNA. $^{*}P < 0.05$ vs. siCon, $^{\#}P < 0.05$ vs. siCon + OGD; CF, cytosolic fraction; CN, cytosolic nuclei; $n = 4$. **(E)** Left panel: representative immunoblots of coimmunoprecipitation of ENOPH1 and ADI1 from whole cell lysates of control cultures or OGD-treated cells with anti-ENOPH1 antibody or normal anti-IgG; right panel: quantitative data showed that OGD enhanced interaction of ENOPH1 with ADI1. $^{*}P < 0.05$ vs. Con; $n = 4$.

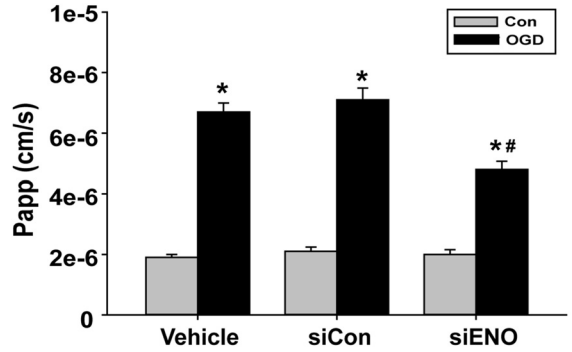


FIGURE 8 | Knockdown of ENOPH1 with siRNA reduces OGD induced blood brain barrier (BBB) disruption *in vitro*. The permeability of fluorescein isothiocyanate (FITC)-dextran across bEND3 monolayers was significantly increased after 6 h OGD. This was partially inhibited by pretreating cells with ENOPH1 siRNA. The permeability of the endothelial monolayer was assessed by calculating the transfer rate of FITC-dextran from the luminal compartment to the abluminal compartment, and was expressed as the apparent permeability coefficient (Papp, cm/s). $^{*}P < 0.05$ vs. Con, $^{\#}P < 0.05$ vs. siCon + OGD; $n = 4$. Papp [cm/s] = dQ/(dt*A*Co), dQ: the amount of FITC-dextran getting into the abluminal compartment; dt : duration of OGD treatment; dQ/dt : the rate of transfer (ng/s); A: surface area (cm^2); Co: the initial concentration in the luminal chamber (ng/cm^3).

of ENOPH1 with CRISPR-activated plasmid potentiates these changes; (3) knockdown of ENOPH1 attenuates OGD-induced ROS generation and ER stress; (4) OGD potentiates the interaction between ENOPH1 and its downstream molecule ADI1, increases ADI1 expression and promotes ADI1's translocation into the cytosol; (5) ENOPH1 appears to restrain ADI1's translocation from the nucleus to the cytosol, but does not affect OGD-induced ADI1 upregulation; and (6) knockdown of ENOPH1 attenuates OGD-induced endothelial barrier disruption.

Currently, the biological functions of ENOPH1 are largely unknown. A few recent studies have shown that ENOPH1 is widely expressed in the brain and is associated with neurodevelopmental disorders and anxiety (Barth et al., 2014; Komlósi et al., 2015). As the first study, here we investigated the role of ENOPH1 in ischemic stroke with a focus on the BBB. Our data show that both *in vivo* and *in vitro* ischemia induce ENOPH1 upregulation in cerebral microvascular endothelial cells. Of note, this change is quite rapid and persistent, as ENOPH1 mRNA expression was found to be increased in endothelial cells at 1 h after OGD treatment and remained high at the end of 6 h OGD exposure. Under our experimental conditions, significant cytotoxicity was only observed for 6 h OGD, but not for 1 h OGD and 3 h OGD, which was consistent with our previous studies (Liu et al., 2012, 2015). Therefore, in this study, 6 h OGD was chosen to test whether ENOPH1 contributes to brain endothelial cell death under ischemic conditions.

Our data show that knockdown of ENOPH1 with siRNA reduces OGD-induced cell death/apoptosis, while overexpressing ENOPH1 by transfecting ENOPH1 CRISPR activation plasmid results in a higher cell death rate in brain endothelial cells, which clearly supports a role of ENOPH1 in OGD-induced endothelial cell death/apoptosis. Moreover, along with ENOPH1 knockdown or overexpression is the inhibition or activation of apoptosis-associated proteins, including cleaved caspase-3, cleaved PARP, Bax and Bcl-2, further supporting a proapoptotic action of ENOPH1.

Increased free radical generation and impaired function of the (ER stress) are two common pathophysiological events occurring in the ischemic brain (Olmez and Ozyurt, 2012; Yu et al., 2016). Here, our data have also shown increased ROS generation in brain endothelial cells after 6 h OGD treatment. Of note, knockdown of ENOPH1 with siRNA markedly attenuated OGD-induced ROS generation in endothelial cells, suggesting that ENOPH1 may contribute to ischemic endothelial cell death via promoting intracellular ROS generation. Under ischemic conditions, there are several enzymatic and non-enzymatic sources of ROS generation, such as NADPH oxidase (Hur et al., 2010), the mitochondrial respiratory chain (Sanderson et al., 2013), and xanthine oxidase (Ono et al., 2009). In this study, we did not further explore the mechanism by which ENOPH1 enhances ROS generation under OGD condition. To our surprise, ischemic brain endothelial cells appear to have suppressed ER stress under our experimental conditions, as 6 h OGD led to suppressed expression of ER-stress associated proteins (Ire-1a, Calnexin, GRP78, and PERK). Moreover, ENOPH1 appears to act as an ER stress suppressor because knockdown of ENOPH1 significantly reverses OGD-induced suppression of ER stress-associated proteins. Our possible explanation for this unexpected observation would be ENOPH1 protein levels could retard ER stress protective effect by inhibiting some ER molecular chaperones (such as GRP78) expression in endothelial cells under OGD conditions.

ADI1 is a downstream molecule of ENOPH1 in methionine salvage pathway and has been shown to be implicated in cell apoptosis, cell growth inhibition, oxidoreductase reaction and virus infection (Hirano et al., 2005; Oram et al., 2007; Cheng et al., 2009). In addition, ADI1 is an oxidoreductase and can combine molecular oxygen donor to generate ROS (Oram et al., 2007). This known evidence has promoted us to hypothesize that ADI1 may mediate ENOPH1's effect on promoting ischemic endothelial cell death. Our data show that OGD induces ADI1 mRNA and protein upregulation in a time-dependent manner similar to ENOPH1. To our surprise, although the coimmunoprecipitation assay clearly shows that ADI1 interacts with ENOPH1, knockdown of ENOPH1 does not affect OGD-induced ADI1 upregulation. Another interesting finding is that OGD increases the translocation of ADI1 from the nucleus to the cytosol and knockdown of ENOPH1 enhances this translocation. Our data raise several important questions: (i) what promotes the translocation of ADI1 to the cytosol under ischemic conditions; (ii) what translocated ADI1 does in the cytosol; and (iii) how ENOPH1 potentiates ADI1 translocation to the cytosol. Future studies are warranted to answer these questions.

Last, our data that knockdown of ENOPH1 partially inhibits OGD-induced permeability increase of the endothelial

monolayer support an important role of ENOPH1 in ischemic BBB injury. It is well known that the brain capillary endothelial cells and the tight junctions between adjacent endothelial cells are the two most important structural components that maintain the integrity of the BBB (ElAli et al., 2011; Jumnongprakhon et al., 2016). Our current study was only focused the effect of ENOPH1 on ischemic endothelial injury and did not study its impact on the tight junctions. As a fact, ENOPH1's downstream molecule ADI1 can bind to and inhibit the activity of membrane-type matrix metalloproteinase (MT1-MMP; Uekita et al., 2004; Chang et al., 2016), and our data demonstrate that ENOPH1 enhances the translocation of ADI1 to the nucleus under OGD conditions. As thus, there is a great possibility that ENOPH1 may act as a separator to push ADI1 away from the MT1-MMP (located in cell membrane and the cytosol), and thus activate MT1-MMP. The latter in turn activates MMP-2 to mediate the degradation of tight junction proteins and the components of the basal membrane, leading to BBB disruption (Yang et al., 2007). Future studies are needed to test this possibility.

Taken together, our present study defines a novel role of ENOPH1 in ischemic brain injury, in which cerebral ischemia triggers ENOPH1 upregulation in brain endothelial cells, leading to increased ROS generation and the activation of apoptosis associated molecules to contribute to ischemic cell death. Moreover, under OGD condition, ENOPH1 promotes the translocation of its downstream molecule ADI1 from the cytoplasm to the nucleus, future studies are needed to investigate the mechanisms underlying this change and its biological consequence.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: WL (Wenlan) and YZ. Performed the experiments: YZ, TW and KY. Analyzed the data: YZ, JX and LR. Contributed reagents/materials/analysis tools: TW and KY. Wrote the article: WL (Wenlan) and WL (Weiping).

FUNDING

The work was supported by grants from National Natural Science Foundation of China (81571149); by China Postdoctoral Science Foundation (2015M572387), and Project Funded by Shenzhen Science and Technology Innovation Commission (JCYJ20150330102401097, KQCX20140521101427034, ZDSYS20140509173142601).

ACKNOWLEDGMENTS

We thank Prof. Xinchun Jin for animal experimental support.

REFERENCES

Barth, A., Bilkei-Gorzo, A., Drews, E., Otte, D. M., Diaz-Lacava, A., Varadarajulu, J., et al. (2014). Analysis of quantitative trait loci in mice suggests a role of Enoph1 in stress reactivity. *J. Neurochem.* 128, 807–817. doi: 10.1111/jnc.12517

Chang, M. L., Huang, Y. H., Cheng, J. C., and Yeh, C. T. (2016). Interaction between hepatic membrane type 1 matrix metalloproteinase and acireductone dioxygenase 1 regulates hepatitis C virus infection. *J. Viral Hepat.* 23, 256–266. doi: 10.1111/jvh.12486

Cheng, J. C., Yeh, Y. J., Pai, L. M., Chang, M. L., and Yeh, C. T. (2009). 293 cells over-expressing human ADI1 and CD81 are permissive for serum-derived hepatitis C virus infection. *J. Med. Virol.* 81, 1560–1568. doi: 10.1002/jmv.21495

Duan, B., Wang, Y. Z., Yang, T., Chu, X. P., Yu, Y., Huang, Y., et al. (2011). Extracellular spermine exacerbates ischemic neuronal injury through sensitization of ASIC1a channels to extracellular acidosis. *J. Neurosci.* 31, 2101–2112. doi: 10.1523/jneurosci.4351-10.2011

ElAli, A., Doeppner, T. R., Zechariah, A., and Hermann, D. M. (2011). Increased blood-brain barrier permeability and brain edema after focal cerebral ischemia induced by hyperlipidemia: role of lipid peroxidation and calpain-1/2, matrix metalloproteinase-2/9 and RhoA overactivation. *Stroke* 42, 3238–3244. doi: 10.1161/strokeaha.111.615559

Grabovac, V., and Bernkop-Schnürch, A. (2006). Improvement of the intestinal membrane permeability of low molecular weight heparin by complexation with stem bromelain. *Int. J. Pharm.* 326, 153–159. doi: 10.1016/j.ijpharm.2006.06.042

Hawkins, K. E., DeMars, K. M., Singh, J., Yang, C., Cho, H. S., Frankowski, J. C., et al. (2014). Neurovascular protection by post-ischemic intravenous injections of the lipoxin A4 receptor agonist, BML-111, in a rat model of ischemic stroke. *J. Neurochem.* 129, 130–142. doi: 10.1111/jnc.12607

Hirano, W., Gotoh, I., Uekita, T., and Seiki, M. (2005). Membrane-type 1 matrix metalloproteinase cytoplasmic tail binding protein-1 (MTCBP-1) acts as an eukaryotic aci-reductone dioxygenase (ARD) in the methionine salvage pathway. *Genes Cells* 10, 565–574. doi: 10.1111/j.1365-2443.2005.00859.x

Hur, J., Lee, P., Kim, M. J., Kim, Y., and Cho, Y. W. (2010). Ischemia-activated microglia induces neuronal injury via activation of gp91phox NADPH oxidase. *Biochem. Biophys. Res. Commun.* 391, 1526–1530. doi: 10.1016/j.bbrc.2009.12.114

Jumnongprakhon, P., Govitrapong, P., Tocharus, C., and Tocharus, J. (2016). Melatonin promotes blood-brain barrier integrity in methamphetamine -induced inflammation in primary rat brain microvascular endothelial cells. *Brain Res.* 1646, 182–192. doi: 10.1016/j.brainres.2016.05.049

Kaur, C., and Ling, E. A. (2008). Antioxidants and neuroprotection in the adult and developing central nervous system. *Curr. Med. Chem.* 15, 3068–3080. doi: 10.2174/092986708786848640

Kim, G. H., Komotar, R. J., McCullough-Hicks, M. E., Otten, M. L., Starke, R. M., Kellner, C. P., et al. (2009). The role of polyamine metabolism in neuronal injury following cerebral ischemia. *Can. J. Neurol. Sci.* 36, 14–19. doi: 10.1017/s0317167100006247

Komlósi, K., Duga, B., Hadzsiev, K., Czakó, M., Kosztolányi, G., Fogarasi, A., et al. (2015). Phenotypic variability in a Hungarian patient with the 4q21 microdeletion syndrome. *Mol. Cytogenet.* 8:16. doi: 10.1186/s13039-015-0118-7

Li, J., Doyle, K. M., and Tatlisumak, T. (2007). Polyamines in the brain: distribution, biological interactions and their potential therapeutic role in brain ischaemia. *Curr. Med. Chem.* 14, 1807–1813. doi: 10.2174/092986707781058841

Liu, W., Hendren, J., Qin, X. J., Shen, J., and Liu, K. J. (2009). Normobaric hyperoxia attenuates early blood-brain barrier disruption by inhibiting MMP-9-mediated occludin degradation in focal cerebral ischemia. *J. Neurochem.* 108, 811–820. doi: 10.1111/j.1471-4159.2008.05821.x

Liu, J., Jin, X., Liu, K. J., and Liu, W. (2012). Matrix metalloproteinase-2-mediated occludin degradation and caveolin-1-mediated claudin-5 redistribution contribute to blood-brain barrier damage in early ischemic stroke stage. *J. Neurosci.* 32, 3044–3057. doi: 10.1523/jneurosci.6409-11.2012

Liu, W., Sood, R., Chen, Q., Sakoglu, U., Hendren, J., Cetin, O., et al. (2008). Normobaric hyperoxia inhibits NADPH oxidase-mediated matrix metalloproteinase-9 induction in cerebral microvessels in experimental stroke. *J. Neurochem.* 107, 1196–1205. doi: 10.1111/j.1471-4159.2008.05664.x

Liu, J., Weaver, J., Jin, X., Zhang, Y., Xu, J., Liu, K. J., et al. (2015). Nitric oxide interacts with caveolin-1 to facilitate autophagy-lysosome-mediated claudin-5 degradation in oxygen-glucose deprivation-treated endothelial cells. *Mol. Neurobiol.* doi: 10.1007/s12035-015-9504-8 [Epub ahead of print].

Olmez, I., and Ozyurt, H. (2012). Reactive oxygen species and ischemic cerebrovascular disease. *Neurochem. Int.* 60, 208–212. doi: 10.1016/j.neuint.2011.11.009

Ono, T., Tsuruta, R., Fujita, M., Aki, H. S., Kutsuna, S., Kawamura, Y., et al. (2009). Xanthine oxidase is one of the major sources of superoxide anion radicals in blood after reperfusion in rats with forebrain ischemia/reperfusion. *Brain Res.* 1305, 158–167. doi: 10.1016/j.brainres.2009.09.061

Oram, S. W., Ai, J., Pagani, G. M., Hitchens, M. R., Stern, J. A., Eggener, S., et al. (2007). Expression and function of the human androgen-responsive gene ADI1 in prostate cancer. *Neoplasia* 9, 643–651. doi: 10.1593/neo.07415

Sanderson, T. H., Reynolds, C. A., Kumar, R., Przyklenk, K., and Hüttemann, M. (2013). Molecular mechanisms of ischemia-reperfusion injury in brain: pivotal role of the mitochondrial membrane potential in reactive oxygen species generation. *Mol. Neurobiol.* 47, 9–23. doi: 10.1007/s12035-012-8344-z

Sandoval, K. E., and Witt, K. A. (2008). Blood-brain barrier tight junction permeability and ischemic stroke. *Neurobiol. Dis.* 32, 200–219. doi: 10.1016/j.nbd.2008.08.005

Sauter, M., Moffatt, B., Saechao, M. C., Hell, R., and Wirtz, M. (2013). Methionine salvage and S-adenosylmethionine: essential links between sulfur, ethylene and polyamine biosynthesis. *Biochem. J.* 451, 145–154. doi: 10.1042/bj20121744

Takano, K., Ogura, M., Nakamura, Y., and Yoneda, Y. (2005). Neuronal and glial responses to polyamines in the ischemic brain. *Curr. Neurovasc. Res.* 2, 213–223. doi: 10.2174/1567202054368335

Uekita, T., Gotoh, I., Kinoshita, T., Itoh, Y., Sato, H., Shiomi, T., et al. (2004). Membrane-type 1 matrix metalloproteinase cytoplasmic tail-binding protein-1 is a new member of the Cupin superfamily. A possible multifunctional protein acting as an invasion suppressor down-regulated in tumors. *J. Biol. Chem.* 279, 12734–12743. doi: 10.1074/jbc.m309957200

Wang, Z., Leng, Y., Tsai, L. K., Leeds, P., and Chuang, D. M. (2011). Valproic acid attenuates blood-brain barrier disruption in a rat model of transient focal cerebral ischemia: the roles of HDAC and MMP-9 inhibition. *J. Cereb. Blood Flow Metab.* 31, 52–57. doi: 10.1038/jcbfm.2010.195

Wen, X., Li, Y., and Liu, Y. (2010). Opposite action of peroxisome proliferator-activated receptor-gamma in regulating renal inflammation: functional switch by its ligand. *J. Biol. Chem.* 285, 29981–29988. doi: 10.1074/jbc.m110.110908

Yang, Y., Estrada, E. Y., Thompson, J. F., Liu, W., and Rosenberg, G. A. (2007). Matrix metalloproteinase-mediated disruption of tight junction proteins in cerebral vessels is reversed by synthetic matrix metalloproteinase inhibitor in focal ischemia in rat. *J. Cereb. Blood Flow Metab.* 27, 697–709. doi: 10.1038/sj.jcbfm.9600375

Yang, F., Wang, Z., Wei, X., Han, H., Meng, X., Zhang, Y., et al. (2014). NLRP3 deficiency ameliorates neurovascular damage in experimental ischemic stroke. *J. Cereb. Blood Flow Metab.* 34, 660–667. doi: 10.1038/jcbfm.2013.242

Yu, Z., Sheng, H., Liu, S., Zhao, S., Glembotski, C. C., Warner, D. S., et al. (2016). Activation of the ATF6 branch of the unfolded protein response in neurons improves stroke outcome. *J. Cereb. Blood Flow Metab.* doi: 10.1177/0271678x16650218 [Epub ahead of print].

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Molecular Neuroscience

REVIEW
published: 16 June 2016
doi: 10.3389/fnmol.2016.00047

Energy and Potassium Ion Homeostasis during Gamma Oscillations

Oliver Kann[1,2]*, *Jan-Oliver Hollnagel*[1,2], *Shehabeldin Elzoheiry*[1,2] and *Justus Schneider*[1,2]

[1] Institute of Physiology and Pathophysiology, University of Heidelberg, Heidelberg, Germany, [2] Interdisciplinary Center for Neurosciences (IZN), University of Heidelberg, Heidelberg, Germany

Fast neuronal network oscillations in the gamma frequency band (30–100 Hz) occur in various cortex regions, require timed synaptic excitation and inhibition with glutamate and GABA, respectively, and are associated with higher brain functions such as sensory perception, attentional selection and memory formation. However, little is known about energy and ion homeostasis during the gamma oscillation. Recent studies addressed this topic in slices of the rodent hippocampus using cholinergic and glutamatergic receptor models of gamma oscillations (GAM). Methods with high spatial and temporal resolution were applied *in vitro*, such as electrophysiological recordings of local field potential (LFP) and extracellular potassium concentration ($[K^+]_o$), live-cell fluorescence imaging of nicotinamide adenine dinucleotide (phosphate) and flavin adenine dinucleotide [NAD(P)H and FAD, respectively] (cellular redox state), and monitoring of the interstitial partial oxygen pressure (pO_2) in depth profiles with microsensor electrodes, including mathematical modeling. The main findings are: (i) GAM are associated with high oxygen consumption rate and significant changes in the cellular redox state, indicating rapid adaptations in glycolysis and oxidative phosphorylation; (ii) GAM are accompanied by fluctuating elevations in $[K^+]_o$ of less than 0.5 mmol/L from baseline, likely reflecting effective K^+-uptake mechanisms of neuron and astrocyte compartments; and (iii) GAM are exquisitely sensitive to metabolic stress induced by lowering oxygen availability or by pharmacological inhibition of the mitochondrial respiratory chain. These findings reflect precise cellular adaptations to maintain adenosine-5′-triphosphate (ATP), ion and neurotransmitter homeostasis and thus neural excitability and synaptic signaling during GAM. Conversely, the exquisite sensitivity of GAM to metabolic stress might significantly contribute the exceptional vulnerability of higher brain functions in brain disease.

Keywords: cognition, extracellular potassium concentration, GABA-A receptor, membrane ion transport, mitochondria, Na^+/K^+-ATPase, neural information processing, tissue oxygen tension

OPEN ACCESS

Edited by:
Jochen C. Meier, Technical University Braunschweig, Germany

Reviewed by:
Jakob Wolfart, University of Rostock, Germany
Rüdiger Köhling, Rostock University Medical Center, Germany

***Correspondence:**
Oliver Kann
oliver.kann@physiologie.uni-heidelberg.de

Received: *18 February 2016*
Accepted: *30 May 2016*
Published: *16 June 2016*

Citation:
Kann O, Hollnagel J-O, Elzoheiry S and Schneider J (2016) Energy and Potassium Ion Homeostasis during Gamma Oscillations. Front. Mol. Neurosci. 9:47. doi: 10.3389/fnmol.2016.00047

GAMMA OSCILLATIONS AND HIGHER BRAIN FUNCTIONS

Neuronal information processing is primarily executed by principal cells, such as granule and pyramidal neurons that release excitatory neurotransmitter, glutamate (Bliss and Lømo, 1973; Miles and Wong, 1987; LoTurco et al., 1988; Malenka et al., 1989). It is generally thought that these projection neurons process, transfer, store and retrieve information and therefore, underlie the emergence of higher brain functions such as sensory perception, attentional selection, motor behavior, and memory formation (Buzsáki, 2006; Kullmann and Lamsa, 2007; Hájos and Paulsen, 2009; Ho et al., 2011).

Neuronal information processing, however, depends on the coordination of principal cell activity in cortical networks (Buzsáki, 2006; Traub and Whittington, 2010). Such coordination can be provided by neuronal network oscillations that show a wide spectrum of frequencies, ranging from about 0.05 Hz to 600 Hz (Buzsáki and Draguhn, 2004). Prominent examples are network oscillations in the theta (4–12 Hz), beta (13–30 Hz) and gamma (30–100 Hz) bands, which are associated with different cognitive and behavioral states (Buzsáki, 2006; Hájos and Paulsen, 2009; Uhlhaas and Singer, 2010; Watrous et al., 2015). Of course, this does not preclude the importance of slower oscillations for higher brain functions (Buzsáki, 2006; Schroeder and Lakatos, 2009).

Gamma oscillations (GAM) (30–100 Hz) have been found in many mammalian brain regions, such as visual, auditory, somatosensory and motor systems, and in the hippocampus (Kreiter and Singer, 1992; Murthy and Fetz, 1992; Franowicz and Barth, 1995; Lebedev and Nelson, 1995; Whittington et al., 1995; Gray and Viana Di Prisco, 1997). GAM are associated with rhythmic fluctuations of the membrane potential of 5–10 mV in excitatory pyramidal cells and fast-spiking inhibitory interneurons, reflecting precisely timed incidence of excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs) (Whittington et al., 1995; Penttonen et al., 1998; Fischer et al., 2002; Salkoff et al., 2015). These rhythmic fluctuations support the synchronized generation of action potentials (neuronal "spiking") in principal cells with great precision (Buzsáki, 2006; Hájos and Paulsen, 2009; Watrous et al., 2015) and thus, permit the coordinated activation of defined sets of neurons, i.e., functional ensembles that are thought to represent the information-carrying multicellular subsets of neuronal networks (Buzsáki and Chrobak, 1995; Whittington et al., 1997; Fries et al., 2007; Traub and Whittington, 2010). GAM have a role in higher brain functions, such as voluntary movement, visual and auditory perception, attentional selection as well as memory formation (Gray et al., 1989; Pantev et al., 1991; Paulsen and Moser, 1998; Haenschel et al., 2000; Melloni et al., 2007; Montgomery and Buzsáki, 2007; Cheyne et al., 2008; Lisman and Buzsáki, 2008; van Vugt et al., 2010; Zhang et al., 2012; Popa et al., 2013). GAM *in vivo* occur transiently on the 100 ms time scale upon sensory input (Pantev et al., 1991; Bragin et al., 1995; Franowicz and Barth, 1995). In the human brain and dependent on the task, however, they can last for prolonged times in the range of minutes (Lehmann et al., 2001; Lutz et al., 2004). A summary of some key features of cortical GAM is given in **Table 1**.

TABLE 1 | Features of cortical gamma oscillations (GAM).

Features of gamma oscillations	Reference
Presence in most cortical areas	Murthy and Fetz (1992); Haenschel et al. (2000); van Vugt et al. (2010); and Popa et al. (2013)
Strong relationship to higher brain functions	Gray et al. (1989); Pantev et al. (1991); Lutz et al. (2004); and Zhang et al. (2012)
Fast rhythmic inhibition by interneurons	Traub et al. (2001); Hájos et al. (2004); Cardin et al. (2009); and Gulyás et al. (2010)
High oxygen consumption rate	Niessing et al. (2005); Kann et al. (2011); and Huchzermeyer et al. (2013)
Increase in $[K^+]_o$ of <0.5 mmol/L	Huchzermeyer et al. (2008); and Kann et al. (2011)
Exquisite sensitivity to metabolic stress	Huchzermeyer et al. (2008); Hájos et al. (2009); Barth and Mody (2011); and Whittaker et al. (2011)

GAM (30–100 Hz) have been related to sensory perception, attentional selection, motor behavior, and memory formation. These oscillations strongly depend on fast-spiking GABAergic interneurons, such as parvalbumin-positive basket cells, that exert fast rhythmic perisomatic inhibition on principal cells in local networks of the hippocampus and the neocortex. Only some key references are given. See details in the text.

INVESTIGATING GAMMA OSCILLATIONS *IN VITRO*: EXPERIMENTAL MODELS AND METHODS

Experimental Models in Hippocampal Slice Preparations

GAM in cortical tissue *in vitro* can be reliably induced by various methods, such as electrical stimulation or bath application of cholinergic or glutamatergic receptor agonists. In many studies, acute slices or organotypic slice cultures of the hippocampus have been used (Whittington et al., 1995; Fisahn et al., 1998; Hájos et al., 2009; Kann et al., 2011). An overview about the induction and features of GAM under various recording conditions, i.e., in different models, is given in **Table 2**. Pharmacologically induced hippocampal GAM *in vitro* share many features with GAM *in vivo*, such as intrinsic generation of GAM in the CA3 region, reversal of the phase of the local field potential (LFP) between stratum pyramidale (cell body layer of pyramidal cells) and stratum radiatum (apical dendritic compartment) of CA3, similar current source density profiles, and highest spiking probability of pyramidal cells at the negative peak of oscillation cycles (CA3, stratum pyramidale) that is followed by spiking of perisomatic inhibitory interneurons within 2 ms, consistent with monosynaptic excitation (Bragin et al., 1995; Penttonen et al., 1998; Csicsvari et al., 2003; Hájos et al., 2004; Hájos and Paulsen, 2009). However, in the majority of *in vitro* studies GAM are persistent for tens of minutes, show a frequency around 40 Hz, and are rarely associated with an additional, slower network rhythm (**Table 2**).

The synaptic mechanisms that underlie the generation of GAM have been reviewed in detail, and they largely depend on the ratio of neuronal excitation and inhibition (Bartos et al., 2007; Hájos and Paulsen, 2009). In the cortex, synaptic inhibition is mainly mediated by neurotransmitter, gamma-aminobutyric acid (GABA) that is released from the heterogeneous group of GABAergic interneurons (Mann and Paulsen, 2007; Klausberger and Somogyi, 2008; Fritschy and Panzanelli, 2014; Kaila et al., 2014). Notably, the transient activation of GABA-A receptors has a key role for the generation of GAM. This is because GAM are completely blocked by GABA-A receptor antagonist, bicuculline in various *in vitro* models, and studies also using transgenic mice show that synaptic

TABLE 2 | Features of hippocampal gamma oscillations (GAM) *in vitro*.

Slice type	Animal	Age	Recording condition	Energy substrate	Induction	Oxygen fraction	Frequency	Duration	Temperature	Reference
Culture	Rat	p6, div21–42	Submerged	Glucose 5.6 mM	mAChR agonist	95%	40 Hz	Persistent with theta	32°C	Fischer et al. (2002)
Culture	Rat	p7–9, div5–9	Interface	Glucose 10 mM	mAChR agonist	20%	49 Hz	Persistent	34 ± 1°C	Huchzermeyer et al. (2013)
Culture	Rat	p7–9, div7–21	Interface	†	mAChR agonist	95%	40 Hz	Persistent	34 ± 1°C	Galow et al. (2014)
Culture	Rat	p7–9, div7–28	Interface	Glucose 10 mM	mAChR agonist/GluR agonist	95%	*24–52 Hz	Persistent	28–36°C	Schneider et al. (2015)
Acute	Rat	adult	Interface	Glucose 10 mM	Electrical stimulation/PE of GluR agonists	95%	40 Hz	Transient	36°C	Whittington et al. (1995)
Acute	Rat	p15–25/adult	Interface	Glucose 10 mM	mAChR agonist	95%	39 Hz	Persistent with theta	34°C	Fisahn et al. (1998)
Acute	Rat	p20–30	Submerged	Glucose 10 mM	†mAChR agonist	95%	38–61 Hz	Transient with theta, delta or none	31–32°C	Fellous and Sejnowski (2000)
Acute	Rat	adult	Interface	Glucose 10 mM	PE of GluR agonist	95%	†	Transient	35.8 ± 0.5°C	Pöschel et al. (2002)
Acute	Mouse	p18–25	Submerged	Glucose 10 mM	PE of GluR agonist	95%	33 Hz	Transient	29°C	Gloveli et al. (2005)
Acute	Rat	p13–20	Submerged	Glucose 10 mM	mAChR agonist	95%	28 Hz	Persistent with beta	29–33°C	Oren et al. (2006)
Culture/ acute	Rat/ mouse	p7–9, div7–12/ p20–30/adult	Interface/ submerged	Glucose 10 mM	mAChR agonist	95%	29–42 Hz	Persistent	34 ± 1°C/ 25 ± 1°C	Kann et al. (2011)
Acute	Rat	p42–56	Interface	Glucose 10 mM	†mAChR agonist/ACh esterase inhibitor	95%	30–47 Hz	Persistent	36 ± 0.5°C	Hollnagel et al. (2015)
Acute	Rat	p2–5/ p6–21	Modified interface	Glucose 11 mM	GluR agonist	95%	–/24–35 Hz	Persistent/ transient with slow waves	33.5 ± 34°C	Tsintsadze et al. (2015)

*Acute slices have a thickness of around 400 μm; the residual thickness of organotypic hippocampal slice cultures is around 200 μm. In most studies, muscarinic acetylcholine receptors (mAChR) were activated with acetylcholine or carbachol, glutamate receptors (GluR) with glutamate or kainic acid. The frequency of GAM also varies with temperature, i.e., ~3.5 Hz increase per degree Celsius for the range of 28–36°C (Schneider et al., 2015). *4 Hz increase when 2 mmol/L lactate was added to 5 mmol/L glucose at 34 ± 1°C. †Various substrates, concentrations or frequencies. div, days in vitro; PE, pressure ejection; p, postnatal day.*

excitation of fast-spiking, parvalbumin-positive interneurons is required for the generation of normal GAM *in vitro* and *in vivo* (Whittington et al., 1995; Fuchs et al., 2007; Cardin et al., 2009; Sohal et al., 2009; Gulyás et al., 2010; Korotkova et al., 2010; Oren et al., 2010). During hippocampal GAM, individual pyramidal cells generate action potentials at 1–3 Hz *in vitro* and *in vivo*, whereas fast-spiking perisomatic interneurons generate action potentials phase-coupled at almost every gamma cycle (Csicsvari et al., 2003; Hájos et al., 2004; Kann et al., 2014).

Recordings of LFP, $[K^+]_o$, pO_2 and Redox State

The LFP, which is also known as micro-, depth or intracranial electroencephalogram (EEG), has been frequently used to monitor neuronal network oscillations *in vitro* and *in vivo*. LFP electrodes are small-sized, have usually a resistance of about 1–2 MOhm and are positioned in the extracellular space. The recorded extracellular potentials arise from all transmembrane ionic fluxes that underlie cellular electrical events, ranging from fast action potentials and postsynaptic potentials in neurons to slow membrane potential fluctuations in glial cells (Buzsáki et al., 2012; Einevoll et al., 2013; Hales and Pockett, 2014). Recent estimates suggest that >95% of the LFP originates in the vicinity of about 250 μm of the electrode tip (Katzner et al., 2009). Thus, the LFP recording represents a spatial average of all electrical events in a confined volume of neuronal tissue at a given point in time. Although still under debate, the prominent influence of tip geometry and impedance has not been proven yet (Nelson and Pouget, 2010).

The $[K^+]_o$ can be determined with double-barreled microelectrodes in neuronal tissue. The reference barrel (LFP) is filled with 154 mM NaCl solution, and the ion-sensitive barrel with an ion-exchanger (K^+ ionophore cocktail) and 100 mM KCl ($[K^+]_o$; Heinemann and Lux, 1975; Gorji and Speckmann, 2009; Papageorgiou et al., 2016). Recordings of $[K^+]_o$ have been used to determine the level of neuronal activation and K^+-homeostasis, including the functions of glial cells (see below). K^+-sensitive microelectrodes measure

the accumulation of K^+ in a restricted extracellular space, irrespective of whether K^+ is released from dendrites, somata or axons. The microelectrodes detect changes in $[K^+]_o$ from the surrounding tissue microenvironment of less than 100 μm for most conditions of experimental K^+-electrophoresis or electrical stimulation (Lux, 1974; Heinemann et al., 1986; Lux et al., 1986; Kann et al., 2003a,b).

The interstitial partial oxygen pressure (pO_2) or the oxygen concentration in neuronal tissue can be determined with Clark-type oxygen microsensors, which are polarographic electrodes. Because oxygen is the final electron acceptor at the mitochondrial respiratory chain, oxygen consumption provides a valuable indirect measure of the metabolic rate in tissues (Rolfe and Brown, 1997). During the recording, oxygen diffuses from the adjacent tissue through a silicone membrane at the sensor tip and is reduced at a gold cathode within the microsensor. The resulting current is measured with a picoammeter and converted into mmHg (or mmol/L) according to calibration curves (Revsbech, 1989; Lecoq et al., 2009; Thomsen et al., 2009). Oxygen microsensors measure quite locally, i.e., with a spatial resolution of 1–2 times the outside tip diameter (8–12 μm). Oxygen consumption rates (mmol/L per min) can be calculated by recording pO_2 depth profiles in slice preparations and by applying mathematical models that consider convective transport, diffusion, and activity-dependent oxygen consumption (Hall and Attwell, 2008; Huchzermeyer et al., 2013).

The cellular redox state is a useful and non-invasive tool to get insight into neuronal energy metabolism. For this purpose, live-cell fluorescence imaging of nicotinamide adenine dinucleotide (phosphate) and flavin adenine dinucleotide [NAD(P)H and FAD, respectively] have been applied. These dinucleotides serve in cellular energy transfer and have been used to get insight into activity-dependent changes in cytosolic and mitochondrial redox state, and thus adaptations in energy metabolism (Kann et al., 2003a,b; Shuttleworth et al., 2003; Kasischke et al., 2004; Brennan et al., 2006; Ivanov et al., 2011). When excited with ultraviolet light the reduced forms (NADH and NADPH) are fluorescent, while the oxidized forms are non-fluorescent. Investigators often refer to changes in NAD(P)H fluorescence because the emission spectra of NADPH and NADH overlap, and their redox states are coupled via nicotinamide nucleotide transhydrogenase (Schuchmann et al., 2001; Kann and Kovács, 2007). Cellular NAD(P)H fluorescence is primarily governed by activities of the respiratory chain (electron transport chain) and the tricarboxylic acid (TCA) cycle in mitochondria. However, relative changes in NAD(P)H fluorescence in brain slices are influenced by a variety of additional factors and need careful interpretation (Kann and Kovács, 2007; Berndt et al., 2015). Electron transport flavoproteins and α-lipoamide dehydrogenase contribute to about 75% of the flavin fluorescence in neurons (Kann and Kovács, 2007). Because both of them are also closely linked to the mitochondrial NADH pool FAD fluorescence provides insight into the mitochondrial redox state (Shuttleworth et al., 2003; Kann and Kovács, 2007). Because here the oxidized form is fluorescent, changes in FAD fluorescence are opposite to NAD(P)H fluorescence. In most studies using epifluorescence recordings (epi-illuminating light source and CCD camera), the changes in NAD(P)H fluorescence originate from neuronal and glial compartments of virtually all layers (*z*-axis) of the slice (Shuttleworth et al., 2003; Foster et al., 2005; Kann and Kovács, 2007; Huchzermeyer et al., 2008).

ENERGY METABOLISM DURING GAMMA OSCILLATIONS

Recent experimental studies have started to define the bioenergetics of cortical GAM. The available experimental evidence from many *in vitro* and some *in vivo* studies in animals and humans indicates that GAM in the hippocampus and the neocortex are associated with significant cellular adaptations to maintain energy, ion and neurotransmitter homeostasis and thus neuronal excitability and synaptic signaling. Below, we discuss the homeostatic adaptations underlying GAM, with emphasis on activity-dependent changes in pO_2, cellular redox state [NAD(P)H and FAD] and $[K^+]_o$.

Recordings of LFP and pO_2 with high temporal and spatial resolution revealed a positive correlation between the power of GAM and the local decrease in pO_2 in acute slices and slice cultures of the hippocampus, reflecting the increase in activity-dependent oxygen consumption (Kann et al., 2011). Intriguingly, the local decrease in pO_2 during GAM was significantly larger compared to repetitive electrical stimulation (10 s, 20 Hz) and close to the decrease in pO_2 associated with pathological activity, i.e., seizure-like events. These findings were supported by a second study in slice cultures of the rat hippocampus demonstrating the about twofold increase in local oxygen consumption rate during GAM (about 11 mmol/L per min) compared with spontaneous asynchronous neuronal network activity (about 5 mmol/L per min). This follow-up study was based on depth profiles of local pO_2 with high spatial resolution and mathematical modeling of convective transport, diffusion and activity-dependent consumption of oxygen (Huchzermeyer et al., 2013). Notably, the oxygen microsensor measured locally in stratum pyramidale, i.e., the layer where the somata of pyramidal cells are densely packed and mainly receive strong perisomatic inhibition from the complex axon arbors of GABAergic parvalbumin-positive basket cells (Sik et al., 1993; Hu et al., 2014; Kann et al., 2014). Thus, the data reflect oxygen consumption related to postsynaptic inhibition and action potential generation in pyramidal cells as well as axonal action potentials and fast rhythmic release of GABA from basket cells (Kann et al., 2014). Further experimental studies are required to determine the fractions of energy utilization in pyramidal cells (perisomatic postsynaptic potentials and action potentials at the axon initial segment) vs. GABAergic basket cells (action potentials and GABA release in the complex axon arbor).

The oxygen consumption rates during persistent GAM are higher than those obtained during spontaneous asynchronous activity or repetitive electrical stimulation in acute hippocampal and cerebellar slices reported to range from 0.7 to 1.3 mmol/L per min (Hall and Attwell, 2008; Ivanov and Zilberter, 2011;

Hall et al., 2012). Several aspects need to be considered when comparing oxygen consumption rates from different experimental studies *in vitro* and *in vivo*. The high oxygen consumption rate (Huchzermeyer et al., 2013) was determined during agonist-induced persistent GAM in the pyramidal cell layer (perisomatic region) of the CA3 network in slice cultures that feature high tissue preservation and connectivity (Zhao et al., 2012; Studer et al., 2014; Schneider et al., 2015), thus reflecting a high activity state. In addition, the local CA3 network is a generator of GAM of high power within the hippocampus, and the electrophysiological and bioenergetical features of this network may not entirely apply to other neuronal networks or cortical regions (Buzsáki, 2006; Traub and Whittington, 2010; Kann et al., 2014). Another aspect is the spatial resolution. "Local pO_2" in the depth profiles as determined with oxygen microsensors in slice preparations refers to a distance of about 0.2 mm (Hall and Attwell, 2008; Huchzermeyer et al., 2013). For comparison, the resolution of functional magnetic resonance imaging (fMRI) technologies *in vivo* is in the order of 1 mm^3 and more. However, the *in vitro* studies suggest that GAM are associated with high energy expenditure.

These findings are in line with studies in humans using positron emission tomography with 2-deoxy-2-[18F] fluoro-D-glucose. The studies demonstrated stimulus-dependent increases in glucose metabolic rate in primary and associative visual cortices of about 40% and 60%, respectively (Phelps et al., 1981) as well as a positive correlation between spectral amplitudes of GAM and the regional glucose uptake, which was determined during seizure-free intervals in patients with non-lesional focal epilepsy (Nishida et al., 2008). Remarkably, the correlations between glucose uptake and oscillations in other human frequency bands, such as theta (4–7 Hz), alpha (8–12 Hz) and beta (16–32 Hz) were found to be either minor or absent. The tight correlation between GAM and energy expenditure is supported by studies using fMRI as a measure of neurovascular coupling. The power of GAM positively correlated with the hemodynamic fMRI response in the cat visual cortex (Niessing et al., 2005). Positive correlations between GAM and fMRI signals were also described for the human cortex during specific tasks and for nearly the entire cerebral cortex of the monkey (Lachaux et al., 2007; Schölvinck et al., 2010; Scheeringa et al., 2011). In general, there appears to be a linear relationship between neuronal activity, energy metabolism and hemodynamic responses (Sheth et al., 2004; Martin et al., 2006; Viswanathan and Freeman, 2007; Hyder et al., 2013). However, details about the relationships between GAM in local networks, energy expenditure and adaptations in blood flow need to be defined in future studies (Sumiyoshi et al., 2012).

Recently, the utilization of various energy substrates during persistent GAM was explored in rat slice cultures. It was demonstrated that GAM can be powered by various energy-rich substrates, with glucose being most effective (Galow et al., 2014). Notably, the high concentration (20 mmol/L) of either lactate or pyruvate was necessary to maintain GAM. The amplitude of the GAM, however, was significantly reduced. In another study, the addition of lactate (2 mmol/L) to lowered glucose concentration (5 mmol/L) exclusively increased the frequency by about 4 Hz, whereas the power of GAM was unchanged (Schneider et al., 2015). Therefore, lactate appears to be much less beneficial to fuel fast neuronal network oscillations compared with neuronal activity that was evoked by electrical stimulation in slice preparations (Schurr et al., 1988; Izumi et al., 1997; Schurr and Payne, 2007; Ivanov et al., 2011; Barros, 2013).

Using FAD and NAD(P)H fluorescence imaging during GAM, the concomitant changes in the mitochondrial redox state were investigated in the CA3 network of hippocampal slice cultures. GAM were associated with a shift towards reduction of the dinucleotides although the interstitial pO_2 was hyperoxic (Huchzermeyer et al., 2008; Kann et al., 2011; **Figure 1A**). This finding might reflect an increase in the availability of energy-rich carbon molecules as a result of enhanced glycolysis in neuronal and astrocytic compartments (Kasischke et al., 2004; Brennan et al., 2006; Hertz et al., 2007; Galow et al., 2014) or an imbalance in the activities of neuronal TCA cycle and mitochondrial respiratory chain. Moreover, repetitive electrical stimulation (20 Hz, 10 s) superimposed on GAM resulted in significantly smaller shifts towards oxidation of the dinucleotides compared to controls (Kann et al., 2011; **Figure 1B**). This suggests that GAM are associated with near-limit utilization of mitochondrial oxidative capacity and provides further evidence for the high energy expenditure during GAM. The data might also imply that rapid and sufficient supply of oxygen and nutrients through changes in blood flow is a fundamental prerequisite for the maintenance of ion and energy homeostasis and therefore, the capability of local neuronal networks to express fast oscillations (Scheeringa et al., 2011; Sumiyoshi et al., 2012; Huchzermeyer et al., 2013; Kann et al., 2014). However, prolonged performance of neuronal mitochondria carries an inherent risk of increased generation of superoxide anion at complexes I and III of the mitochondrial respiratory chain (Morán et al., 2012; Kann, 2016). Increased superoxide anion levels can favor the accumulation of hydrogen peroxide and the generation of other reactive oxygen and nitrogen species (ROS and RNS, respectively). Some of them are reactive molecules with high biological toxicity owing to the capability to oxidize macromolecules such as lipids, DNA, and proteins (Kann and Kovács, 2007; Morán et al., 2012). In particular, fast-spiking, parvalbumin-positive basket cells might transiently generate high ROS levels because the unique electrophysiological and bioenergetical characteristics may frequently result in mismatching changes of metabolic state, Ca^{2+}-load and pO_2 in mitochondria (Kann, 2016). Such mismatches have been discussed to promote the generation of superoxide anion (Kann and Kovács, 2007; Nicholls, 2008; Adam-Vizi and Starkov, 2010). Detailed experimental studies are required to determine free radical generation and oxidative stress in excitatory and inhibitory neurons during GAM.

Intriguingly, the electrophysiological and bioenergetic features of the hippocampal CA3 network, i.e., highest levels in gamma oscillation power, oxygen consumption, and mitochondrial performance, also correlated with the highest

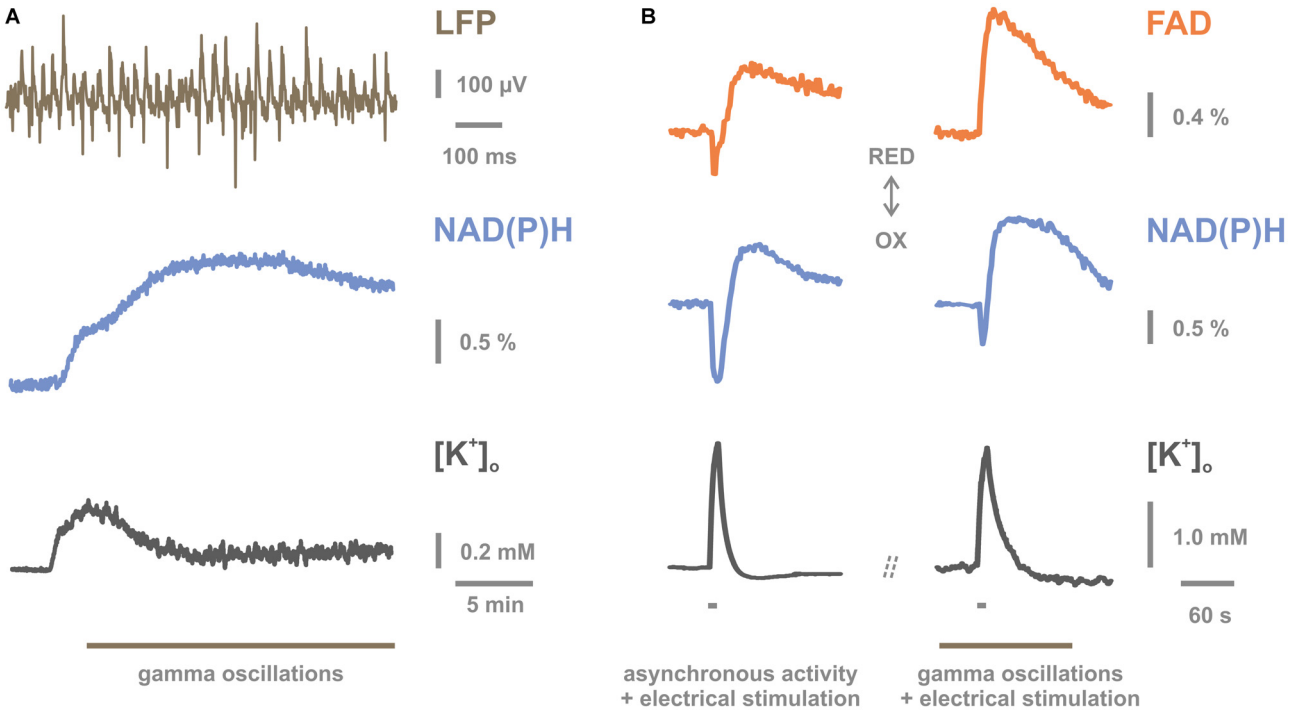


FIGURE 1 | Mitochondrial redox state during gamma oscillations (GAM) *in vitro*. (A) Persistent GAM at around 40 Hz are generated in the presence of acetylcholine and physostigmine in the CA3 region of rat hippocampal slice cultures as revealed by local field potential (LFP) recordings (upper trace). Persistent GAM are associated with a long-lasting elevation in nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) fluorescence (middle trace). This significant shift in the redox state towards reduction likely reflects the activation of reducing processes (tricarboxylic acid cycle and glycolysis) and/or the limitation of oxidizing processes (mitochondrial respiratory chain). Persistent GAM (symbolized by gray line) are associated with a fluctuating elevation in $[K^+]_o$ of less than 0.2 mmol/L (lower trace). Note the higher temporal resolution of the upper trace illustrating GAM. **(B)** Simultaneous recordings of flavin adenine dinucleotide (FAD) (upper traces) and NAD(P)H (middle traces) fluorescence and $[K^+]_o$ (lower traces) during spontaneous asynchronous activity (left) and persistent GAM (right). Recordings were made at identical locations during the two network activity states in a given slice culture. Superimposed repetitive electrical stimulation (10 s, 20 Hz, gray bars) resulted in FAD and NAD(P)H fluorescence transients with biphasic shapes relative to pre-stimulation baseline as well as transient changes in $[K^+]_o$. Upward deflections in fluorescence traces indicate shifts in the redox state towards reduction (gray arrow, RED), and downward deflections towards oxidation (gray arrow, OX; "REDOX" state). Biphasic transients are illustrated such that downward deflections in FAD and NAD(P)H indicate increase and decrease in emission fluorescence intensity, respectively. Note that amplitudes and kinetics of downward deflections in FAD and NAD(P)H fluorescence are clearly altered during GAM (right), likely indicating near-limit utilization of mitochondrial oxidative capacity. The K^+-sensitive microelectrode was placed in stratum pyramidale, i.e., the layer where the somata of pyramidal cells are densely packed and receive strong perisomatic inhibition from the complex axon arbors of basket cells. Note that the decay time of $[K^+]_o$ transients is prolonged during GAM (right). See details in the text. This figure is reproduced and modified from Kann et al. (2011) (Figures 5C,7A,C) by permission of Oxford University Press on behalf of The Guarantors of Brain. This material is published under a Standard License and is not covered by any Creative Commons License. For commercial and non-commercial reuse, please, seek the permission of journals.permissions@oup.com.

expression of complex I subunits (Kann et al., 2011; Wirtz and Schuelke, 2011). Complex I (NADH:ubiquinone oxidoreductase) is a member of the respiratory chain in mitochondria. It is composed of up to 46 subunits that are encoded by nuclear and mitochondrial DNA (Distelmaier et al., 2009). Complex I has been discussed to strongly control oxidative phosphorylation in mitochondria, and to be involved in the pathogenesis of various neurodegenerative diseases (Pathak and Davey, 2008; Koopman et al., 2013). The pattern of complex I gene expression in the hippocampus might reflect unique enzymatic properties of neuronal mitochondria in the CA3 network to meet the homeostatic challenges that are associated with the generation of GAM (Kann, 2012).

Conversely, a variety of studies demonstrated the fast decline of GAM in hippocampal slice preparations during metabolic stress, i.e., (i) by lowering the oxygen fraction in the ambient atmosphere to the normoxic range in the semi-interface recording condition (Huchzermeyer et al., 2008); (ii) by lowering the application rate of oxygenated recording solution in the submerged recording condition (Hájos et al., 2009); and (iii) by induction of hypoxic events (Fano et al., 2007; Pietersen et al., 2009). The fast decline of GAM was also found during pharmacological inhibition of the respiratory chain by rotenone (acting on complex I) or potassium cyanide (acting on cytochrome *c* oxidase in complex IV), and in the presence of protonophores that exert mitochondrial membrane uncoupling (Kann et al., 2011; Whittaker et al., 2011). Moreover, the exquisite sensitivity of GAM to mitochondrial dysfunction has been identified because other types of neuronal activity, such as electrical stimulus-evoked neuronal activation and pathological seizure-like events were more resistant to both, respiratory chain inhibition and low interstitial pO_2 (Huchzermeyer et al.,

2008; Kann et al., 2011). Similar observations were reported for unilateral hippocampal ischemia *in vivo* (Barth and Mody, 2011). These studies consistently show that hippocampal GAM are rapidly impaired during metabolic stress and are in line with data on high energy expenditure during GAM. Mechanistically, dysfunction of fast-spiking GABAergic interneurons, such as parvalbumin-positive basket cells, might be mainly responsible for this rapid impairment. This is likely because these inhibitory interneurons: (i) are crucial for the generation of GAM; and (ii) show unique bioenergetic, biophysical and electrophysiological properties (Kageyama and Wong-Riley, 1982; Gulyás et al., 2006; Hu and Jonas, 2014; Takács et al., 2015). Further details were recently summarized and discussed in reviews about the "interneuron energy hypothesis" (Kann et al., 2014; Kann, 2016).

POTASSIUM ION HOMEOSTASIS DURING GAMMA OSCILLATIONS

The high energy expenditure of neurons during GAM is most likely caused by increased rates of action potentials and postsynaptic potentials. In particular, the significant and widespread increase in rates of EPSPs and IPSPs in the local network elicits strong ion fluxes across the neuronal membrane of both excitatory principal cells and inhibitory interneurons (Wong-Riley, 1989; Hájos and Paulsen, 2009; Kann et al., 2014). These ion fluxes tend to dissipate the gradients of sodium, calcium, potassium and chloride ions, ultimately utilizing potential energy. In order to keep homeostasis and neuronal excitability and thus, ensure maintenance of neuronal information processing, these ionic gradients have to be continuously restored by ion pumps, such as Na^+/K^+-ATPase and Ca^{2+}-ATPase, and transporters, such as Na^+/Ca^{2+}-exchanger, Na^+/H^+-exchanger, and K^+/Cl^--cotransporter (Somjen, 2002; Buzsáki et al., 2007; Kann et al., 2014). In addition, synthesis, release and uptake of neurotransmitters and precursors require various transport processes across neuronal and glial membranes (Bak et al., 2006; Roth and Draguhn, 2012). These transport processes are ultimately powered by cellular energy carrier, adenosine-5′-triphosphate (ATP) that is generated to a large extent by oxidative phosphorylation in neuronal mitochondria (Attwell and Laughlin, 2001; Erecińska and Silver, 2001).

However, experimental studies that explored the ion homeostasis during naturally occurring, fast neuronal network oscillations are rare. The few available studies focused on changes in $[K^+]_o$ in hippocampal slice preparations (see below). $[K^+]_o$ was monitored by double-barreled microelectrodes (see above). $[K^+]_o$ is normally between 2.7 to 3.8 mmol/L in neuronal tissue *in vivo*, which is lower than in the other extracellular fluids of the body (Prince et al., 1973; Lux, 1974; Heinemann and Lux, 1977; Somjen, 2002; Gorji and Speckmann, 2009). Keeping $[K^+]_o$ in this narrow range protects central neurons from undue influences on excitability and synaptic transmission because the elevation in $[K^+]_o$ generally depolarizes neuronal membranes, and substantial $[K^+]_o$ elevation has drastic consequences, such as the pathological occurrence of epileptic activity (Zuckermann and Glaser, 1968; Heinemann et al., 1986; Janigro et al., 1997; Somjen, 2002; Jandová et al., 2006; Steinhäuser et al., 2012). Normally, the generation of action potentials and postsynaptic potentials results in K^+-release from neurons, e.g., through voltage-gated K^+-channels and non-selective cation channels (Somjen, 2002; Buzsáki et al., 2007; **Figure 2**). At inhibitory synapses, K^+-release can be evoked by pre- and postsynaptic GABA-B receptors that open K^+-channels. In addition, at the postsynaptic membrane K^+-release drives the extrusion of chloride through K^+/Cl^--cotransporters, such as KCC2, to reverse the Cl^--influx that underlies the hyperpolarizing action of GABA-A receptors (Mann and Paulsen, 2007; Blaesse et al., 2009; Kaila et al., 2014). The activation of GABA-A receptors, however, might also cause shunting inhibition in both principal cells and interneurons (Alger and Nicoll, 1979; Andersen et al., 1980; Bartos et al., 2007; Mann and Paulsen, 2007). K^+-uptake occurs mainly through Na^+/K^+-ATPase of neurons and astrocytes. Under certain conditions, it might be supported by astrocytic K^+-transporters, K^+-channels and gap-junctions that permit K^+-buffering and spatial redistribution within the astrocyte syncytium (Somjen, 2002; Steinhäuser et al., 2012). There is first evidence for the role of astrocytes in GAM because

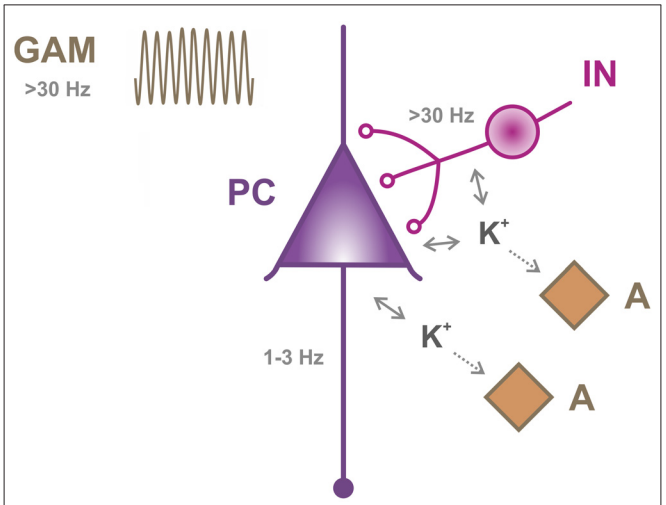


FIGURE 2 | K^+-homeostasis during gamma oscillations (GAM) *in vitro*. The schematic representation illustrates key features of GAM (30–100 Hz) in stratum pyramidale of hippocampal slice preparations. GAM require precise synaptic interactions of excitatory principal cells, such as pyramidal cells (PC), and fast-spiking, inhibitory interneurons (IN), such as parvalbumin-positive basket cells, which feature complex axon arbors. In the hippocampus, fast-spiking interneurons exert fast rhythmic inhibition on the perisomatic region of pyramidal cells by phasic release of GABA from presynaptic terminals (open circles). Interactions with other subtypes of interneurons and excitation of interneurons by pyramidal cells are not illustrated. During GAM, enhanced K^+-release in stratum pyramidale occurs at several neuronal sites: (i) axons of interneurons and pyramidal cells that fire action potentials at different frequencies; (ii) presynaptic endings of interneurons; and (iii) perisomatic postsynaptic membranes of pyramidal cells. The K^+-uptake occurs mainly in neurons and might be supported by adjacent astrocytes (A). Note that $[K^+]_o$ does not exceed 3.5 mmol/L during GAM. Note that GAM are associated with a high oxygen consumption rate, indicating adaptations of adenosine-5′-triphosphate (ATP) generation in mitochondria to power ion pumps and secondary ion transport. See details in the text.

functional manipulation of astrocytes markedly decreased the EEG power in the gamma frequency band in awake-behaving mice, whereas neuronal synaptic activity remained intact. The reduction in cortical GAM was accompanied by altered behavioral performance in the novel object recognition test (Lee et al., 2014). However, the details about the nature of the role of astrocytes, for example, in ion and/or neurotransmitter homeostasis, are less clear and require further experimental studies.

Changes in $[K^+]_o$ associated with GAM were explored in the CA3 network of hippocampal slice cultures (**Figure 2**). The ion-sensitive microelectrodes measured $[K^+]_o$ locally in stratum pyramidale, i.e., the layer where the somata of pyramidal cells are densely packed and receive strong perisomatic inhibition from the complex axon arbors of basket cells (Kann et al., 2014). It was shown that the pharmacological induction of GAM by bath application of acetylcholine at low micromolar concentrations evoked an initial transient increase in $[K^+]_o$ of about 0.5 mmol/L from the baseline of 3 mmol/L. This was followed by a fluctuating elevation in $[K^+]_o$ of less than 0.2 mmol/L when persistent GAM were present (Huchzermeyer et al., 2008; Kann et al., 2011; **Figure 1A**). In addition, repetitive electrical stimulation was superimposed on persistent GAM to get further insight into K^+-homeostasis during fast neuronal network oscillations (Kann et al., 2011; **Figure 1B**). In these experiments, the amplitude of the electrically evoked $[K^+]_o$ transient did not differ in the absence (i.e., spontaneous asynchronous activity) and presence of GAM, indicating the same level of superimposed neuronal activation. By contrast, the decay time of the evoked $[K^+]_o$ transients was prolonged. This might reflect that K^+-uptake mechanisms, such as Na^+/K^+-ATPase activity and glial K^+-buffering, operate near limit during persistent GAM. Similar to GAM, sharp wave-ripples (SPW-Rs) were associated with a transient increase in $[K^+]_o$ of about 0.1 mmol/L in the CA3 network of acute hippocampal slices (Behrens et al., 2007). In this model, SPW-Rs that were induced by repetitive electrical stimulation lasted for about 70 ms, with an incidence of about 8/min (Behrens et al., 2007; Hollnagel et al., 2014). The superimposed ripples had a frequency of about 190 Hz. SPW-Rs represent a different type of fast and highly synchronous neuronal network oscillations (Draguhn et al., 1998; Maier et al., 2003; Behrens et al., 2005; Schönberger et al., 2014). They occur during consummatory behaviors and non-REM sleep and are thought to represent stored information that is transferred to, for example, the neocortex during memory consolidation (Behrens et al., 2005; Hollnagel et al., 2014; Buzsáki, 2015).

These findings provide first experimental evidence that $[K^+]_o$ indeed does not exceed the upper limit of about 3.5 mmol/L during fast neuronal network oscillations. This is in line with *in vivo* studies showing that optical stimuli transiently elevated $[K^+]_o$ by about 0.5 mmol/L in the cat visual cortex (Singer and Lux, 1975; Connors et al., 1979; Somjen, 2002). Slow neuronal network oscillations, such as sleep oscillations (<1 Hz) in the cat neocortex, were associated with periodic elevations in $[K^+]_o$ of about 1.8 mmol/L (Amzica and Steriade, 2000). These data contrast with *in vitro* and *in vivo* studies, in which repetitive electrical stimulation (up to 100 Hz, up to 60 s) was applied as a tool to activate neurons. In these studies much larger $[K^+]_o$ transients of up to 10 mmol/L from baseline were described (Heinemann and Lux, 1975, 1977; Lothman and Somjen, 1975; Gabriel et al., 1998; Kann et al., 2003b; Huchzermeyer et al., 2008). The most likely explanation is that the artificial and robust electrical stimulation evokes action potentials at unphysiological frequencies in most of the excitatory and inhibitory neurons adjacent to the stimulation electrode (Heinemann and Lux, 1977; Janigro et al., 1997)—note that during hippocampal GAM, for example, excitatory pyramidal cells and fast-spiking interneurons generate action potentials at 1–3 Hz and >30 Hz, respectively (Kann et al., 2014). Although there is depression or attenuation of spiking according to the biophysical properties of the neuronal subtype (Wong and Prince, 1981; Madison and Nicoll, 1984; Martina and Jonas, 1997; Kann et al., 2003b; Kim et al., 2012), the resulting bulk K^+-release from neurons presumably exceeds the K^+-uptake mechanisms in neurons and glial cells during repetitive electrical stimulation, which is reflected by the characteristic stimulus-induced $[K^+]_o$ transients. However, further experimental studies are required to determine the contribution of specific ion channels, transporters and pumps in neurons and glial cells to K^+-homeostasis during different network activity states.

The maintenance of K^+-homeostasis during naturally occurring fast network oscillations is likely achieved by strongly enhanced ATP generation in mitochondria—reflected by high oxygen consumption rate and near-limit utilization of oxidative capacity during GAM (see above)—to fuel ion pumps and secondary ion transport.

CONCLUSION

Fast neuronal network oscillations in the gamma frequency band (30–100 Hz) occur in various regions of the cortex and have been implicated in higher brain functions such as sensory perception, attentional selection and memory formation. These GAM are associated with precise cellular adaptations to maintain energy and ion homeostasis and thus neuronal excitability and synaptic signaling. Homeostasis is apparently safeguarded by strongly enhanced oxidative phosphorylation in mitochondria to generate ATP. Conversely, metabolic stress and/or ion channel dysfunction might contribute to the exceptional vulnerability of GAM and thus higher brain functions in brain disease.

AUTHOR CONTRIBUTIONS

OK, J-OH, SE and JS wrote the manuscript. OK created the figures.

ACKNOWLEDGMENTS

The authors thank Andrea Lewen for text editing assistance. This work was funded by the German Research Foundation (DFG) within the Collaborative Research Center (SFB) 1134 (project B02).

REFERENCES

Adam-Vizi, V., and Starkov, A. A. (2010). Calcium and mitochondrial reactive oxygen species generation: how to read the facts. *J. Alzheimers Dis.* 20, S413–S426. doi: 10.3233/JAD-2010-100465

Alger, B. E., and Nicoll, R. A. (1979). GABA-mediated biphasic inhibitory responses in hippocampus. *Nature* 281, 315–317. doi: 10.1038/281315a0

Amzica, F., and Steriade, M. (2000). Neuronal and glial membrane potentials during sleep and paroxysmal oscillations in the neocortex. *J. Neurosci.* 20, 6648–6665.

Andersen, P., Dingledine, R., Gjerstad, L., Langmoen, I. A., and Mosfeldt Laursen, A. (1980). Two different responses of hippocampal pyramidal cells to application of gamma-amino butyric acid. *J. Physiol.* 305, 279–296. doi: 10.1113/jphysiol.1980.sp013363

Attwell, D., and Laughlin, S. B. (2001). An energy budget for signaling in the grey matter of the brain. *J. Cereb. Blood Flow Metab.* 21, 1133–1145. doi: 10.1097/00004647-200110000-00001

Bak, L. K., Schousboe, A., and Waagepetersen, H. S. (2006). The glutamate/GABA-glutamine cycle: aspects of transport, neurotransmitter homeostasis and ammonia transfer. *J. Neurochem.* 98, 641–653. doi: 10.1111/j.1471-4159.2006.03913.x

Barros, L. F. (2013). Metabolic signaling by lactate in the brain. *Trends Neurosci.* 36, 396–404. doi: 10.1016/j.tins.2013.04.002

Barth, A. M., and Mody, I. (2011). Changes in hippocampal neuronal activity during and after unilateral selective hippocampal ischemia *in vivo*. *J. Neurosci.* 31, 851–860. doi: 10.1523/JNEUROSCI.5080-10.2011

Bartos, M., Vida, I., and Jonas, P. (2007). Synaptic mechanisms of synchronized gamma oscillations in inhibitory interneuron networks. *Nat. Rev. Neurosci.* 8, 45–56. doi: 10.1038/nrn2044

Behrens, C. J., van den Boom, L. P., de Hoz, L., Friedman, A., and Heinemann, U. (2005). Induction of sharp wave-ripple complexes *in vitro* and reorganization of hippocampal networks. *Nat. Neurosci.* 8, 1560–1567. doi: 10.1038/nn1571

Behrens, C. J., van den Boom, L. P., and Heinemann, U. (2007). Effects of the $GABA_A$ receptor antagonists bicuculline and gabazine on stimulus-induced sharp wave-ripple complexes in adult rat hippocampus *in vitro*. *Eur. J. Neurosci.* 25, 2170–2181. doi: 10.1111/j.1460-9568.2007.05462.x

Berndt, N., Kann, O., and Holzhütter, H.-G. (2015). Physiology-based kinetic modeling of neuronal energy metabolism unravels the molecular basis of NAD(P)H fluorescence transients. *J. Cereb. Blood Flow Metab.* 35, 1494–1506. doi: 10.1038/jcbfm.2015.70

Blaesse, P., Airaksinen, M. S., Rivera, C., and Kaila, K. (2009). Cation-chloride cotransporters and neuronal function. *Neuron* 61, 820–838. doi: 10.1016/j.neuron.2009.03.003

Bliss, T. V. P., and Lømo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J. Physiol.* 232, 331–356. doi: 10.1113/jphysiol.1973.sp010273

Bragin, A., Jandó, G., Nádasdy, Z., Hetke, J., Wise, K., and Buzsáki, G. (1995). Gamma (40–100 Hz) oscillation in the hippocampus of the behaving rat. *J. Neurosci.* 15, 47–60.

Brennan, A. M., Connor, J. A., and Shuttleworth, C. W. (2006). NAD(P)H fluorescence transients after synaptic activity in brain slices: predominant role of mitochondrial function. *J. Cereb. Blood Flow Metab.* 26, 1389–1406. doi: 10.1038/sj.jcbfm.9600292

Buzsáki, G. (2006). *Rhythms of the Brain*. New York, NY: Oxford University Press.

Buzsáki, G. (2015). Hippocampal sharp wave-ripple: a cognitive biomarker for episodic memory and planning. *Hippocampus* 25, 1073–1088. doi: 10.1002/hipo.22488

Buzsáki, G., Anastassiou, C. A., and Koch, C. (2012). The origin of extracellular fields and currents - EEG, ECoG, LFP and spikes. *Nat. Rev. Neurosci.* 13, 407–420. doi: 10.1038/nrn3241

Buzsáki, G., and Chrobak, J. J. (1995). Temporal structure in spatially organized neuronal ensembles: a role for interneuronal networks. *Curr. Opin. Neurobiol.* 5, 504–510. doi: 10.1016/0959-4388(95)80012-3

Buzsáki, G., and Draguhn, A. (2004). Neuronal oscillations in cortical networks. *Science* 304, 1926–1929. doi: 10.1126/science.1099745

Buzsáki, G., Kaila, K., and Raichle, M. (2007). Inhibition and brain work. *Neuron* 56, 771–783. doi: 10.1016/j.neuron.2007.11.008

Cardin, J. A., Carlén, M., Meletis, K., Knoblich, U., Zhang, F., Deisseroth, K., et al. (2009). Driving fast-spiking cells induces gamma rhythm and controls sensory responses. *Nature* 459, 663–667. doi: 10.1038/nature08002

Cheyne, D., Bells, S., Ferrari, P., Gaetz, W., and Bostan, A. C. (2008). Self-paced movements induce high-frequency gamma oscillations in primary motor cortex. *Neuroimage* 42, 332–342. doi: 10.1016/j.neuroimage.2008.04.178

Connors, B., Dray, A., Fox, P., Hilmy, M., and Somjen, G. (1979). LSD's effect on neuron populations in visual cortex gauged by transient responses of extracellular potassium evoked by optical stimuli. *Neurosci. Lett.* 13, 147–150. doi: 10.1016/0304-3940(79)90032-6

Csicsvari, J., Jamieson, B., Wise, K. D., and Buzsáki, G. (2003). Mechanisms of gamma oscillations in the hippocampus of the behaving rat. *Neuron* 37, 311–322. doi: 10.1016/s0896-6273(02)01169-8

Distelmaier, F., Koopman, W. J., van den Heuvel, L. P., Rodenburg, R. J., Mayatepek, E., Willems, P. H., et al. (2009). Mitochondrial complex I deficiency: from organelle dysfunction to clinical disease. *Brain* 132, 833–842. doi: 10.1093/brain/awp058

Draguhn, A., Traub, R. D., Schmitz, D., and Jefferys, J. G. R. (1998). Electrical coupling underlies high-frequency oscillations in the hippocampus *in vitro*. *Nature* 394, 189–192. doi: 10.1038/28184

Einevoll, G. T., Kayser, C., Logothetis, N. K., and Panzeri, S. (2013). Modelling and analysis of local field potentials for studying the function of cortical circuits. *Nat. Rev. Neurosci.* 14, 770–785. doi: 10.1038/nrn3599

Erecińska, M., and Silver, I. A. (2001). Tissue oxygen tension and brain sensitivity to hypoxia. *Respir. Physiol.* 128, 263–276. doi: 10.1016/s0034-5687(01)00306-1

Fano, S., Behrens, C. J., and Heinemann, U. (2007). Hypoxia suppresses kainate-induced γ-oscillations in rat hippocampal slices. *Neuroreport* 18, 1827–1831. doi: 10.1097/wnr.0b013e3282f13e4f

Fellous, J.-M., and Sejnowski, T. J. (2000). Cholinergic induction of oscillations in the hippocampal slice in the slow (0.5–2 Hz), theta (5–12 Hz) and gamma (35–70 Hz) bands. *Hippocampus* 10, 187–197. doi: 10.1002/(SICI)1098-1063(2000)10:2<187::AID-HIPO8>3.0.CO;2-M

Fisahn, A., Pike, F. G., Buhl, E. H., and Paulsen, O. (1998). Cholinergic induction of network oscillations at 40 Hz in the hippocampus *in vitro*. *Nature* 394, 186–189. doi: 10.1038/28179

Fischer, Y., Wittner, L., Freund, T. F., and Gähwiler, B. H. (2002). Simultaneous activation of gamma and theta network oscillations in rat hippocampal slice cultures. *J. Physiol.* 539, 857–868. doi: 10.1113/jphysiol.2001.013050

Foster, K. A., Beaver, C. J., and Turner, D. A. (2005). Interaction between tissue oxygen tension and NADH imaging during synaptic stimulation and hypoxia in rat hippocampal slices. *Neuroscience* 132, 645–657. doi: 10.1016/j.neuroscience.2005.01.040

Franowicz, M. N., and Barth, D. S. (1995). Comparison of evoked potentials and high-frequency (gamma-band) oscillating potentials in rat auditory cortex. *J. Neurophysiol.* 74, 96–112.

Fries, P., Nikolić, D., and Singer, W. (2007). The gamma cycle. *Trends Neurosci.* 30, 309–316. doi: 10.1016/j.tins.2007.05.005

Fritschy, J.-M., and Panzanelli, P. (2014). $GABA_A$ receptors and plasticity of inhibitory neurotransmission in the central nervous system. *Eur. J. Neurosci.* 39, 1845–1865. doi: 10.1111/ejn.12534

Fuchs, E. C., Zivkovic, A. R., Cunningham, M. O., Middleton, S., LeBeau, F. E. N., Bannerman, D. M., et al. (2007). Recruitment of parvalbumin-positive interneurons determines hippocampal function and associated behavior. *Neuron* 53, 591–604. doi: 10.1016/j.neuron.2007.01.031

Gabriel, S., Eilers, A., Kivi, A., Kovacs, R., Schulze, K., Lehmann, T.-N., et al. (1998). Effects of barium on stimulus induced changes in extracellular potassium concentration in area CA1 of hippocampal slices from normal and pilocarpine-treated epileptic rats. *Neurosci. Lett.* 242, 9–12. doi: 10.1016/s0304-3940(98)00012-3

Galow, L. V., Schneider, J., Lewen, A., Ta, T. T., Papageorgiou, I. E., and Kann, O. (2014). Energy substrates that fuel fast neuronal network oscillations. *Front. Neurosci.* 8:398. doi: 10.3389/fnins.2014.00398

Gloveli, T., Dugladze, T., Saha, S., Monyer, H., Heinemann, U., Traub, R. D., et al. (2005). Differential involvement of oriens/pyramidale interneurones in hippocampal network oscillations *in vitro*. *J. Physiol.* 562, 131–147. doi: 10.1113/jphysiol.2004.073007

Gorji, A., and Speckmann, E.-J. (2009). Epileptiform EEG spikes and their functional significance. *Clin. EEG Neurosci.* 40, 230–233. doi: 10.1177/155005940904000404

Gray, C. M., König, P., Engel, A. K., and Singer, W. (1989). Oscillatory responses in cat visual cortex exhibit inter-columnar synchronization which reflects global stimulus properties. *Nature* 338, 334–337. doi: 10.1038/338334a0

Gray, C. M., and Viana Di Prisco, G. (1997). Stimulus-dependent neuronal oscillations and local synchronization in striate cortex of the alert cat. *J. Neurosci.* 17, 3239–3253.

Gulyás, A. I., Buzsáki, G., Freund, T. F., and Hirase, H. (2006). Populations of hippocampal inhibitory neurons express different levels of cytochrome *c*. *Eur. J. Neurosci.* 23, 2581–2594. doi: 10.1111/j.1460-9568.2006.04814.x

Gulyás, A. I., Szabó, G. G., Ulbert, I., Holderith, N., Monyer, H., Erdélyi, F., et al. (2010). Parvalbumin-containing fast-spiking basket cells generate the field potential oscillations induced by cholinergic receptor activation in the hippocampus. *J. Neurosci.* 30, 15134–15145. doi: 10.1523/JNEUROSCI.4104-10.2010

Haenschel, C., Baldeweg, T., Croft, R. J., Whittington, M., and Gruzelier, J. (2000). Gamma and beta frequency oscillations in response to novel auditory stimuli: a comparison of human electroencephalogram (EEG) data with *in vitro* models. *Proc. Natl. Acad. Sci. U S A* 97, 7645–7650. doi: 10.1073/pnas.120162397

Hájos, N., Ellender, T. J., Zemankovics, R., Mann, E. O., Exley, R., Cragg, S. J., et al. (2009). Maintaining network activity in submerged hippocampal slices: importance of oxygen supply. *Eur. J. Neurosci.* 29, 319–327. doi: 10.1111/j.1460-9568.2008.06577.x

Hájos, N., Pálhalmi, J., Mann, E. O., Németh, B., Paulsen, O., and Freund, T. F. (2004). Spike timing of distinct types of GABAergic interneuron during hippocampal gamma oscillations *in vitro*. *J. Neurosci.* 24, 9127–9137. doi: 10.1523/JNEUROSCI.2113-04.2004

Hájos, N., and Paulsen, O. (2009). Network mechanisms of gamma oscillations in the CA3 region of the hippocampus. *Neural Netw.* 22, 1113–1119. doi: 10.1016/j.neunet.2009.07.024

Hales, C. G., and Pockett, S. (2014). The relationship between local field potentials (LFPs) and the electromagnetic fields that give rise to them. *Front. Syst. Neurosci.* 8:233. doi: 10.3389/fnsys.2014.00233

Hall, C. N., and Attwell, D. (2008). Assessing the physiological concentration and targets of nitric oxide in brain tissue. *J. Physiol.* 586, 3597–3615. doi: 10.1113/jphysiol.2008.154724

Hall, C. N., Klein-Flügge, M. C., Howarth, C., and Attwell, D. (2012). Oxidative phosphorylation, not glycolysis, powers presynaptic and postsynaptic mechanisms underlying brain information processing. *J. Neurosci.* 32, 8940–8951. doi: 10.1523/JNEUROSCI.0026-12.2012

Heinemann, U., Konnerth, A., Pumain, R., and Wadman, W. J. (1986). Extracellular calcium and potassium concentration changes in chronic epileptic brain tissue. *Adv. Neurol.* 44, 641–661.

Heinemann, U., and Lux, H. D. (1975). Undershoots following stimulus-induced rises of extracellular potassium concentration in cerebral cortex of cat. *Brain Res.* 93, 63–76. doi: 10.1016/0006-8993(75)90286-3

Heinemann, U., and Lux, H. D. (1977). Ceiling of stimulus induced rises in extracellular potassium concentration in the cerebral cortex of cat. *Brain Res.* 120, 231–249. doi: 10.1016/0006-8993(77)90903-9

Hertz, L., Peng, L., and Dienel, G. A. (2007). Energy metabolism in astrocytes: high rate of oxidative metabolism and spatiotemporal dependence on glycolysis/glycogenolysis. *J. Cereb. Blood Flow Metab.* 27, 219–249. doi: 10.1038/sj.jcbfm.9600343

Ho, V. M., Lee, J. A., and Martin, K. C. (2011). The cell biology of synaptic plasticity. *Science* 334, 623–638. doi: 10.1126/science.1209236

Hollnagel, J. O., Maslarova, A., ul Haq, R., and Heinemann, U. (2014). $GABA_B$ receptor dependent modulation of sharp wave-ripple complexes in the rat hippocampus *in vitro*. *Neurosci. Lett.* 574, 15–20. doi: 10.1016/j.neulet.2014.04.045

Hollnagel, J. O., ul Haq, R., Behrens, C. J., Maslarova, A., Mody, I., and Heinemann, U. (2015). No evidence for role of extracellular choline-acetyltransferase in generation of gamma oscillations in rat hippocampal slices *in vitro*. *Neuroscience* 284, 459–469. doi: 10.1016/j.neuroscience.2014.10.016

Hu, H., Gan, J., and Jonas, P. (2014). Fast-spiking, parvalbumin$^+$ GABAergic interneurons: from cellular design to microcircuit function. *Science* 345:1255263. doi: 10.1126/science.1255263

Hu, H., and Jonas, P. (2014). A supercritical density of Na^+ channels ensures fast signaling in GABAergic interneuron axons. *Nat. Neurosci.* 17, 686–693. doi: 10.1038/nn.3678

Huchzermeyer, C., Albus, K., Gabriel, H. J., Otáhal, J., Taubenberger, N., Heinemann, U., et al. (2008). Gamma oscillations and spontaneous network activity in the hippocampus are highly sensitive to decreases in pO2 and concomitant changes in mitochondrial redox state. *J. Neurosci.* 28, 1153–1162. doi: 10.1523/JNEUROSCI.4105-07.2008

Huchzermeyer, C., Berndt, N., Holzhütter, H.-G., and Kann, O. (2013). Oxygen consumption rates during three different neuronal activity states in the hippocampal CA3 network. *J. Cereb. Blood Flow Metab.* 33, 263–271. doi: 10.1038/jcbfm.2012.165

Hyder, F., Rothman, D. L., and Bennet, M. R. (2013). Cortical energy demand of signaling and nonsignaling components in brain are conserved across mammalian species and activity levels. *Proc. Natl. Acad. Sci. U S A* 110, 3549–3554. doi: 10.1073/pnas.1214912110

Ivanov, A., Mukhtarov, M., Bregestovski, P., and Zilberter, Y. (2011). Lactate effectively covers energy demands during neuronal network activity in neonatal hippocampal slices. *Front. Neuroenergetics* 3:2. doi: 10.3389/fnene.2011.00002

Ivanov, A., and Zilberter, Y. (2011). Critical state of energy metabolism in brain slices: the principal role of oxygen delivery and energy substrates in shaping neuronal activity. *Front. Neuroenergetics* 3:9. doi: 10.3389/fnene.2011.00009

Izumi, Y., Benz, A. M., Katsuki, H., and Zorumski, C. F. (1997). Endogenous monocarboxylates sustain hippocampal synaptic function and morphological integrity during energy deprivation. *J. Neurosci.* 17, 9448–9457.

Jandová, K., Päsler, D., Antonio, L. L., Raue, C., Ji, S., Njunting, M., et al. (2006). Carbamazepine-resistance in the epileptic dentate gyrus of human hippocampal slices. *Brain* 129, 3290–3306. doi: 10.1093/brain/awl218

Janigro, D., Gasparini, S., D'Ambrosio, R., McKhann, G. II, and DiFrancesco, D. (1997). Reduction of K^+ uptake in glia prevents long-term depression maintenance and causes epileptiform activity. *J. Neurosci.* 17, 2813–2824.

Kageyama, G. H., and Wong-Riley, M. T. (1982). Histochemical localization of cytochrome oxidase in the hippocampus: correlation with specific neuronal types and afferent pathways. *Neuroscience* 7, 2337–2361. doi: 10.1016/0306-4522(82)90199-3

Kaila, K., Price, T. J., Payne, J. A., Puskarjov, M., and Voipio, J. (2014). Cation-chloride cotransporters in neuronal development, plasticity and disease. *Nat. Rev. Neurosci.* 15, 637–654. doi: 10.1038/nrn3819

Kann, O. (2012). The energy demand of fast neuronal network oscillations: insights from brain slice preparations. *Front. Pharmacol.* 2:90. doi: 10.3389/fphar.2011.00090

Kann, O. (2016). The interneuron energy hypothesis: implications for brain disease. *Neurobiol. Dis.* 90, 75–85. doi: 10.1016/j.nbd.2015.08.005

Kann, O., Huchzermeyer, C., Kovács, R., Wirtz, S., and Schuelke, M. (2011). Gamma oscillations in the hippocampus require high complex I gene expression and strong functional performance of mitochondria. *Brain* 134, 345–358. doi: 10.1093/brain/awq333

Kann, O., and Kovács, R. (2007). Mitochondria and neuronal activity. *Am. J. Physiol. Cell Physiol.* 292, C641–C657. doi: 10.1152/ajpcell.00222.2006

Kann, O., Kovács, R., and Heinemann, U. (2003a). Metabotropic receptor-mediated $Ca2^+$ signaling elevates mitochondrial $Ca2^+$ and stimulates oxidative metabolism in hippocampal slice cultures. *J. Neurophysiol.* 90, 613–621. doi: 10.1152/jn.00042.2003

Kann, O., Schuchmann, S., Buchheim, K., and Heinemann, U. (2003b). Coupling of neuronal activity and mitochondrial metabolism as revealed by NAD(P)H fluorescence signals in organotypic hippocampal slice cultures of the rat. *Neuroscience* 119, 87–100. doi: 10.1016/s0306-4522(03)00026-5

Kann, O., Papageorgiou, I. E., and Draguhn, A. (2014). Highly energized inhibitory interneurons are a central element for information processing in cortical networks. *J. Cereb. Blood Flow Metab.* 34, 1270–1282. doi: 10.1038/jcbfm.2014.104

Kasischke, K. A., Vishwasrao, H. D., Fisher, P. J., Zipfel, W. R., and Webb, W. W. (2004). Neural activity triggers neuronal oxidative metabolism followed by astrocytic glycolysis. *Science* 305, 99–103. doi: 10.1126/science.1096485

Katzner, S., Nauhaus, I., Benucci, A., Bonin, V., Ringach, D. L., and Carandini, M. (2009). Local origin of field potentials in visual cortex. *Neuron* 61, 35–41. doi: 10.1016/j.neuron.2008.11.016

Kim, E., Owen, B., Holmes, W. R., and Grover, L. M. (2012). Decreased afferent excitability contributes to synaptic depression during high-frequency stimulation in hippocampal area CA1. *J. Neurophysiol.* 108, 1965–1976. doi: 10.1152/jn.00276.2011

Klausberger, T., and Somogyi, P. (2008). Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. *Science* 321, 53–57. doi: 10.1126/science.1149381

Koopman, W. J., Distelmaier, F., Smeitink, J. A., and Willems, P. H. (2013). OXPHOS mutations and neurodegeneration. *EMBO J.* 32, 9–29. doi: 10.1038/emboj.2012.300

Korotkova, T., Fuchs, E. C., Ponomarenko, A., von Engelhardt, J., and Monyer, H. (2010). NMDA receptor ablation on parvalbumin-positive interneurons impairs hippocampal synchrony, spatial representations and working memory. *Neuron* 68, 557–569. doi: 10.1016/j.neuron.2010.09.017

Kreiter, A. K., and Singer, W. (1992). Oscillatory neuronal responses in the visual cortex of the awake macaque monkey. *Eur. J. Neurosci.* 4, 369–375. doi: 10.1111/j.1460-9568.1992.tb00884.x

Kullmann, D. M., and Lamsa, K. P. (2007). Long-term synaptic plasticity in hippocampal interneurons. *Nat. Rev. Neurosci.* 8, 687–699. doi: 10.1038/nrn2207

Lachaux, J. P., Fonlupt, P., Kahane, P., Minotti, L., Hoffmann, D., Bertrand, O., et al. (2007). Relationship between task-related gamma oscillations and BOLD signal: new insights from combined fMRI and intracranial EEG. *Hum. Brain Mapp.* 28, 1368–1375. doi: 10.1002/hbm.20352

Lebedev, M. A., and Nelson, R. J. (1995). Rhythmically firing (20–50 Hz) neurons in monkey primary somatosensory cortex: activity patterns during initiation of vibratory-cued hand movements. *J. Comput. Neurosci.* 2, 313–334. doi: 10.1007/bf00961443

Lecoq, J., Tiret, P., Najac, M., Shepherd, G. M., Greer, C. A., and Charpak, S. (2009). Odor-evoked oxygen consumption by action potential and synaptic transmission in the olfactory bulb. *J. Neurosci.* 29, 1424–1433. doi: 10.1523/JNEUROSCI.4817-08.2009

Lee, H. S., Ghetti, A., Pinto-Duarte, A., Wang, X., Dziewczapolski, G., Galimi, F., et al. (2014). Astrocytes contribute to gamma oscillations and recognition memory. *Proc. Natl. Acad. Sci. U S A* 111, E3343–E3352. doi: 10.1073/pnas.1410893111

Lehmann, D., Faber, P. L., Achermann, P., Jeanmonod, D., Gianotti, L. R., and Pizzagalli, D. (2001). Brain sources of EEG gamma frequency during volitionally meditation-induced, altered states of consciousness and experience of the self. *Psychiatry Res.* 108, 111–121. doi: 10.1016/s0925-4927(01)00116-0

Lisman, J., and Buzsáki, G. (2008). A neural coding scheme formed by the combined function of gamma and theta oscillations. *Schizophr. Bull.* 34, 974–980. doi: 10.1093/schbul/sbn060

Lothman, E. W., and Somjen, G. G. (1975). Extracellular potassium activity, intracellular and extracellular potential responses in the spinal cord. *J. Physiol.* 252, 115–136. doi: 10.1113/jphysiol.1975.sp011137

LoTurco, J. L., Coulter, D. A., and Alkon, D. L. (1988). Enhancement of synaptic potentials in rabbit CA1 pyramidal neurons following classical conditioning. *Proc. Natl. Acad. Sci. U S A* 85, 1672–1676. doi: 10.1073/pnas.85.5.1672

Lutz, A., Greischar, L. L., Rawlings, N. B., Ricard, M., and Davidson, R. J. (2004). Long-term meditators self-induce high-amplitude gamma synchrony during mental practice. *Proc. Natl. Acad. Sci. U S A* 101, 16369–16373. doi: 10.1073/pnas.0407401101

Lux, H. D. (1974). Fast recording ion specific microelectrodes: their use in pharmacological studies in the CNS. *Neuropharmacology* 13, 509–517. doi: 10.1016/0028-3908(74)90140-3

Lux, H. D., Heinemann, U., and Dietzel, I. (1986). Ionic changes and alterations in the size of the extracellular space during epileptic activity. *Adv. Neurol.* 44, 619–639.

Madison, D. V., and Nicoll, R. A. (1984). Control of the repetitive discharge of rat CA 1 pyramidal neurones *in vitro*. *J. Physiol.* 354, 319–331. doi: 10.1113/jphysiol.1984.sp015378

Maier, N., Nimmrich, V., and Draguhn, A. (2003). Cellular and network mechanisms underlying spontaneous sharp wave-ripple complexes in mouse hippocampal slices. *J. Physiol.* 550, 873–887. doi: 10.1113/jphysiol.2003.044602

Malenka, R. C., Kauer, J. A., Perkel, D. J., Mauk, M. D., Kelly, P. T., Nicoll, R. A., et al. (1989). An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. *Nature* 340, 554–557. doi: 10.1038/340554a0

Mann, E. O., and Paulsen, O. (2007). Role of GABAergic inhibition in hippocampal network oscillations. *Trends Neurosci.* 30, 343–349. doi: 10.1016/j.tins.2007.05.003

Martin, C., Martindale, J., Berwick, J., and Mayhew, J. (2006). Investigating neural-hemodynamic coupling and the hemodynamic response function in the awake rat. *Neuroimage* 32, 33–48. doi: 10.1016/j.neuroimage.2006.02.021

Martina, M., and Jonas, P. (1997). Functional differences in Na^+ channel gating between fast-spiking interneurones and principal neurones of rat hippocampus. *J. Physiol.* 505, 593–603. doi: 10.1111/j.1469-7793.1997.593ba.x

Melloni, L., Molina, C., Pena, M., Torres, D., Singer, W., and Rodriguez, E. (2007). Synchronization of neural activity across cortical areas correlates with conscious perception. *J. Neurosci.* 27, 2858–2865. doi: 10.1523/jneurosci.4623-06.2007

Miles, R., and Wong, R. K. S. (1987). Latent synaptic pathways revealed after tetanic stimulation in the hippocampus. *Nature* 329, 724–726. doi: 10.1038/329724a0

Montgomery, S. M., and Buzsáki, G. (2007). Gamma oscillations dynamically couple hippocampal CA3 and CA1 regions during memory task performance. *Proc. Natl. Acad. Sci. U S A* 104, 14495–14500. doi: 10.1073/pnas.0701826104

Morán, M., Moreno-Lastres, D., Marín-Buera, L., Arenas, J., Martín, M. A., and Ugalde, C. (2012). Mitochondrial respiratory chain dysfunction: implications in neurodegeneration. *Free Radic. Biol. Med.* 53, 595–609. doi: 10.1016/j.freeradbiomed.2012.05.009

Murthy, V. N., and Fetz, E. E. (1992). Coherent 25- to 35-Hz oscillations in the sensorimotor cortex of awake behaving monkeys. *Proc. Natl. Acad. Sci. U S A* 89, 5670–5674. doi: 10.1073/pnas.89.12.5670

Nelson, M. J., and Pouget, P. (2010). Do electrode properties create a problem in interpreting local field potential recordings? *J. Neurophysiol.* 103, 2315–2317. doi: 10.1152/jn.00157.2010

Nicholls, D. G. (2008). Oxidative stress and energy crises in neuronal dysfunction. *Ann. N Y Acad. Sci.* 1147, 53–60. doi: 10.1196/annals.1427.002

Niessing, J., Ebisch, B., Schmidt, K. E., Niessing, M., Singer, W., and Galuske, R. A. W. (2005). Hemodynamic signals correlate tightly with synchronized gamma oscillations. *Science* 309, 948–951. doi: 10.1126/science.1110948

Nishida, M., Juhász, C., Sood, S., Chugani, H. T., and Asano, E. (2008). Cortical glucose metabolism positively correlates with gamma-oscillations in nonlesional focal epilepsy. *Neuroimage* 42, 1275–1284. doi: 10.1016/j.neuroimage.2008.06.027

Oren, I., Hájos, N., and Paulsen, O. (2010). Identification of the current generator underlying cholinergically induced gamma frequency field potential oscillations in the hippocampal CA3 region. *J. Physiol.* 588, 785–797. doi: 10.1113/jphysiol.2009.180851

Oren, I., Mann, E. O., Paulsen, O., and Hájos, N. (2006). Synaptic currents in anatomically identified CA3 neurons during hippocampal gamma oscillations *in vitro*. *J. Neurosci.* 26, 9923–9934. doi: 10.1523/jneurosci.1580-06.2006

Pantev, C., Makeig, S., Hoke, M., Galambos, R., Hampson, S., and Gallen, C. (1991). Human auditory evoked gamma-band magnetic fields. *Proc. Natl. Acad. Sci. U S A* 88, 8996–9000. doi: 10.1073/pnas.88.20.8996

Papageorgiou, I. E., Lewen, A., Galow, L. V., Cesetti, T., Scheffel, J., Regen, T., et al. (2016). TLR4-activated microglia require IFN-γ to induce severe neuronal dysfunction and death *in situ*. *Proc. Natl. Acad. Sci. U S A* 113, 212–217. doi: 10.1073/pnas.1513853113

Pathak, R. U., and Davey, G. P. (2008). Complex I and energy thresholds in the brain. *Biochim. Biophys. Acta* 1777, 777–882. doi: 10.1016/j.bbabio.2008.05.443

Paulsen, O., and Moser, E. I. (1998). A model of hippocampal memory encoding and retrieval: GABAergic control of synaptic plasticity. *Trends Neurosci.* 21, 273–278. doi: 10.1016/s0166-2236(97)01205-8

Penttonen, M., Kamondi, A., Acsády, L., and Buzsáki, G. (1998). Gamma frequency oscillation in the hippocampus of the rat: intracellular analysis *in vivo*. *Eur. J. Neurosci.* 10, 718–728. doi: 10.1046/j.1460-9568.1998.00096.x

Phelps, M. E., Kuhl, D. E., and Mazziota, J. C. (1981). Metabolic mapping of the brain's response to visual stimulation: studies in humans. *Science* 211, 1445–1448. doi: 10.1126/science.6970412

Pietersen, A. N., Lancaster, D. M., Patel, N., Hamilton, J. B., and Vreugdenhil, M. (2009). Modulation of gamma oscillations by endogenous adenosine through A1 and A2A receptors in the mouse hippocampus. *Neuropharmacology* 56, 481–492. doi: 10.1016/j.neuropharm.2008.10.001

Popa, D., Spolidoro, M., Proville, R. D., Guyon, N., Belliveau, L., and Léna, C. (2013). Functional role of the cerebellum in gamma-band synchronization of the sensory and motor cortices. *J. Neurosci.* 33, 6552–6556. doi: 10.1523/JNEUROSCI.5521-12.2013

Pöschel, B., Draguhn, A., and Heinemann, U. (2002). Glutamate-induced gamma oscillations in the dentate gyrus of rat hippocampal slices. *Brain Res.* 938, 22–28. doi: 10.1016/s0006-8993(02)02477-0

Prince, D. A., Lux, H. D., and Neher, E. (1973). Measurement of extracellular potassium activity in cat cortex. *Brain Res.* 50, 489–495. doi: 10.1016/0006-8993(73)90758-0

Revsbech, N. P. (1989). An oxygen microsensor with a guard cathode. *Limnol. Oceanogr.* 34, 474–478. doi: 10.4319/lo.1989.34.2.0474

Rolfe, D. F., and Brown, G. C. (1997). Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiol. Rev.* 77, 731–758.

Roth, F. C., and Draguhn, A. (2012). GABA metabolism and transport: effects on synaptic efficacy. *Neural Plast.* 2012:805830. doi: 10.1155/2012/805830

Salkoff, D. B., Zagha, E., Yüzgeç, Ö., and McCormick, D. A. (2015). Synaptic mechanisms of tight spike synchrony at gamma frequency in cerebral cortex. *J. Neurosci.* 35, 10236–10251. doi: 10.1523/JNEUROSCI.0828-15.2015

Scheeringa, R., Fries, P., Petersson, K. M., Oostenveld, R., Grothe, I., Norris, D. G., et al. (2011). Neuronal dynamics underlying high- and low-frequency EEG oscillations contribute independently to the human BOLD signal. *Neuron* 69, 572–583. doi: 10.1016/j.neuron.2010.11.044

Schneider, J., Lewen, A., Ta, T. T., Galow, L. V., Isola, R., Papageorgiou, I. E., et al. (2015). A reliable model for gamma oscillations in hippocampal tissue. *J. Neurosci. Res.* 93, 1067–1078. doi: 10.1002/jnr.23590

Schölvinck, M. L., Maier, A., Ye, F. Q., Duyn, J. H., and Leopold, D. A. (2010). Neural basis of global resting-state fMRI activity. *Proc. Natl. Acad. Sci. U S A* 107, 10238–10243. doi: 10.1073/pnas.0913110107

Schönberger, J., Draguhn, A., and Both, M. (2014). Lamina-specific contribution of glutamatergic and GABAergic potentials to hippocampal sharp wave-ripple complexes. *Front. Neural Circuits* 8:103. doi: 10.3389/fncir.2014.00103

Schroeder, C. E., and Lakatos, P. (2009). Low-frequency neuronal oscillations as instruments of sensory selection. *Trends Neurosci.* 32, 9–18. doi: 10.1016/j.tins.2008.09.012

Schuchmann, S., Kovacs, R., Kann, O., Heinemann, U., and Buchheim, K. (2001). Monitoring NAD(P)H autofluorescence to assess mitochondrial metabolic functions in rat hippocampal-entorhinal cortex slices. *Brain Res. Brain Res. Protoc.* 7, 267–276. doi: 10.1016/s1385-299x(01)00080-0

Schurr, A., and Payne, R. S. (2007). Lactate, not pyruvate, is neuronal aerobic glycolysis end product: an *in vitro* electrophysiological study. *Neuroscience* 147, 613–619. doi: 10.1016/j.neuroscience.2007.05.002

Schurr, A., West, C. A., and Rigor, B. M. (1988). Lactate-supported synaptic function in the rat hippocampal slice preparation. *Science* 240, 1326–1328. doi: 10.1126/science.3375817

Sheth, S. A., Nemoto, M., Guiou, M., Walker, M., Pouratian, N., and Toga, A. W. (2004). Linear and nonlinear relationships between neuronal activity, oxygen metabolism and hemodynamic responses. *Neuron* 42, 347–355. doi: 10.1016/s0896-6273(04)00221-1

Shuttleworth, C. W., Brennan, A. M., and Connor, J. A. (2003). NAD(P)H fluorescence imaging of postsynaptic neuronal activation in murine hippocampal slices. *J. Neurosci.* 23, 3196–3208.

Sik, A., Tamamaki, N., and Freund, T. F. (1993). Complete axon arborization of a single CA3 pyramidal cell in the rat hippocampus and its relationship with postsynaptic parvalbumin-containing interneurons. *Eur. J. Neurosci.* 5, 1719–1728. doi: 10.1111/j.1460-9568.1993.tb00239.x

Singer, W., and Lux, H. D. (1975). Extracellular potassium gradients and visual receptive fields in the cat striate cortex. *Brain Res.* 96, 378–383. doi: 10.1016/0006-8993(75)90751-9

Sohal, V. S., Zhang, F., Yizhar, O., and Deisseroth, K. (2009). Parvalbumin neurons and gamma rhythms enhance cortical circuit performance. *Nature* 459, 698–702. doi: 10.1038/nature07991

Somjen, G. G. (2002). Ion regulation in the brain: implications for pathophysiology. *Neuroscientist* 8, 254–267. doi: 10.1177/1073858402008003011

Steinhäuser, C., Seifert, G., and Bedner, P. (2012). Astrocyte dysfunction in temporal lobe epilepsy: K^+ channels and gap junction coupling. *Glia* 60, 1192–1202. doi: 10.1002/glia.22313

Studer, D., Zhao, S., Chai, X., Jonas, P., Graber, W., Nestel, S., et al. (2014). Capture of activity-induced ultrastructural changes at synapses by high-pressure freezing of brain tissue. *Nat. Protoc.* 9, 1480–1495. doi: 10.1038/nprot.2014.099

Sumiyoshi, A., Suzuki, H., Ogawa, T., Riera, J. J., Shimokawa, H., and Kawashima, R. (2012). Coupling between gamma oscillation and fMRI signal in the rat somatosensory cortex: its dependence on systemic physiological parameters. *Neuroimage* 60, 738–746. doi: 10.1016/j.neuroimage.2011.12.082

Takács, V. T., Szönyi, A., Freund, T. F., Nyiri, G., and Gulyás, A. I. (2015). Quantitative ultrastructural analysis of basket and axo-axonic cell terminals in the mouse hippocampus. *Brain Struct. Funct.* 220, 919–940. doi: 10.1007/s00429-013-0692-6

Thomsen, K., Piilgaard, H., Gjedde, A., Bonvento, G., and Lauritzen, M. (2009). Principal cell spiking, postsynaptic excitation and oxygen consumption in the rat cerebellar cortex. *J. Neurophysiol.* 102, 1503–1512. doi: 10.1152/jn.00289.2009

Traub, R. D., Kopell, N., Bibbig, A., Buhl, E. H., LeBeau, F. E. N., and Whittington, M. A. (2001). Gap junctions between interneuron dendrites can enhance synchrony of gamma oscillations in distributed networks. *J. Neurosci.* 21, 9478–9486.

Traub, R. D., and Whittington, M. A. (2010). *Cortical Oscillations in Health and Disease*. New York, NY: Oxford University Press.

Tsintsadze, V., Minlebaev, M., Suchkov, D., Cunningham, M. O., and Khazipov, R. (2015). Ontogeny of kainate-induced gamma oscillations in the rat CA3 hippocampus *in vitro*. *Front. Cell. Neurosci.* 9:195. doi: 10.3389/fncel.2015.00195

Uhlhaas, P. J., and Singer, W. (2010). Abnormal neural oscillations and synchrony in schizophrenia. *Nat. Rev. Neurosci.* 11, 100–113. doi: 10.1038/nrn2774

van Vugt, M. K., Schulze-Bonhage, A., Litt, B., Brandt, A., and Kahana, M. J. (2010). Hippocampal gamma oscillations increase with memory load. *J. Neurosci.* 30, 2694–2699. doi: 10.1523/JNEUROSCI.0567-09.2010

Viswanathan, A., and Freeman, R. D. (2007). Neurometabolic coupling in cerebral cortex reflects synaptic more than spiking activity. *Nat. Neurosci.* 10, 1308–1312. doi: 10.1038/nn1977

Watrous, A. J., Fell, J., Ekstrom, A. D., and Axmacher, N. (2015). More than spikes: common oscillatory mechanisms for content specific neural representations during perception and memory. *Curr. Opin. Neurobiol.* 31, 33–39. doi: 10.1016/j.conb.2014.07.024

Whittaker, R. G., Turnbull, D. M., Whittington, M. A., and Cunningham, M. O. (2011). Impaired mitochondrial function abolishes gamma oscillations in the hippocampus through an effect on fast-spiking interneurons. *Brain* 134:e180. doi: 10.1093/brain/awr018

Whittington, M. A., Traub, R. D., Faulkner, H. J., Stanford, I. M., and Jefferys, J. G. R. (1997). Recurrent excitatory postsynaptic potentials induced by synchronized fast cortical oscillations. *Proc. Natl. Acad. Sci. U S A* 94, 12198–12203. doi: 10.1073/pnas.94.22.12198

Whittington, M. A., Traub, R. D., and Jefferys, J. G. R. (1995). Synchronized oscillations in interneuron networks driven by metabotropic glutamate receptor activation. *Nature* 373, 612–615. doi: 10.1038/373612a0

Wirtz, S., and Schuelke, M. (2011). Region-specific expression of mitochondrial complex I genes during murine brain development. *PLoS One* 6:e18897. doi: 10.1371/journal.pone.0018897

Wong, R. K. S., and Prince, D. A. (1981). Afterpotential generation in hippocampal pyramidal cells. *J. Neurophysiol.* 45, 86–97.

Wong-Riley, M. T. T. (1989). Cytochrome oxidase: an endogenous metabolic marker for neuronal activity. *Trends Neurosci.* 12, 94–101. doi: 10.1016/0166-2236(89)90165-3

Zhang, Z. G., Hu, L., Hung, Y. S., Mouraux, A., and Iannetti, G. D. (2012). Gamma-band oscillations in the primary somatosensory cortex - a direct and obligatory correlate of subjective pain intensity. *J. Neurosci.* 32, 7429–7438. doi: 10.1523/JNEUROSCI.5877-11.2012

Zhao, S., Studer, D., Chai, X., Graber, W., Brose, N., Nestel, S., et al. (2012). Structural plasticity of hippocampal mossy fiber synapses as revealed by high-pressure freezing. *J. Comp. Neurol.* 520, 2340–2351. doi: 10.1002/cne.23040

Zuckermann, E. C., and Glaser, G. H. (1968). Hippocampal epileptic activity induced by localized ventricular perfusion with high-potassium cerebrospinal fluid. *Exp. Neurol.* 20, 87–110. doi: 10.1016/0014-4886(68)90126-x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Molecular Neuroscience

REVIEW
published: 16 March 2017
doi: 10.3389/fnmol.2017.00073

Emerging Roles for Glycogen in the CNS

*Alice E. Waitt[1], Liam Reed[1], Bruce R. Ransom[2] and Angus M. Brown[1,2]**

[1]School of Life Sciences, University of Nottingham, Nottingham, UK, [2]Department of Neurology, University of Washington, Seattle, WA, USA

The ability of glycogen, the depot into which excess glucose is stored in mammals, to act as a source of rapidly available energy substrate, has been exploited by several organs for both general and local advantage. The liver, expressing the highest concentration of glycogen maintains systemic normoglycemia ensuring the brain receives a supply of glucose in excess of demand. However the brain also contains glycogen, although its role is more specialized. Brain glycogen is located exclusively in astrocytes in the adult, with the exception of pathological conditions, thus in order to benefit neurons, and energy conduit (lactate) is trafficked inter-cellularly. Such a complex scheme requires cell type specific expression of a variety of metabolic enzymes and transporters. Glycogen supports neural elements during withdrawal of glucose, but once the limited buffer of glycogen is exhausted neural function fails and irreversible injury ensues. Under physiological conditions glycogen acts to provide supplemental substrates when ambient glucose is unable to support function during increased energy demand. Glycogen also supports learning and memory where it provides lactate to neurons during the conditioning phase of *in vitro* long-term potentiation (LTP), an experimental correlate of learning. Inhibiting the breakdown of glycogen or intercellular transport of lactate in *in vivo* rat models inhibits the retention of memory. Our current understanding of the importance of brain glycogen is expanding to encompass roles that are fundamental to higher brain function.

Keywords: glycogen, glucose, lactate, astrocyte, potassium, memory, aglycemia, exercise

OPEN ACCESS

Edited by:
Susan A. Masino,
Trinity College, USA

Reviewed by:
Johannes Hirrlinger,
Leipzig University, Germany
María Llorens-Martín,
Consejo Superior de Investigaciones Científicas (CSIC), Spain
Lasse Kristoffer Bak,
University of Copenhagen, Denmark

****Correspondence:***
Angus M. Brown
ambrown@nottingham.ac.uk

Received: *16 January 2017*
Accepted: *03 March 2017*
Published: *16 March 2017*

Citation:
Waitt AE, Reed L, Ransom BR and Brown AM (2017) Emerging Roles for Glycogen in the CNS. Front. Mol. Neurosci. 10:73. doi: 10.3389/fnmol.2017.00073

INTRODUCTION

The lack of appreciation for the role(s) that brain glycogen contributes to brain function cannot be attributed to its recent discovery, as its presence, detected by both biochemical assay (Koizumi, 1974) and electron microscopy (Koizumi and Shiraishi, 1970), has been known for decades. Unlike skeletal muscle or liver, where glycogen is expressed uniformly throughout a homogenous cell population (Stryer, 1995), the cellular (and sub-cellular) location of glycogen within the brain provides clues to its function (Cataldo and Broadwell, 1986; Oe et al., 2016), but detailed information regarding the intricacies of metabolic cell-to-cell signaling within the brain (Dringen et al., 1995; Magistretti and Pellerin, 1997; Dienel, 2009) were required before potential roles for glycogen could be proposed and experimentally tested (Brown et al., 2003; Suzuki et al., 2011). This disregard for brain glycogen was compounded by the low concentration with which glycogen is expressed in the brain; up to 100 times lower than liver and skeletal muscle (Stryer, 1995). Although never consigned as a vestigial entity glycogen was quietly ignored, and in a neurocentric world there were few advocates proposing it warranted detailed investigation.

SYSTEMIC HYPOGLYCEMIA

The presence of glycogen in the brain may be viewed as at odds with established facts regarding whole body metabolism. Given the brains exclusive reliance on glucose as its sole energy support, and the complex endocrine machinery that secures blood glucose levels within a narrow concentration range (Frier and Fisher, 2007), any need for glycogen within the brain initially seems superfluous. The liver breaks down glycogen in response to falling systemic glucose levels ensuring adequate delivery of glucose to the brain for up to 24 h (Stryer, 1995). Skeletal muscle glycogen provides a localized energy reserve for muscle (Champe and Harvey, 2008; although even this is a simplification, with recent studies suggesting a highly complex and coordinated reciprocal metabolism of glucose and lactate between brain and muscle, dependent upon immediate localized requirements; Dalsgaard et al., 2004; Dalsgaard, 2006). What is not in doubt is the exquisite sensitivity that the brain displays when confronted with a shortfall in glucose delivery (Frier and Fisher, 2007). Consistent with the homeostatic hormonal response that maintains normoglycemia, in event of decreased systemic glucose (hypoglycemia) the brain responds via the autonomic nervous system, with a series of signals (sweating, tremor, decreased temperature, decreased intraocular pressure, increased gastric emptying) that warn the sufferer of an impending hypoglycemic attack (Frier and Fisher, 2007). The critical aspect of this autonomic response is that its threshold lies above that of the glycopenic symptoms (confusion, drowsiness, odd behavior, speech difficulty, lack of co-ordination) of hypoglycemia characterized by confusion. However repeated hypoglycemic episodes compromise the threshold for the autonomic symptoms, a process called hypoglycemia unawareness (Cryer, 2002), such that they dip below those of the glycopenic effects rendering the sufferer incapable of responding appropriately to the hypoglycemia, with potentially fatal consequences (**Figure 1**).

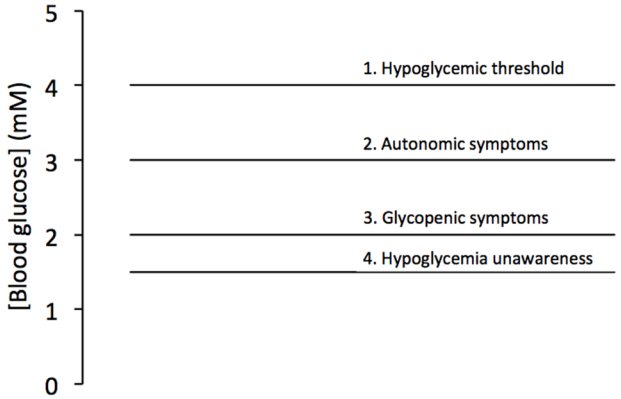


FIGURE 1 | Blood glucose levels associated with hypoglycemia. Blood glucose below 4 mM is considered hypoglycemic (1), with autonomic symptoms (2) triggered at about 3 mM and glycopenic symptoms triggered at about 2 mM (3). However the condition hypoglycemia unawareness leads to a fall in the threshold of the autonomic symptoms below that of the glycopenic threshold (4). Adapted from Figure 7.1 (Frier and Fisher, 2007).

GLUCOSE IS THE MAIN ENERGY SUPPORT FOR BRAIN FUNCTION

In the last 20 years attempts to deduce the role(s) of brain glycogen have been played out against a background of confusion, dead ends and polarized opinion, which stems from the simple fact that we know little about critical aspects of metabolism in the brain at the cellular level (Ames, 2000). The scenario can be simplified as follows; we do not know which energy substrates cells use under particular conditions. This rather simplistic statement conceals a degree of complexity that is initially difficult to appreciate when one considers that all we are considering is which of two energy substrates (glucose or lactate) two cell types (neurons and glia) use. To begin to unravel this, we must first review evidence that led to the dogmatic view of brain energy metabolism, which states that glucose is the energy substrate used by all cells in the brain (Dwyer, 2002). This has substantial support based on the following facts: (1) during insulin-induced hypoglycemia there is no systemic alternate energy substrate that can substitute for glucose; (2) complex homeostatic mechanisms have evolved to maintain normoglycemia (no such equivalent systems exist for alternate substrates such as lactate); (3) non-glucose substrates are converted, in the process of gluconeogenesis, into glucose, implying glucose is a universally preferred substrate; (4) metabolites of glucose are detected in the brain using NMR spectroscopic methods; and (5) the arterial-venous difference for brain glucose is positive, implying glucose uptake into the brain. Although it has been known for decades that the brain releases lactate (Abi-Saab et al., 2002), historically this was considered a waste product of glucose metabolism, and of no metabolic consequence. This dismissal of lactate as a viable energy substrate was reconsidered in the face of evidence that suggested it might be actively taken up and metabolized by neurons. However the conditions under which this would occur were obscured due to the technical difficulties involved in measuring energy usage in single cells. An additional complication is the role that glycogen plays. Glycogen, the storage form of glucose in the body (Champe and Harvey, 2008), is a molecule comprising dehydrated glucosyl molecules, which can be liberated as lactate (Shulman et al., 2001).

SUB-CELLULAR LOCATION OF GLYCOGEN

Since the advent of the electron microscope it has been known that in the adult rodent brain glycogen is stored almost exclusively in astrocytes (Cataldo and Broadwell, 1986). Due to a lack of appreciation of the role that glial cells contribute to brain function that was prevalent in previous decades, such a location for glycogen, compounded by its low concentrations, led to dismissal of any importance attached to the compound (Stryer, 1995). We should not judge our predecessors for their lack of insight, as it is only in recent years, where the

intercellular metabolic signaling pathways have been identified, that a role for glycogen has become apparent (Brown et al., 2003; Suzuki et al., 2011). Initial studies, which reported that glycogen was located in astrocyte processes abutting synapses (Koizumi and Shiraishi, 1970; Phelps, 1972; Koizumi, 1974), have been confirmed with more advanced microscopic techniques that allow 3D reconstruction of very fine astrocytic processes (Cali et al., 2016). These studies suggest not only an intimate relationship exists between astrocytes and neurons, but in particular between astrocytes and synapses, with mitochondria expressed in high density in these processes (Cali et al., 2016).

Until recently the regional expression of brain glycogen was unclear. This was primarily because the technique used to assess glycogen expression (biochemical glycogen assay) was incapable of the resolution required to accurately measure the glycogen concentration in small areas of brain (Passonneau et al., 1967). A refined technique that uses immunohistochemical assessment of glycogen has recently been used to accurately visualize glycogen expression throughout the rodent brain (Oe et al., 2016). The report confirmed that in the adult glycogen is expressed almost exclusively in astrocytes and is located in the astrocytic processes rather than in the soma. The highest expression of glycogen occurred in the hippocampus, striatum, cortex and cerebellar molecular layer, a region of high synaptic activity, but was lower in white matter and sub-cortical areas. It is interesting to note that the regions with the highest expression of glycogen also have higher metabolic demand.

The dogmatic view of an exclusively astrocytic domain for glycogen must be considered against the evidence of neuronally located glycogen. Such a location was associated with pathological conditions such as Lafora's disease, a form of epilepsy, where such an over-abundance of neuronal glycogen is considered a side effect of the disease and has no physiological function (Vilchez et al., 2007). In the sciatic nerve glycogen related enzymes are shown to be present in axons (Pfeiffer-Guglielmi et al., 2006, 2007), and glycogen is present in both axons and Schwann cells, but only under pathological conditions (Powell et al., 1985; Katsuragi et al., 1988; Kalichman et al., 1998). A recent article has described neuronal glycogen that acts to protect axons during periods of hypoxia, as glycogen phosphorylase (GP) is present in nerves facilitating glycogen metabolism (Saez et al., 2014).

ASTROCYTIC SUPPORT OF CULTURED NEURONS

Initial studies that investigated the role of neuronal cells were carried out in tissue culture conditions. This reductionist approach was justified in the light of the difficulty of gaining meaningful data from *in situ* tissue (Kettenmann and Grantyn, 1992). These studies yielded bountiful information, but it was only as the studies progressed that questions were raised as to their validity i.e., did what occurred in the artificial nature of the tissue culture dish have any relation to what occurred *in vivo*. A key example of this was the viability of neurones in culture, where the yield of neurones cultured in isolation was poor. As the neurones cultured were from neo-natal animals, these neurones had to develop in the absence of any support from the *in vivo* environment they would normally develop in. Glial cells were added to the culture in an attempt to increase neurone yield, which did indeed occur although the reasons were unknown (Whatley et al., 1981). We now know that neurones are reliant on glial cells for a multitude of reasons; release of trophic factors to guide developing neurones, to provide myelin, to buffer extracellular compounds such as potassium and glutamate. In addition the extracellular space is essentially infinite in culture conditions, whereas *in vivo* it is relatively small (15% of brain volume) and provides a very small buffer zone between neighboring cells (Swanson and Choi, 1993). However studies using tissue culture from the Waagepetersen laboratory confirm that glia-neuron transfer of metabolites interactions can occur (Sickmann et al., 2005). The glia and other surrounding neurones would also provide structural support to maintain the neuron in a fixed location within the brain parenchyma. Although not the focus of this review, oligodendrocytes are implied to play a role in supplying lactate to axons (Lee et al., 2012).

Whereas the behavior of neurones may have been compromised in tissue culture conditions, it was a serendipitous approach that yielded preliminary information on the metabolic support afforded neurones by astrocytes (Swanson and Choi, 1993). Neurones that were co-cultured with astrocytes displayed increased viability compared to neurones that were cultured in isolation. However it was discovered that it was the presence of glycogen in the astrocytes that was the critical factor in promoting neuronal survival, as astrocytes with depleted glycogen did not significantly prolong neuronal survival (Swanson and Choi, 1993). This was an intriguing result that posed more questions than it answered, most notably, what was the form in which glycogen provided support?

THE ROLE OF GLYCOGEN DURING AGLYCEMIA

From studies of glycogen in the liver and skeletal muscle the nature of the glycogen molecule and its regulation are well established (Stryer, 1995), information that can reasonably be applied to brain glycogen. In the periphery glycogen acts as a storage depot for excess glucose during periods of plenty, to be liberated in time of need. In the liver glycogen is liberated in the form of glucose to maintain normoglycemic blood glucose levels (Stryer, 1995), whereas in the muscle glycogen is glycolytically metabolized (Stryer, 1995), with the apparent waste product lactate liberated into the bloodstream. The nature of the support offered by astrocytic glycogen to neurons was presumably metabolic, thus how this was achieved was a prime focus of investigation. The simplest explanation was that glycogen supported solely astrocytes, relieving the metabolic burden from neurones, i.e., astrocytes are fueled exclusively be glycogen sparing interstitial glucose for exclusive use by neurons (DiNuzzo et al., 2010). Whilst this is a theory as simplistic as it

is attractive, it is not the case. Studies carried out in cultured astrocytes indicated that when astrocytes were incubated in the presence of glucose they released lactate (Dringen et al., 1993b), whereas neurones did not release lactate; in fact they consumed it (Dringen et al., 1993a). This indicated that astrocytes might release lactate, which is then taken up and used by neurons as an energy substrate to supplement glucose uptake (Dringen et al., 1993a). This is where the origins of a contentious and continuing dispute arose. Whilst it can be insightful to study how processes work under tissue culture conditions, they may not apply *in vivo*. This is especially important where the interstitial compartment is proposed to be the medium of exchange through which putative metabolic compounds are passed from astrocyte to neurons. However under tissue culture conditions where the interstitial space is infinitely large, this is unrealistic and alternate models were required.

Given the complexity of metabolic interactions in gray matter the Ransom lab chose to use a simple white matter model, the rodent optic nerve, to investigate the role of glycogen (Ransom et al., 1994). These studies were prompted by comparison of the effects of anoxia or aglycemia on the stimulus evoked compound action potential (CAP). The tissue is accessible to recordings of the stimulus evoked CAP, a monitor of axon conduction, whose area is proportional to the number of conducting axons (Cummins et al., 1979). The CAP area recorded in 10 mM glucose provides a baseline measure of axon conduction against which the post-insult CAP can be compared (Stys et al., 1991). In addition to electrophysiological recordings, the tissue is amenable to biochemical assay, simultaneous recordings of lactate release from the tissue and immunohistochemical staining. In the presence of oxygen but with glucose withdrawn (aglycemia) the CAP lasted up to 30 min before failing (Ransom and Fern, 1997). This suggested that there was an endogenous energy reserve present within the tissue that supported axon conduction in the absence of exogenously applied glucose, but that the reserve was limited and could only support conduction for a limited period of time; once the reserve was exhausted conduction failed. The most likely candidate for this energy reserve is glycogen, which has been located in astrocytes by electron microscopy in both the rat (Wender et al., 2000) and mouse optic nerve (MON; Brown et al., 2003). Given that glycogen supports axon conduction in the absence of glucose, one would reasonably predict that increasing glycogen content should increase the latency to CAP failure, whereas depleting glycogen content would attenuate latency to CAP failure, during a subsequent period of aglycemia. This was indeed found to be the case (Brown et al., 2003). The macromolecule glycogen is too large to be transported intercellularly, thus there must exist a glycogen-derived metabolic conduit that supports axon conduction. This is most likely lactate, since astrocytes in culture release lactate in the presence of glucose (Dringen et al., 1993a,b). If lactate were released from the breakdown of glycogen, then lactate, in the absence of glucose, should be able to support MON axon conduction. In addition, blocking lactate release from astrocytes, or uptake into axons with pharmacological blockers should also attenuate the CAP: all of these predictions were shown to occur experimentally (Brown et al., 2003; Tekkok et al., 2005). Immunohistochemical studies to investigate the presence of monocarboxylate transporters (MCTs) on cells membranes, demonstrated MCT1 immunoreactivity on astrocyte membranes and MCT2 immunoreactivity on axon membranes (Tekkok et al., 2005). The MCTs facilitate the movement of lactate across cell membranes, thus their presence is an absolute requirement for trans-membrane flux of lactate. Preventing glycogen breakdown by pharmacologically inhibiting GP also attenuated the latency to CAP failure during aglycemia (Brown et al., 2004; **Figure 2**).

The sciatic nerve was studied in order to determine whether there was an equivalent metabolic cell-to-cell signaling pathway in the peripheral nervous system (PNS). Electron microscopic images revealed that Schwann cells did express glycogen, which was measured by biochemical assay at a concentration about twice that in the optic nerve (Brown et al., 2012). The sciatic nerve contains two broad classes of axons, large myelinated A fibers, and smaller unmyelinated C fibers grouped within Remak bundles (Landon, 1976). Introduction of aglycemia resulted in the C peak failing within about 30 min, whereas the larger myelinated A fibers could sustain conduction for up to two and a half hours before they began to fail (Brown et al., 2012). Such a difference could in part be related to the greater metabolic rate of small axons (per unit volume) compared to

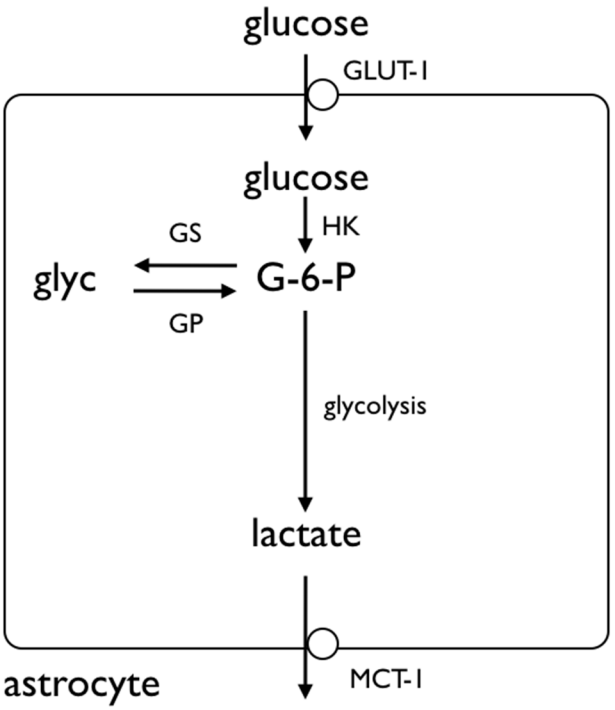


FIGURE 2 | Glycogen storage in the astrocyte. Glucose is taken up by astrocytes via the GLUT-1 glucose transporter, where it is phosphorylated by hexokinase (HK) to glucose-6-phosphate (G-6-P). The G-6-P can either proceed along the glycolytic pathway, or be added to the glycogen molecule by glycogen synthase (GS). The glycogen is broken down by glycogen phosphorylase (GP), ultimately to lactate, which crosses the astrocyte membrane by monocarboxylate transporter (MCT). Adapted from Figure 1 (Fryer and Brown, 2015).

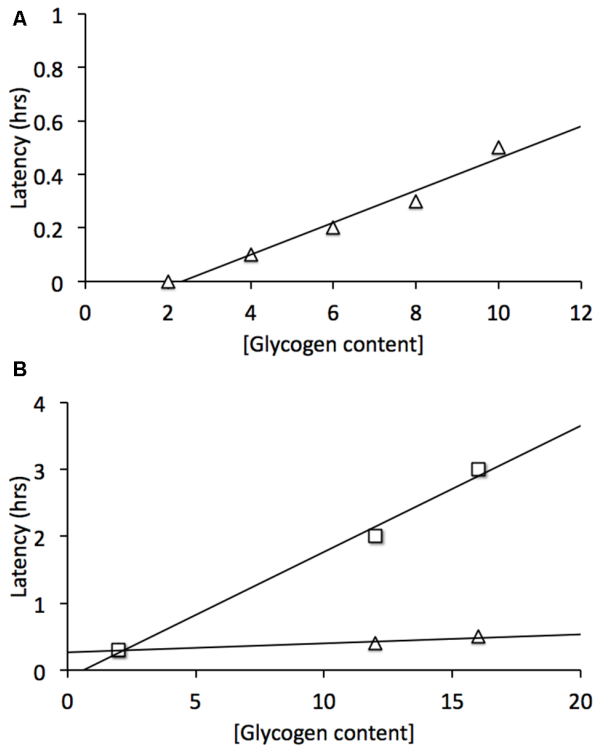


FIGURE 3 | Glycogen content determines latency to failure in optic nerve axons and Schwann cell A fibers but not C fibers. (A) In mouse optic nerve (MON) glycogen content determines latency to failure during subsequent period of aglycemia (△). **(B)** In Schwann cells elevated glycogen content benefits A fibers (□) but not C fibers (△). Note elevated (glycogen) does not prolong C fiber latency to failure. Adapted from Figure 2 (Brown et al., 2003) and Figure 5 (Brown et al., 2012).

larger axons (Perge et al., 2012). However when glycogen content was increased there was no increase in the latency to failure in the C peak, whereas the latency to failure of A fibers was increased (**Figure 3**). Thus glycogen expressed in Schwann cells only benefitted the A fibers and had no effect on the C fibers. Glycogen was broken down to lactate in the Schwann cell and transported intercellularly via MCTs (Domènech-Estévez et al., 2015) to the A fibers (Brown et al., 2012). Thus, there are striking similarities between the central and PNSs with regard to the metabolic provision of glycogen-derived lactate, where glycogen, located in glial cells, is broken down to lactate, which acts as a metabolic conduit to support axon conduction during periods of shortfall in glucose delivery to the tissue. However what is more physiologically relevant is the role(s) that glycogen plays under normal, non-pathological conditions, thus the next logical step in the MON studies was to investigate the role of glycogen in supporting physiological activity.

THE ROLE OF GLYCOGEN DURING HIGH FREQUENCY STIMULUS

Imposing a short period of high intensity stimulus was very effective at revealing key information. At frequencies between 10 Hz and 100 Hz, i.e., an action potential was evoked between every 100 ms and 10 ms, the increasing metabolic demand imposed on the tissue exceeded its ability to support conduction, in the presence of 10 mM glucose aCSF (Brown et al., 2003). In the first instance imposing a 4 min period of 100 Hz stimulus on the MON caused an elongation of the CAP profile, no doubt due to accumulation of interstitial K^+ (Connors et al., 1982) exceeding the ability of the Na^+-K^+ ATPase to maintain homeostatic trans-membrane ion concentrations (Ransom et al., 2000), but the CAP area did not fall below baseline (Brown et al., 2003). However a decrease in glycogen content after only 4 min of the high intensity stimulus occurred in the presence of 10 mM glucose (Brown et al., 2003), suggesting that even this high concentration of glucose was unable to support the CAP, and glycogen derived lactate was released as a supplemental energy substrate. Extended exposure to 100 Hz stimulus (on the order or tens of minutes) caused a decrease in the CAP area that could be reversed if the MON was supplied with 30 mM glucose aCSF (Brown et al., 2003). This reveals a very important point, which is that the MON will continue to conduct action potentials during periods of increased energy demand if excess substrate is applied, i.e., if the increased energy demand required by the increased stimulus is met by increased supply of energy substrate then CAP conduction will be maintained. Thus, there is no absolute threshold with regard to conduction failure; rather it is determined on the basis of whether the energy demand is matched by supply of substrate. This point was further demonstrated by exposing MONs to 2 mM glucose aCSF. Under baseline conditions of stimulus every 30 s the CAP area was maintained at baseline levels. However depleting glycogen with a short period of exposure to 0 mM glucose resulted in subsequent exposure to 2 mM glucose being unable to support conduction (Brown et al., 2003), implying that under hypoglycemic conditions supplementary glycogen-derived lactate is required to fully support function. The relationship between the ability of the MON to support conduction under the duress of increased energy demand was shown when MONs were exposed to 100 Hz stimulus in 2 mM glucose, where the CAP fell rapidly on imposition of the stimulus (Brown et al., 2003). These experiments provided the first information regarding potential role(s) of glycogen under physiological conditions, namely to act as an energy buffer to supply substrate during short-term mis-match between ambient glucose and energy demand.

A key issue that was missing from these studies was identification of the signaling mechanism whereby neurones inform astrocytes of their metabolic requirements. Such a mechanism would have to adhere to the following requirements: (1) it must be localized; (2) it must be capable of invoking rapid glycogen breakdown; (3) the intensity of the signal must be proportionate to the stimulus intensity; (4) the intensity of the signal must dissipate as the stimulus intensity declines; and (5) if the signal is not universal, then comparable localized equivalent systems must exist across the nervous system. A potential mechanism was revealed via studies investigating the neurotransmitter modulation of glycogen content.

THE ASTROCYTE NEURON LACTATE SHUTTLE HYPOTHESIS

One important aspect of glycogen's metabolism in the brain is the means by which it is modified. Whereas in the periphery glycogen is modulated by the hormones insulin and glucagon, in the brain parenchyma the influence of these hormones appear to be attenuated and neurotransmitters contribute regulatory roles. Pierre Magistretti's group started investigating this phenomenon in the early 1990's. Their goal was to identify physiologically relevant modulators, and these initial studies identified VIP, nor-epinephrine and adenosine as well as K^+ (see later) as controlling glycogen levels (Hof et al., 1988; Sorg and Magistretti, 1991). However their studies investigating the putative modulatory role of the most abundant excitatory CNS neurotransmitter, glutamate, yielded unexpected results that were adapted as the basis of the astrocyte neuron lactate shuttle hypothesis (ANLSH; Pellerin and Magistretti, 1994). This hypothesis had its origins in studies carried out in the honeybee retina, which is metabolically and morphologically compartmentalized. Light stimulus induces glucose uptake in the honeybee retinal glial cells, but these cells show no increased oxygen uptake. However the photoreceptors take up alanine derived from the astrocytic glucose and increase oxygen consumption (Tsacopoulos et al., 1994). This metabolic compartmentalization clearly displays a division of labor between glial cells and neurons, in which the glial cells take up glucose and glycolytically convert it to alanine, which is then transported to the photoreceptors via the interstitial space for oxidative metabolism. Magistretti and Pellerin (1997) work proposed a similar compartmentalized scenario in the mammalian brain, which is set in motion when neuronal activity results in elevated interstitial glutamate. The glutamate is co-transported with Na^+ into the astrocyte, where the glutamate is converted to glutamine, then shuttled to the neurones for conversion to glutamate by glutaminase. However the excess Na^+ must be pumped out of the astrocyte via the Na^+-K^+ ATPase and conversion of glutamate to glutamine requires ATP. The energy debt is met via astrocytic uptake of glucose followed by glycolytic conversion to lactate, yielding two molecules of ATP. One molecule of ATP fuels the Na^+-K^+ ATPase, the other glutamate conversion. The product of the glycolysis, lactate, is then transported out of the astrocyte and into the neurone where it is oxidatively metabolized (Pellerin and Magistretti, 1994). This is a superficially stoichiometrically pleasing scheme but has come in for intense scrutiny, key criticisms being that this scheme obviously only applies to glutamatergic brain areas; what occurs, for example, in white matter, and if all glucose and glycogen goes to lactate what fuels other processes? The debate is too convoluted and voluminous to describe in detail here, but the following reviews may be consulted to provide a balanced view of both sides of the argument with those opposed to the hypothesis proposing arguments that cannot be ignored (Chih et al., 2001; Dienel and Hertz, 2001; Chih and Roberts, 2003; Pellerin and Magistretti, 2003, 2012; Aubert et al., 2005; Dienel, 2010; DiNuzzo et al., 2010).

ELEVATED INTERSTITIAL K^+ PROMOTES GLYCOGENOLYSIS

A recent study has linked elevations in extracellular K^+, as would occur during increased neuronal activity, to promotion of astrocytic glycogenolysis (Choi et al., 2012). This is a more universally applicable mechanism as all neurons/axons, be they central or peripheral, release K^+ into the interstitium as a result of activity (Kandel et al., 2000). The greater the intensity of the activity the greater the increase in K^+, and as demonstrated in rat optic nerve the K^+ increase in the interstitium is buffered very efficiently by astrocytes (Ransom et al., 2000), and retreats towards baseline in the order of seconds. In the sciatic nerve the stimulus induced increases in K^+ are attenuated, and the elevations persist for longer, suggesting less efficient buffering than occurs in central tissue (Hoppe et al., 1991). The interstitial K^+ activates a Na^+-HCO_3^- co-transporter on the astrocyte membrane, whose activation results in intracellular alkalization. This change in intracellular HCO_3 results in increased soluble adenylyl cyclase (sAC) activity, an enzyme that causes elevated cAMP, leading to glycogenolysis, and production of intracellular lactate (Choi et al., 2012). The lactate is transported out the cell to the neurones, where it acts as an energy substrate for oxidative phosphorylation (**Figure 4**).

In a complementary study interstitial K^+ elevations were shown to have a separate but related effect, activating a channel on the astrocyte membrane through which pools of astrocytic lactate could flow into the interstitium, in parallel with the established route of MCTs (Sotelo-Hitschfeld et al., 2015). This is a very important route for astrocytic lactate release, since it is coupled to the membrane potential and allows lactate release against a concentration gradient, whereas the MCT is electro-neutral and net flux is governed by the trans-membrane concentrations of H^+ and lactate (**Figure 5**).

GLYCOGEN AND MEMORY

It is germane at this point to reflect upon the properties of glycogen and how these can confer certain metabolic advantages. Glycogen is an energy buffer, present in astrocytes that can be mobilized rapidly, without the need to expend ATP, as is the case with glucose. As such glycogen stands as a readily metabilizable energy source that would rapidly provide energy substrate. Compare this to glucose, which is delivered via the blood stream implying delivery of excess glucose would require delivery of excess blood, a process called functional hyperemia. This is a comparatively slow process (Gordon et al., 2008), and would be unable to deliver the instant energy required by neurones. This is worth bearing in mind in the following discussion of the role of glycogen during memory formation.

In studies dating back two decades the role of glycogen in supporting one of the most important brain functions, learning and memory commenced. Learning and memory are functions that can be tested in live animals via a series of standard tests (Kandel et al., 2000). The underlying cellular mechanisms are

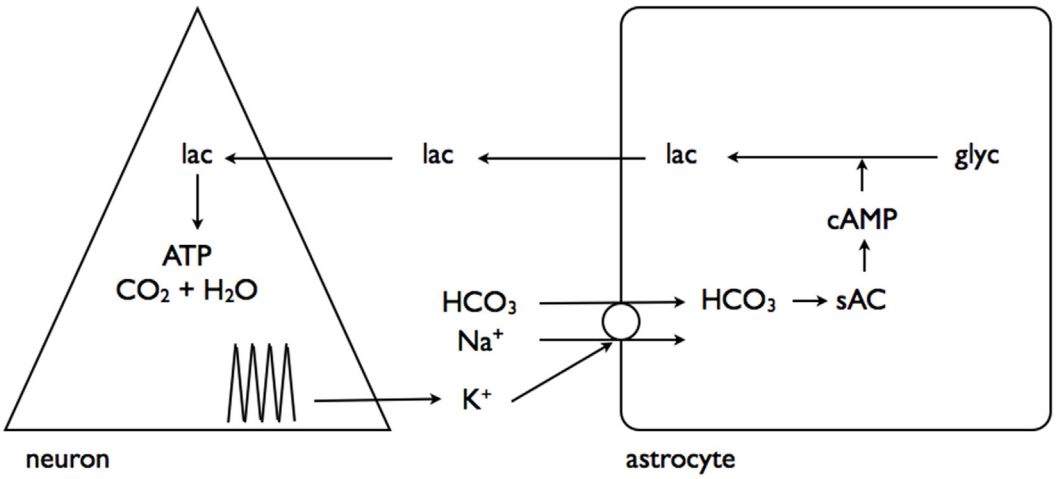


FIGURE 4 | Interstitial K^+ stimulates astrocytic glycogenolysis. Increased neural activity promotes elevated interstitial K^+, which activates the Na^+-HCO_3^- coupled transporter leading to alkalization of the astrocyte cytoplasm. This activates soluble adenylyl cyclase (sAC), which in turn elevates cytoplasmic cAMP leading to glycogenolysis and transfer of lactate out of the astrocyte for neuronal oxidative metabolism. Adapted from Figure 7 (Choi et al., 2012).

rather more difficult to pin down, but in a variety of animal models, including chick and rats, these have been ascribed to long-term potentiation (LTP) in the hippocampus. This process is quantifiable by using electrophysiological techniques, where a brief, high frequency conditioning stimulus is applied to axonal input to the CA1 pyramidal cell dendritic field. The resulting synaptic response is enhanced post-conditioning, an indication of "learning" occurring at the synapse (Malenka, 2003). The initial studies took place with the chick as the animal model. The chick was exposed to two beads, red or blue in color. The red bead was coated in an aversant compound and the chicks choice of pecking was noted. The ratio between the number of pecks of red beads and blue was measured, with an increased ratio indicative of red bead avoidance and hence learning (Hertz et al., 1996). The concentration of glycogen in the forebrain decreased concomitant with an increase in glutamate (Hertz et al., 2003). The learning process could be attenuated by inhibiting glycolysis or glycogenolysis and restored by applying exogenous aspartate and acetate, which is metabolized in the astrocytes, or application of glutamine (Gibbs et al., 2006a,b). These studies demonstrated robust learning in the chick, which appeared to involve breakdown of glycogen to ultimately produce glutamate.

The main findings of these chick experiments were extended in studies on rodents to encompass not only live animal studies on learning and memory, but investigation of the cellular

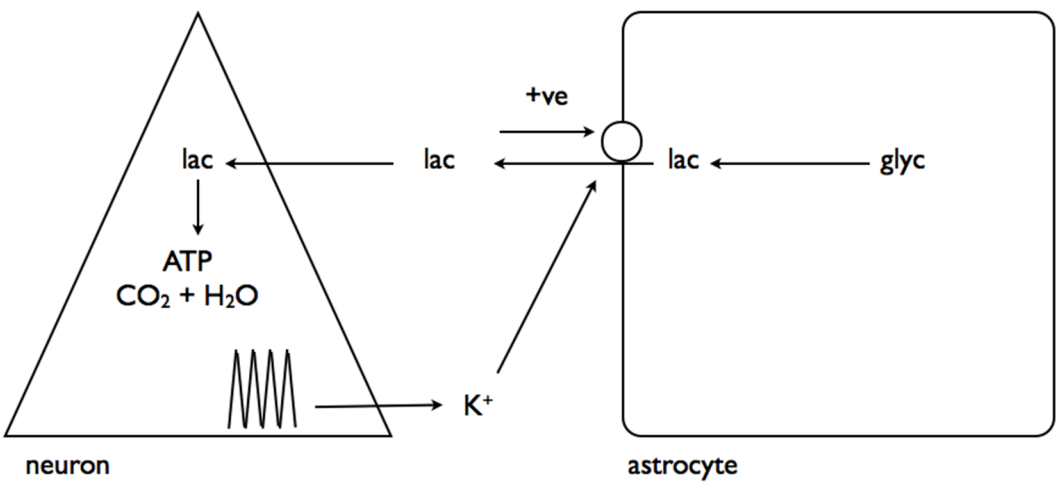


FIGURE 5 | Interstitial K^+ promotes astrocytic lactate release via the lactate channel. As described in **Figure 4**, neural activity results in elevated interstitial K^+. This activates a channel leading to release of the cytoplasmic lactate pool into the interstitium, which, via a positive feedback loop, causes further lactate release. Adapted from Figure 8 (Sotelo-Hitschfeld et al., 2015).

mechanism underlying these processes using *in vitro* techniques. Rats were placed in a white chamber, separated from another chamber by a door. The experimenter opened the door, and when the rat entered the new chamber it received an electric footshock, and was then placed back in the white chamber. The time taken for the rat to re-enter the second chamber was taken as an index of memory, with increased latency signifying that the rat had learned to avoid the chamber (Taubenfeld et al., 2001). The rats were tested daily after the conditioning shock, and the latency to re-enter the second chamber was stable at about 300 s for up to 7 days. Injection of the GP inhibitor DAB into the rat's brain decreased the latency indicating that memory was impaired. Exogenously dialyzed lactate into the hippocampus circumvented the inhibitory effects of DAB and augmented the latency. Electrophysiological recordings of LTP in hippocampal slices showed that in the presence of DAB the synaptic efficacy was initially enhanced, but this rapidly fell, indicative of no long-term enhancement of synaptic efficacy occurring (Suzuki et al., 2011). These data confirm a role for glycogen in the process of memory. This data appears to suggest there is a metabolic aspect to LTP, that glycogen-derived lactate is an integral component of the process (Suzuki et al., 2011). The speed with which glycogen can be metabolized makes it an ideal energy buffer, capable of rapid delivery of lactate to the neuronal elements that exhibit very rapid augmentation of synaptic efficacy in response to high frequency conditioning pulses.

This conclusion was further complemented by a study on mice where brain glycogen synthase (GS) was knocked out. Biochemical assay revealed that liver and muscle glycogen were unaffected. In these knockout animals learning and memory was impaired compared to control animals (Duran et al., 2013). Whilst initially in agreement with the previous studies, these data must be considered in the context of the role brain glycogen plays during physiological activity. The data suggests that only learning and memory were affected, since animals appeared to be normal, suggesting that brain glycogen is not vital for life, and that its absence does not lead to any significant observable pathology. Obviously more detailed studies on the effect of brain GS knockouts are required.

GLYCOGEN AND EXERCISE

A role for glycogen during exercise is emerging. Its role as an energy reserve has important consequences during exercise with skeletal muscle glycogen used to provide energy. The effects, if any, of exercise on brain glycogen were unknown. Initial studies demonstrated that in rats exercising on a treadmill, the skeletal muscle glycogen fell by up to 90%, but super-compensated to ∼45% after 24 h recovery. A similar scenario occurred in the brain, with a decrease of over 50%, with regional specific decreases, followed by super-compensation (Matsui et al., 2012). Interestingly with substantial exercise training the brain glycogen baseline level increased, which may be due to the increased energy demands required by the brain during exercise. However in these studies there was significant systemic hypoglycemia. Thus experiments were carried out in animals in which no hypoglycemia was achieved. In this instance brain glycogen fell, but lactate in the brain increased, implying glycogen plays a role in the brain during exercise that is not affected by hypoglycemia. Exercise results in increased serotonin turnover, which may promote glycogenolysis (Matsui et al., 2015). The same group have reported that type 2 diabetic patients had higher glycogen in the hippocampus, hypothalamus and cortex, and that MCT2 protein levels decreased, suggesting less efficient lactate shuttling (Shima et al., 2016). This effect has been correlated with exercise, which was shown to increase glycogen levels, MCT2 expression and improve cognitive performance. Thus these data support a link between glycogen levels and cognitive performance.

CONCLUSION

The role(s) of nervous system glycogen are slowly emerging. Far from being a mere metabolic curiosity, it appears to act as an energy buffer, capable of the rapid delivery of the energy substrate lactate, to fuel neural function. Established occasions when this occurs are during the absence of glucose, or during hypoglycemia where glycogen derived lactate acts as a supplemental energy substrate to fuel neuronal function. As such a balance appears to be struck between ambient glucose and glycogen-derived lactate, when the former, is insufficient to meet immediate energy requirements delivery of the latter is increased. Brain glycogen also plays a pivotal role in learning and memory, where it's presence is an absolute requirement for the transformation of short term learning into storage as memories. As such glycogen is positioned to play the key role as a buffer to supply energy substrate for short term increases in energy demand as occurs during the cellular mechanisms underlying LTP.

AUTHOR CONTRIBUTIONS

AMB and BRR conceived the review; edited the final version of the manuscript. AEW, LR and AMB wrote the first draft of the manuscript.

FUNDING

The review was funded in part by the University of Nottingham.

REFERENCES

Abi-Saab, W. M., Maggs, D. G., Jones, T., Jacob, R., Srihari, V., Thompson, J., et al. (2002). Striking differences in glucose and lactate levels between brain extracellular fluid and plasma in conscious human subjects: effects of hyperglycemia and hypoglycemia. *J. Cereb. Blood Flow Metab.* 22, 271–279. doi: 10.1097/00004647-200203000-00004

Ames, A. III. (2000). CNS energy metabolism as related to function. *Brain Res. Rev.* 34, 42–68. doi: 10.1016/s0165-0173(00)00038-2

Aubert, A., Costalat, R., Magistretti, P. J., and Pellerin, L. (2005). Brain lactate kinetics: modeling evidence for neuronal lactate uptake upon activation. *Proc. Natl. Acad. Sci. U S A* 102, 16448–16453. doi: 10.1073/pnas.0505427102

Brown, A. M., Baltan Tekkok, S., and Ransom, B. R. (2004). Energy transfer from astrocytes to axons: the role of CNS glycogen. *Neurochem. Int.* 45, 529–536. doi: 10.1016/j.neuint.2003.11.005

Brown, A. M., Evans, R. D., Black, J., and Ransom, B. R. (2012). Schwann cell glycogen selectively supports myelinated axon function. *Ann. Neurol.* 72, 406–418. doi: 10.1002/ana.23607

Brown, A. M., Tekkök, S. B., and Ransom, B. R. (2003). Glycogen regulation and functional role in mouse white matter. *J. Physiol.* 549, 501–512. doi: 10.1113/jphysiol.2003.042416

Cali, C., Baghabra, J., Boges, D. J., Holst, G. R., Kreshuk, A., Hamprecht, F. A., et al. (2016). Three-dimensional immersive virtual reality for studying cellular compartments in 3D models from EM preparations of neural tissues. *J. Comp. Neurol.* 524, 23–38. doi: 10.1002/cne.23852

Cataldo, A. M., and Broadwell, R. D. (1986). Cytochemical identification of cerebral glycogen and glucose-6-phosphatase activity under normal and experimental conditions. I. Neurons and glia. *J. Electron Microsc. Tech.* 3, 413–437. doi: 10.1002/jemt.1060030406

Champe, P. C., and Harvey, R. A. (2008). *Biochemistry*. Baltimore, MD: Lippincott Williams & Wilkins.

Chih, C.-P., Lipton, P., and Roberts, E. L. (2001). Do active cerebral neurons really use lactate rather than glucose? *Trends Neurosci.* 24, 573–578. doi: 10.1016/s0166-2236(00)01920-2

Chih, C.-P., and Roberts, E. L. (2003). Energy substrates for neurons during neural activity: a critical review of the astrocyte-neuron lactate shuttle hypothesis. *J. Cereb. Blood Flow Metab.* 23, 1263–1281. doi: 10.1097/01.wcb.0000081369.51727.6f

Choi, H. B., Gordon, G. R., Zhou, N., Tai, C., Rungta, R. L., Martinez, J., et al. (2012). Metabolic communication between astrocytes and neurons via bicarbonate-responsive soluble adenylyl cyclase. *Neuron* 75, 1094–1104. doi: 10.1016/j.neuron.2012.08.032

Connors, B. W., Ransom, B. R., Kunis, D. M., and Gutnick, M. J. (1982). Activity-dependent K^+ accumulation in the developing rat optic nerve. *Science* 216, 1341–1343. doi: 10.1126/science.7079771

Cryer, P. E. (2002). Hypoglycaemia: the limiting factor in the glycaemic management of type I and type II diabetes. *Diabetologia* 45, 937–948. doi: 10.1007/s00125-002-0822-9

Cummins, K. L., Perkel, D. H., and Dorfman, L. J. (1979). Nerve fiber conduction-velocity distributions. I. Estimation based on the single-fiber and compound action potentials. *Electroencephalogr. Clin. Neurophysiol.* 46, 634–646. doi: 10.1016/0013-4694(79)90101-9

Dalsgaard, M. K. (2006). Fuelling cerebral activity in exercising man. *J. Cereb. Blood Flow Metab.* 26, 731–750. doi: 10.1038/sj.jcbfm.9600256

Dalsgaard, M. K., Quistorff, B., Danielsen, E. R., Selmer, C., Vogelsang, T., and Secher, N. H. (2004). A reduced cerebral metabolic ratio in exercise reflects metabolism and not accumulation of lactate within the human brain. *J. Physiol.* 554, 571–578. doi: 10.1113/jphysiol.2003.055053

Dienel, G. A. (2009). "Energy metabolism in the brain," in *From Molecules to Networks: An Introduction to Cellular and Molecular Neuroscience*, 2nd Edn. eds J. H. Byrne and J. L. Roberts (New York, NY: Academic Press), 49–110.

Dienel, G. A. (2010). Astrocytes are 'good scouts': being prepared also helps neighboring neurons. *J. Cereb. Blood Flow Metab.* 30, 1893–1894. doi: 10.1038/jcbfm.2010.152

Dienel, G. A., and Hertz, L. (2001). Glucose and lactate metabolism during brain activation. *J. Neurosci. Res.* 66, 824–838. doi: 10.1002/jnr.10079

DiNuzzo, M., Mangia, S., Maraviglia, B., and Giove, F. (2010). Glycogenolysis in astrocytes supports blood-borne glucose channeling not glycogen-derived lactate shuttling to neurons: evidence from mathematical modeling. *J. Cereb. Blood Flow Metab.* 30, 1895–1904. doi: 10.1038/jcbfm.2010.151

Domènech-Estévez, E., Baloui, H., Repond, C., Rosafio, K., Médard, J.-J., Tricaud, N., et al. (2015). Distribution of monocarboxylate transporters in the peripheral nervous system suggests putative roles in lactate shuttling and myelination. *J. Neurosci.* 35, 4151–4156. doi: 10.1523/JNEUROSCI.3534-14.2015

Dringen, R., Gebhardt, R., and Hamprecht, B. (1993a). Glycogen in astrocytes: possible function as lactate supply for neighboring cells. *Brain Res.* 623, 208–214. doi: 10.1016/0006-8993(93)91429-v

Dringen, R., Schmoll, D., Cesar, M., and Hamprecht, B. (1993b). Incorporation of radioactivity from [^{14}C] lactate into the glycogen of cultured mouse astroglial cells. Evidence for gluconeogenesis in brain cells. *Biol. Chem. Hoppe Seyler* 374, 343–347. doi: 10.1515/bchm3.1993.374.1-6.343

Dringen, R., Peters, H., Wiesinger, H., and Hamprecht, B. (1995). Lactate transport in cultured glial cells. *Dev. Neurosci.* 17, 63–69. doi: 10.1159/000111275

Duran, J., Saez, I., Gruart, A., Guinovart, J. J., and Delgado-Garcia, J. M. (2013). Impairment in long-term memory formation and learning-dependent synaptic plasticity in mice lacking glycogen synthase in the brain. *J. Cereb. Blood Flow Metab.* 33, 550–556. doi: 10.1038/jcbfm.2012.200

Dwyer, D. S. (2002). *Glucose Metabolism in the Brain*. London: Academic Press.

Frier, B. M., and Fisher, B. M. (2007). *Hypoglycaemia in Clinical Diabetes*. New York, NY: John Wiley & Sons, Ltd.

Fryer, K. L., and Brown, A. M. (2015). Pluralistic roles for glycogen in the central and peripheral nervous systems. *Metab. Brain Dis.* 30, 299–306. doi: 10.1007/s11011-014-9516-5

Gibbs, M. E., Anderson, D. G., and Hertz, L. (2006a). Inhibition of glycogenolysis in astrocytes interrupts memory consolidation in young chickens. *Glia* 54, 214–222. doi: 10.1002/glia.20377

Gibbs, M. E., O'Dowd, B. S., Hertz, E., and Hertz, L. (2006b). Astrocytic energy metabolism consolidates memory in young chicks. *Neuroscience* 141, 9–13. doi: 10.1016/j.neuroscience.2006.04.038

Gordon, G. R., Choi, H. B., Rungta, R. L., Ellis-Davies, G. C., and MacVicar, B. A. (2008). Brain metabolism dictates the polarity of astrocyte control over arterioles. *Nature* 456, 745–749. doi: 10.1038/nature07525

Hertz, L., Gibbs, M. E., O'Dowd, B. S., Sedman, G. L., Robinson, S. R., Sykova, E., et al. (1996). Astrocyte-neuron interaction during one-trial aversive learning in the neonate chick. *Neurosci. Biobehav. Rev.* 20, 537–551. doi: 10.1016/0149-7634(95)00020-8

Hertz, L., O'Dowd, B. S., Ng, K. T., and Gibbs, M. E. (2003). Reciprocal changes in forebrain contents of glycogen and of glutamate/glutamine during early memory consolidation in the day-old chick. *Brain Res.* 994, 226–233. doi: 10.1016/j.brainres.2003.09.043

Hof, P. R., Pascale, E., and Magistretti, P. J. (1988). K^+ at concentrations reached in the extracellular space during neuronal activity promotes a Ca^{2+}-dependent glycogen hydrolysis in mouse cerebral cortex. *J. Neurosci.* 8, 1922–1928.

Hoppe, D., Chvatal, A., Kettenmann, H., Orkand, R. K., and Ransom, B. R. (1991). Characteristics of activity-dependent potassium accumulation in mammalian peripheral nerve *in vitro*. *Brain Res.* 552, 106–112. doi: 10.1016/0006-8993(91)90666-j

Kalichman, M. W., Powell, H. C., and Mizisin, A. P. (1998). Reactive, degenerative, and proliferative Schwann cell responses in experimental galactose and human diabetic neuropathy. *Acta Neuropathol.* 95, 47–56. doi: 10.1007/s004010050764

Kandel, E. R., Schwartz, J. H., and Jessell, T. M. (2000). *Principles of Neural Science*. New York, NY: McGraw-Hill.

Katsuragi, S., Shimoji, A., Watanabe, K., and Miyakawa, T. (1988). Pathological findings of the sural nerve in mitochondrial encephalomyopathy. *Jpn. J. Psychiatry Neurol.* 42, 307–313. doi: 10.1111/j.1440-1819.1988.tb01981.x

Kettenmann, H., and Grantyn, R. (1992). *Practical Electrophysiological Methods*. New York, NY: Wiley Liss.

Koizumi, J. (1974). Glycogen in the central nervous system. *Prog. Histochem. Cytochem.* 6, 1–35. doi: 10.1016/s0079-6336(74)80003-3

Koizumi, J., and Shiraishi, H. (1970). Ultrastructural appearance of glycogen in the hypothalamus of the rabbit following chlorpromazine administration. *Exp. Brain Res.* 10, 276–282. doi: 10.1007/bf00235051

Landon, D. N. (1976). *The Peripheral Nerve*. London: Wiley & Sons.

Lee, Y., Morrison, B. M., Li, Y., Lengacher, S., Farah, M. H., Hoffman, P. N., et al. (2012). Oligodendroglia metabolically support axons and contribute to neurodegeneration. *Nature* 487, 443–448. doi: 10.1038/nature11314

Magistretti, P. J., and Pellerin, L. (1997). Metabolic coupling during activation. A cellular view. *Adv. Exp. Med. Biol.* 413, 161–166. doi: 10.1007/978-1-4899-0056-2_18

Malenka, R. C. (2003). The long-term potential of LTP. *Nat. Rev. Neurosci.* 4, 923–926. doi: 10.1038/nrn1258

Matsui, T., Ishikawa, T., Ito, H., Okamoto, M., Inoue, K., Lee, M. C., et al. (2012). Brain glycogen supercompensation following exhaustive exercise. *J. Physiol.* 590, 607–616. doi: 10.1113/jphysiol.2011.217919

Matsui, T., Soya, S., Kawanaka, K., and Soya, H. (2015). Brain glycogen decreases during intense exercise without hypoglycemia: the possible involvement of serotonin. *Neurochem. Res.* 40, 1333–1340. doi: 10.1007/s11064-015-1594-1

Oe, Y., Baba, O., Ashida, H., Nakamura, K. C., and Hirase, H. (2016). Glycogen distribution in the microwave-fixed mouse brain reveals heterogeneous astrocytic patterns. *Glia* 64, 1532–1545. doi: 10.1002/glia.23020

Passonneau, J. V., Gatfield, P. D., Schulz, D. W., and Lowry, O. H. (1967). An enzymic method for measurement of glycogen. *Anal. Biochem.* 19, 315–326. doi: 10.1016/0003-2697(67)90167-4

Pellerin, L., and Magistretti, P. J. (1994). Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. *Proc. Natl. Acad. Sci. U S A* 91, 10625–10629. doi: 10.1073/pnas.91.22.10625

Pellerin, L., and Magistretti, P. J. (2003). Food for thought: challenging the dogmas. *J. Cereb. Blood Flow Metab.* 23, 1282–1286. doi: 10.1097/01.WCB.0000096064.12129.3d

Pellerin, L., and Magistretti, P. J. (2012). Sweet sixteen for ANLS. *J. Cereb. Blood Flow Metab.* 32, 1152–1166. doi: 10.1038/jcbfm.2011.149

Perge, J. A., Niven, J. E., Mugnaini, E., Balasubramanian, V., and Sterling, P. (2012). Why do axons differ in caliber? *J. Neurosci.* 32, 626–638. doi: 10.1523/JNEUROSCI.4254-11.2012

Pfeiffer-Guglielmi, B., Coles, J. A., Francke, M., Reichenbach, A., Fleckenstein, B., Jung, G., et al. (2006). Immunocytochemical analysis of rat vagus nerve by antibodies against glycogen phosphorylase isozymes. *Brain Res.* 1110, 23–29. doi: 10.1016/j.brainres.2006.06.080

Pfeiffer-Guglielmi, B., Francke, M., Reichenbach, A., and Hamprecht, B. (2007). Glycogen phosphorylase isozymes and energy metabolism in the rat peripheral nervous system—an immunocytochemical study. *Brain Res.* 1136, 20–27. doi: 10.1016/j.brainres.2006.12.037

Phelps, C. H. (1972). Barbiturate-induced glycogen accumulation in brain. An electron microscopic study. *Brain Res.* 39, 225–234. doi: 10.1016/0006-8993(72)90797-4

Powell, H. C., Haas, R., Hall, C. L., Wolff, J. A., Nyhan, W., and Brown, B. I. (1985). Peripheral nerve in type III glycogenosis: selective involvement of unmyelinated fiber Schwann cells. *Muscle Nerve* 8, 667–671. doi: 10.1002/mus.880080808

Ransom, B. R., and Fern, R. (1997). Does astrocytic glycogen benefit axon function and survival in CNS white matter during glucose deprivation? *Glia* 21, 134–141. doi: 10.1002/(SICI)1098-1136(199709)21:1<134::AID-GLIA15>3.3.CO;2-P

Ransom, C. B., Ransom, B. R., and Sontheimer, H. (2000). Activity-dependent extracellular K^+ accumulation in rat optic nerve: the role of glial and axonal Na^+ pumps. *J. Physiol.* 522, 427–442. doi: 10.1111/j.1469-7793.2000.00427.x

Ransom, B. R., Waxman, S. G., and Stys, P. K. (1994). "Anoxic injury of central myelinated axons: nonsynaptic ionic mechanisms," in *Cerebral Ischemia and Basic Mechanisms*, eds A. Hartman, F. Yatsu and W. Kuschinsky (Heidelberg: Springer-Verlag), 77–90.

Saez, I., Duran, J., Sinadinos, C., Beltran, A., Yanes, O., Tevy, M. F., et al. (2014). Neurons have an active glycogen metabolism that contributes to tolerance to hypoxia. *J. Cereb. Blood Flow Metab.* 34, 945–955. doi: 10.1038/jcbfm.2014.33

Shima, T., Jesmin, S., Matsui, T., Soya, M., and Soya, H. (2016). Differential effects of type 2 diabetes on brain glycometabolism in rats: focus on glycogen and monocarboxylate transporter 2. *J. Physiol. Sci.* doi: 10.1007/s12576-016-0508-6 [Epub ahead of print].

Shulman, R. G., Hyder, F., and Rothman, D. L. (2001). Cerebral energetics and the glycogen shunt: neurochemical basis of functional imaging. *Proc. Natl. Acad. Sci. U S A* 98, 6417–6422. doi: 10.1073/pnas.101129298

Sickmann, H. M., Schousboe, A., Fosgerau, K., and Waagepetersen, H. S. (2005). Compartmentation of lactate originating from glycogen and glucose in cultured astrocytes. *Neurochem. Res.* 30, 1295–1304. doi: 10.1007/s11064-005-8801-4

Sorg, O., and Magistretti, P. J. (1991). Characterization of the glycogenolysis elicited by vasoactive intestinal peptide, noradrenaline and adenosine in primary cultures of mouse cerebral cortical astrocytes. *Brain Res.* 563, 227–233. doi: 10.1016/0006-8993(91)91538-c

Sotelo-Hitschfeld, T., Niemeyer, M. I., Mächler, P., Ruminot, I., Lerchundi, R., Wyss, M. T., et al. (2015). Channel-mediated lactate release by K^+-stimulated astrocytes. *J. Neurosci.* 35, 4168–4178. doi: 10.1523/JNEUROSCI.5036-14.2015

Stryer, L. (1995). *Biochemistry.* New York, NY: W.H. Freeman and Co.

Stys, P. K., Ransom, B. R., and Waxman, S. G. (1991). Compound action potential of nerve recorded by suction electrode: a theoretical and experimental analysis. *Brain Res.* 546, 18–32. doi: 10.1016/0006-8993(91)91154-s

Suzuki, A., Stern, S. A., Bozdagi, O., Huntley, G. W., Walker, R. H., Magistretti, P. J., et al. (2011). Astrocyte-neuron lactate transport is required for long-term memory formation. *Cell* 144, 810–823. doi: 10.1016/j.cell.2011.02.018

Swanson, R. A., and Choi, D. W. (1993). Glial glycogen stores affect neuronal survival during glucose deprivation *in vitro*. *J. Cereb. Blood Flow Metab.* 13, 162–169. doi: 10.1097/00008506-199304000-00017

Taubenfeld, S. M., Milekic, M. H., Monti, B., and Alberini, C. M. (2001). The consolidation of new but not reactivated memory requires hippocampal C/EBPβ. *Nat. Neurosci.* 4, 813–818. doi: 10.1038/90520

Tekkok, S. B., Brown, A. M., Westenbroek, R., Pellerin, L., and Ransom, B. R. (2005). Transfer of glycogen-derived lactate from astrocytes to axons via specific monocarboxylate transporters supports mouse optic nerve activity. *J. Neurosci. Res.* 81, 644–652. doi: 10.1002/jnr.20573

Tsacopoulos, M., Veuthey, A. L., Saravelos, S. G., Perrottet, P., and Tsoupras, G. (1994). Glial cells transform glucose to alanine, which fuels the neurons in the honeybee retina. *J. Neurosci.* 14, 1339–1351.

Vilchez, D., Ros, S., Cifuentes, D., Pujadas, L., Vallès, J., García-Fojeda, B., et al. (2007). Mechanism suppressing glycogen synthesis in neurons and its demise in progressive myoclonus epilepsy. *Nat. Neurosci.* 10, 1407–1413. doi: 10.1038/nn1998

Wender, R., Brown, A. M., Fern, R., Swanson, R. A., Farrell, K., and Ransom, B. R. (2000). Astrocytic glycogen influences axon function and survival during glucose deprivation in central white matter. *J. Neurosci.* 20, 6804–6810.

Whatley, S. A., Hall, C., and Lim, L. (1981). Hypothalamic neurons in dissociated cell culture: the mechanism of increased survival times in the presence of non-neuronal cells. *J. Neurochem.* 36, 2052–2056. doi: 10.1111/j.1471-4159.1981.tb10833.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Molecular Neuroscience

REVIEW
published: 23 November 2016
doi: 10.3389/fnmol.2016.00124

RNA Editing—Systemic Relevance and Clue to Disease Mechanisms?

Jochen C. Meier[1], Svenja Kankowski[1], Heinz Krestel[2] and Florian Hetsch[1]*

[1] Cell Physiology, Technische Universität Braunschweig, Braunschweig, Germany, [2] Neurology, Universitätsspital und Universität Bern, Bern, Switzerland

Recent advances in sequencing technologies led to the identification of a plethora of different genes and several hundreds of amino acid recoding edited positions. Changes in editing rates of some of these positions were associated with diseases such as atherosclerosis, myopathy, epilepsy, major depression disorder, schizophrenia and other mental disorders as well as cancer and brain tumors. This review article summarizes our current knowledge on that front and presents glycine receptor C-to-U RNA editing as a first example of disease-associated increased RNA editing that includes assessment of disease mechanisms of the corresponding gene product in an animal model.

Keywords: RNA editing, epilepsy, cancer, mental disorders, glycine receptor, glutamate receptor, potassium channels, serotonin

INTRODUCTION

The conversion of genetic information from the DNA to the protein level includes the transcription of a given gene into RNA and its subsequent translation. Protein expression is tightly regulated at each step, and depends on the presence and activity of a bulk of proteins, which in most cases are themselves regulated by further proteins, hormones, metabolites or other modulators. Thus, any cell may generate nearly infinite expression profiles being able e.g., to differentiate in a tissue-dependent manner during development or to adapt to changes in environmental conditions. The variability of a genome is further increased by posttranscriptional modifications that change the genetic information during expression and lead to alternative variants of a given protein. Posttranscriptional modifications may occur at each step of gene expression and are carried out by evolutionarily conserved mechanisms. Commonly known posttranscriptional modifications are the splicing events in the nucleus, RNA editing and the specific insertion of a selenocysteine instead of a cysteine during translation. This review article focuses on RNA editing of nuclear transcripts in higher eukaryotes, its mechanisms, systemic relevance and association with development and disease.

RNA EDITING—ENZYMES AND MECHANISMS

RNA editing was described for the first time in *Trypanosoma* where the mRNA of the mitochondrial gene coding for cox-2 was found to differ from its expected sequence in terms of four missing uridines at the 5′ end in comparison to the respective DNA sequence (Benne et al., 1986). Subsequently to this intriguing discovery, it became clear that this "RNA editing" event occurs much more frequently, and that about 60% of the genomic information in mitochondria of *Trypanosoma* is edited by insertion or deletion of uridines. Nuclear mRNA of higher eukaryotes was also found to be edited in a reproducible fashion. However, unlike *Trypanosoma*, editing of nuclear mRNA in higher eukaryotes is rather a consequence of amination or

OPEN ACCESS

Edited by:
Nicola Maggio,
The Chaim Sheba Medical Center, Israel

Reviewed by:
Luis Gerardo Aguayo,
University of Concepción, Chile
Maximilian Lenz,
University of Düsseldorf, Germany

***Correspondence:**
Jochen C. Meier
jochen.meier@tu-braunschweig.de

Received: 11 September 2016
Accepted: 04 November 2016
Published: 23 November 2016

Citation:
Meier JC, Kankowski S, Krestel H and Hetsch F (2016) RNA Editing—Systemic Relevance and Clue to Disease Mechanisms? Front. Mol. Neurosci. 9:124. doi: 10.3389/fnmol.2016.00124

deamination of purines and pyrimidines than a consequence of nucleotide insertion or deletion. The prevalent type of RNA editing in higher eukaryotes results from two types of hydrolytic enzymatic deamination reactions. As discussed below in more detail, deamination of cytidine leads to the conversion to uridine (C-to-U), which is dependent on the APOBEC ("apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like") gene family and auxiliary proteins. Enzymatic deamination of adenosine yields inosine (A-to-I) and is dependent on the ADAR ("adenosine deaminase acting on RNA") enzyme gene family. U-to-C and G-to-A RNA editing may also occur due to amination of the respective bases (Sharma et al., 1994; Grohmann et al., 2010; Niavarani et al., 2015; Knie et al., 2016).

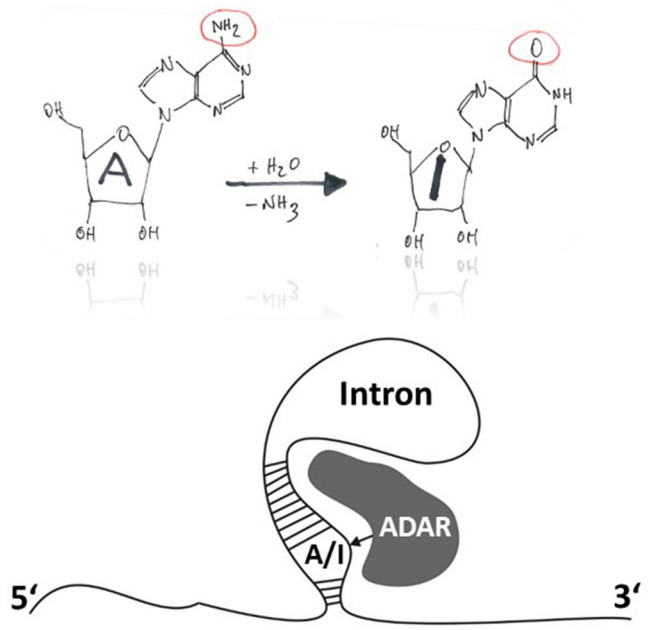
THE ADAR GENE FAMILY

Members of the ADAR family catalyze the deamination of adenosine to inosine except in protozoa, yeast and plants (Jin et al., 2009). Inosin is interpreted as guanosine (G) during mRNA translation. ADAR principle function and sites of action are shown in **Figure 1**. ADAR enzymes are highly conserved among invertebrates and vertebrates. Three different *ADAR* genes (*ADAR1–3*) were identified in the mammalian genome with *ADAR1* being the first one (O'Connell and Keller, 1994; Kim et al., 1994a). During evolution, the ADAR family putatively arose from adenosine deaminases acting on tRNA (ADAT), which are conserved from yeast to man and also have a bacterial ortholog (TadA). Interestingly, the evolutionary predecessors of adenosine deaminases (ADAT and ADAR) are cytidine and not adenosine deaminases acting on mononucleotides, classifying ADARs, like APOBECs, into the cytidine deaminase family. This viewpoint is corroborated by comparison of the X-ray structures of the ADAR and APOBEC catalytic domains (for mechanisms of APOBEC-dependent RNA editing see below). The deamination motif of human ADAR2 bears two α-helices (α2 and α5) and four ß-strands that form a structure similar to the core of cytidine deaminase catalytic domains (Macbeth et al., 2005). Another similarity in the structural implications of APOBEC and ADAR is Zn^{2+} complexation at the active site of ADAR that is mediated by the histidine and glutamate residues of an HAE motif (aa 394–396 in human ADAR2) and the more distally located cysteine residues C451 and C516 (human ADAR2). Unlike APOBEC, members of the ADAR family are able to catalyze deamination without the aid of additional auxiliary proteins (Nishikura, 2010). This independent mode of action is perhaps intrinsic to several RNA binding domains located in the N-terminal part of the ADAR proteins. Thus, investigation of deamination mechanisms and the search for *in vivo* substrates may be more straightforward than in the case of the APOBEC enzymes. Catalytic activity has only been shown for the ubiquitously expressed ADAR1 and ADAR2 (Kim et al., 1994b; Higuchi et al., 2000) but not for brain specific ADAR3 which may be a regulatory component of the A-to-I editing machinery as it binds single and double stranded RNA and was shown to inhibit *in vitro* the activities of RNA editing enzymes of the ADAR gene family (Chen et al., 2000; Nishikura, 2010). Though most of the A-to-I RNA editing sites were attributed to non-coding genome regions such as *Alu* repeats or L1 LINE (Neeman et al., 2006), an increasing number of A-to-I RNA editing sites in protein coding regions is identified using advanced sequencing techniques (Levanon et al., 2005; Li et al., 2009; see http://www.rnaedit.com). Furthermore, there are some important indications that ADAR-mediated RNA editing modulates the efficiency of RNAi pathways including the generation of microRNAs (miRNAs) and the processing of small interfering RNAs (siRNAs) (Nishikura, 2006). However, 17 different amino acid recoding events can occur due to A-to-I changes within codons (**Figure 1**).

RNA and DNA binding of ADAR1 have been attributed to Z-DNA binding domains, which might confer target site specificity of editing of viral RNA templates (Brown et al., 2000) and, thus, play a virus selective role in the host response to infection (George and Samuel, 2011). When ADAR1 or ADAR2 is incubated with double-stranded RNA (dsRNA; >20 bp), about 50% of the adenosine residues will be edited in a promiscuous fashion (Bass and Weintraub, 1987; Nishikura et al., 1991). However, dsRNA molecules with imperfectly matched base pairs lead to a more selective action of ADAR, indicating that the secondary structure of the RNA substrate is crucial for site specificity of an ADAR-mediated editing event (**Figure 1**). Thus, cellular editing by ADAR occurs preferentially in double stranded RNA with mismatches, bulges and internal loops, suggesting that target site selectivity is based on conformation rather than on a particular sequence (Bass, 1997). The type of mismatch greatly influences A-to-I editing efficiency, with A:C being preferred over A:A, A:G or A:U (Wong et al., 2001). Nonetheless, in some cases A-to-I RNA editing does not occur although the target position seems to be in the right context (Lehmann and Bass, 2000). Actually, epigenetic regulation can contribute to ADAR-dependent A-to-I RNA editing, as was recently revealed by investigation of the cellular epitranscriptome and identification of methyl-6-adenosine RNA modification (Saletore et al., 2013), which blocks ADAR-dependent RNA editing (Véliz et al., 2003) and may be a ground-breaking discovery for personalized medical care (see below for a discussion of A-to-I RNA editing-dependent mechanisms in disease). Another possibly promising pharmaceutical approach may consist in targeting RNA splicing. In fact, ADAR2 activity can lead to creation of new RNA splice sites in ADAR2-coding mRNA and lead to alternative ADAR splice variants with altered RNA editing capacity (Rueter et al., 1999).

ADAR-DEPENDENT RNA EDITING IN DEVELOPMENT

Already 30 years ago, it was postulated that A-to-I RNA editing may play an important role in development (Bass and Weintraub, 1987). Indeed, recently, down-regulation of α3-GABA(A)R expression was shown to result from A-to-I RNA editing by ADAR1 or ADAR2 at the I/M site (AUA-to-AUG coding for isoleucine [I] and methionine [M], respectively,



Arg	> Gly	(GluA2)
Asn	> Asp	(HT2C-R)
Asn	> Ser	(HT2C-R)
Asp	> Gly	(HT2C-R)
Glu	> Gly	
Glu	> Arg	
Gln	> Arg	(GluA2)
His	> Arg	
Ile	> Met	(HT2C-R, $GABA_AR$ α3)
Ile	> Val	(Kv1.1, HT2C-R)
Lys	> Arg	
Lys	> Glu	
Met	> Val	
Ser	> Gly	
Thr	> Ala	
Tyr	> Cys	
X	> Trp	

FIGURE 1 | Principles of A-to-I RNA editing. The hand-drawing top left provides a schematic illustration of the deamination of adenosine to inosine (A-to-I). In contrast to APOBEC-dependent C-to-U RNA editing, ADARs do not need auxiliary proteins for deamination but rely on secondary mRNA structure motifs within introns (bottom-left). A-to-I RNA editing can lead to indicated (right-hand) amino acid recoding events. Selected edited products and their possible involvement in disease are discussed in this review article.

Figure 1), which impairs GABA(A)R α3 surface expression, suggesting that by trafficking control of α3-containing receptors RNA editing may facilitate the switch of subunit compositions during development and affect the subcellular distribution of α subunits in the adult brain (Ohlson et al., 2007; Daniel et al., 2011). Switching subunit compositions may accelerate GABAergic synaptic response kinetics and enhance spike-timing precision at more mature developmental states, as GABA(A)R α3 expression is developmentally downregulated and GABA(A)R α1-coding mRNA cannot be edited at the I/M position (because AUU encodes isoleucine in mouse, rat and human; Lavoie et al., 1997; Jüttner et al., 2001).

Due to the advances in sequencing techniques a recent study could survey changes in the global landscape of A-to-I RNA editing in human brain tissues and revealed many more gene products that undergo developmental and disease-specific changes in gene expression. Thereby a spatiotemporal atlas of RNA editing was created that revealed a dynamic profile of RNA editing (Hwang et al., 2016). This pioneering study revealed three patterns of uniquely regulated RNA editing sites during cortical development from fetal to old age comprising stably high, stably low, and increasing editing at given sites. The increasing pattern of A-to-I RNA editing included sites in vesicle or organelle membrane-related genes and glutamate signaling pathways. In two selected disorders, namely spinal cord injury and glioblastoma, perturbed A-to-I RNA editing could be demonstrated, as discussed below in more detail.

ADAR-DEPENDENT RNA EDITING IN DISEASE

The pioneer studies by Peter H. Seeburg revealed a critical position that, amongst others, determines the calcium permeability of the AMPA-type ionotropic glutamate receptor channel and is situated in the hairpin loop between transmembrane domains 1 and 3 (Seeburg et al., 2001). In this case, A-to-I RNA editing at the Q/R site (CAG-to-CGG) of the AMPA receptor subunit GluA2 occurs at a rate of virtually 100% (**Figure 1**). Genetically engineered mice that carry an editing resistant Q/R site in *Gria2*—with 70% GluA2 mRNA expression from wild-type and 75% editing vs. 100% in wild-type—develop severe epilepsy with generalized seizures and premature postnatal lethality (Brusa et al., 1995). A conditional mouse model with deficient GluA2 Q/R editing only in forebrain had its seizure origin in the hippocampus (Krestel et al., 2004). To test the hypothesis whether editing deficiency at the GluA2 Q/R site contributes to human mesial temporal lobe epilepsy (TLE), hippocampi from patients who had undergone surgery for pharmacotherapy-resistant epilepsy were analyzed. It turned out that: (i) the GluA2 Q/R site editing belongs to the stably high editing pattern with 100% editing in all patients analyzed aged from 5 years to 57 years at the time of surgery; (ii) epilepsy could not perturb this stably high pattern; and (iii) epilepsy in mice due to deficient GluA2 Q/R site editing probably was an artifact of genetic engineering (Krestel et al., 2013).

In patients with amyotrophic lateral sclerosis, decreased RNA editing at the Q/R site was found to occur in motor neurons (Kwak and Kawahara, 2005; **Figure 2**). In fact, ADAR2 gene targeting in motor neurons provokes a decline in motor function and the phenotype can be reverted if the mice express RNA edited GluA2 (Higuchi et al., 2000; Hideyama et al., 2010). Reduced A-to-I RNA editing at the Q/R site of GluA2 was furthermore shown to be associated with glioblastoma (Maas et al., 2001; **Figure 2**). However, in spinal cord injury and glioblastoma, the developmentally increasing pattern of A-to-I RNA editing of many gene transcripts seems to be disrupted (Hwang et al., 2016), raising the question about underlying mechanisms of these diseases (Fu et al., 2016), including investigation of "master" genes that regulate function of down-stream genes and thus govern signaling cascades involved in disease. Regarding GluA2 under-editing, which was more pronounced in glioblastoma compared to neighboring non-tumor tissue (Hwang et al., 2016), and taking into account that brain tumor cells can release glutamate by transferrin-mediated iron accumulation (Chirasani et al., 2009), reduced RNA editing and increased calcium signaling through GluA2 may contribute to aggressiveness of tumor growth and expansion. Regarding spinal cord injury in a mouse model (Chen et al., 2013), decreased RNA editing of GluA2 in the epicenter of the injured site actually points to a disease-promoting mechanism, as cellular calcium overload should promote neurodegeneration.

Moreover, changes in ADAR-mediated A-to-I RNA editing were associated with several other types of cancer, including breast cancer, neuroblastoma and hepatocellular carcinoma; for a review see Fu et al. (2016), suggesting that some common master gene products may be involved in the pathogenesis of these diverse diseases. Finally, in human postmortem brains of patients with schizophrenia and bipolar disorder ADAR2 expression tended to be decreased, and decreased ADAR2 expression was significantly correlated with decreased editing of the R/G sites of AMPA receptors (Kubota-Sakashita et al., 2014; **Figures 1**, **2**). All these examples point to a crucial role of AMPA-type glutamate receptor RNA editing in many disease conditions, but the underlying mechanisms still remain obscured and need to be addressed. Although RNA splicing of ADAR2 may provide correlative mechanistic insights into the interrelation between enzyme function and RNA editing (Fu et al., 2016), identification of a general downstream mechanism of disease manifestation, e.g., changes in calcium signaling, would eventually reveal promising strategies to tackle these diseases early in their pathomechanisms.

Serotonergic neurotransmission was shown to be influenced by RNA editing of serotonin receptors (5-HT2C-R; Gurevich et al., 2002a; **Figures 1**, **2**). A-to-I RNA editing of 5-HT2C-R at up to five positions decreases the apparent receptor affinity to serotonin, with codon 158 (AAU-to-AGU [C site] or –GAU [E site] or –GGU [C and E sites]) in the intracellular loop between transmembrane domains 3 and 4 producing the strongest effects (up to a 20-fold decrease; Fitzgerald et al., 1999). Editing of 5-HT2C-R thus seems to be a versatile tool for neurons to dynamically adjust receptor response properties to alterations in serotonin levels. This is an economic way of regulation as

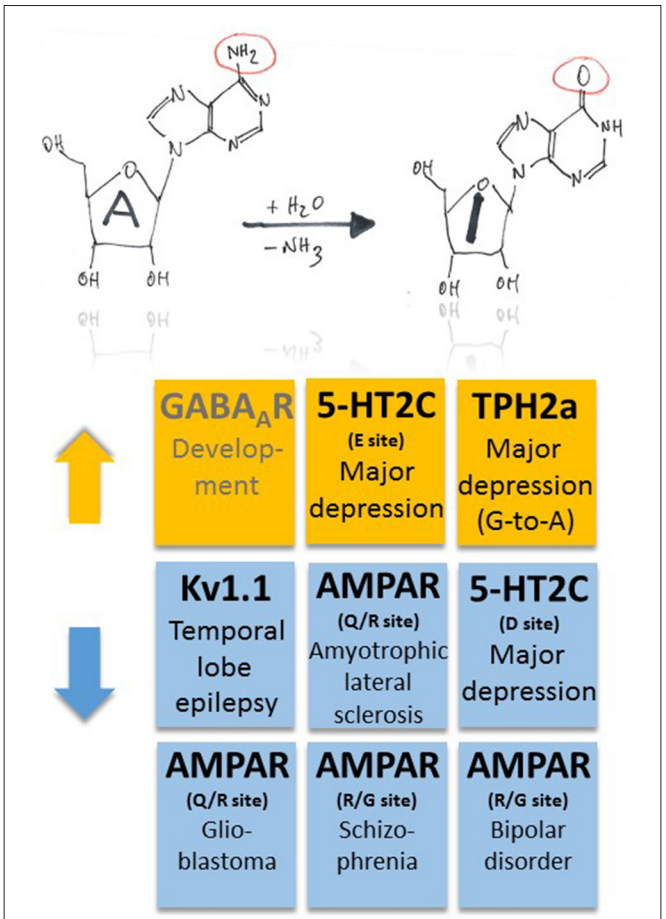


FIGURE 2 | Pathophysiological implications of altered ADAR-dependent RNA editing in disease. Both up- and down-regulation of A-to-I RNA editing (indicated by orange and blue colors, respectively) were associated with several, different diseases. Note that up-regulation of GABA$_A$R α3 RNA editing makes an exception as it plays a role in development (indicated by gray text color).

it bypasses the need for supplementary genes (or exons that could be alternatively spliced) and associated regulation of gene expression. However, the mechanism may also fail as a complex pattern of altered RNA editing in depressed suicide victims was suggested to exacerbate the effects of low serotonin (Gurevich et al., 2002b; **Figure 2**). Furthermore, G-to-A RNA editing occurs in transcripts coding for tryptophan hydroxylase 2 (TPH2a) that controls brain serotonin synthesis (Grohmann et al., 2010). In this case, G-to-A editing leads to amino acid substitution R441H that decreases TPH2a enzyme activity and, thus, may also contribute to major depression disorder (Grohmann et al., 2010; **Figure 2**). In fact, coincident changes in RNA editing of both 5-HT2C-R and TPH2 may result in a cumulative decrease in 5-HT signaling, constituting a worst-case scenario for the patients.

Potassium channels also undergo A-to-I RNA editing which substitutes valine for isoleucine at position 400 (I400V) within the transmembrane domain 6 of human Kv1.1 (Bhalla et al., 2004). The resulting accelerated recovery from inactivation and

increase in K^+ outward current upon membrane depolarization (Bhalla et al., 2004) can stabilize repolarization through Kv1.1 potassium channels and thus control neuronal excitability. The Kv1.1 I/V site belongs to the "increasing pattern" observed in human brain development (Hwang et al., 2016), as mentioned above. On the other side, a decrease of RNA editing may translate into destabilized repolarization and may contribute to the maintenance of neuronal hyperexcitability. Remarkable in this context is the observation that the RNA editing rate decreases with epilepsy duration in the removed hippocampus of patients who underwent surgery for intractable mesial TLE with hippocampal sclerosis but not with other clinical parameters (**Figure 2**). A specific association of the decrease with either the epileptic process itself or its antiepileptic medication history was suggested (Krestel et al., 2013).

THE APOBEC GENE FAMILY

The successful cloning of *APOBEC-1*, the first identified member of the *APOBEC* gene family of cytidine deaminases (Navaratnam et al., 1993; Teng et al., 1993), signified an important hallmark in research of the molecular components of the mammalian C-to-U editosome. APOBEC is a family of evolutionarily conserved proteins. As of today, 10 additional cytidine deaminases homologous to APOBEC-1 and thus contributing to the *APOBEC* gene family were identified (APOBEC-1, -2, -3A, -3B, -3C, -3D, 3E, -3F, -3G, -3H, -4; activation-induced cytidine deaminase (AID); Bransteitter et al., 2009). While members of the APOBEC enzyme family are responsible for editing of pre-mRNA, single stranded DNA (ssDNA) and genomic DNA, AID seems to mostly edit ssDNA and contribute to the adaptive immune response by introducing dC-to-dU mutations into the VDJ region of the immunoglobulin gene (Conticello, 2008). APOBEC-1 is the best characterized member of the *APOBEC* gene family. Thanks to the pioneer work of Harold C. Smith, the role of APOBEC-1 and auxiliary proteins in pre-mRNA editing of apolipoprotein B (ApoB) was elucidated in great detail (Backus and Smith, 1992; Schock et al., 1996; Dance et al., 2002; Smith, 2007). The role of APOBEC-2 was discovered more recently. APOBEC-2 was shown to be involved in C-to-U RNA editing of eukaryotic translation initiation factor 4 gamma 2 as well as phosphatase and tensin homolog (PTEN) genes and to be associated with tumorigenesis (Okuyama et al., 2012). In addition, APOBEC-2 plays a crucial role in muscle development (Sato et al., 2010) and in the TGFß-mediated manifestation of internal organ left-right asymmetry during development (Vonica et al., 2011). APOBEC-3A-H can inhibit the propagation of HIV and human papillomavirus (HPV) by editing the viral double-stranded cDNA intermediates that serve as template for the expression of viral proteins (Conticello, 2008; McDougall et al., 2011). APOBEC-3B may preferentially edit genomic DNA and is implicated in cancer as its expression correlates with increased DNA damage and thus represents an enzymatic source of mutation in breast cancer (Burns et al., 2013). APOBEC-3G edits ssDNA under certain conditions (McDougall et al., 2011). By deaminating C to U and thus inserting mutations, APOBEC-3G might protect the mammalian genome against the spread of retroviruses. Supportive evidence comes from the upregulated expression of APOBEC-3G in inflammatory skin disorders such as *Lichen planus*. This disease is believed to be associated with activation of quiescent human endogenous retroviruses, and upregulation of APOBEC-3G is perceived as an endogenous defense mechanism (Nogueira et al., 2015). APOBEC-4 was found by *in silico* methods (Rogozin et al., 2005) and appears to influence HIV-1 expression (Marino et al., 2016).

A common feature of all members of the APOBEC family is an N-terminal catalytic domain comprising the sequence $HXEX_{27/28}PCXXC$ (Jarmuz et al., 2002) that coordinates a Zn^{2+} ion within the active center of the enzymes. The tertiary structure of APOBEC catalytic domains, derived from X-ray and NMR studies on APOBEC-2 (Prochnow et al., 2007) and APOBEC-3G (CD2, a C-terminal additional catalytic domain only present in some APOBEC isoforms; Chen et al., 2008; Holden et al., 2008), is composed of a five ß-strands containing ß-sheet that is surrounded by six α-helices. In the case of full-length APOBEC-2, homo-tetramerization has been shown to prevent the active site of the catalytic domains to be accessible to nucleic acids (Prochnow et al., 2007), counteracting the catalytic activity of the enzyme. This self-inhibitory action of APOBEC-2 might explain why demonstration of *in vivo* deamination activity of this isoform is rather challenging. However, the C-to-U RNA editing mediated by APOBEC-1 depends on many auxiliary proteins (Blanc and Davidson, 2010; **Table 1** and **Figure 3**). Although APOBEC-1 itself can accommodate RNA substrates involving zinc-finger domains and, additionally, two critical phenylalanines (Anant et al., 1995; Navaratnam et al., 1995), APOBEC-1 complementation factor (ACF) is essential for editing activity (Schock et al., 1996; Mehta et al., 2000). ACF contains RNA recognition motifs (RRM) that direct APOBEC-1 to the target sites (Lellek et al., 2000; Mehta et al., 2000; Henderson et al., 2001). These RRM domains bind to the mooring sequence UGAUCAGUAUA located downstream of the edited position and thus confer target specificity (Smith, 2007), while the C-terminal auxiliary domain of ACF is required for interaction with APOBEC-1 (Mehta and Driscoll, 2002). Furthermore, the RNA-binding protein CUGBP2 is part of the APOBEC-1 holoenzyme and specifically targets AU-rich elements immediately upstream of the edited position (Anant et al., 2001). Many other factors have also been shown to modulate ApoB RNA editing, although in these cases the mechanisms are less well understood (**Table 1**). *APOBEC-1* knockout mice do not edit ApoB mRNA (Hirano et al., 1996; Nakamuta et al., 1996), even though these mice express other members of the *APOBEC* gene family, indicating the intriguing specificity of this editing process. APOBEC-1 and ACF are present in both cytoplasm and nucleus but within the progress of protein expression, ApoB mRNA editing appears to be not cytosolic but intra-nuclear and post-transcriptional (Lau et al., 1991; Blanc and Davidson, 2010). A detailed review of the

TABLE 1 | APOBEC-1 auxiliary proteins.

Name	Function	Effect on C-to-U RNA editing	Reference
ACF	RNA binding (mooring sequence UGAUCAGU-AUA) and interaction with APOBEC-1	↑	Mehta and Driscoll (2002) Sowden et al. (2004)
CUGBP2	RNA binding (AU-rich elements) and ACF interaction	↑	Anant et al. (2001)
GRY-RBP	RNA binding and interaction with ACF and APOBEC-1	↓	Blanc et al. (2001)
hnRNP C1	RNA binding and interaction with APOBEC-1	↓	Greeve et al. (1998)
ABBP-2	Interaction with APOBEC-1	↑	Lau et al. (2001)
BAG-4	Interaction with APOBEC-1 and re-routing to the perinucleolar compartment	↓	Lau and Chan (2003)
AUX240	Editosome assembly	↑	Schock et al. (1996)

current knowledge about ApoB mRNA editing by APOBEC-1 was recently published by Harold C. Smith (Prohaska et al., 2014).

C-to-U RNA editing can lead to 13 different amino acid recoding events, with two of them leading to a STOP codon, as shown in **Figure 3**. In the following section, examples of amino acid recoding C-to-U RNA editing associated with disease will be presented.

APOBEC-DEPENDENT RNA EDITING IN DISEASE

The mRNA coding for ApoB is C-to-U RNA edited (Boström et al., 1990). By regulating low and high density lipoprotein metabolism, RNA editing and the resulting biosynthesis of truncated ApoB48 protein is a critical regulator of plasma cholesterol content (Nakamuta et al., 1996), and dysregulation of APOBEC-1-dependent ApoB48 expression results in hypercholesterolemia and atherosclerosis (Fu et al., 2004; **Figure 4**).

As mentioned above, G-to-A editing leads to amino acid substitution R441H that decreases TPH2a enzyme activity (Grohmann et al., 2010). However, the alternatively spliced TPH2b enzyme can be C-to-U RNA-edited within the splice insert exon 3b, leading to c.385C>T and a truncated protein variant due to Q129X substitution (Grohmann et al., 2010). Editing at this position of TPH2b transcripts of suicides and patients with schizophrenia decreased substantially by 50% and 30%, respectively (Grohmann et al., 2010; **Figure 4**).

Changes in C-to-U RNA editing were also associated with several other diseases. In particular, APOBEC-2 deficient mice showed a markedly increased ratio of slow to fast fibers in soleus muscle and exhibited a reduction in body mass from birth onwards, with elderly mutant animals revealing clear histological evidence of a mild myopathy (Sato et al., 2010; **Figure 4**). Increased expression of APOBEC-2 in the liver resulted in

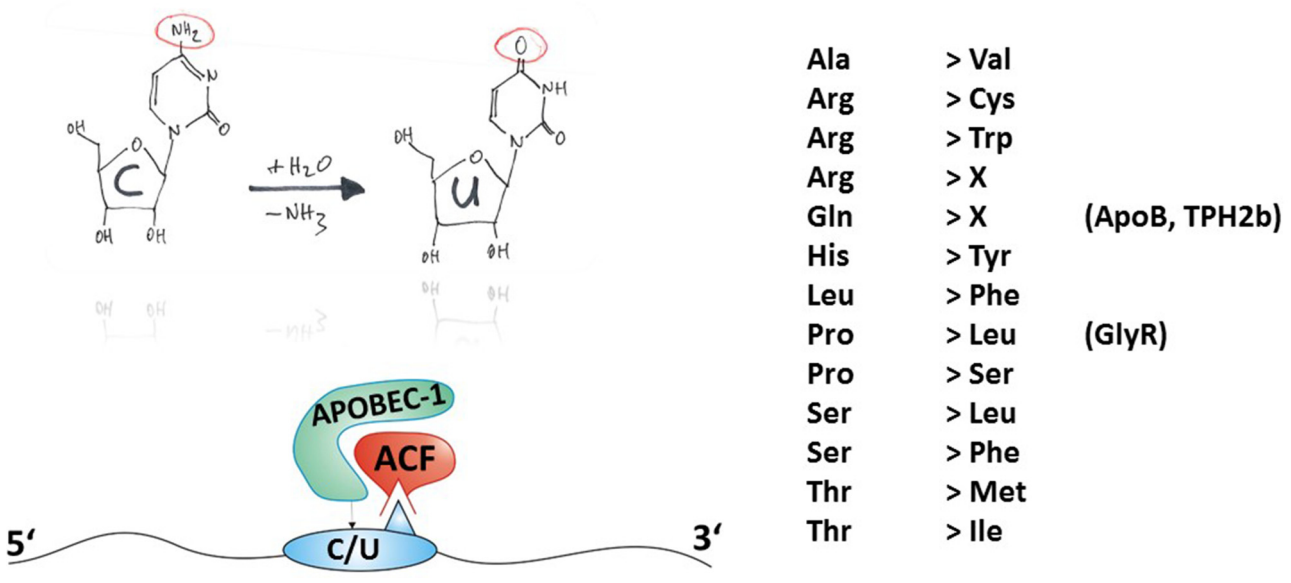


FIGURE 3 | Principles of C-to-U RNA editing. The hand-drawing top left provides a schematic illustration of the deamination of cytidine to uridine (C-to-U). In contrast to ADAR-dependent A-to-I RNA editing, APOBEC needs auxiliary proteins for deamination as detailed in **Table 1** and exemplified in the bottom-left panel. Thus, the mature mRNA can be RNA-edited. C-to-U RNA editing can lead to indicated (right-hand) amino acid recoding events. Some selected gene products associated with disease (in brackets) and their possible involvement in disease are discussed in this review article.

significantly high frequencies of nucleotide alterations in the transcripts of eukaryotic translation initiation factor 4 gamma 2 as well as PTEN and was accompanied by hepatocellular carcinoma in 10% of 72 weeks old animals as well as lung tumors in 35% of transgenic mice analyzed (Okuyama et al., 2012; **Figure 4**).

APOBEC-3B seems to be an enzymatic source of mutation in breast cancer (Burns et al., 2013). In particular, the DNA cytosine deaminase activity of APOBEC-3B was shown to be up-regulated in primary breast tumors and breast cancer cell lines (**Figure 4**), suggesting that APOBEC3B-catalyzed deamination provides a chronic source of DNA damage in breast cancers that could select TP53 inactivation and explain how some tumors evolve rapidly and manifest heterogeneity. (Burns et al., 2013). Surprisingly, in the case of *Wilms Tumor 1*, APOBEC-3A was associated with a novel form of G-to-A editing, perhaps opening a way to further investigations into the mechanisms of other potential mRNA changes and helping us to redefine the RNA editing paradigm in both health and disease (Niavarani et al., 2015).

Although APOBEC-4 did not show any deamination activity, it was shown to enhance the replication of HIV-1, suggesting a natural role in modulating host promoters or endogenous long terminal repeat (LTR) promoters rather than being a cytosine deaminase (Marino et al., 2016).

Again, the description of global changes in C-to-U RNA editing due to changes in APOBEC-2, APOBEC-3 or APOBEC-4 function is not sufficient and can just be a starting point for the investigation of the critical targets that govern disease progression in the various kinds of diseases described here.

Like GABA type A receptors the neurotransmitter receptors for glycine (GlyRs) belong to the ligand-gated ion channel gene superfamily and are glycine-gated chloride channels which were involved in TLE, inflammatory pain sensitization, autism spectrum disorder and glioblastoma (Harvey et al., 2004; Eichler et al., 2008; Förstera et al., 2014; Pilorge et al., 2016). GlyRs are C-to-U RNA edited although the mooring sequence recognized by ACF is not very well conserved (Meier et al., 2005). In this case, C-to-U RNA editing leads to a gain-of-function as the resulting amino acid substitutions P185L (in GlyR α1 and α3 subunits) and P192L (in GlyR α2) increase apparent agonist affinities of the neurotransmitter receptors (see Eichler et al., 2008; Legendre et al., 2009; and **Figures 5A,B**). Actually, C-to-U RNA editing of GlyRs was shown to be increased in the hippocampus of patients with pharmacoresistant TLE (Eichler et al., 2008; **Figures 4**, **5**), suggesting that it plays a critical role in this disease. To test this hypothesis, a corresponding animal model was generated and allowed investigation of neuron type-specific mechanisms of RNA-edited GlyR action (Winkelmann et al., 2014). In sharp contrast to the still prevailing dogma that GlyRs are exclusively located at the postsynaptic site of synaptic signaling (Tyagarajan and Fritschy, 2014), we found that RNA-edited GlyRs are expressed at the presynaptic terminals of hippocampal neurons (Winkelmann et al., 2014). This is due to RNA splicing of the GlyR α3 subunit (Nikolic et al., 1998; Eichler et al., 2009) and substantiated

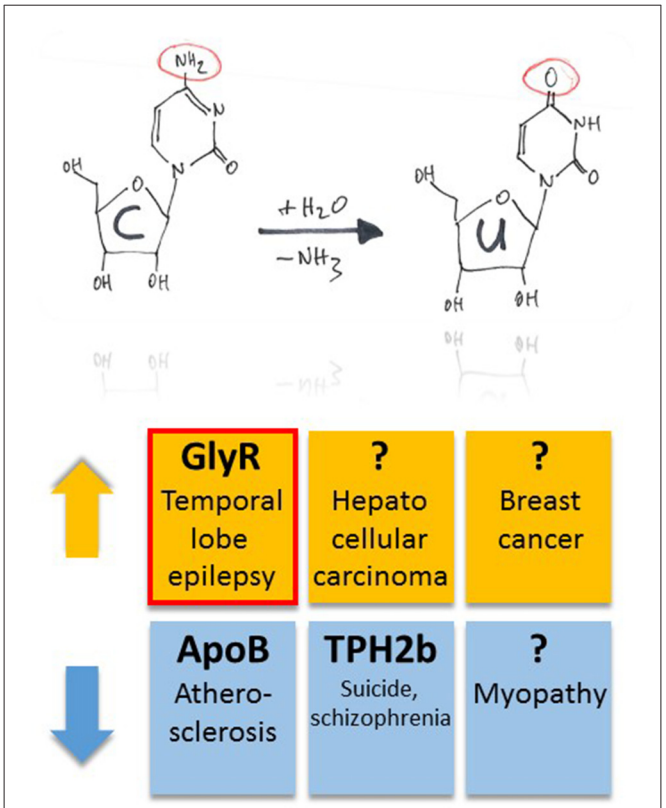


FIGURE 4 | Pathophysiological implications of altered APOBEC-dependent RNA editing in disease. Both up- and down-regulation of C-to-U RNA editing (indicated by orange and blue colors, respectively) were associated with several, different diseases. However, compared to diseases that have been associated with altered A-to-I RNA editing, this list is rather short. In particular, in half of the cases, the question marks indicate global changes in C-to-U RNA editing due to changes in enzyme expression without identification of specific gene products that were associated with up- or down-regulation of the APOBEC enzymes in animal models or tissue samples of patients. The red framed example is discussed in more detail in this review article.

for other GlyR subunits due to the absence of GlyR β protein in the hippocampus (Weltzien et al., 2012). Actually, other researchers also highlight the importance of presynaptic GlyRs (for examples see Lee et al., 2009; Kubota et al., 2010; Waseem and Fedorovich, 2010). However, even if GlyRs are expressed at both presynaptic and postsynaptic sites, a small number of presynaptic RNA-edited gain-of-function GlyRs even a single cluster of the non-RNA-edited GlyR α3L splice variant, which contains up to 200 receptor channels (Notelaers et al., 2012), will have a greater impact on the presynaptic membrane potential due to the much smaller volume and hence electrical capacity of this compartment compared to the somatodendritic compartment (Meier et al., 2014). Notably in this context, application of a low glycine concentration (10 μM) to hippocampal slice preparations enhanced the occurrence of epileptiform activity whereas a high glycine concentration (100 μM) attenuated recurrent epileptiform discharge (Chen et al., 2014). These divergent effects can

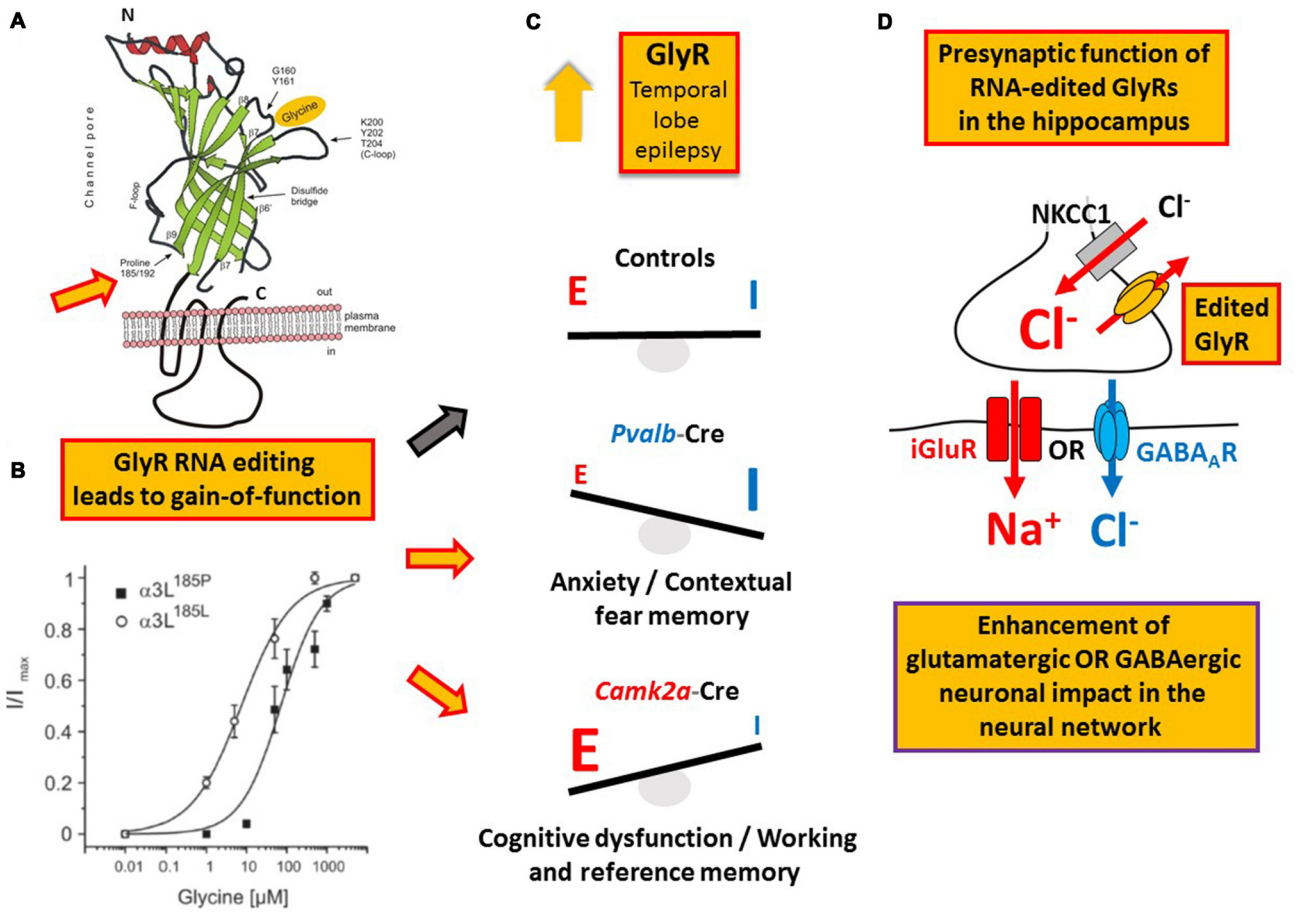


FIGURE 5 | Example of a RNA editing-dependent bedside-to-bench approach. (A) GlyR C-to-U RNA editing leads to amino acid recoding in the ligand binding domain (P185L or P192L, indicated by the orange filled red framed arrow); modified from Legendre et al. (2009). **(B)** The amino acid recoding increases apparent GlyR affinity to glycine irrespectively of the subunit composition as discussed in the main text. Panel **(B)** is reproduced from Legendre et al. (2009) and shown here again for the purpose of clarity. **(C)** Increased RNA editing of GlyR-coding mRNA in patients with temporal lobe epilepsy (TLE) as documented in 2008 by Eichler and colleagues. In a corresponding mouse model of TLE RNA-edited GlyRs elicited different neuropsychiatric symptoms depending on the neuron type that expressed these receptor variants, compared to control animals without expression of RNA-edited GlyR and indicated by red framed yellow arrows and the black framed gray arrow, respectively, placed between **(B,C)**. **(D)** It is the presynaptic function of RNA-edited GlyRs that is responsible for increased neuronal impact on network function and the neuron type-specific neuropsychiatric symptoms described in the main text and shown in panel **(C)**.

be explained by preponderant functional impact of low glycine on presynaptic GlyRs expressed at glutamatergic terminals, resulting in facilitated glutamate release, and massive recruitment of somatodendritic GlyR activation by 100 μM glycine, resulting in tonic inhibition, respectively. Based on current evidence we believe that the presynaptic compartment is particularly vulnerable to maladaptive changes in neurotransmitter receptor signaling in disease (for a review see Meier et al., 2014). In the context of the rather low neuronal ambient glycine concentration in the hippocampus, presynaptic RNA-edited GlyRs were indeed shown to facilitate neurotransmitter release and contribute to gain-of-function of the affected neuron types, which elicited neuropsychiatric symptoms like cognitive dysfunction or persistence of contextual fear memory in our animal model. The different symptoms depended on the neuron type that expressed the RNA-edited GlyR variant, namely glutamatergic principle neurons (Camk2a-Cre) and parvalbumin-positive neurons (Pvalb-Cre), respectively (Winkelmann et al., 2014; Çalışkan et al., 2016; **Figures 5C,D**). As these symptoms of the genetically targeted mice are reminiscent of the disease symptomatology of TLE patients, we are currently investigating whether novel molecular and chemical tools (antagonists of RNA-edited GlyRs) are able to identify the neuron types with increased GlyR RNA editing in the hippocampus of patients with TLE and to counterbalance GlyR-dependent changes in neural network excitability. However, and most importantly, these studies demonstrate that it is not sufficient to study changes in RNA editing using bulk material because the same RNA-edited gene product (i.e., GlyR) can elicit completely different symptoms, depending on the neuron type that expresses the pathogenic RNA-edited protein variant.

CONCLUSION AND PERSPECTIVE

RNA editing is an evolutionarily conserved process that has several advantages over gene mutation. Like alternative RNA splicing, the extent of RNA editing may be regulated, resulting in increased genomic variance. Furthermore, mRNA variability can be regulated whereas gene mutations are permanent. It seems as if there is reasonable evidence supporting altered RNA processing in a wide range of disease, including paroxysmal and neoplastic disorders of the brain, neuromuscular disease, as well as lung and liver disease and breast cancer. The examples discussed in this review article actually point to a critical role of deviation from normal RNA processing/editing in disease ontology and/or the epitranscriptomics of disease. However, there are several hundreds more amino acid recoding RNA editing events in many different gene products including gene regulatory transcripts (see http://www.rnaedit.com) that require investigation at the functional level; some of which may turn out to be the master regulatory targets of the editing machinery and disease-promoting cellular programs. Thus, we are just at begining of a new era of research that will ultimately need to identify functional changes of gene products due to amino acid recoding RNA editing and identify molecular key players that are masters and hence could signal upstream of, and regulate RNA processing machines. Furthermore, using animal models, in depth characterization of the functional consequences at both molecular and cellular levels are required to identify mechanisms that are responsible for the diverse phenotypes and disease symptomatology. The fact that there is an increasing number of patients with unknown cause of disease further underscores the need for characterization of causes and effects of epitranscriptional pathophysiological deviation from normal RNA processing, hopefully providing a future good starting point for the development of novel, genuine, therapeutic concepts.

AUTHOR CONTRIBUTIONS

All authors contributed to writing the manuscript.

ACKNOWLEDGMENTS

Funding by the Bundesministerium für Bildung und Forschung (BMBF; Era-Net NEURON II CIPRESS to JCM) and the Deutsche Forschungsgemeinschaft (DFG; Priority Programme SPP 1784 ME2075/7-1 to JCM) is acknowledged.

REFERENCES

Anant, S., Henderson, J. O., Mukhopadhyay, D., Navaratnam, N., Kennedy, S., Min, J., et al. (2001). Novel role for RNA-binding protein CUGBP2 in mammalian RNA editing. CUGBP2 modulates C to U editing of apolipoprotein B mRNA by interacting with apobec-1 and ACF, the apobec-1 complementation factor. *J. Biol. Chem.* 276, 47338–47351. doi: 10.1074/jbc.m104911200

Anant, S., MacGinnitie, A. J., and Davidson, N. O. (1995). Apobec-1, the catalytic subunit of the mammalian apolipoprotein B mRNA editing enzyme, is a novel RNA-binding protein. *J. Biol. Chem.* 270, 14762–14767. doi: 10.1074/jbc.270.24.14762

Backus, J. W., and Smith, H. C. (1992). Three distinct RNA sequence elements are required for efficient apolipoprotein B (apoB) RNA editing *in vitro*. *Nucleic Acids Res.* 20, 6007–6014. doi: 10.1093/nar/20.22.6007

Bass, B. L. (1997). RNA editing and hypermutation by adenosine deamination. *Trends Biochem. Sci.* 22, 157–162. doi: 10.1016/s0968-0004(97)01035-9

Bass, B. L., and Weintraub, H. (1987). A developmentally regulated activity that unwinds RNA duplexes. *Cell* 48, 607–613. doi: 10.1016/0092-8674(87)90239-x

Benne, R., Van den Burg, B. J., Brakenhoff, J. P., Sloof, P., Van Boom, J. H., and Tromp, M. C. (1986). Major transcript of the frameshifted coxII gene from trypanosome mitochondria contains four nucleotides that are not encoded in the DNA. *Cell* 46, 819–826. doi: 10.1016/0092-8674(86)90063-2

Bhalla, T., Rosenthal, J. J. C., Holmgren, M., and Reenan, R. (2004). Control of human potassium channel inactivation by editing of a small mRNA hairpin. *Nat. Struct. Mol. Biol.* 11, 950–956. doi: 10.1038/nsmb825

Blanc, V., and Davidson, N. O. (2010). APOBEC-1-mediated RNA editing. *Wiley Interdiscip. Rev. Syst. Biol. Med.* 2, 594–602. doi: 10.1002/wsbm.82

Blanc, V., Navaratnam, N., Henderson, J. O., Anant, S., Kennedy, S., Jarmuz, A., et al. (2001). Identification of GRY-RBP as an apolipoprotein B RNA-binding protein that interacts with both apobec-1 and apobec-1 complementation factor to modulate C to U editing. *J. Biol. Chem.* 276, 10272–10283. doi: 10.1074/jbc.M006435200

Boström, K., Garcia, Z., Poksay, K. S., Johnson, D. F., Lusis, A. J., and Innerarity, T. L. (1990). Apolipoprotein B mRNA editing. Direct determination of the edited base and occurrence in non-apolipoprotein B-producing cell lines. *J. Biol. Chem.* 265, 22446–22452.

Bransteitter, R., Prochnow, C., and Chen, X. S. (2009). The current structural and functional understanding of APOBEC deaminases. *Cell. Mol. Life Sci.* 66, 3137–3147. doi: 10.1007/s00018-009-0070-y

Brown, B. A., Lowenhaupt, K., Wilbert, C. M., Hanlon, E. B., and Rich, A. (2000). The zalpha domain of the editing enzyme dsRNA adenosine deaminase binds left-handed Z-RNA as well as Z-DNA. *Proc. Natl. Acad. Sci. U S A* 97, 13532–13536. doi: 10.1073/pnas.240464097

Brusa, R., Zimmermann, F., Koh, D. S., Feldmeyer, D., Gass, P., Seeburg, P. H., et al. (1995). Early-onset epilepsy and postnatal lethality associated with an editing-deficient GluR-B allele in mice. *Science* 270, 1677–1680. doi: 10.1126/science.270.5242.1677

Burns, M. B., Lackey, L., Carpenter, M. A., Rathore, A., Land, A. M., Leonard, B., et al. (2013). APOBEC3B is an enzymatic source of mutation in breast cancer. *Nature* 494, 366–370. doi: 10.1038/nature11881

Çalışkan, G., Müller, I., Semtner, M., Winkelmann, A., Raza, A. S., Hollnagel, J. O., et al. (2016). Identification of parvalbumin interneurons as cellular substrate of fear memory persistence. *Cereb. Cortex* 26, 2325–2340. doi: 10.1093/cercor/bhw001

Chen, C.-X., Cho, D.-S., Wang, Q., Lai, F., Carter, K. C., and Nishikura, K. (2000). A third member of the RNA-specific adenosine deaminase gene family, ADAR3, contains both single- and double-stranded RNA binding domains. *RNA* 6, 755–767. doi: 10.1017/s1355838200000170

Chen, K., Deng, S., Lu, H., Zheng, Y., Yang, G., Kim, D., et al. (2013). RNA-seq characterization of spinal cord injury transcriptome in acute/subacute phases: a resource for understanding the pathology at the systems level. *PLoS One* 8:e72567. doi: 10.1371/journal.pone.0072567

Chen, K.-M., Harjes, E., Gross, P. J., Fahmy, A., Lu, Y., Shindo, K., et al. (2008). Structure of the DNA deaminase domain of the HIV-1 restriction factor APOBEC3G. *Nature* 452, 116–119. doi: 10.1038/nature06638

Chen, R., Okabe, A., Sun, H., Sharopov, S., Hanganu-Opatz, I. L., Kolbaev, S. N., et al. (2014). Activation of glycine receptors modulates spontaneous epileptiform activity in the immature rat hippocampus. *J. Physiol.* 592, 2153–2168. doi: 10.1113/jphysiol.2014.271700

Chirasani, S. R., Markovic, D. S., Synowitz, M., Eichler, S. A., Wisniewski, P., Kaminska, B., et al. (2009). Transferrin-receptor-mediated iron accumulation controls proliferation and glutamate release in glioma cells. *J. Mol. Med. (Berl)* 87, 153–167. doi: 10.1007/s00109-008-0414-3

Conticello, S. G. (2008). The AID/APOBEC family of nucleic acid mutators. *Genome Biol.* 9:229. doi: 10.1186/gb-2008-9-6-229

Dance, G. S. C., Sowden, M. P., Cartegni, L., Cooper, E., Krainer, A. R., and Smith, H. C. (2002). Two proteins essential for apolipoprotein B mRNA editing are expressed from a single gene through alternative splicing. *J. Biol. Chem.* 277, 12703–12709. doi: 10.1074/jbc.m111337200

Daniel, C., Wahlstedt, H., Ohlson, J., Björk, P., and Ohman, M. (2011). Adenosine-to-inosine RNA editing affects trafficking of the γ-aminobutyric acid type A ($GABA_A$) receptor. *J. Biol. Chem.* 286, 2031–2040. doi: 10.1074/jbc.m110.130096

Eichler, S. A., Förstera, B., Smolinsky, B., Jüttner, R., Lehmann, T. N., Fähling, M., et al. (2009). Splice-specific roles of glycine receptor α3 in the hippocampus. *Eur. J. Neurosci.* 30, 1077–1091. doi: 10.1111/j.1460-9568.2009.06903.x

Eichler, S. A., Kirischuk, S., Jüttner, R., Schafermeier, P. K., Legendre, P., Lehmann, T. N., et al. (2008). Glycinergic tonic inhibition of hippocampal neurons with depolarising GABAergic transmission elicits histopathological signs of temporal lobe epilepsy. *J. Cell. Mol. Med.* 12, 2848–2866. doi: 10.1111/j.1582-4934.2008.00357.x

Fitzgerald, L. W., Iyer, G., Conklin, D. S., Krause, C. M., Marshall, A., Patterson, J. P., et al. (1999). Messenger RNA editing of the human serotonin 5-HT2C receptor. *Neuropsychopharmacology* 21, 82S–90S. doi: 10.1016/s0893-133x(99)00004-4

Förstera, B., a Dzaye, O. D., Winkelmann, A., Semtner, M., Benedetti, B., Markovic, D. S., et al. (2014). Intracellular glycine receptor function facilitates glioma formation *in vivo*. *J. Cell Sci.* 127, 3687–3698. doi: 10.1242/jcs.146662

Fu, T., Mukhopadhyay, D., Davidson, N. O., and Borensztajn, J. (2004). The peroxisome proliferator-activated receptor α (PPARα) agonist ciprofibrate inhibits apolipoprotein B mRNA editing in low density lipoprotein receptor-deficient mice: effects on plasma lipoproteins and the development of atherosclerotic lesions. *J. Biol. Chem.* 279, 28662–28669. doi: 10.1074/jbc.m403271200

Fu, Y., Zhao, X., Li, Z., Wei, J., and Tian, Y. (2016). Splicing variants of ADAR2 and ADAR2-mediated RNA editing in glioma. *Oncol. Lett.* 12, 788–792. doi: 10.3892/ol.2016.4734

George, C. X., and Samuel, C. E. (2011). Host response to polyoma virus infection is modulated by RNA adenosine deaminase ADAR1 but not by ADAR2. *J. Virol.* 85, 8338–8347. doi: 10.1128/JVI.02666-10

Greeve, J., Lellek, H., Rautenberg, P., and Greten, H. (1998). Inhibition of the apolipoprotein B mRNA editing enzyme-complex by hnRNP C1 protein and 40S hnRNP complexes. *Biol. Chem.* 379, 1063–1073. doi: 10.1515/bchm.1998.379.8-9.1063

Grohmann, M., Hammer, P., Walther, M., Paulmann, N., Büttner, A., Eisenmenger, W., et al. (2010). Alternative splicing and extensive RNA editing of human TPH2 transcripts. *PLoS One* 5:e8956. doi: 10.1371/journal.pone.0008956

Gurevich, I., Englander, M. T., Adlersberg, M., Siegal, N. B., and Schmauss, C. (2002a). Modulation of serotonin 2C receptor editing by sustained changes in serotonergic neurotransmission. *J. Neurosci.* 22, 10529–10532.

Gurevich, I., Tamir, H., Arango, V., Dwork, A. J., Mann, J. J., and Schmauss, C. (2002b). Altered editing of serotonin 2C receptor pre-mRNA in the prefrontal cortex of depressed suicide victims. *Neuron* 34, 349–356. doi: 10.1016/s0896-6273(02)00660-8

Harvey, R. J., Depner, U. B., Wässle, H., Ahmadi, S., Heindl, C., Reinold, H., et al. (2004). GlyR α3: an essential target for spinal PGE2-mediated inflammatory pain sensitization. *Science* 304, 884–887. doi: 10.1126/science.1094925

Henderson, J. O., Blanc, V., and Davidson, N. O. (2001). Isolation, characterization and developmental regulation of the human apobec-1 complementation factor (ACF) gene. *Biochim. Biophys. Acta* 1522, 22–30. doi: 10.1016/s0167-4781(01)00295-0

Hideyama, T., Yamashita, T., Suzuki, T., Tsuji, S., Higuchi, M., Seeburg, P. H., et al. (2010). Induced loss of ADAR2 engenders slow death of motor neurons from Q/R site-unedited GluR2. *J. Neurosci.* 30, 11917–11925. doi: 10.1523/JNEUROSCI.2021-10.2010

Higuchi, M., Maas, S., Single, F. N., Hartner, J., Rozov, A., Burnashev, N., et al. (2000). Point mutation in an AMPA receptor gene rescues lethality in mice deficient in the RNA-editing enzyme ADAR2. *Nature* 406, 78–81. doi: 10.1038/35017558

Hirano, K., Young, S. G., Farese, R. V. Jr., Ng, J., Sande, E., Warburton, C., et al. (1996). Targeted disruption of the mouse apobec-1 gene abolishes apolipoprotein B mRNA editing and eliminates apolipoprotein B48. *J. Biol. Chem.* 271, 9887–9890. doi: 10.1074/JBC.271.17.9887

Holden, L. G., Prochnow, C., Chang, Y. P., Bransteitter, R., Chelico, L., Sen, U., et al. (2008). Crystal structure of the anti-viral APOBEC3G catalytic domain and functional implications. *Nature* 456, 121–124. doi: 10.1038/nature07357

Hwang, T., Park, C. K., Leung, A. K., Gao, Y., Hyde, T. M., Kleinman, J. E., et al. (2016). Dynamic regulation of RNA editing in human brain development and disease. *Nat. Neurosci.* 19, 1093–1099. doi: 10.1038/nn.4337

Jarmuz, A., Chester, A., Bayliss, J., Gisbourne, J., Dunham, I., Scott, J., et al. (2002). An anthropoid-specific locus of orphan C to U RNA-editing enzymes on chromosome 22. *Genomics* 79, 285–296. doi: 10.1006/geno.2002.6718

Jin, Y., Zhang, W., and Li, Q. (2009). Origins and evolution of ADAR-mediated RNA editing. *IUBMB Life* 61, 572–578. doi: 10.1002/iub.207

Jüttner, R., Meier, J., and Grantyn, R. (2001). Slow IPSC kinetics, low levels of α subunit expression and paired-pulse depression are distinct properties of neonatal inhibitory GABAergic synaptic connections in the mouse superior colliculus. *Eur. J. Neurosci.* 13, 2088–2098. doi: 10.1046/j.0953-816x.2001.01587.x

Kim, U., Garner, T. L., Sanford, T., Speicher, D., Murray, J. M., and Nishikura, K. (1994a). Purification and characterization of double-stranded RNA adenosine deaminase from bovine nuclear extracts. *J. Biol. Chem.* 269, 13480–13489.

Kim, U., Wang, Y., Sanford, T., Zeng, Y., and Nishikura, K. (1994b). Molecular cloning of cDNA for double-stranded RNA adenosine deaminase, a candidate enzyme for nuclear RNA editing. *Proc. Natl. Acad. Sci. U S A* 91, 11457–11461. doi: 10.1073/pnas.91.24.11457

Knie, N., Grewe, F., Fischer, S., and Knoop, V. (2016). Reverse U-to-C editing exceeds C-to-U RNA editing in some ferns–a monilophyte-wide comparison of chloroplast and mitochondrial RNA editing suggests independent evolution of the two processes in both organelles. *BMC Evol. Biol.* 16:134. doi: 10.1186/s12862-016-0707-z

Krestel, H., Raffel, S., von Lehe, M., Jagella, C., Moskau-Hartmann, S., Becker, A., et al. (2013). Differences between RNA and DNA due to RNA editing in temporal lobe epilepsy. *Neurobiol. Dis.* 56, 66–73. doi: 10.1016/j.nbd.2013.04.006

Krestel, H. E., Shimshek, D. R., Jensen, V., Nevian, T., Kim, J., Geng, Y., et al. (2004). A genetic switch for epilepsy in adult mice. *J. Neurosci.* 24, 10568–10578. doi: 10.1523/JNEUROSCI.4579-03.2004

Kubota, H., Alle, H., Betz, H., and Geiger, J. R. (2010). Presynaptic glycine receptors on hippocampal mossy fibers. *Biochem. Biophys. Res. Commun.* 393, 587–591. doi: 10.1016/j.bbrc.2010.02.019

Kubota-Sakashita, M., Iwamoto, K., Bundo, M., and Kato, T. (2014). A role of *ADAR2* and RNA editing of glutamate receptors in mood disorders and schizophrenia. *Mol. Brain* 7:5. doi: 10.1186/1756-6606-7-5

Kwak, S., and Kawahara, Y. (2005). Deficient RNA editing of GluR2 and neuronal death in amyotropic lateral sclerosis. *J. Mol. Med. (Berl)* 83, 110–120. doi: 10.1007/s00109-004-0599-z

Lau, P. P., and Chan, L. (2003). Involvement of a chaperone regulator, Bcl2-associated athanogene-4, in apolipoprotein B mRNA editing. *J. Biol. Chem.* 278, 52988–52996. doi: 10.1074/jbc.M310153200

Lau, P. P., Villanueva, H., Kobayashi, K., Nakamuta, M., Chang, B. H., and Chan, L. (2001). A DnaJ protein, apobec-1-binding protein-2, modulates apolipoprotein B mRNA editing. *J. Biol. Chem.* 276, 46445–46452. doi: 10.1074/jbc.M109215200

Lau, P. P., Xiong, W. J., Zhu, H. J., Chen, S. H., and Chan, L. (1991). Apolipoprotein B mRNA editing is an intranuclear event that occurs posttranscriptionally coincident with splicing and polyadenylation. *J. Biol. Chem.* 266, 20550–20554.

Lavoie, A. M., Tingey, J. J., Harrison, N. L., Pritchett, D. B., and Twyman, R. E. (1997). Activation and deactivation rates of recombinant $GABA_A$ receptor channels are dependent on α-subunit isoform. *Biophys. J.* 73, 2518–2526. doi: 10.1016/s0006-3495(97)78280-8

Lee, E. A., Cho, J. H., Choi, I. S., Nakamura, M., Park, H. M., Lee, J. J., et al. (2009). Presynaptic glycine receptors facilitate spontaneous glutamate release onto hilar neurons in the rat hippocampus. *J. Neurochem.* 109, 275–286. doi: 10.1111/j.1471-4159.2009.05960.x

Legendre, P., Förstera, B., Jüttner, R., and Meier, J. C. (2009). Glycine receptors caught between genome and proteome–Functional implications of RNA editing and splicing. *Front. Mol. Neurosci.* 2:23. doi: 10.3389/neuro.02.023.2009

Lehmann, K. A., and Bass, B. L. (2000). Double-stranded RNA adenosine deaminases ADAR1 and ADAR2 have overlapping specificities. *Biochemistry* 39, 12875–12884. doi: 10.1021/bi001383g

Lellek, H., Kirsten, R., Diehl, I., Apostel, F., Buck, F., and Greeve, J. (2000). Purification and molecular cloning of a novel essential component of the apolipoprotein B mRNA editing enzyme-complex. *J. Biol. Chem.* 275, 19848–19856. doi: 10.1074/jbc.M001786200

Levanon, E. Y., Hallegger, M., Kinar, Y., Shemesh, R., Djinovic-Carugo, K., Rechavi, G., et al. (2005). Evolutionarily conserved human targets of adenosine to inosine RNA editing. *Nucleic Acids Res.* 33, 1162–1168. doi: 10.1093/nar/gki239

Li, J. B., Levanon, E. Y., Yoon, J. K., Aach, J., Xie, B., Leproust, E., et al. (2009). Genome-wide identification of human RNA editing sites by parallel DNA capturing and sequencing. *Science* 324, 1210–1213. doi: 10.1126/science.1170995

Maas, S., Patt, S., Schrey, M., and Rich, A. (2001). Underediting of glutamate receptor GluR-B mRNA in malignant gliomas. *Proc. Natl. Acad. Sci. U S A* 98, 14687–14692. doi: 10.1073/pnas.251531398

Macbeth, M. R., Schubert, H. L., Vandemark, A. P., Lingam, A. T., Hill, C. P., and Bass, B. L. (2005). Inositol hexakisphosphate is bound in the ADAR2 core and required for RNA editing. *Science* 309, 1534–1539. doi: 10.1126/science.1113150

Marino, D., Perković, M., Hain, A., Jaguva Vasudevan, A. A., Hofmann, H., Hanschmann, K. M., et al. (2016). APOBEC4 enhances the replication of HIV-1. *PLoS One* 11:e0155422. doi: 10.1371/journal.pone.0155422

McDougall, W. M., Okany, C., and Smith, H. C. (2011). Deaminase activity on ssDNA occurred *in vitro* when APOBEC3G forms homotetramers and higher-order complexes. *J. Biol. Chem.* 286, 30655–30661. doi: 10.1074/jbc.M111.269506

Mehta, A., and Driscoll, D. M. (2002). Identification of domains in apobec-1 complementation factor required for RNA binding and apolipoprotein-B mRNA editing. *RNA* 8, 69–82. doi: 10.1017/s1355838202015649

Mehta, A., Kinter, M. T., Sherman, N. E., and Driscoll, D. M. (2000). Molecular cloning of apobec-1 complementation factor, a novel RNA-binding protein involved in the editing of apolipoprotein B mRNA. *Mol. Cell Biol.* 20, 1846–1854. doi: 10.1128/mcb.20.5.1846-1854.2000

Meier, J. C., Henneberger, C., Melnick, I., Racca, C., Harvey, R. J., Heinemann, U., et al. (2005). RNA editing produces glycine receptor α3P185L resulting in high agonist potency. *Nat. Neurosci.* 8, 736–744. doi: 10.1038/nn1467

Meier, J. C., Semtner, M., Winkelmann, A., and Wolfart, J. (2014). Presynaptic mechanisms of neuronal plasticity and their role in epilepsy. *Front. Cell. Neurosci.* 8:164. doi: 10.3389/fncel.2014.00164

Nakamuta, M., Chang, B. H., Zsigmond, E., Kobayashi, K., Lei, H., Ishida, B. Y., et al. (1996). Complete phenotypic characterization of apobec-1 knockout mice with a wild-type genetic background, and a human apolipoprotein B transgenic background and restoration of apolipoprotein B mRNA editing by somatic gene transfer of Apobec-1. *J. Biol. Chem.* 271, 25981–25988. doi: 10.1074/JBC.271.42.25981

Navaratnam, N., Bhattacharya, S., Fujino, T., Patel, D., Jarmuz, A. L., and Scott, J. (1995). Evolutionary origins of apoB mRNA editing: catalysis by a cytidine deaminase that has acquired a novel RNA-binding motif at its active site. *Cell* 81, 187–195. doi: 10.1016/0092-8674(95)90328-3

Navaratnam, N., Morrison, J. R., Bhattacharya, S., Patel, D., Funahashi, T., Giannoni, F., et al. (1993). The p27 catalytic subunit of the apolipoprotein B mRNA editing enzyme is a cytidine deaminase. *J. Biol. Chem.* 268, 20709–20712.

Neeman, Y., Levanon, E. Y., Jantsch, M. F., and Eisenberg, E. (2006). RNA editing level in the mouse is determined by the genomic repeat repertoire. *RNA* 12, 1802–1809. doi: 10.1261/rna.165106

Niavarani, A., Currie, E., Reyal, Y., Anjos-Afonso, F., Horswell, S., Griessinger, E., et al. (2015). APOBEC3A is implicated in a novel class of G-to-A mRNA editing in WT1 transcripts. *PLoS One* 10:e0120089. doi: 10.1371/journal.pone.0120089

Nikolic, Z., Laube, B., Weber, R. G., Lichter, P., Kioschis, P., Poustka, A., et al. (1998). The human glycine receptor subunit α3. Glra3 gene structure, chromosomal localization, and functional characterization of alternative transcripts. *J. Biol. Chem.* 273, 19708–19714. doi: 10.1074/JBC.273.31.19708

Nishikura, K. (2006). Editor meets silencer: crosstalk between RNA editing and RNA interference. *Nat. Rev. Mol. Cell Biol.* 7, 919–931. doi: 10.1038/nrm2061

Nishikura, K. (2010). Functions and regulation of RNA editing by ADAR deaminases. *Annu. Rev. Biochem.* 79, 321–349. doi: 10.1146/annurev-biochem-060208-105251

Nishikura, K., Yoo, C., Kim, U., Murray, J. M., Estes, P. A., Cash, F. E., et al. (1991). Substrate specificity of the dsRNA unwinding/modifying activity. *EMBO J.* 10, 3523–3532.

Nogueira, M. A., Gavioli, C. F., Pereira, N. Z., de Carvalho, G. C., Domingues, R., Aoki, V., et al. (2015). Human endogenous retrovirus expression is inversely related with the up-regulation of interferon-inducible genes in the skin of patients with lichen planus. *Arch. Dermatol. Res.* 307, 259–264. doi: 10.1007/s00403-014-1524-0

Notelaers, K., Smisdom, N., Rocha, S., Janssen, D., Meier, J. C., Rigo, J. M., et al. (2012). Ensemble and single particle fluorimetric techniques in concerted action to study the diffusion and aggregation of the glycine receptor α3 isoforms in the cell plasma membrane. *Biochim. Biophys. Acta* 1818, 3131–3140. doi: 10.1016/j.bbamem.2012.08.010

O'Connell, M. A., and Keller, W. (1994). Purification and properties of double-stranded RNA-specific adenosine deaminase from calf thymus. *Proc. Natl. Acad. Sci. U S A* 91, 10596–10600. doi: 10.1073/pnas.91.22.10596

Ohlson, J., Pedersen, J. S., Haussler, D., and Ohman, M. (2007). Editing modifies the $GABA_A$ receptor subunit α3. *RNA* 13, 698–703. doi: 10.1261/rna.349107

Okuyama, S., Marusawa, H., Matsumoto, T., Ueda, Y., Matsumoto, Y., Endo, Y., et al. (2012). Excessive activity of apolipoprotein B mRNA editing enzyme catalytic polypeptide 2 (APOBEC2) contributes to liver and lung tumorigenesis. *Int. J. Cancer* 130, 1294–1301. doi: 10.1002/ijc.26114

Pilorge, M., Fassier, C., Le Corronc, H., Potey, A., Bai, J., De Gois, S., et al. (2016). Genetic and functional analyses demonstrate a role for abnormal glycinergic signaling in autism. *Mol. Psychiatry* 21, 936–945. doi: 10.1038/mp.2015.139

Prochnow, C., Bransteitter, R., Klein, M. G., Goodman, M. F., and Chen, X. S. (2007). The APOBEC-2 crystal structure and functional implications for the deaminase AID. *Nature* 445, 447–451. doi: 10.1038/nature05492

Prohaska, K. M., Bennett, R. P., Salter, J. D., and Smith, H. C. (2014). The multifaceted roles of RNA binding in APOBEC cytidine deaminase functions. *Wiley Interdiscip. Rev. RNA* 5, 493–508. doi: 10.1002/wrna.1226

Rogozin, I. B., Basu, M. K., Jordan, I. K., Pavlov, Y. I., and Koonin, E. V. (2005). APOBEC4, a new member of the AID/APOBEC family of polynucleotide (deoxy)cytidine deaminases predicted by computational analysis. *Cell Cycle* 4, 1281–1285. doi: 10.4161/cc.4.9.1994

Rueter, S. M., Dawson, T. R., and Emeson, R. B. (1999). Regulation of alternative splicing by RNA editing. *Nature* 399, 75–80. doi: 10.1038/19992

Saletore, Y., Chen-Kiang, S., and Mason, C. E. (2013). Novel RNA regulatory mechanisms revealed in the epitranscriptome. *RNA Biol.* 10, 342–346. doi: 10.4161/rna.23812

Sato, Y., Probst, H. C., Tatsumi, R., Ikeuchi, Y., Neuberger, M. S., and Rada, C. (2010). Deficiency in APOBEC2 leads to a shift in muscle fiber type, diminished body mass, and myopathy. *J. Biol. Chem.* 285, 7111–7118. doi: 10.1074/jbc.M109.052977

Schock, D., Kuo, S. R., Steinburg, M. F., Bolognino, M., Sparks, J. D., Sparks, C. E., et al. (1996). An auxiliary factor containing a 240-kDa protein complex is involved in apolipoprotein B RNA editing. *Proc. Natl. Acad. Sci. U S A* 93, 1097–1102. doi: 10.1073/pnas.93.3.1097

Seeburg, P. H., Single, F., Kuner, T., Higuchi, M., and Sprengel, R. (2001). Genetic manipulation of key determinants of ion flow in glutamate receptor

channels in the mouse. *Brain Res.* 907, 233–243. doi: 10.1016/s0006-8993(01)02445-3

Sharma, P. M., Bowman, M., Madden, S. L., Rauscher, F. J. III, and Sukumar, S. (1994). RNA editing in the Wilms' tumor susceptibility gene, WT1. *Genes Dev.* 8, 720–731. doi: 10.1101/gad.8.6.720

Smith, H. C. (2007). Measuring editing activity and identifying cytidine-to-uridine mRNA editing factors in cells and biochemical isolates. *Methods Enzymol.* 424, 389–416. doi: 10.1016/S0076-6879(07)24018-2

Sowden, M. P., Lehmann, D. M., Lin, X., Smith, C. O., and Smith, H. C. (2004). Identification of novel alternative splice variants of APOBEC-1 complementation factor with different capacities to support apolipoprotein B mRNA editing. *J. Biol. Chem.* 279, 197–206. doi: 10.1074/jbc.M307920200

Teng, B., Burant, C. F., and Davidson, N. O. (1993). Molecular cloning of an apolipoprotein B messenger RNA editing protein. *Science* 260, 1816–1819. doi: 10.1126/science.8511591

Tyagarajan, S. K., and Fritschy, J. M. (2014). Gephyrin: a master regulator of neuronal function? *Nat. Rev. Neurosci.* 15, 141–156. doi: 10.1038/nrn3670

Véliz, E. A., Easterwood, L. M., and Beal, P. A. (2003). Substrate analogues for an RNA-editing adenosine deaminase: mechanistic investigation and inhibitor design. *J. Am. Chem. Soc.* 125, 10867–10876. doi: 10.1021/ja029742d

Vonica, A., Rosa, A., Arduini, B. L., and Brivanlou, A. H. (2011). APOBEC2, a selective inhibitor of TGFβ signaling, regulates left-right axis specification during early embryogenesis. *Dev. Biol.* 350, 13–23. doi: 10.1016/j.ydbio.2010.09.016

Waseem, T. V., and Fedorovich, S. V. (2010). Presynaptic glycine receptors influence plasma membrane potential and glutamate release. *Neurochem. Res.* 35, 1188–1195. doi: 10.1007/s11064-010-0174-7

Weltzien, F., Puller, C., O'Sullivan, G. A., Paarmann, I., and Betz, H. (2012). Distribution of the glycine receptor β-subunit in the mouse CNS as revealed by a novel monoclonal antibody. *J. Comp. Neurol.* 520, 3962–3981. doi: 10.1002/cne.23139

Winkelmann, A., Maggio, N., Eller, J., Caliskan, G., Semtner, M., Häussler, U., et al. (2014). Changes in neural network homeostasis trigger neuropsychiatric symptoms. *J. Clin. Invest.* 124, 696–711. doi: 10.1172/JCI71472

Wong, S. K., Sato, S., and Lazinski, D. W. (2001). Substrate recognition by ADAR1 and ADAR2. *RNA* 7, 846–858. doi: 10.1017/s135583820101007x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Molecular Neuroscience

PERSPECTIVE
published: 15 December 2016
doi: 10.3389/fnmol.2016.00147

APP Causes Hyperexcitability in Fragile X Mice

Cara J. Westmark[1], Shih-Chieh Chuang[2†], Seth A. Hays[3†], Mikolaj J. Filon[1], Brian C. Ray[1], Pamela R. Westmark[4], Jay R. Gibson[3], Kimberly M. Huber[3] and Robert K. S. Wong[2]*

[1] Department of Neurology, University of Wisconsin-Madison, Madison, Madison, WI, USA, [2] Department of Physiology and Pharmacology, State University of New York Downstate Medical Center, Brooklyn, NY, USA, [3] Department of Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX, USA, [4] Department of Medicine, University of Wisconsin-Madison, Madison, Madison, WI, USA

Amyloid-beta protein precursor (APP) and metabolite levels are altered in fragile X syndrome (FXS) patients and in the mouse model of the disorder, *Fmr1*^{KO} mice. Normalization of APP levels in *Fmr1*^{KO} mice (*Fmr1*^{KO}/*APP*^{HET} mice) rescues many disease phenotypes. Thus, APP is a potential biomarker as well as therapeutic target for FXS. Hyperexcitability is a key phenotype of FXS. Herein, we determine the effects of APP levels on hyperexcitability in *Fmr1*^{KO} brain slices. *Fmr1*^{KO}/*APP*^{HET} slices exhibit complete rescue of UP states in a neocortical hyperexcitability model and reduced duration of ictal discharges in a CA3 hippocampal model. These data demonstrate that APP plays a pivotal role in maintaining an appropriate balance of excitation and inhibition (E/I) in neural circuits. A model is proposed whereby APP acts as a rheostat in a molecular circuit that modulates hyperexcitability through $mGluR_5$ and FMRP. Both over- and under-expression of APP in the context of the *Fmr1*^{KO} increases seizure propensity suggesting that an APP rheostat maintains appropriate E/I levels but is overloaded by $mGluR_5$-mediated excitation in the absence of FMRP. These findings are discussed in relation to novel treatment approaches to restore APP homeostasis in FXS.

Keywords: amyloid-beta, amyloid-beta precursor protein, fragile X mental retardation protein, fragile X syndrome, hyperexcitability

OPEN ACCESS

Edited by:
Susan A. Masino, Trinity College, USA

Reviewed by:
Barbara Bardoni, CNRS UMR7275 Institut de Pharmacologie Moléculaire et Cellulaire, France
Niraj S. Desai, University of Texas at Austin, USA

****Correspondence:***
Cara J. Westmark
westmark@facstaff.wisc.edu

† These authors have contributed equally to this work.

Received: *26 September 2016*
Accepted: *01 December 2016*
Published: *15 December 2016*

Citation:
Westmark CJ, Chuang S-C, Hays SA, Filon MJ, Ray BC, Westmark PR, Gibson JR, Huber KM and Wong RKS (2016) APP Causes Hyperexcitability in Fragile X Mice. Front. Mol. Neurosci. 9:147. doi: 10.3389/fnmol.2016.00147

INTRODUCTION

Amyloid-beta protein precursor (APP) levels are dysregulated in numerous neurological disorders that are comorbid with a seizure phenotype including fragile X syndrome (FXS) (Westmark, 2013). FXS is a trinucleotide repeat disorder caused by a CGG repeat expansion at the 5′-end of the *FMR1* gene. Hypermethylation of the repeat expansion results in transcriptional silencing of the *FMR1* gene and loss of expression of fragile X mental retardation protein (FMRP) (Jin and Warren, 2000). FMRP is an RNA binding protein (RBP) that plays a pivotal role in synaptic function. It is one of numerous RBP that interact with *amyloid precursor protein* (*App*) mRNA to regulate post-transcriptional and/or translational events involved in the synthesis of APP (Westmark and Malter, 2012). Specifically, FMRP binds to a guanine-rich region in the coding region of *App* mRNA and regulates APP translation through a metabotropic glutamate receptor 5 ($mGluR_5$)-dependent pathway (Westmark and Malter, 2007). We hypothesize that altered expression of APP in FXS contributes to disease severity. In support of this hypothesis, genetic knockout of one *App* allele in *Fmr1*^{KO} mice (*Fmr1*^{KO}/*APP*^{HET} mice) reduces APP expression in the *Fmr1*^{KO} to wild type (WT) levels and rescues audiogenic-induced seizures (AGS), the percentage of mature spines, open field

and marble burying behavioral phenotypes, and mGluR-LTD (Westmark et al., 2011). APP and metabolite levels are altered in *Fmr1*^{KO} mice and FXS patients (Sokol et al., 2006; Westmark et al., 2011; Erickson et al., 2014; Pasciuto et al., 2015; Ray et al., 2016). Thus, APP is a potential therapeutic target as well as blood-based biomarker for FXS (Berry-Kravis et al., 2013; Westmark et al., 2016), and it is of interest to determine the effect(s) of APP levels on additional disease phenotypes. Herein, we ascertain the effects of *App* knockdown on hyperexcitability in the *Fmr1*^{KO} mouse.

GENETIC REDUCTION OF *APP* RESCUES HYPEREXCITABILITY IN *Fmr1*^{KO} MICE

The psychiatric phenotype of FXS includes hyperexcitability traits such as tactile defensiveness, attention deficits, hyperactivity, and hyperarousal to sensory stimulation (Tranfaglia, 2011). There is high comorbidity of epilepsy in FXS with electroencephalogram (EEG) patterns most often consisting of a centrotemporal spike pattern resembling Benign Focal Epilepsy of Childhood (BFEC) (Berry-Kravis, 2002). Hyperexcitability can be modeled in the *Fmr1*^{KO} mice both *in vivo* and *in vitro* (brain slices). *In vivo*, the *Fmr1*^{KO} mice are susceptible to AGS (Chen and Toth, 2001). In the AGS model, mice are exposed to 110 dB siren, which elicits out-of-control (wild) running and jumping followed by convulsive seizures and often death. There is substantial evidence that dysregulated APP expression alters seizure propensity. AGS are exacerbated by overexpression of APP in the *Fmr1*^{KO} mouse (FRAXAD mice) and partially rescued by reduced expression of APP in *Fmr1*^{KO}/*APP*^{HET} mice (Westmark et al., 2010, 2011). Alzheimer's disease (Tg2576) and Down syndrome (Ts65Dn) mice, which overexpress human and mouse APP respectively, are highly susceptible to AGS (Westmark et al., 2010). Numerous mouse models that express altered APP or metabolite levels exhibit elevated rates of spontaneous or provoked seizures (Moechars et al., 1996; Steinbach et al., 1998; Del Vecchio et al., 2004; Lalonde et al., 2005; Palop et al., 2007; Kobayashi et al., 2008; Westmark et al., 2008; Minkeviciene et al., 2009; Ziyatdinova et al., 2011; Sanchez et al., 2012) while suppression of transgenic APP in Alzheimer's disease mice during postnatal development delays the onset of EEG abnormalities (Born et al., 2014).

In brain slices, hyperexcitability can be measured by recording UP states and epileptiform discharges. UP states are short periods of local network activity that generate a steady-state level of depolarization and synchronous firing among groups of neighboring neurons (Gibson et al., 2008). *Fmr1*^{KO} mice exhibit an increased duration of the UP state, consistent with network hyperexcitability (Gibson et al., 2008; Goncalves et al., 2013). Specifically, spontaneously occurring UP states are 38-67% longer in *Fmr1*^{KO} than in WT slices (Hays et al., 2011). Deletion of *Fmr1* selectively in excitatory neurons mimics the prolonged UP states whereas knockdown of mGluR$_5$ rescues the hyperexcitability in the *Fmr1*^{KO} with no effect in WT (Hays et al., 2011). To determine if hyperexcitability was rescued in *Fmr1*^{KO} mice by knockdown of *App*, we recorded UP states in *Fmr1*^{KO}/*App*^{HET} mice and littermate controls per previously described methods (Gibson et al., 2008). Briefly, *Fmr1*^{HET}/*App*^{HET} females were bred with *App*^{HET} males to generate WT, *Fmr1*^{KO}, *App*^{HET} and *Fmr1*^{KO}/*App*^{HET} male littermates. Thalamocortical slices (400 μm) from postnatal day 24–28 (P24-P28) males were transected parallel to the pia mater to remove the thalamus and midbrain, and spontaneously generated UP states were recorded in layer 4 of the somatosensory cortex. The increased duration of the UP states observed in the *Fmr1*^{KO} was completely rescued in *Fmr1*^{KO}/*APP*^{HET} mice (**Figures 1A,B**) where UP state duration decreased from 931 ± 55 milliseconds (ms) in *Fmr1*^{KO} to 597 ± 30 ms in *Fmr1*^{KO}/*APP*^{HET}, ($p < 0.001$). UP state duration was not significantly different between *APP*^{HET} and WT slices suggesting that rescue was not a consequence of a general reduction in excitability due to lower APP levels.

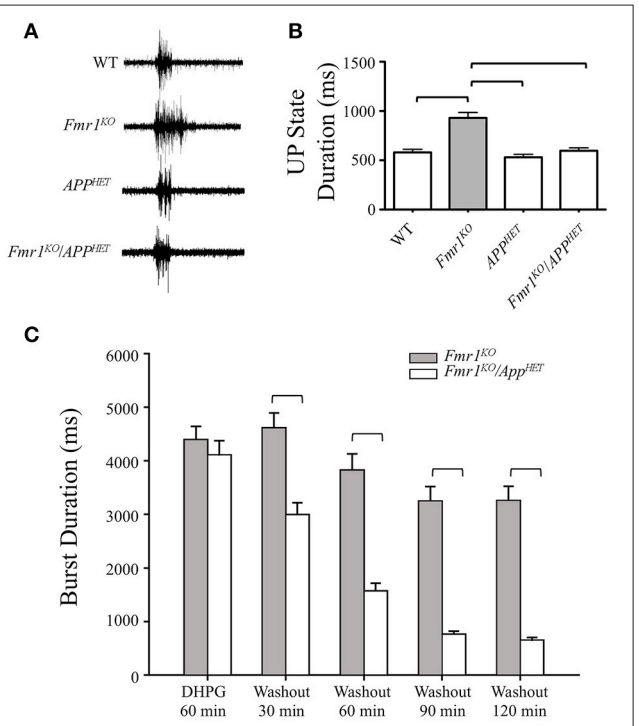


FIGURE 1 | Rescue of hyperexcitability in *Fmr1*^{KO} mice by genetic manipulation of *APP*. Thalamocortical slices from WT ($n = 17$), *Fmr1*^{KO} ($n = 22$), *App*^{HET} ($n = 13$), and *Fmr1*^{KO}/*App*^{HET} ($n = 13$) male mice were assessed for neocortical hyperexcitability. **(A)** Trace recordings and **(B)** histogram depicting a significant increase in UP state activity in *Fmr1*^{KO} slices compared to WT, which was completely rescued in the *Fmr1*^{KO}/*App*^{HET}. Error bars represent SEM. Horizontal bars denote statistically different levels by one-way ANOVA and Bonferroni's multiple comparison test ($P < 0.0001$). DHPG-induced prolonged epileptiform discharges were assessed in hippocampal slices from *Fmr1*^{KO} and *Fmr1*^{KO}/*App*^{HET} male mice ($n = 6$ mice per cohort). The recordings were continuous for 3 or more hours in a single slice per animal. **(C)** Summary frequency histogram of synchronized epileptiform discharges from *Fmr1*^{KO} and *Fmr1*^{KO}/*App*^{HET} slices in the presence of DHPG (60 min) and after DHPG washout at the indicated times up to 2 h. The mean durations of epileptiform discharges in *Fmr1*^{KO}/*App*^{HET} slices at 30, 60, 90, and 120 min after DHPG washout are significantly shorter than those in *Fmr1*^{KO} for all times tested ($P < 0.001$).

Hyperexcitability can also be evaluated in slices of the CA3 region of the hippocampus in *Fmr1*^{KO} mice. Prolonged epileptic bursts can be induced by group 1 mGluR agonists in both WT and *Fmr1*^{KO} mice and with a GABAergic antagonist only in *Fmr1*^{KO} (Chuang et al., 2005; Zhong et al., 2009). In WT slices, DHPG elicits short (∼500 ms) synchronized discharges that gradually extend to reach an average duration of 4.4 ± 0.14 s at 60 min; and in untreated *Fmr1*^{KO} slices, bicuculline elicits short <1 ms synchronized discharges that progressively increase in duration over 60 min (average duration 2.3 ïĆś 0.13 s) (Osterweil et al., 2013). These prolonged epileptiform discharges resemble the ictal discharges observed in the CA3 region in epilepsy (Merlin and Wong, 1997; Wong et al., 2004). The number and duration of ictal-like discharges were assessed by intracellular CA3 recordings in juvenile *Fmr1*^{KO} and *Fmr1*^{KO}/*App*^{HET} slices in the presence of DHPG (60 min) and after DHPG washout for up to 2 h as previously described (Chuang et al., 2005) (**Figure 1C**, Supplementary Figure 1). In the presence of DHPG, a distinct population of ictal-like discharges (burst duration > 1500 ms) occurred in both *Fmr1*^{KO} and *Fmr1*^{KO}/*App*^{HET} slices. After DHPG washout, the ictal-like discharges remained distinct for the duration of the recording (up to 2 h post-DHPG washout) in the *Fmr1*^{KO}, but not in the *Fmr1*^{KO}/*App*^{HET} slices. Thus, a major difference between *Fmr1*^{KO} and *Fmr1*^{KO}/*App*^{HET} slices is that while ictal-like discharges were transiently expressed in both genotypes, they were not maintained in the *Fmr1*^{KO}/*App*^{HET} upon termination of receptor stimulation. The seizure activity modeled in the hippocampal slice paradigm is congruent with the AGS phenotype observed in *Fmr1*^{KO}/*App*^{HET} mice where wild running and seizures are attenuated but not completely rescued to WT levels (Westmark et al., 2011). The two critical components of plasticity include the initiating factors required for induction of the modification and the downstream effectors that maintain expression of the enhanced response (Bianchi et al., 2012). Our data suggest that genetic reduction of *App* in the *Fmr1*^{KO} background does not prevent the induction of seizure activity, but can attenuate progression; thus, APP appears to be a downstream effector that maintains hyperexcitability in the context of the *Fmr1*^{KO}.

The complete rescue of hyperexcitability in the neocortex compared to the partial rescue in the hippocampus in the *Fmr1*^{KO}/*App*^{HET} mice is in accord with studies in immature mice demonstrating that the hippocampus has a lower seizure threshold compared to neocortex (Abdelmalik et al., 2005). This could be due differential expression and/or activity of group 1 mGluRs (mGluR$_1$ and mGluR$_5$) in the respective neurons under study. In fast spiking inhibitory neurons (neocortical slice model), mGluR$_1$ is more highly expressed than mGluR$_5$ (Sun et al., 2009); however reduced expression of mGluR$_5$ or APP in the *Fmr1*^{KO} completely rescues neocortical hyperexcitability whereas UP states are still longer in the *Fmr1*^{KO} after treatment with the mGluR$_1$ inhibitor LY367385 (Hays et al., 2011). These data suggest that mGluR$_5$ is the critical group 1 mGluR that modulates *Fmr1*-dependent hyperexcitability in the neocortex. Alternatively, in CA3 hippocampal neurons, both group 1 mGluR subtypes are involved in the induction and maintenance of mGluR-mediated bursts, but mGluR$_5$ plays a greater role in the induction and mGluR$_1$ in the maintenance of the prolonged epileptic bursts (Merlin, 2002). As burst duration but not induction are rescued in the *Fmr1*^{KO}/*APP*^{HET}, these data suggest that the hyperexcitability elicited by elevated APP expression in the *Fmr1*^{KO} CA3 region is dependent on mGluR$_1$.

Synaptic dysfunction occurs when the appropriate balance of excitation and inhibition (E/I) in neural circuits is not maintained (Gatto and Broadie, 2010). The absence of FMRP during postnatal development results in an E/I imbalance dominated by excitation. Our results demonstrate that E/I balance is predominantly restored when APP expression is reduced to WT levels in the *Fmr1*^{KO}. Thus, APP plays a critical role in modulating excitability. The other half of E/I balance is the inhibitory feedback on circuits. FMRP normally binds to multiple GABA$_A$R mRNAs, and their expression is decreased in juvenile *Fmr1*^{KO} (Braat et al., 2015) resulting in delay of the developmental GABA switch in *Fmr1*^{KO} (He et al., 2014). Selective deletion of *Fmr1* in inhibitory neurons has no effect on prolonged UP states suggesting that impaired GABA$_A$R signaling in FXS does not account for increased hyperexcitability in the neocortex (Hays et al., 2011). Conversely, a competitive antagonist of GABA$_A$R, bicuculline, elicits epileptiform discharges in the CA3 region of the hippocampus (Osterweil et al., 2013). These findings suggest that inhibitory feedback is differentially regulated in the neocortex and hippocampus in *Fmr1*^{KO}. Overall, the neocortical hyperexcitability and hippocampal epileptiform discharge slice models share the features of prolonged activity states and dependence on mGluR$_5$, FMRP, and APP, but differ in induction mode (neocortical slices exhibit baseline excitation vs. hippocampal slices require pharmacological stimulation), inhibitory feedback (hippocampal slices are dependent of GABA$_A$R), and protein synthesis requirements (CA3 bursts require extracellular signal-regulated kinase (ERK)1/2 activation and new protein synthesis) (Zhao et al., 2004; Chuang et al., 2005; Hays et al., 2011).

A MODEL FOR AN APP-INDUCED SHORT CIRCUIT IN FRAGILE X

Regarding possible mechanisms for APP-mediated hyperexcitability, (Westmark, 2013) APP or a metabolite could interfere with cell surface receptor activation. For example, Aβ oligomers cause redistribution of mGluR$_5$to synapses (Renner et al., 2010) and trigger multiple distinct signaling events through mGluR$_5$/prion protein complexes (Um et al., 2013; Hu et al., 2014; Haas and Strittmatter, 2016). In neurons that overexpress APP, Aβ depresses excitatory synaptic transmission (Kamenetz et al., 2003). In *Fmr1*^{KO} mice, Aβ levels are elevated in older mice but reduced in juvenile mice compared to WT controls (Westmark et al., 2011; Pasciuto et al., 2015). Thus, increased α-secretase and/or decreased BACE1 processing during postnatal development could result in decreased Aβ levels and increased synaptic transmission (Jin and Warren, 2000) Altered APP expression could affect scaffolding protein interactions at the postsynaptic density. For example, APP co-immunoprecipitates

with Homer2 and Homer3 (Parisiadou et al., 2008). These scaffolding proteins inhibit APP processing, reduce cell surface APP expression, and prevent maturation of BACE1 (Parisiadou et al., 2008). Uncoupled Homer1-mGluR$_5$ interactions underlie *Fmr1*^{KO} phenotypes, and genetic deletion of Homer1a rescues prolonged UP states in *Fmr1*^{KO} mice similar to the complete rescue observed herein in the *Fmr1*^{KO}/*APP*^{HET} mice (Ronesi et al., 2012). APP does not co-immunoprecipitate with Homer1 (Parisiadou et al., 2008); however, Aβ induces disassembly of Homer1b and Shank1 clusters (Roselli et al., 2009). (Westmark and Malter, 2012) APP or metabolites could alter the activity of intracellular signaling pathways such as ERK and mTOR (Young et al., 2009; Ma et al., 2010; Caccamo et al., 2011; Chasseigneaux et al., 2011; Pasciuto et al., 2015). Both of these pathways play pivotal roles in FXS pathology (Osterweil et al., 2010; Hoeffer et al., 2012). And Westmark and Malter (2007) APP metabolites could function in feedback loops to regulate the aforementioned pathways or even the transcription of the APP and APP processing enzymes. Aβ binds to the promoter regions of the APP and BACE1 genes and may function as a transcription factor to regulate its own production and/or processing (Bailey et al., 2011). Thus, there are numerous molecular junctures where altered expression of APP or metabolites could interfere with synaptic function and lead to a hyperexcitable circuit.

Overall, these data suggest a model whereby mGluR$_5$ inhibitors act as a circuit breaker, FMRP as an automatic transfer switch and APP as a rheostat in a circuit that controls hyperexcitability (**Figure 2**). *The mGluR$_5$ circuit breaker*: Genetic reduction of mGluR$_5$ in the *Fmr1*^{KO} mouse rescues plasticity (ocular dominance plasticity, neocortical hyperexcitability), dendritic spines (density on cortical pyramidal neurons), protein synthesis, behavior (inhibitory avoidance extinction), and AGS (Dolen et al., 2007; Hays et al., 2011). Pharmaceutical inhibition of mGluR$_5$ likewise rescues numerous *Fmr1*^{KO} phenotypes (Michalon et al., 2012, 2014). Thus, inhibiting mGluR$_5$ appears to break a circuit that mediates hyperexcitability in the *Fmr1*^{KO} mouse. *The FMRP automatic transfer switch*: mGluR$_5$ activation causes a rapid dephosphorylation of FMRP, which permits protein synthesis (Ceman et al., 2003; Narayanan et al., 2007), as well as a biphasic change in FMRP levels (initial decrease followed by increase) (Zhao et al., 2011). Thus, FMRP appears to function as an automatic transfer switch downstream of mGluR$_5$ to control protein synthesis in response to receptor activation. In FXS models, loss of the FMRP switch that modulates mGluR$_5$ signaling permits a constitutively-on circuit. *The APP rheostat*: Born and colleagues demonstrated that juvenile overexpression of APP contributes to sharp wave EEG discharges in APP transgenic mice, and proposed that APP expression functions as a rheostat that regulates synaptic balance in the brain (Born et al., 2014). We have observed that both over- and under-expression of APP increases seizure propensity in juvenile *Fmr1*^{KO} mice suggesting that tight regulation of this protein may be necessary to mitigate hyperexcitability in FXS (Westmark et al., 2010, 2011). Genetic reduction of APP in *Fmr1*^{KO} mice rescues plasticity (mGluR-LTD, neocortical hyperexcitability, epileptiform discharge duration but not induction), dendritic spines (percent mature spines but not dendritic spine length), protein synthesis, behavior (open field, marble burying), and AGS (Westmark et al., 2011; Pasciuto et al., 2015). The partial rescue of dendritic spines and epileptiform discharges in the *Fmr1*^{KO}/*APP*^{HET} suggests that APP is necessary but not sufficient to maintain synaptic homeostasis. Thus, in the context of WT mice, an APP variable resistor is capable of maintaining an appropriate E/I balance, but in *Fmr1*^{KO} and some APP transgenic mice, excess APP appears to cause a short circuit through overload of the APP rheostat resulting in hyperexcitability. Likewise, complete loss of APP would bypass the APP rheostat. *Fmr1*^{KO}/*App*^{KO} mice exhibit an extremely strong AGS phenotype (97%, $n = 36$ mice) (Westmark et al., 2013a), which is not observed in *App*^{KO} mice (11%, $n = 36$ mice). These data suggest that exacerbated hyperexcitability is a result of the combined loss of both FMRP and APP.

APP and metabolites play key roles in regulating synaptic activity with both Aβ and sAPPα implicated in positive feedback loops that facilitate mGluR$_5$ signaling (Casley et al., 2009; Renner et al., 2010; Ferreira and Klein, 2011; Westmark et al., 2011, 2013b; Pasciuto et al., 2015). Thus, the APP rheostat may provide a graded response to mGluR$_5$ activation through feedback loops involving amyloidogenic and non-amyloidogenic secretase processing. We found that AGS are attenuated in *Fmr1*^{KO} mice with BACE1 inhibitor treatment (Supplementary Figure 2). Prox and colleagues found that seizures are increased in the ADAM10 conditional knockout mouse (loss of α-secretase processing) (Prox et al., 2013). The effect of sAPPα overexpression on hyperexcitability, which could be studied in TgAPPα (Bailey et al., 2013) and TgAPPα/*Fmr1*^{KO} mice, remains to be determined. Thus, multiple APP fragments may play roles in hyperexcitability and seizure susceptibility. A caveat to this model is that over-expression of APP alone is not sufficient to increase seizure propensity in either WT or *Fmr1*^{KO} mice. We tested seizures in two alternative Alzheimer's disease mouse models, R1.40 and J20, which exhibit elevated APP expression (Lamb et al., 1997; Mucke et al., 2000). Neither strain exhibited a strong AGS phenotype (Supplementary Figures 3, 4) in contrast to Tg2576 and Ts65Dn (Westmark et al., 2010). A genetic cross of J20 with *Fmr1*^{KO} mice that produced mice over-expressing human APP in the context of the *Fmr1*^{KO} background did not result in exacerbated AGS rates in comparison to *Fmr1*^{KO} unlike the FRAXAD mice (cross of Tg2576 with *Fmr1*^{KO}) (Westmark et al., 2010). The inclusion of flanking sequences in the transgenic constructs used for the R1.40 and J20 mice are expected to affect posttranscriptional regulation of the *APP* gene, which could alter the temporal and spatial expression of APP and metabolites and thus their contribution to seizure threshold. Of note, *Fmr1*^{HET}/J20 female mice exhibited a 50% wild running rate, which was significantly higher than WT, *Fmr1*^{HET} and J20 controls, supporting the assertion that APP works in synergy with FMRP to regulate hyperexcitability (Supplementary Figure 4). This synergistic effect is also observed in mGluR-LTD studies where loss of FMRP and APP modulate synaptic transmission in opposite directions (Westmark et al., 2011). The *Fmr1*^{KO}/*APP*^{HET} mice used herein were a constitutive *App* knockdown. It remains to be determined how conditional knockdown of *App* during development affects *Fmr1* phenotypes.

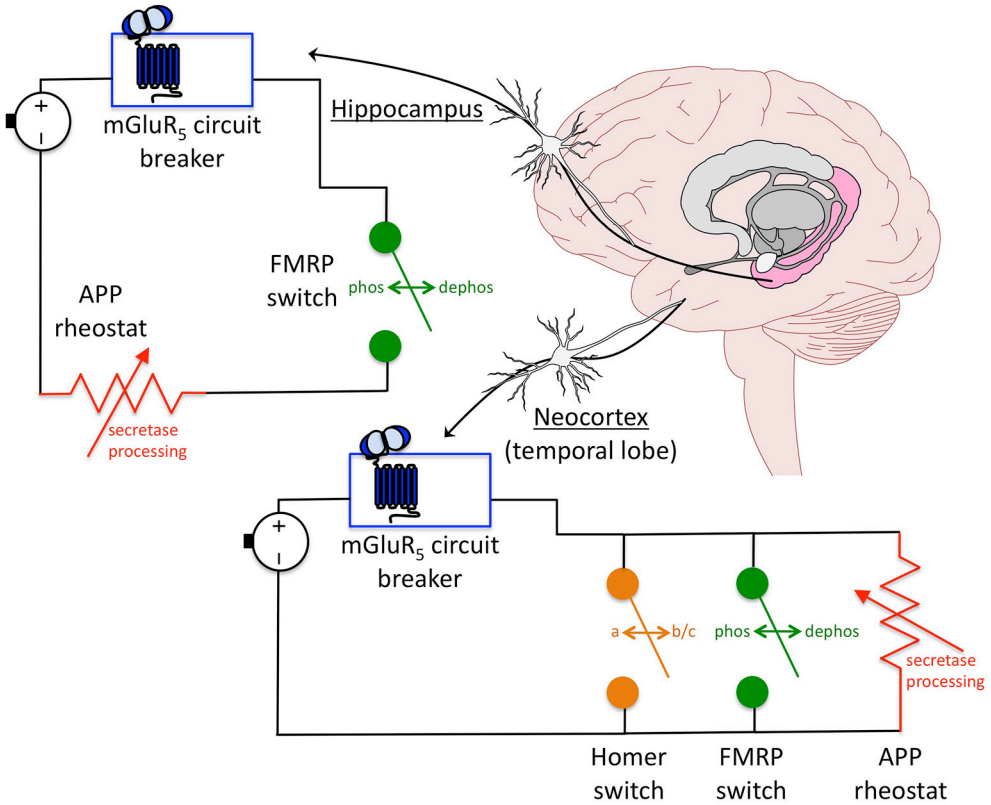


FIGURE 2 | Model for an APP-induced short circuit in FXS. APP acts as a rheostat (i.e., variable resistor, dimmer switch) in a circuit where $mGluR_5$ inhibitors are a circuit breaker and FMRP is an automatic transfer switch that regulate neuronal excitability. The FMRP switch is dependent on a rapid dephosphorylation reaction in response to $mGluR_5$ activation. In the absence of FMRP, the circuit is constitutively on. In the presence of $mGluR_5$ inhibitors, the circuit is shut down. The downstream APP circuitry appears to be wired differently dependent on brain region. In the neocortex, knockdown of individual proteins including $mGluR_5$, Homer and APP completely rescues excitability levels suggesting that these components are arranged in a parallel circuit whereby there is more than one continuous signaling pathway between $mGluR_5$ activation and excitability output. Rescue of any one of the parallel components is sufficient to restore synaptic homeostasis. In the hippocampus, ictal burst duration, but not induction, is rescued in $Fmr1^{KO}/App^{HET}$ slices in response to DHPG treatment. This incomplete rescue suggests that APP and FMRP are wired in series downstream of $mGluR_5$, and that the APP rheostat is overloaded in the absence of FMRP resulting in a short circuit. This model has important implications for therapeutic development and suggests that APP should be considered as a drug target for FXS as part of a multi-drug therapeutic strategy.

RELEVANCE TO THERAPEUTIC DEVELOPMENT

All major $Fmr1^{KO}$ phenotypes can be corrected by inhibition or knockdown of $mGluR_5$ in mice; however, neural circuitry is likely more complicated in humans and it may be necessary to employ pharmaceutical cocktails for disease treatment. Drugs under study for FXS such as acamprosate, AFQ056, donepezil, ganaxolone, lithium, lovastatin, memantine, minocycline and sertraline exhibit on- and/or off-site effects that are expected to modulate APP, Aβ, BACE1, and/or ADAM10 (Westmark et al., 2013b). Targeting APP and metabolites in FXS may allow fine tuning of excitability levels as part of a multi-drug therapeutic approach. Both amyloidogenic and non-amyloidogenic therapies have been proposed to treat FXS (Westmark et al., 2013b; Pasciuto et al., 2015). Both amyloidogenic ($Aβ_{1-42}$) and non-amyloidogenic (sAPPα) metabolites of APP stimulate phosphorylation of ERK and modulate synthesis of multiple synaptic proteins predicted to be regulated through $mGluR_5$/FMRP and to contribute to altered synaptic plasticity (Westmark et al., 2011; Pasciuto et al., 2015). Thus, it may be necessary to simultaneously modulate both α- and β-secretase processing to attain homeostatic levels of APP metabolites and rescue hyperexcitability in FXS.

AUTHOR CONTRIBUTIONS

CW, JG, KH, RW conceived and designed the experiments. CW, SC, SH, MF, BR, PW acquired data. CW, SC, SH, JG, KH, RW interpreted data. CW drafted the manuscript.

FUNDING

This work was funded by FRAXA Research Foundation and NIH R21AG044714 (CW), and NIH 1R01HD056370 (JG).

ACKNOWLEDGMENTS

The authors thank the Research Animal Resources Centers (RARC) staffs at the University of Wisconsin, the State University of New York Downstate Medical Center and the University of Texas Southwestern for expert animal care and assistance with transport between facilities. All mouse procedures were performed in accordance with NIH guidelines and approved University of Wisconsin-Madison, State University of New York Downstate Medical Center and University of Texas Southwestern Medical Center animal care protocols administered through their RARC.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnmol.2016.00147/full#supplementary-material

REFERENCES

Abdelmalik, P. A., Burnham, W. M., and Carlen, P. L. (2005). Increased seizure susceptibility of the hippocampus compared with the neocortex of the immature mouse brain *in vitro*. *Epilepsia* 46, 356–366. doi: 10.1111/j.0013-9580.2005.34204.x

Bailey, A. R., Hou, H., Song, M., Obregon, D. F., Portis, S., Barger, S., et al. (2013). GFAP expression and social deficits in transgenic mice overexpressing human sAPPalpha. *Glia* 61, 1556–1569. doi: 10.1002/glia.22544

Bailey, J. A., Maloney, B., Ge, Y. W., and Lahiri, D. K. (2011). Functional activity of the novel Alzheimer's amyloid beta-peptide interacting domain (AbetaID) in the APP and BACE1 promoter sequences and implications in activating apoptotic genes and in amyloidogenesis. *Gene* 488, 13–22. doi: 10.1016/j.gene.2011.06.017

Berry-Kravis, E. (2002). Epilepsy in fragile X syndrome. *Dev. Med. Child Neurol.* 44, 724–728. doi: 10.1111/j.1469-8749.2002.tb00277.x

Berry-Kravis, E., Hessl, D., Abbeduto, L., Reiss, A. L., Beckel-Mitchener, A., Urv, T. K., et al. (2013). Outcome measures for clinical trials in fragile x syndrome. *J. Dev. Behav. Pediatr.* 34, 508–522. doi: 10.1097/DBP.0b013e31829d1f20

Bianchi, R., Wong, R. K. S., and Merlin, L. R. (2012). "Glutamate receptors in epilepsy: group I mGluR-mediated epileptogenesis," in *Jasper's Basic Mechanisms of the Epilepsies, 4th Edn.,* eds J. L. Noebels, M. Avoli, M. A. Rogawski, R. W. Olsen, and A. V. Delgado-Escueta (Bethesda, MD: Oxford University Press). doi: 10.1093/med/9780199746545.003.0011

Born, H. A., Kim, J. Y., Savjani, R. R., Das, P., Dabaghian, Y. A., Guo, Q., et al. (2014). Genetic suppression of transgenic APP rescues Hypersynchronous network activity in a mouse model of Alzheimer's disease. *J. Neurosci.* 34, 3826–3840. doi: 10.1523/JNEUROSCI.5171-13.2014

Braat, S., D'Hulst, C., Heulens, I., De Rubeis, S., Mientjes, E., Nelson, D. L., et al. (2015). The GABAA receptor is an FMRP target with therapeutic potential in fragile X syndrome. *Cell Cycle* 14, 2985–2995. doi: 10.4161/15384101.2014.989114

Caccamo, A., Maldonado, M. A., Majumder, S., Medina, D. X., Holbein, W., Magri, A., et al. (2011). Naturally secreted amyloid-beta increases mammalian target of rapamycin (mTOR) activity via a PRAS40-mediated mechanism. *J. Biol. Chem.* 286, 8924–8932. doi: 10.1074/jbc.M110.180638

Casley, C. S., Lakics, V., Lee, H. G., Broad, L. M., Day, T. A., Cluett, T., et al. (2009). Up-regulation of astrocyte metabotropic glutamate receptor 5 by amyloid-β peptide. *Brain Res.* 1260, 65–75. doi: 10.1016/j.brainres.2008.12.082

Ceman, S., O'Donnell, W. T., Reed, M., Patton, S., Pohl, J., and Warren, S. T. (2003). Phosphorylation influences the translation state of FMRP-associated polyribosomes. *Hum. Mol. Genet.* 12, 3295–3305. doi: 10.1093/hmg/ddg350

Chasseigneaux, S., Dinc, L., Rose, C., Chabret, C., Coulpier, F., Topilko, P., et al. (2011). Secreted amyloid precursor protein beta and secreted amyloid precursor protein alpha induce axon outgrowth *in vitro* through Egr1 signaling pathway. *PLoS ONE* 6:e16301. doi: 10.1371/journal.pone.0016301

Chen, L., and Toth, M. (2001). Fragile X mice develop sensory hyperreactivity to auditory stimuli. *Neuroscience* 103, 1043–1050. doi: 10.1016/S0306-4522(01)00036-7

Chuang, S. C., Zhao, W., Bauchwitz, R., Yan, Q., Bianchi, R., and Wong, R. K. (2005). Prolonged epileptiform discharges induced by altered group I metabotropic glutamate receptor-mediated synaptic responses in hippocampal slices of a fragile X mouse model. *J. Neurosci.* 25, 8048–8055. doi: 10.1523/JNEUROSCI.1777-05.2005

Del Vecchio, R. A., Gold, L. H., Novick, S. J., Wong, G., and Hyde, L. A. (2004). Increased seizure threshold and severity in young transgenic CRND8 mice. *Neurosci. Lett.* 367, 164–167. doi: 10.1016/j.neulet.2004.05.107

Dolen, G., Osterweil, E., Rao, B. S., Smith, G. B., Auerbach, B. D., Chattarji, S., et al. (2007). Correction of Fragile X Syndrome in Mice. *Neuron* 56, 955–962. doi: 10.1016/j.neuron.2007.12.001

Erickson, C. A., Ray, B., Maloney, B., Wink, L. K., Bowers, K., Schaefer, T. L., et al. (2014). Impact of acamprosate on plasma amyloid-beta precursor protein in youth: a pilot analysis in fragile X syndrome-associated and idiopathic autism spectrum disorder suggests a pharmacodynamic protein marker. *J. Psychiatr. Res.* 59, 220–228. doi: 10.1016/j.jpsychires.2014.07.011

Ferreira, S. T., and Klein, W. L. (2011). The Abeta oligomer hypothesis for synapse failure and memory loss in Alzheimer's disease. *Neurobiol. Learn. Mem.* 96, 529–543. doi: 10.1016/j.nlm.2011.08.003

Gatto, C. L., and Broadie, K. (2010). Genetic controls balancing excitatory and inhibitory synaptogenesis in neurodevelopmental disorder models. *Front. Synaptic Neurosci.* 2:4. doi: 10.3389/fnsyn.2010.00004

Gibson, J. R., Bartley, A. F., Hays, S. A., and Huber, K. M. (2008). Imbalance of neocortical excitation and inhibition and altered UP states reflect network hyperexcitability in the mouse model of fragile X syndrome. *J. Neurophysiol.* 100, 2615–2626. doi: 10.1152/jn.90752.2008

Goncalves, J. T., Anstey, J. E., Golshani, P., and Portera-Cailliau, C. (2013). Circuit level defects in the developing neocortex of Fragile X mice. *Nat. Neurosci.* 16, 903–909. doi: 10.1038/nn.3415

Haas, L. T., and Strittmatter, S. M. (2016). Oligomers of amyloid beta prevent physiological activation of the cellular prion protein-metabotropic glutamate receptor 5 complex by glutamate in Alzheimer disease. *J. Biol. Chem.* 291, 17112–17121. doi: 10.1074/jbc.M116.720664

Hays, S. A., Huber, K. M., and Gibson, J. R. (2011). Altered neocortical rhythmic activity states in Fmr1 KO mice are due to enhanced mGluR5 signaling and involve changes in excitatory circuitry. *J. Neurosci.* 31, 14223–14234. doi: 10.1523/JNEUROSCI.3157-11.2011

He, Q., Nomura, T., Xu, J., and Contractor, A. (2014). The developmental switch in GABA polarity is delayed in fragile X mice. *J. Neurosci.* 34, 446–450. doi: 10.1523/JNEUROSCI.4447-13.2014

Hoeffer, C. A., Sanchez, E., Hagerman, R. J., Mu, Y., Nguyen, D. V., Wong, H., et al. (2012). Altered mTOR signaling and enhanced CYFIP2 expression levels in subjects with fragile X syndrome. *Genes Brain Behav.* 11, 332–341. doi: 10.1111/j.1601-183X.2012.00768.x

Hu, N. W., Nicoll, A. J., Zhang, D., Mably, A. J., O'Malley, T., Purro, S. A., et al. (2014). mGlu5 receptors and cellular prion protein mediate amyloid-beta-facilitated synaptic long-term depression *in vivo*. *Nat. Commun.* 5:3374. doi: 10.1038/ncomms4374

Jin, P., and Warren, S. T. (2000). Understanding the molecular basis of fragile X syndrome. *Hum. Mol. Genet.* 9, 901–908. doi: 10.1093/hmg/9.6.901

Kamenetz, F., Tomita, T., Hsieh, H., Seabrook, G., Borchelt, D., Iwatsubo, T., et al. (2003). APP processing and synaptic function. *Neuron* 37, 925–937. doi: 10.1016/S0896-6273(03)00124-7

Kobayashi, D., Zeller, M., Cole, T., Buttini, M., McConlogue, L., Sinha, S., et al. (2008). BACE1 gene deletion: impact on behavioral function in a model of Alzheimer's disease. *Neurobiol. Aging* 29, 861–873. doi: 10.1016/j.neurobiolaging.2007.01.002

Lalonde, R., Dumont, M., Staufenbiel, M., and Strazielle, C. (2005). Neurobehavioral characterization of APP23 transgenic mice with the SHIRPA primary screen. *Behav. Brain Res.* 157, 91–98. doi: 10.1016/j.bbr.2004.06.020

Lamb, B. T., Call, L. M., Slunt, H. H., Bardel, K. A., Lawler, A. M., Eckman, C. B., et al. (1997). Altered metabolism of familial Alzheimer's disease-linked amyloid precursor protein variants in yeast artificial chromosome transgenic mice. *Hum. Mol. Genet.* 6, 1535–1541. doi: 10.1093/hmg/6.9.1535

Ma, T., Hoeffer, C. A., Capetillo-Zarate, E., Yu, F., Wong, H., Lin, M. T., et al. (2010). Dysregulation of the mTOR pathway mediates impairment of synaptic plasticity in a mouse model of Alzheimer's disease. *PLoS ONE* 5:e12845. doi: 10.1371/journal.pone.0012845

Merlin, L. R. (2002). Differential roles for mGluR1 and mGluR5 in the persistent prolongation of epileptiform bursts. *J. Neurophysiol.* 87, 621–625.

Merlin, L. R., and Wong, R. K. (1997). Role of group I metabotropic glutamate receptors in the patterning of epileptiform activities *in vitro*. *J. Neurophysiol.* 78, 539–544.

Michalon, A., Bruns, A., Risterucci, C., Honer, M., Ballard, T. M., Ozmen, L., et al. (2014). Chronic metabotropic glutamate receptor 5 inhibition corrects local alterations of brain activity and improves cognitive performance in fragile X mice. *Biol. Psychiatry* 75, 189–197. doi: 10.1016/j.biopsych.2013.05.038

Michalon, A., Sidorov, M., Ballard, T. M., Ozmen, L., Spooren, W., Wettstein, J. G., et al. (2012). Chronic pharmacological mGlu5 inhibition corrects fragile X in adult mice. *Neuron* 74, 49–56. doi: 10.1016/j.neuron.2012.03.009

Minkeviciene, R., Rheims, S., Dobszay, M. B., Zilberter, M., Hartikainen, J., Fülöp, L., et al. (2009). Amyloid beta-induced neuronal hyperexcitability triggers progressive epilepsy. *J. Neurosci.* 29, 3453–3462. doi: 10.1523/JNEUROSCI.5215-08.2009

Moechars, D., Lorent, K., De Strooper, B., Dewachter, I., and Van Leuven, F. (1996). Expression in brain of amyloid precursor protein mutated in the alpha-secretase site causes disturbed behavior, neuronal degeneration and premature death in transgenic mice. *EMBO J.* 15, 1265–1274.

Mucke, L., Masliah, E., Yu, G. Q., Mallory, M., Rockenstein, E. M., Tatsuno, G., et al. (2000). High-level neuronal expression of Abeta 1-42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J. Neurosci.* 20, 4050–4058.

Narayanan, U., Nalavadi, V., Nakamoto, M., Pallas, D. C., Ceman, S., Bassell, G. J., et al. (2007). FMRP phosphorylation reveals an immediate-early signaling pathway triggered by group I mGluR and mediated by PP2A. *J. Neurosci.* 27, 14349–14357. doi: 10.1523/JNEUROSCI.2969-07.2007

Osterweil, E. K., Chuang, S. C., Chubykin, A. A., Sidorov, M., Bianchi, R., Wong, R. K., et al. (2013). Lovastatin corrects excess protein synthesis and prevents epileptogenesis in a mouse model of fragile x syndrome. *Neuron* 77, 243–250. doi: 10.1016/j.neuron.2012.01.034

Osterweil, E. K., Krueger, D. D., Reinhold, K., and Bear, M. F. (2010). Hypersensitivity to mGluR5 and ERK1/2 leads to excessive protein synthesis in the hippocampus of a mouse model of fragile X syndrome. *J. Neurosci.* 30, 15616–15627. doi: 10.1523/JNEUROSCI.3888-10.2010

Palop, J. J., Chin, J., Roberson, E. D., Wang, J., Thwin, M. T., Bien-Ly, N., et al. (2007). Aberrant excitatory neuronal activity and compensatory remodeling of inhibitory hippocampal circuits in mouse models of Alzheimer's disease. *Neuron* 55, 697–711. doi: 10.1016/j.neuron.2007.07.025

Parisiadou, L., Bethani, I., Michaki, V., Krousti, K., Rapti, G., and Efthimiopoulos, S. (2008). Homer2 and Homer3 interact with amyloid precursor protein and inhibit Abeta production. *Neurobiol. Dis.* 30, 353–364. doi: 10.1016/j.nbd.2008.02.004

Pasciuto, E., Ahmed, T., Wahle, T., Gardoni, F., D'Andrea, L., Pacini, L., et al. (2015). Dysregulated ADAM10-mediated processing of APP during a critical time window leads to synaptic deficits in fragile X syndrome. *Neuron* 87, 382–398. doi: 10.1016/j.neuron.2015.06.032

Prox, J., Bernreuther, C., Altmeppen, H., Grendel, J., Glatzel, M., D'Hooge, R., et al. (2013). Postnatal disruption of the disintegrin/metalloproteinase ADAM10 in brain causes epileptic seizures, learning deficits, altered spine morphology, and defective synaptic functions. *J. Neurosci.* 33, 12915–12928. doi: 10.1523/jneurosci.5910-12.2013

Ray, B., Sokol, D. K., Maloney, B., and Lahiri, D. K. (2016). Finding novel distinctions between the sAPPalpha-mediated anabolic biochemical pathways in Autism Spectrum Disorder and Fragile X Syndrome plasma and brain tissue. *Sci. Rep.* 6:26052. doi: 10.1038/srep26052

Renner, M., Lacor, P. N., Velasco, P. T., Xu, J., Contractor, A., Klein, W. L., et al. (2010). Deleterious effects of amyloid beta oligomers acting as an extracellular scaffold for mGluR5. *Neuron* 66, 739–754. doi: 10.1016/j.neuron.2010.04.029

Ronesi, J. A., Collins, K. A., Hays, S. A., Tsai, N.-P., Guo, W., Birnbaum, S. G., et al. (2012). Disrupted Homer scaffolds mediate abnormal mGluR5 function in a mouse model of fragile X syndrome. *Nat. Neurosci.* 15, 431–440. doi: 10.1038/nn.3033

Roselli, F., Hutzler, P., Wegerich, Y., Livrea, P., and Almeida, O. F. (2009). Disassembly of shank and homer synaptic clusters is driven by soluble beta-amyloid(1-40) through divergent NMDAR-dependent signalling pathways. *PLoS ONE* 4:e6011. doi: 10.1371/journal.pone.0006011

Sanchez, P. E., Zhu, L., Verret, L., Vossel, K. A., Orr, A. G., Cirrito, J. R., et al. (2012). Levetiracetam suppresses neuronal network dysfunction and reverses synaptic and cognitive deficits in an Alzheimer's disease model. *Proc. Natl. Acad. Sci. U.S.A.* 109, E2895–E2903. doi: 10.1073/pnas.1121081109

Sokol, D. K., Chen, D., Farlow, M. R., Dunn, D. W., Maloney, B., Zimmer, J. A., et al. (2006). High levels of Alzheimer beta-amyloid precursor protein (APP) in children with severely autistic behavior and aggression. *J. Child Neurol.* 21, 444–449.

Steinbach, J. P., Müller, U., Leist, M., Li, Z. W., Nicotera, P., and Aguzzi, A. (1998). Hypersensitivity to seizures in beta-amyloid precursor protein deficient mice. *Cell Death Differ.* 5, 858–866. doi: 10.1038/sj.cdd.4400391

Sun, Q. Q., Zhang, Z., Jiao, Y., Zhang, C., Szabo, G., and Erdelyi, F. (2009). Differential metabotropic glutamate receptor expression and modulation in two neocortical inhibitory networks. *J. Neurophysiol.* 101, 2679–2692. doi: 10.1152/jn.90566.2008

Tranfaglia, M. R. (2011). The psychiatric presentation of fragile x: evolution of the diagnosis and treatment of the psychiatric comorbidities of fragile X syndrome. *Dev. Neurosci.* 33, 337–348. doi: 10.1159/000329421

Um, J. W., Kaufman, A. C., Kostylev, M., Heiss, J. K., Stagi, M., Takahashi, H., et al. (2013). Metabotropic glutamate receptor 5 is a coreceptor for Alzheimer Abeta oligomer bound to cellular prion protein. *Neuron* 79, 887–902. doi: 10.1016/j.neuron.2013.06.036

Westmark, C. J. (2013). What's hAPPening at synapses? The role of amyloid β-protein precursor and beta-amyloid in neurological disorders. *Mol. Psychiatry* 18, 425–434. doi: 10.1038/mp.2012.122

Westmark, C. J., Berry-Kravis, E. M., Ikonomidou, C., Yin, J. C., and Puglielli, L. (2013b). Developing BACE-1 inhibitors for FXS. *Front. Cell. Neurosci.* 7:77. doi: 10.3389/fncel.2013.00077

Westmark, C. J., and Malter, J. S. (2007). FMRP mediates mGluR5-dependent translation of amyloid precursor protein. *PLoS Biol.* 5:e52. doi: 10.1371/journal.pbio.0050052

Westmark, C. J., and Malter, J. S. (2012). The regulation of AβPP expression by RNA-binding proteins. *Ageing Res. Rev.* 11, 450–459. doi: 10.1016/j.arr.2012.03.005

Westmark, C. J., Sokol, D. K., Maloney, B., and Lahiri, D. K. (2016). Novel roles of amyloid-beta precursor protein metabolites in fragile X syndrome an autism. *Mol. Psychiatry* 21, 1333–1341. doi: 10.1038/mp.2016.134

Westmark, C. J., Westmark, P. R., Beard, A. M., Hildebrandt, S. M., and Malter, J. S. (2008). Seizure susceptibility and mortality in mice that over-express amyloid precursor protein. *Int. J. Clin. Exp. Pathol.* 1, 157–168.

Westmark, C. J., Westmark, P. R., and Malter, J. S. (2010). Alzheimer's disease and Down syndrome rodent models exhibit audiogenic seizures. *J. Alzheimers. Dis.* 20, 1009–1013. doi: 10.3233/JAD-2010-100087

Westmark, C. J., Westmark, P. R., and Malter, J. S. (2013a). Soy-based diet exacerbates seizures in mouse models of neurological disease. *J. Alzheimers. Dis.* 33, 797–805. doi: 10.3233/JAD-2012-121426

Westmark, C. J., Westmark, P. R., O'Riordan, K. J., Ray, B. C., Hervey, C. M., Salamat, M. S., et al. (2011). Reversal of Fragile X Phenotypes by Manipulation of AbetaPP/Abeta Levels in Fmr1 Mice. *PLoS ONE* 6:e26549. doi: 10.1371/journal.pone.0026549

Wong, R. K., Chuang, S. C., and Bianchi, R. (2004). Plasticity mechanisms underlying mGluR-induced epileptogenesis. *Adv. Exp. Med. Biol.* 548, 69–75. doi: 10.1007/978-1-4757-6376-8_5

Young, K. F., Pasternak, S. H., and Rylett, R. J. (2009). Oligomeric aggregates of amyloid beta peptide 1-42 activate ERK/MAPK in SH-SY5Y cells via the alpha7 nicotinic receptor. *Neurochem. Int.* 55, 796–801. doi: 10.1016/j.neuint.2009.08.002

Zhao, W., Bianchi, R., Wang, M., and Wong, R. K. (2004). Extracellular signal-regulated kinase 1/2 is required for the induction of group I metabotropic glutamate receptor-mediated epileptiform discharges. *J. Neurosci.* 24, 76–84. doi: 10.1523/JNEUROSCI.4515-03.2004

Zhao, W., Chuang, S. C., Bianchi, R., and Wong, R. K. (2011). Dual regulation of fragile X mental retardation protein by group I metabotropic glutamate receptors controls translation-dependent epileptogenesis in the hippocampus. *J. Neurosci.* 31, 725–734. doi: 10.1523/JNEUROSCI.2915-10.2011

Zhong, J., Chuang, S. C., Bianchi, R., Zhao, W., Lee, H., Fenton, A. A., et al. (2009). BC1 regulation of metabotropic glutamate receptor-mediated neuronal excitability. *J. Neurosci.* 29, 9977–9986. doi: 10.1523/JNEUROSCI.3893-08.2009

Ziyatdinova, S., Gurevicius, K., Kutchiashvili, N., Bolkvadze, T., Nissinen, J., Tanila, H., et al. (2011). Spontaneous epileptiform discharges in a mouse model of Alzheimer's disease are suppressed by antiepileptic drugs that block sodium channels. *Epilepsy Res.* 94, 75–85. doi: 10.1016/j.eplepsyres.2011.01.003

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Molecular Neuroscience

ORIGINAL RESEARCH
published: 08 December 2016
doi: 10.3389/fnmol.2016.00138

Specific Metabolomics Adaptations Define a Differential Regional Vulnerability in the Adult Human Cerebral Cortex

Rosanna Cabré[1], Mariona Jové[1], Alba Naudí[1], Victoria Ayala[1], Gerard Piñol-Ripoll[2], Maria P. Gil-Villar[2], Mayelin Dominguez-Gonzalez[3], Èlia Obis[1], Rebeca Berdun[1], Natalia Mota-Martorell[1], Manuel Portero-Otin[1], Isidre Ferrer[3,4] and Reinald Pamplona[1]**

[1] Department of Experimental Medicine, University of Lleida-Institute for Research in Biomedicine of Lleida (UdL-IRBLleida), Lleida, Spain, [2] Neurology Division, Hospital Santa María of Lleida, Lleida, Spain, [3] Institute of Neuropathology, University of Barcelona, Barcelona, Spain, [4] Center for Biomedical Research on Neurodegenerative Diseases, Instituto de Salud Carlos III - ISCIII, Barcelona, Spain

Brain neurons offer diverse responses to stresses and detrimental factors during development and aging, and as a result of both neurodegenerative and neuropsychiatric disorders. This multiplicity of responses can be ascribed to the great diversity among neuronal populations. Here we have determined the metabolomic profile of three healthy adult human brain regions—entorhinal cortex, hippocampus, and frontal cortex—using mass spectrometry-based technologies. Our results show the existence of a lessened energy demand, mitochondrial stress, and lower one-carbon metabolism (particularly restricted to the methionine cycle) specifically in frontal cortex. These findings, along with the better antioxidant capacity and lower mTOR signaling also seen in frontal cortex, suggest that this brain region is especially resistant to stress compared to the entorhinal cortex and hippocampus, which are more vulnerable regions. Globally, our results show the presence of specific metabolomics adaptations in three mature, healthy human brain regions, confirming the existence of cross-regional differences in cell vulnerability in the human cerebral cortex.

Keywords: energy metabolism, mammalian target of rapamycin (mTOR), metabolomics, methionine cycle, mitochondrial stress, nucleotide metabolism, one-carbon metabolism, selective neuronal vulnerability

OPEN ACCESS

Edited by:
Detlev Boison, Legacy Health, USA

Reviewed by:
Daniela Kaufer, University of California, Berkeley, USA
Munjal M. Acharya, University of California, Irvine School of Medicine, USA

****Correspondence:***
Isidre Ferrer
8082ifa@gmail.com
Reinald Pamplona
reinald.pamplona@mex.udl.cat

Received: *03 August 2016*
Accepted: *22 November 2016*
Published: *08 December 2016*

Citation:
Cabré R, Jové M, Naudí A, Ayala V, Piñol-Ripoll G, Gil-Villar MP, Dominguez-Gonzalez M, Obis È, Berdun R, Mota-Martorell N, Portero-Otin M, Ferrer I and Pamplona R (2016) Specific Metabolomics Adaptations Define a Differential Regional Vulnerability in the Adult Human Cerebral Cortex. Front. Mol. Neurosci. 9:138. doi: 10.3389/fnmol.2016.00138

INTRODUCTION

Human evolution is associated with rapid expansion of brain size and complexity, a prerequisite for the emergence of cognitive functions. These evolutionary changes have been linked to and supported by adaptations in brain metabolism, especially with respect to increased energy supply (Mink et al., 1981; Cáceres et al., 2003; Uddin et al., 2004; Fu et al., 2011; Somel et al., 2013). Thus, neurons in the human nervous system can perform a wide array of motor, sensory, regulatory, behavioral, and cognitive functions. This functional diversity is expressed in the central nervous system (CNS) by a complex organization in different regions that groups neuronal populations with a diversity of neural cells. The morphological and functional diversity among neurons suggests that each neuron type, and by extension each brain region, has its own genomic expression profile in addition to the 'housekeeping' genes necessary for the basal function of all cells, which are

essentially related to cellular metabolism (Lein et al., 2007; Hawrylycz et al., 2012). This gene expression profile determines a proteomic pattern which, in turn, configures a regional neuron-specific metabolomic profile. Because each level of organization of the '-omics' depends on the other, and a perturbation in one network can affect another, the phenotypic properties of different brain regions are ultimately the product of distinctive combinations of expressed gene products and their regulation, resulting in the metabolome as the informative modality to define cellular diversity in the CNS.

The fact that specific regions of the CNS exhibit differential vulnerabilities to aging and various neurodegenerative (NDD) and neuropsychiatric diseases (NPD) also reinforces the idea of the heterogeneity in neuronal responses to cell-damaging processes, in addition to specificity in the etiology of each pathology (Mattson and Magnus, 2006; Domínguez et al., 2016). So, in order to better understand the mechanisms which are involved in neuronal resistance/sensitivity to stress and death, it is crucial to define the cell vulnerability of the different brain regions in physiological conditions.

This study focuses on the prospects that an 'omic' approach offers for the identification of traits that define the selective neuronal vulnerability (SNV) for a given brain region, and their potential involvement in the neuronal aging process and the development of NDD and NPD. To date no metabolomic studies investigating cross-regional differences in the human brain have been reported. To overcome this limitation, here we use mass spectrometry-based technology (ESI-TQ-MS/MS) to measure the concentrations of 37 specific metabolites of three different regions of the adult human cerebral cortex.

We designed a panel of metabolites mostly belonging to the one-carbon metabolism, as an integrative network of nutrient status and energy metabolism which involves three pathways: the folate cycle, the methionine cycle, and the *trans*-sulfuration pathway (Locasale, 2013). In addition to the metabolomic analysis, we have also measured, using western blot, different factors associated with stress resistance and cell survival such as the antioxidants catalase and SOD1, the FOXO transcriptional factor FOXO1, the repressor element 1-silencing transcription factor REST (Lu et al., 2014), and the master regulator that senses cell nutrient and energy status, the mechanistic target of rapamycin mTOR (Perluigi et al., 2015).

Using this approach, we studied metabolic differences in three functionally and evolutionarily distinct brain regions: entorhinal cortex, hippocampus, and frontal cortex. The frontal cortex is a brain region that appeared recently during primate evolution and which is implicated in complex associative functions, while the entorhinal-hippocampus system functions as a hub in a widespread network for memory. We also focused on hippocampus, entorhinal and frontal cortex areas because of their importance in aging, NDDs such as Alzheimer's disease (AD), and NPDs such as schizophrenia. This allowed us to examine how resistance to stress determines region-specific vulnerability in the adult human cerebral cortex.

TABLE 1 | Cases examined.

Case	Age (*y*)	Gender	Post-mortem delay	Cause of death	Entorhinal cortex (*n* = 11)	Hippocampus (*n* = 9)	Frontal cortex (*n* = 11)
1	43	Male	4 h 35 min	Respiratory failure			x
2	43	Male	5 h 55 min	Multiorgan failure			x
3	47	Male	4 h 55 min	Cardiac arrest			x
4	48	Female	4 h	Respiratory failure			x
5	50	Male	17 h 15 min	Cardiac arrest	x		
6	52	Male	4 h 04 min	Myocardial infarction	x		
7	52	Male	4 h 40 min	Broncho-pneumonia			x
8	53	Male	7 h 25 min	Heart failure			x
9	54	Female	14 h 25 min	Bilateral pneumonia	x	x	
10	54	Male	10 h 35 min	Pneumonia	x	x	
11	56	Male	3 h 45 min	Renal failure	x	x	x
12	56	Male	8 h 50 min	Myocardial infarction	x	x	
13	57	Male	20 h 30 min	Respiratory failure	x	x	
14	58	Male	3 h 10 min	Massive intestinal ischaemia	x	x	
15	58	Male	4 h	Respiratory failure			x
16	58	Male	8 h 05 min	Pneumonia	x	x	
17	59	Male	4 h 15 min	Multiorgan failure	x	x	
18	61	Male	3 h 55 min	Multiorgan failure		x	x
19	64	Female	5 h	Heart failure			x
20	66	Female	4 h 15 min	Respiratory failure	x		x
Mean post-mortem delay (in hours)					9.01 ± 1.81	8.61 ± 1.94	4.76 ± 0.32#
Mean age* (in years)					56.36 ± 1.26	57.00 ± 0.76	53.72 ± 2.41

**Values are expressed as mean ± SEM. Non-significant differences were observed for the variable 'age' between regions. Non-significant differences were observed for 'post-mortem delay' between regions, with the exception of the comparison entorhinal cortex vs frontal cortex (#p = 0.048).*

SUBJECTS/MATERIALS AND METHODS

Chemicals

Unless otherwise specified, all reagents were from Sigma–Aldrich, and of the highest purity available.

Human Samples

Brain samples were obtained from the Institute of Neuropathology Brain Bank following the guidelines of the local ethics committee, and in accordance with recently published criteria of sample quality (Ferrer, 2015). The study of human samples was carried out according to the Spanish Law of Science and accompanying guidelines and with the approval of the local ethics committee of the Bellvitge University Hospital (Barcelona, Spain). The selection of cases examined in the present study corresponded to a consecutive series of donations (see **Table 1**). The brains of adult healthy subjects were obtained at from 3 to 20 h after death, and were immediately prepared for morphological and biochemical studies, as previously described Ferrer et al. (2008).

Briefly, at autopsy, one hemisphere was fixed in 4% buffered formalin for about 3 weeks while the other hemisphere was cut in coronal sections 1 cm thick. Selected samples of the brain were dissected and placed in labeled plastic bags, immediately frozen on dry ice, and stored at −80°C until use. The neuropathological study was carried out on formalin-fixed, paraffin-embedded samples of the frontal, primary motor, primary sensory, parietal, temporal superior, temporal inferior, anterior cingulated, anterior insular, and primary and associative visual cortices; entorhinal cortex and hippocampus; caudate, putamen, and globus pallidus; medial and posterior thalamus; subthalamus; Meynert nucleus; amygdala; midbrain (two levels), pons, and medulla oblongata; and cerebellar cortex and dentate nucleus. De-waxed sections, 5-μm thick, were stained with haematoxylin and eosin, and Klü Barrera or processed for immunohistochemistry to β-amyloid, phosphorylated tau, α-synuclein, ubiquitin, p62, TDP43, glial fibrillary protein, and microglia markers.

Selected cases did not show lesions on the neuropathological examination including any kind of β-amyloid, tau, hypoxic, or vascular pathology. Following the initial screening, the present series includes 20 cases: 16 men and 4 women, aged from 43 to 66 years with post-mortem delay ranging from 3 h 10 min to 20 h 30 min (**Table 1**). Frozen samples of the entorhinal cortex ($n = 11$), hippocampus ($n = 9$), and frontal cortex area 8 ($n = 11$) were used for metabolomics and western blot studies. Samples from the three regions were processed in parallel.

Metabolomic Analysis

An important technical concern is the accuracy of metabolite measurements made in postmortem brain tissue for *in vivo* metabolite concentrations. The criteria applied for the selection of cases ensure the quality of the samples and the preservation of the concentration of *in vivo* metabolites measured. Reinforcing this, previous studies demonstrated that the concentration of several metabolites (such as myo-inositol, creatine, glutamine, glutamate, *N*-acetylaspartate, taurine, spermine, spermidine, and putrescine) remained stable in postmortem brain tissue over long-term intervals (Perry et al., 1981; Petroff et al., 1988; Michaelis et al., 1996; Chen et al., 2009; Opstad et al., 2010). Because most of these metabolites belong to metabolic pathways associated with one-carbon metabolites, we understand that metabolites analyzed in the present study are stable; consequently, we excluded postmortem delay as a confounding factor in the present study.

Metabolite Extraction from Brain Samples

Tissue samples (40 mg) were homogenized in cold methanol (20 v/w) containing 1 μg/mL of phenylalanine C13 as internal standard and 1 μM butylhydroxytoluene as antioxidant, obtaining a final concentration of 50 mg tissue/mL. Then, samples were incubated at 20°C for 1 h and centrifuged at 12000 *g* for 3 min, and the supernatants were subjected to mass spectrometry analysis.

Triple Quadrupole Mass Spectrometry

For analysis, we have developed a new method (using a targeted approach based on LC ESI-TQ MS/MS) to detect and quantify a metabolomic panel including 37 metabolites belonging to energy metabolism and one-carbon metabolism in human brain tissue (see **Table 2**). Samples were decoded and randomized before injection. Every 5 samples, internal and external standards were injected as a quality control. Data were finally normalized according to deuterated internal standard content, and expressed as MS counts.

Samples were analyzed with liquid chromatography (UPLC 1290, Agilent Technologies, San Jose, CA, USA) coupled with electrospray ionization on a triple quadrupole mass spectrometer (ESI-TQ MS/MS, Agilent Technologies 6420, San Jose, CA, USA). For analysis 6 μL of the extract was injected. Chromatographic separation was achieved on a reversed phase C18 (2.1 × 50 mm, 1.8 μm particles; Agilent Technologies, San Jose, CA, USA) column using a flow rate of 0.2 mL/min during a 19 min gradient (0–5 min 0% B, 5–8 min from 0% B to 30% B, 8–9 min from 30% B to 100% B, 8–12 min 100% B, 12–13 min from 100% B to 0% B, 13–19 min 0% B), while using the solvents A, 0.1% formic acid, and B, acetonitril 0.1% formic acid. Electrospray ionization was performed in both positive and negative ion mode (depending on the target metabolite) using N_2 at a pressure of 50 psi for the nebulizer with a flow of 12 L/min and a temperature of 325°C, respectively.

To detect the individual metabolites, multiple reaction monitoring (MRM) in negative and in positive ion mode was performed with individually optimized fragmentor voltage and collision energies (Optimizer Application, MassHunter, Agilent Technologies, San Jose, CA, USA). Most of the MRM parameters were achieved by flow injection of pure standards and the MassHunter Optimizer software (Agilent Technologies, San Jose, CA, USA). However, some of metabolites required manual optimization using MassHunter Qualitative Analyses (Agilent Technologies, San Jose, CA, USA). All the MRM parameters obtained from optimization were compared to the literature when available for certain compounds. Finally, a chromatographic system was applied to determine retention time

TABLE 2 | Analytical traits of the panel of metabolites designed to be measured in the samples of cerebral cortex from healthy adults.

	Dynamic MRM							
Compound name	**Precursor ion**	**Product ion**	**Ret time (min)**	**Delta ret time**	**Fragmentor**	**Collision energy**	**Cell accelerator voltage**	**Polarity**
3 P-Glycerate (*)	184.9	96.9	0.82	1	75	12	7	Negative
Cis-Aconitate (*)	175.03	139	1.5	1	100	15	7	Positive
Fumarate	115	71	1.8	1	60	4	7	Negative
Glutamic acid	146	102.1	0.8	1	75	12	7	Negative
Glutamine	147.1	84	0.79	1	70	16	7	Positive
Glyceraldehyde 3P	168.99	96.9	0.8	1	119	4	7	Negative
Leucine	132.1	90.3	0.9	1	82	12	7	Positive
L-Carnitine	162.1	60.1	0.81	1	107	16	7	Positive
NADH (*)	666.9	136	2.4	1	124	44	7	Positive
NADPH (*)	746.1	302	1.57	1	129	36	7	Positive
Phosphoenolpyruvate (*)	166.97	78.9	0.84	1	55	8	7	Negative
Proline	116.07	70.1	0.81	1	75	16	7	Positive
Pyruvate (*)	87	43.1	1.4	1	35	4	7	Negative
Succinate	117	73	2.2	1	65	8	7	Negative
Tryptophan	205.1	188	9.8	1	70	4	7	Positive
Betaine	118.09	58.1	0.82	1	107	28	7	Positive
Choline	105.12	61.1	0.78	1	92	16	7	Positive
Glycine	76	48.1	0.78	1	35	0	7	Positive
L-Serine	106.05	60.1	0.79	1	60	8	7	Positive
Sarcosine	90.06	44.1	0.8	1	40	16	7	Positive
Threonine	120.1	74.1	0.8	1	65	4	7	Positive
5-Methyl-THF (*)	460.2	313.1	13.3	2	104	12	7	Positive
Cysteine	122.03	59	0.85	1	129	24	7	Positive
Cystathionine	223.08	88	0.8	1	77	24	7	Positive
Folate acid (*)	442.15	295.1	9.35	2	92	8	7	Positive
GSH	308.09	84	1.6	1	97	28	7	Positive
GSSG (*)	613.16	355	1.4	1	161	16	7	Positive
Homocysteine	136	90	0.88	1	65	4	7	Positive
Methionine	150.06	56.1	1.4	1	70	12	7	Positive
PLP (*)	248	94	1.6	1	110	28	7	Positive
Pyridoxal	168.1	150	1.6	1	70	8	7	Positive
Pyridoxamine	169.1	134	0.82	1	100	20	7	Positive
SAH (*)	385.13	136	4.39	1	97	12	7	Positive
SAM	399.15	250	0.8	1	100	8	7	Positive
Spermidine	146.2	72.1	0.71	1	75	12	7	Positive

(Continued)

TABLE 2 | Continued

	Dynamic MRM							
Compound name	**Precursor ion**	**Product ion**	**Ret time (min)**	**Delta ret time**	**Fragmentor**	**Collision energy**	**Cell accelerator voltage**	**Polarity**
Taurine	124	80	0.8	1	102	20	7	Negative
Adenosine	268.1	136	6	1	92	12	7	Positive
ADP (*)	426.02	158.9	1.33	1	134	20	7	Negative
AMP	348.07	136	1.8	1	102	16	7	Positive
Deoxyguanosine	268.1	43.1	9.3	1	168	56	7	Positive
Deoxyguanosine 5MP (*)	348.07	152	3.58	1	85	8	7	Positive
Guanine (*)	152	135	1.46	1	107	16	7	Positive
Guanosine 5MP	364	152	0.86	1	87	8	7	Positive
Hypoxanthine	137.05	55.1	2	1	109	32	7	Positive
Inosine	269.09	137	7.3	1	70	4	7	Positive
Inosine 5MP	349.06	137	2.08	1	72	4	7	Positive
Inosine 5DP (*)	427	158.9	1.2	2	119	24	7	Negative
Ribose 5-P	231.02	79.1	0.67	1	100	15	7	Negative
Xanthine	153	110	2.54	1	92	16	7	Positive
Xanthosine	285.1	153	7.6	2	65	4	7	Positive
Myo-inositol	179.05	87	0.75	1	102	16	7	Negative
N-Acetyl-Asp-Glut	305.1	148	2.9	1	70	4	7	Positive
N-Acetyl-Asp acid (*)	174	88	1.49	1	75	12	7	Positive
Phenilalanine-^{13}C (standard)	167.09	120.1	5.9	2	70	8	7	Positive

*Of the initial 53 metabolites included in and optimized for the analysis, 16 (marked by *) were finally excluded because they were absent after the extraction method was applied. So the final analyzed panel was composed of 37 metabolites.*

of each standard. Peak determination and peak area integration were carried out with MassHunter Qualitative Analyses (Agilent Technologies, San Jose, CA, USA).

Mass Spectrometry Analysis of 2-SC

2-SC was determined as trifluoroacetic acid methyl ester (TFAME) derivatives in acid-hydrolysed, delipidated, and reduced brain protein samples with GC/MS using a HP6890 Series II gas chromatograph (Agilent, Barcelona, Spain) with an MSD5973A Series detector and a 7683 Series automatic injector, an HP-5MS column (30 m × 0.25 mm × 0.25 μm), and the described temperature program (Naudí et al., 2013). Quantification was performed with internal and external standardization using standard curves constructed from mixtures of deuterated and non-deuterated standards. Analyses were carried out with selected ion-monitoring GC/MS (SIM-GC/MS). The ions used were lysine and [$^2H_8$]lysine, m/z 180 and 187, respectively, and 2-SC and [$^2H_2$]SC, m/z 284 and 286. The amount of product was expressed as μmoles of 2-SC per mol of lysine.

Western Blot Analysis

The amounts of different factors associated with stress resistance and cell survival such as the antioxidants catalase and SOD1, the FOXO transcriptional factor FOXO1, the repressor element 1-silencing transcription factor REST, and the master regulator that senses cell nutrient and energy status, mechanistic target of rapamycin mTOR, were estimated using western blot analyses in samples from brain tissue.

Brain tissue (50 mg from each specific brain region) was homogenized in a buffer containing 180 mM KCl, 5 mM MOPS, 2 mM EDTA, 1 mM diethylenetriaminepentaacetic acid, 1 μM butylated hydroxyltoluene, protease inhibitor mix (80-6501-23, Amersham Biosciences), and phosphatase inhibitors (Na3VO4 1 mM, NaF 1 mM). A brief centrifugation (1000 rpm at 4°C for 3 min) to pellet and remove cellular debris was performed. The protein concentration was measured using the Bradford method (Bio-Rad Protein Assay 500-0006). Proteins were separated with one-dimensional SDS-PAGE. Samples were mixed with sample buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 20% 2-β-mercaptoethanol and 0.02% bromophenol blue) and heated at 95°C for 5 min. Proteins (35 μg) were subjected to electrophoresis on 10% SDS-polyacrylamide minigels. For immunodetection, proteins were transferred, using a Mini Trans-Blot Transfer Cell (Bio Rad) in a buffer containing 25 mM TRIS, 192 mM Glycine, and 20% methanol, to polyvinylidene difluoride (PVDF) membranes (Immobilon-P Millipore, Bedford, MA, USA). The membranes were immersed in blocking solution (0.5% BSA Sigma-Aldrich A4503, 0.1% Tween in TBS) at room temperature for 1 h. After blocking, the membranes were washed two times using 0.05% TBS-T buffer. Afterward, they were incubated in primary solution using specific antibodies: anti-GFAP (1:1000, ref. ab7260), anti-catalase (1:1000, ref. ab16731), anti-SOD1 (1:5000, ref. ab52950), anti-FoxO1 (1:1000, ref. 2880 Cell Signaling), anti-REST (1:1000, ref. ab21635), and anti-phospho-mTOR and anti-mTOR (1:1000 in both cases, ref. 2971s and 2972-Cell Signaling Technology, respectively). An antibody to actin (1:5000, ref. A5441 Sigma) was also used in each analysis to determine the amount of the different factors in reference to total protein mass. Primary antibody specificity was tested by incubating only with the secondary antibody.

The primary antibody was incubated at 4°C for 16 h. Then, the membrane was washed three times in 0.05% TBS-T buffer and incubated at room temperature for 1 h with the appropriate secondary antibodies [ECL Anti-mouse IgG, horseradish Peroxidase linked whole antibody-NA93IV GE Healthcare (1:50000) and ImmunoPure Goat Anti-Rabbit IgG peroxidase conjugated-31460 Pierce Biotechnology (1:100000)]. After five washes with 0.05% TBS-T buffer, bands were visualized using an enhanced chemiluminescence HRP substrate (Millipore, Bedford, MA, USA). Signal quantification and recording was performed with ChemiDoc equipment (Bio-Rad Laboratories, Inc., Barcelona, Spain). The amounts of the determined factors were specifically calculated from the ratio of their densitometry values in reference to the densitometry values of their own actin content. Ratio of phospho-mTOR to total-mTOR was calculated. The amounts of REST and GFAP were specifically calculated from the ratio of their densitometry values in reference to the densitometry values of their gallyas stain protein.

Statistical Analysis

All statistic calculations were performed using the SPSS software (SPSS Inc, Chicago, IL, USA). Values were expressed as means ± standard error of the mean (SEM). Comparisons between groups were made with ANOVA followed by DMS tests for paired groups. The minimum level of statistical significance was set at $p < 0.05$ in all the analyses.

RESULTS

Evidence from comparative studies of gene expression and evolution between humans and anthropoid primate species suggests that neurons from human neocortex are characterized by high energy metabolism, along with an increase in neuroglial cell density in order to support greater metabolic demands (Sherwood et al., 2006). We have extended this idea to three different regions of the adult human brain cerebral cortex to evaluate possible differences in the density of glial cells relative to neurons, as an indirect indication of region-specific adaptation to neuronal metabolic demands. Our results demonstrate that frontal cortex shows an increased density of neurons along with a concomitant decrease in glial cells compared to entorhinal cortex ($p < 0.01$ and $p < 0.05$, respectively) and hippocampus ($p < 0.05$ and $p < 0.01$, respectively) (**Figure 1**), suggesting lower neuronal metabolic demands specifically in frontal cortex.

To explore the bioenergetic demands of the different regions of the human cerebral cortex, we measured, using a TQMS approach, a myriad of metabolites which directly (succinate and fumarate) or indirectly (leucine, tryptophan, glutamate, glutamine, proline, carnitine, and glyceraldehyde-3-phosphate) are associated with or involved in Krebs cycle (**Figure 2**). No interregional differences were detected for leucine, tryptophan, carnitine, glutamate, or glutamine. In contrast, the

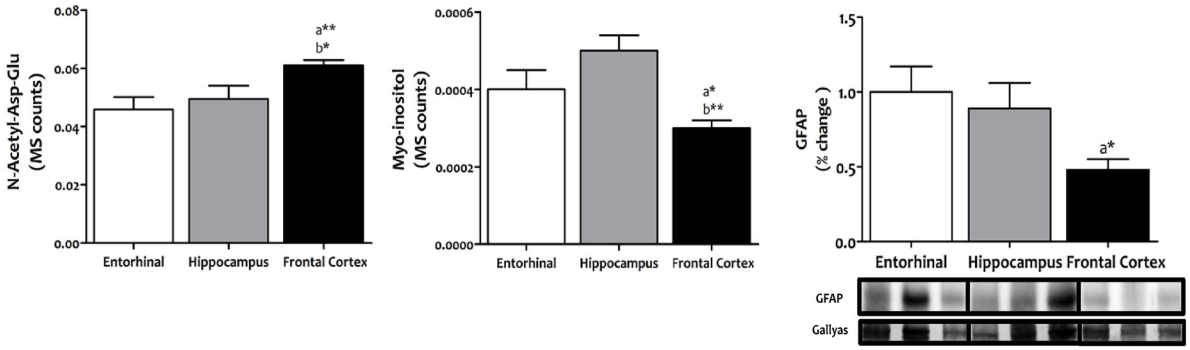


FIGURE 1 | Content of neurons and glial cells in different regions of the adult human cerebral cortex. *N*-acetyl-Asp-Glu was used as a marker for neuronal content, and myoinositol and GFAP as markers for glial cell content. *N*-acetyl-Asp-Glu and myoinositol were determined with TQMS, while GFAP was measured with western blot. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. a, significant differences with respect to entorhinal cortex; b, significant differences with respect to hippocampus.

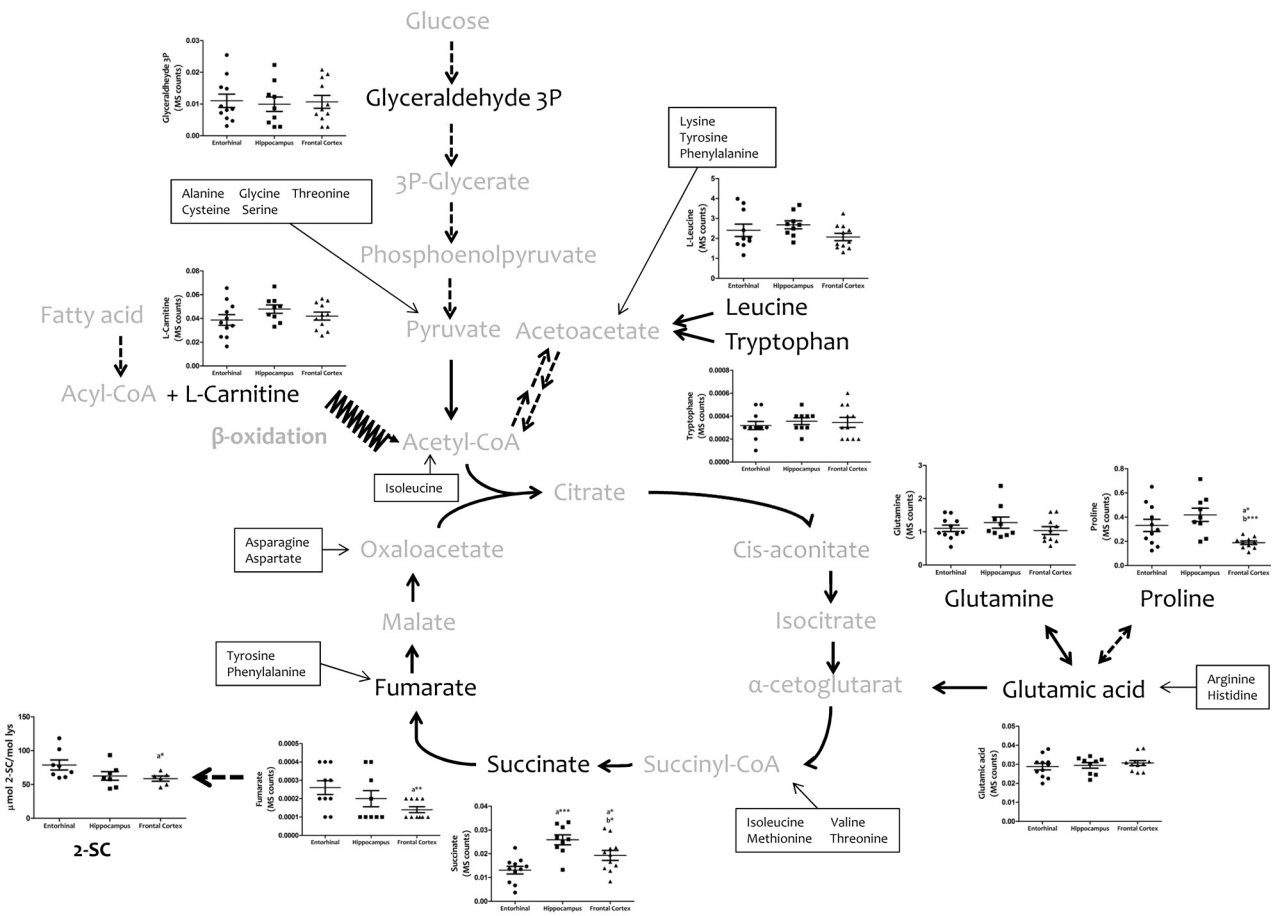


FIGURE 2 | Concentrations of metabolites involved in bioenergetics metabolism in different regions of the adult human cerebral cortex. Metabolites were detected and quantified with TQMS. The steady-state level of 2-SC was measured with GC-MS. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. a, significant differences with respect to entorhinal cortex; b, significant differences with respect to hippocampus.

concentrations of succinate and fumarate, and of proline, were significantly lower in frontal cortex compared to entorhinal cortex and hippocampus, while no differences were observed between entorhinal cortex and hippocampus.

Since mitochondria play a key role in cell bioenergetics, we detected and measured 2-SC as a biomarker of mitochondrial stress in order to test for potential cross-regional differences. 2-SC [S-(2-succino)cysteine] is a chemical modification

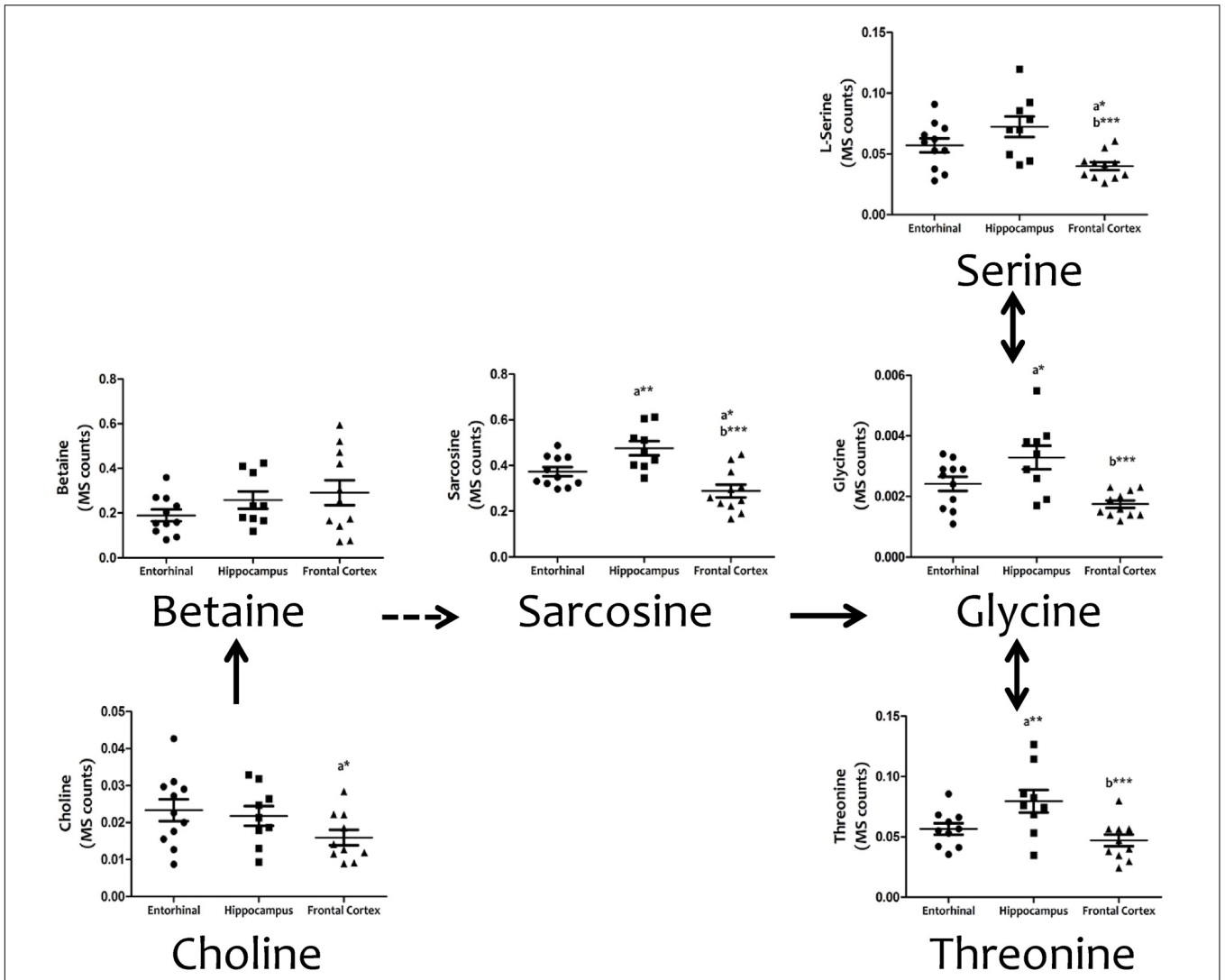


FIGURE 3 | Tissue concentrations of metabolites from the serine, glycine and threonine metabolism in different regions of the adult human cerebral cortex. All metabolites were measured with TQMS. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. a, significant differences with respect to entorhinal cortex; b, significant differences with respect to hippocampus.

of cysteine in proteins by the Krebs cycle intermediate, fumarate, via a succination reaction. Recent studies suggest that succination is a mechanistic link between mitochondrial dysfunction, oxidative and ER stress, and cellular progression toward apoptosis (Merkley et al., 2014). Our results show that the steady-state levels of 2-SC are significantly lower in frontal cortex compared to entorhinal cortex, with no differences between entorhinal cortex and hippocampus (**Figure 2**).

Cell physiology requires the biosynthesis of a diversity of cellular components (including proteins, lipids, and nucleic acids), as well the maintenance of cell redox status, and genetic and epigenetic status. Amino acid metabolism involving serine and glycine, and the carbon units that they provide, covers many of these requirements. **Figure 3** demonstrates the existence of significant inter-regional differences in the concentrations of serine, glycine, and threonine, and related metabolites such as choline, betaine, and sarcosine, thereby verifying that the concentrations of all these metabolites are significantly lower in frontal cortex, and higher in hippocampus, compared to entorhinal cortex.

We then investigated interregional differences of one-carbon metabolism in human cerebral cortex. In particular, we focused our analysis on metabolites belonging to the methionine metabolism (including the methionine cycle and the *trans*-sulfuration pathway) and purine metabolism. To assess these pathways, we measured, with TQMS, the metabolites (directly or closely related to) that follow: (a) for methionine cycle: methionine, *S*-adenosyl-methionine, and homocysteine, as well as spermidine and proline; (b) for the *trans*-sulfuration pathway: cystationine, cysteine, gluthatione, taurine, and vitamin B6 (pyridoxal and pyridoxamine); and

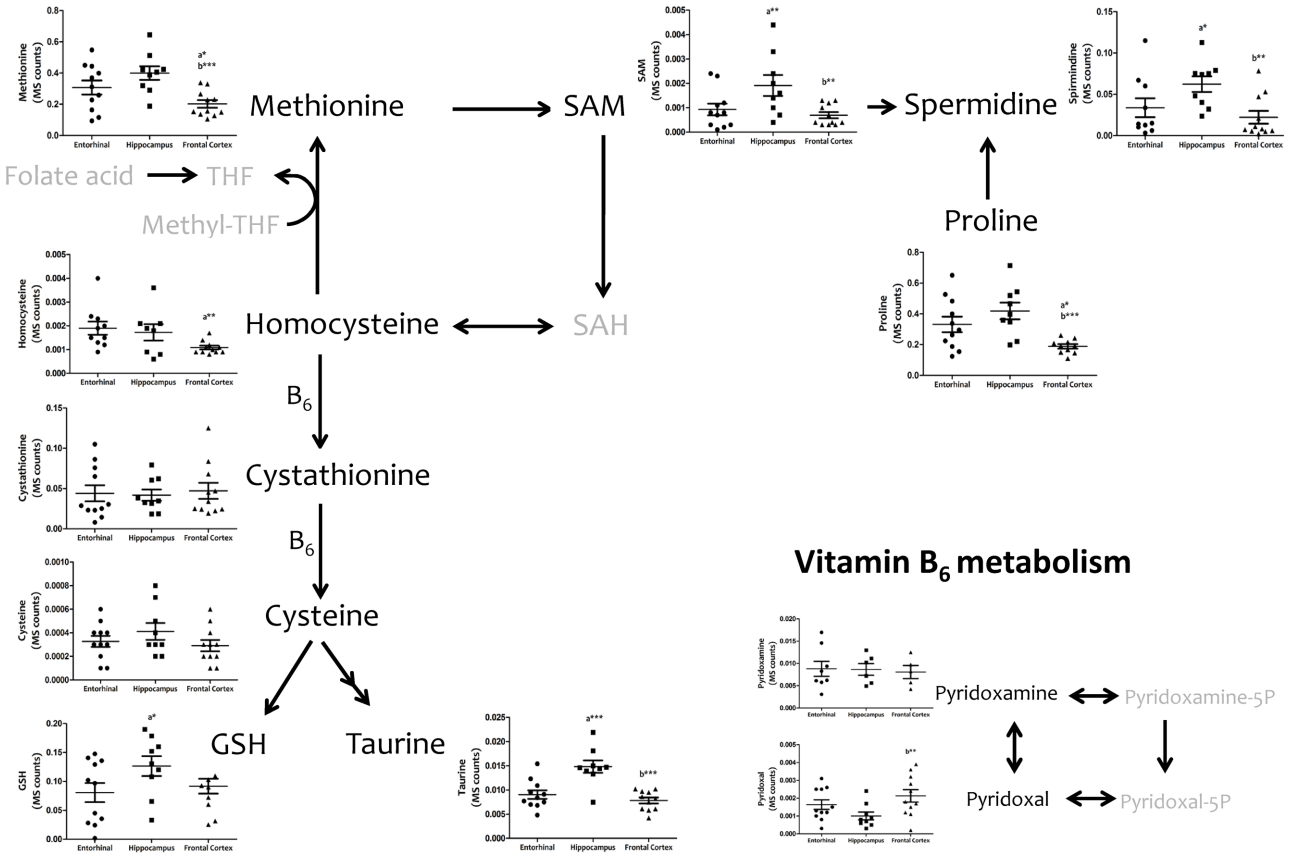


FIGURE 4 | Tissue concentrations of metabolites belonging to the methionine metabolism, including the methionine cycle and the *trans*-sulfuration pathway, in different regions of the adult human cerebral cortex. All metabolites were measured with TQMS. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. a, significant differences with respect to entorhinal cortex; b, significant differences with respect to hippocampus.

finally, (c) for purine metabolism: inosine monophosphate (IMP), adenosine monophosphate (AMP), guanosine monophosphate (GMP), adenosine, inosine, xanthosine, deoxyguanosine, hypoxanthine, and xanthine, and we included the metabolite from pentose phosphate pathway ribose-5-phosphate. TQMS analysis showed a marked decrease in the concentration of all metabolites of the methionine cycle in frontal cortex with respect to hippocampus and/or entorhinal cortex, while significantly higher concentrations of SAM and spermidine in hippocampus compared to entorhinal cortex were detected (**Figure 4**). No interregional changes were detected for metabolites of the *trans*-sulfuration pathway, with the exception of taurine, which was significantly higher in hippocampus compared to entorhinal cortex, and again lower in frontal cortex compared to hippocampus (**Figure 4**). No interregional differences were detected for the metabolites of the purine metabolism, with the exception of xanthosine and hypoxhantine, which were significantly lower in frontal cortex (**Figure 5**).

We then considered whether this interregionally differing metabolic status might be associated with changes in cellular systems linked to stress resistance and cell survival. To this end, we measured the antioxidant enzymes catalase and SOD1, the FOXO transcriptional factor FOXO1, the repressor element 1-silencing transcription factor REST, and the master regulator that senses cell nutrient and energy status, mechanistic target of rapamycin mTOR (**Figure 6**). Western blot analysis showed that catalase and SOD1 were significantly and specifically increased in frontal cortex, that FOXO1 and REST did not show interregional differences, and that the activation of mTOR was significantly lower in frontal cortex.

DISCUSSION

Although metabolic pathways important to brain function are conserved across diverse taxa (Peregrín-Alvarez et al., 2009), current findings show that brain metabolism experienced relevant changes in the human species (Somel et al., 2013). In addition to evolutionary considerations, brain neurons offer a diverse response to stresses during the physiological aging process or as a result of both NDD and NPD (Mattson and Magnus, 2006; Jové et al., 2014; Naudí et al., 2015). The morphological and functional diversity among neuronal cells, the temporal trajectory of functional losses during

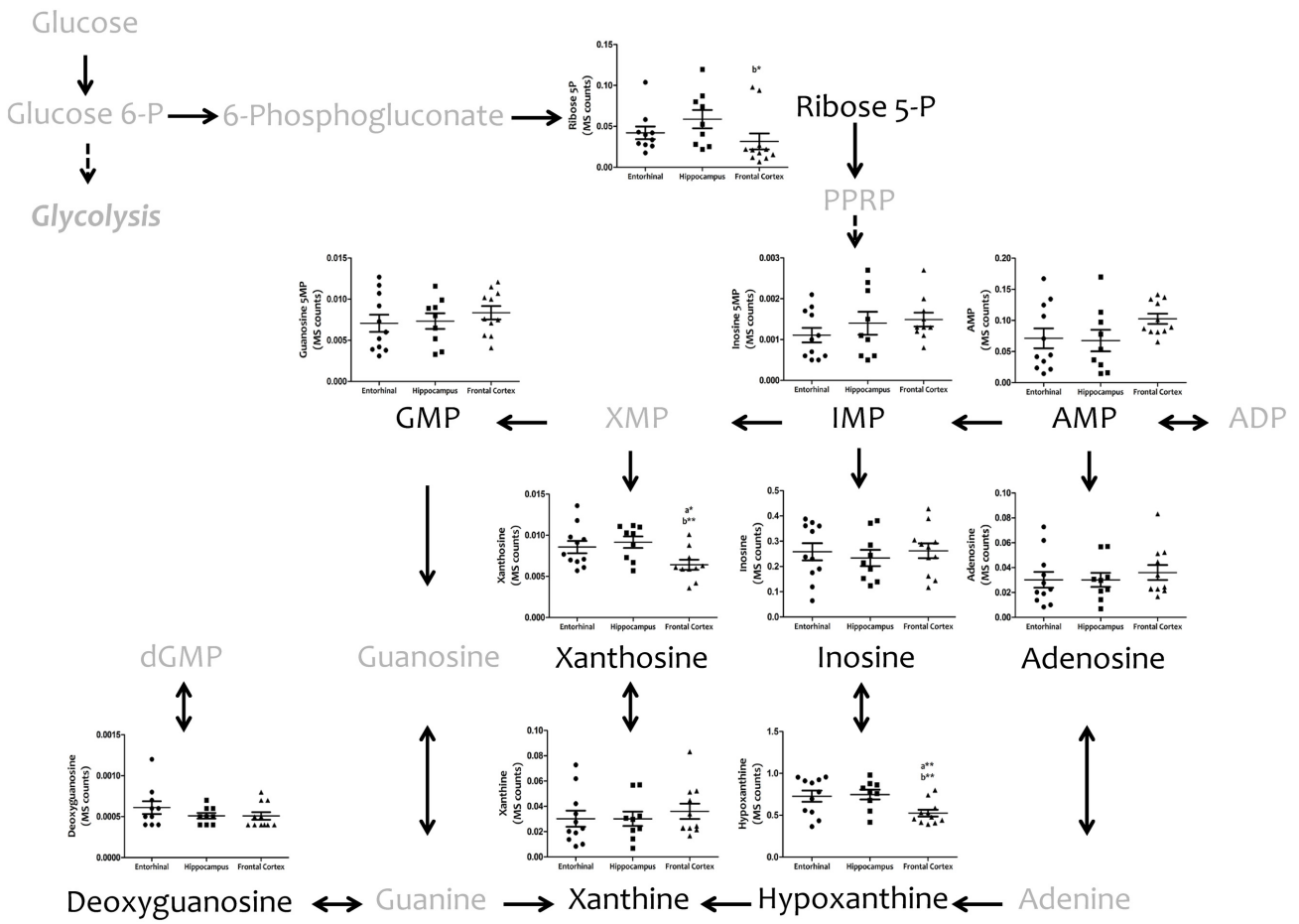


FIGURE 5 | Tissue concentrations of metabolites from the purine metabolism in different regions of the adult human cerebral cortex. All metabolites were measured with TQMS. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. a, significant differences with respect to entorhinal cortex; b, significant differences with respect to hippocampus.

the aging process, and the temporal pattern and specificity in the appearance and development of each NDD and NPD, as well as the heterogeneity in neuronal responses to detrimental processes associated with each of the pathologies, all confirm the existence of a cross-regional SNV (Mattson and Magnus, 2006; Jové et al., 2014; Naudí et al., 2015). This SNV could be expressed through a neuron(region)-specific metabolomic profile. Hence, metabolomics can help to define and improve understanding of cellular (regional) diversity in the CNS. From an inter-regional comparative perspective there is, however, a lack of studies focused on outlining the specific metabolomics of the distinctly functional sub-regions of the brain. To shed light on this, we performed a comparative metabolomic analysis of three healthy human brain regions: entorhinal cortex, hippocampus, and frontal cortex.

Our results show the existence of reduced energy demand, mitochondrial stress, and one-carbon metabolism (particularly restricted to the methionine cycle) specifically in frontal cortex. These findings, along with a better antioxidant capacity and lower mTOR signaling as well in frontal cortex, suggest that this brain region is especially resistant to stress compared to the entorhinal cortex and hippocampus, which are more vulnerable regions.

The one-carbon metabolism can be considered as an integrative network of nutrient status. Thus, inputs in the form of amino acids (which donate carbon units) enter the metabolic network, are metabolized, and then become output for diverse biological functions which include biosynthesis of cell components, regulation of redox status, regulation of methylation reactions, and regulation of nucleotide pools. The partitioning of carbon units into these different cellular outputs basically involves three interconnected pathways: the folate cycle, the methionine cycle, and the *trans*-sulfuration pathway (Locasale, 2013). Several studies have shown that defects in one-carbon metabolism in brain induce deep disturbances in cell physiology as a consequence of the relevant pathways where one-carbon metabolism is involved, and also, more importantly, through the toxic effects derived from the metabolites which shape the core of the methionine cycle. Thus, a connection

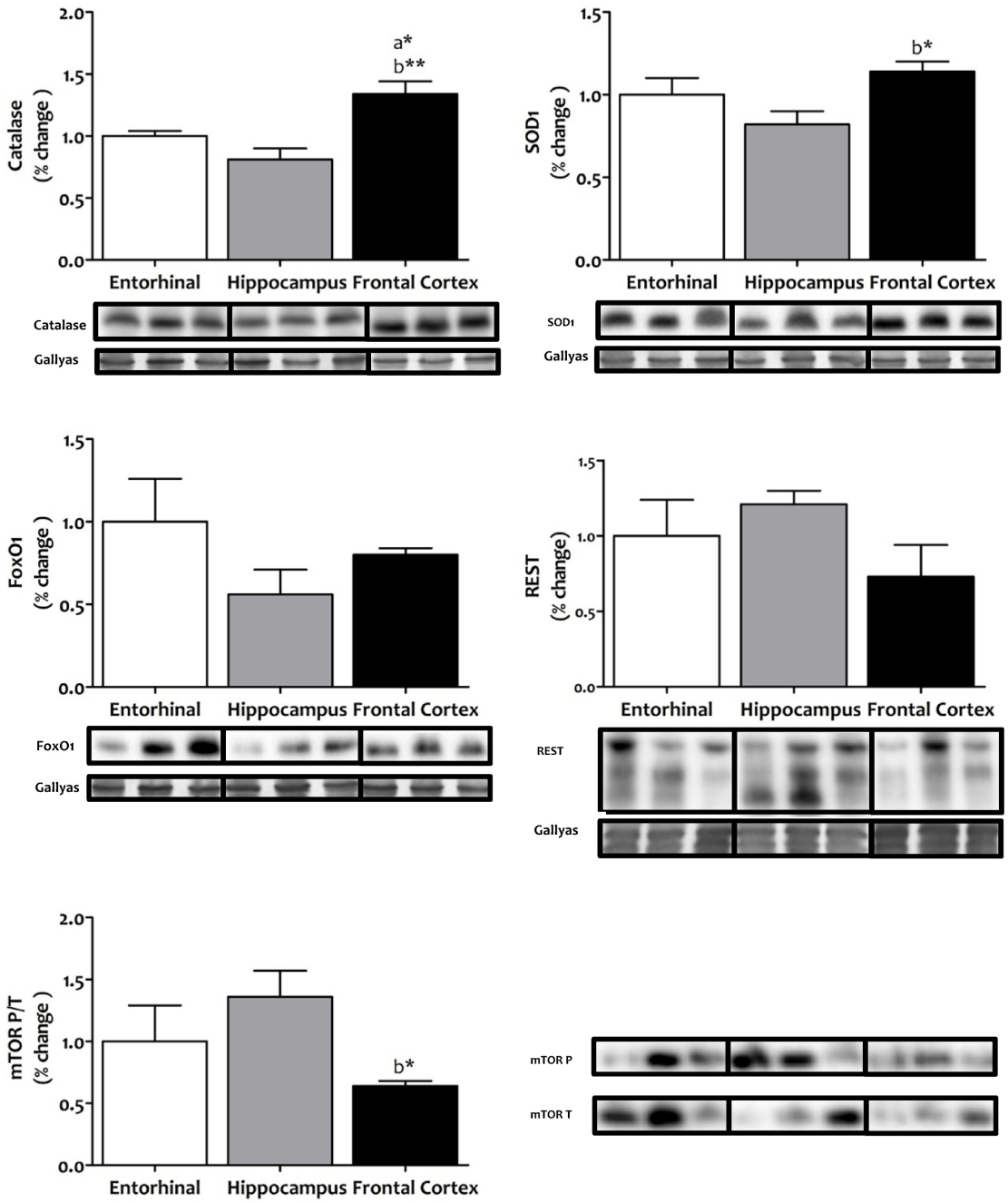


FIGURE 6 | Tissue protein expression of factors associated with stress resistance and cell survival in different regions of the adult human cerebral cortex. The antioxidants catalase and SOD1, the FOXO transcriptional factor FOXO1, the repressor element 1-silencing transcription factor REST, and the master regulator that senses cell nutrient and energy status, mechanistic target of rapamycin mTOR, where all determined with western blot. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. a, significant differences with respect to entorhinal cortex; b, significant differences with respect to hippocampus. All western blots can be found as **Supplementary Figure S1**.

has been established between high levels of homocysteine and cognitive function, from mild cognitive decline to vascular dementia and AD (Miller, 2003). In contrast, low methionine and derived metabolite content, either constitutively or induced by nutritional intervention, is associated with resistance to stress and a longer lifespan (Pamplona and Barja, 2006, 2011; Naudí et al., 2007). Hence, we may infer that the lower one-carbon metabolism observed in frontal cortex is a physiological adaptation which confers resistance to stress on this region.

mTOR is a conserved serine/threonine kinase which regulates metabolism in response to nutrients, growth factors, and cellular energy conditions. Available evidence indicates that the mTOR signaling pathway is involved in brain aging and age-related NDD diseases (Garelick and Kennedy, 2011; Bockaert and Marin, 2015; Perluigi et al., 2015). In this line, an increasing number of studies show that disruption in mTOR signaling in the brain affects multiple pathways including glucose metabolism, energy production, mitochondrial function, and autophagy. Conversely, attenuation of the mTOR signal, through pharmacological or nutritional intervention, increases longevity and is associated with a healthy lifespan, including improvement in brain function (Garelick and Kennedy, 2011; Perluigi et al., 2015). Consequently, we may infer that the lower mTOR signaling observed in frontal cortex is a physiological adaptation which confers resistance to stress on this region.

CONCLUSION

Our results define the existence of metabolomic differences in three different regions of the mature, healthy human brain, confirming the existence of cross-regional differences in the brain. We must note, however, that although our study covers key cellular metabolic pathways, it is far from being comprehensive. Nevertheless, our findings indicate that the metabolomic signature is an optimized feature associated with diversity among neuronal populations in brain cortex, allowing us to hypothesize that the metabolic optimization of some physiological traits, such as resistance to stress, is region-specific. However, it is evident that more studies are needed to draw a metabolomic-wide atlas of metabolites in the adult human brain.

AUTHOR CONTRIBUTIONS

IF and RP designed the experiments. RC, MJ, AN, MD-G, and RP analyzed the data. RC, VA, GP-R, MG-V, EO, RB, NM-M, and MP-O performed the experiments. RP supervised the design and data interpretation. The manuscript was written by IF and RP and edited by AN and RP. All authors discussed the results and commented on the manuscript.

ACKNOWLEDGMENTS

This research was funded by the Spanish Ministry of Economy and Competitiveness, Institute Carlos III (FIS grants PI14/00757 and PI14/00328), and the Autonomous Government of Catalonia (2014SGR69 and 2014SGR168) to IF and RP. This study was co-financed by FEDER funds from the European Union ('A way to build Europe'). RC received predoctoral fellowships from the Autonomous Government of Catalonia. We thank T. Yohannan for editorial help.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnmol.2016.00138/full#supplementary-material

FIGURE S1 | Western blots of tissue protein expression of factors associated with stress resistance and cell survival in different regions of the adult human cerebral cortex.

REFERENCES

Bockaert, J., and Marin, P. (2015). mTOR in brain physiology and pathologies. *Physiol. Rev.* 95, 1157–1187. doi: 10.1152/physrev.00038.2014

Cáceres, M., Lachuer, J., Zapala, M. A., Redmond, J. C., Kudo, L., Geschwind, D. H., et al. (2003). Elevated gene expression levels distinguish human from non-human primate brains. *Proc. Natl. Acad. Sci. U.S.A.* 100, 13030–13035. doi: 10.1073/pnas.2135499100

Chen, G. G., Turecki, G., and Mamer, O. A. (2009). A quantitative GC-MS method for three major polyamines in postmortem brain cortex. *J. Mass Spectrom.* 44, 1203–1210. doi: 10.1002/jms.1597

Domínguez, M., de Oliveira, E., Odena, M. A., Portero, M., Pamplona, R., and Ferrer, I. (2016). Redox proteomic profiling of neuroketal-adducted proteins in human brain: regional vulnerability at middle age increases in the elderly. *Free Radic. Biol. Med.* 95, 1–15. doi: 10.1016/j.freeradbiomed.2016.02.034

Ferrer, I. (2015). Selection of controls in the study of human neurodegenerative diseases in old age. *J. Neural Transm.* 122, 941–947. doi: 10.1007/s00702-014-1287-y

Ferrer, I., Martinez, A., Boluda, S., Parchi, P., and Barrachina, M. (2008). Brain banks: benefits, limitations and cautions concerning the use of post-mortem brain tissue for molecular studies. *Cell Tissue Bank.* 9, 181–194. doi: 10.1007/s10561-008-9077-0

Fu, X., Giavalisco, P., Liu, X., Catchpole, G., Fu, N., Ning, Z.-B., et al. (2011). Rapid metabolic evolution in human prefrontal cortex. *Proc. Natl. Acad. Sci. U.S.A.* 108, 6181–6186. doi: 10.1073/pnas.1019164108

Garelick, M. G., and Kennedy, B. K. (2011). TOR on the brain. *Exp. Gerontol.* 46, 155–163. doi: 10.1016/j.exger.2010.08.030

Hawrylycz, M. J., Lein, E. S., Guillozet-Bongaarts, A. L., Shen, E. H., Ng, L., Miller, J. A., et al. (2012). An anatomically comprehensive atlas of the adult human brain transcriptome. *Nature* 489, 391–399. doi: 10.1038/nature11405

Jové, M., Portero-Otín, M., Naudí, A., Ferrer, I., and Pamplona, R. (2014). Metabolomics of human brain aging and age-related neurodegenerative diseases. *J. Neuropathol. Exp. Neurol.* 73, 640–657. doi: 10.1097/NEN.0000000000000091

Lein, E. S., Hawrylycz, M. J., Ao, N., Ayres, M., Bensinger, A., Bernard, A., et al. (2007). Genome-wide atlas of gene expression in the adult mouse brain. *Nature* 445, 168–176. doi: 10.1038/nature05453

Locasale, J. W. (2013). Serine, glycine and one-carbon units: cancer metabolism in full circle. *Nat. Rev. Cancer* 13, 572–583. doi: 10.1038/nrc3557

Lu, T., Aron, L., Zullo, J., Pan, Y., Kim, H., Chen, Y., et al. (2014). REST and stress resistance in ageing and Alzheimer's disease. *Nature* 507, 448–454. doi: 10.1038/nature13163

Mattson, M. P., and Magnus, T. (2006). Ageing and neuronal vulnerability. *Nat. Rev. Neurosci.* 7, 278–294. doi: 10.1038/nrn1886

Merkley, E. D., Metz, T. O., Smith, R. D., Baynes, J. W., and Frizzell, N. (2014). The succinated proteome. *Mass Spectrom. Rev.* 33, 98–109. doi: 10.1002/mas.21382

Michaelis, T., Helms, G., and Frahm, J. (1996). Metabolic alterations in brain autopsies: proton NMR identification of free glycerol. *NMR Biomed.* 9, 121–124. doi: 10.1002/(SICI)1099-1492(199605)9:3<121::AID-NBM409>3.0.CO;2-F

Miller, A. L. (2003). The methionine-homocysteine cycle and its effects on cognitive diseases. *Altern. Med. Rev.* 8, 7–19.

Mink, J. W., Blumenschine, R. J., and Adams, D. B. (1981). Ratio of central nervous system to body metabolism in vertebrates: its constancy and functional basis. *Am. J. Physiol.* 241, R203–R212.

Naudí, A., Cabré, R., Jové, M., Ayala, V., Gonzalo, H., Portero-Otín, M., et al. (2015). Lipidomics of human brain aging and Alzheimer's Disease pathology. *Int. Rev. Neurobiol.* 122, 133–189. doi: 10.1016/bs.irn.2015.05.008

Naudí, A., Caro, P., Jove, M., Gomez, J., Boada, J., Ayala, V., et al. (2007). Methionine restriction decreases endogenous oxidative molecular damage and increases mitochondrial biogenesis and uncoupling protein 4 in rat brain. *Rejuvenation Res.* 10, 473–484. doi: 10.1089/rej.2007.0538

Naudí, A., Jové, M., Cacabelos, D., Ayala, V., Cabre, R., Caro, P., et al. (2013). Formation of S-(carboxymethyl)-cysteine in rat liver mitochondrial proteins: effects of caloric and methionine restriction. *Amino Acids* 44, 361–371. doi: 10.1007/s00726-012-1339-2

Opstad, K. S., Wright, A. J., Bell, B. A., Griffiths, J. R., and Howe, F. A. (2010). Correlations between in vivo 1 H MRS and ex vivo 1 H HRMAS metabolite measurements in adult human gliomas. *J. Magn. Reson. Imaging* 31, 289–297. doi: 10.1002/jmri.22039

Pamplona, R., and Barja, G. (2006). Mitochondrial oxidative stress, aging and caloric restriction: the protein and methionine connection. *Biochim. Biophys. Acta* 1757, 496–508. doi: 10.1016/j.bbabio.2006.01.009

Pamplona, R., and Barja, G. (2011). An evolutionary comparative scan for longevity-related oxidative stress resistance mechanisms in homeotherms. *Biogerontology* 12, 409–435. doi: 10.1007/s10522-011-9348-1

Peregrín-Alvarez, J. M., Sanford, C., and Parkinson, J. (2009). The conservation and evolutionary modularity of metabolism. *Genome Biol.* 10:R63. doi: 10.1186/gb-2009-10-6-r63

Perluigi, M., Di Domenico, F., and Butterfield, D. A. (2015). mTOR signaling in aging and neurodegeneration: at the crossroad between metabolism dysfunction and impairment of autophagy. *Neurobiol. Dis.* 84, 39–49. doi: 10.1016/j.nbd.2015.03.014

Perry, T. L., Hansen, S., and Gandham, S. S. (1981). Postmortem changes of amino compounds in human and rat brain. *J. Neurochem.* 36, 406–410. doi: 10.1111/j.1471-4159.1981.tb01608.x

Petroff, O. A., Ogino, T., and Alger, J. R. (1988). High-resolution proton magnetic resonance spectroscopy of rabbit brain: regional metabolite levels and postmortem changes. *J. Neurochem.* 51, 163–171. doi: 10.1111/j.1471-4159.1988.tb04850.x

Sherwood, C. C., Stimpson, C. D., Raghanti, M. A., Wildman, D. E., Uddin, M., Grossman, L. I., et al. (2006). Evolution of increased glia-neuron ratios in the human frontal cortex. *Proc. Natl. Acad. Sci. U.S.A.* 103, 13606–13611. doi: 10.1073/pnas.0605843103

Somel, M., Liu, X., and Khaitovich, P. (2013). Human brain evolution: transcripts, metabolites and their regulators. *Nat. Rev. Neurosci.* 14, 112–127. doi: 10.1038/nrn3372

Uddin, M., Wildman, D. E., Liu, G., Xu, W., Johnson, R. M., Hof, P. R., et al. (2004). Sister grouping of chimpanzees and humans as revealed by genome-wide phylogenetic analysis of brain gene expression profiles. *Proc. Natl. Acad. Sci. U.S.A.* 101, 2957–2962. doi: 10.1073/pnas.0308725100

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Molecular Neuroscience

ORIGINAL RESEARCH
published: 09 March 2017
doi: 10.3389/fnmol.2017.00044

Cerebrospinal Fluid Cortisol Mediates Brain-Derived Neurotrophic Factor Relationships to Mortality after Severe TBI: A Prospective Cohort Study

Miranda J. Munoz[1,2], *Raj G. Kumar*[1,3], *Byung-Mo Oh*[1,4], *Yvette P. Conley*[1,3], *Zhensheng Wang*[5,6], *Michelle D. Failla*[7] *and Amy K. Wagner*[1,6,8,9]*

[1] Department of Physical Medicine and Rehabilitation, University of Pittsburgh, Pittsburgh, PA, USA, [2] Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA, USA, [3] Department of Epidemiology, University of Pittsburgh, Pittsburgh, PA, USA, [4] Department of Rehabilitation Medicine, Seoul National University Hospital, Seoul, South Korea, [5] Department of Nursing, University of Pittsburgh, Pittsburgh, PA, USA, [6] Safar Center for Resuscitation Research, University of Pittsburgh, Pittsburgh, PA, USA, [7] Department of Psychiatry, Vanderbilt University Medical Center, Nashville, TN, USA, [8] Department of Neuroscience, University of Pittsburgh, Pittsburgh, PA, USA, [9] Center for Neuroscience, University of Pittsburgh, Pittsburgh, PA, USA

Distinct regulatory signaling mechanisms exist between cortisol and brain derived neurotrophic factor (BDNF) that may influence secondary injury cascades associated with traumatic brain injury (TBI) and predict outcome. We investigated concurrent CSF BDNF and cortisol relationships in 117 patients sampled days 0–6 after severe TBI while accounting for BDNF genetics and age. We also determined associations between CSF BDNF and cortisol with 6-month mortality. *BDNF* variants, rs6265 and rs7124442, were used to create a gene risk score (GRS) in reference to previously published hypothesized risk for mortality in "younger patients" (<48 years) and hypothesized BDNF production/secretion capacity with these variants. Group based trajectory analysis (TRAJ) was used to create two cortisol groups (high and low trajectories). A Bayesian estimation approach informed the mediation models. Results show CSF BDNF predicted patient cortisol TRAJ group ($P = 0.001$). Also, GRS moderated BDNF associations with cortisol TRAJ group. Additionally, cortisol TRAJ predicted 6-month mortality ($P = 0.001$). In a mediation analysis, BDNF predicted mortality, with cortisol acting as the mediator ($P = 0.011$), yielding a mediation percentage of 29.92%. Mediation effects increased to 45.45% among younger patients. A BDNF*GRS interaction predicted mortality in younger patients ($P = 0.004$). Thus, we conclude 6-month mortality after severe TBI can be predicted through a mediation model with CSF cortisol and BDNF, suggesting a regulatory role for cortisol with BDNF's contribution to TBI pathophysiology and mortality, particularly among younger individuals with severe TBI. Based on the literature, cortisol modulated BDNF effects on mortality after TBI may be related to known hormone and neurotrophin relationships to neurological injury severity and autonomic nervous system imbalance.

Keywords: cortisol, BDNF, TBI, biomarkers, genetics, cerebrospinal fluid

OPEN ACCESS

Edited by:
Susan A. Masino,
Trinity College, USA

Reviewed by:
Weiwen Wang,
Institute of Psychology (CAS), China
Chris Dulla,
Tufts University School of Medicine, USA
Munjal M. Acharya,
University of California, Irvine, USA

***Correspondence:**
Amy K. Wagner
wagnerak@upmc.edu

Received: *23 October 2016*
Accepted: *09 February 2017*
Published: *09 March 2017*

Citation:
Munoz MJ, Kumar RG, Oh B-M, Conley YP, Wang Z, Failla MD and Wagner AK (2017) Cerebrospinal Fluid Cortisol Mediates Brain-Derived Neurotrophic Factor Relationships to Mortality after Severe TBI: A Prospective Cohort Study. Front. Mol. Neurosci. 10:44. doi: 10.3389/fnmol.2017.00044

INTRODUCTION

Although death rates have decreased over time, ∼2.5 million Americans experience traumatic brain injury (TBI) yearly, with more than 50,000 associated fatalities (Centers for Disease Control Prevention, 2016). TBI results in several acute secondary injury cascades, that include aseptic inflammation (Kumar et al., 2015, 2016), excitotoxicity (Wagner et al., 2005), monaminergic dysfunction (Wagner et al., 2007), neurotrophin abnormalities (Failla et al., 2016), and stress induced steroidogenesis (Wagner et al., 2011a; Santarsieri et al., 2014) that result in blood brain barrier disruption and CNS damage (Wagner et al., 2011b; Goyal et al., 2013); representative biomarkers for each of these pathways have been identified in CSF and serum for clinical populations with moderate/severe TBI. However, individual biomarker studies have limitations with predicting therapeutic treatment response (Maas et al., 2010), which may be due to the focus of current research on single biomarker relationships, rather than the interactions of several. Further, many clinical intervention studies have not included biomarker characterization to assess treatment effects.

Under certain conditions glucocorticoids, cortisol in humans and corticosterone in animals, can be neuroprotective, anti-inflammatory, and anticonvulsive, in order to restore homeostasis after injury, as the end product of the stress-activated HPA axis (McEwan, 1999; Jeanneteau et al., 2008; Joëls, 2008). Based on this premise, the Corticosteroid Randomization after Significant Head Injury (CRASH) trial administered the glucocorticoid methylprednisolone acutely after TBI. This trial was stopped prematurely when treatment had a higher mortality rate than placebo (Roberts et al., 2004). Recent work suggests elevated cortisol levels, when linked to prolonged stress, can impair synaptic plasticity and can result in neuronal cell death (Antonawich et al., 1999; Rothman and Mattson, 2013). Also, our previous work shows endogenous CSF cortisol levels are markedly increased after TBI, and we showed that sustained elevations in CSF cortisol profiles are associated with poorer outcomes (Santarsieri et al., 2014).

Brain derived neurotrophic factor (BDNF) is well-known for its roles in neuronal survival, neuronal maintenance, and neural plasticity. Despite these benefits, BDNF administration after experimental TBI was not protective against structural or functional deficits (Blaha et al., 2000). Subsequent work suggests that BDNF effects on TBI pathophysiology may be target receptor dependent, with the pro-apoptotic p75 receptor upregulation post-injury contributing to cell death (Rostami et al., 2014; Sebastiani et al., 2015). Our previous clinical work suggests CSF BDNF levels are elevated after TBI and associated with earlier time until death (Failla et al., 2016).

Brain derived neurotrophic factor (BDNF) and cortisol regulation are interconnected via multiple mechanisms (Rothman and Mattson, 2013), and levels of each are modified under stress, which are relevant in the context of TBI, a major pathophysiological stressor. While the exact pathways by which cortisol and BDNF interact are not completely known, some work shows glucocorticoid response elements are located in the *BDNF* gene promoter region, suggesting transcriptional control over BDNF production (Rothman and Mattson, 2013). Other indirect mechanisms propose glucocorticoid regulation of BDNF through CREB, MAPK/ERK, or Shp2 (Kumamaru et al., 2008, 2011; Alboni et al., 2011). BDNF, through synaptic modulation in the nucleus of the solitary tract (Clark et al., 2011) and hypothalamus (Tapia-Arancibia et al., 2004), may also regulate HPA-axis reactivity and cortisol releasing hormone (Jeanneteau et al., 2012). Interactive signaling between BDNF and cortisol may significantly influence HPA-reactivity immediately after TBI. The literature on regulatory effects between cortisol and BDNF is sparse, yet one experimental TBI study, involving young adult rats, does suggest that both injury and adrenalectomy independently resulted in acute (within 4 h of injury) elevations in hippocampal BDNF mRNA expression, and the effects of both TBI and adrenalectomy on BDNF elevations are cumulative. Alternatively, corticosterone replacement prevented this increase among adrenalectomized rats (Grundy et al., 2000). Together, these results suggest that cortisol can have acute modulatory effects that limit increased BDNF expression after TBI. Similar effects are noted with NT3 (Grundy et al., 2001).

Both cortisol and BDNF exhibit age-dependent changes that may impact their role with damage and recovery after TBI, especially as older age increases risk for mortality after TBI (Roozenbeek et al., 2012). Positive correlations between high cortisol levels and older age are likely due to age-associated HPA axis changes, including decreased cortisol clearance and impaired glucocorticoid negative feedback (Ferrari et al., 2001; Pal et al., 2014). Older age is also linked to lower BDNF levels (Erickson et al., 2012) and relative brain increases in the pro-apoptotic p75 receptor (Webster et al., 2006; Tapia-Arancibia et al., 2008). In populations with TBI, both cortisol and BDNF levels vary by age (Santarsieri et al., 2014; Failla et al., 2016), and our previous work characterizes interactions between *BDNF* gene variation and age that predict mortality post-TBI, wherein risk variants at a particular gene locus differ by age (Failla et al., 2016).

The, *BDNF* genotypes (Val/Val) for rs6265 causes an amino-acid substitution of valine to methionine at amino-acid residue 66 (Val66Met) (Tapia-Arancibia et al., 2004). This substitution alters intracellular trafficking and packaging of pro-BDNF and secretion of the mature peptide (Egan et al., 2003). Also, rs7124442 (T/T homozygotes) are associated with activity dependent BDNF secretion and BDNF mRNA trafficking (Egan et al., 2003; Orefice et al., 2013). In our previous work (Failla et al., 2015), a gene risk score (GRS) was used, ranking genetic variants associated with decreased BDNF production or secretion, as risk variants for mortality after TBI. These high risk genotypes were associated with increased mortality only among younger

Abbreviations: BBB, blood brain barrier; BDNF, brain-derived neurotropic factor; CREB, cAMP response element binding protein; ELISA, enzyme-linked immunosorbent assay; GCS, Glasgow Coma Scale; GOS, Glasgow Outcome Scale; GRS, gene risk score; HPA axis, hypothamic-pituitary-adrenal axis; HRV, heart rate variability; MAPK/ERK, microtubule associated protein kinase/extracellular signal-regulated kinases; NT3, Neurotrophin-3; RCS, restricted cubic spline analysis; RIA, radioimmunoassay; Shp2, Src Homology Phosphatase 2; SNP, single nucleotide polymorphism; TBI, traumatic brain injury; TRAJ, group-based trajectory analysis; TrkB, tropomyosin receptor kinase B.

individuals with TBI (age <45 years). However, for the low-risk genotype there was an interaction by age, wherein younger individuals with low-risk variants (high secretion/trafficking) were protected against mortality, while older individuals with low-risk variants were at an increased risk for mortality after TBI.

The literature evaluating cortisol actions in acute TBI pathophysiology among clinical populations are limited. Our previous work demonstrates that serum cortisol levels are elevated over the first week after severe TBI, particularly among older individuals, as a physiological response to the acute stress of associated with severe injury and associated critical illness (Wagner et al., 2011a). Although, some develop acute adrenal insufficiency and a relative state of hypocortisolemia after injury (Wagner et al., 2011a). In contrast, our previous work also shows that acute CSF cortisol profiles are ∼8–10X that observed among healthy controls over the first week after severe TBI. Further, persistent high acute CSF cortisol levels are associated both increased mortality as well as worse survivor based outcomes (Santarsieri et al., 2014). To date there are no clinical studies delineating possible regulatory effects cortisol may have on BDNF in the context of TBI.

Given the regulatory relationships between CORT and BDNF, and their known individual relationships to TBI outcome, we hypothesized that CSF cortisol would mediate CSF BDNF associations with 6-month mortality in a model that also considered age and BDNF genetics (e.g., as covariates or as points of cohort stratification). We also hypothesized that *BDNF* genetics would moderate the relationship between CSF BDNF and cortisol trajectories. Our results confirm these hypotheses and showed that CSF BDNF predicts, and BDNF GRS moderates, patient cortisol group-based trajectory analysis (TRAJ) group membership. Also, cortisol TRAJ predicted 6-month mortality. In mediation analysis, BDNF predicted mortality, with cortisol acting as the mediator, and CSF cortisol mediation effects were stronger among younger individuals (age <48 years) compared to older individuals. Together, the findings suggest a possible regulatory role for cortisol with BDNF's contribution to TBI pathophysiology and mortality, particularly among younger individuals with severe TBI.

MATERIALS AND METHODS

Participants

This study was approved by the University of Pittsburgh Institutional Review Board, and this cohort represents a subset of patients enrolled in a larger study examining biomarker and genetic relationships to outcome after TBI. Participants were 16–73 years old and had a diagnosis of severe TBI [GCS≤8, with positive findings consistent with TBI on CT scan]. Self-reported non-White individuals were excluded as BDNF levels and genetics vary based on race (Freedman et al., 2004; Nettiksimmons et al., 2014). As shown in **Figure 1**, 216 participants had either BDNF *or* acute cortisol measurements; specifically, 185 had BDNF and 155 had cortisol. There were 131 participants with both BDNF and cortisol. Fourteen were excluded due to missing *BDNF* genotyping, GCS or mortality data. The final sample size was 117 participants with TBI. CSF

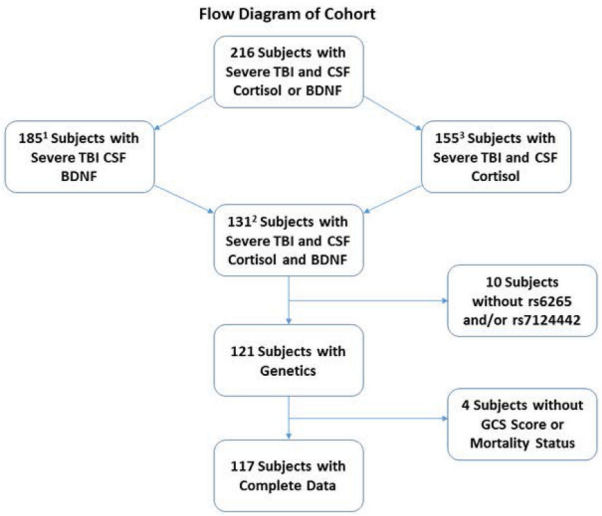


FIGURE 1 | Diagram depicting how the cohort was derived for analysis. The cohort was restricted to self-reported White individuals with complete demographic, clinical, genetics and biomarker data. There were a total of 216 White severe TBI subjects with CSF Cortisol and BDNF. Of these, 185 and 155 subjects had CSF BDNF and cortisol, respectively. A total of 131 subjects had both CSF cortisol and BDNF. A total of 14 subjects were removed for missing BDNF genetics or GCS or mortality data, leaving a final cohort of 117.
[1]Used for estimating prior distributions in the mediation analysis path for BDNF-mortality (total effect).
[2]Used for estimating prior distributions in the mediation analysis path of BDNF-cortisol, cortisol-mortality, and BDNF-mortality (direct effect).
[3]Used to generate CSF cortisol trajectories in the most inclusive cohort with CSF cortisol.

from 13 healthy subjects was obtained and used to generate a control reference level for cortisol.

CSF Sample Collection and Processing

Brain-derived neurotropic factor (BDNF) and Cortisol were measured from CSF samples collected the first week post-injury. Samples were collected up to twice daily via EVD (extraventricular drainage) catheter placed for routine clinical care. For our injury cohort, 455 CSF samples were analyzed for BDNF, and 400 CSF samples were analyzed for cortisol. Healthy control subjects ($N = 13$) had CSF collected via lumbar puncture at ∼7 a.m. After collection, samples were stored at 4°C until processing. Samples were then centrifuged, aliquoted, and stored at −80°C until batch analysis. CSF BDNF levels were measured via ELISA kit (RayBiotech), previously detailed (Failla et al., 2016). The inter/intra-assay coefficient of variation (CV) was <10% and <12%, respectively; assay sensitivity was 80 pg/mL.

CSF cortisol levels were measured in with solid-phase 125I RIA, Coat-A-Count® *in vitro* Diagnostic Test Kit (Siemens Healthcare Diagnostics). Inter- and intra-assay CVs were <10%. Samples with levels below the given range were assigned the detection limit value, and samples with undetectable levels were assigned values of 0.001 for analysis purposes. Additional samples were measured with a commercial ELISA kit (1-3002, Salimetrics) according to manufacturer instructions, with some adaptation for CSF measurement. To avoid matrix effects and to

fit within the range of the kit, a 1:4 sample dilution was used. The inter-/intra-assay CV was <10% and <16%, respectively. Validation experiments with CSF also showed excellent linearity with serial dilution and recovery from spiking (90%–110%). Additionally, we measured cortisol levels by ELISA in 20 CSF samples previously measured by RIA to determine the correlation between the two measurement methods. A linear regression fit the relationship between the measured concentrations by RIA and ELISA. The ELISA results were then converted using this equation ($C_{RIA} = 6.32 + 0.76 \times C_{ELISA}$) before pooling data for analyses.

Mortality

Six-month mortality status was examined using survival analysis. GOS scores were obtained by research-trained neuropsychometrists blinded to genetic and biomarker information. The Social Security Death Index (http://www.genealogybank.com/gbnk/ssdi/) was utilized to determine mortality status, and time post-injury until death.

Genotyping and SNP Selection

DNA was either isolated from blood using a simple salting out procedure or from CSF using the Qiamp DNA extraction protocol (Qiagen). Two *BDNF* SNPs were genotyped by TaqMan allele discrimination assay using commercial Assay-on-Demand reagents (Applied Biosystems Incorporated). Genotype data calls from two individuals, blinded to all phenotype data, were compared; discrepancies were settled by reviewing the raw data and rerunning samples when necessary. Hardy-Weinberg equilibrium was confirmed for all allele frequencies.

Brain derived neurotrophic factor *(BDNF)* rs6265 and rs7124442 were selected based on previous data correlating these SNPs to mortality. Both rs6265 and rs7124442 have been reported as functional with a minor allele frequency of 38.9 and 39.1%, respectively. Each SNP represents a different haplotype block of BDNF covering variation corresponding to BDNF isoform A (Failla et al., 2015). A risk allele count, GRS, was used as previously published (Failla et al., 2015) with the hypothesized risk alleles, for young individuals with TBI, being rs6265 Met and rs7124442 C carriers (Egan et al., 2003; Orefice et al., 2013). The GRS is fundamentally based upon the idea that genetic risk is cumulative. The cumulative GRS for the two identified *BDNF* variants ranged from 0 to 2 and was calculated by summing the number of risk alleles for these two SNPs. GRS = 0 was considered the no risk group (Val/Val, T/T), GRS = 1 contained carriers for one risk allele (Val/Val, C-carrier or T/T, Met-carrier), and GRS = 2 included carriers of both risk alleles (Met-carrier, C-carrier). This method for GRS formulation is consistent with GRIPS (Genetic RIsk Prediction Studies) guidelines (Janssens et al., 2011).

Statistical Analysis

Analysis was performed with SAS (Statistical Analysis Software) version 9.4 and the SPSS (Statistical Package for Social Sciences) version 23. Descriptive statistics included means, standard error of the mean (SEM), and frequencies. Mean comparisons for CSF biomarkers and continuous demographic and clinical variables were assessed using the Mann-Whitney *U*-test. Associations between categorical demographic and clinical variables by survival status were conducted using the Chi-square test, or Fisher's exact test (if applicable). The daily levels for CSF BDNF and cortisol were also graphically represented by survival status. Weekly average for BDNF were calculated by survival status and GRS.

Trajectory Analysis

To assess temporal (repeated measures) CSF cortisol profiles, we applied TRAJ (Niyonkuru et al., 2013) as previously reported (Santarsieri et al., 2014, 2015) to describe multiple longitudinal patterns of change in order to identify distinct subgroups within the population. For cortisol, TRAJ was conducted on natural log transformed values from 7 time points (D0–D6 post-TBI). Cortisol group TRAJ membership identified individuals with "*high*" vs. "*low*" cortisol level over this time frame.

For BDNF levels, we initially applied TRAJ procedures and found an unbalanced group membership (>85% participants belonged to the "*low*" group), indicating poor discrimination of participants into homogeneous subgroups for longitudinal BDNF levels. Also, upon inspecting daily levels, we observed stable BDNF concentrations over time; given the minimal temporal variation, we characterized mean BDNF above/below the 75th percentile for analysis.

A Bayesian Approach to Inform and Internally Validate Mediation Model

Attrition in sample size was a concern as the primary analysis was restricted to subjects with samples of BDNF, cortisol, BDNF genetics, GCS, and age. To protect against a selection bias of only using information from subjects with complete data, we used prior knowledge of the BDNF/mortality association to inform the current study analysis. Specifically, we applied the Bayes estimation using Monte Carlo Markov Chain (MCMC) method to assess primary relationships. The MCMC method, a stochastic procedure that estimates parameters of interest using random generated samples, can be used with Bayes estimation (Hamra et al., 2013). Using the MCMC method, we obtained exact parameter distributions of interest instead of asymptotic normal distribution requiring a medium or large sample size under maximum likelihood estimation. Final Bayes estimates rested between the prior distribution and the current effect size obtained from the study cohort for our mediation analysis. The MCMC procedure incorporates re-sampling of regions from prior distributions, to increase estimate precision and validity.

Mediation and Regression Analyses

Mediation is used to test whether a factor is in the causal pathway between an exposure of interest and a selected outcome. We hypothesized that cortisol trajectory can mediate the relationship between BDNF and mortality. To formally test the hypothesis, we applied the Baron and Kenny method of mediation analysis (Baron and Kenny, 1986). In this method, a series of regression models were performed to adjust for other potential confounders.

To examine whether the association between acute CSF BDNF levels and mortality was mediated by acute CSF cortisol levels, we established if the following four criteria for mediation effects were met: (1) BDNF levels were associated with mortality (total effect); (2) BDNF levels were associated with cortisol levels; (3) cortisol levels were associated with mortality after adjusting for BDNF levels; and (4) the association between BDNF levels and mortality was attenuated after adjusting for cortisol level (indirect effect).

We performed logistic regression to evaluate the significance of criteria 2, and generated Cox proportional hazard models to evaluate the significance of criteria 1, 3, and 4. Time of follow-up was computed from injury and death date or 180 days (6-months) after injury, which represents the right censored time point. Regression and mediation analyses were performed for the entire population and after stratifying the population at the 75th percentile for age. Cox proportional hazard models were adjusted for binary 75th percentile age (<48 vs. ≥48 years old), GCS and GRS.

To investigate the moderating effects of GRS on BDNF level associations with cortisol TRAJ group, we performed a logistic regression model, stratified by GRS group (0 vs. 1 vs. 2). A two-way interaction term between BDNF level and GRS was created and fit into each model to assess the statistical significance of interactions.

The mediation percentage was calculated by considering the natural logarithm (ln) of the odds (or hazard) ratios (OR) with the following equation: Mediation percentage $= \{[\ln(OR_{TotalEffect}) - \ln(OR_{DirectEffect})]/\ln(OR_{TotalEffect})\}^{*}100\%$. Conceptually, the relationship between BDNF and mortality may be mediated by cortisol TRAJ, but this effect typically does not account for 100% of that relationship, and there may be other unmeasured factors that also mediate the relationship to some degree. The direct effect, corresponds to the effect of BDNF on mortality, adjusting for cortisol TRAJ. That is, if the mortality relationship through cortisol TRAJ is removed, the remaining effect represents BDNF effects on mortality without adjustment for cortisol TRAJ. The indirect effect, or the mediation effect, is derived by subtracting the total effect from the direct effect.

RESULTS

Cohort Demographics

There were 117 individuals with TBI in the final analytic sample (**Figure 1**). The clinical and demographics for the whole cohort, and stratified by survival is provided in **Table 1**. The age range for this cohort was 16–74, with the mean age 36 years old. Most subjects were men (85.3%). The median GCS was 7. The primary mechanisms of injury were automobile (52.2%) and motorcycle accidents (24.3%). The mean non-head Injury Severity Score was 13.26, while the average length of acute hospital stay was 21.2 days. The most prevalent neurological injury types were subarachnoid hemorrhage (SAH, 70.1%) and subdural hematoma (SDH, 69.2%). Individuals with TBI that were non-survivors were significantly older, had lower GCS scores (more severe injuries), fall mechanism of injuries, shorter length of stays, and were more likely to have contusions and less likely to have DAI in CT scans ($p = 0.05$ for all comparisons). No other demographic and clinical variables were significantly different by survival status.

CSF Cortisol Trajectory Profile

Trajectory analysis identified two different TRAJ group profiles: *high* and *low* CSF cortisol (**Figure 2**). CSF cortisol levels for the *high* group were higher than the *low* group on days 1–6 post-injury ($p < 0.01$ all comparisons). CSF cortisol levels in the *high* group peaked on day 2 post-injury and then declined, while levels for the *low* group were highest on day 1, followed by a modest decrease in levels. Also *high* CSF cortisol TRAJ group was associated with increased mortality ($P = 0.001$) when adjusting for age (<48 vs. ≥ 48 years old), GCS, and GRS. In the *high* cortisol TRAJ group, there were significant differences in CSF cortisol levels over time, with significant differences noted for day 0 vs. days 1–6 ($P = 0.012$ all comparisons). In the *low* cortisol TRAJ group, we observed a stably low level from day 0 to 6 with a small spike from day 0 to day 1 and a subsequent decline. There was no significant difference of average day 1–6 levels compared with day 0 ($P = 0.219$).

Characterization of Cortisol and BDNF Profiles by Survival Status

In **Figure 3A**, daily cortisol levels were graphed by survival status. In days 2, 3, and 4, non-survivors had significantly elevated cortisol levels compared to survivors ($p < 0.05$ all comparisons). Daily levels of CSF BDNF were plotted by survival status (**Figure 3B**). Levels were significantly elevated among non-survivors compared to survivors at day 3 and 5 ($p < 0.05$). There was a trend toward lower BDNF levels for non-survivors compared to survivors at day 0 ($p = 0.078$). No other day was significantly different with respect to BDNF levels by survival status.

Cortisol Trajectory Mediates BDNF Associations with Mortality

We obtained prior knowledge of the coefficient of each corresponding pathway in the mediation analysis from our original study cohort of all subjects with BDNF ($n = 185$). The summary of the prior knowledge used is provided in **Table 2**. Prior information showed those with a mean CSF BDNF level ≥75th percentile were more often in the *high* cortisol TRAJ group ($HR = 4.12$, 95% CI: 1.86, 9.24, $P = 0.001$), after adjusting for age, GCS and GRS. Mediation analysis, adjusting for binary age, GCS, and GRS, showed CSF BDNF levels dichotomized at the 75th percentile were associated with mortality (total effect) ($HR = 1.94$, 95% CI: 1.19, 3.12, $P = 0.011$). The CSF BDNF-mortality association was attenuated after adjusting for CSF cortisol TRAJ membership (direct effect) ($P = 0.106$), possibly suggesting a full mediation of CSF BDNF-mortality association through CSF cortisol. However, the proportion of mediation was ∼30% (see **Table 3**); therefore, we report partial mediation effects of CSF cortisol. The mediation effect was stronger among younger individuals (≤48 years old) (mediation ∼45%) when the population was stratified and evaluated in separate models (**Table 3**). There were no significant mediation effects among older individuals, although this group has a small sample size ($N = 27$).

TABLE 1 | Demographic and clinical characterization of the cohort.

	Overall ($n = 117$)	Survivors ($n = 91$)	Non-survivors ($n = 26$)	*P*-value
Age in years (mean ± SE)	36.0 ± 1.5	32.40 (1.4)	48.42 (3.7)	**<0.001***
Sex, Men (%)	99 (85.3)	77 (85.6)	22 (84.6)	0.905
GCS, median (IQR)	7 (6-7)	7 (6-8)	6 (6-7)	**0.043***
Mechanism of injury, *n* (%)				**0.034***
Automobile	60 (52.2)	41 (45.6)	7 (26.9)	
Motorcycle	28 (24.3)	22 (24.4)	6 (23.1)	
Fall/jump	19 (16.5)	10 (11.1)	9 (34.6)	
Other	8 (7.0)	17 (18.9)	4 (15.4)	
Length of hospital stay in days (mean ± SE)	21.2 ± 1.0	23.01 (1.1)	14.68 (1.7)	**0.002***
Injury Severity Score Non-head (mean ± SE)	13.26 (1.0)	12.99 (1.0)	14.18 (2.3)	0.822
Neurological injury type, *n* (%)				
SDH	81 (69.2)	60 (65.9)	21 (80.8)	0.148
DAI	36 (30.8)	33 (36.3)	3 (11.5)	**0.016***
EDH	14 (12.0)	11 (12.1)	3 (11.5)	0.939
Contusion	52 (44.4)	35 (38.5)	17 (65.4)	**0.015***
IVH	39 (33.3)	32 (35.2)	7 (30.0)	0.432
ICH	40 (34.2)	30 (33.0)	10 (38.5)	0.602
SAH	82 (70.1)	61 (67.0)	21 (80.8)	0.177
rs6265, *n* (%)				0.617
Val/Val	68 (58.1)	54 (59.3)	14 (53.9)	
Met-carrier	49 (41.9)	37 (40.7)	12 (46.2)	
rs7124442, *n* (%)				0.520
T/T	61 (52.1)	46 (50.6)	15 (57.7)	
C-carrier	56 (47.9)	45 (49.5)	11 (42.3)	
BDNF mean (SE)	0.19 (0.01)	0.18 (0.01)	0.21 (0.02)	**0.045***
Cortisol mean (SE)	24.28 (1.3)	21.01 (1.20)	35.75 (3.45)	**<0.0001***

*$p \leq 0.05$.

BDNF Biomarker Associations with Cortisol Are Dependent upon BDNF Genotype

We generated separate models among those with GRS = 0, GRS = 1, and GRS = 2 to evaluate the predictive capacity of CSF BDNF with regard to CSF cortisol TRAJ group membership. These age-adjusted models showed that, among subjects with GRS = 1, being in the high BDNF group (>75th percentile) was associated with *high* CSF cortisol TRAJ group membership (OR = 6.50, 95% CI: 1.70, 24.81, $P = 0.006$) (**Table 4**). In **Figure 4**, average BDNF levels were calculated by GRS in each cortisol TRAJ group. In GRS = 1 group, there is a significant difference in BDNF levels in CORT TRAJ high vs. low ($p = 0.011$). There is no statistically significant difference for GRS 0 or 2. These findings are consistent with the results from **Table 4**, wherein the GRS = 2 group was relatively small in sample size.

BDNF Genetic Interactions with CSF BDNF Level Mortality Relationships May Be Age Dependent

An age stratified analysis, with separate models for age <48 vs. ≥48 years old, revealed younger individuals had a significant CSF BDNF*GRS interaction in this mortality model (**Table 5**, $P = 0.028$). This model was adjusted for GCS and cortisol TRAJ group. For younger people, this interaction shows the association between high BDNF and mortality varies by *BDNF* genotype such that those with higher secretion genotypes (GRS = 0) have a higher mortality risk.

In **Figure 5A**, average BDNF levels were calculated by survival status and GRS. In non-survivors, there is a significant difference in mean BDNF levels by GRS ($p = 0.015$), such that individuals with GRS of 2 had the lowest BDNF levels and individuals with GRS of 0 had the highest BDNF. Also, non-survivors with a GRS of 0 had significantly increased BDNF compared to survivors with a GRS of 0 ($p = 0.007$). In **Figure 5B**, BDNF levels by survival status and GRS were calculated among just individuals <48 years old. In non-survivors, there was a significant difference in BDNF levels by GRS, with individuals with GRS of 2 having the lowest BDNF ($p = 0.032$). Among individuals with GRS of 0, BDNF levels were significantly elevated among non-survivors compared to survivors ($p = 0.015$). There were no significant differences in BDNF levels by GRS among either survivors or non-survivors over the age of 48 (data not shown). Together, these data are consistent with the multivariate model in **Table 5** and suggest CSF cortisol has a regulatory influence on younger individuals with high BDNF levels and a low BDNF secretion genotype.

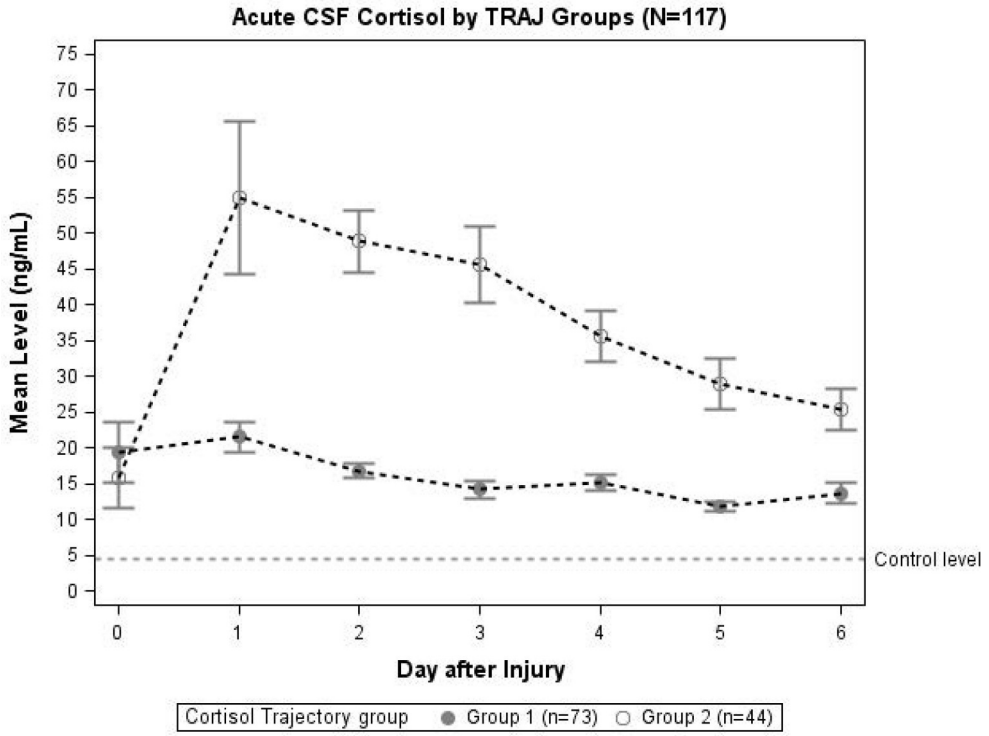


FIGURE 2 | Daily mean CSF cortisol levels for CSF cortisol TRAJ groups ($N = 117$). Daily mean CSF cortisol levels for the cortisol TRAJ groups. Error bars indicate standard error of the mean. There was a significant difference in CSF cortisol levels between the two groups on days 1–6 ($p < 0.01$). CSF cortisol values are provided for $N = 13$ healthy controls as a reference.

DISCUSSION

Cortisol and BDNF are crucial biomarkers to characterize in the context of TBI, as they can have both potentially useful and harmful effects in the brain. This study investigated the relationship between CSF cortisol and BDNF as gene- and age-dependent biomarkers that predict mortality after TBI. We found cortisol mediates BDNF effects on mortality after TBI and that genetics and age also influence this mediation effect. While our previous work shows cortisol and BDNF are predictive biomarkers for outcome post-TBI (Santarsieri et al., 2014; Failla et al., 2016), no studies have investigated the inter-relationships between these biomarkers, and their combined effects on clinical outcome. These data show the direct effects of high BDNF levels on mortality are more pronounced among younger individuals with TBI who also have high secretion *BDNF* genotypes. However, the regulatory (mediating) effects of high CSF cortisol on BDNF mortality risk occurs among those with low BDNF secretion genotypes.

We also found increased cortisol levels are associated with mortality after TBI. Elevated CSF cortisol is linked to an increased inflammatory response among adults with severe TBI (Santarsieri et al., 2015). While traditionally considered as having anti-inflammatory properties (Barnes, 1979), recent work suggests under certain conditions, CNS cortisol creates a more permissive, pro-inflammatory state (Sapolsky et al., 2000; Yeager et al., 2004). In addition to inflammation, cortisol has signaling and regulatory influences on neurotrophins (McEwan, 2012), particularly BDNF (Rothman and Mattson, 2013).

A prolonged, elevated cortisol state, which occurs with ongoing stress and is further perpetuated by impaired negative feedback secondary to the stress response, can result in reduced synaptic plasticity, mediated by glucocorticoid downregulation of BDNF expression (Duman et al., 1997; Numakawa et al., 2009). The focus on BDNF effects on learning and memory has garnered significant attention for BDNF due to its role in plasticity and neural maintenance, but BDNF is a viable biomarker for mortality as first reported by Failla et al. (2015).

Many studies have shown an inverse relationship between cortisol and BDNF in the setting of chronic stress and in experiments with glucocorticoid injections, where elevated cortisol leads to BDNF reductions (Smith et al., 1995) and adrenalectomy results in elevated BDNF levels (Stranahan et al., 2011). Our current study found positive associations between elevated CSF cortisol and BDNF levels. A closer, temporal examination of the stress literature demonstrates that acute stress increases BDNF (Marmigère et al., 2003; Tapia-Arancibia et al., 2004), while chronic stress reduces BDNF (Smith et al., 1995; Liston and Gan, 2011). This acute increase in BDNF has been confirmed in experimental TBI models across multiple brain regions (Yang et al., 1996; Hicks et al., 1997; Grundy et al., 2000; Rostami et al., 2014), while regional reductions in BDNF have also been documented at chronic time points after experimental TBI (Chen et al., 2005).

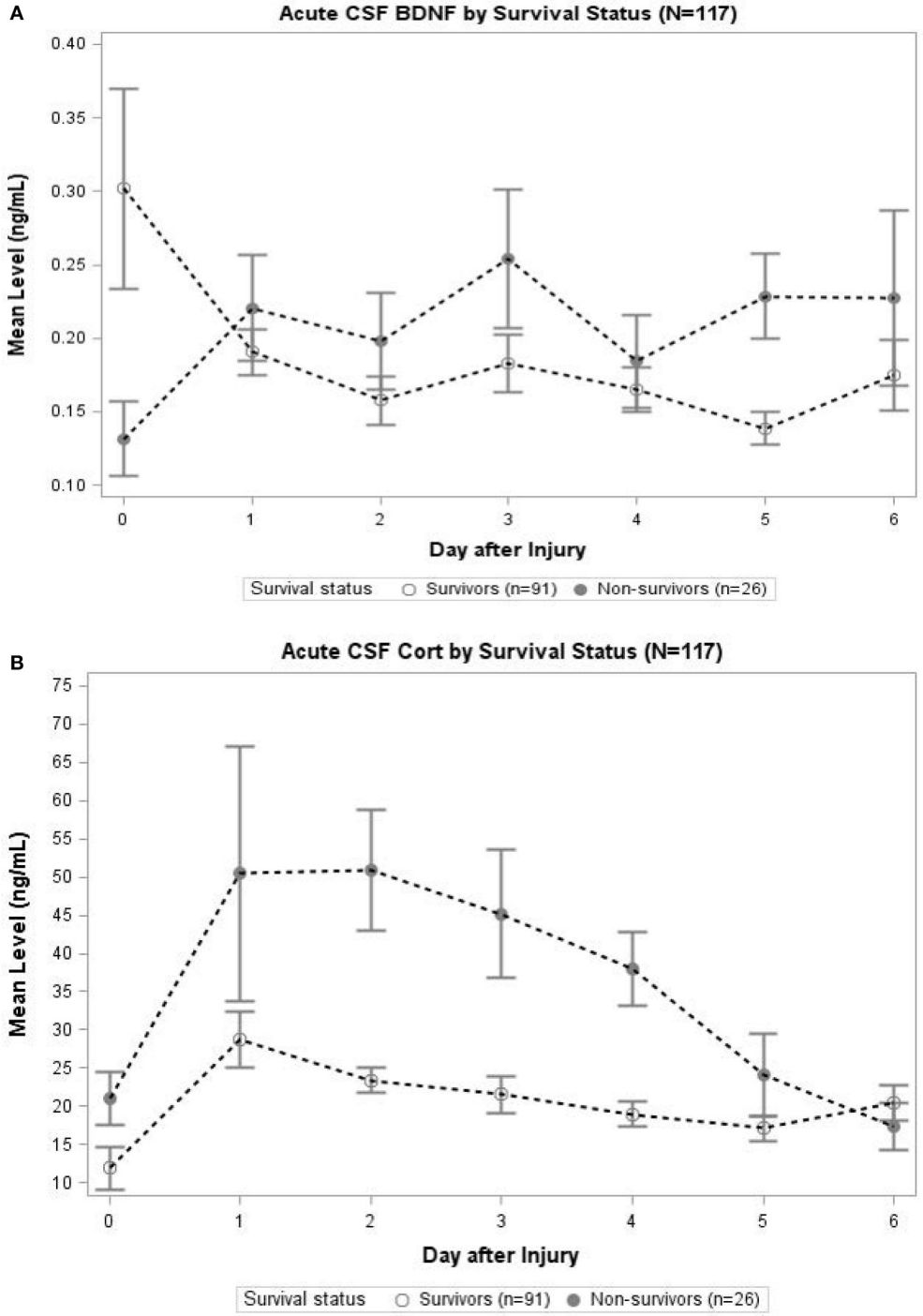


FIGURE 3 | (A) Acute CSF Cortisol by Survival Status ($N = 117$). The open circles indicate survivors and closed circles are non-survivors. Data for CSF Cortisol (ng/mL) were averaged within survival category each day. Error bars indicate standard error of the mean. On days 2–4, CSF Cortisol levels were significantly ($p < 0.05$) elevated among non-survivors compared to survivors. **(B)** Acute CSF BDNF by Survival Status ($N = 117$). The open circles indicate survivors and closed circles are non-survivors. Data for CSF BDNF (ng/mL) were averaged within survival category each day. Error bars indicate standard error of the mean. On days 3 and 5, CSF BDNF levels were significantly ($p < 0.05$) elevated among non-survivors compared to survivors. There was a trend toward significant difference in levels on day 0 ($p = 0.078$); there were no significant differences in CSF BDNF levels for any other day.

This initial increase in BDNF may represent a physiological response to influence HPA reactivity and attempt to maintain homeostasis within the brain in response to initial/acute stress (Marmigère et al., 2003; McEwan, 2015). However, BDNF increases can be pathological in the setting of high and/or prolonged pathophysiological stress (e.g., TBI). Clinically,

TABLE 2 | Prior knowledge of BDNF and mortality association mediated by cortisol level among all subjects and stratified by 75th quartile of age.

	All subjects (*N* = 185)		Age ≤ 48 years (*N* = 139)		Age >48 years (*N* = 46)	
Pathway[a]	**β (SE)**	***P*-value**	**β (SE)**	***P*-value**	**β (SE)**	***P*-value**
BDNF to Cortisol (Logistic)[b]	1.35 (0.48)	**0.005**	1.94 (0.61)	**0.001**	0.30 (0.75)	0.694
Cortisol to mortality (Cox)	1.13 (0.40)	**0.005**	1.00 (0.56)	0.095	1.27 (0.57)	**0.026**
BDNF to mortality-Total Effect (Cox)	0.65 (0.30)	**0.033**	1.03 (0.42)	**0.015**	0.30 (0.41)	0.457
BDNF to mortality-Direct Effect (Cox)[c]	0.47 (0.38)	0.217	0.54 (0.65)	0.407	0.48 (0.49)	0.324

[a]*All pathways adjusted for 75th percentile age (at 48 year old).*
[b]*Weekly average CSF BDNF in ng/mL (≥75th percentile, <75th percentile); CSF cortisol using trajectory analysis grouping (low vs. high).*
[c]*Also adjusted for cortisol trajectory grouping (low vs. high).*
Bolded p-values indicate statistical significance.

TABLE 3 | Bayes estimation of BDNF and mortality association mediated by cortisol level among all subjects and by 75th percentile of age using Monte Carlo Markov Chain (MCMC) method[a].

	All subjects (N = 117)		Age ≤ 48 years (*N* = 90)		Age >48 years (*N* = 27)	
Pathway[b]	**HR/OR (95% CI)**	***P*-value**	**HR/OR (95% CI)**	***P*-value**	**HR/OR (95% CI)**	***P*-value**
BDNF to Cortisol (Logistic)	4.12 (1.86–9.24)	**0.001**	7.48 (3.22–17.92)	**<0.001**	1.44 (0.43–4.80)	0.185
Cortisol to mortality (Cox)	3.05 (1.70–5.45)	**<0.001**	2.99 (1.27–7.00)	**0.017**	3.61 (1.53–8.97)	**0.006**
BDNF to mortality-Total Effect (Cox)	1.94 (1.19–3.12)	**0.011**	2.89 (1.42–5.70)	**0.004**	1.47 (0.73–2.95)	0.221
BDNF to mortality-Direct Effect (Cox)	1.58 (0.90–2.78)	0.106	1.78 (0.72–4.42)	0.185	1.72 (0.78–3.76)	0.159
Mediation percentage (95% CI)[c]	29.92% (19.58–40.25%)		45.45% (34.05–56.85%)		−41.10% (−93.52%, 11.33%)	

[a]*Analysis was performed with weekly average CSF BDNF in ng/mL (≥75th percentile, <75th percentile); cortisol using trajectory analysis grouping (low vs. high); Prior distribution of coefficients for each corresponding pathway (**Table 2**) was incorporated in MCMC Bayes estimation, which resamples prior distributions to derive a more precise and valid parameter estimate.*
[b]*All pathways were adjusted for 75th percentile age (<48 vs. ≥48 years old), Glasgow Coma Scale (GCS) and Gene Risk Score (GRS). For "BDNF to mortality-Direct Effect," cortisol using trajectory analysis grouping (low vs. high) was additionally adjusted.*
[c]*The mediation percentage was calculated with the following equation: Mediation percentage = {[ln(OR$_{Total\ Effect}$) − ln(OR$_{Direct\ Effect}$)]/ln(OR Total Effect)}*100.*
Bolded p-values indicate statistical significance.

TABLE 4 | Odds Ratios and 95% Confidence Intervals for CSF BDNF and cortisol levels.

	GRS = 0 (*N* = 29)[a]		GRS = 1 (*N* = 71)		GRS = 2 (*N* = 17)	
	Cortisol TRAJ Group 2 vs. 1[b]	**OR (95% CI)[c]**	**Cortisol TRAJ Group 2 vs. 1[b]**	**OR (95% CI)**	**Cortisol TRAJ Group 2 vs. 1[b]**	**OR (95% CI)**
BDNF 75th pct.	15/14	2.46 (0.30, 16.13)	23/48	6.50 (1.70, 24.81)	6/11	9.00 (0.28, 285.51)
P trend		0.285		**0.006**		0.213

Individual Models stratified by Gene Risk Scores.
[a]*GRS score: 0 = Val/Val (rs6265) and T/T (rs7124442); 1 = Val/Val (rs6265), C-carriers (rs7124442) or Met-Carriers (rs6265), T/T (rs7124442); 2 = Met-Carriers (rs6265) and C-Carriers (rs7124442).*
[b]*Number of subjects in each cortisol trajectory group (Group 2 has higher cortisol level).*
cAdjusted for binary age (≤48 vs. >48 years old).
Bolded p-value indicates statistical significance.

increased CSF BDNF may be due to BBB disruption after TBI. In addition to brain production, BDNF is synthesized in the periphery (Caporali and Emanueli, 2009) and can be stored and released from platelets (Nakahashi et al., 2000) after tissue injury (Fujimura et al., 2002). Failla et al. (2015) demonstrated that serum BDNF levels are reduced post-TBI, hypothesizing that reduced serum levels may be due BDNF transit into the CNS, resulting in an acute increase in CSF BDNF. In either case, increased CSF BDNF may represent failed compensatory mechanisms that result in higher risk for mortality.

Acutely elevated CSF BDNF levels may not represent neuroprotective processes due to injury induced regional changes in TrkB vs. p75 target receptor expression. BDNF is released as proBDNF, which binds to the pro-apoptotic $p75^{NTR}$. When cleaved by plasmin producing mature BDNF, this molecule binds to the pro-survival TrkB receptor. Experimental

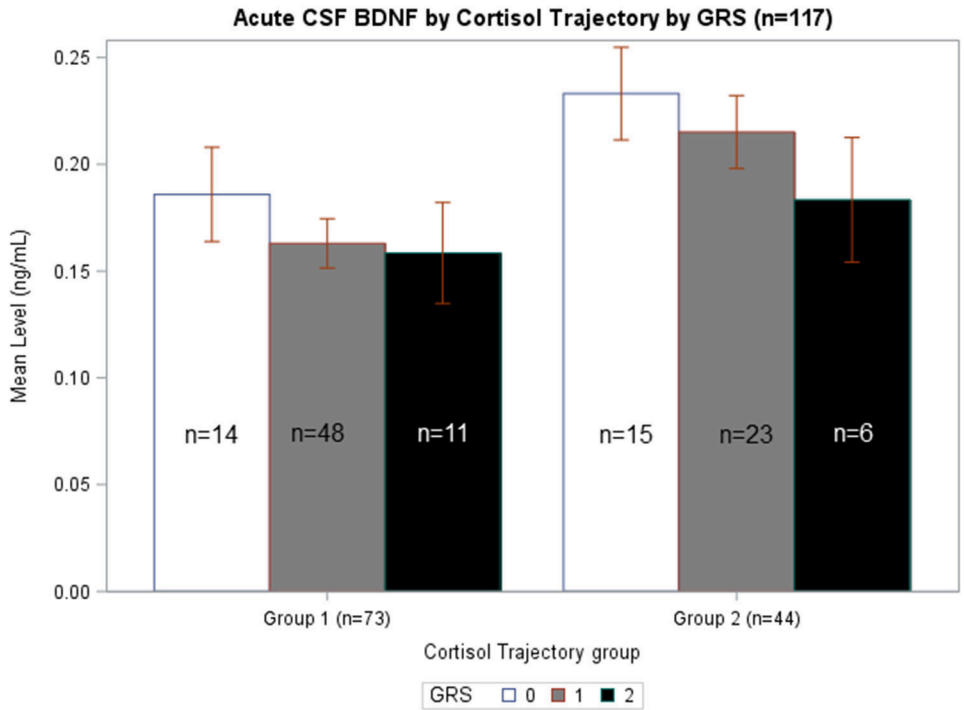


FIGURE 4 | Acute CSF BDNF by Cortisol Trajectory Group by GRS. Weekly average CSF BDNF (ng/mL) is reported. Error bars indicate standard error of the mean. White, gray, and black corresponds to GRS scores of 0, 1, and 2, respectively. Results are stratified among the high ($n = 44$) and low ($n = 73$) cortisol TRAJ groups. Among individuals with GRS = 1, those in the high cortisol TRAJ group had significantly elevated BDNF levels compared to those in the low cortisol TRAJ group $p = 0.011$. There were no significant differences in BDNF levels by cortisol TRAJ group for individuals with GRS of 0 or 2.

TABLE 5 | Cox proportional hazards model for the association between BDNF and mortality[a].

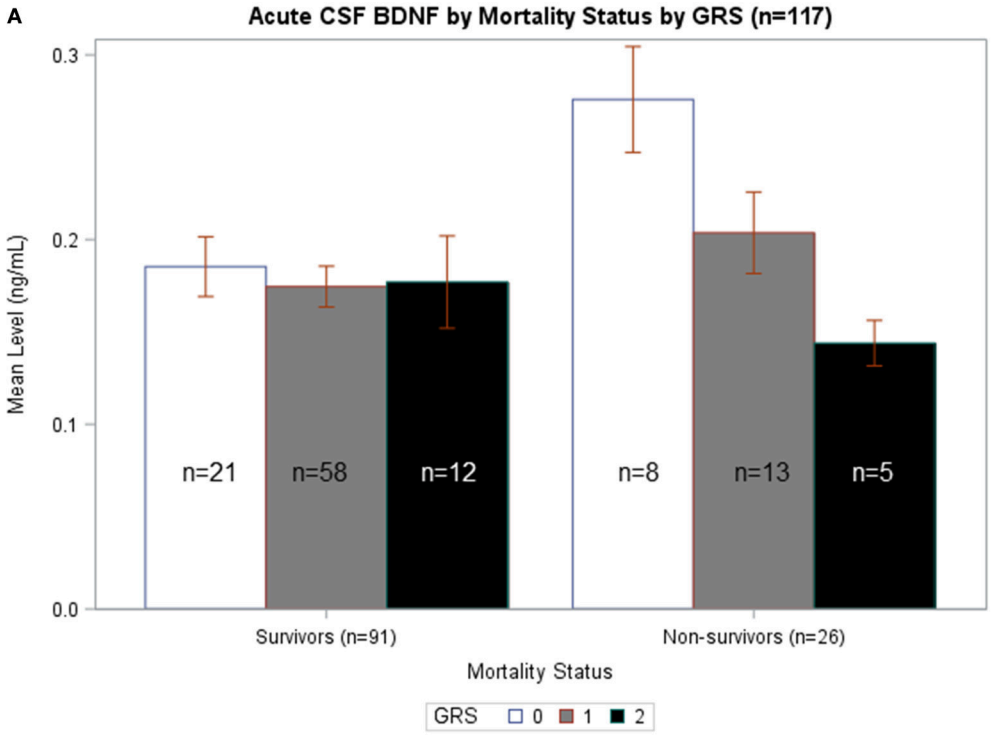
	Age below 75th percentile (≤48 years) (*N* = 90)		Age above 75th percentile (>48 years) (*N* = 27)	
	Coefficient (SE)	***P*-value**	**Coefficient (SE)**	***P*-value**
BDNF*GRS	−2.28 (1.04)	**0.028**	−0.26 (1.13)	0.819

Individual models are stratified by age.
[a]Covariates included in the model: GCS, cortisol (trajectory group).
Bolded p-value indicates statistical significance.

excitotoxic conditions, characteristic of TBI, have demonstrated a downregulation of full-length tyrosine kinase receptor (TrkB.FL) and an upregulation of the inactive truncated form of the receptor (TrkB.T) (Gomes et al., 2012; Vidaurre et al., 2012). A regionally specific increase in $p75^{NTR}$ receptors additionally contributes to a shift from pro-survival to pro-apoptotic BDNF function, possibly contributing to increased mortality after injury. Aging also can detrimentally shift the BDNF receptor ratios, along with causing regional reductions in BDNF and TrkB mRNA expression (Romanczyk et al., 2002; Webster et al., 2006), including in the hypothalamus (Tapia-Arancibia et al., 2004). This age-related decline in BDNF pro-survival signaling may possibly hinder neuronal survival and maintenance, therefore increasing mortality risk. Age, and possibly also injury, related increases in $p75^{NTR}$ receptors may contribute to less apparent cortisol mediation effects on BDNF associations with mortality.

Cortisol mediation of BDNF effects on mortality may be more prominent among those with high BDNF levels and concurrent low BDNF secretion genotypes, and the reported mediation effect suggest that there may be a mechanism connecting these two signaling pathways in the context of TBI mortality. One plausible mechanism might involve post-TBI cortisol elevations inhibiting the interaction of Shp2 with TrkB, affecting neurological injury severity. Cortisol mediated inhibition of this signaling pathway suppresses the MAPK/ERK pathway (Kumamaru et al., 2011), essential for transcribing certain neural plasticity genes (Arango-Lievano et al., 2015). Disrupted TrkB signaling could increase brain tissue damage even while elevated glucocorticoid levels are simultaneously increasing pro-BDNF and tissue plasminogen activator expression (Revest et al., 2014), to generate mature BDNF.

Another possible mechanism for cortisol mediated BDNF mortality effects is through autonomic instability after injury. TBI results in a marked disruption in autonomic balance and stability. Evidence of reduced HRV after TBI implies reduced parasympathetic tone (Baguley et al., 2006). With TBI, HRV has been shown to predict brain death and cerebrovascular



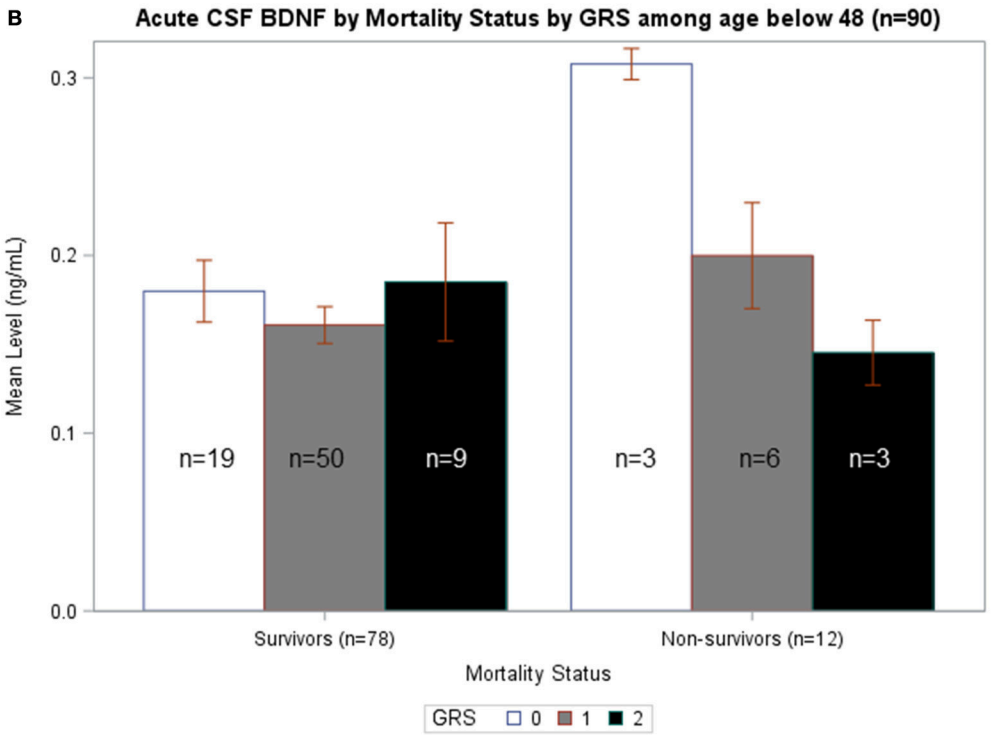


FIGURE 5 | (A) Acute CSF BDNF by Mortality Status by GRS. Weekly average CSF BDNF (ng/mL) is reported. Error bars indicate standard error of the mean. White, gray, and black corresponds to GRS scores of 0, 1, and 2, respectively. Results are stratified among survivors ($n = 91$) and non-survivors ($n = 26$). Among non-survivors, there is a significant difference in BDNF levels by GRS, such that higher GRS scores have lowest BDNF levels ($p = 0.015$). There is no difference in BDNF levels by GRS in survivors-only. Only among individuals with GRS scores of 0 is there a significant difference in BDNF levels in survivors compared to non-survivors ($p = 0.007$). **(B)** Acute CSF BDNF by Mortality Status by GRS among age below 48. Weekly average CSF BDNF (ng/mL) in reported after restricting population to only individuals below age 48. Error bars indicate standard error of the mean. White, gray, and black corresponds to GRS scores of 0, 1, and 2,

(Continued)

FIGURE 5 | Continued
respectively. Results are stratified among survivors ($n = 78$) and non-survivors ($n = 12$). Among non-survivors, there is a significant difference in BDNF levels by GRS, such that higher GRS scores have lowest BDNF levels ($p = 0.032$). There is no difference in BDNF levels by GRS in survivors-only. Only among individuals with GRS scores of 0 is there a significant difference in BDNF levels in survivors compared to non-survivors ($p = 0.015$).

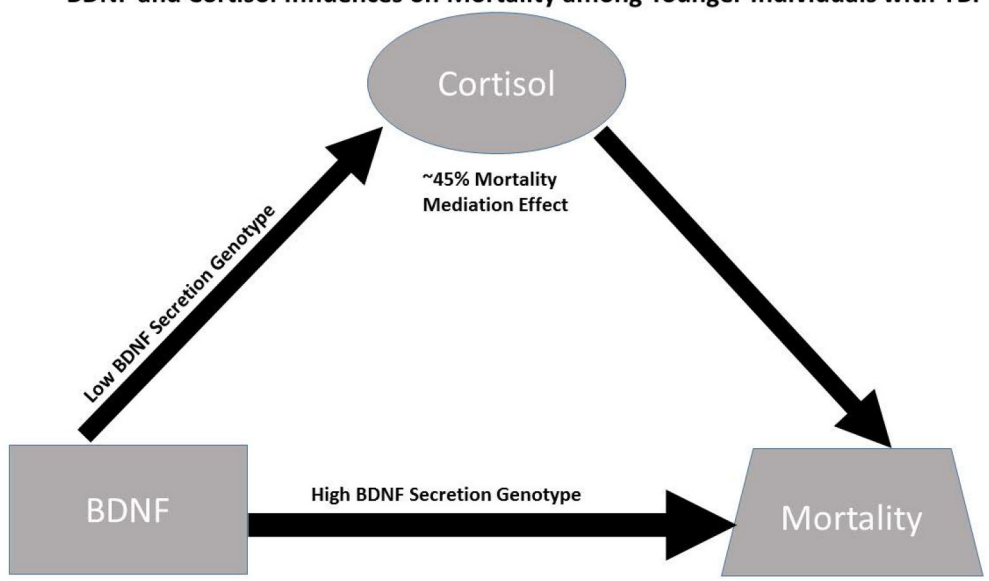


FIGURE 6 | Conceptual figure capturing biomarker relationships among younger individuals (≤48 years. Old, *N* = 90): (1) cortisol has a significant regulatory (mediating) effect on BDNF's influence on mortality, accounting for ~45% of BDNF's mortality effects; (2) the association between BDNF above/below the 75th percentile and high cortisol TRAJ is only significant among individuals with low BDNF secretion genotypes; and (3) the direct association between BDNF above/below the 75th percentile and mortality risk is stronger among individuals with the high BDNF secretion genotype (GRS = 0) compared to the low secretion genotypes. Taken together, the data support a conceptual model for younger individuals with TBI wherein high BDNF levels among those with high BDNF secretion phenotypes directly increases mortality risk, while mortality risk for those with high BDNF levels paired with low BDNF secretion genotypes is regulated (mediated) by cortisol.

dysregulation (Ryan et al., 2011), and HRV is associated with autonomic dysfunction in multiple other pathological states including sepsis, shock, and adrenal insufficiency (Morris et al., 2007; Werdan et al., 2009), each of which can co-occur in TBI. Further work suggests that HRV can be an effective mortality predictor, among hemodynamically stable patients with TBI (Ryan et al., 2011). Importantly, there is evidence that BDNF levels (Pal et al., 2014), *BDNF* genetic variability (Yang et al., 2010), and cortisol levels (Pal et al., 2014) are associated with HRV, making autonomic instability a plausible mechanism by which cortisol regulation of BDNF affects mortality risk.

Our previous work defined "risk alleles" as the genetic variations of the *BDNF* SNPs rs6265 and rs7124442 that result in lower activity-dependent BDNF secretion and impaired BDNF mRNA trafficking, respectively (Egan et al., 2003; Orefice et al., 2013), to create a GRS (Failla et al., 2015). Interestingly, higher GRSs were only associated with BDNF related mortality among younger subjects. A more nuanced look at the relationship between cortisol and BDNF in our population showed very high BDNF levels are associated with high cortisol levels among people who have a low secretion "risk allele" (rs6265 Met or rs7124442 C). Among these individuals, high CSF BDNF levels were associated with *high* CSF CORT TRAJ group membership. Our previous work suggests these risk variants are associated with lower serum BDNF. Previous work elaborated that, in addition to lower BDNF secretion capacity and autonomic mediated suppression of serum BDNF, low serum BDNF post-TBI may be due to reactive platelet BDNF dumping systemically and more BBB BDNF transit into the CNS (Tanno et al., 1992; Failla et al., 2016). Taken together, cortisol may have a regulatory influence (i.e., mediation effect) under conditions where innate BDNF production capacity is reduced, yet CNS BDNF levels are high due to injury severity factors, such as cortisol induced suppression of Shp2 associated MAPK/ERK signaling, reactive platelet dumping of BDNF systemically, and increased serum-to-CSF BBB transit. Our work is consistent with previous studies where those with rs6265 Met/Met homozygosity possessed significantly higher HPA axis reactivity, determined by serum cortisol elevations in response to physical stress (Schüle et al., 2006).

Our study demonstrates age contributes to biomarker associations on outcome after TBI. Age can affect many different biomarkers, including stress hormones (Wagner et al., 2011a; Santarsieri et al., 2014; Ranganathan et al., 2016), sex hormones (Ranganathan et al., 2016), inflammation (Kumar et al., 2016), and BDNF (Failla et al., 2015). Experimental models show

decreased BDNF mRNA, BDNF protein, and TrkB.FL mRNA in many different brain regions with older age (Romanczyk et al., 2002; Webster et al., 2006; Erickson et al., 2012). Our study demonstrated that in younger subjects (age < 48 year), CSF BDNF effects on mortality can differ as a function of genetic variation, while this BDNF*GRS interaction is not significant among older subjects. Lower sample size for the older subgroup, and for the GRS = 2 subgroup, are potential alternative reasons for this finding. While specific mechanisms are difficult to discern with these clinical biomarker data, age related differences in BDNF secretion capacity (perhaps due to age-dependent risk factors like hypertension, cerebral hypoperfusion, and poor glucose metabolism) (Kennedy et al., 2009; Erickson et al., 2012), age related increases in CSF BDNF acutely (Failla et al., 2015), and also target receptor milieu (Erickson et al., 2012) may contribute to this finding. What the data do suggest though is that, in the context of TBI, factors other than genetics contribute to CSF BDNF profiles observed in this age group.

While BDNF secretion capacity is reduced with aging, CSF BDNF levels are elevated after TBI and are increased with age (Failla et al., 2015). Although CSF cortisol relationships to CSF BDNF were studied, both BDNF and cortisol are synthesized in the periphery and likely contribute to the CSF profiles observed. Notably, BDNF levels are reduced in serum after TBI and are associated with mortality (Failla et al., 2015), while serum cortisol profiles are not associated with mortality (Wagner et al., 2011a). Thus, it is unclear at this point, how serum relationships between BDNF and cortisol might differ from CSF relationships between these two markers and why. Presumably, variable levels of BBB dysfunction, acute adrenal insufficiency associated with the critical illness that accompanies severe TBI, and personal biology may influence these relationships, creating the possibility for potentially heterogeneous or dynamic relationships with the role that CSF cortisol plays in the causal pathway between CSF BDNF and mortality.

The study has several limitations, including small sample size, particularly for age stratified analyses and for the BDNF cut-point. Dividing the age groups at the 75th percentile left the model underpowered to fully explore GRS*CSF BDNF interactions in the older age group, and larger future studies focusing on this older population would be beneficial. We used a data driven approach to determine the 75th percentile as the cut point for BDNF analyses. Specifically, the 75th percentile cutoff was based on our preliminary analysis where we used a larger, and presumably more representative cohort, to define the cut-point to be used with the Bayesian model. We believe the 75th percentile is a good starting estimate for examining high BDNF levels in other populations. It is possible that the actual cut point may vary in other populations (70th, 80th, percentile, etc.). However, our main interpretation of this work is that cortisol levels mediate associations between high BDNF levels and mortality.

Due to effects of race on BDNF allelic frequencies, we limited our analyses to self-reported Whites. Also, the group with a GRS = 2 is small, making significant associations with cortisol TRAJ challenging despite the large odds ratio. Despite this, some issues with low sample size are overcome by using the MCMC method of Bayes estimation, which uses prior knowledge to inform a posterior probability based on the current study data; it also incorporates a validation process to internally re-sample prior distributions to arrive at more precise and valid parameters. Future studies could consider a weighted GRS score formulation to better quantify the relative contribution each variant contributes to genetic associations identified in this study. Additionally, our small sample size limited our ability to differentiate *BDNF* heterozygote gene effects from variant homozygote gene effects on mortality. Examining *BDNF* heterozygote gene effects, including unique interactions with cortisol, may be relevant to TBI pathophysiology (Kim et al., 2011; Notaras et al., 2016).

Including only self-reported whites is an additional limitation as the results may not be generalizable to other races. However, the literature is clear that racial genetic differences can confound the relationships assessed (Freedman et al., 2004; Nettiksimmons et al., 2014). Only two *BDNF* gene variants were used to construct the GRS, which likely underestimates the total variance explained by *BDNF* genetics on cortisol profiles, BDNF profiles, and mortality; nevertheless, the chosen variants are both functional and affect BDNF (Egan et al., 2003; Orefice et al., 2013) and cortisol (Alexander et al., 2010; Armbruster et al., 2016) levels clinically. Additionally, our BDNF ELISA does not differentiate between the proBDNF and mature BDNF isoforms.

Despite these limitations, this study suggests causal effects of cortisol on mortality for those with high BDNF that are more pronounced among younger individuals with low BDNF secretion genotypes. While this causal relationship is important for mortality prediction post-TBI, specific mechanisms facilitating this relationship have yet to be discerned; additionally the high BDNF secretion genotype directly increases mortality risk among younger individuals with high BDNF levels (see **Figure 6**). In addition to injury effects on both CSF BDNF and cortisol biomarker levels (e.g., BBB damage), mechanisms by which these biomarkers interact with each other, age, and genetics to impact mortality are likely varied, but may reflect both neurological injury severity and autonomic instability. Future experimentation should evaluate how our mediation effects translate mechanistically to brain tissue BDNF and BDNF target receptor signaling after TBI. Additional trauma to body regions outside the head is common in the population with severe TBI. These injuries, as well as other systemic complications, such as hospital acquired pneumonia, sepsis, and shock can be non-neurological contributors to mortality. Thus, future work considering if/how serum BDNF or Cortisol reflect the peripheral injury response, and its effects on TBI pathophysiology is warranted. Clinically, it would be interesting to further characterize BDNF cortisol relationships, with survivor based outcomes, like learning and memory, from the viewpoint of post-acute TBI serving as a chronic stress paradigm influencing regulatory relationships between cortisol and BDNF. Understanding these dynamic relationships of secondary injury occurring after TBI may facilitate more effective, personalized interventions after injury.

ETHICS STATEMENT

All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved

by the University of Pittsburgh Institutional Review Board.

AUTHOR CONTRIBUTIONS

AW: Contributed to hypothesis formulation, literature review, results interpretation, writing and editing of manuscript. MM: Contributed to literature review, results interpretation, data generation, writing and editing of manuscript. MF: Contributed to hypothesis formulation, literature review, data generations, editing of manuscript. RK: Contributed to data analysis, results interpretation, writing and editing of manuscript. ZW: Contributed to data analysis, results interpretation, writing and editing of manuscript. YC: Contributed to data generation, results interpretation, and editing of manuscript. BO: Contributed to data generation, results interpretation and editing of manuscript.

FUNDING

This work was supported in part by the Department of Defense (DOD W81XWH-071-0701); National Institutes of Health (NIH R01 HD048162, R01NR008424, 5P01NS030318; Centers for Disease Control (CDC) R49 CCR 323155); National Institute for Disability, Independent Living, and Rehabilitation Research (NIDILRR 90DP0041).

REFERENCES

Alboni, S., Tascedda, F., Corsini, D., Benatti, C., Caggia, F., Capone, G., et al. (2011). Stress induces altered CRE/CREB pathway activity and BDNF expression in the hippocampus of glucocorticoid receptor-impaired mice. *Neuropharmacology* 60, 1337–1346. doi: 10.1016/j.neuropharm.2011.01.050

Alexander, N., Osinsky, R., Schmitz, A., Mueller, E., Kuepper, Y., and Hennig, J. (2010). The BDNF Val66Met polymorphism affects HPA-axis reactivity to acute stress. *Psychoneuroendocrinology* 35, 949–953. doi: 10.1016/j.psyneuen.2009.12.008

Antonawich, F. J., Miller, G., Rigsby, D. C., and Davis, J. N. (1999). Regulation of ischemic cell death by glucocorticoids and adrenocorticotropic hormone. *Neuroscience* 88, 319–325. doi: 10.1016/S0306-4522(98)00213-9

Arango-Lievano, M., Lambert, W. M., Bath, K. G., Garabedian, M. J., Chao, M. V., and Jeanneteau, F. (2015). Neurotrophic-priming of glucocorticoid receptor signaling is essential for neuronal plasticity to stress and antidepressant treatment. *Proc. Natl. Acad. Sci. U.S.A.* 112, 15737–15742. doi: 10.1073/pnas.1509045112

Armbruster, D., Müller-Alcazar, A., Strobel, A., Lesch, K.-P., Kirschbaum, C., and Brocke, B. (2016). BDNF val^{66}met genotype shows distinct associations with the acoustic startle reflex and the cortisol stress response in young adults and children. *Psychoneuroendocrinology* 66, 39–46. doi: 10.1016/j.psyneuen.2015.12.020

Baguley, I. J., Heriseanu, R. E., Felmingham, K. L., and Cameron, I. D. (2006). Dysautonomia and heart rate variability following severe traumatic brain injury. *Brain Inj.* 20, 437–444. doi: 10.1080/02699050600664715

Barnes, P. J. (1979). Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clin. Sci. Lond. Engl.* 94, 557–572.

Baron, R. M., and Kenny, D. A. (1986). The moderator-mediator variable distinction in social psychological research: conceptual, strategic, and statistical considerations. *J. Pers. Soc. Psychol.* 51, 1173–1182. doi: 10.1037/0022-3514.51.6.1173

Blaha, G. R., Raghupathi, R., Saatman, K. E., and McIntosh, T. K. (2000). Brain-derived neurotrophic factor administration after traumatic brain injury in the rat does not protect against behavioral or histological deficits. *Neuroscience* 99, 483–493. doi: 10.1016/S0306-4522(00)00214-1

Caporali, A., and Emanueli, C. (2009). Cardiovascular actions of neurotrophins. *Physiol. Rev.* 89, 279–308. doi: 10.1152/physrev.00007.2008

Centers for Disease Control and Prevention (2016). *Traumatic Brain Injury in the United States: Emergency Department Visits, Hospitalizations and Deaths 2002–2006*. Available online at: http://www.cdc.gov/traumaticbraininjury/tbi_ed.html (Accessed April 6, 2016).

Chen, X., Li, Y., Kline, A. E., Dixon, C. E., Zafonte, R. D., and Wagner, A. K. (2005). Gender and environmental effects on regional brain-derived neurotrophic factor expression after experimental traumatic brain injury. *Neuroscience* 135, 11–17. doi: 10.1016/j.neuroscience.2005.05.041

Clark, C. G., Hasser, E. M., Kunze, D. L., Katz, D. M., and Kline, D. D. (2011). Endogenous brain-derived neurotrophic factor in the nucleus tractus solitarius tonically regulates synaptic and autonomic function. *J Neurosci.* 31, 12318–12329. doi: 10.1523/JNEUROSCI.0746-11.2011

Duman, R. S., Heninger, G. R., and Nestler, E. J. (1997). A molecular and cellular theory of depression. *Arch. Gen. Psychiatry* 54, 597–606. doi: 10.1001/archpsyc.1997.01830190015002

Egan, M. F., Kojima, M., Callicott, J. H., Goldberg, T. E., Kolachana, B. S., Bertolino, A., et al. (2003). The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell* 112, 257–269. doi: 10.1016/S0092-8674(03)00035-7

Erickson, K. I., Miller, D. L., and Roecklein, K. A. (2012). The aging hippocampus: interactions between exercise, depression, and BDNF. *Neuroscientist* 18, 82–97. doi: 10.1177/1073858410397054

Failla, M. D., Conley, Y. P., and Wagner, A. K. (2016). Brain-Derived Neurotrophic Factor (BDNF) in traumatic brain injury-related mortality: interrelationships between genetics and acute systemic and central nervous system BDNF profiles. *Neurorehabil. Neural Repair.* 30, 83–93. doi: 10.1177/1545968315586465

Failla, M. D., Kumar, R. G., Peitzman, A. B., Conley, Y. P., Ferrell, R. E., and Wagner, A. K. (2015). Variation in the BDNF gene interacts with age to predict mortality in a prospective, longitudinal cohort with severe TBI. *Neurorehabil. Neural Repair.* 29, 234–246. doi: 10.1177/1545968314542617

Ferrari, E., Cravello, L., Muzzoni, B., Casarotti, D., Paltro, M., Solerte, S. B., et al. (2001). Age-related changes of the hypothalamic-pituitary-adrenal axis: pathophysiological correlates. *Eur. J. Endocrinol.* 144, 319–329. doi: 10.1530/eje.0.1440319

Freedman, M. L., Reich, D., Penney, K. L., McDonald, G. J., Mignault, A. A., Patterson, N., et al. (2004). Assessing the impact of population stratification on genetic association studies. *Nat. Genet.* 36, 388–393. doi: 10.1038/ng1333

Fujimura, H., Altar, C. A., Chen, R., Nakamura, T., Nakahasi, T., Kambayashi, J., et al. (2002). Brain-derived neurotrophic factor is stored in human platelets and released by agonist stimulation. *Thromb. Haemost.* 87, 728–734.

Gomes, J. R., Costa, J. T., Melo, C. V., Felizzi, F., Monteiro, P., Pinto, M. J., et al. (2012). Excitotoxicity downregulates TrkB.FL signaling and upregulates the neuroprotective truncated TrkB receptors in cultured hippocampal and striatal neurons. *J. Neurosci.* 32, 4610–4622. doi: 10.1523/JNEUROSCI.0374-12.2012

Goyal, A., Failla, M. D., Niyonkuru, C., Amin, K., Fabio, A., Berger, R. P., et al. (2013). S100b as a prognostic biomarker in outcome prediction for patients with severe traumatic brain injury. *J Neurotrauma.* 30, 946–957. doi: 10.1089/neu.2012.2579

Grundy, P. L., Patel, N., Harbuz, M. S., Lightman, S. L., and Sharples, P. M. (2000). Glucocorticoids modulate BDNF mRNA expression in the rat hippocampus after traumatic brain injury. *Neuroreport* 11, 3381–3384. doi: 10.1097/00001756-200010200-00023

Grundy, P. L., Patel, N., Harbuz, M. S., Lightman, S. L., and Sharples, P. M. (2001). Glucocorticoids modulate the NGF mRNA response in the rat hippocampus after traumatic brain injury. *Brain Res.* 892, 386–390. doi: 10.1016/S0006-8993(00)03258-3

Hamra, G., MacLehose, R., and Richardson, D. (2013). Markov chain Monte Carlo: an introduction for epidemiologists. *Int. J. Epidemiol.* 42, 627–634. doi: 10.1093/ije/dyt043

Hicks, R. R., Numan, S., Dhillon, H. S., Prasad, M. R., and Seroogy, K. B. (1997). Alterations in BDNF and NT-3 mRNAs in rat hippocampus after experimental brain trauma. *Brain Res. Mol. Brain. Res.* 48, 401–406. doi: 10.1016/S0169-328X(97)00158-7

Janssens, A. C. J. W., Ioannidis, J. P. A., van Duijn, C. M., Little, J., Khoury, M. J., and GRIP Group (2011). Strengthening the reporting of genetic risk prediction studies: The GRIPS Statement. *Ann. Intern. Med.* 154, 421–425. doi: 10.7326/0003-4819-154-6-201103150-00008

Jeanneteau, F. D., Lambert, W. M., Ismaili, N., Bath, K. G., Lee, F. S., Garabedian, M. J., et al. (2012). BDNF and glucocorticoids regulate corticotrophin-releasing hormone (CRH) homeostasis in the hypothalamus. *Proc. Natl. Acad. Sci. U.S.A.* 109, 1305–1310. doi: 10.1073/pnas.1114122109

Jeanneteau, F., Garabedian, M. J., and Chao, M. V. (2008). Activation of Trk neurotrophin receptors by glucocorticoids provides a neuroprotective effect. *Proc. Natl. Acad. Sci. U.S.A.* 105, 4862–4867. doi: 10.1073/pnas.0709102105

Joëls, M. (2008). Functional actions of corticosteroids in the hippocampus. *Eur. J. Pharmacol.* 583, 312–321. doi: 10.1016/j.ejphar.2007.11.064

Kennedy, K. M., Rodrigue, K. M., Land, S. J., and Raz, N. (2009). BDNF Val66Met polymorphism influences age differences in microstructure of the Corpus Callosum. *Front. Hum. Neurosci.* 3:19. doi: 10.3389/neuro.09.019.2009

Kim, J.-M., Stewart, R., Bae, K.-Y., Kim, S.-W., Yang, S.-J., Park, K.-H., et al. (2011). Role of BDNF val66met polymorphism on the association between physical activity and incident dementia. *Neurobiol. Aging* 32, 551.e5–e12. doi: 10.1016/j.neurobiolaging.2010.01.018

Kumamaru, E., Numakawa, T., Adachi, N., and Kunugi, H. (2011). Glucocorticoid suppresses BDNF-stimulated MAPK/ERK pathway via inhibiting interaction of Shp2 with TrkB. *FEBS Lett.* 585, 3224–3228. doi: 10.1016/j.febslet.2011.09.010

Kumamaru, E., Numakawa, T., Adachi, N., Yagasaki, Y., Izumi, A., Niyaz, M., et al. (2008). Glucocorticoid prevents brain-derived neurotrophic factor-mediated maturation of synaptic function in developing hippocampal neurons through reduction in the activity of mitogen-activated protein kinase. *Mol. Endocrinol.* 22, 546–558. doi: 10.1210/me.2007-0264

Kumar, R. G., Diamond, M. L., Boles, J. A., Berger, R. P., Tisherman, S. A., Kochanek, P. M., et al. (2015). Acute CSF interleukin-6 trajectories after TBI: associations with neuroinflammation, polytrauma, and outcome. *Brain Behav. Immun.* 45, 253–262. doi: 10.1016/j.bbi.2014.12.021

Kumar, R. G., Rubin, J. E., Berger, R. P., Kochanek, P. M., and Wagner, A. K. (2016). Principal components derived from CSF inflammatory profiles predict outcome in survivors after severe traumatic brain injury. *Brain Behav. Immun.* 53, 183–193. doi: 10.1016/j.bbi.2015.12.008

Liston, C., and Gan, W.-B. (2011). Glucocorticoids are critical regulators of dendritic spine development and plasticity *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 16074–16079. doi: 10.1073/pnas.1110444108

Maas, A. I. R., Roozenbeek, B., and Manley, G. T. (2010). Clinical trials in traumatic brain injury: past experience and current developments. *Neurother. J. Am. Soc. Exp. Neurother.* 7, 115–126. doi: 10.1016/j.nurt.2009.10.022

Marmigère, F., Givalois, L., Rage, F., Arancibia, S., and Tapia-Arancibia, L. (2003). Rapid induction of BDNF expression in the hippocampus during immobilization stress challenge in adult rats. *Hippocampus* 13, 646–655. doi: 10.1002/hipo.10109

McEwan, B. S. (1999). Stress and the aging hippocampus. *Front. Neuroendocrinol.* 20, 49–70. doi: 10.1006/frne.1998.0173

McEwan, B. S. (2012). The ever-changing brain: cellular and molecular mechanisms for the effects of stressful experiences. *Dev. Neurobiol.* 72, 878–890. doi: 10.1002/dneu.20968

McEwan, B. S. (2015). Preserving neuroplasticity: role of glucocorticoids and neurotrophins via phosphorylation. *Proc. Natl. Acad. Sci. U.S.A.* 112, 15544–15545. doi: 10.1073/pnas.1521416112

Morris, J. A., Norris, P. R., Waitman, L. R., Ozdas, A., Guillamondegui, O. D., and Jenkins, J. M. (2007). Adrenal insufficiency, heart rate variability, and complex biologic systems: a study of 1,871 critically ill trauma patients. *J. Am. Coll. Surg.* 204, 885–892. doi: 10.1016/j.jamcollsurg.2007.01.019

Nakahashi, T., Fujimura, H., Altar, C. A., Li, J., Kambayashi, J., Tandon, N. N., et al. (2000). Vascular endothelial cells synthesize and secrete brain-derived neurotrophic factor. *FEBS Lett.* 470, 113–117. doi: 10.1016/S0014-5793(00)01302-8

Nettiksimmons, J., Simonsick, E. M., Harris, T., McDonald, G. J., Andre Mignault, A. A., Patterson, N., et al. (2014). The associations between serum brain-derived neurotrophic factor, potential confounders, and cognitive decline: a longitudinal study. *PLoS ONE* 9:e91339. doi: 10.1371/journal.pone.0091339

Niyonkuru, C., Wagner, A. K., Ozawa, H., Amin, K., Goyal, A., and Fabio, A. (2013). Group-based trajectory analysis applications for prognostic biomarker model development in severe TBI: a practical example. *J. Neurotrauma.* 30, 938–945. doi: 10.1089/neu.2012.2578

Notaras, M. J., Hill, R. A., Gogos, J. A., and van den Buuse, M. (2016). BDNF Val66Met genotype interacts with a history of simulated stress exposure to regulate sensorimotor gating and startle reactivity. *Schizophr. Bull.* doi: 10.1093/schbul/sbw077. [Epub ahead of print].

Numakawa, T., Kumamaru, E., Adachi, N., Yagasaki, Y., Izumi, A., and Kunugi, H. (2009). Glucocorticoid receptor interaction with TrkB promotes BDNF-triggered PLC-gamma signaling for glutamate release via a glutamate transporter. *Proc. Natl. Acad. Sci. U.S.A.* 106, 647–652. doi: 10.1073/pnas.0800888106

Orefice, L. L., Waterhouse, E. G., Partridge, J. G., Lalchandani, R. R., Vicini, S., and Xu, B. (2013). Distinct roles for somatically and dendritically synthesized brain-derived neurotrophic factor in morphogenesis of dendritic spines. *J. Neurosci.* 33, 11618–11632. doi: 10.1523/JNEUROSCI.0012-13.2013

Pal, R., Singh, S. N., Chatterjee, A., and Saha, M. (2014). Age-related changes in cardiovascular system, autonomic functions, and levels of BDNF of healthy active males: role of yogic practice. *Age (Dordr)* 36:9683. doi: 10.1007/s11357-014-9683-7

Ranganathan, P., Kumar, R. G., Davis, K., McCullough, E. H., Berga, S. L., and Wagner, A. K. (2016). Longitudinal sex and stress hormone profiles among reproductive age and post-menopausal women after severe TBI: a case series analysis. *Brain Inj.* 30, 452–461. doi: 10.3109/02699052.2016.1144081

Revest, J.-M., Le Roux, A., Roullot-Lacarrière, V., Kaouane, N., Vallee, M., Kasanetz, F., et al. (2014). BDNF-TrkB signaling through Erk1/2 MAPK phosphorylation mediates the enhancement of fear memory induced by glucocorticoids. *Mol. Psychiatry.* 19, 1001–1009. doi: 10.1038/mp.2013.134

Roberts, I., Yates, D., Sandercock, P., Farrell, B., Wasserberg, J., Lomas, G., et al. (2004). Effect of intravenous corticosteroids on death within 14 days in 10008 adults with clinically significant head injury (MRC CRASH trial): randomised placebo-controlled trial. *Lancet Lond Engl.* 364, 1321–1328. doi: 10.1016/S0140-6736(04)17188-2

Romanczyk, T. B., Weickert, C. S., Webster, M. J., Herman, M. M., Akil, M., and Kleinman, J. E. (2002). Alterations in trkB mRNA in the human prefrontal cortex throughout the lifespan. *Eur. J. Neurosci.* 15, 269–280. doi: 10.1046/j.0953-816x.2001.01858.x

Roozenbeek, B., Chiu, Y.-L., Lingsma, H. F., Gerber, L. M., Steyerberg, E. W., Ghajar, J., et al. (2012). Predicting 14-day mortality after severe traumatic brain injury: application of the IMPACT models in the brain trauma foundation TBI-trac® New York State database. *J Neurotrauma.* 29, 1306–1312. doi: 10.1089/neu.2011.1988

Rostami, E., Krueger, F., Plantman, S., Davidsson, J., Agoston, D., Grafman, J., et al. (2014). Alteration in BDNF and its receptors, full-length and truncated TrkB and p75(NTR) following penetrating traumatic brain injury. *Brain Res.* 1542, 195–205. doi: 10.1016/j.brainres.2013.10.047

Rothman, S. M., and Mattson, M. P. (2013). Activity-dependent, stress-responsive BDNF signaling and the quest for optimal brain health and resilience throughout the lifespan. *Neuroscience* 239, 228–240. doi: 10.1016/j.neuroscience.2012.10.014

Ryan, M. L., Ogilvie, M. P., Pereira, B. M. T., Gomez-Rodriguez, J. C., Manning, R. J., Vargas, P. A., et al. (2011). Heart rate variability is an independent predictor of morbidity and mortality in hemodynamically stable trauma patients. *J. Trauma.* 70, 1371–1380. doi: 10.1097/TA.0b013e31821858e6

Santarsieri, M., Kumar, R. G., Kochanek, P. M., Berga, S., and Wagner, A. K. (2015). Variable neuroendocrine-immune dysfunction in individuals with unfavorable outcome after severe traumatic brain injury. *Brain Behav Immun.* 45, 15–27. doi: 10.1016/j.bbi.2014.09.003

Santarsieri, M., Niyonkuru, C., McCullough, E. H., Dobos, J. A., Dixon, E. C., Berga, S. L., et al. (2014). Cerebrospinal fluid cortisol and progesterone profiles and outcomes prognostication after severe traumatic brain injury. *J. Neurotrauma.* 31, 699–712. doi: 10.1089/neu.2013.3177

Sapolsky, R. M., Romero, L. M., and Munck, A. U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr. Rev.* 21, 55–89. doi: 10.1210/edrv.21.1.0389

Schüle, C., Zill, P., Baghai, T. C., Eser, D., Zwanzger, P., Wenig, N., et al. (2006). Brain-derived neurotrophic factor Val66Met polymorphism and dexamethasone/CRH test results in depressed patients. *Psychoneuroendocrinology* 31, 1019–1025. doi: 10.1016/j.psyneuen.2006.06.002

Sebastiani, A., Gölz, C., Werner, C., Schäfer, M. K. E., Engelhard, K., and Thal, S. C. (2015). Proneurotrophin binding to P75 neurotrophin receptor (P75ntr) is essential for brain lesion formation and functional impairment after experimental traumatic brain injury. *J Neurotrauma.* 32, 1599–1607. doi: 10.1089/neu.2014.3751

Smith, M. A., Makino, S., Kvetnansky, R., and Post, R. M. (1995). Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. *J. Neurosci.* 15(3 Pt 1), 1768–1777.

Stranahan, A. M., Arumugam, T. V., and Mattson, M. P. (2011). Lowering corticosterone levels reinstates hippocampal brain-derived neurotropic factor and Trkb expression without influencing deficits in hypothalamic brain-derived neurotropic factor expression in leptin receptor-deficient mice. *Neuroendocrinology* 93, 58–64. doi: 10.1159/000322808

Tanno, H., Nockels, R. P., Pitts, L. H., and Noble, L. J. (1992). Breakdown of the blood-brain barrier after fluid percussive brain injury in the rat. Part 1: Distribution and time course of protein extravasation. *J. Neurotrauma.* 9, 21–32. doi: 10.1089/neu.1992.9.21

Tapia-Arancibia, L., Aliaga, E., Silhol, M., and Arancibia, S. (2008). New insights into brain BDNF function in normal aging and Alzheimer disease. *Brain Res. Rev.* 59, 201–220. doi: 10.1016/j.brainresrev.2008.07.007

Tapia-Arancibia, L., Rage, F., Givalois, L., and Arancibia, S. (2004). Physiology of BDNF: focus on hypothalamic function. *Front. Neuroendocrinol.* 25, 77–107. doi: 10.1016/j.yfrne.2004.04.001

Vidaurre, O. G., Gascón, S., Deogracias, R., Sobrado, M., Cuadrado, E., Montaner, J., et al. (2012). Imbalance of neurotrophin receptor isoforms TrkB-FL/TrkB-T1 induces neuronal death in excitotoxicity. *Cell Death Dis.* 3:e256. doi: 10.1038/cddis.2011.143

Wagner, A. K., Amin, K. B., Niyonkuru, C., Postal, B. A., McCullough, E. H., Ozawa, H., et al. (2011b). CSF Bcl-2 and cytochrome C temporal profiles in outcome prediction for adults with severe TBI. *J. Cereb. Blood Flow Metab.* 31, 1886–1896. doi: 10.1038/jcbfm.2011.31

Wagner, A. K., Fabio, A., Puccio, A. M., Hirschberg, R., Li, W., Zafonte, R. D., et al. (2005). Gender associations with cerebrospinal fluid glutamate and lactate/pyruvate levels after severe traumatic brain injury. *Crit. Care Med.* 33, 407–413. doi: 10.1097/01.CCM.0000153931.23488.DD

Wagner, A. K., McCullough, E. H., Niyonkuru, C., Ozawa, H., Loucks, T. L., Dobos, J. A., et al. (2011a). Acute serum hormone levels: characterization and prognosis after severe traumatic brain injury. *J Neurotrauma.* 28, 871–888. doi: 10.1089/neu.2010.1586

Wagner, A. K., Ren, D., Conley, Y. P., Ma, X., Kerr, M. E., Zafonte, R. D., et al. (2007). Sex and genetic associations with cerebrospinal fluid dopamine and metabolite production after severe traumatic brain injury. *J. Neurosurg.* 106, 538–547. doi: 10.3171/jns.2007.106.4.538

Webster, M. J., Herman, M. M., Kleinman, J. E., and Shannon Weickert, C. (2006). BDNF and trkB mRNA expression in the hippocampus and temporal cortex during the human lifespan. *Gene Expr. Patterns* 6, 941–951. doi: 10.1016/j.modgep.2006.03.009

Werdan, K., Schmidt, H., Ebelt, H., Zorn-Pauly, K., Koidl, B., Hoke, R. S., et al. (2009). Impaired regulation of cardiac function in sepsis, SIRS, and MODS. *Can. J. Physiol. Pharmacol.* 87, 266–274. doi: 10.1139/Y09-012

Yang, A. C., Chen, T.-J., Tsai, S.-J., Hong, C. J., Kuo, C. H., Yang, C. H., et al. (2010). BDNF Val66Met polymorphism alters sympathovagal balance in healthy subjects. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 153B, 1024–1030. doi: 10.1002/ajmg.b.31069

Yang, K., Perez-Polo, J. R., Mu, X. S., Yan, H. Q., Xue, J. J., Iwamoto, Y., et al. (1996). Increased expression of brain-derived neurotrophic factor but not neurotrophin-3 mRNA in rat brain after cortical impact injury. *J Neurosci Res.* 44, 157–164. doi: 10.1002/(SICI)1097-4547(19960415)44:2<157::AID-JNR8>3.0.CO;2-C

Yeager, M. P., Guyre, P. M., and Munck, A. U. (2004). Glucocorticoid regulation of the inflammatory response to injury. *Acta Anaesthesiol. Scand.* 48, 799–813. doi: 10.1111/j.1399-6576.2004.00434.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Molecular Neuroscience

REVIEW
published: 01 Novemeber 2016
doi: 10.3389/fnmol.2016.00112

Metabolic Therapy for Temporal Lobe Epilepsy in a Dish: Investigating Mechanisms of Ketogenic Diet using Electrophysiological Recordings in Hippocampal Slices

Masahito Kawamura Jr.[1]*, *David N. Ruskin*[2] *and Susan A. Masino*[2]

[1] *Department of Pharmacology, Jikei University School of Medicine, Tokyo, Japan,* [2] *Department of Psychology and Neuroscience Program, Trinity College, Hartford, CT, USA*

The hippocampus is prone to epileptic seizures and is a key brain region and experimental platform for investigating mechanisms associated with the abnormal neuronal excitability that characterizes a seizure. Accordingly, the hippocampal slice is a common *in vitro* model to study treatments that may prevent or reduce seizure activity. The ketogenic diet is a metabolic therapy used to treat epilepsy in adults and children for nearly 100 years; it can reduce or eliminate even severe or refractory seizures. New insights into its underlying mechanisms have been revealed by diverse types of electrophysiological recordings in hippocampal slices. Here we review these reports and their relevant mechanistic findings. We acknowledge that a major difficulty in using hippocampal slices is the inability to reproduce precisely the *in vivo* condition of ketogenic diet feeding in any *in vitro* preparation, and progress has been made in this *in vivo/in vitro* transition. Thus far at least three different approaches are reported to reproduce relevant diet effects in the hippocampal slices: (1) direct application of ketone bodies; (2) mimicking the ketogenic diet condition during a whole-cell patch-clamp technique; and (3) reduced glucose incubation of hippocampal slices from ketogenic diet–fed animals. Significant results have been found with each of these methods and provide options for further study into short- and long-term mechanisms including Adenosine triphosphate (ATP)-sensitive potassium (K_{ATP}) channels, vesicular glutamate transporter (VGLUT), pannexin channels and adenosine receptors underlying ketogenic diet and other forms of metabolic therapy.

Keywords: ketone bodies, adenosine receptors, pannexin channels, ATP-sensitive potassium channels, vesicular glutamate transporter, temporal lobe epilepsy

OPEN ACCESS

Edited by:
Jakob Wolfart,
University of Rostock, Germany

Reviewed by:
Oliver Kann,
Heidelberg University, Germany
Axel Neu,
University Medical Center Hamburg-Eppendorf, Germany

***Correspondence:**
Masahito Kawamura Jr.
m.kawamura@jikei.ac.jp

Received: *26 August 2016*
Accepted: *17 October 2016*
Published: *01 Novemeber 2016*

Citation:
Kawamura M Jr., Ruskin DN and Masino SA (2016) Metabolic Therapy for Temporal Lobe Epilepsy in a Dish: Investigating Mechanisms of Ketogenic Diet using Electrophysiological Recordings in Hippocampal Slices. Front. Mol. Neurosci. 9:112. doi: 10.3389/fnmol.2016.00112

HIPPOCAMPUS: A KEY BRAIN REGION TO INVESTIGATE KETOGENIC DIET MECHANISMS

The hippocampus is well-known as a brain area involved in learning and memory and also as the key region underlying the form of epilepsy known as mesial temporal lobe epilepsy. The temporal lobe refers to the ventrolateral middle part of cerebral cortex and abnormal neuronal discharge or a lesion affecting this lobe causes seizures (Gastaut, 1973). There are two main types of temporal

lobe epilepsy classified by the epileptic focus: mesial and lateral. The epileptic focus of mesial temporal lobe epilepsy is the hippocampus, amygdala or parahippocampal gyrus and the focus of lateral temporal lobe epilepsy is in neocortex. Over 80% of patients with temporal lobe epilepsy have the mesial form (Schramm et al., 2001; Quarato et al., 2005) and it is often resistant to pharmacological treatment. The typical symptom of mesial temporal lobe epilepsy is complex partial seizures, which have a high probability of an accompanying characteristic aura. For an individual patient the aura may present as epigastric discomfort sometimes described as nausea, or psychiatric symptoms including fear. Complex partial seizures often begin with arrest of motor activities or staring. Autonomic motor behaviors are usually oroalimentary automatisms or complex automatisms. Dystonic posturing lasting for 1–2 min often occurs involving the arm contralateral to the ictal discharge (Engel, 2001). In a majority of patients, mesial temporal lobe epilepsies are associated with hippocampal sclerosis (Watson, 2003), which is atrophy with global gliosis and loss of CA1 and/or CA3 pyramidal neurons in the hippocampus (Thom, 2009).

The basic structure of hippocampus is simple. It includes principal cells (granular cells of dentate gyrus and CA1-4 pyramidal neurons) and surrounding interneurons. The principal cells form an excitatory circuit which is modulated by inhibitory interneurons. Notably, CA3 pyramidal neurons are connected to each other by excitatory recurrent collaterals. Thus, the hippocampal circuit is regulated by a balance between excitation from recurrent collaterals and inhibition from interneurons. When the balance collapses, the hippocampal circuit becomes hyper excitable and susceptible to seizures. Therefore, atrophy in the hippocampus is thought to be one of the main focuses of mesial temporal lobe epilepsy and excision of hippocampal sclerosis with selective amygdalohippocampectomy successfully improves ∼70% of surgical patients (Wiebe et al., 2001; Paglioli et al., 2006). Unfortunately, even though a majority experience significant improvement after surgery, at least half of patients relapse and do not experience permanent and complete seizure control (McIntosh et al., 2004; de Tisi et al., 2011; Najm et al., 2013). Based on clinical and experimental evidence, the hippocampus is a good experimental target for investigating epileptogenesis and therapeutic interventions for temporal lobe epilepsy.

The ketogenic diet was designed in the 1920s to treat epilepsy by mimicking the metabolic changes induced by fasting (Wilder, 1921). Ketogenic diet is effective against many types of seizures. It is used more frequently against generalized seizures such as myoclonic, atonic and absence seizures. However, it is also reported that ketogenic diet has been used successfully to treat focal seizures such as simple and complex partial seizures as effectively as generalized seizures [Freeman et al., 1998; Maydell et al., 2001; but one study reported that the ketogenic diet was less effective in patients with epileptiform discharges in the temporal region (Beniczky et al., 2010)]. Despite almost 100 years of clinical use, however, the mechanisms underlying the success of ketogenic diet therapy are not well understood. In recent decades, ketogenic diet has increasingly been noted as a useful therapy for medically refractory epilepsy in adults (Sirven et al., 1999; Mosek et al., 2009) and children (Hallböök et al., 2007). Patients with temporal lobe epilepsy are well-known to be frequently resistant to antiepileptic drugs (Wiebe and Jette, 2012). As mentioned above, the first choice of treatment for medically refractory mesial temporal lobe epilepsy associated with hippocampal sclerosis is surgery (anterior temporal lobectomy or selective amygdalohippocampectomy) because of good therapeutic outcomes (Tanriverdi et al., 2008). For temporal lobe epilepsy patients who are not good candidates for surgery, however, ketogenic diet is one of the therapeutic options (Ray and Wyllie, 2005; Klein et al., 2010). Thus, a natural question is how the ketogenic diet produces its beneficial effects in temporal lobe epilepsy. Broad reviews on the diet's mechanisms are available (Lutas and Yellen, 2013; Rogawski et al., 2016); here, we focus on work from multiple laboratories studying ketogenic diet's antiseizure mechanisms using acute hippocampal slice preparations.

ADVANTAGES OF THE HIPPOCAMPAL SLICE PREPARATION IN STUDYING ANTIEPILEPTIC MECHANISMS OF KETOGENIC DIET

Because epilepsy is caused by abnormal neuronal discharges in the brain, electrophysiological measurements are the most direct and useful approach for researching epilepsy and its treatments. There are two approaches for electrophysiological recordings of any brain region: *in vivo* and *in vitro*. *In vivo* electrophysiological recording of hippocampus is usually done by extracellular recording of electrically-evoked activity (Stewart and Reid, 1993), or continuous recording of spontaneous field activity (Li et al., 2008), single-cell intracellular sharp electrodes (Henze and Buzsáki, 2001) or multiple unit activity (Lin et al., 2006); these preparations can be acute or chronic. The technique for *in vivo* patch-clamp recording was also developed recently (Pernia-Andrade and Jonas, 2014). *In vitro* electrophysiological recording is done using single-cell intracellular sharp electrodes (Abe and Ogata, 1981) or patch-clamp electrodes (Kawamura et al., 2004), or extracellular field recording with single electrodes (Masino and Dunwiddie, 1999) or electrode arrays (Knowles et al., 1987) using acute slices of hippocampus. Compared with *in vivo* hippocampal recordings, the advantages of hippocampal slices are several-fold: (1) Ease of use and tissue access: acute brain slices must be maintained by perfusion with oxygenated artificial cerebrospinal fluid (Sakmann et al., 1989). Continuous perfusion allows for changing the extracellular fluid, making it easy to apply and wash out agonists and/or antagonists of various proteins such as ion channels, receptors and transporters. Furthermore, it is easy to examine in detail the functional mechanisms and dynamics of neuronal activity; (2) Efficient use of resources: we usually make 3–6 brain slices from one rodent, potentially obtaining 3–6 recordings, and thus allowing us to reduce the number of animals used; (3) History: a huge number of electrophysiological experiments have been done

using hippocampal slice preparations in the last half-century. Several methods for causing seizure-like bursting *in vitro* have been used in the hippocampal slice preparation including electrical kindling (Sayin et al., 1999), kainic acid treatment (Congar et al., 2000; Smith and Dudek, 2001), inhibition of GABA receptors (Köhling et al., 2000; Stafstrom et al., 2009), inhibition of potassium ion channels (Stafstrom et al., 2009) and neuronal hyperexcitability by high extracellular potassium concentrations (Congar et al., 2000; Stafstrom et al., 2009) or low extracellular magnesium concentrations (Dulla et al., 2005; Kovács et al., 2005); and (4) Potential use of human tissue: experimental techniques from rodent hippocampal slice preparations are also applicable to acutely resected hippocampal tissue obtained from patients with surgically approachable epilepsy (Schroder et al., 2000). All these approaches support the use of hippocampal slice preparations to elucidate epileptic mechanisms.

Among these advantages lurk some disadvantages. One unavoidable pitfall of *in vitro* recording is that the environment of acute brain slice preparations is inherently different from *in vivo* condition. Cutting brain tissue causes acute traumatic injury such as excitatory GABA signaling caused by increased intracellular chloride concentration in acute hippocampal preparations (Dzhala et al., 2012). It is known that gliosis occurs in the hippocampal slice cultures (Lossi et al., 2009). Recently, it has been reported that the early stage of reactive gliosis already occurs in acute hippocampal slice preparations (Takano et al., 2014). We usually cut slices at 300–500 μm, necessarily limiting neuronal networks and three-dimensional morphologies to this thickness. Also, artificial cerebrospinal fluid does not and cannot reproduce actual cerebrospinal fluid exactly. Thus, results from *in vitro* hippocampal slice preparations should be confirmed by *in vivo* electrophysiological recordings or behavioral tests as much as possible. For that reason both *in vivo* and *in vitro* electrophysiological recordings are useful and both are essential for epilepsy research.

Ketogenic diet therapy presumably alters aspects of blood and cerebrospinal fluid to produce an anticonvulsant effect; in making and supporting brain slices, it is necessary to replace blood and cerebrospinal fluid with a bathing solution. Thus, the major difficulty in using acute hippocampal slices for ketogenic diet research is the inability to precisely reproduce or maintain the metabolic condition induced by diet therapy in this *in vitro* preparation. The lack of a standard protocol for a "diet in a dish" is evident in the diversity of experimental procedures, the prevalence of mixed results and the relative dearth of studies given the importance of delineating the key mechanisms underlying an enduring and successful treatment for a common and challenging neurological condition. *In vivo* recording clearly does not have this problem because it uses the whole body of experimental animals and diet-altered metabolism is maintained (Koranda et al., 2011; Masino et al., 2011). Several special strategies have been implemented for examining mechanisms of the ketogenic diet using hippocampal slice preparations; we focus on reviewing three of these approaches (**Figures 1–3**).

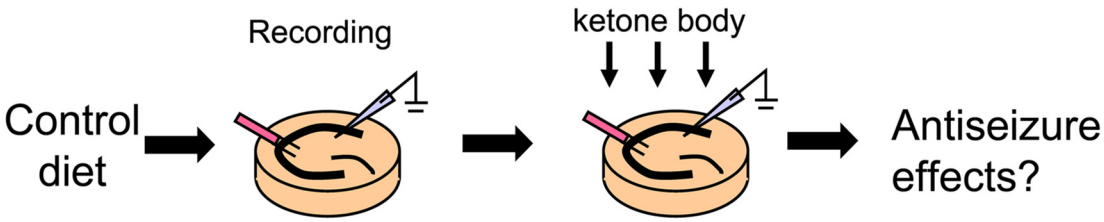
THREE STRATEGIES FOR INVESTIGATING KETOGENIC DIET MECHANISMS IN HIPPOCAMPAL SLICES

Direct Application of Ketone Bodies to Hippocampal Slices From Control Diet-Fed Rodents

The ketogenic diet was developed to mimic fasting, which alleviates epileptic seizures (Wilder, 1921). Ketogenic diet feeding increases ketone bodies (β-hydroxybutyrate (βHB), acetoacetate (AA), acetone) to usually over 1 mM in blood in humans (Bergqvist et al., 2005; Than et al., 2005) and rodents (Hartman et al., 2008; Linard et al., 2010) synthesized from free fatty acids in the liver (Masino and Rho, 2012) and then used for energy in the brain instead of glucose (Masino et al., 2009). Chronic ketosis (increased levels of ketone bodies) is the eponymous metabolic hallmark of the ketogenic diet. Therefore, one approach to reproducing a ketogenic diet in a hippocampal slice is a direct application of ketone bodies to determine if and how ketone bodies modulate neuronal activity directly.

In this paradigm, hippocampal slices are taken from control diet-fed animals and dissolved ketone bodies are applied in an extracellular solution such as artificial cerebrospinal fluid (**Figure 1**). Slice preparation protocols, electrophysiological recording methods, rodent model (different strains of rats or mice), and mixtures of ketone bodies can be found among the laboratories. Not surprisingly, results among the studies were mixed.

A number of studies found negative results. Thio et al. (2000) reported that direct application of ketone bodies had no effect on synaptic activity in acute hippocampal slices from Sprague-Dawley (SD) rats. They recorded evoked field excitatory postsynaptic potentials (fEPSP) and population spikes (PS) in the CA1 region stimulated by Schaffer collateral fibers and applied mixed ketone bodies (1 mM AA and 2 mM βHB) to the slices. A 20 min application of ketone bodies did not change either fEPSP slope or PS amplitude. They also recorded potassium channel blocker 4-aminopyridine-induced epileptiform discharges from the dentate granule cell layer and CA3 region and reported that application for 105 min did not change the frequency or duration of these ictal events. Kimura et al. (2012) also reported that application of mixed ketone bodies (1 mM each AA and βHB) for 20 min did not change fEPSP slope and an 80 min application did not change the high-frequency tetanic stimulation-induced long-term potentiation (LTP) recorded from CA1 region in acute hippocampal slices from Wistar rats. Similar results were reported that mixed ketone bodies (1 mM AA and 2 mM βHB) had no effect on CA1 region synaptic transmission or theta burst-induced LTP in SD rat acute hippocampal slices (Youssef, 2015). A unique approach was used by Samoilova et al. (2010). They made organotypic hippocampal slices which were cultured with low glucose and 10 mM βHB medium for at least 3 days. This chronic *in vitro* ketosis, however, did not alleviate intrinsic or induced epileptiform discharges (but was neuroprotective).

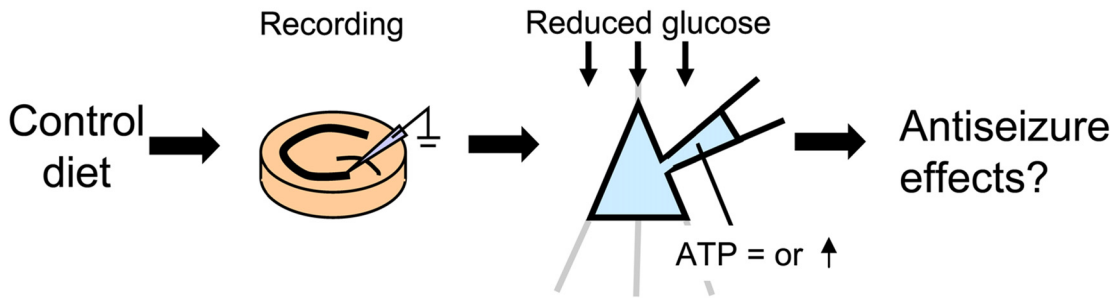


Juge et al., 2010	C57BL/6 mouse	Whole-cell patch clamp	**10 mM AA**	**positive**
Tanner et al., 2011	C57BL/6 mouse	Single channel	**2 mM β HB**	**positive**
Kim et al., 2015	*Kcna1*-null mouse	Spikes using multielectrode	**1 mM AA and 5 mM β HB**	**positive**
Thio et al., 2000	SD rat	fEPSP and PS	**1 mM AA and 2 mM β HB**	negative
Samoilova et al., 2010	Wistar rat	PS	**10 mM β HB**	negative
Kimura et al., 2012	Wistar rat	fEPSP	**1 mM AA and 1 mM β HB**	negative
Youssef, 2015	SD rat	fEPSP	**1 mM AA and 2 mM β HB**	negative

FIGURE 1 | Direct application of ketone bodies to hippocampal slices From control diet-fed animals. Reference list is shown at bottom. Three of seven references report positive effects for antiseizure with direct application of ketone bodies. AA, acetoacetate; βHB, β-hydroxybutyrate; fEPSP, field postsynaptic potential; PS, population spike.

All of these studies concluded that ketone bodies do not directly affect synaptic transmission, seizure-like activity or LTP in the rat hippocampal slice.

Other studies, however, have found positive results and even revealed new mechanisms. Juge et al. (2010) made acute hippocampal slices from C57BL/6 mice and incubated the slices with 10 mM AA for over 2 h, after which they recorded EPSPs from CA1 pyramidal neurons using whole-cell patch clamp. Frequency and amplitude of miniature EPSPs (mEPSP) from AA-incubated slices were significantly reduced compared with control slices. Ketone bodies inhibited valinomycin-evoked glutamate uptake by the purified vesicular glutamate transporter (VGLUT), suggesting that ketone bodies inhibit synaptic transmission with reduction of glutamate release via direct ketone body-induced suppression of glutamate uptake into vesicles. Importantly, they also investigated the behavioral effects of ketone bodies. Seizures in Wistar rats induced by intrahippocampal 4-aminopyridine were moderated by intrahippocampal 10 mM AA, both infused by microdialysis. These results clearly show that direct application of ketone bodies modulates synaptic transmission in hippocampal slices and reduces seizure activity *in vivo* (Juge et al., 2010). In addition, Tanner et al. (2011) recorded single channel activity from dentate granule neurons after incubating acute hippocampal slices from C57BL/6 mice with 2 mM βHB for over 40 min. Preincubation with this ketone body increased steady-state and stimulus-elevated open probability of Adenosine triphosphate (ATP)-sensitive potassium (K_{ATP}) channels, which contribute to the slow afterhyperpolarization after action potential bursts to modulate spontaneous firing, suggesting that direct ketone body-mediated opening of K_{ATP} channels in dentate granule neurons may act as a seizure gate in the hippocampus. Similar results were reported from neurons of the substantia nigra in coronal midbrain slices of rats and mice from same laboratory (Ma et al., 2007). Kim et al. (2015) recorded from organotypic hippocampal slices which were cultured with 5 mM βHB and 1 mM AA medium for 2 weeks. They used *Kcna1*-null mice (C3HeB/FeJ background) lacking voltage-gated potassium ($K_v1.1$) channels, which is thought be a model for several types of epilepsy including human temporal lobe epilepsy. Extracellular multielectrode array recordings showed spontaneous seizure-like events in organotypic hippocampal slice cultures from *Kcna1*-null mice. The application of ketone bodies for 2 weeks attenuated the seizure-like events in the mutant tissue. They



Kawamura et al., 2010	SD rat and C57BL/6 mouse	Whole-cell patch clamp	**$[ATP]_i$= 2-5 mM $[glucose]_e$ = 3 mM**	**positive**

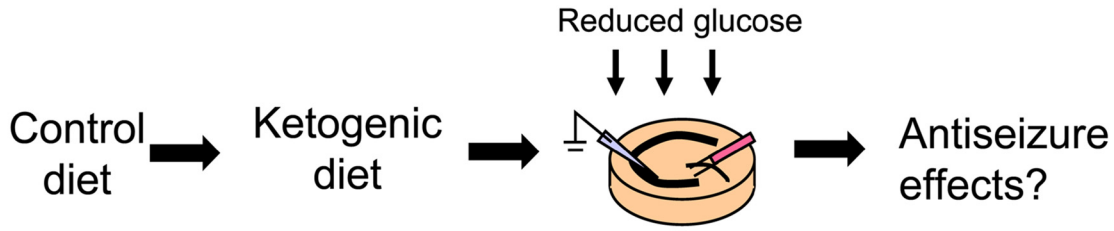
FIGURE 2 | Controlling glucose and adenosine triphosphate (ATP) levels by using whole-cell patch clamp recording of a single cell in a hippocampal slice from control diet-fed animals. To mimic the ketogenic diet milieu, extracellular glucose is reduced and intracellular ATP is increased. Reference is shown at bottom. $[ATP]_i$, intracellular ATP concentration; $[glucose]_e$, extracellular glucose concentration.

also applied 5 mM βHB *in vivo* using subcutaneously implanted osmotic minipumps to *Kcna1*-null mice and reported that administration of this ketone body reduced the number of seizures (Kim et al., 2015). In typical studies, slice preparations are maintained by higher extracellular glucose concentration than is physiological; up to 25–30 mM extracellular glucose concentration for acute hippocampal slices (Bischofberger et al., 2006) and 10–12 mM extracellular glucose for hippocampal slice cultures [Galow et al., 2014; but electrophysiological recording can be done in 5–10 mM glucose (Schneider et al., 2015)]. Since it is reported that complete replacement from glucose to ketone bodies decreases neuronal activity (Arakawa et al., 1991; Wada et al., 1997), all experiments for direct application of ketone bodies were done by adding ketone bodies to the usual *in vitro* glucose concentration for slice preparations. Experiments using purified VGLUT by Juge et al. (2010) were done with glucose-free conditions and the results showed the direct effect of ketone bodies clearly. Ma et al. (2007) compared the effect of ketone body application ranging between 12 mM and 5 mM extracellular glucose concentration in coronal midbrain slices and found the effect was not changed by reduced glucose. These reports suggest that the effects of ketone bodies might not be correlated with glucose concentration.

In sum, several studies have used direct application of ketone bodies in hippocampal acute slices or organotypic cultures, and both positive and negative results have been found. Negative and positive studies used rats and mice, respectively, so a simple explanation is that the discrepancy arises from species differences. This seems unlikely because ketogenic diet is known to reduce behavioral seizures in both rats (Appleton and DeVivo, 1974; Hori et al., 1997; Bough et al., 2002, 2006; Zhao et al., 2004), and mice (Uhlemann and Neims, 1972; Rho et al., 1999; Noh et al., 2003; Hartman et al., 2008; Kwon et al., 2008; but see Linard et al., 2010). The methods for applying ketone bodies varied in these reports including concentration of ketone bodies, time for application and application pathway (perfusion or preincubation) and these might contribute to inter-study variation. Aligning technical details for direct application of ketone bodies may be useful for finding common mechanisms.

Changing Intracellular ATP and Extracellular Glucose With Whole-Cell Patch Clamp to Mimic Ketogenic Diet

A less common approach mimics the altered metabolism found during ketogenic diet treatment using single-cell patch clamp recording. Fasting and ketogenic diet are thought to cause anticonvulsant effect by changing brain metabolism, and this approach attempts to mimic a key metabolic "end point." The other metabolic hallmark (besides ketosis) of the ketogenic diet is a stable, mild hypoglycemia in humans (Huttenlocher, 1976; Noakes et al., 2006; Nuttall et al., 2015) and rodents (Bough et al., 2006). It is also reported that ketogenic diet decreases glucose concentration in the hippocampal extracellular fluid by 30% compared to control diet measured by *in vivo* microdialysis in mice (although lactate did not change; Samala et al., 2011). Interestingly, plasma glucose level correlates with the antiepileptic effect of the ketogenic diet (Mantis et al., 2004), indicating that extracellular glucose is mechanistically relevant. Intracellular conditions are also thought to be changed by ketogenic diet-altered metabolism and product major goal of brain energy metabolism is the generation of sufficient ATP. Several studies have reported that ketogenic diet increases brain ATP levels in humans (Pan et al., 1995) and rodents (DeVivo et al., 1978; Nakazawa et al., 1983; Nylen et al., 2009), thought to be caused by mitochondrial biogenesis leading from chronic ketosis (Bough et al., 2006). Therefore, intracellular ATP is a promising mechanism for mimicking the ketogenic diet *in vitro*. From these reports, reducing and increasing extracellular glucose and intracellular ATP, respectively, might in combination



Stafstrom et al., 1999	SD rat	**Ketogenic diet (4.8:1) for 6-8 weeks**	fEPSP and PS	**positive**
Bough et al., 2006	SD rat	**Ketogenic diet (6:1) for 20 days**	fEPSP	**positive**
Kawamura et al., 2014	SD rat and C57BL/6 mouse	**Ketogenic diet (6:1) for 13-18 days**	PS	**positive**
Simeone et al., 2015	*Kcna1*-null mouse	**Ketogenic diet (6:1) for 11-15 days**	Spikes using multielectrode	**positive**

FIGURE 3 | Acute hippocampal slices from ketogenic diet-fed animals. Extracellular glucose is reduced during incubation and recording to maintain the *in vivo* effect of the ketogenic diet. Reference list is shown at bottom. fEPSP, field postsynaptic potential; PS, population spike. Ketogenic diet (4.8:1) or (6:1) means fat: [protein + carbohydrate] ratio.

reproduce ketogenic diet conditions in acute hippocampal slices (**Figure 2**). As mentioned in "Advantages of the Hippocampal Slice Preparation in Studying Antiepileptic Mechanisms of Ketogenic Diet" Section, it is easy to change extracellular solution with an *in vitro* brain slice (including moderately lowering glucose), but trickier to change the intracellular milieu. The whole-cell patch clamp technique is one of the methods for recording a single cell (Kawamura et al., 2004). This technique allows physical exchange between the intracellular fluid and the artificial intracellular solution in the recording pipette, enabling experimental modification of the intracellular fluid of the recorded neuron (**Figure 2**) including elevating intracellular ATP. We recorded from CA3 pyramidal neurons with the whole-cell patch clamp technique in acute hippocampal slices from control diet-fed SD rats or C57BL/6 mice. We found that increased intracellular ATP (comparing 0.5, 1, 2 and 5 mM ATP concentration in the intracellular solutions) and reduced extracellular glucose (from 11 mM to 3 mM) caused an outward current (hyperpolarization when recording membrane potential) in CA3 pyramidal neurons (Kawamura et al., 2010). The direction and magnitude of this current was dose-dependent for both extracellular glucose and intracellular ATP, and importantly it was found in both rats and mice. Pharmacological and genetic experiments demonstrated that when intracellular ATP was sufficient or increased, reducing extracellular glucose opened pannexin-1 channels and released intracellular ATP to the extracellular space. Released ATP was rapidly hydrolyzed to adenosine which activated adenosine A_1 receptors (A_1R) with subsequent opening of K_{ATP} channels. Opening of these potassium channels caused hyperpolarization and reduced excitability. These results indicate that mimicking the ketogenic diet condition with increased ATP and reduced glucose reduces excitability in hippocampal CA3 pyramidal neurons with autocrine modulation via adenosine A_1R, and this might be a one of the key mechanisms of the anticonvulsant effects of the ketogenic diet *in vivo* (**Figure 4**). This approach for reproducing ketogenic diet conditions in acute hippocampal slice is useful to elucidate detailed mechanisms within single neurons. However, it mimics only two of the aspects of ketogenic diet feeding. Further examinations using behavioral tests and recordings from *in vivo* ketogenic diet-fed animals are needed to link the results from this approach to the effects of ketogenic diet feeding.

Hippocampal Slices From Ketogenic Diet-Fed Rodents

A third approach is possibly the most direct and useful way for investigating mechanisms of ketogenic diet because it uses acute hippocampal slices from ketogenic diet-fed animals (**Figure 3**). In patients, ketogenic diet is applied through three meals with snacks and the typical fat: (protein+carbohydrate) ratio is 3:1 or 4:1 in children (Hartman and Vining, 2007; Zupec-Kania and Spellman, 2008) and down to 1:1 in adults. On the other hand ketogenic diet for rodents is usually applied by free access to food with more strict ketogenic ratio 4.8:1 or 6:1 (da Silveira et al., 2010). One question about this approach is whether or not the intra- and extracellular milieu produced by ketogenic diet feeding can be maintained after making and incubating brain slices. However, four reports show that it can work successfully.

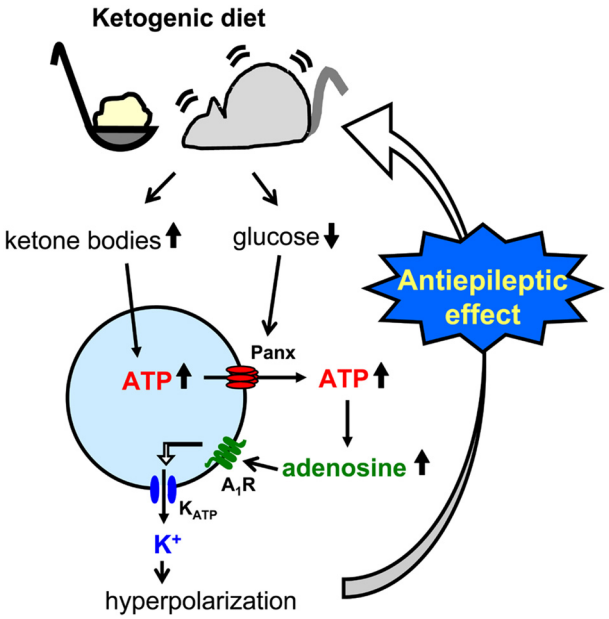


FIGURE 4 | Schematic of pannexin-1 channel-adenosine A_1 receptor-K_{ATP} channel autocrine regulation in CA3 pyramidal neurons and relationship to ketogenic diet. Ketogenic diet-fed animal increases ketone body levels and reduces glucose levels. Ketone bodies might increase intracellular ATP concentration, and increased ATP is released to the extracellular space through reduced glucose-induced opening of pannexin-1 channels (panx). After breakdown to adenosine by nucleotidases, activated adenosine A_1 receptors (A_1R) open ATP-sensitive potassium (K_{ATP}) channels which hyperpolarizes CA3 pyramidal neurons. This hyperpolarization reduces neuronal hyperexcitability and causes the ketogenic diet's antiseizure effects.

Stafstrom et al. (1999) reported that a ketogenic diet (4.8:1 ratio) induced antiseizure effects in acute hippocampal slices from kainic acid-treated rats. They recorded fEPSP and PS from area CA1 from SD rats fed a ketogenic diet for 6–8 weeks. Synaptic transmission was not significantly different between slices from control diet-fed and ketogenic diet-fed rats. However the frequency of kainic acid-induced spontaneous seizures was significantly lower in slices from ketogenic diet-fed rats than from control diet-fed rats. The slices were incubated by normal artificial cerebrospinal fluid not including ketone bodies. The authors concluded that the effects observed in the slices from ketogenic diet-fed rats would be independent of direct ketone body action (Stafstrom et al., 1999). Similar results were reported by Simeone et al. (2014) using extracellular multielectrode array recordings in acute hippocampal slices from ketogenic diet-fed *Knca1*-null mice. The pathologic seizure-like events generated in *Knca1*-null mice slice were diminished by ketogenic diet (6:1 ratio) treatment for 11–15 days. Mossy fiber-CA3 dendritic field potential slopes and fiber volley amplitudes of mossy fiber were not significantly different between slices from control diet-fed and ketogenic diet-fed *Knca1*-null mice, however ketogenic diet increased paired-pulse facilitation ratios and the half maximal stimulation intensities of field potential slope which are decreased by Kv1.1 knock out. They suggested that the improvement of mitochondria function by ketogenic diet might restore the hyperexcitability of CA3 neurons in *Knca1*-null mice and decrease seizure-like events (Simeone et al., 2014). Bough et al. (2006) recorded medial perforant pathway-evoked fEPSPs from the dentate molecular layer in acute hippocampal slices from SD rats fed with a ketogenic diet (6:1 ratio) for over 20 days. Reducing extracellular glucose concentration from 10 to 2 mM depressed the slope of fEPSP reversibly in slices from control diet-fed rats and this depression was inhibited in slice from ketogenic diet-fed rats. The effects were lost after slices were incubated in 10 mM glucose for over 3.5 h. This result, however, strongly suggests that intracellular metabolic changes with ketogenic diet can be maintained over 3 h after changing extracellular conditions into artificial cerebrospinal fluid. They concluded that synaptic transmissions in hippocampal slices from ketogenic diet-fed rats were more resistant to reduced glucose than slices from control diet-fed rats with facilitation of mitochondrial biogenesis (Bough et al., 2006). We also reported that ketogenic diet caused antiseizure effects in acute hippocampal slices of rats and mice (Kawamura et al., 2014). We recorded PS and GABA receptor blocker bicuculline-induced seizure-like bursting from the CA3 region in acute hippocampal slices from SD rats or C57BL/6 mice fed a ketogenic diet (6:1 ratio) for 13–18 days. Excitability and bicuculline-induced bursting were significantly inhibited by reduced extracellular glucose concentration in slices from ketogenic diet-fed rats and mice but were not changed by reduced extracellular glucose in slices from control diet-fed rodents. In this study, the effect of ketogenic diet feeding is maintained for at least 6 h after making hippocampal slices. Ketogenic diet-induced suppression of bicuculline-induced bursting was inhibited by adenosine A_1R antagonist and did not occur in slices from adenosine A_1R knock-out mice. Antagonism of K_{ATP} channels or pannexin-1 channels inhibited the ketogenic diet-induced suppression of bicuculline-induced bursting. These results suggest that ketogenic diet causes antiseizure effects through a pannexin-1 channel-adenosine A_1R-K_{ATP} channel autocrine pathway (**Figure 4**), the same pathway revealed by the whole-cell patch clamp technique for mimicking ketogenic diet conditions as described in "Changing Intracellular ATP and Extracellular Glucose with Whole-Cell Patch Clamp to Mimic Ketogenic Diet" Section (Kawamura et al., 2010).

These studies used acute hippocampal slices from ketogenic diet-fed rodents successfully to elucidate altered neuronal activity underlying this treatment. Interestingly, reducing extracellular glucose concentration is thought be one of the most important points for reproducing the effects of ketogenic diet in this approach. Synaptic transmission in hippocampal slices from ketogenic diet-fed rodents were not different from slices from control diet-fed rodents when extracellular glucose concentration in artificial cerebrospinal fluid is standard in all three reports [however, evidence is mounting that this standard glucose concentration for acute brain slices is higher than physiological brain glucose levels (Shram et al., 1997; Lowry and Fillenz, 2001; Kealy et al., 2013)]. Reduced glucose reveals the difference between ketogenic diet- and control diet-fed animals in two of these studies (Bough et al., 2006; Kawamura et al., 2014), which parallels the finding that the anticonvulsant effect of the

ketogenic diet is correlated with plasma glucose levels (Mantis et al., 2004). Therefore it would be useful to make extracellular glucose concentrations lower than standard to reproduce or maintain effects of the ketogenic diet in acute hippocampal slices.

CONCLUSION

Here we describe three approaches for researching anticonvulsant mechanisms of ketogenic diets by using electrophysiological recording from hippocampal slices. The usefulness of hippocampal slices is that it is easy to elucidate the details of neuronal modulation by ketogenic diet as shown in **Figure 4**. All three approaches have contributed to finding detailed potential mechanisms underlying ketogenic diet effects including VGLUT modulation, K_{ATP} opening, activation of adenosine receptors, and ATP release from pannexin channels. Complementary *in vivo* work has provided additional evidence for some of these mechanisms (Masino et al., 2011; Giménez-Cassina et al., 2012). Taken together, it is clear that electrophysiological recordings from hippocampal slices is a good tool for ketogenic diet research. However, all three approaches need experimental manipulations for reproducing ketogenic diet effects *in vitro* such as ketone application, reduced extracellular glucose and increased intracellular ATP, and this requirement may explain the lack of a standardized protocol and a robust literature in this area. Finally, electrophysiology in slices is not a direct measurement of the *in vivo* anticonvulsant effects of ketogenic diet, and a combination of both *in vivo* and *in vitro* recordings is the best approach to provide further insight into the key anticonvulsant mechanisms underlying ketogenic diet and other metabolic therapies.

AUTHOR CONTRIBUTIONS

MK, DNR and SAM wrote the article.

ACKNOWLEDGMENTS

The authors acknowledge the support of The Mother and Child Health Foundation and NIH (NS066392, AT008742 and NS 065957).

REFERENCES

Abe, H., and Ogata, N. (1981). Effects of penicillin on electrical activities of neurons in guinea-pig hippocampal slices. *Jpn. J. Pharmacol.* 31, 661–675. doi: 10.1254/jjp.31.661

Appleton, D. B., and DeVivo, D. C. (1974). An animal model for the ketogenic diet. *Epilepsia* 15, 211–227. doi: 10.1111/j.1528-1157.1974.tb04943.x

Arakawa, T., Goto, T., and Okada, Y. (1991). Effect of ketone body (D-3-hydroxybutyrate) on neural activity and energy metabolism in hippocampal slices of the adult guinea pig. *Neurosci. Lett.* 130, 53–56. doi: 10.1016/0304-3940(91)90225-i

Beniczky, S., Jose Miranda, M., Alving, J., Heber Povlsen, J., and Wolf, P. (2010). Effectiveness of the ketogenic diet in a broad range of seizure types and EEG features for severe childhood epilepsies. *Acta Neurol. Scand.* 121, 58–62. doi: 10.1111/j.1600-0404.2009.01303.x

Bergqvist, A. G., Schall, J. I., Gallagher, P. R., Cnaan, A., and Stallings, V. A. (2005). Fasting versus gradual initiation of the ketogenic diet: a prospective, randomized clinical trial of efficacy. *Epilepsia* 46, 1810–1819. doi: 10.1111/j.1528-1167.2005.00282.x

Bischofberger, J., Engel, D., Li, L., Geiger, J. R., and Jonas, P. (2006). Patch-clamp recording from mossy fiber terminals in hippocampal slices. *Nat. Protoc.* 1, 2075–2081. doi: 10.1038/nprot.2006.312

Bough, K. J., Gudi, K., Han, F. T., Rathod, A. H., and Eagles, D. A. (2002). An anticonvulsant profile of the ketogenic diet in the rat. *Epilepsy Res.* 50, 313–325. doi: 10.1016/s0920-1211(02)00086-4

Bough, K. J., Wetherington, J., Hassel, B., Pare, J. F., Gawryluk, J. W., Greene, J. G., et al. (2006). Mitochondrial biogenesis in the anticonvulsant mechanism of the ketogenic diet. *Ann. Neurol.* 60, 223–235. doi: 10.1002/ana.20899

Congar, P., Gaïarsa, J. L., Popovici, T., Ben-Ari, Y., and Crépel, V. (2000). Permanent reduction of seizure threshold in post-ischemic CA_3 pyramidal neurons. *J. Neurophysiol.* 83, 2040–2046.

da Silveira, V. G., de Paula Cognato, G., Müller, A. P., Figueiró, F., Bonan, C. D., Perry, M. L., et al. (2010). Effect of ketogenic diet on nucleotide hydrolysis and hepatic enzymes in blood serum of rats in a lithium-pilocarpine-induced status epilepticus. *Metab. Brain Dis.* 25, 211–217. doi: 10.1007/s11011-010-9198-6

de Tisi, J., Bell, G. S., Peacock, J. L., McEvoy, A. W., Harkness, W. F., Sander, J. W., et al. (2011). The long-term outcome of adult epilepsy surgery, patterns of seizure remission and relapse: a cohort study. *Lancet* 378, 1388–1395. doi: 10.1016/S0140-6736(11)60890-8

DeVivo, D. C., Leckie, M. P., Ferrendelli, J. S., and McDougal, D. B. Jr. (1978). Chronic ketosis and cerebral metabolism. *Ann. Neurol.* 3, 331–337. doi: 10.1002/ana.410030410

Dulla, C. G., Dobelis, P., Pearson, T., Frenguelli, B. G., Staley, K. J., and Masino, S. A. (2005). Adenosine and ATP link PCO_2 to cortical excitability via pH. *Neuron* 48, 1011–1023. doi: 10.1016/j.neuron.2005.11.009

Dzhala, V., Valeeva, G., Glykys, J., Khazipov, R., and Staley, K. (2012). Traumatic alterations in GABA signaling disrupt hippocampal network activity in the developing brain. *J. Neurosci.* 32, 4017–4031. doi: 10.1523/JNEUROSCI.5139-11.2012

Engel, J. Jr. (2001). Mesial temporal lobe epilepsy: what have we learned? *Neuroscientist* 7, 340–352. doi: 10.1177/107385840100700410

Freeman, J. M., Vining, E. P., Pillas, D. J., Pyzik, P. L., Casey, J. C., and Kelly, L. M. (1998). The efficacy of the ketogenic diet—1998: A prospective evaluation of intervention in 150 children. *Pediatrics* 102, 1358–1363. doi: 10.1542/peds.102.6.1358

Galow, L. V., Schneider, J., Lewen, A., Ta, T.-T., Papageorgiou, I. E., and Kann, O. (2014). Energy substrates that fuel fast neuronal network oscillations. *Front. Neurosci.* 8:398. doi: 10.3389/fnins.2014.00398

Gastaut, H. (Ed.) (1973). *Dictionary of Epilepsy. Part 1. Definition.* Geneva: World Health Organization.

Giménez-Cassina, A., Martínez-François, J. R., Fisher, J. K., Szlyk, B., Polak, K., Wiwczar, J., et al. (2012). BAD-dependent regulation of fuel metabolism and K_{ATP} channel activity confers resistance to epileptic seizures. *Neuron* 74, 719–730. doi: 10.1016/j.neuron.2012.03.032

Hallböök, T., Köhler, S., Rosén, I., and Lundgren, J. (2007). Effects of ketogenic diet on epileptiform activity in children with therapy resistant epilepsy. *Epilepsy Res.* 77, 134–140. doi: 10.1016/j.eplepsyres.2007.09.008

Hartman, A. L., Lyle, M., Rogawski, M. A., and Gasior, M. (2008). Efficacy of the ketogenic diet in the 6-Hz seizure test. *Epilepsia* 49, 334–339. doi: 10.1111/j.1528-1167.2007.01430.x

Hartman, A. L., and Vining, E. P. (2007). Clinical aspects of the ketogenic diet. *Epilepsia* 48, 31–42. doi: 10.1111/j.1528-1167.2007.00914.x

Henze, D. A., and Buzsáki, G. (2001). Action potential threshold of hippocampal pyramidal cells *in vivo* is increased by recent spiking activity. *Neuroscience* 105, 121–130. doi: 10.1016/s0306-4522(01)00167-1

Hori, A., Tandon, P., Holmes, G. L., and Stafstrom, C. E. (1997). Ketogenic diet: effects on expression of kindled seizures and behavior in adult rats. *Epilepsia* 38, 750–758. doi: 10.1111/j.1528-1157.1997.tb01461.x

Huttenlocher, P. R. (1976). Ketonemia and seizures: metabolic and anticonvulsant effects of two ketogenic diets in childhood epilepsy. *Pediatr. Res.* 10, 536–540. doi: 10.1203/00006450-197605000-00006

Juge, N., Gray, J. A., Omote, H., Miyaji, T., Inoue, T., Hara, C., et al. (2010). Metabolic control of vesicular glutamate transport and release. *Neuron* 68, 99–112. doi: 10.1016/j.neuron.2010.09.002

Kawamura, M., Gachet, C., Inoue, K., and Kato, F. (2004). Direct excitation of inhibitory interneurons by extracellular ATP mediated by P2Y1 receptors in the hippocampal slice. *J. Neurosci.* 24, 10835–10845. doi: 10.1523/jneurosci.3028-04.2004

Kawamura, M. Jr., Ruskin, D. N., Geiger, J. D., Boison, D., and Masino, S. A. (2014). Ketogenic diet sensitizes glucose control of hippocampal excitability. *J. Lipid Res.* 55, 2254–2260. doi: 10.1194/jlr.M046755

Kawamura, M. Jr., Ruskin, D. N., and Masino, S. A. (2010). Metabolic autocrine regulation of neurons involves cooperation among pannexin hemichannels, adenosine receptors and KATP channels. *J. Neurosci.* 30, 3886–3895. doi: 10.1523/JNEUROSCI.0055-10.2010

Kealy, J., Bennett, R., and Lowry, J. P. (2013). Simultaneous recording of hippocampal oxygen and glucose in real time using constant potential amperometry in the freely-moving rat. *J. Neurosci. Methods* 215, 110–120. doi: 10.1016/j.jneumeth.2013.02.016

Kim, D. Y., Simeone, K. A., Simeone, T. A., Pandya, J. D., Wilke, J. C., Ahn, Y., et al. (2015). Ketone bodies mediate antiseizure effects through mitochondrial permeability transition. *Ann. Neurol.* 78, 77–87. doi: 10.1002/ana.24424

Kimura, R., Ma, L.-Y., Wu, C., Turner, D., Shen, J. X., Ellsworth, K., et al. (2012). Acute exposure to the mitochondrial complex I toxin rotenone impairs synaptic long-term potentiation in rat hippocampal slices. *CNS Neurosci. Ther.* 18, 641–646. doi: 10.1111/j.1755-5949.2012.00337.x

Klein, P., Janousek, J., Barber, A., and Weissberger, R. (2010). Ketogenic diet treatment in adults with refractory epilepsy. *Epilepsy Behav.* 19, 575–579. doi: 10.1016/j.yebeh.2010.09.016

Knowles, W. D., Traub, R. D., and Strowbridge, B. W. (1987). The initiation and spread of epileptiform bursts in the *in vitro* hippocampal slice. *Neuroscience* 21, 441–455. doi: 10.1016/0306-4522(87)90134-5

Köhling, R., Vreugdenhil, M., Bracci, E., and Jefferys, J. G. (2000). Ictal epileptiform activity is facilitated by hippocampal $GABA_A$ receptor-mediated oscillations. *J. Neurosci.* 20, 6820–6829.

Koranda, J. L., Ruskin, D. N., Masino, S. A., and Blaise, J. H. (2011). A ketogenic diet reduces long-term potentiation in the dentate gyrus of freely behaving rats. *J. Neurophysiol.* 106, 662–666. doi: 10.1152/jn.00001.2011

Kovács, R., Kardos, J., Heinemann, U., and Kann, O. (2005). Mitochondrial calcium ion and membrane potential transients follow the pattern of epileptiform discharges in hippocampal slice cultures. *J. Neurosci.* 25, 4260–4269. doi: 10.1523/JNEUROSCI.4000-04.2005

Kwon, Y. S., Jeong, S.-W., Kim, D. W., Choi, E. S., and Son, B. K. (2008). Effects of the ketogenic diet on neurogenesis after kainic acid-induced seizures in mice. *Epilepsy Res.* 78, 186–194. doi: 10.1016/j.eplepsyres.2007.11.010

Li, T., Ren, G., Lusardi, T., Wilz, A., Lan, J. Q., Iwasato, T., et al. (2008). Adenosine kinase is a target for the prediction and prevention of epileptogenesis in mice. *J. Clin. Invest.* 118, 571–582. doi: 10.1172/jci33737

Lin, L., Chen, G., Xie, K., Zaia, K. A., Zhang, S., and Tsien, J. Z. (2006). Large-scale neural ensemble recording in the brains of freely behaving mice. *J. Neurosci. Methods* 155, 28–38. doi: 10.1016/j.jneumeth.2005.12.032

Linard, B., Ferrandon, A., Koning, E., Nehlig, A., and Raffo, E. (2010). Ketogenic diet exhibits neuroprotective effects in hippocampus but fails to prevent epileptogenesis in the lithium-pilocarpine model of mesial temporal lobe epilepsy in adult rats. *Epilepsia* 51, 1829–1836. doi: 10.1111/j.1528-1167.2010.02667.x

Lossi, L., Alasia, S., Salio, C., and Merighi, A. (2009). Cell death and proliferation in acute slices and organotypic cultures of mammalian CNS. *Prog. Neurobiol.* 88, 221–245. doi: 10.1016/j.pneurobio.2009.01.002

Lowry, J. P., and Fillenz, M. (2001). Real-time monitoring of brain energy metabolism *in vivo* using microelectrochemical sensors: the effects of anesthesia. *Bioelectrochemistry* 54, 39–47. doi: 10.1016/s1567-5394(01)00109-8

Lutas, A., and Yellen, G. (2013). The ketogenic diet: metabolic influences on brain excitability and epilepsy. *Trends Neurosci.* 36, 32–40. doi: 10.1016/j.tins.2012.11.005

Ma, W., Berg, J., and Yellen, G. (2007). Ketogenic diet metabolites reduce firing in central neurons by opening K_{ATP} channels. *J. Neurosci.* 27, 3618–3625. doi: 10.1523/JNEUROSCI.0132-07.2007

Mantis, J. G., Centeno, N. A., Todorova, M. T., McGowan, R., and Seyfried, T. N. (2004). Management of multifactorial idiopathic epilepsy in EL mice with caloric restriction and the ketogenic diet: role of glucose and ketone bodies. *Nutr. Metab. (Lond)* 1:11. doi: 10.1186/1743-7075-1-11

Masino, S. A., and Dunwiddie, T. V. (1999). Temperature-dependent modulation of excitatory transmission in hippocampal slices is mediated by extracellular adenosine. *J. Neurosci.* 19, 1932–1939.

Masino, S. A., Kawamura, M., Wasser, C. A., Pomeroy, L. T., and Ruskin, D. N. (2009). Adenosine, ketogenic diet and epilepsy: the emerging therapeutic relationship between metabolism and brain activity. *Curr. Neuropharmacol.* 7, 257–268. doi: 10.2174/157015909789152164

Masino, S. A., Li, T., Theofilas, P., Sandau, U. S., Ruskin, D. N., Fredholm, B. B., et al. (2011). A ketogenic diet suppresses seizures in mice through adenosine A(1) receptors. *J. Clin. Invest.* 121, 2679–2683. doi: 10.1172/JCI57813

Masino, S. A., and Rho, J. M. (2012). "Mechanisms of ketogenic diet action," in *Jasper's Basic Mechanisms of the Epilepsies*, 4th Edn. eds J. L. Noebels, M. Avoli, M. A. Rogawski, R. W. Olsen and A. V. Delgado-Escueta (Bethesda, MD: National Center for Biotechnology Information), 1003–1024.

Maydell, B. V., Wyllie, E., Akhtar, N., Kotagal, P., Powaski, K., Cook, K., et al. (2001). Efficacy of the ketogenic diet in focal versus generalized seizures. *Pediatr. Neurol.* 25, 208–212. doi: 10.1016/s0887-8994(01)00310-1

McIntosh, A. M., Kalnins, R. M., Mitchell, L. A., Fabinyi, G. C., Briellmann, R. S., and Berkovic, S. F. (2004). Temporal lobectomy: long-term seizure outcome, late recurrence and risks for seizure recurrence. *Brain* 127, 2018–2030. doi: 10.1093/brain/awh221

Mosek, A., Natour, H., Neufeld, M. Y., Shiff, Y., and Vaisman, N. (2009). Ketogenic diet treatment in adults with refractory epilepsy: a prospective pilot study. *Seizure* 18, 30–33. doi: 10.1016/j.seizure.2008.06.001

Najm, I., Jehi, L., Palmini, A., Gonzalez-Martinez, J., Paglioli, E., and Bingaman, W. (2013). Temporal patterns and mechanisms of epilepsy surgery failure. *Epilepsia* 54, 772–782. doi: 10.1111/epi.12152

Nakazawa, M., Kodama, S., and Matsuo, T. (1983). Effects of ketogenic diet on electroconvulsive threshold and brain contents of adenosine nucleotides. *Brain Dev.* 5, 375–380. doi: 10.1016/s0387-7604(83)80042-4

Noakes, M., Foster, P. R., Keogh, J. B., James, A. P., Mamo, J. C., and Clifton, P. M. (2006). Comparison of isocaloric very low carbohydrate/high saturated fat and high carbohydrate/low saturated fat diets on body composition and cardiovascular risk. *Nutr. Metab. (Lond)* 3:7. doi: 10.1186/1743-7075-3-7

Noh, H. S., Kim, Y. S., Lee, H. P., Chung, K. M., Kim, D. W., Kang, S. S., et al. (2003). The protective effect of a ketogenic diet on kainic acid-induced hippocampal cell death in the male ICR mice. *Epilepsy Res.* 53, 119–128. doi: 10.1016/s0920-1211(02)00262-0

Nuttall, F. Q., Almokayyad, R. M., and Gannon, M. C. (2015). Comparison of a carbohydrate-free diet vs. fasting on plasma glucose, insulin and glucagon in type 2 diabetes. *Metabolism* 64, 253–262. doi: 10.1016/j.metabol.2014.10.004

Nylen, K., Velazquez, J. L., Sayed, V., Gibson, K. M., Burnham, W. M., Snead, O. C. III., et al. (2009). The effects of a ketogenic diet on ATP concentrations and the number of hippocampal mitochondria in *Aldh5a1*$^{(-/-)}$ mice. *Biochim. Biophys. Acta* 1790, 208–212. doi: 10.1016/j.bbagen.2008.12.005

Paglioli, E., Palmini, A., Portuguez, M., Azambuja, N., da Costa, J. C., da Silva Filho, H. F., et al. (2006). Seizure and memory outcome following temporal lobe surgery: selective compared with nonselective approaches for hippocampal sclerosis. *J. Neurosurg.* 104, 70–78. doi: 10.3171/jns.2006.104.1.70

Pan, W. J., Osmanović, S. S., and Shefner, S. A. (1995). Characterization of the adenosine A1 receptor-activated potassium current in rat locus ceruleus neurons. *J. Pharmacol. Exp. Ther.* 273, 537–544.

Pernia-Andrade, A. J., and Jonas, P. (2014). Theta-gamma-modulated synaptic currents in hippocampal granule cells *in vivo* define a mechanism for network oscillations. *Neuron* 81, 140–152. doi: 10.1016/j.neuron.2013.09.046

Quarato, P. P., Di Gennaro, G., Mascia, A., Grammaldo, L. G., Meldolesi, G. N., Picardi, A., et al. (2005). Temporal lobe epilepsy surgery: different surgical strategies after a non-invasive diagnostic protocol. *J. Neurol. Neurosurg. Psychiatry* 76, 815–824. doi: 10.1136/jnnp.2004.044016

Ray, A., and Wyllie, E. (2005). Treatment options and paradigms in childhood temporal lobe epilepsy. *Expert Rev. Neurother.* 5, 785–801. doi: 10.1586/14737175.5.6.785

Rho, J. M., Kim, D. W., Robbins, C. A., Anderson, G. D., and Schwartzkroin, P. A. (1999). Age-dependent differences in flurothyl seizure sensitivity in mice treated with a ketogenic diet. *Epilepsy Res.* 37, 233–240. doi: 10.1016/s0920-1211(99)00068-6

Rogawski, M. A., Löscher, W., and Rho, J. M. (2016). Mechanisms of ation of atiseizure dugs and the ktogenic det. *Cold Spring Harb. Perspect. Med.* 6:a022780. doi: 10.1101/cshperspect.a022780

Sakmann, B., Edwards, F., Konnerth, A., and Takahashi, T. (1989). Patch clamp techniques used for studying synaptic transmission in slices of mammalian brain. *Q. J. Exp. Physiol.* 74, 1107–1118. doi: 10.1113/expphysiol.1989.sp003336

Samala, R., Klein, J., and Borges, K. (2011). The ketogenic diet changes metabolite levels in hippocampal extracellular fluid. *Neurochem. Int.* 58, 5–8. doi: 10.1016/j.neuint.2010.10.011

Samoilova, M., Weisspapir, M., Abdelmalik, P., Velumian, A. A., and Carlen, P. L. (2010). Chronic *in vitro* ketosis is neuroprotective but not anti-convulsant. *J. Neurochem.* 113, 826–835. doi: 10.1111/j.1471-4159.2010.06645.x

Sayin, U., Rutecki, P., and Sutula, T. (1999). NMDA-dependent currents in granule cells of the dentate gyrus contribute to induction but not permanence of kindling. *J. Neurophysiol.* 81, 564–574.

Schneider, J., Lewen, A., Ta, T. T., Galow, L. V., Isola, R., Papageorgiou, I. E., et al. (2015). A reliable model for gamma oscillations in hippocampal tissue. *J. Neurosci. Res.* 93, 1067–1078. doi: 10.1002/jnr.23590

Schramm, J., Kral, T., Grunwald, T., and Blümcke, I. (2001). Surgical treatment for neocortical temporal lobe epilepsy: clinical and surgical aspects and seizure outcome. *J. Neurosurg.* 94, 33–42. doi: 10.3171/jns.2001.94.1.0033

Schroder, W., Hinterkeuser, S., Seifert, G., Schramm, J., Jabs, R., Wilkin, G. P., et al. (2000). Functional and molecular properties of human astrocytes in acute hippocampal slices obtained from patients with temporal lobe epilepsy. *Epilepsia* 41, S181–S184. doi: 10.1111/j.1528-1157.2000.tb01578.x

Shram, N. F., Netchiporouk, L. I., Martelet, C., Jaffrezic-Renault, N., and Cespuglio, R. (1997). Brain glucose: voltammetric determination in normal and hyperglycaemic rats using a glucose microsensor. *Neuroreport* 8, 1109–1112. doi: 10.1097/00001756-199703240-00009

Simeone, T. A., Samson, K. K., Matthews, S. A., and Simeone, K. A. (2014). *In vivo* ketogenic diet treatment attenuates pathologic sharp waves and high frequency oscillations in *in vitro* hippocampal slices from epileptic $K_v1.1\alpha$ knockout mice. *Epilepsia* 55, e44–e49. doi: 10.1111/epi.12603

Sirven, J., Whedon, B., Caplan, D., Liporace, J., Glosser, D., O'Dwyer, J., et al. (1999). The ketogenic diet for intractable epilepsy in adults: preliminary results. *Epilepsia* 40, 1721–1726. doi: 10.1111/j.1528-1157.1999.tb01589.x

Smith, B. N., and Dudek, F. E. (2001). Short- and long-term changes in CA1 network excitability after kainate treatment in rats. *J. Neurophysiol.* 85, 1–9.

Stafstrom, C. E., Ockuly, J. C., Murphree, L., Valley, M. T., Roopra, A., and Sutula, T. P. (2009). Anticonvulsant and antiepileptic actions of 2-deoxy-D-glucose in epilepsy models. *Ann. Neurol.* 65, 435–447. doi: 10.1002/ana.21603

Stafstrom, C. E., Wang, C., and Jensen, F. E. (1999). Electrophysiological observations in hippocampal slices from rats treated with the ketogenic diet. *Dev. Neurosci.* 21, 393–399. doi: 10.1159/000017389

Stewart, C., and Reid, I. (1993). Electroconvulsive stimulation and synaptic plasticity in the rat. *Brain Res.* 620, 139–141. doi: 10.1016/0006-8993(93)90280-z

Takano, T., He, W., Han, X., Wang, F., Xu, Q., Wang, X., et al. (2014). Rapid manifestation of reactive astrogliosis in acute hippocampal brain slices. *Glia* 62, 78–95. doi: 10.1002/glia.22588

Tanner, G. R., Lutas, A., Martínez-François, J. R., and Yellen, G. (2011). Single K ATP channel opening in response to action potential firing in mouse dentate granule neurons. *J. Neurosci.* 31, 8689–8696. doi: 10.1523/JNEUROSCI.5951-10.2011

Tanriverdi, T., Olivier, A., Poulin, N., Andermann, F., and Dubeau, F. (2008). Long-term seizure outcome after mesial temporal lobe epilepsy surgery: corticalamygdalohippocampectomy versus selective amygdalohippocampectomy. *J. Neurosurg.* 108, 517–524. doi: 10.3171/JNS/2008/108/3/0517

Than, K. D., Kossoff, E. H., Rubenstein, J. E., Pyzik, P. L., McGrogan, J. R., and Vining, E. P. (2005). Can you predict an immediate, complete and sustained response to the ketogenic diet? *Epilepsia* 46, 580–582. doi: 10.1111/j.0013-9580.2005.53304.x

Thio, L. L., Wong, M., and Yamada, K. A. (2000). Ketone bodies do not directly alter excitatory or inhibitory hippocampal synaptic transmission. *Neurology* 54, 325–331. doi: 10.1212/wnl.54.2.325

Thom, M. (2009). Hippocampal sclerosis: progress since Sommer. *Brain Pathol.* 19, 565–572. doi: 10.1111/j.1750-3639.2008.00201.x

Uhlemann, E. R., and Neims, A. H. (1972). Anticonvulsant properties of the ketogenic diet in mice. *J. Pharmacol. Exp. Ther.* 180, 231–238.

Wada, H., Okada, Y., Nabetani, M., and Nakamura, H. (1997). The effects of lactate and β-hydroxybutyrate on the energy metabolism and neural activity of hippocampal slices from adult and immature rat. *Dev. Brain Res.* 101, 1–7. doi: 10.1016/s0165-3806(97)00007-2

Watson, C. (2003). Hippocampal sclerosis and the syndrome of medial temporal lobe epilepsy. *Expert Rev. Neurother.* 3, 821–828. doi: 10.1002/pmic.200300361

Wiebe, S., Blume, W. T., Girvin, J. P., and Eliasziw, M. (2001). A randomized, controlled trial of surgery for temporal-lobe epilepsy. *N. Engl. J. Med.* 345, 311–318. doi: 10.1056/nejm200108023450501

Wiebe, S., and Jette, N. (2012). Pharmacoresistance and the role of surgery in difficult to treat epilepsy. *Nat. Rev. Neurol.* 8, 669–677. doi: 10.1038/nrneurol.2012.181

Wilder, R. M. (1921). The effects of ketonemia on the course of epilepsy. *Mayo Clin. Proc.* 2, 307–308.

Youssef, F. F. (2015). Ketone bodies attenuate excitotoxic cell injury in the rat hippocampal slice under conditions of reduced glucose availability. *Neurol. Res.* 37, 211–216. doi: 10.1179/1743132814Y.0000000430

Zhao, Q., Stafstrom, C. E., Fu, D. D., Hu, Y., and Holmes, G. L. (2004). Detrimental effects of the ketogenic diet on cognitive function in rats. *Pediatr. Res.* 55, 498–506. doi: 10.1203/01.pdr.0000112032.47575.d1

Zupec-Kania, B. A., and Spellman, E. (2008). An overview of the ketogenic diet for pediatric epilepsy. *Nutr. Clin. Pract.* 23, 589–596. doi: 10.1177/0884533608326138

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Molecular Neuroscience

ORIGINAL RESEARCH
published: 06 December 2016
doi: 10.3389/fnmol.2016.00137

Exogenous Ketone Supplements Reduce Anxiety-Related Behavior in Sprague-Dawley and Wistar Albino Glaxo/Rijswijk Rats

Csilla Ari[1], Zsolt Kovács[2], Gabor Juhasz[3], Cem Murdun[1], Craig R. Goldhagen[1], Andrew P. Koutnik[1], Angela M. Poff[1], Shannon L. Kesl[1] and Dominic P. D'Agostino[1]*

[1] Department of Molecular Pharmacology and Physiology, Hyperbaric Biomedical Research Laboratory, Morsani College of Medicine, University of South Florida, Tampa, FL, USA, [2] Department of Zoology, University of West Hungary, Szombathely, Hungary, [3] Proteomics Laboratory, Eotvos Lorand University, Budapest, Hungary

Nutritional ketosis has been proven effective for seizure disorders and other neurological disorders. The focus of this study was to determine the effects of ketone supplementation on anxiety-related behavior in Sprague-Dawley (SPD) and Wistar Albino Glaxo/Rijswijk (WAG/Rij) rats. We tested exogenous ketone supplements added to food and fed chronically for 83 days in SPD rats and administered sub-chronically for 7 days in both rat models by daily intragastric gavage bolus followed by assessment of anxiety measures on elevated plus maze (EPM). The groups included standard diet (SD) or SD + ketone supplementation. Low-dose ketone ester (LKE; 1,3-butanediol-acetoacetate diester, ∼10 g/kg/day, LKE), high dose ketone ester (HKE; ∼25 g/kg/day, HKE), beta-hydroxybutyrate-mineral salt (βHB-S; ∼25 g/kg/day, KS) and βHB-S + medium chain triglyceride (MCT; ∼25 g/kg/day, KSMCT) were used as ketone supplementation for chronic administration. To extend our results, exogenous ketone supplements were also tested sub-chronically on SPD rats (KE, KS and KSMCT; 5 g/kg/day) and on WAG/Rij rats (KE, KS and KSMCT; 2.5 g/kg/day). At the end of treatments behavioral data collection was conducted manually by a blinded observer and with a video-tracking system, after which blood βHB and glucose levels were measured. Ketone supplementation reduced anxiety on EPM as measured by less entries to closed arms (sub-chronic KE and KS: SPD rats and KSMCT: WAG/Rij rats), more time spent in open arms (sub-chronic KE: SPD and KSMCT: WAG/Rij rats; chronic KSMCT: SPD rats), more distance traveled in open arms (chronic KS and KSMCT: SPD rats) and by delayed latency to entrance to closed arms (chronic KSMCT: SPD rats), when compared to control. Our data indicates that chronic and sub-chronic ketone supplementation not only elevated blood βHB levels in both animal models, but reduced anxiety-related behavior. We conclude that ketone supplementation may represent a promising anxiolytic strategy through a novel means of inducing nutritional ketosis.

Keywords: anxiety, exogenous ketone supplements, ketones, elevated plus maze, animal models

Abbreviations: 5-HT, Serotonin; AD, Alzheimer's disease; βHB, beta-hydroxybutyrate; CNS, central nervous system; EPM, elevated plus maze; GLUT1 DS, Glucose transporter type-1 deficiency syndrome; HKE, high-dose ketone ester; KD, ketogenic diet; LKE, low-dose ketone ester; MCT, medium chain triglyceride; SD, standard diet; SPD rats, Sprague-Dawley rats; WAG/Rij rats, Wistar Albino Glaxo/Rijswijk rats.

OPEN ACCESS

Edited by:
Susan A. Masino, Trinity College, USA

Reviewed by:
David Ruskin, Trinity College, USA
Mark Beenhakker, University of Virginia, USA

***Correspondence:**
Csilla Ari
cdrari@health.usf.edu;
csari2000@yahoo.com

Received: *15 September 2016*
Accepted: *22 November 2016*
Published: *06 December 2016*

Citation:
Ari C, Kovács Z, Juhasz G, Murdun C, Goldhagen CR, Koutnik AP, Poff AM, Kesl SL and D'Agostino DP (2016) Exogenous Ketone Supplements Reduce Anxiety-Related Behavior in Sprague-Dawley and Wistar Albino Glaxo/Rijswijk Rats. Front. Mol. Neurosci. 9:137. doi: 10.3389/fnmol.2016.00137

INTRODUCTION

Anxiety disorders, such as generalized anxiety disorder, phobia and panic disorder, are the most prevalent type of mental disorders (Li, 2012). Anxiety can be associated with psychiatric morbidity, disability, increased healthcare burden and mortality in the general population (Teri et al., 1999). These symptoms can cause significant distress interfering with a person's quality of life, while they commonly occur along with other mental or physical illnesses, which may mask anxiety symptoms or aggravate them. Some symptoms, like fear and worry, occur in all anxiety disorders including generalized anxiety disorders, panic disorder and social anxiety disorder (Stahl, 2003; Mula, 2013). Our knowledge relating to exact cause and pathomechanism(s) of anxiety disorders is far from complete; however, it is known that the amygdala is determinant in the experience of fear and anxiety by mediating the autonomic and endocrine responses through the output to the hypothalamus, and avoidance behavior through the output to the periaqueductal gray matter (Stahl, 2003; Engin and Treit, 2008; Li, 2012; Mula, 2013). Previous studies also show that serotonergic, glutamatergic as well as GABAergic system have a role in the regulation of anxiety (Nagy et al., 1979; Kakui et al., 2009; Li, 2012; Dias et al., 2013). Anxiety and depression are common problems affecting people with epilepsy and Alzheimer's disease (AD), and can exacerbate symptoms of Glucose transporter type-1 deficiency syndrome (GLUT1 DS). Comorbidity between anxiety, depression and AD has been recognized (Teri et al., 1999; Hamid et al., 2011; Mula, 2013), and anxiety plays a key role in suicidality among patients with depression (Placidi et al., 2000). Interestingly, the same brain regions involved in a significant proportion of patients with focal epilepsy, such as the amygdala and the hippocampus, also play a key role in the neurobiology of anxiety (Li, 2012; Dias et al., 2013).

Anecdotal reports suggest that nutritional ketosis can promote a reduction in anxiety, although there is currently no convincing evidence to indicate that elevated ketone levels would reduce anxiety in humans (Ehrenreich, 2006). The elevation of ketones such as beta-hydroxybutyrate (βHB) and acetoacetate (AcAc) associated with nutritional ketosis causes a fundamental shift in metabolic physiology and brain neuropharmacology that is associated with preservation of brain homeostasis (Bough and Rho, 2007; Yudkoff et al., 2007; D'Agostino et al., 2013). Ketosis can be achieved by prolonged fasting (Owen et al., 1967) or by strict adherence to a ketogenic diet (KD), which is a metabolic-based therapy, a high-fat (70%–85% kcal) and low carbohydrate (3%–5%) diet (Kwiterovich et al., 2003; De Giorgis and Veggiotti, 2013). Maintaining long-term ketosis has proven to be beneficial in epileptic patients by decreasing the frequency and severity of seizures (Kossoff et al., 2011). Nutritional ketosis has also confirmed beneficial effects in animal models and human patients with AD, GLUT1 DS and cancer (Poff et al., 2013, 2014; Veggiotti and De Giorgis, 2014; Newport et al., 2015). GLUT1 DS results from impaired glucose transport into the brain (Klepper and Voit, 2002), however, ketones use another transporter to enter the central nervous system (CNS) providing an alternative source of fuel. Therefore, nutritional ketosis is used as a treatment option in GLUT1 patients (De Giorgis and Veggiotti, 2013) effectively correcting the impaired brain energy metabolism, reducing the frequency of the seizures (Leen et al., 2010) and improving behavior in autism spectrum disorder (Herbert and Buckley, 2013). Despite the proven and emerging therapeutic applications of the KD, many patients experience difficulties with compliance or experience a loss of effectiveness over time; therefore, new therapeutic strategies are needed.

The Wistar Albino Glaxo/Rijswijk (WAG/Rij) rat strain was originally developed as an animal model of human absence epilepsy, as the animals show spontaneous spike-wave discharges in the EEG (Coenen and Van Luijtelaar, 2003). Nevertheless, WAG/Rij rats are often used for investigation of different CNS diseases, such as anxiety, similar to Sprague-Dawley (SPD) rats by means of elevated plus maze (EPM; Sarkisova et al., 2003; Kovács et al., 2006, 2012, 2015; Sarkisova and Kulikov, 2006; Sarkisova and van Luijtelaar, 2011; Rebuli et al., 2015). EPM is a widely used behavioral assay for rodents, and it has been validated to assess the anxiety responses of rodents (Pellow et al., 1985; Walf and Frye, 2007). This test relies upon rodents' proclivity toward dark enclosed spaces (approach) and an unconditioned fear of heights/open spaces (avoidance; Barnett, 1975; Walf and Frye, 2007). It is a widely-used animal model and investigation method of anxiety that is primarily sensitive to benzodiazepine-type anxiolytics (e.g., diazepam; Paslawski et al., 1996). Anti-anxiety behavior (increased open arm time and/or open arm entries) can be determined, which reflects the rodent's preference for protected areas (e.g., closed arms) and their innate motivation to explore novel environments (Walf and Frye, 2007). Consequently, EPM assay on SPD and WAG/Rij rats is a suitable method for investigate the effect of ketone supplementation-evoked changes on anxiety level.

We hypothesized that ketone supplementation would decrease measures of anxiety-related behavior assessed with EPM behavioral assay in two rat strains. We have previously characterized the effects of ketone supplementation on blood ketone levels given *via* intragastric gavage (D'Agostino et al., 2013; Kesl et al., 2015) in rats and *via* chronic feeding mice (Poff et al., 2014). Ketone supplementation causes a rapid and sustained increase in blood ketone level, which may evoke anxiolytic effect by the increase of the GABAergic effects (Yudkoff et al., 2007; Li, 2012) or through numerous neuropharmacological pathways (Rho, 2015). To ensure that our results were not strain dependent, we assessed the effects of ketone supplementation on anxiety in SPD rats as well as in WAG/Rij rats, which have reduced activity of GABAergic system (Luhmann et al., 1995). Thus, the focus of this study was to test and determine the effects of several forms of ketone supplementation on anxiety-related behavior by using EPM behavioral assay in two rat strains. Exogenous ketone supplements were fed chronically to SPD rats and administered sub-chronically (gavage bolus) to SPD rats and WAG/Rij rats prior to assessment of anxiety measures.

MATERIALS AND METHODS

Animals

Two months old male SPD (n = 87) and 8 months old male WAG/Rij (n = 32) rats were used in the experiments. The animals were housed at Department of Molecular Pharmacology and Physiology (Hyperbaric Biomedical Research Laboratory, Morsani College of Medicine, University of South Florida, Tampa, FL, USA) and the Department of Zoology (University of West Hungary, Savaria Campus, Szombathely, Hungary). Animals were kept in groups of 2–4 under standard laboratory conditions (12:12 h light-dark cycle, light was on from 08:00 AM to 08:00 PM) in air-conditioned rooms at 22 ± 2°C.

Animal treatment and measuring procedures were performed in accordance with the University of South Florida Institutional Animal Care and Use Committee (IACUC) guidelines (Protocol #0006R) and with the local ethical rules in accordance with the Hungarian Act of Animal Care and Experimentation (1998. XXVIII. Section 243/1998) in conformity with the regulations for animal experimentation in the European Communities Council Directive of 24 November 1986 (86/609/EEC). All experiments were approved by the University of South Florida IACUC and all efforts were made to reduce the number of animals used.

Synthesis and Formulation of Ketone Precursors

Ketone ester (KE; 1,3-butanediol-acetoacetate diester) was synthesized as previously described (D'Agostino et al., 2013). Ketone salt (KS, which is Na^+/K^+–β-hydroxybutyrate mineral salt) is a novel agent that was mixed into a 50% solution supplying approximately 375 mg/g of pure βHB and 125 mg/g of Na^+/K^+ in a 1:1 ratio. Both KE and KS were developed and synthesized in collaboration with Savind Inc. Human food grade medium chain triglyceride (MCT) oil (∼60% caprylic triglyceride/40% capric triglyceride) was purchased from Now Foods (Bloomingdale, IL, USA). KS was mixed with MCT in a 1:1 ratio (KSMCT) at the University of South Florida (USF, Tampa, FL, USA).

Ketone Supplementation

In order to determine the effect of different administration forms, we tested chronic administration, when the ketone supplementation was mixed into the regular rodent chow, which the animals had access to all day for several weeks, and sub-chronic administration when the ketone supplementation was gavaged orally at a single time point daily for only 7 days.

Chronic Administration

A total of 48 male SPD rats were fed for 83 days with either standard rodent chow (2018 Teklad Global 18% Protein Rodent Diet (#2018), Harlan) standard diet (SD)/control; n = 9) or SD + ketone supplementation. Four treatment animal groups included low-dose KE (∼10 g/kg b.w./day, Low-dose ketone ester (LKE); n = 10), high-dose KE (∼25 g/kg b.w./day, high dose ketone ester (HKE); n = 10), KS (∼25 g/kg b.w./day, KS; n = 9) and KSMCT (∼25 g/kg b.w./day, KSMCT; n = 10). Higher dose was used for chronic administration, as the rats were consuming food-integrated ketone supplementation throughout the day, not at a single time point.

Sub-Chronic Oral Gavage

In order to familiarize the animals to the intragastric gavage method, water was gavaged for 5 days before ketone supplementation. Following the adaptation period to the intragastric gavage method, 39 male SPD rats were fed with standard diet, described in previous studies (Poff et al., 2013) and gavaged daily with 5 g/kg b.w./day water (SD/control; n = 11) or ketone supplements KE (n = 9), KS (n = 9), KSMCT (n = 10) sub-chronically for 7 days.

In addition, following the adaptation period to the intragastric gavage method, WAG/Rij male rats (n = 32) were fed with SD and gavaged sub-chronically with ∼2.5 g/kg b.w./day water (SD/control; n = 8), KE (n = 8), KS (n = 8) or KSMCT (n = 8) for 7 days. For the sub-chronic gavage administration the gavage dose was used that induced desired elevation of blood ketone based on our previous studies (Kesl et al., 2016).

Anxiety Assay

EPM (Coulbourn Instruments) was used to assess anxiety-related behavior of the rats after 83 days of chronic feeding or after 7 days of oral gavage. EPM experiments were carried out under non-stress conditions (in dimly lit and quiet room) between 12.00 h and 14.00 h.

The rats were transferred in their home cage to the experimental room 30 min prior to beginning the experiment. Briefly, rats were placed in the intersection of the four arms of the EPM, facing the open arm opposite to where the experimenter was and their behavior was recorded for 5 min. The amount of time spent and number of entries made on the open arms, closed arms and the center zones were video recorded. Latency to entry into the closed arms and the distance traveled in each zones was also measured in chronically treated SPD rats. Only those behaviors are discussed at each experimental scenario where significant difference was found. At the end of the 5 min test the rats were removed from the maze and placed back into their home cage. The maze was cleaned with 70% alcohol and later with tap water and dried with paper towel between rats. The primary method for data collection was a video-tracking system with computer interface and video camera (SMART V3.0 PLATFORM, Panlab, Harvard Apparatus, Holliston, MA, USA), to automatically collect behavioral data in SPD rats. A blinded observer was present in the testing room separated from the maze by a curtain, and collected EPM data in both SPD and WAG/Rij animals.

Blood Analyses and Weight Measurement

In the chronic feeding study, blood βHB and glucose levels were measured 24 h before the 1st day of ketone treatments (baseline

levels) and at 13th week after the EPM experiment. In the 7 day oral gavage studies, blood βHB and glucose levels were measured 24 h before the 1st day of ketone treatments (baseline levels; SPD and WAG/Rij rats), 24 h after the first gavage and 60 min after gavage on the 7th day (SPD and WAG/Rij rats). Whole blood samples (10 μL) were taken from the saphenous vein for analysis of blood glucose (mg/dl) and βHB (mmol/l) levels with the commercially available glucose and ketone (βHB) monitoring system Precision Xtra^{TM} (Abbott Laboratories, Abbott Park, IL, USA).

The body weight of all animals was recorded before the first ketone treatment (before) and on the last day of the ketone treatment (after).

Statistics

All data are presented as the mean ± standard error of the mean (SEM). We compared the effects of ketone supplementations on anxiety-related behavior as well as on blood βHB and glucose levels to control or/and baseline levels. Data analysis was performed using GraphPad PRISM version 6.0a. Results were considered significant when $p < 0.05$. Significance was determined by one-way analysis of variance (ANOVA) with Fisher's LSD test for the behavioral data. Blood ketone, blood glucose and body weight change were compared using a two-way ANOVA with Tukey's multiple comparisons test.

RESULTS

Ketone Supplementation Reduced Anxiety on Elevated Plus Maze

More Time Spent in Open Arms with Ketone Supplements

After chronic feeding of ketone supplementation in SPD rats the time spent in the open arms was significantly more in KSMCT group ($p = 0.0094$), while time spent in the closed arms was significantly less in LKE, KS and KSMCT groups ($p = 0.0389$, 0.0077 and 0.0019, respectively), compared to the control (SD) in SPD rats. Time spent in the center was significantly more in KS group ($p = 0.0239$; **Figure 1A**).

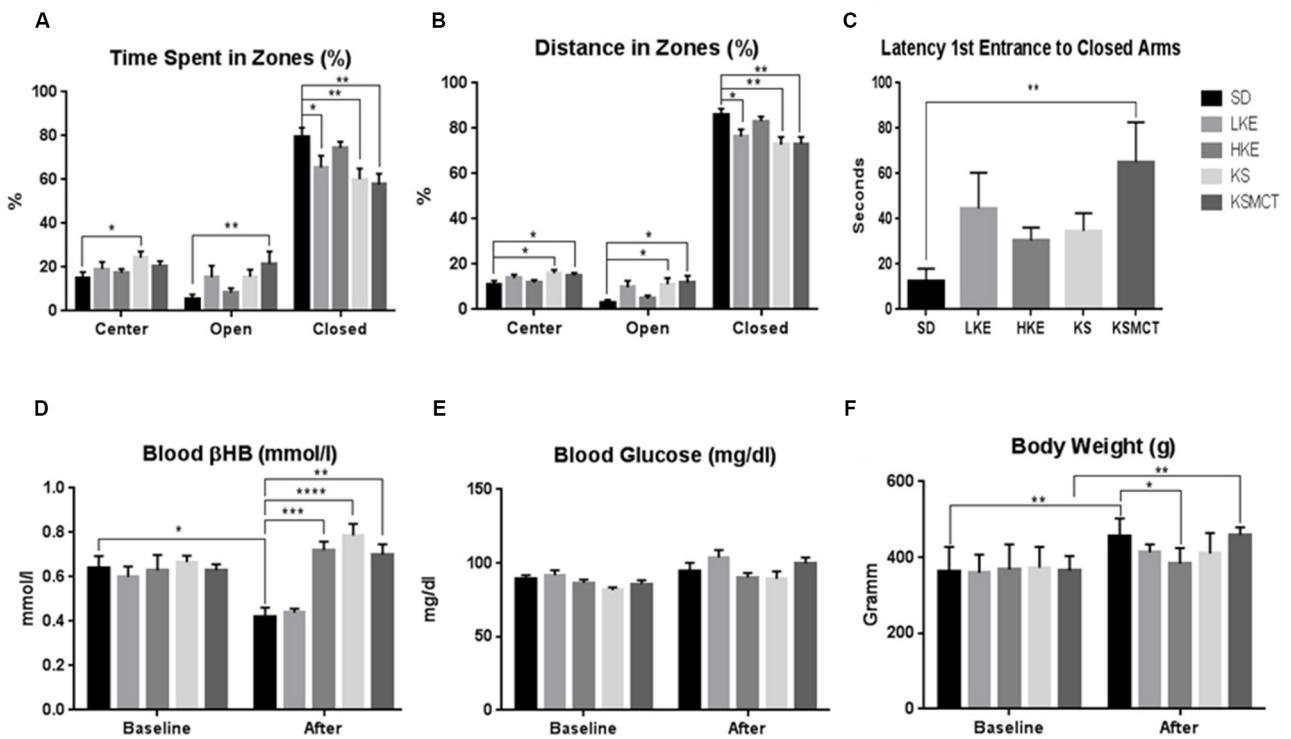


FIGURE 1 | Response of Sprague-Dawley (SPD) rats to chronic feeding of exogenous ketone supplementation. (A) Rats consuming KSMCT supplements spent more time in open arms (open), Low-dose ketone ester (LKE), KS and KSMCT groups spent less time in closed arms (closed), showing reduced anxiety compared to control (SD) group; **(B)** Rats consuming ketone supplements traveled more distance in open arms (KS and KSMCT) and less in closed arms (LKE, KS and KSMCT), showing reduced anxiety compared to control group; **(C)** Rats consuming KSMCT entered the closed arms later, showing reduced anxiety compared to control group; **(D)** Rats consuming high dose ketone ester (HKE), KS and KSMCT showed elevated blood ketone levels after 13 weeks (after) compared to control group; **(E)** Blood glucose levels did not change significantly were lower in HKE and KSMCT groups compared to control after 13 weeks; and **(F)** body weight was lower in HKE group after 13 weeks compared to control. Abbreviations: SD, standard rodent chow + water (~25 g/kg b.w. water/day); LKE, SD + LKE (1,3-butanediol-acetoacetate diester, ~10 g/kg b.w./day); HKE, SD + HKE (~25 g/kg b.w./day); KS, SD + beta-hydroxybutyrate-mineral salt (βHB-S; ~25 g/kg b.w./day); KSMCT, SD + βHB-S+medium chain triglyceride (MCT; ~25 g/kg b.w./day); (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

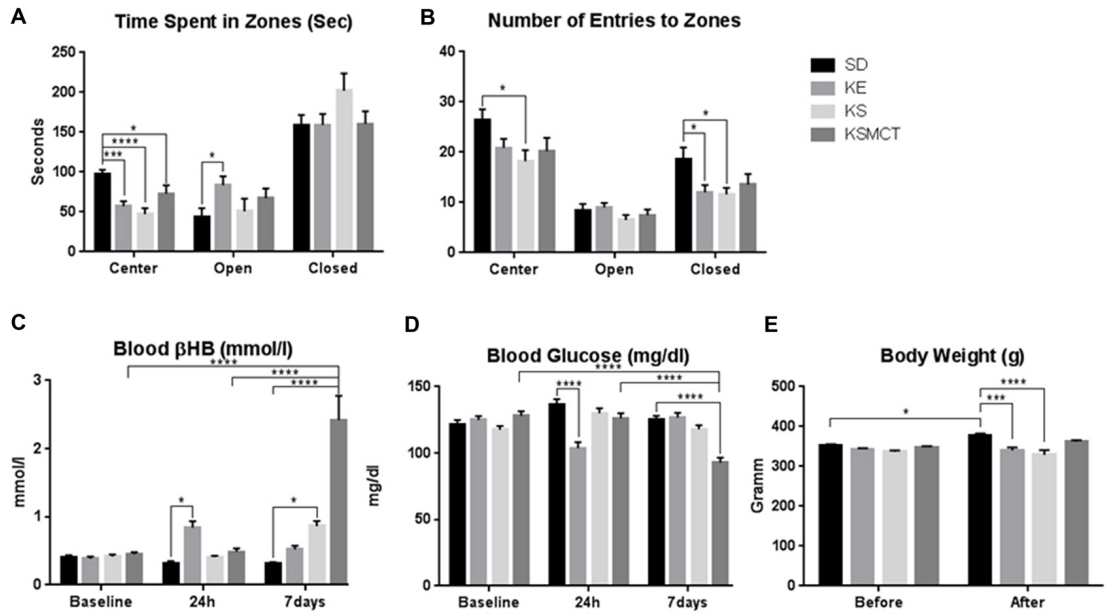


FIGURE 2 | Response of SPD rats to 7 days oral administration (gavage) of exogenous ketone supplementation. (A) More time spent in open arms (open) by KE group and less time spent in center by KE, KS and KSMCT groups were detected compared to control; **(B)** Less entries in closed arms (closed) by KE and KS groups; **(C)** Blood βHB levels were higher in KE group after 24 h and in KS and KSMCT groups after 7 days, compared to control; **(D)** Blood glucose levels were lower in KE group after 24 h and in KSMCT group compared to baseline, control and 24 h; **(E)** Body weight was lower in KE and KS groups compared to control after 7 days. Abbreviations: SD, standard rodent chow + water (~5 g/kg b.w. water/day); KE, SD + ketone ester (1,3-butanediol-acetoacetate diester, ~5 g/kg b.w./day); KS, SD + beta-hydroxybutyrate-mineral salt (βHB-S; ~5 g/kg b.w./day); KSMCT, SD + βHB-S + MCT (~5 g/kg b.w./day); (*$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$).

After 7 days of gavage administration in SPD rats, the time spent in the open arms increased in the KE group ($p = 0.0281$), whereas time spent in the center decreased in KE, KS and KSMCT groups ($p = 0.0005$, <0.0001 and 0.023, respectively; **Figure 2A**). In WAG/Rij rats the KSMCT treated rats spent more time in the open arms ($p = 0.0018$) and less time in the closed arms ($p = 0.0003$), whereas KE treated rats spent more time in the center ($p = 0.0027$), compared to the control (SD) group (**Figure 3A**).

Less Entries to Closed Arms with Ketone Supplements

Entries to the closed arms were less frequent with KE and KS treatment ($p = 0.0436$, 0.0234, respectively) in SPD and with KSMCT treatment ($p = 0.0014$) in WAG/Rij rat models, respectively, after 7 days of administration (**Figures 2B**, **3B**). SPD rats also entered fewer times to the center when treated with KS (**Figure 2A**; $p = 0.0193$), compared to control (SD) animals. Conversely, WAG/Rij rats made less entries to open arms in KE treated group ($p = 0.0318$).

More Distance Traveled in Open Arms, Less in Closed Arms and Delayed Latency of Entrance to Closed Arms with Ketone Supplements

After chronic feeding in SPD rats, the distance traveled in the open arms was significantly greater in KS and KSMCT groups ($p = 0.036$ and 0.0165), and distance traveled in the closed arms was significantly less in LKE, KS and KSMCT groups ($p = 0.0252$, 0.00041 and 0.0032), compared to the control (SD). Distance traveled in the center was more in KS and KSMCT groups ($p = 0.0206$ and 0.0482; **Figure 1B**).

The latency to first entrance of closed arms was significantly greater in KSMCT group after chronic feeding ($p = 0.0038$; **Figure 1C**).

Elevation of Blood βHB Levels with Ketone Supplements

After 83 days of chronic feeding in SPD rats, blood βHB levels remained significantly elevated in HKE, KS and KSMCT groups, compared to control ($p = 0.0004$, <0.0001, 0.0014; **Figure 1D**) while it decreased in SD compared to baseline ($p = 0.0307$).

Blood βHB levels were elevated in SPD rats after 24 h of a single gavage in KE group (**Figure 2C**; $p = 0.0325$), compared to control.

In SPD rats βHB was elevated in KSMCT groups at 7 days compared to their level at 24 h and baseline ($p < 0.0001$; **Figure 2C**). Blood βHB was also elevated in KS and KSMCT treatment groups compared to control group ($p = 0.0194$, <0.0001; **Figure 2C**). After 7 days of gavage, blood βHB was elevated in KE, KS and KSMCT groups in WAG/Rij rats ($p < 0.0001$) compared to baseline, 24 h and control (**Figure 3C**).

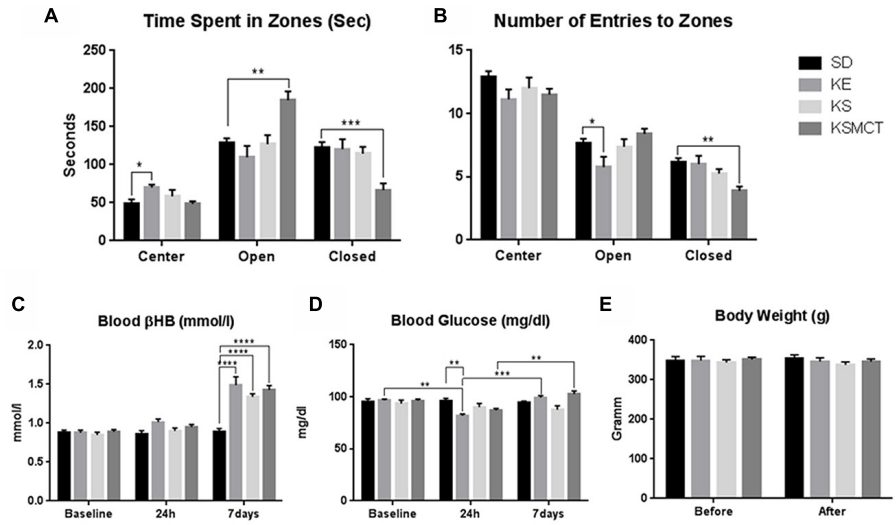


FIGURE 3 | Response of Wistar Albino Glaxo/Rijswijk (WAG/Rij) rats to 7 days oral administration of exogenous ketone supplementation. (A) More time spent in open arms (open) and less time spent in closed arms (closed) by KSMCT group were demonstrated compared to control; **(B)** Less entries in closed arms by KSMCT group and in open arms by KE group; **(C)** Blood βHB levels were higher in all treatment groups (KE, KS and KSMCT) after 7 days, compared to baseline, 24h and control. **(D)** Blood glucose levels decreased after 24 h in KE group compared to control and baseline, but increased after 7 days compared to 24 h in KE and KSMCT group; **(E)** Body weight did not change significantly in either groups. Abbreviations: SD, standard rodent chow + water (∼2.5 g/kg b.w. water/day); KE, SD + ketone ester (1,3-butanediol-acetoacetate diester, ∼2.5 g/kg b.w./day); KS, SD + beta-hydroxybutyrate-mineral salt (βHB-S; ∼2.5 g/kg b.w./day); KSMCT, SD + beta-hydroxybutyrate-mineral salt (BHB-S) + MCT (KSMCT; ∼2.5 g/kg b.w./day); (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

Ketone Supplementation and Blood Glucose Levels

After 13 weeks of chronic feeding in SPD rats blood glucose did not change significantly in any groups (**Figure 1E**).

However, in SPD rats, after sub-chronic ketone treatments, blood glucose levels were lower at 24 h in KE group compared to control ($p < 0.0001$; **Figure 2D**). After 7 days of oral gavage blood glucose was lower in KSMCT compared to control, to baseline and to the level at 24 h in SPD rats ($p < 0.0001$; **Figure 2D**).

In WAG/Rij rats the KE group had lower glucose levels after 24 h, compared to baseline levels ($p = 0.0064$), however after 7 days their level were elevated again, compared to the level at 24 h ($p = 0.0006$; **Figure 3D**). Moreover, glucose levels were also elevated after 7 days compared to 24 h in KSMCT group (**Figure 3D**).

Differences in Changes of Blood Ketone and Glucose Levels Between the Two Animal Models

There was significant difference in βHB levels between the two animal models in KE and KSMCT groups at 7 days only (**Figure 4A**). The glucose levels were different between the two animal models in each treatment groups at each time points, except in KSMCT group at 7 days (**Figure 4B**).

Body Weight Changes during Ketone Supplementation

After chronic feeding, the body weight of SPD rats was lower in HKE group compared to the control ($p = 0.0366$). The body weight increased in SD and KSMCT groups, compared to their baseline ($p = 0.0015$, 0.0012; **Figure 1F**).

After 7 days of treatment the body weight of SPD rats increased in SD group, compared to its baseline ($p = 0.0297$). The body weight was lower in KE and KS treatment groups after 7 days, compared to control ($p = 0.0005$, <0.0001; **Figure 2E**). In WAG/Rij rats the body weight did not change significantly in either group during the treatment period (**Figure 3E**).

DISCUSSION

The current study demonstrated the anxiolytic effect of chronic (13 weeks) and sub-chronic (7 days) administration of several forms of ketone supplementation in both SPD and WAG/Rij rats. Anxiolytic effect was assessed by means of EPM and measured by less entries and time spent in closed arms, more entries and time spent in open arms, more distance traveled in open arms, and delayed latency to entrance into closed arms.

Ketones are produced naturally in the liver only under certain physiological conditions associated with the suppression of the hormone insulin: starvation, fasting, calorie restriction, prolonged exercise, or during the consumption of high fat, low carbohydrate KD. The restrictive nature of these states has limited the clinical applicability of therapeutic ketosis due to practical considerations. In an effort to circumvent this dilemma, researchers have recently developed a number of exogenous ketogenic supplements, ketogenic precursors that are metabolized to produce a dose-dependent elevation of βHB and AcAc in the blood (Veech, 2004; Clarke et al., 2012; Kesl et al.,

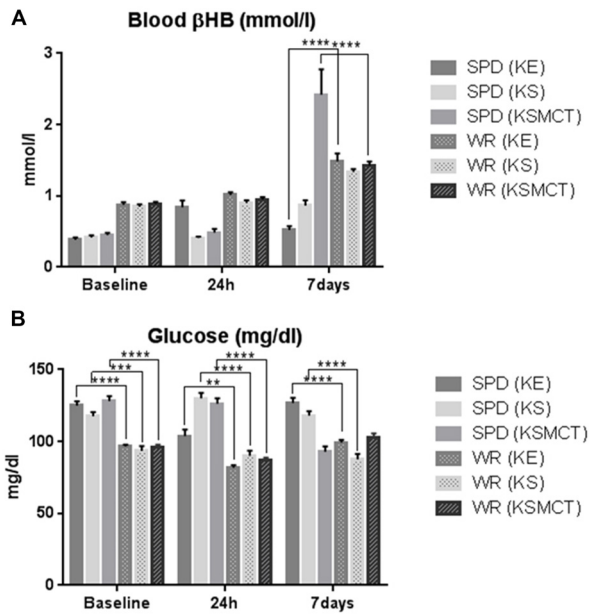


FIGURE 4 | Blood βHB and glucose levels compared between the two animal models. (A) Blood βHB levels were higher at 7 days in WAG/Rij rats after KE and KSMCT treatment. **(B)** Blood glucose levels were significantly lower in WAG/Rij rats, except in KSMCT group at 7 days. (**$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

2016). Induction of hyperketonemia produces acute and chronic changes in metabolic physiology and neuropharmacological pathways that provide therapeutic effects in varied disease states. The ketone supplements tested in this study allowed for a rapid and controlled induction of physiologic ketosis without the need for fasting or severe dietary restrictions. Previous studies have demonstrated the use of exogenous ketones as a means to induce a dose-dependent hyperketonemia (1–7 mM) in rats, mice, dogs, pigs and humans (Desrochers et al., 1992, 1995; Ciraolo et al., 1995; Brunengraber, 1997; Puchowicz et al., 2000; Clarke et al., 2012; Srivastava et al., 2012). Exogenous ketogenic supplementation mimics the metabolic and physiologic effects of the KD, including enhancing mitochondrial biogenesis, anaplerosis, suppression of glycolysis and increasing ATP and adenosine production, all thought to mediate the therapeutic effects of KD in epilepsy (Veech, 2004; Srivastava et al., 2012; Kesl et al., 2014; Poff et al., 2015). Since many of the benefits of ketosis are mechanistically attributable to the ketone bodies, it is possible that exogenous ketone supplementation could mimic the therapeutic efficacy of the KD for certain disorders, including anxiety disorders, or other disorders, such as seizure disorders and AD that have a comorbidity of anxiety.

The differences in blood βHB and glucose levels between the two animal models both before and after the treatment highlight the need to examine the response to ketone supplementation in more rodent models with different pathologies. Both in chronic and sub-chronic KE treatment resulted in lower body weight in SPD animals, however, the body weight did not change significantly during the treatment period in WAG/Rij rats.

Previous studies have shown a clear anxiolytic effect in the EPM when the antidepressant/antipanic drug phenelzine, agonists and/or antagonists of different neurotransmitter systems (e.g., GABAergic and glutamatergic system) were given acutely to rats (Paslawski et al., 1996; Engin and Treit, 2008). Although, there is no compelling evidence that the KD or elevated blood ketone levels would induce global changes in GABA levels (Hartman et al., 2007), the exogenous ketone supplementation tested in the present study similarly increased the open arms exploration in the EPM and increased the latency to enter the closed arms, showing anxiolytic effect.

In summary, LKE decreased time spent in closed arms and reduced distance traveled in closed arms after chronic treatment. Moreover, KE increased time spent in open arms, decreased time spent in center and decreased number of entries in closed arms after sub-chronic treatment in SPD rats. KS was proven to be effective in reducing time spent and distance traveled in closed arms and increase distance traveled in open arms after chronic treatment. It also reduced time spent and number of entries to center, while decreasing number of entries in closed arms after sub-chronic treatment in SPD rats. KSMCT effectively increased time spent and distance traveled in open arms and decreased time spent and distance traveled in closed arms, as well as delayed latency to first entrance to closed arms after chronic treatment in SPD rats. In WAG/Rij rats KSMCT successfully increased time spent in open arms and decreased time spent and number of entries in closed arms after sub-chronic treatment. Differences could be observed between the effects of different ketone supplements on anxiety. These results indicate that KS and KSMCT are the most effective after chronic treatment, while KE and KS seem to be the most effective after sub-chronic treatment in rats without pathology (SPD). In rats with pathology (WAG/Rij) KSMCT was the most effective treatment after sub-chronic administration.

Previous studies showed that the percentage of open arm entries linearly increase with age (Lynn and Brown, 2010) and the aging-related changes in EPM behavior are strain-specific (Ferguson and Gray, 2005), therefore the rats used in the present study involved two strains and animals of different age. In other studies, those rats that were pre-treated with amphetamine exhibited increased anxiety-like behavior on the EPM, which was successfully reversed by paroxetine, a selective serotonin (5-HT) reuptake inhibitor (Tu et al., 2014). Those results suggested that 5-HT levels in the ventral hippocampus are critical for regulating anxiety behavior and that increasing 5-HT levels may be an effective strategy for reducing anxiety (Tu et al., 2014). Therefore, we speculate that the ketone supplements reduced anxiety-related behavior not solely by elevating blood ketone levels, but may also effect the regulation of 5-HT levels. In spite of that WAG/Rij rats show different anxiety behavior compared to SPD rats (e.g., WAG/Rij rats spent approximately equal times in the closed and open arms without ketone supplementation: **Figures 2A**, **3A**), sub-chronic ketone supplementation was effective not only in SPD rats but also

in WAG/Rij rats (**Figures 2A,B**, **3A,B**). Thus, our results on WAG/Rij rats strengthened the hypothesis on anxiolytic effect of exogenous ketone supplementation found in SPD rats.

Previous studies demonstrated that 18% of Americans and 14% of Europeans may be affected by one or more anxiety disorders (Kessler et al., 2005) that are associated with high financial costs, increased risk of mortality and morbidity as well as impaired workplace performance (Greenberg et al., 1999; Albert et al., 2005). Ketone supplementation may be a potential therapeutic intervention in treatment of anxiety disorders (Yudkoff et al., 2007; Engin and Treit, 2008; Masino et al., 2012; Lutas and Yellen, 2013), while very little is known about the link between ketone application-evoked changes in CNS and anxiety disorders. However, it has been demonstrated that KD may: (i) decrease extracellular glutamate release/level by means of inhibition of vesicular glutamate transporter; (ii) increase adenosine level; and (iii) augment the GABAergic effects by $GABA_A$ receptors (Yudkoff et al., 2007; Engin and Treit, 2008; Masino et al., 2012; Lutas and Yellen, 2013). It has been demonstrated that serotonergic, glutamatergic and GABAergic system of different brain areas such as hippocampus and/or amygdala have a role in the regulation of anxiety: 5-HT transporters, 5-HT receptors (e.g., 5-HT1A), N-methyl-D-aspartate (NMDA) receptors and GABA receptors (e.g., $GABA_A$ receptors) are potential targets in the treatment of anxiety disorders (Nagy et al., 1979; Kakui et al., 2009; Li, 2012; Sankar, 2012; Dias et al., 2013). It was concluded that GABAergic system may have critical role in the modulation of the level of anxiety: increased GABAergic transmission may evoke anxiolytic effect (Engin and Treit, 2008; Li, 2012). Thus, augmentation of the GABAergic effects by means of KD *via* $GABA_A$ receptors may evoke a decrease in anxiety level. It has also been demonstrated that: (i) KD may increase extracellular adenosine level (Masino et al., 2012; Lutas and Yellen, 2013); (ii) inhibition of adenosine receptors (A_1R and $A_{2A}R$) by means of caffeine promotes anxious behavior (Klein et al., 1991); (iii) A_1R- or $A_{2A}R$-knockout mice showed anxiogenic-like behaviors (Ledent et al., 1997; Johansson et al., 2001); and (iv) modulation of adenosine receptor activity might be an effective treatment strategy for patients with anxiety disorders (Kovács and Dobolyi, 2013). In addition, as KD may evoke decreased extracellular glutamate level (Lutas and Yellen, 2013) and NMDA receptor antagonists may have anxiolytic effects (Guimarães et al., 1991; Engin and Treit, 2008) KD may exert its alleviating effect on anxiety level *via* glutamatergic system. Thus, theoretically, as ketone supplementation may generate similar changes in brain neurotransmitter systems as KD by means of ketosis (**Figures 1**–**3**), chronic and sub-chronic ketone supplementation-provoked anxiolytic effects may be evoked by means of glutamatergic and/or GABAergic as well as adenosinergic system in SPD and WAG/Rij rats. Indeed, a recent study supports the effect of ketone esters increasing the brain GABA/Glutamate ratio in an animal model of Angelman's syndrome (Ciarlone et al., 2016). However, our knowledge is not sufficient at present to explain the mechanism(s), by which ketone supplementation exerts its anti-anxiety effects.

We measured higher βHB levels after sub-chronic ketone supplementation in WAG/Rij rats (KE and KSMCT) compared to SPD rats (**Figure 4A**). This result and the reduced activity of GABAergic system in WAG/Rij rat brain (Luhmann et al., 1995) may explain that half doses of KE and KSMCT (∼2.5 g/kg b.w./day) than applied in SPD rats (∼5 g/kg b.w./day) effectively decreased the anxiety level in WAG/Rij rats. Higher basal ketone levels (and its putative anti-anxiety effects) in WAG/Rij rats may also cause lower basal anxiety levels compared to SPD rats (e.g., WAG/Rij rats spent more time in the open arms compared to SPD rats before ketone supplements; **Figures 2A**, **3A**).

In conclusion, based on the present study, we can conclude that chronic and sub-chronic administration of exogenous ketone supplementation may be an effective way to reduce anxiety. Achieving nutritional ketosis with exogenous ketone supplementation while maintaining a normal diet might be an alternative to the KD, or may further augment the therapeutic efficacy of the KD. Therefore, it is important to understand the long-term effects of these supplements fed chronically. These preliminary data show that chronic and sub-chronic feeding of ketone supplements not only elevated blood ketone levels, but also reduced anxiety-related behavior, which can be highly beneficial for patients managing diseases like epilepsy and AD with nutritional ketosis. Since achieving nutritional ketosis requires strict dietary restrictions, compliance is a major difficulty in this treatment. The administration of exogenous ketone supplements that increase ketone levels in the blood without dietary restrictions may be an effective option to improve compliance. We propose that exogenous ketone supplementation could provide an alternative method to reduce anxiety for healthy individuals, as well as those with disorders that are metabolically managed with the KD. Therefore, further studies are needed to determine the molecular basis of ketone supplementation-induced anxiolytic changes, and how this strategy can be implemented in human clinical trials with patients suffering anxiety disorders.

AUTHOR CONTRIBUTIONS

CA: conception and design of experiments, data collection, analysis and interpretation of data, writing manuscript; ZK: design of experiments, data collection, analysis and interpretation of data, writing manuscript; APK, AMP and SLK: data collection, revising manuscript; CM and CRG: data collection; GJ and DPD: design of experiments, revising manuscript.

ACKNOWLEDGMENTS

This work was supported by Office of Naval Research (ONR) Grant N000141310062 (to DPD), the National Development Agency of Hungary (under Grant No. TIOP-1.3.1.-07/2-2F-2009-2008; ZK), the National Development Agency of Hungary TÁMOP 4.2.1./B-09/1/KMR-2010-0003 and the KTIA_NAP_13-2014-0023 (to GJ). We wish to thank Tamás Török (NYME SEK) for the technical assistance.

REFERENCES

Albert, C. M., Chae, C. U., Rexrode, K. M., Manson, J. E., and Kawachi, I. (2005). Phobic anxiety and risk of coronary heart disease and sudden cardiac death among women. *Circulation* 111, 480–487. doi: 10.1161/01.cir.0000153813.64165.5d

Barnett, S. A. (1975). *The Rat—A Study in Behavior.* Chicago, IL: University of Chicago Press.

Bough, K. J., and Rho, J. M. (2007). Anticonvulsant mechanisms of the ketogenic diet. *Epilepsia* 48, 43–58. doi: 10.1111/j.1528-1167.2007.00915.x

Brunengraber, H. (1997). Potential of ketone body esters for parenteral and oral nutrition. *Nutrition* 13, 233–235. doi: 10.1016/s0899-9007(96)00409-1

Ciarlone, S. L., Grieco, J. C., D'Agostino, D. P., and Weeber, E. J. (2016). Ketone ester supplementation attenuates seizure activity and improves behavior and hippocampal synaptic plasticity in an Angelman syndrome mouse model. *Neurobiol. Dis.* 96, 38–46. doi: 10.1016/j.nbd.2016.08.002

Ciraolo, S. T., Previs, S. F., Fernandez, C. A., Agarwal, K. C., David, F., Koshy, J., et al. (1995). Model of extreme hypoglycemia in dogs made ketotic with (R,S)-1,3-butanediol acetoacetate esters. *Am. J. Physiol.* 269, E67–E75.

Clarke, K., Tchabanenko, K., Pawlosky, R., Carter, E., Todd King, M., Musa-Veloso, K., et al. (2012). Kinetics, safety and tolerability of (R)-3-hydroxybutyl (R)-3-hydroxybutyrate in healthy adult subjects. *Regul. Toxicol. Pharmacol.* 63, 401–408. doi: 10.1016/j.yrtph.2012.04.008

Coenen, A. M. L., and Van Luijtelaar, E. L. J. M. (2003). Genetic animal models for absence epilepsy: a review of the WAG/Rij strain of rats. *Behav. Genet.* 33, 635–655. doi: 10.1023/A:1026179013847

D'Agostino, D. P., Pilla, R., Held, H. E., Landon, C. S., Puchowicz, M., Brunengraber, H., et al. (2013). Therapeutic ketosis with ketone ester delays central nervous system oxygen toxicity seizures in rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 304, R829–R836. doi: 10.1152/ajpregu.00506.2012

De Giorgis, V., and Veggiotti, P. (2013). GLUT1 deficiency syndrome 2013: current state of the art. *Seizure* 22, 803–811. doi: 10.1016/j.seizure.2013.07.003

Desrochers, S., David, F., Garneau, M., Jetté, M., and Brunengraber, H. (1992). Metabolism of R- and S-1,3-butanediol in perfused livers from meal-fed and starved rats. *Biochem. J.* 285, 647–653. doi: 10.1042/bj2850647

Desrochers, S., Quinze, K., Dugas, H., Dubreuil, P., Bomont, C., David, F., et al. (1995). R,S-1,3-butanediol acetoacetate esters, potential alternates to lipid emulsions for total parenteral nutrition. *J. Nutr. Biochem.* 6, 111–118. doi: 10.1016/0955-2863(94)00011-a

Dias, B. G., Banerjee, S. B., Goodman, J. V., and Ressler, K. J. (2013). Towards new approaches to disorders of fear and anxiety. *Curr. Opin. Neurobiol.* 23, 346–352. doi: 10.1016/j.conb.2013.01.013

Ehrenreich, M. J. (2006). A case of the re-emergence of panic and anxiety symptoms after initiation of a high-protein, very low carbohydrate diet. *Psychosomatics* 47, 178–179. doi: 10.1176/appi.psy.47.2.178

Engin, E., and Treit, D. (2008). The effects of intra-cerebral drug infusions on animals' unconditioned fear reactions: a systematic review. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 32, 1399–1419. doi: 10.1016/j.pnpbp.2008.03.020

Ferguson, S. A., and Gray, E. P. (2005). Aging effects on elevated plus maze behavior in spontaneously hypertensive, Wistar-Kyoto and Sprague-Dawley male and female rats. *Physiol. Behav.* 85, 621–628. doi: 10.1016/j.physbeh.2005.06.009

Greenberg, P. E., Sisitsky, T., Kessler, R. C., Finkelstein, S. N., Berndt, E. R., Davidson, J. R., et al. (1999). The economic burden of anxiety disorders in the 1990s. *J. Clin. Psychiatry* 60, 427–435. doi: 10.4088/jcp.v60n0702

Guimarães, F. S., Carobrez, A. P., De Aguiar, J. C., and Graeff, F. G. (1991). Anxiolytic effect in the elevated plus-maze of the NMDA receptor antagonist AP7 microinjected into the dorsal periaqueductal grey. *Psychopharmacology (Berl)* 103, 91–94. doi: 10.1007/bf02244080

Hamid, H., Ettinger, A. B., and Mula, M. (2011). Anxiety symptoms in epilepsy: salient issues for future research. *Epilepsy Behav.* 22, 63–68. doi: 10.1016/j.yebeh.2011.04.064

Hartman, A. L., Gasior, M., Vining, E. P., and Rogawski, M. A. (2007). The neuropharmacology of the ketogenic diet. *Pediatr. Neurol.* 36, 281–292. doi: 10.1016/j.pediatrneurol.2007.02.008

Herbert, M. R., and Buckley, J. A. (2013). Autism and dietary therapy case report and review of the literature. *J. Child Neurol.* 28, 975–982. doi: 10.1177/0883073813488668

Johansson, B., Halldner, L., Dunwiddie, T. V., Masino, S. A., Poelchen, W., Giménez-Llort, L., et al. (2001). Hyperalgesia, anxiety and decreased hypoxic neuroprotection in mice lacking the adenosine A1 receptor. *Proc. Natl. Acad. Sci. U S A* 98, 9407–9412. doi: 10.1073/pnas.161292398

Kakui, N., Yokoyama, F., Yamauchi, M., Kitamura, K., Imanishi, T., Inoue, T., et al. (2009). Anxiolytic-like profile of mirtazapine in rat conditioned fear stress model: functional significance of 5-hydroxytryptamine 1A receptor and α1-adrenergic receptor. *Pharmacol. Biochem. Behav.* 92, 393–398. doi: 10.1016/j.pbb.2008.12.022

Kesl, S., Poff, A., Ward, N., Fiorelli, T., Ari, C., Van Putten, A., et al. (2015). Effect of sustaining dietary ketosis on the hippocampal and serum metabolome of Sprague-Dawley rats. *FASEB J.* 29, Suppl. 745.4

Kesl, S. L., Poff, A. M., Ward, N. P., Fiorelli, T. N., Ari, C., Van Putten, A. J., et al. (2016). Effects of exogenous ketone supplementation on blood ketone, glucose, triglyceride, and lipoprotein levels in Sprague-Dawley rats. *Nutr. Metab. (Lond)* 13:9. doi: 10.1186/s12986-016-0069-y

Kesl, S., Prather, J., Sherwood, J., Gould, L., and D'Agostino, P. D. (2014). Sustaining dietary ketosis to improve blood flow and wound healing in young and aged Fisher rats. *FASEB J.* 28:734.7, Suppl. 734.7

Kessler, R. C., Chiu, W. T., Demler, O., Merikangas, K. R., and Walters, E. E. (2005). Prevalence, severity and comorbidity of 12-month DSM-IV disorders in the national comorbidity survey replication. *Arch. Gen. Psychiatry* 62, 617–627. doi: 10.1001/archpsyc.62.6.617

Klein, E., Zohar, J., Geraci, M. F., Murphy, D. L., and Uhde, T. W. (1991). Anxiogenic effects of m-CPP in patients with panic disorder: comparison to caffeine's anxiogenic effects. *Biol. Psychiatry* 30, 973–984. doi: 10.1016/0006-3223(91)90119-7

Klepper, J., and Voit, T. (2002). Facilitated glucose transporter protein type 1 (GLUT1) deficiency syndrome: impaired glucose transport into brain– a review. *Eur. J. Pediatr.* 161, 295–304. doi: 10.1007/s00431-002-0939-3

Kossoff, E. H., Freeman, J. M., Turner, Z., and Rubenstein, J. E. (2011). *Ketogenic Diets: Treatments for Epilepsy and Otherdisorders.* 5th Edn. New York, NY: Demos Medical.

Kovács, Z., Czurkó, A., Kékesi, K. A., and Juhász, G. (2012). Neonatal tricyclic antidepressant clomipramine treatment reduces the spike-wave discharge activity of the adultWAG/Rij rat. *Brain Res. Bull.* 89, 102–107. doi: 10.1016/j.brainresbull.2012.07.010

Kovács, Z., and Dobolyi, A. (2013). "Anatomical distribution of nucleoside system in the human brain and implications for therapy," in *Adenosine: A Key Link Between Metabolism and Brain Activity*, eds S. A. Masino and D. Boison (New York, NY: Springer Science, Business Media), 621–656.

Kovács, Z., Kékesi, K. A., Dobolyi, Á., Lakatos, R., and Juhász, G. (2015). Absence epileptic activity changing effects of non-adenosine nucleoside inosine, guanosine and uridine in Wistar Albino Glaxo Rijswijk rats. *Neuroscience* 300, 593–608. doi: 10.1016/j.neuroscience.2015.05.054

Kovács, Z., Kékesi, K. A., Szilágyi, N., Abrahám, I., Székács, D., Király, N., et al. (2006). Facilitation of spike-wave discharge activity by lipopolysaccharides in Wistar Albino Glaxo/Rijswijk rats. *Neuroscience* 140, 731–742. doi: 10.1016/j.neuroscience.2006.02.023

Kwiterovich, P. O. Jr., Vining, E., Pyzik, P., Skolasky, R. Jr., and Freeman, J. (2003). Effect of a high-fat ketogenic diet on plasma levels of lipids, lipoproteins and apolipoproteins in children. *JAMA* 290, 912–920. doi: 10.1001/jama.290.7.912

Ledent, C., Vaugeois, J. M., Schiffmann, S. N., Pedrazzini, T., El Yacoubi, M., and Vanderhaeghen, J. J. (1997). Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor. *Nature* 388, 674–678. doi: 10.1038/41771

Leen, W. G., Klepper, J., Verbeek, M. M., Leferink, M., Hofste, T., van Engelen, B. G., et al. (2010). Glucose transporter-1 deficiency syndrome: the expanding clinical and genetic spectrum of a treatable disorder. *Brain* 133, 655–670. doi: 10.1093/brain/awp336

Li, X. (2012). Using the conditioned fear stress (CFS) animal model to understand the neurobiological mechanisms and pharmacological treatment of anxiety.

Shanghai Arch. Psychiatry 24, 241–249. doi: 10.3969/j.issn.1002-0829.2012.05.001

Luhmann, H. J., Mittmann, T., van Luijtelaar, G., and Heinemann, U. (1995). Impairment of intracortical GABAergic inhibition in a rat model of absence epilepsy. *Epilepsy Res.* 22, 43–51. doi: 10.1016/0920-1211(95)00032-6

Lutas, A., and Yellen, G. (2013). The ketogenic diet: metabolic influences on brain excitability and epilepsy. *Trends Neurosci.* 36, 32–40. doi: 10.1016/j.tins.2012.11.005

Lynn, D. A., and Brown, G. R. (2010). The ontogeny of anxiety-like behavior in rats from adolescence to adulthood. *Dev. Psychobiol.* 52, 731–739. doi: 10.1002/dev.20468

Masino, S. A., Kawamura, M. Jr., Ruskin, D. N., Geiger, J. D., and Boison, D. (2012). Purines and neuronal excitability: links to the ketogenic diet. *Epilepsy Res.* 100, 229–238. doi: 10.1016/j.eplepsyres.2011.07.014

Mula, M. (2013). Treatment of anxiety disorders, in epilepsy: an evidence-based approach. *Epilepsia* 54, 13–18. doi: 10.1111/epi.12101

Nagy, J., Zámbó, K., and Decsi, L. (1979). Anti-anxiety action of diazepam after intra-amygdaloid application in the rat. *Neuropharmacology* 18, 573–576. doi: 10.1016/0028-3908(79)90104-7

Newport, M. T., VanItallie, T. B., Kashiwaya, Y., King, M. T., and Veech, R. L. (2015). A new way to produce hyperketonemia: use of ketone ester in a case of Alzheimer's disease. *Alzheimers Dement.* 11, 99–103. doi: 10.1016/j.jalz.2014.01.006

Owen, O. E., Morgan, A. P., Kemp, H. G., Sullivan, J. M., Herrera, M. G., and Cahill, G. F. Jr. (1967). Brain metabolism during fasting. *J. Clin. Invest.* 46, 1589–1595. doi: 10.1172/JCI105650

Paslawski, T., Treit, D., Baker, G. B., George, M., and Coutts, R. T. (1996). The antidepressant drug phenelzine produces antianxiety effects in the plus-maze and increases in rat brain GABA. *Psychopharmacology (Berl)* 127, 19–24. doi: 10.1007/bf02805970

Pellow, S., Chopin, P., File, S. E., and Briley, M. (1985). Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J. Neurosci. Methods* 14, 149–167. doi: 10.1016/0165-0270(85)90031-7

Placidi, G. P. A., Oquendo, M. A., Malone, K. M., Brodsky, B., Ellis, S. P., and Mann, J. J. (2000). Anxiety in major depression: relationship to suicide attempts. *Am. J. Psychiatry* 157, 1614–1618. doi: 10.1176/appi.ajp.157.10.1614

Poff, A. M., Ari, C., Arnold, P., Seyfried, T. N., and D'Agostino, D. P. (2014). Ketone supplementation decreases tumor cell viability and prolongs survival of mice with metastatic cancer. *Int. J. Cancer* 135, 1711–1720. doi: 10.1002/ijc.28809

Poff, A. M., Ari, C., Seyfried, T., and D'Agostino, D. (2013). The ketogenic diet and hyperbaric oxygen therapy prolong survival in mice with systemic metastatic cancer. *PLoS One* 8:e65522. doi: 10.1371/journal.pone.0065522

Poff, A. M., Ward, N., Seyfried, T. N., Arnold, P., and D'Agostino, D. P. (2015). Non-toxic metabolic management of metastatic cancer in VM mice: novel combination of ketogenic diet, ketone supplementation and hyperbaric oxygen therapy. *PLoS One* 10:e0127407. doi: 10.1371/journal.pone.0127407

Puchowicz, M. A., Smith, C. L., Bomont, C., Koshy, J., David, F., and Brunengraber, H. (2000). Dog model of therapeutic ketosis induced by oral administration of R,S-1,3-butanediol diacetoacetate. *J. Nutr. Biochem.* 11, 281–287. doi: 10.1016/s0955-2863(00)00079-6

Rebuli, M. E., Camacho, L., Adonay, M. E., Reif, D. M., Aylor, D., and Patisaul, H. B. (2015). Impact of low dose oral exposure to bisphenol A (BPA) on juvenile and adult ratexploratory and anxiety behavior: a CLARITY-BPA consortium study. *Toxicol. Sci.* 148, 341–354. doi: 10.1093/toxsci/kfv163

Rho, J. M. (2015). How does the ketogenic diet induce anti-seizure effects? *Neurosci. Lett.* doi: 10.1016/j.neulet.2015.07.034 [Epub ahead of print]

Sankar, R. (2012). $GABA_A$ receptor physiology and its relationship to the mechanism of action of the 1,5-benzodiazepine clobazam. *CNS Drugs* 26, 229–244. doi: 10.2165/11599020-000000000-00000

Sarkisova, K. Y., and Kulikov, M. A. (2006). Behavioral characteristics of WAG/Rij rats susceptible and non-susceptible to audiogenic seizures. *Behav. Brain Res.* 166, 9–18. doi: 10.1016/j.bbr.2005.07.024

Srivastava, S., Kashiwaya, Y., King, M., Baxa, U., Tam, J., Niu, G., et al. (2012). Mitochondrial biogenesis and increased uncoupling protein 1 in brown adipose tissue of mice fed a ketone ester diet. *FASEB J.* 26, 2351–2362. doi: 10.1096/fj.11-200410

Sarkisova, K. Y., Midzianovskaia, I. S., and Kulikov, M. A. (2003). Depressive-like behavioral alterations and c-fos expression in the dopaminergic brain regions in WAG/Rij rats with genetic absence epilepsy. *Behav. Brain Res.* 144, 211–226. doi: 10.1016/s0166-4328(03)00090-1

Sarkisova, K., and van Luijtelaar, G. (2011). The WAG/Rij strain: a genetic animal model of absence epilepsy with comorbidity of depression. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 35, 854–876. doi: 10.1016/j.pnpbp.2010.11.010

Stahl, S. M. (2003). Brainstorms: symptoms and circuits, part 2: anxiety disorders. *J. Clin. Psychiatry*. 64, 1408–1409.

Teri, L., Ferretti, L. E., Gibbons, R. G., Logsdon, S. M., McCurry, W. A., Kukull, W. C., et al. (1999). Anxiety in Alzheimer's disease: prevalence and comorbidity. *J. Gerontol. A Biol. Sci. Med. Sci.* 54, M348–M352. doi: 10.1093/gerona/54.7.m348

Tu, W., Cook, A., Scholl, J. L., Mears, M., Watt, M. J., Renner, K. J., et al. (2014). Serotonin in the ventral hippocampus modulates anxiety-like behavior during amphetamine withdrawal. *Neuroscience* 281C, 35–43. doi: 10.1016/j.neuroscience.2014.09.019

Veech, R. L. (2004). The therapeutic implications of ketone bodies: the effects of ketone bodies in pathological conditions: ketosis, ketogenic diet, redox states, insulin resistance and mitochondrial metabolism. *Prostaglandins Leukot. Essent. Fatty Acids* 70, 309–319. doi: 10.1016/j.plefa.2003.09.007

Veggiotti, P., and De Giorgis, V. (2014). Dietary treatments and new therapeutic perspective in GLUT1 deficiency syndrome. *Curr. Treat. Options Neurol.* 16:291. doi: 10.1007/s11940-014-0291-8

Walf, A. A., and Frye, C. A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat. Protoc.* 2, 322–328. doi: 10.1038/nprot.2007.44

Yudkoff, M., Daikhin, Y., Melø, T. M., Nissim, I., Sonnewald, U., and Nissim, I. (2007). The ketogenic diet and brain metabolism of amino acids: relationship to the anticonvulsant effect. *Annu. Rev. Nutr.* 27, 415–430. doi: 10.1146/annurev.nutr.27.061406.093722

Conflict of Interest Statement: International Patent # PCT/US2014/031237, University of South Florida, D.P. D'Agostino, S. Kesl, P. Arnold, "*Compositions and Methods for Producing Elevated and Sustained Ketosis*". P. Arnold (Savind) has received financial support (ONR N000140610105 and N000140910244) from D.P. D'Agostino (USF) to synthesize ketone esters. Provisional patent # 16A007, University of South Florida, C. Ari, P. Arnold, D.P. D'Agostino "*Exogenous Ketone Supplements for Reducing Anxiety-Related Behavior*". The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer DR and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

frontiers in Molecular Neuroscience

ORIGINAL RESEARCH
published: 15 May 2017
doi: 10.3389/fnmol.2017.00129

Nutritional Ketosis Affects Metabolism and Behavior in Sprague-Dawley Rats in Both Control and Chronic Stress Environments

Milene L. Brownlow[1,2]*, *Seung H. Jung*[1,2], *Raquel J. Moore*[1,3], *Naomi Bechmann*[1,3] and *Ryan Jankord*[1]

[1] *Applied Neuroscience Branch, Warfighter Interface Division, Air Force Research Laboratory, Wright-Patterson Air Force Base, Dayton, OH, USA,* [2] *Research Associateship Program, National Research Council, National Academies of Science, Washington DC, USA,* [3] *Infoscitex, Inc., Dayton, OH, USA*

Nutritional ketosis may enhance cerebral energy metabolism and has received increased interest as a way to improve or preserve performance and resilience. Most studies to date have focused on metabolic or neurological disorders while anecdotal evidence suggests that ketosis may enhance performance in the absence of underlying dysfunction. Moreover, decreased availability of glucose in the brain following stressful events is associated with impaired cognition, suggesting the need for more efficient energy sources. We tested the hypotheses that ketosis induced by endogenous or exogenous ketones could: (a) augment cognitive outcomes in healthy subjects; and (b) prevent stress-induced detriments in cognitive parameters. Adult, male, Sprague Dawley rats were used to investigate metabolic and behavioral outcomes in 3 dietary conditions: ketogenic (KD), ketone supplemented (KS), or NIH-31 control diet in both control or chronic stress conditions. Acute administration of exogenous ketones resulted in reduction in blood glucose and sustained ketosis. Chronic experiments showed that in control conditions, only KD resulted in pronounced metabolic alterations and improved performance in the novel object recognition test. The hypothalamic-pituitary-adrenal (HPA) axis response revealed that KD-fed rats maintained peripheral ketosis despite increases in glucose whereas no diet effects were observed in ACTH or CORT levels. Both KD and KS-fed rats decreased escape latencies on the third day of water maze, whereas only KD prevented stress-induced deficits on the last testing day and improved probe test performance. Stress-induced decrease in hippocampal levels of β-hydroxybutyrate was attenuated in KD group while both KD and KS prevented stress effects on BDNF levels. Mitochondrial enzymes associated with ketogenesis were increased in both KD and KS hippocampal samples and both endothelial and neuronal glucose transporters were affected by stress but only in the control diet group. Our results highlight the complex relationship between peripheral metabolism, behavioral performance and biochemical changes in the hippocampus. Endogenous ketosis improved behavioral and metabolic parameters associated with energy metabolism and cognition while ketone supplementation replicated the biochemical effects within the hippocampus but only showed modest effects on behavioral improvements.

Keywords: nutritional ketosis, behavioral performance, metabolism, hippocampus, ketone supplements, stress

OPEN ACCESS

Edited by:
Susan A. Masino,
Trinity College, USA

Reviewed by:
Zsolt Kovacs,
University of West Hungary, Hungary
Mariano Ruiz-Gayo,
CEU San Pablo University, Spain
Cliff H. Summers,
University of South Dakota, USA

***Correspondence:**
Milene L. Brownlow
milene_lara@yahoo.com

Received: *23 February 2017*
Accepted: *18 April 2017*
Published: *15 May 2017*

Citation:
Brownlow ML, Jung SH, Moore RJ, Bechmann N and Jankord R (2017) Nutritional Ketosis Affects Metabolism and Behavior in Sprague-Dawley Rats in Both Control and Chronic Stress Environments. Front. Mol. Neurosci. 10:129. doi: 10.3389/fnmol.2017.00129

INTRODUCTION

In creased abundance of food choices has resulted in widespread consumption of convenient meals that often lack nutritional value, contributing to the epidemics of obesity, diabetes and metabolic disorders. Clinical studies indicate a strong association between stressful events with adiposity, increased body mass index and weight gain (Dallman et al., 2005; Block et al., 2009). These metabolic changes are due, in part, to higher circulating levels of glucose, insulin and insulin resistance induced by cumulative stress (Sinha and Jastreboff, 2013). In non-diabetic individuals, higher levels of peripheral glucose were predictive of cognitive decline within 5 years whereas in diabetic patients, higher glucose levels were related to increased risk of dementia (Crane et al., 2013).

In the brain, prolonged periods of physiological and psychological stress are associated with decreased glucose availability, leading to impaired decision making abilities, reaction time, changes in attention and memory and learning deficits (Baran et al., 2009; Yuen et al., 2012; Olver et al., 2015). In rodents, performance in challenging tasks reduced glucose availability in several brain areas, particularly in the hippocampus (McNay et al., 2000), with more demanding tasks resulting in greater decreases compared to less demanding tasks (McNay et al., 2001). Therefore, nutrient availability and the inability to effectively utilize alternative fuel sources may contribute to deteriorating performance during physically and cognitively taxing settings. This underscores the importance of optimizing metabolic interventions targeting the homeostatic control of brain networks involved in efficient energy utilization.

The beneficial effects of low carbohydrate, ketogenic diets (KDs) on metabolism have been extensively described (Vernon et al., 2004; Westman et al., 2007). KDs enhanced athletic outcomes (Phinney et al., 1983; Volek et al., 2016) and reversed motor deficits in a model of amyotrophic lateral sclerosis (Zhao et al., 2012). Recently, KDs have been considered a potential therapeutic approach to neurological disorders (Kashiwaya et al., 2000; Prins, 2008; Puchowicz et al., 2008; Kelley and Hartman, 2011; Kim et al., 2012; Choi et al., 2016). Its neuroprotective effects can be associated with the ability of ketone bodies to act as additional energy substrates (Keon et al., 1995), replacing glucose as the brain's main energy source (Cahill, 2006; Masino et al., 2009; Courchesne-Loyer et al., 2013). Furthermore, recent studies are unraveling ketone bodies' role as potent signaling molecules in the brain (Shimazu et al., 2013; Newman and Verdin, 2014a,b), leading to adaptive cellular responses to environmental stimuli such as fasting, exercise and dietary interventions (Marosi and Mattson, 2014).

Chronic use of KD can be challenging due to limitations such as restricted food choices, unbalanced macronutrient profile and gastrointestinal side effects. Therefore, the low compliance to this diet led to the development of supplements such as synthetic ketone esters that mimic ketosis achieved with KD or prolonged starvation (Veech, 2004; D'agostino et al., 2013; Kashiwaya et al., 2013). Nutritional ketosis resulting from adherence to KD is often referred to as endogenous ketosis in contrast to peripheral ketosis induced by dietary supplements, referred to as exogenous ketosis. Ketone supplementation caused rapid and sustained elevation of blood ketones (1–5 mmol/L β-hydroxybutyrate) for hours after oral administration (D'agostino et al., 2013; Kesl et al., 2016). If demonstrated that exogenous ketones are capable of mimicking the beneficial effects of KD, dietary supplementation may be used to augment cognitive and physiological performance, bypassing the need for such a strict nutritional regimen.

While KD-induced benefits have been described in disease models, cognitive augmentation in healthy individuals still warrant investigation. Moreover, we sought to investigate the role of endogenous or exogenous ketosis following challenges known to result in functional, morphological and cognitive impairments. For instance, persistent stress and elevated corticosteroids are known to suppress neurogenesis and expression of brain-derived neurotrophic factor (BDNF) in the hippocampus (Rothman and Mattson, 2013) in addition to atrophy of dendritic branching (Sapolsky, 1985) and neuronal loss (Watanabe et al., 1992).

Taken together, the role of ketones in augmenting performance and cognition in the absence of disorders has only recently been the subject of investigation and merely anecdotal evidence is currently available. Thus, our aim was to test the hypothesis that ketone bodies are capable of preserving cognition during performance of cognitively demanding tasks or following exposure to stressful events. We investigated whether endogenous or exogenous ketones modulated metabolic, behavioral and biochemical events relevant to cognitive performance in young rats with no underlying neurological or metabolic dysfunction.

MATERIAL AND METHODS

Ethical Approval

All animal handling and procedures were approved by the Wright-Patterson Air Force Base Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Animals

Adult, male Sprague-Dawley rats (6–8 weeks old) were purchased from Charles River (Wilmington, MA). Animals were allowed to acclimate to Wright Patterson Air Force Base (WPAFB) animal facility (7–10 days) prior to experiments. Throughout experimental procedure, subjects were singly housed in clear Plexiglas cages (10.5W × 10L × 8H in) with *ad libitum* access to food and water. Ambient housing conditions were controlled for temperature (18–24°C), humidity (30–70%), and a standard 12 h light/dark cycle (0600–1800). All experiments were performed during the light phase (between 0700 and 1600). Animals were handled routinely from the time of their arrival to minimize any effects of handling stress on experimental measurements.

Experimental Procedure

Figure 1 summarizes the experimental design adopted. All animals were individually housed before the commencement of the study. Food intake and body weight were assessed three times a week for 3 weeks before assignment into diet groups. Metabolic and behavioral data gathered during baseline (**Table 1**) were used

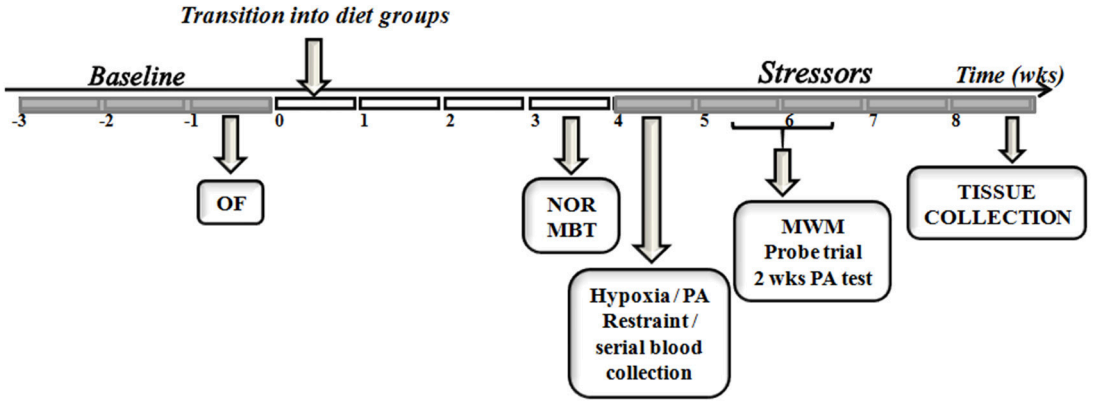


FIGURE 1 | Study timeline. The experimental design used in the comparison between endogenous and exogenous ketones prior to and during exposure to control or chronic stress paradigm is depicted. OF, open field; NOR, novel object recognition; MBT, marble burying test; PA, passive avoidance; MWM, morris water maze; wks, weeks.

TABLE 1 | Baseline parameters prior to assignment into dietary treatment groups.

Groups	Diets (*n*)			*p*-value
	NIH-31 (20)	KD (20)	KS (20)	
Body weight (g)	312.7 ± 4.3	321.1 ± 3.8	316.0 ± 4.4	0.36
Food intake (g/day)	27.1 ± 0.6	28.5 ± 0.5	27.7 ± 0.7	0.28
Estimated metabolic rate (Fl/BW)	0.09 ± 0.004	0.09 ± 0.001	0.09 ± 0.002	0.57
Glucose (mg/dl)	119.6 ± 2.6	121.6 ± 3.78	119.0 ± 3.2	0.88
β-hydroxybutyrate (mM)	0.5 ± 0.03	0.5 ± 0.02	0.5 ± 0.05	0.99
Open Field				
Distance traveled (m)	21.0 ± 0.6	22.4 ± 0.6	22.4 ± 0.7	0.22
Speed (cm/s)	7.5 ± 0.2	8.0 ± 0.2	8.0 ± 0.2	0.27
Time Center/Total Time	0.03 ± 0.005	0.03 ± 0.004	0.03 ± 0.004	0.72

to evenly distribute animals into dietary treatments ($n = 20$/diet). Peripheral blood was collected by tail clipping (between 0700 and 0900) and non-fasting levels of glucose and β-hydroxybutyrate (BHB) were measured using a glucose/ketone meter (Precision Xtra™, Abbott Laboratories, Abbott Park, IL).

Subjects were progressively transitioned into new dietary groups, while access to previous diet was gradually reduced, allowing habituation to the new food. Animals were allowed to adjust to new diets for a 3 week period prior to behavioral testing. Subsequently, each diet group was subdivided into control or stress ($n = 10$/group) to investigate whether prolonged exposure to endogenous vs. exogenous ketones could mitigate challenge-induced detriments on behavioral performance. The stress paradigm adopted was chosen according the published studies on chronic variable stress on rodents (Herman et al., 2008; Jankord and Herman, 2008; Shea et al., 2015) and consisted of once-daily exposure to randomly assigned stressors. Restraint stress was performed by inserting the animals into a custom made well-ventilated, flat bottom clear plastic rodent restrainer for 1 h. Cold exposure consisted of placing cages (with no food, bedding or water) in a cold room at 4°C for 30 min. Constant motion was performed by placing cages onto an orbital shaker set at 100 rpm for 1 h. Novel housing consisted of placing animals into a novel cage (different dimensions – 16W × 20L × 8H in; different bedding with *ad libitum* access to food and water) overnight. Exposure to hypoxic conditions was performed by placing groups of rats in chambers with a solid lid containing inlet and outlets ports; ambient air was slowly replaced by high nitrogen, low oxygen gas mixture until it reached concentrations in the range of 8–12% O_2. Animals were monitored throughout the hypoxia procedure (1 h). Stressors were randomly presented daily throughout the experimental paradigm. Serial blood collection was carried out during a 1 h restraint challenge to assess plasma levels of the stress hormones adrenocorticotrophic hormone (ACTH) and corticosterone (CORT). Moreover, all rats were fasted for 4 h, followed by *ad libitum* access to food for 2 h, after which food intake was calculated along with tail blood collection for measurements of postprandial levels of glucose, ketones and insulin. Animals were monitored daily for signs of distress and body weight was assessed three times a week to confirm normal growth rates for all animals.

Diets

Three different diets were utilized for this study and the macronutrient distribution in each diet is detailed in **Table 2**. Briefly, the ketogenic diet (KD, Teklad, Madison, WI) consisted of a low carbohydrate, medium chain triglyceride diet (carbohydrates: 0.5%; proteins: 22.4%; fats: 77.1%; 4.7 kcal/g). This diet has been shown to successfully achieve ketosis in rodents (Brownlow et al., 2013), without introducing high amounts of omega-6 or hydrogenated fats when compared to the standard rodent NIH-31 diet (carbohydrates: 62.2%; proteins: 23.8%; fats: 14%; 3.0 kcal/g). We performed a preliminary study to ensure that a mixture of the commercially available ketone supplements Caprylic Triglyceride (CT, a medium chain

TABLE 2 | Nutritional information of diets used.

Diets	NIH-31	Ketogenic diet (KD)	Ketone supplementation (KS)
Ingredient	**grams/kg**	**grams/kg**	**grams/kg**
Casein	210	300	210
L-Cystine	3	2.86	3
Sucrose	200	0	200
Maltodextrin	100	0	100
Corn starch	369	0	369
Cellulose (fiber)	40	245.31	40
MCT oil (medium chain triglycerides)	0	270	0
Flaxseed Oil	21	70	21
Canola Oil	19	60	19
Mineral mix Ca-P deficient (79055)	13.4	18.5	13.4
Calcium phosphate dibasic CaHPO4	7	8.5	7
Calcium carbonate CaCO3	7.3	10.75	7.3
40060 VM, Teklad	10	14	10
Ethoxyquin (Liquid)	0.1	0.08	0.1
β-hydroxybutyrate (BHB)	0	0	12.3
Caprylic triglyceride (CT)	0	0	80
Total	1,000	1,000	1,000
Protein, % of kcal	23.8	22.4	21.8
Carbohydrate, % of kcal	62.2	0.5	59.5
Fat, % by kcal	14	77.1	9.1
Ketone supplements	0	0	10.2
Vitamin mix, % of kcal	1.3	1.2	1.2
kcal/g	3.0	4.7	4.3

triglyceride—MCT- purchased from Parrillo Performance, Fairfield, OH) and KetoCaNa (Prototype Nutrition, Urbana, IL; 20 g of KetoCaNa mixed into 100 ml of CT) successfully modulated peripheral levels of glucose and ketone bodies following intragastric administration at a 10 g/kg dose (**Figures 2A,B**). A mixture of ketone salts and MCTs was considered due to the finding of immediate induction of ketosis elicited by the salts coupled with a slower, more sustained increase following MCT processing in the liver, resulting in increased ketone production (Kesl et al., 2016). The proportion of supplements used was chosen based on suggestions by colleagues and experimental testing in pilot studies. We adjusted the final numbers based on how well the salts stayed in solution after being mixed with CT. Hence, the ketone supplemented diet (KS; carbohydrates: 59.5%; proteins: 21.8%; fats: 9.1%; ketone supplements: 10.2%; 4.3 kcal/g) consisted of adding the mixture of ketone supplements (described above) at a 10% concentration (w/v) into the powdered NIH-31 diet (CTL, Teklad, Madison, WI). Due to the soft consistency of both KD and KS, these diets could not be pelleted and were placed in jars on the bottom of the animal's cage. Food was replaced three times a week to ensure freshness and *ad libitum* consumption.

Behavioral Testing

The *open field* (OF) test was used as a standard test of general activity. Animals were placed for 5 min in an arena (40.5 × 45 × 36 cm Plexiglas) while their locomotor activity was monitored and quantified using EthoVision XT system (version 7.0.418, Noldus Information Technology, Leesburg, VT).

Short-term memory was assessed by the *novel object recognition* (NOR) test. Two identical objects were placed along the center line of the arena and animals were allowed to explore for 5 min. After each trial, the arena and objects were cleaned to minimize olfactory cues. Twenty four hours following the acclimation trial, one of the objects was replaced with a novel object. Animals were given a 5 min exploratory trial and working memory was evaluated by the percentage of exploration index (time exploring the novel object divided by the combined time spent exploring both novel and familiar objects multiplied by 100).

To assess memory acquisition and consolidation following hypoxia, *passive avoidance* (PA) was performed using classical conditioning chambers (Med Associates Inc., St. Albans, VT), as previously described (Sandusky et al., 2013). Briefly, animals in the stress groups were exposed for 1 h to hypoxic conditions (8–12% O_2), prior to being placed in the brightly-lit side of the testing chamber for 30 s, after which a door opened, allowing entry to a dark chamber. Upon entry to the dark chamber on day 1, animals immediately received a mild foot shock (1.0 mA, 2s), ending their training trial and being subsequently placed back in the hypoxia chamber for 1 h prior to returning to their home cages. On day 2, animals were again placed in the brightly-lit chamber for 10s before the door was opened and latency to cross into the dark compartment (maximum of 10 min) after the door opened was taken as a measure of memory for the aversive experience.

The *Morris Water Maze* (MWM) tests spatial navigation and memory measured by the latency to find the escape platform (Morris, 1984). Water temperature was maintained at 19–23°C and extra-maze cues were placed on the walls. A clear platform (6 cm diameter) was located approximately 1.0 cm below the water in the southwest (SW) quadrant. Four alternating training days were completed, each with four 60 s trials with randomized starting positions. Inter-trial intervals were increased by testing all animals before the start of the next trial. On the first training day, animals that did not reach the platform within 60 s were gently guided to the platform. Probe trial was performed 48 h following the last (4th) training day, in which the platform was removed. Swim path, position, speed and latency to reach platform were recorded using EthoVision XT system (version 7.0.418, Noldus Information Technology, Leesburg, VT).

Tissue Processing

Rats were killed with pentobarbital (100 mg/kg) and transcardially perfused with 0.1M phosphate buffered saline (PBS). Interscapular brown adipose tissue (BAT), white adipose tissue (epididymal and perirenal fat pads), adrenals, thymus, and spleen were carefully harvested and weighed. Briefly, BAT was visually located after separating the skin on the back of the neck and dissected, cleaned from muscle and connective tissue

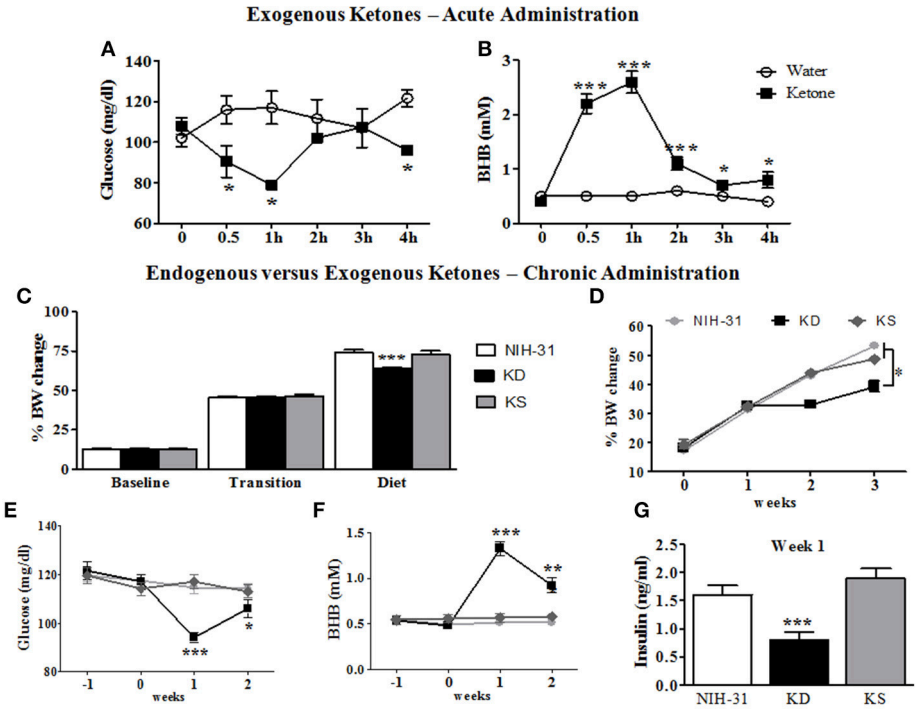


FIGURE 2 | Metabolic and behavioral changes induced by endogenous vs. exogenous ketosis. Intragastric administration of exogenous ketones (mixture of KetoCaNa + Caprylic Triglyceride, CT) induces a reduction in blood glucose **(A)** while increasing and sustaining higher blood ketone levels for up to 4 h **(B)**. **(C)** No differences in body weight gain were observed during baseline assessment or transition into new diets whereas decreased body weight gain was observed in KD-fed rats following the start of dietary interventions. **(D)** KD-fed rats showed slower weight gain compared to control or KS diets after onset of chronic administration. **(E)** Blood glucose levels were significantly reduced in KD group 1 week after the start of dietary treatment. **(F)** KD feeding resulted in significantly elevated blood levels of the ketone body β-hydroxybutyrate (BHB) starting 1 week following dietary treatments. **(G)** Blood insulin levels at week 1 were significantly decreased in KD group. Data presented as mean ± S.E.M. (n = 6–8/group). *$p \leq 0.05$; **$p < 0.01$; ***$p < 0.001$.

prior to being weighed. Epididymal fat pads were dissected after opening the abdominal cavity, locating white adipose tissue in the inguinal anatomical region (slightly lateral to midline). After gently pulling the fat pad upward, the epididymus was visualized and gently separated from white adipose tissue so epididymal fat pad could be weighed. This was done on both sides. After locating the kidneys, perirenal fat pads were identified as the white adipose tissue located around the kidneys and extracted by carefully pulling and separating the fat from the kidney. Brains were collected immediately following perfusion; one hemisphere was dissected and immediately frozen on dry ice for biochemical analysis. The other hemisphere was immersion fixed in 4% phosphate-buffered paraformaldehyde for 24 h and cryoprotected in 30% sucrose solution for at least 24 h prior to sectioning on the coronal plane (25 μm thickness) on a sliding microtome with a freezing plate (Leica SM2010R). Sections were placed into cryoprotectant solution and stored at −20°C until further processing. Freshly frozen hippocampi were quickly minced and homogenized in RIPA homogenization buffer (pH 7.2) containing phosphatase and protease inhibitors (Sigma Aldrich, St Louis, MO) and soluble (cytosolic) fraction was separated following centrifugation at 20,000 rpm for 30 min. Pellets were then treated with Mem-Per Solubilization buffer, according to manufacturer's instructions (ThermoFisher Scientific, Waltham, MA) for extraction of membrane fraction. Hippocampal samples were used for further biochemical analysis by enzymatic or western blotting assays.

Biochemical Analysis

All enzymatic assays were run in duplicate, analyzed within the same assay and performed according to manufacturer's instructions. Blood samples were centrifuged at 4,000 × g for 15 min at 4°C and plasma was stored at −20°C until processed. Plasma levels of ACTH and CORT were measured using a rat stress hormone magnetic multiplex bead assay (RSHMAG-69K, EMD Millipore, Temecula, CA). Insulin concentrations were determined by ELISA (Crystal Chem., Downers Grove, IL). Protein content in hippocampal homogenates was determined using BCA protein assay kit (Pierce, Rockford, IL); BDNF levels were quantified in the cytosolic fraction using ELISA (Bolster Biological, Pleasanton, CA) and normalized to total protein content. BHB concentration was measured using a commercially available kit (Cayman Chemicals, MI).

Western Blotting

Following tissue homogenization and determination of protein concentration, equal amounts of proteins (12 μg/well) were

loaded in each well of a 4–20% SDS PAGE gel and transferred onto a 0.2 μm pore size nitrocellulose membrane. Membranes were blocked in 5% blocking solution (Bio-Rad Laboratories, Inc. Hercules, CA) in 0.01% PBS-Tween and immunoblotted overnight (4°C on shaker) with different primary antibodies. Blots were washed with 0.01% PBS-T and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (ThermoFisher, Waltham, MA) for 1 h at room temperature. After incubation, membranes were washed before visualization using enhanced chemiluminescence (SuperSignal West Femto, ThermoFisher Scientific, Waltham, MA). Primary antibodies used were all rabbit polyclonal: BDH1 (Proteintech, 1:1,000); ACAT1 (Proteintech, 1:1,000); GLUT1 (Proteintech, 1:1,000); GLUT3 (Abcam, 1:2,000); COX IV (Abcam, 1:1,000) and β-actin (Invitrogen, 1:5,000). Band intensities were quantified by densitometry analysis using ImageJ software; corrected for background intensities and normalized to levels of β-actin (soluble fraction) or cytochrome oxygenase IV (COX IV, for mitochondrial enzymes) used as loading controls.

Statistical Analysis

Statistical analysis was carried out using SigmaPlot (SigmaPlot 11.0, San Jose, CA) by ANOVA followed by Fisher's Least Significant Difference (FLSD) *post-hoc* analysis for all comparisons performed. One-way ANOVA was performed with diet as independent variable; two-way ANOVA with diet and stress or time points as independent variables and three-way ANOVA with diet, stress and test session (training, testing, or 2 week test for passive avoidance test and testing days for MWM). One-, two-, or three-way repeated measures (RM) ANOVA were used when necessary and are indicated in corresponding results section. Statistical significance was established with $p \leq 0.05$ for all tests. Graphs were generated using Graph Pad Prism 5.01 (La Jolla, CA).

RESULTS

Acute Administration of Exogenous Ketones Rapidly Changed Blood Glucose and Ketones

An initial time course experiment was performed to ensure that ketone supplements successfully induced and maintained ketosis in rodents. Administration of exogenous ketones by oral gavage reduced blood glucose within the first 30 min (95.3 mg/dl; two-way RM ANOVA treatment and time interaction, $p = 0.02$, **Figure 2A**), when compared to animals gavaged with water (116.0 mg/dl). Peripheral levels of BHB seemed to peak 1 h following administration and were significantly elevated for the duration of testing (ketone: 2.6 mM vs. water: 0.5 mM; two-way RM ANOVA main effects of treatment: $p = 0.0002$; time: $p < 0.0001$ and treatment and time interaction: $p < 0.0001$; **Figure 2B**).

KD, but not KS, Impacted Body Weight and Blood Levels of Glucose, Ketones, and Insulin

Body weight data collected was averaged for different experimental phases (3 weeks of baseline, transition week and 3 weeks of dietary interventions) and percentage of body weight change from initial week (wk -3) was calculated (**Figure 2C**). No differences in body weight gain were observed during the initial 3 weeks of baseline assessments (one-way RM ANOVA, $p = 0.64$) or during the transition week ($p = 0.80$) and a main effect of diet (one-way RM ANOVA, $p < 0.001$) was observed following the start of diet treatments. Body weight gain was significantly slower in KD-fed rats when compared to both control and KS diets (FLSD, $p < 0.001$ for both comparisons). One-way ANOVA revealed a main effect of diet ($p < 0.0001$), starting at week 2, when KD was compared to both the NIH-31 and KS groups, depicted in **Figure 2D**.

After transitioning into new dietary groups, a significant decrease in blood glucose levels was found in KD-fed rats (one-way RM ANOVA, main effect of diet, $p = 0.007$, **Figure 2E**) together with increased peripheral levels of BHB (one-way RM ANOVA, main effect of diet, $p < 0.0001$, **Figure 2F**). Plasma insulin levels from rats fed KD were lower than other groups ($p < 0.001$ when compared to both NIH-31 and KS groups, week 1 time point, **Figure 2G**), showing a 50% reduction in comparison to the NIH-31-fed group (FLSD, $p < 0.001$). Ketone supplementation did not affect insulin levels when compared to the NIH-31 diet (FLSD, $p = 0.21$). On week 3, behavioral testing was performed to assess diet-induced changes in short-term memory and tail blood collection was not performed to avoid possible confounding variables in both behavioral and biochemical measures.

24 h Retrieval in the Novel Object Recognition Test Was Enhanced in KD Fed Rats

Short-term memory was assessed by the novel object recognition test. One-way ANOVA revealed a main effect of diet ($p = 0.03$); **Figure 3A** highlights the *post-hoc* comparison showing that the KD-fed group exhibited significantly greater percentage of novel object exploration compared to NIH-31-fed group (FLSD, $p = 0.009$). No differences were observed between KD-fed and either KS (FLSD, $p = 0.11$) or between KS vs. NIH-31 (FLSD, $p = 0.29$) groups.

24 h Performance in the Passive Avoidance Was Negatively Affected by Hypoxia

The passive avoidance test was used to investigate learning and memory performance following exposure to stress induced by exposure to hypoxia prior to and immediately after training trials. All animals entered the dark chamber rapidly on training day and two-way ANOVA revealed no diet ($p = 0.74$) or hypoxia ($p = 0.63$) differences when latency to cross was analyzed (**Figure 3B**, clear bars). Three-way RM ANOVA (diet, hypoxia and testing session) revealed main effects of hypoxia (gray box, $p = 0.05$), testing session ($p < 0.0001$) and significant interaction between

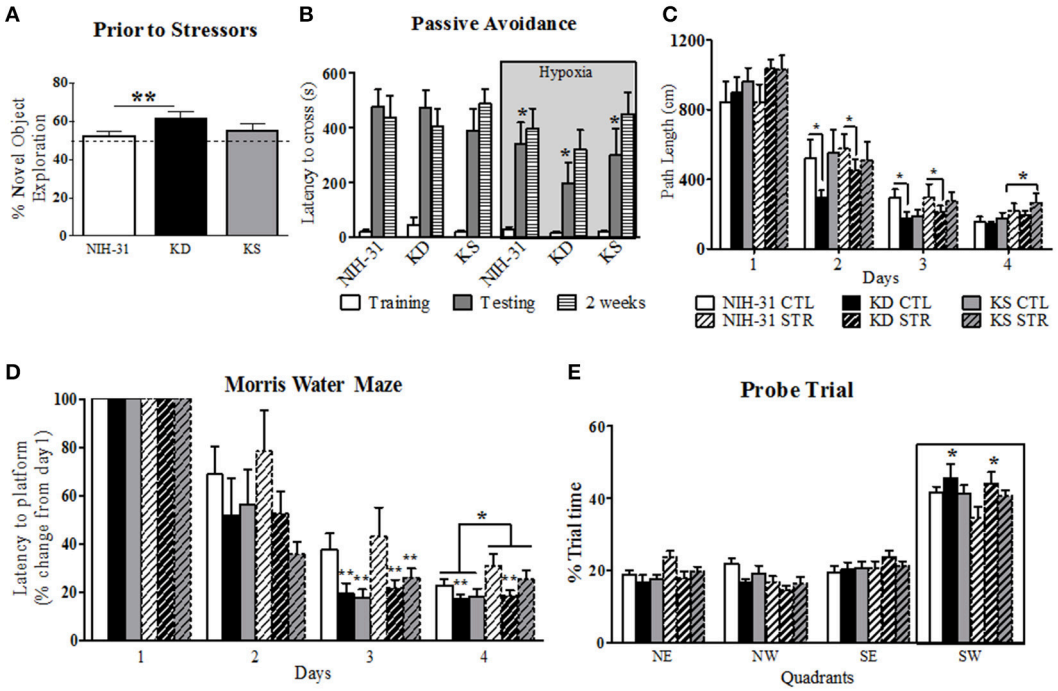


FIGURE 3 | Diet and stress-induced effects on behavioral performance prior to and during daily exposure to stressors. (A) KD-fed rats showed greater percentage of novel object exploration compared to NIH-31 diet. This improvement occurred regardless of changes in locomotor activity (not shown). No differences in total distance traveled or speed were observed in any of the groups. The dotted line represents chance exploratory preference. **(B)** Hypoxia exposure prior to and following training trials significantly decreased latency to cross 24 h later (shadedbars) in the passive avoidance test. This impairment, however, was not present 2 weeks later (dashed bars). **(C)** KD-fed rats displayed shorter path lengths on days 2 and 3 of testing in the Morris water maze. Stress significantly increased path length on day 4 of testing, regardless of diet. **(D)** Morris water maze performance was significantly improved in both KD and KS groups on day 3, regardless of stress, and was impaired by stress on day 4. KD feeding prevented stress-induced impairments in performance on day 4 **(E)** Percentage of trial time spent in each quadrant during probe trial. KD-fed rats spent more time in target quadrant (SW) than NIH-31-fed groups. Data are presented as mean ± S.E.M. (n = 9–10/group). *$p \leq 0.05$; **$p < 0.01$.

testing session and hypoxia ($p = 0.02$). Specifically, groups that were exposed to hypoxia showed significantly shorter latencies to cross over to the dark chamber when compared to animals kept in normoxic conditions during testing session (two-way RM ANOVA $p = 0.01$), indicating poorer performance. This detrimental effect was, however, no longer present when animals were tested 2 weeks later ($p = 0.45$; **Figure 3B**, striped bars). There were no significant effects of diet during training, 24 h or 2 weeks testing for the passive avoidance test (three-way RM ANOVA, $p = 0.61$).

Performance in the MWM Was Modulated by Both Diet and Stress

Spatial learning and memory were assessed by the MWM after multi-modal stress exposure. All animals swam without difficulty and no differences in swim speed were observed between diets or stress ($p = 0.95$ and $p = 0.92$, respectively, data not shown). A non-significant trend was observed for a main effect of stress on path length ($p = 0.07$), suggesting that exposure to stress resulted in a trend for increased swim distance. This effect reached statistical significance on day 4 (two-way ANOVA, main effect of stress, $p = 0.02$, **Figure 3C**). Individual analysis of daily path length revealed that KD-fed rats swam significantly shorter distances when compared to NIH-31-fed rats on both days 2 and 3 ($p = 0.05$ and $p = 0.04$, respectively) regardless of exposure to stressors.

As evidenced by **Figure 3D**, all groups improved with gradual decreases in latency to reach the escape platform (three-way RM ANOVA main effect of days, $p < 0.0001$). Three-way ANOVA revealed a main effect of stress ($p = 0.05$), although no main effects of diet ($p = 0.22$) were observed when averaged latencies were compared across all 4 days. Stress-induced increase in latency to reach platform was most pronounced on day 4 of testing ($p = 0.02$). Significant differences between groups were observed within days when latency to reach platform on testing days was compared to initial performance on Day 1 (three-way RM ANOVA main effect of diet, $p = 0.003$). For instance, on Day 2 groups fed control diet showed a non-significant trend for increased latencies when compared to both KD (FLSD, $p = 0.1$) and KS groups (FLSD, $p = 0.06$). This difference reached statistical significance on Day 3 (main effect of diet, $p = 0.005$), with KD and KS groups displaying shorter latencies than control diet groups (FLSD, $p = 0.004$, and $p = 0.006$, respectively). Main effects of both diet ($p = 0.03$) and stress ($p = 0.05$) were observed on Day 4. KD-fed animals displayed significantly shorter

latencies compared to the control diet (FLSD, $p = 0.008$, **Figure 3D**).

All animals spent the majority of probe testing time in the target quadrant (outlined by boxed area in **Figure 3E**). No differences in swim speed or distance were observed (data not shown). Three-way RM ANOVA (diet, stress, and quadrants) revealed a main effect of quadrant ($p < 0.0001$) and a significant interaction between quadrant and diet ($p = 0.03$) vs. a non-significant trend for quadrant and stress interaction ($p = 0.07$). KD-fed animals spent significantly more time in the target quadrant than NIH-31-fed groups (FLSD, $p = 0.02$).

Neither Diet Affected Hypothalamic-Pituitary-Adrenal Axis Response to Acute Stressor

Diet-induced changes in stress hormone levels were assessed by a 1 h restraint challenge, during which blood samples were collected at 0 (baseline), 30 and 60 min and then after 1 h recovery (120 min). **Figure 4** summarizes restraint-induced changes in peripheral levels of glucose (**Figure 4A**), BHB (**Figure 4B**), ACTH (**Figure 4C**), and CORT (**Figure 4D**). Glucose levels steadily increased (two-way RM ANOVA main effect of time $p < 0.0001$, **Figure 4A**) but no main effects of diet were observed ($p = 0.15$). Area under the curve analysis (AUC, **Figure 4A** inset) showed that KD group exhibited lower overall levels of glucose than NIH-31 group ($p = 0.05$). Blood glucose levels in KS group were intermediate and did not differ from either NIH-31 or KD groups (AUC analysis: FLSD, $p = 0.28$ and $p = 0.36$, respectively). Two-way RM ANOVA revealed main effects of time ($p < 0.0001$) and diet ($p < 0.0001$) when ketone levels were analyzed throughout all time points, suggesting that ketone levels gradually dropped after the onset of the stressor, concomitantly with the increase in glucose levels. *Post-hoc* analysis showed that this difference was due to KD-fed group presenting higher levels of ketone bodies at all time points tested when compared to other dietary groups [**Figure 4B**: KD vs. NIH-31, FLSD baseline ($p = 0.02$), 30 min ($p = 0.001$), 60 min ($p = 0.0002$) and 120 min ($p < 0.0001$); KD vs. KS, FLSD baseline ($p = 0.01$), 30 min ($p = 0.004$), 60 min ($p = 0.007$) and 120 min ($p = 0.0001$)]. Accordingly, area under the curve analysis (**Figure 4B** inset) revealed a main effect of diet ($p < 0.001$ and FLSD, $p < 0.001$) when compared to both groups.

No differences were observed in the total levels or area under the curve analysis for ACTH (**Figure 4C** and inset) or CORT (**Figure 4D** and inset) between groups. ACTH secretion profile in both KD and KS groups seemed slightly different, with peak levels appearing somewhat faster (at 30 min) and lower than control counterparts. However, these differences did not reach statistical significance which may be due in part to great individual variability in the biochemical measurements of ACTH in plasma samples.

Postprandial Levels of Glucose, Ketones, and Insulin

In order to determine diet-induced metabolic changes, all animals had food removed for 4 h (0800–1,200) followed by *ad libitum* access to respective diets for 2 h (1,200–1,400), after which food consumption was calculated and blood was collected for assessments of glucose, ketones and plasma insulin levels (**Figure 5**). Two-way ANOVA revealed main effects of both diet ($p = 0.01$) and stress ($p = 0.04$) on glucose levels 2 h after feeding. Stressed rats had greater postprandial glucose levels than control rats (FLSD, $p = 0.03$). Consistent with previous measurements, postprandial glucose measurements were attenuated in KD

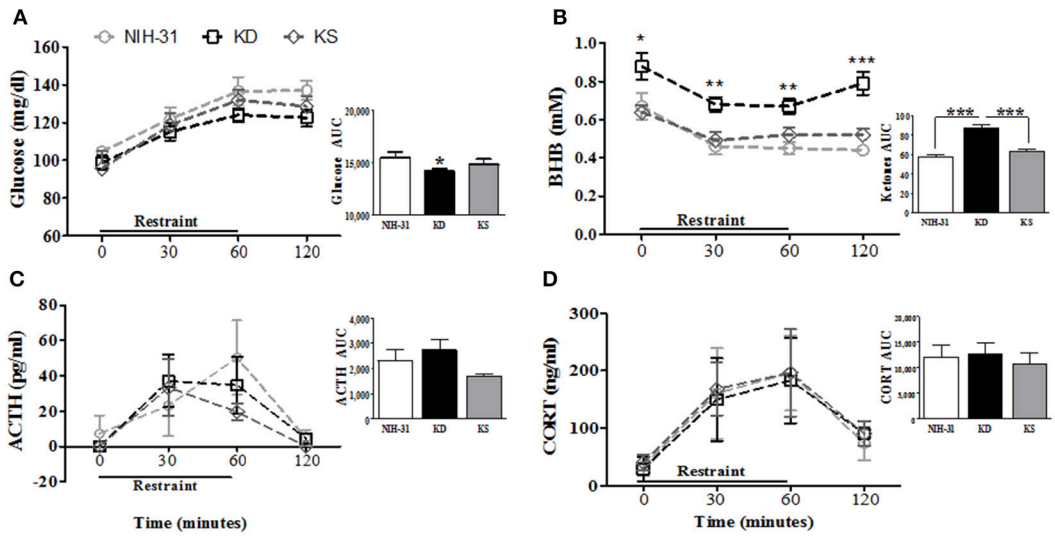


FIGURE 4 | Effects of endogenous (KD) vs. exogenous (KS) ketones on blood chemistry and the stress response. (A) Blood glucose and **(B)** ketone levels during and following 1 h restraint challenge ($n = 9$–10/group). Plasma levels of the stress hormones **(C)** ACTH and **(D)** corticosterone were unchanged by either KD or KS. Insets represent area under the curve (AUC) analysis for each measurement. Data shown as mean ± S.E.M. ($n = 6$–8/group). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

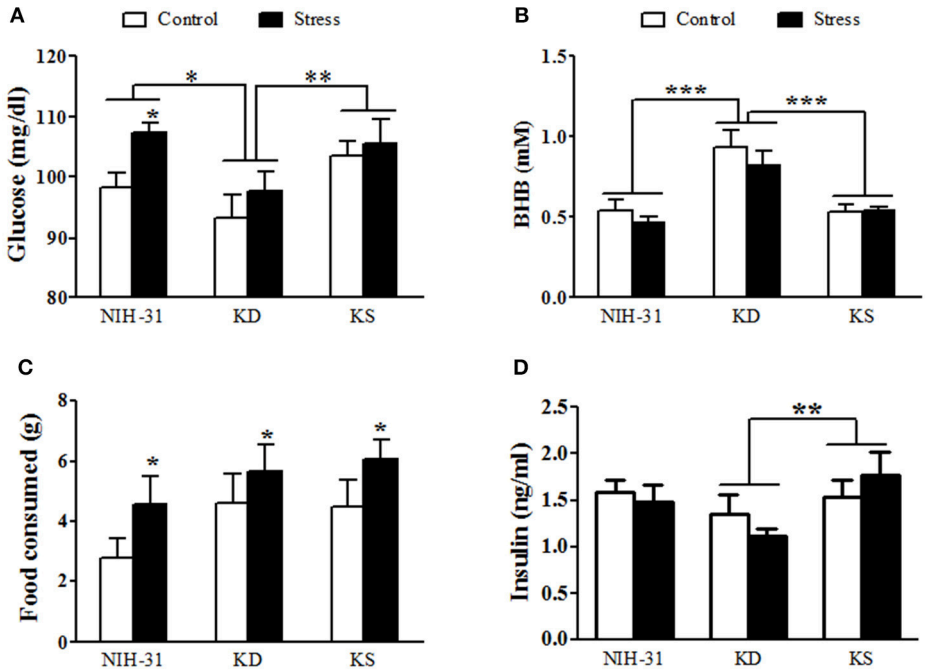


FIGURE 5 | Postprandial measurements of glucose, ketones, food intake and insulin. (A) Blood glucose was reduced in KD-fed groups and elevated in stressed groups. **(B)** Ketone (BHB) levels were elevated in KD groups only and not affected by stress. **(C)** Food intake was not different across diets but was elevated in stressed groups. **(D)** Non-fasting insulin levels were reduced by KD. Data shown as mean ± S.E.M. (n = 8–10/group). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

groups in comparison to both NIH-31 (FLSD, $p = 0.01$) and KS (FLSD, $p = 0.004$) groups (**Figure 5A**). Postprandial levels of BHB were increased in KD-fed group only (two-way ANOVA main effect of diet, $p < 0.0001$; FLSD, $p < 0.0001$ when compared to both other diets; **Figure 5B**), regardless of stress ($p = 0.26$). Of note, at the time point investigated, consumption of ketone supplements in the context of a normal carbohydrate diet was not effective in inducing significant ketosis when compared to NIH-31 diet alone (FLSD, $p = 0.53$). Food consumption was not affected by diet ($p = 0.14$) but was significantly increased in stressed groups ($p = 0.04$, **Figure 5C**).

Non-fasting insulin levels revealed a main effect of diet ($p = 0.04$, **Figure 5D**). *Post-hoc* comparisons showed that this effect was due to a pronounced decrease in insulin levels in KD-fed groups when compared to KS-fed (FLSD, $p = 0.01$) while displaying a non-significant trend in comparison to NIH-31-fed groups (FLSD, $p = 0.10$).

Neither Dietary Treatment Prevented Body Weight Changes during Chronic Stress

Weekly assessments (three-way RM ANOVA with diet, stress and weeks as independent variables) showed main effects of both stress ($p = 0.04$) and diet ($p < 0.0001$). All groups exposed to stressful conditions displayed smaller body weight changes throughout the 4 weeks of daily exposure to stressors (**Figure 6A**). The diet effect observed was due to KD-fed groups remaining significantly smaller than animals from the other dietary groups. Averaged body weight changes during the last 4 experimental weeks were analyzed to investigate the combined effects of both diet and stress (**Figure 6B**). Two-way ANOVA revealed main effects of both diet ($p < 0.001$) and stress ($p = 0.04$) on percentage of body weight change with *post-hoc* analysis indicating that KD-fed rats presented significantly smaller body weight changes when compared to both NIH-31 and KS diets (FLSD, $p < 0.001$ for both comparisons, **Figure 6B**). Importantly, chronic supplementation with ketones did not affect body weight gain in comparison to the control diet (FLSD, $p = 0.75$).

Chronic Effects of Both Diet and Stress on Anatomy

No differences in brain (diet: $p = 0.12$ and stress: $p = 0.99$), adrenal (diet: $p = 0.39$ and stress: $p = 0.93$) or spleen (diet: $p = 0.90$ and stress: $p = 0.15$) raw weights were observed. Thymus involution was observed in stressed animals, regardless of dietary group ($p = 0.04$). In addition to raw tissue weights, brown adipose tissue (BAT), perirenal, and epididymal fat pads are also shown as percentage of final body weight. Normalized BAT weight was significantly increased in KS groups (main effect of diet, $p = 0.01$, **Figure 6C**) and this effect was observed when compared to KD groups (FLSD, $p = 0.0025$). No stress-induced differences were observed in raw values ($p = 0.87$) or normalized BAT weight ($p = 0.53$). Perirenal and epididymal fat pads were dissected and weighed to assess chronic effects of diet and stress on peripheral energy deposits. Two-way ANOVA revealed a significant effect of diet on normalized perirenal fat pad weight ($p = 0.002$). **Figure 6D** highlights the *post-hoc*

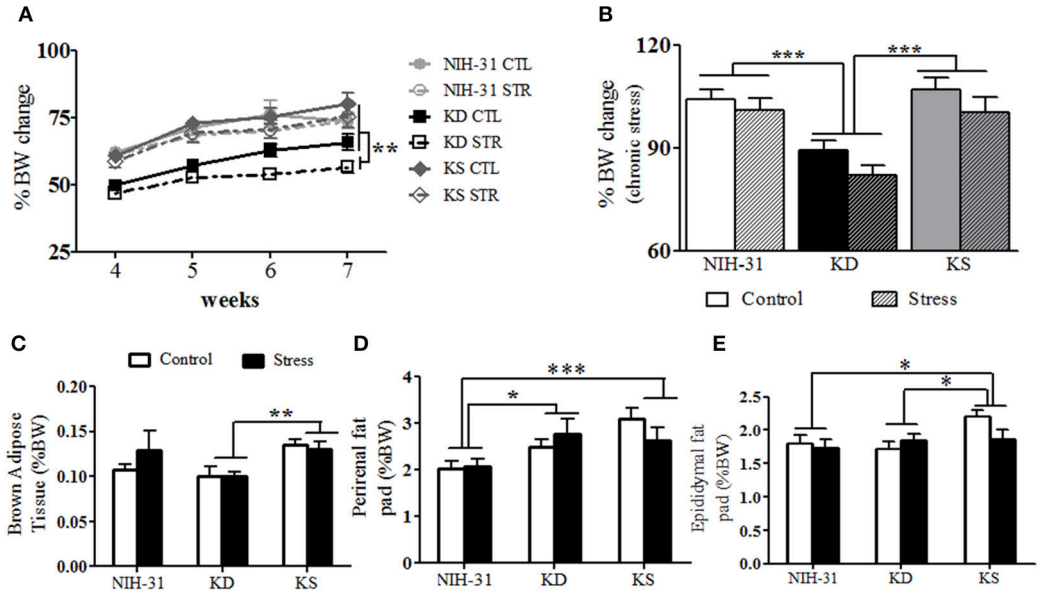


FIGURE 6 | Anatomical changes at euthanasia following chronic exposure to stressors. (A) Following exposure to stressors, main effects of diet ($p < 0.0001$) and stress ($p = 0.04$) were observed, with KD-fed rats exposed to stress displaying significantly lower body weight changes. **(B)** Averaged percentage of body weight change values during the stress exposure period showed main effects of diet ($p < 0.001$) and stress ($p = 0.04$) and showed that KD groups showed consistent lower weight gain. **(C)** KS-fed rats showed increased brown adipose tissue weight (BAT, normalized to body weight) in comparison to KD-fed group ($p = 0.002$). **(D)** Normalized perirenal fat pad weights were larger in both KD and KS in comparison to NIH-31-fed groups ($p = 0.01$ and $p = 0.0006$, respectively). **(E)** A trend for main effect of diet ($p = 0.07$) was observed with normalized epididymal fat pad weights, with increased weights in KS groups, compared to both NIH-31 ($p = 0.03$) and KD ($p = 0.04$) groups. Data represented as mean ± S.E.M. ($n = 9$–10/group). *$p < 0.05$; **$p < 0.01$, and ***$p < 0.001$.

comparisons showing that perirenal fat pads obtained from NIH-31-fed animals were significantly smaller than the ones dissected from both KD (FLSD, $p = 0.01$) and KS (FLSD, $p = 0.0006$) groups. When analyzing normalized epididymal fat pad weights, a trend for diet-induced differences was observed ($p = 0.07$, **Figure 6E**). Overall, KS groups displayed larger epididymal fat pads when compared to both NIH-31 (FLSD, $p = 0.03$) and KD (FLSD, $p = 0.05$) groups. No stress effects were observed in either perirenal ($p = 0.77$) or epididymal ($p = 0.38$) fat pads, regardless of dietary treatment.

Stress-Induced Reduction in Hippocampal BHB and BDNF were Attenuated by KD and Both KD and KS, respectively

Two-way ANOVA revealed a main effect of stress ($p < 0.001$) on hippocampal levels of BHB, with all diet groups showing lower levels following exposure to stressors (**Figure 7A**). While *post-hoc* comparisons did not find differences between any of the dietary groups in the control condition, stressed animals fed KD showed significant attenuation of decrements in hippocampal BHB levels, when compared to stressed NIH-31-fed group (FLSD, $p = 0.02$). **Figure 7B** depicts changes in the ratio of BDNF levels to hippocampal weight. Two-way ANOVA revealed a main effect of stress ($p = 0.03$), while *post-hoc* comparisons showed that NIH-31 fed groups showed a 13% reduction BDNF levels after repeated exposure to stressors (FLSD, $p = 0.008$) but no effect of stress was observed in either the KD or KS groups (**Figure 7B**).

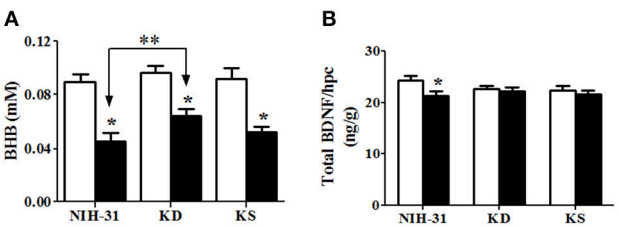


FIGURE 7 | Hippocampal levels of β-hydroxybutyrate (BHB) and brain derived neurotrophic factor (BDNF). (A) Daily exposure to stress resulted in decreased hippocampal levels of BHB. This decrease, however, was attenuated in KD-fed rats compared with stressed rats on control diet (indicated by bracket with arrows). **(B)** Decreased BDNF levels were observed in stressed NIH-31 fed rats compared with non-stressed NIH-31 fed rats. However, both KD and KS groups showed a non-significant trend ($p = 0.10$) for lower levels of hippocampal BDNF. Data shown as mean ± S.E.M. ($n = 8$–10/group). *$p \leq 0.05$; **$p < 0.01$.

Diet and Stress-Induced Changes in the Protein Levels of Mitochondrial Enzymes and Glucose Transporters

β-hydroxybutyrate dehydrogenase-1 (BDH1), the enzyme that catabolizes BHB in the brain, was significantly increased in the hippocampi of rats fed either KD or KS (two-way ANOVA main effect of diet, $p < 0.001$, **Figure 8A**). After stress, a further increase was more prominent in KD when compared

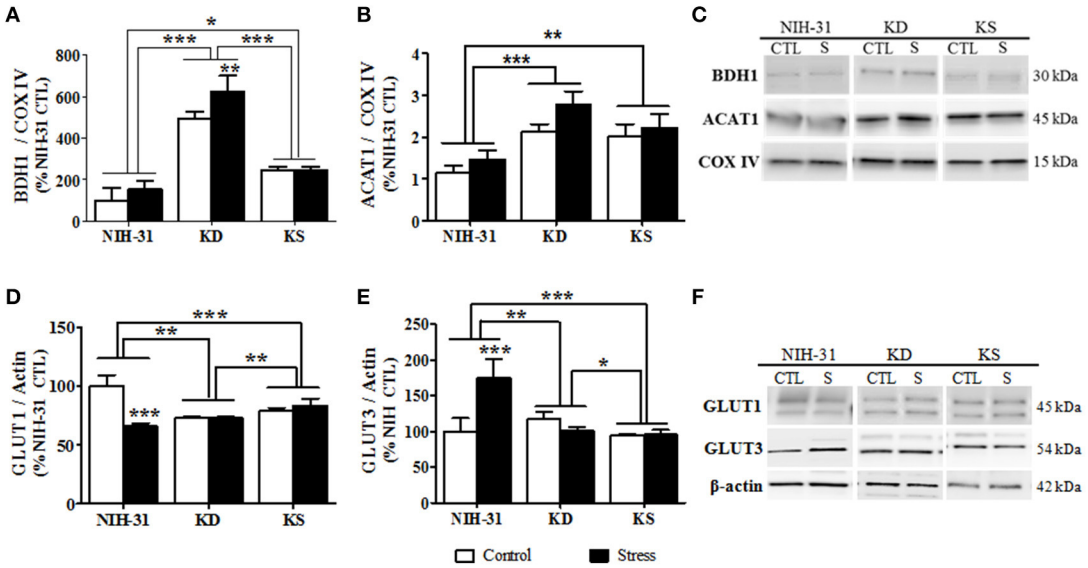


FIGURE 8 | Diet and stress-induced biochemical changes in mitochondrial enzymes and glucose transporters. Hippocampal levels of the mitochondrial enzymes **(A)** 3-hydroxybutyrate dehydrogenase, type 1 (BDH1), and **(B)** acetyl-Coenzyme A acetyltransferase 1 following nutritional interventions and exposure to stressful conditions in healthy adult male rats. **(C)** Representative blots and corresponding loading control (COX IV). Diet and stress-induced changes in the levels of glucose receptors GLUT1 **(D)** and GLUT3 **(E). (F)** Representative immunoblots used and loading control (β-actin). Data shown as mean ± S.E.M. (n = 8–10/group). $^{*}p < 0.05$; $^{**}p < 0.01$, and $^{***}p < 0.001$.

to both KS and NIH-31 groups (FLSD, $p < 0.001$ for both comparisons). *Post-hoc* comparison between KS and NIH-31 groups showed a significant increase in BDH1 levels (FLSD, $p = 0.03$). Despite the lack of main effects of stress, *post-hoc* analysis showed a significant increase in stress-induced levels of BDH1 levels in KD group (FLSD, $p = 0.005$). **Figure 8B** illustrates changes in acetyl-CoA transferase (ACAT1), a mitochondrial enzyme that catalyzes the reversible formation of acetoacetyl-CoA from two molecules of acetyl-CoA. Here we show that both endogenous and exogenous ketones induced a significant increase in hippocampal ACAT1 levels (main effect of diet, $p < 0.001$), when compared to control diet (FLSD, $p < 0.001$ and $p = 0.003$, respectively). Furthermore, a non-significant trend for a main effect of stress was observed ($p = 0.06$), mostly driven by the increase within KD-fed group (FLSD, $p = 0.08$). **Figure 8C** depicts representative blots for all groups probed with either BDH1 or ACAT1 and loading control (COX IV).

Two-way ANOVA revealed significant diet ($p = 0.008$), stress ($p < 0.001$) and diet and stress interaction ($p < 0.001$) in the levels of vascular endothelium glucose transporter (GLUT1, **Figure 8D**). Stress-induced decrease was only observed in the NIH-31 fed group (FLSD, $p < 0.001$). However, KD and KS groups displayed lower baseline levels of hippocampal GLUT1 when compared to NIH-31 group (FLSD, $p < 0.001$), regardless of stress exposure. Similarly, main effects of diet ($p < 0.001$), stress ($p = 0.009$) and diet × stress interaction ($p < 0.001$) were observed in the levels of neuronal glucose transporter (GLUT3, **Figure 8E**). **Figure 8F** depicts representative blots for all groups probed with either GLUT1 or GLUT3 and loading control (β-actin).

DISCUSSION

In this study, we investigated the effects of a ketogenic (KD) or ketone supplemented (KS) diet on metabolic, behavioral and biochemical outcomes associated with cognitive performance. Our results show that the KD, but not KS (at the dose tested here), elicited pronounced metabolic effects, and prevented some of the stress-induced effects on behavioral performance. Both KD and KS ameliorated decrements during day 3 of the water maze testing while improved performance during the probe trial was only observed in the KD group. During restraint stress, the KD group maintained peripheral ketosis despite increased glucose levels. The KD elicited biochemical changes in hippocampal levels of mitochondrial enzymes, glucose transporters and BDNF. Of interest, these biochemical changes were also observed in the KS group despite lack of peripheral ketosis and were not affected by exposure to stressors. **Table 4** summarizes the main findings described in this report, showing that KD had effects on multiple outcomes examined in this study while the ketone supplemented diet studied was only able to replicate a portion of these observations.

To investigate the effects of nutritional treatments on behavior, all rats were tested on the novel object recognition tasks 3 weeks after transitioning into dietary groups and prior to starting the chronic stress paradigm. Our finding of increased novel object exploration in young subjects fed KD suggests that endogenous ketosis augmented short term memory acuity in healthy rats. Ketosis-induced improvement in the novel object recognition test had been previously reported in aged rats kept under normoxic conditions or after 1 day in hypobaric chambers (Xu et al., 2010). Subjects tested in our study did not show

anxious or compulsive-like behaviors assessed by the marble burying test or time spent in the center of arena during novel object recognition testing (data not shown).

Next, we aimed to determine the impact of an acute restraint challenge on peripheral levels of stress hormones. Despite an overall increase in glucose, KD-fed animals sustained lower glucose levels (10% reduction in the area under the curve values) and greater ketosis throughout procedure, compared to both control and KS diets (52 and 38% higher, respectively). Even in the absence of statistically significance, subtle changes were observed in the profile of ACTH secretion, with a tendency for a shift in peak values and quicker return to baseline. These results were, nonetheless, not statistically significant and may benefit from further investigation using different dietary formulations, additional rodent species or continuous measurements to better investigate time course changes. Notably, several ACTH values from both KD and KS samples obtained during baseline and 120 min time points were below detection levels, whereas this was not observed with samples from rats fed the control diet. More studies are needed to better understand how changing cerebral energy metabolism to ketone utilization may affect the response to acute or chronic stressors.

Animals completed two behavioral tasks to determine whether the nutritional interventions adopted would prevent or diminish stress-induced detriments. Altitude sickness due to hypoxic conditions resulted in impaired performance on cognitive tasks in humans (Virues-Ortega et al., 2006) while KD showed neuroprotective properties following 3 weeks of hypoxia (Puchowicz et al., 2005, 2008). Neither KD nor KS prevented the hypoxia-induced impairment in the passive avoidance test. In fact, on testing day, animals on KD showed a slightly shorter latency to cross, suggesting that neuroprotective effects elicited by KD during hypoxic conditions may require prior adaptation, as evidenced by changes in cerebral energy utilization described in Puchowicz et al. (2005).

Previous investigations of KD-induced performance in the water maze have shown protective (Kim et al., 2012), detrimental (Zhao et al., 2004), or inconclusive (Fukushima et al., 2015) effects. Our findings reveal beneficial effects of KD despite repeated exposure to stressors known to elicit detriments in behavioral performance. Surprisingly, these effects were also observed in KS groups on the third day of testing; suggesting that supplementation with ketones may modulate biological pathways relevant to cognitive outcomes, despite lack of observable peripheral effects. Although, stress-induced increases in escape latency were observed, these effects only reached statistical significance on the fourth testing day and were prevented by KD feeding, suggesting that endogenous ketones positively impacted adaptation to the cumulative effect of stress on performance in the water maze. Several mechanisms have been suggested to explain ketone bodies effects on cognition. For instance, brain uptake of ketone bodies is proportional to their circulating levels (Blomqvist et al., 2002; Cahill, 2006), providing a more efficient fuel (Veech et al., 2001) for neuronal and glial cells and bypassing possible deficits in glucose uptake and utilization, prevalent in cases of neurodegenerative diseases (Mosconi et al., 2008), or during challenging situations (McNay et al., 2000).

Ketone bodies have also been reported to increase mitochondrial efficiency and biogenesis (Bough et al., 2006; Kashiwaya et al., 2010), which may also contribute to improved cerebral energy metabolism. Furthermore, ketones have also shown to act as potent signaling molecules, modulating energy metabolism (Marosi et al., 2016), epigenetic events (Shimazu et al., 2013) and neuronal excitability (Masino et al., 2011) (for a review, see Newman and Verdin, 2014b). Accordingly, the novel finding that KD attenuated stress induced decrease in hippocampal BHB levels may suggest that increased cerebral availability of ketone bodies plays a role in brain homeostatic mechanisms, modulating hippocampal energy utilization.

The KD diet was chosen based on its high MCT content, knowing that MCT ingestion can rapidly increase liver production of ketone bodies. Moreover, the MCTs included were chosen with the intent to induce neuroprotective effects, replacing standard saturated and hydrogenated fats present in other commercially available rodent KDs. The combination of canola and flaxseed oil was chosen to generate a 2:1 omega-3 to omega-6 ratio, a ratio suggested to be beneficial in well-formulated ketogenic diets. Given the neuroprotective properties associated with flaxseed oil (rich in 18:3 α-linolenic acid) (reviewed in Piermartiri et al., 2015), we acknowledge that the possibility remains that the increased levels of α-linolenic acid generated could also have contributed to the cognitive improvements observed, independently of the ketone bodies.

Hippocampal BDNF levels and expression can be upregulated following interventions that activate hormetic pathways (voluntary exercise, calorie restriction and environmental enrichment) (Mattson, 2008) or down regulated following chronic stress (for a review, see Rothman and Mattson, 2013). Our findings confirmed stress-induced decrements in hippocampal BDNF levels; however, this effect was only significant in NIH-31 fed rats. Due to its effect on decreased hippocampal excitability (Bough et al., 2003; Kawamura et al., 2014), KD is suggested to reduced BDNF signaling on the brain (Masino and Rho, 2012). This is in contrast with compelling evidence of up regulation of BDNF by strategies that, similarly to KD, reduce glycolytic activity such as caloric restriction (Stranahan et al., 2009) and 2-deoxy glucose treatment (Yao et al., 2011). Although, not statistically different, our findings are indicative of lower BDNF levels in both KD and KS control groups (7 and 8% lower, respectively). One other study described that feeding Wistar rats a KD for 8 weeks reduced BDNF levels in the striatum, but not the hippocampus (Vizuete et al., 2013).

Chronic administration of exogenous ketones via diet did not result in physiological levels of ketone bodies despite pronounced effects following acute administration. Intragastric administration of ketone supplements for 28 days resulted in rapid and sustained elevation of ketone bodies and decreased glucose levels in the absence of changes in lipid biomarkers (Kesl et al., 2016). Given that daily oral administration by gavage is a stressful method, we sought translatable approaches for human applications. Higher doses of ketone supplements may have successfully modulated peripheral metabolism considering

the route of administration adopted in this study. However, incorporating large amounts of ketone supplements into one's diet may not be a feasible alternative, resulting in adverse gastrointestinal effects in addition to being a less palatable and more costly approach.

One advantage of choosing *ad libitum* feeding is that animals in all groups were able to decide how much food they wanted to eat and were not given an additional stressor (limited food availability), which would have been a confound to interpreting our results with chronic stress. We do, however, acknowledge that pair-feeding comparisons between KD and KS groups might address some of the differences reported in our study. Of note, considerable evidence from the literature has reported that KD-fed animals gain less weight despite showing no differences in food intake (Brownlow et al., 2013; Poff et al., 2013; Srivastava et al., 2013). We suggest that the changes described in our study are due to the dietary composition of each diet though the possibility remains that differences in energy intake contributed to our observed results. Although, food intake was not measured, body weight changes were similar between NIH-31 and KD-fed groups suggesting similar total caloric intake between these groups. Moreover, we observed a lack of diet-induced differences in food consumption following 4 h fasting. Exposure to stress, however, increased food intake following a brief period of food withdrawal (**Figure 5C**).

Overall, repeated exposure to stressors resulted in classical biochemical, behavioral and anatomical changes. Accordingly, hallmark features of the general adaptation syndrome (Selye, 1976) were seen with: increased energy expenditure suggested by reduced body weight gain despite increased food intake and thymus involution (**Table 3**). Neither diet was effective in preventing these outcomes, suggesting a possible disconnect between physiological mechanisms underlying the beneficial behavioral outcomes observed. Indeed, ketone supplementation resulted in behavioral improvements (shorter escape latencies on day 3 of MWM testing) and anatomical alterations (changes in peripheral energy deposits such as BAT, perirenal, and epididymal fat pads) being observed even in the absence of peripheral ketosis.

Ketone supplementation resulted in increased brown adipose tissue, although this difference only reached statistical significance in comparison to KD groups. This finding is in agreement with other groups claiming that exogenously delivered ketones resulted in increased resting energy expenditure and sympathetic activity (Srivastava et al., 2012; Veech, 2013), an effect not observed in KD groups. In rodents, increased adiposity has been reported in KD-fed rats (Kinzig and Taylor, 2009), an effect abolished in the presence of voluntary exercise (Kinzig et al., 2010). On the contrary, humans kept on KD display greater weight loss and reduced fat mass. This discrepancy highlights inherent challenges of utilizing rodent models. Lack of fidelity when translating rodent outcomes into human applications has been previously described (Martin et al., 2010; Hodge et al., 2016); thus, caution should be exercised and clinical studies remain necessary.

Next, we sought to investigate the interplay between stress and metabolic control of cerebral energy regulation by assessing hippocampal levels of mitochondrial enzymes involved in ketogenesis pathways and glucose transporters. Both KD and KS groups significantly upregulated key mitochondrial enzymes associated with the catalytic conversion and utilization of ketone bodies (BDH1 and ACAT1). This novel finding indicates that chronic administration of exogenous ketones may be capable of modulating brain energy pathways regardless of changes

TABLE 3 | Brain and peripheral tissue weights at euthanasia.

	NIH-31		KD		KS	
Tissue	**Control (10)**	**Stress (9)**	**Control (10)**	**Stress (10)**	**Control (10)**	**Stress (9)**
Brain	2.24 ± 0.03	2.23 ± 0.01	2.20 ± 0.02	2.22 ± 0.02	2.20 ± 0.02	2.19 ± 0.01
Adrenal	0.06 ± 0.002	0.06 ± 0.003	0.06 ± 0.04	0.07 ± 0.006	0.06 ± 0.002	0.06 ± 0.005
Thymus	0.37 ± 0.03	0.32 ± 0.05*	0.36 ± 0.03	0.32 ± 0.02*	0.43 ± 0.03	0.35 ± 0.03*
Spleen	0.89 ± 0.04	0.81 ± 0.04	0.86 ± 0.04	0.83 ± 0.03	0.87 ± 0.05	0.85 ± 0.03
BAT	0.53 ± 0.03	0.61 ± 0.09	0.48 ± 0.04$^{*,\#\#\#}$	0.47 ± 0.02$^{*,\#\#\#}$	0.69 ± 0.04*	0.64 ± 0.04*
Perirenal	10.04 ± 0.89	10.03 ± 0.82	12.11 ± 0.97	12.97 ± 1.54	16.15 ± 1.36***	13.39 ± 1.88***
Epididymal	8.88 ± 0.63$^{\#}$	8.34 ± 0.61$^{\#}$	8.40 ± 0.74$^{\#}$	8.73 ± 0.51$^{\#}$	11.35 ± 0.74	9.32 ± 1.01
BAT (%BW)	0.11 ± 0.005	0.13 ± 0.02	0.10 ± 0.01$^{\#\#}$	0.10 ± 0.005$^{\#\#}$	0.13 ± 0.006	0.13 ± 0.09
Perirenal (%BW)	2.03 ± 0.17	2.08 ± 0.18	2.49 ± 0.17§	2.77 ± 0.33*	3.12 ± 0.21***	2.63 ± 0.28***
Epididymal (%BW)	1.80 ± 0.12$^{\Delta}$	1.73 ± 0.12$^{\Delta}$	1.72 ± 0.12$^{\Delta}$	1.85 ± 0.11$^{\Delta}$	2.20 ± 0.10	1.86 ± 0.16

Thymus: *Stress effect ($p < 0.05$).
Brown Adipose Tissue (BAT): Diet effect ($p < 0.001$), *different than NIH-31 ($p < 0.05$);
$^{\#\#\#}$different than KS ($p < 0.001$).
Perirenal fat pad: Diet effect ($p < 0.01$), ***different than NIH-31 ($p < 0.001$).
Epididymal fat pad: Diet effect ($p < 0.05$), $^{\#}$different than KS ($p < 0.05$).
BAT, (%BW): Diet effect ($p < 0.05$), $^{\#\#}$ different than KS ($p < 0.01$).
Perirenal (%BW): Diet effect ($p < 0.01$), different than NIH-31, *($p < 0.05$), ***($p < 0.001$).; §different than both NIH-31 and KS control groups ($p < 0.05$).
Epididymal (%BW): Trend for diet effect ($p = 0.07$), $^{\Delta}$different than KS ($p < 0.05$).

TABLE 4 | Summary of main findings.

				DIETS					
		Parameters		NIH-31		KD		KS	
Baseline (wk 0–3)	Metabolism	BW (wks 0–3)		–		↓↓↓*		–	
		Glucose (wk 1)		–		↓↓*		–	
		BHB (wk 1)		–		↑↑↑*		–	
		Insulin (wk 1)		–		↓↓↓*		–	
		Food intake		–		–		–	
		ACTH		–		–		–	
		CORT		–		–		–	
	Behavior	% Exploration novel object (24 h) (wk 3)		–		↑*		–	
				Control	**Stress**	**Control**	**Stress**	**Control**	**Stress**
Stress (wk 4–8)	Behavior	Passive Avoidance (wk 4)	24 h	–	↓^	–	↓^	–	↓^
			2 wks	–	–	–	–	–	–
		MWM (wks 5,6)	Path length	–	↑^	–	↓*	–	↑^
			Latency	↓	↑^	↓	↓↓*	↓	↑^
			Probe	↑	↓^	↑	↑*	↑	↓^
	Biochemistry	BHB		–	↓↓↓^	–	↓*	–	↓↓↓^
		BDNF		–	↓^	–	–	–	–
		BDH1		–	–	↑↑↑*	↑↑↑*^	↑↑↑*	↑↑↑*
		ACAT1		–	–	↑↑↑*	↑↑↑*^	↑*	↑*
		GLUT1		–	↓↓↓^	↓↓↓*	↓↓↓*	↓↓↓*	↓↓↓*
		GLUT3		–	↑↑↑^	↓↓*	↓↓*	↓*	↓*
	Organ weight at Euthanasia	Body weight		–	↓^	↓*	↓↓*^	–	↓^
		Brain weight		–	–	–	–	–	–
		Adrenals		–	–	–	–	–	–
		Thymus		–	↓^	–	↓^	–	↓^
		Spleen		–	–	–	–	–	–
		BAT		–	–	–	–	↑*	↑*
		Epididymal fat pad		–	–	–	–	↑*	↑*
		Perirenal fat pad		–	–	↑↑*	↑↑*	↑↑↑*	↑↑↑*

↑ or ↓ ($p < 0.05$); ↑↑ or ↓↓ ($p < 0.01$) and ↑↑↑ or ↓↓↓ ($p < 0.001$)
* *Main effect of Diet and* ^ *Main effect of Stress.*

in peripheral metabolism. Furthermore, reduced GLUT1 levels following chronic stress observed in control diet group was not present in either KD or KS groups. Taking into consideration that the capacity for glucose transport depends on the concentration of transporter proteins (Simpson et al., 2007), this decrease suggests an impairment in brain glucose availability.

Our findings of stress-induced increase in GLUT3 levels in control diet animals is in agreement with previous findings in rodents (Reagan et al., 1999), suggesting that stress may contribute to increased neuronal energy demands under conditions of high allostatic load. The increase in neuronal glucose transporter, coupled with a decrease in the levels of the vascular glucose transporter highlights a mismatch in energy availability/demand, likely underlying detriments in brain homeostasis following exposure to stressors. This effect was not observed in either dietary condition, suggesting that the presence of an alternative energy substrate may be efficiently buffering the stress-induced imbalance in hippocampal energetic demands.

The observed changes in mitochondrial enzymes and glucose transporters resulting from both endogenous and exogenous ketones support the concept of ketone bodies being readily "pushed" into the brain in direct proportion to circulating levels vs. glucose being actively "pulled" depending on its utilization by neurons and astrocytes, described in Cunnane et al. (2016).

We chose to investigate whether a nutritional intervention increasingly used for its therapeutic properties augmented performance and mitigated challenge-induced deficits in healthy young rodents. Mixed results can often be attributed to methodological approach, such as: rodent model (different strains of rats or mice) (Ari et al., 2016), age (McNay and Gold, 2001), diet formulation and study length. For instance, metabolic effects of a western diet were more pronounced in Wistar rats when compared to Sprague-Dawley rats (Marques et al., 2016). Accordingly, *in vitro* hippocampal slices from Sprague-Dawley rats treated with a mixture of ketone bodies failed to show antiseizure effects or changes in synaptic transmission when

using extracellular glucose concentrations commonly used by acute physiological recordings (Thio et al., 2000; Youssef, 2015), whereas other studies have found significant differences using different rodent models or by changing extracellular glucose concentrations during incubation and recording of slices (for a review, see Kawamura et al., 2016).

Furthermore, different ketone supplements, supplement mixture, dosage or administration route could have also impacted our findings and further studies need to be carried out for optimization purposes. Despite these potential differences in design, our study was able to detect changes in metabolic, behavioral and biochemical parameters associated with cognitive performance in both control and stress conditions in the ketone supplemented group.

Our results highlight the dissociation between metabolic, behavioral and biochemical outcomes reported, with the novel finding that exogenous ketones mimic KD changes in both mitochondrial enzymes and glucose transporters. The exogenous ketone mixture tested, when added to a normal carbohydrate content diet (at the dose adopted), were below detection limits in the periphery. However, changes to biochemical machinery associated with ketogenesis pathways and glucose uptake were modulated similarly by both dietary interventions. We hypothesize that, after digestion and absorption into circulation, the ketone bodies produced were available for brain uptake, possibly displacing glucose as the brain's preferred fuel (Veech et al., 2001; Lamanna et al., 2009) and leading to the biochemical effects described. This intriguing finding warrants further investigation as it is likely a promising mechanism by which brain adaptive responses can be modulated, leading to enhanced performance.

In conclusion, we describe here that endogenous ketosis affected metabolic and behavioral outcomes in both stressed and control conditions, whereas these results were only observed in part with the exogenous ketone supplementation protocol tested. However, we report that, in the hippocampus, both endogenous and exogenous ketones were effective in modulating biochemical parameters associated with metabolic and cognitive responses. Our study advances the current views on the subject of performance optimization through a nutritional approach using ketone bodies to modulate metabolic and cognitive outcomes. Taken together, our findings suggest that ketogenic diets and, to a lesser extent, ketone supplements can modulate brain adaptive responses mediating cognitive performance in healthy young subjects during both control or stressed conditions.

AUTHOR CONTRIBUTIONS

Animal handling, behavioral assessments, and tissue collection were conducted at the WPAFB animal facility. Biochemical assays were performed in WPAFB research laboratories (AFRL). This study was conceptually conceived by MB and RJ. MB, RM, NB, and SJ contributed to the design of animal experimentation, such as scheduling of exposure to stressors and behavioral tasks. MB, RM, NB, and SJ participated in the acquisition, analysis, and interpretation of the data. All authors contributed to the drafting and critically revising the intellectual content included in this manuscript. Additionally, all authors have read and approved the final version of the manuscript.

FUNDING

This work was supported by the Air Force Office of Scientific Research [14RH08COR]. MB is recipient of a postdoctoral fellowship from the National Research Council [FA9550-12-D-0001]. Neither sponsor had any participation in the study design collection, analysis or interpretation of data nor in the writing of this manuscript. Distribution statement: Approved for public release: distribution is unlimited. 88ABW Cleared 09/08/2016; 88ABW-2016-4390.

ACKNOWLEDGMENTS

We would like to express our appreciation to Victoria Dershem, Benjamin Holmes, Kevin Schmidt, and Justin Stafford for technical assistance with animal husbandry, behavioral testing, and tissue collection. We would also like to thank Dr. Marcia Gordon for valuable feedback and comments on the manuscript.

REFERENCES

Ari, C., Kovacs, Z., Juhasz, G., Murdun, C., Goldhagen, C. R., Koutnik, A. M., et al. (2016). Exogenous ketone supplements reduce anxiety-related behavior in sprague-dawley and wistar albino glaxo/rijswijk rats. *Front. Mol. Neurosci.* 9:137. doi: 10.3389/fnmol.2016.00137

Baran, S. E., Armstrong, C. E., Niren, D. C., Hanna, J. J., and Conrad, C. D. (2009). Chronic stress and sex differences on the recall of fear conditioning and extinction. *Neurobiol. Learn. Mem.* 91, 323–332. doi: 10.1016/j.nlm.2008.11.005

Block, J. P., He, Y., Zaslavsky, A. M., Ding, L., and Ayanian, J. Z. (2009). Psychosocial stress and change in weight among US adults. *Am. J. Epidemiol.* 170, 181–192. doi: 10.1093/aje/kwp104

Blomqvist, G., Alvarsson, M., Grill, V., Von Heijne, G., Ingvar, M., Thorell, J. O., et al. (2002). Effect of acute hyperketonemia on the cerebral uptake of ketone bodies in nondiabetic subjects and IDDM patients. *Am. J. Physiol. Endocrinol. Metab.* 283, E20–E28. doi: 10.1152/ajpendo.00294.2001

Bough, K. J., Schwartzkroin, P. A., and Rho, J. M. (2003). Calorie restriction and ketogenic diet diminish neuronal excitability in rat dentate gyrus *in vivo*. *Epilepsia* 44, 752–760. doi: 10.1046/j.1528-1157.2003.55502.x

Bough, K. J., Wetherington, J., Hassel, B., Pare, J. F., Gawryluk, J. W., Greene, J. G., et al. (2006). Mitochondrial biogenesis in the anticonvulsant mechanism of the ketogenic diet. *Ann. Neurol.* 60, 223–235. doi: 10.1002/ana.20899

Brownlow, M. L., Benner, L., D'agostino, D., Gordon, M. N., and Morgan, D. (2013). Ketogenic diet improves motor performance but not cognition in two mouse models of Alzheimer's pathology. *PLoS ONE* 8:e75713. doi: 10.1371/journal.pone.0075713

Cahill, G. F. Jr. (2006). Fuel metabolism in starvation. *Annu. Rev. Nutr.* 26, 1–22. doi: 10.1146/annurev.nutr.26.061505.111258

Choi, I. Y., Piccio, L., Childress, P., Bollman, B., Ghosh, A., Brandhorst, S., et al. (2016). A diet mimicking fasting promotes regeneration and reduces autoimmunity and multiple sclerosis symptoms. *Cell Rep.* 15, 2136–2146. doi: 10.1016/j.celrep.2016.05.009

Courchesne-Loyer, A., Fortier, M., Tremblay-Mercier, J., Chouinard-Watkins, R., Roy, M., Nugent, S., et al. (2013). Stimulation of mild, sustained ketonemia by medium-chain triacylglycerols in healthy humans: estimated

potential contribution to brain energy metabolism. *Nutrition* 29, 635–640. doi: 10.1016/j.nut.2012.09.009

Crane, P. K., Walker, R., and Larson, E. B. (2013). Glucose levels and risk of dementia. *N. Engl. J. Med.* 369, 1863–1864. doi: 10.1056/NEJMoa1215740

Cunnane, S. C., Courchesne-Loyer, A., Vandenberghe, C., St-Pierre, V., Fortier, M., Hennebelle, M., et al. (2016). Can ketones help rescue brain fuel supply in later life? Implications for cognitive health during aging and the treatment of Alzheimer's Disease. *Front. Mol. Neurosci.* 9:53. doi: 10.3389/fnmol.2016.00053

D'agostino, D. P., Pilla, R., Held, H. E., Landon, C. S., Puchowicz, M., Brunengraber, H., et al. (2013). Therapeutic ketosis with ketone ester delays central nervous system oxygen toxicity seizures in rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 304, R829–R836. doi: 10.1152/ajpregu.00506.2012

Dallman, M. F., Pecoraro, N. C., and La Fleur, S. E. (2005). Chronic stress and comfort foods: self-medication and abdominal obesity. *Brain Behav. Immun.* 19, 275–280. doi: 10.1016/j.bbi.2004.11.004

Fukushima, A., Ogura, Y., Furuta, M., Kakehashi, C., Funabashi, T., and Akema, T. (2015). Ketogenic diet does not impair spatial ability controlled by the hippocampus in male rats. *Brain Res.* 1622, 36–42. doi: 10.1016/j.brainres.2015.06.016

Herman, J. P., Flak, J., and Jankord, R. (2008). Chronic stress plasticity in the hypothalamic paraventricular nucleus. *Prog. Brain Res.* 170, 353–364. doi: 10.1016/S0079-6123(08)00429-9

Hodge, R. J., Paulik, M. A., Walker, A., Boucheron, J. A., McMullen, S. L., Gillmor, D. S., et al. (2016). Weight and glucose reduction observed with a combination of nutritional agents in rodent models does not translate to humans in a randomized clinical trial with healthy volunteers and subjects with Type 2 diabetes. *PLoS ONE* 11:e0153151. doi: 10.1371/journal.pone.0153151

Jankord, R., and Herman, J. P. (2008). Limbic regulation of hypothalamo-pituitary-adrenocortical function during acute and chronic stress. *Ann. N. Y. Acad. Sci.* 1148, 64–73. doi: 10.1196/annals.1410.012

Kashiwaya, Y., Bergman, C., Lee, J. H., Wan, R., King, M. T., Mughal, M. R., et al. (2013). A ketone ester diet exhibits anxiolytic and cognition-sparing properties, and lessens amyloid and tau pathologies in a mouse model of Alzheimer's disease. *Neurobiol. Aging* 34, 1530–1539. doi: 10.1016/j.neurobiolaging.2012.11.023

Kashiwaya, Y., Pawlosky, R., Markis, W., King, M. T., Bergman, C., Srivastava, S., et al. (2010). A ketone ester diet increases brain malonyl-CoA and uncoupling proteins 4 and 5 while decreasing food intake in the normal Wistar Rat. *J. Biol. Chem.* 285, 25950–25956. doi: 10.1074/jbc.M110.138198

Kashiwaya, Y., Takeshima, T., Mori, N., Nakashima, K., Clarke, K., and Veech, R. L. (2000). D-beta-hydroxybutyrate protects neurons in models of Alzheimer's and Parkinson's disease. *Proc. Natl. Acad. Sci. U.S.A.* 97, 5440–5444. doi: 10.1073/pnas.97.10.5440

Kawamura, M. J., Ruskin, D. N., and Masino, S. A. (2016). Metabolic therapy for temporal lobe epilepsy in a dish: investigating mechanisms of ketogenic diet using electrophysiological recordings in hippocampal slices. *Front. Mol. Neurosci.* 9:112. doi: 10.3389/fnmol.2016.00112

Kawamura, M. Jr., Ruskin, D. N., Geiger, J. D., Boison, D., and Masino, S. A. (2014). Ketogenic diet sensitizes glucose control of hippocampal excitability. *J. Lipid Res.* 55, 2254–2260. doi: 10.1194/jlr.M046755

Kelley, S. A., and Hartman, A. L. (2011). Metabolic treatments for intractable epilepsy. *Semin. Pediatr. Neurol.* 18, 179–185. doi: 10.1016/j.spen.2011.06.004

Keon, C. A., Tuschiya, N., Kashiwaya, Y., Sato, K., Clarke, K., Radda, G. K., et al. (1995). Substrate dependence of the mitochondrial energy status in the isolated working rat heart. *Biochem. Soc. Trans.* 23:307S. doi: 10.1042/bst023307s

Kesl, S. L., Poff, A. M., Ward, N. P., Fiorelli, T. N., Ari, C., Van Putten, A. J., et al. (2016). Effects of exogenous ketone supplementation on blood ketone, glucose, triglyceride, and lipoprotein levels in Sprague-Dawley rats. *Nutr. Metab.* 13:9. doi: 10.1186/s12986-016-0069-y

Kim, D. Y., Hao, J., Liu, R., Turner, G., Shi, F. D., and Rho, J. M. (2012). Inflammation-mediated memory dysfunction and effects of a ketogenic diet in a murine model of multiple sclerosis. *PLoS ONE* 7:e35476. doi: 10.1371/journal.pone.0035476

Kinzig, K. P., Honors, M. A., Hargrave, S. L., Davenport, B. M., Strader, A. D., and Wendt, D. (2010). Sensitivity to the anorectic effects of leptin is retained in rats maintained on a ketogenic diet despite increased adiposity. *Neuroendocrinology* 92, 100–111. doi: 10.1159/000314180

Kinzig, K. P., and Taylor, R. J. (2009). Maintenance on a ketogenic diet: voluntary exercise, adiposity and neuroendocrine effects. *Int. J. Obes.* 33, 824–830. doi: 10.1038/ijo.2009.109

Lamanna, J. C., Salem, N., Puchowicz, M., Erokwu, B., Koppaka, S., Flask, C., et al. (2009). Ketones suppress brain glucose consumption. *Adv. Exp. Med. Biol.* 645, 301–306. doi: 10.1007/978-0-387-85998-9_45

Marosi, K., Kim, S. W., Moehl, K., Scheibye-Knudsen, M., Cheng, A., Cutler, R., et al. (2016). 3-Hydroxybutyrate regulates energy metabolism and induces BDNF expression in cerebral cortical neurons. *J. Neurochem.* 139, 769–781. doi: 10.1111/jnc.13868

Marosi, K., and Mattson, M. P. (2014). BDNF mediates adaptive brain and body responses to energetic challenges. *Trends Endocrinol. Metab.* 25, 89–98. doi: 10.1016/j.tem.2013.10.006

Marques, C., Meireles, M., Norberto, S., Leite, J., Freitas, J., Pestana, D., et al. (2016). High-fat diet-induced obesity Rat model: a comparison between Wistar and Sprague-Dawley Rat. *Adipocyte* 5, 11–21. doi: 10.1080/21623945.2015.1061723

Martin, B., Ji, S., Maudsley, S., and Mattson, M. P. (2010). Control laboratory rodents are metabolically morbid: why it matters. *Proc. Natl. Acad. Sci. U.S.A.* 107, 6127–6133. doi: 10.1073/pnas.0912955107

Masino, S. A., Kawamura, M., Wasser, C. D., Pomeroy, L. T., and Ruskin, D. N. (2009). Adenosine, ketogenic diet and epilepsy: the emerging therapeutic relationship between metabolism and brain activity. *Curr. Neuropharmacol.* 7, 257–268. doi: 10.2174/157015909789152164

Masino, S. A., Li, T., Theofilas, P., Sandau, U. S., Ruskin, D. N., Fredholm, B. B., et al. (2011). A ketogenic diet suppresses seizures in mice through adenosine A(1) receptors. *J. Clin. Invest.* 121, 2679–2683. doi: 10.1172/JCI57813

Masino, S. A., and Rho, J. M. (2012). "Mechanisms of ketogenic diet action," in *Jasper's Basic Mechanisms of the Epilepsies 4th Edn.,* eds J. L. Noebels, M. Avoli, M. A. Rogawski, R. W. Olsen, and A. V. Delgado-Escueta (Bethesda, MD: National Center for Biotechnology Information), 1483–1515.

Mattson, M. P. (2008). Awareness of hormesis will enhance future research in basic and applied neuroscience. *Crit. Rev. Toxicol.* 38, 633–639. doi: 10.1080/10408440802026406

McNay, E. C., Fries, T. M., and Gold, P. E. (2000). Decreases in rat extracellular hippocampal glucose concentration associated with cognitive demand during a spatial task. *Proc. Natl. Acad. Sci. U.S.A.* 97, 2881–2885. doi: 10.1073/pnas.050583697

McNay, E. C., and Gold, P. E. (2001). Age-related differences in hippocampal extracellular fluid glucose concentration during behavioral testing and following systemic glucose administration. *J. Gerontol. A Biol. Sci. Med. Sci.* 56, B66–B71. doi: 10.1093/gerona/56.2.B66

McNay, E. C., McCarty, R. C., and Gold, P. E. (2001). Fluctuations in brain glucose concentration during behavioral testing: dissociations between brain areas and between brain and blood. *Neurobiol. Learn. Mem.* 75, 325–337. doi: 10.1006/nlme.2000.3976

Morris, R. (1984). Developments of a water-maze procedure for studying spatial learning in the rat. *J. Neurosci. Methods* 11, 47–60. doi: 10.1016/0165-0270(84)90007-4

Mosconi, L., Pupi, A., and De Leon, M. J. (2008). Brain glucose hypometabolism and oxidative stress in preclinical Alzheimer's disease. *Ann. N. Y. Acad. Sci.* 1147, 180–195. doi: 10.1196/annals.1427.007

Newman, J. C., and Verdin, E. (2014a). β-hydroxybutyrate: much more than a metabolite. *Diabetes Res. Clin. Pract.* 106, 173–181. doi: 10.1016/j.diabres.2014.08.009

Newman, J. C., and Verdin, E. (2014b). Ketone bodies as signaling metabolites. *Trends Endocrinol. Metab.* 25, 42–52. doi: 10.1016/j.tem.2013.09.002

Olver, J. S., Pinney, M., Maruff, P., and Norman, T. R. (2015). Impairments of spatial working memory and attention following acute psychosocial stress. *Stress Health* 31, 115–123. doi: 10.1002/smi.2533

Phinney, S. D., Bistrian, B. R., Evans, W. J., Gervino, E., and Blackburn, G. L. (1983). The human metabolic response to chronic ketosis without caloric restriction: preservation of submaximal exercise capability with reduced carbohydrate oxidation. *Metab. Clin. Exp.* 32, 769–776. doi: 10.1016/0026-0495(83)90106-3

Piermartiri, T., Pan, H., Figueiredo, T. H., and Marini, A. M. (2015). α-Linolenic acid, a nutraceutical with pleiotropic properties that targets

endogenous neuroprotective pathways to protect against organophosphate nerve agent-induced neuropathology. *Molecules* 20, 20355–20380. doi: 10.3390/molecules201119698

Poff, A. M., Ari, C., Seyfried, T. N., and D'agostino, D. P. (2013). The ketogenic diet and hyperbaric oxygen therapy prolong survival in mice with systemic metastatic cancer. *PLoS ONE* 8:e65522. doi: 10.1371/journal.pone.0065522

Prins, M. L. (2008). Cerebral metabolic adaptation and ketone metabolism after brain injury. *J. Cereb. Blood Flow Metab.* 28, 1–16. doi: 10.1038/sj.jcbfm.9600543

Puchowicz, M. A., Emancipator, D. S., Xu, K., Magness, D. L., Ndubuizu, O. I., Lust, W. D., et al. (2005). Adaptation to chronic hypoxia during diet-induced ketosis. *Adv. Exp. Med. Biol.* 566, 51–57. doi: 10.1007/0-387-26206-7_8

Puchowicz, M. A., Zechel, J. L., Valerio, J., Emancipator, D. S., Xu, K., Pundik, S., et al. (2008). Neuroprotection in diet-induced ketotic rat brain after focal ischemia. *J. Cereb. Blood Flow Metab.* 28, 1907–1916. doi: 10.1038/jcbfm.2008.79

Reagan, L. P., Magarinos, A. M., Lucas, L. R., Van Bueren, A., McCall, A. L., and McEwen, B. S. (1999). Regulation of GLUT-3 glucose transporter in the hippocampus of diabetic rats subjected to stress. *Am. J. Physiol.* 276, E879–E886.

Rothman, S. M., and Mattson, M. P. (2013). Activity-dependent, stress-responsive BDNF signaling and the quest for optimal brain health and resilience throughout the lifespan. *Neuroscience* 239, 228–240. doi: 10.1016/j.neuroscience.2012.10.014

Sandusky, L. A., Flint, R. W., and McNay, E. C. (2013). Elevated glucose metabolism in the amygdala during an inhibitory avoidance task. *Behav. Brain Res.* 245, 83–87. doi: 10.1016/j.bbr.2013.02.006

Sapolsky, R. M. (1985). A mechanism for glucocorticoid toxicity in the hippocampus: increased neuronal vulnerability to metabolic insults. *J. Neurosci.* 5, 1228–1232.

Selye, H. (1976). Forty years of stress research: principal remaining problems and misconceptions. *Can. Med. Assoc. J.* 115, 53–56.

Shea, C. J., Carhuatanta, K. A., Wagner, J., Bechmann, N., Moore, R., Herman, J. P., et al. (2015). Variable impact of chronic stress on spatial learning and memory in BXD mice. *Physiol. Behav.* 150, 69–77. doi: 10.1016/j.physbeh.2015.06.022

Shimazu, T., Hirschey, M. D., Newman, J., He, W., Shirakawa, K., Le Moan, N., et al. (2013). Suppression of oxidative stress by beta-hydroxybutyrate, an endogenous histone deacetylase inhibitor. *Science* 339, 211–214. doi: 10.1126/science.1227166

Simpson, I. A., Carruthers, A., and Vannucci, S. J. (2007). Supply and demand in cerebral energy metabolism: the role of nutrient transporters. *J. Cereb. Blood Flow Metab.* 27, 1766–1791. doi: 10.1038/sj.jcbfm.9600521

Sinha, R., and Jastreboff, A. M. (2013). Stress as a common risk factor for obesity and addiction. *Biol. Psychiatry* 73, 827–835. doi: 10.1016/j.biopsych.2013.01.032

Srivastava, S., Baxa, U., Niu, G., Chen, X., and Veech, R. L. (2013). A ketogenic diet increases brown adipose tissue mitochondrial proteins and UCP1 levels in mice. *IUBMB Life* 65, 58–66. doi: 10.1002/iub.1102

Srivastava, S., Kashiwaya, Y., King, M. T., Baxa, U., Tam, J., Niu, G., et al. (2012). Mitochondrial biogenesis and increased uncoupling protein 1 in brown adipose tissue of mice fed a ketone ester diet. *FASEB J.* 26, 2351–2362. doi: 10.1096/fj.11-200410

Stranahan, A. M., Lee, K., Martin, B., Maudsley, S., Golden, E., Cutler, R. G., et al. (2009). Voluntary exercise and caloric restriction enhance hippocampal dendritic spine density and BDNF levels in diabetic mice. *Hippocampus* 19, 951–961. doi: 10.1002/hipo.20577

Thio, L. L., Wong, M., and Yamada, K. A. (2000). Ketone bodies do not directly alter excitatory or inhibitory hippocampal synaptic transmission. *Neurology* 54, 325–331. doi: 10.1212/WNL.54.2.325

Veech, R. L. (2004). The therapeutic implications of ketone bodies: the effects of ketone bodies in pathological conditions: ketosis, ketogenic diet, redox states, insulin resistance, and mitochondrial metabolism. *Prostaglandins Leukot. Essent. Fatty Acids* 70, 309–319. doi: 10.1016/j.plefa.2003.09.007

Veech, R. L. (2013). Ketone esters increase brown fat in mice and overcome insulin resistance in other tissues in the rat. *Ann. N. Y. Acad. Sci.* 1302, 42–48. doi: 10.1111/nyas.12222

Veech, R. L., Chance, B., Kashiwaya, Y., Lardy, H. A., and Cahill, G. F. Jr. (2001). Ketone bodies, potential therapeutic uses. *IUBMB Life* 51, 241–247. doi: 10.1080/152165401753311780

Vernon, M. C., Kueser, B., Transue, M., Yates, H. E., Yancy, W. S., and Westman, E. C. (2004). Clinical experience of a carbohydrate-restricted diet for the metabolic syndrome. *Metab. Syndr. Relat. Disord.* 2, 180–186. doi: 10.1089/met.2004.2.180

Virues-Ortega, J., Garrido, E., Javierre, C., and Kloezeman, K. C. (2006). Human behaviour and development under high-altitude conditions. *Dev. Sci.* 9, 400–410. doi: 10.1111/j.1467-7687.2006.00505.x

Vizuete, A. F., De Souza, D. F., Guerra, M. C., Batassini, C., Dutra, M. F., Bernardi, C., et al. (2013). Brain changes in BDNF and S100B induced by ketogenic diets in Wistar rats. *Life Sci.* 92, 923–928. doi: 10.1016/j.lfs.2013.03.004

Volek, J. S., Freidenreich, D. J., Saenz, C., Kunces, L. J., Creighton, B. C., Bartley, J. M., et al. (2016). Metabolic characteristics of keto-adapted ultra-endurance runners. *Metab. Clin. Exp.* 65, 100–110. doi: 10.1016/j.metabol.2015.10.028

Watanabe, Y., Gould, E., and McEwen, B. S. (1992). Stress induces atrophy of apical dendrites of hippocampal CA3 pyramidal neurons. *Brain Res.* 588, 341–345. doi: 10.1016/0006-8993(92)91597-8

Westman, E. C., Feinman, R. D., Mavropoulos, J. C., Vernon, M. C., Volek, J. S., Wortman, J. A., et al. (2007). Low-carbohydrate nutrition and metabolism. *Am. J. Clin. Nutr.* 86, 276–284.

Xu, K., Sun, X., Eroku, B. O., Tsipis, C. P., Puchowicz, M. A., and Lamanna, J. C. (2010). Diet-induced ketosis improves cognitive performance in aged rats. *Adv. Exp. Med. Biol.* 662, 71–75. doi: 10.1007/978-1-4419-1241-1_9

Yao, J., Chen, S., Mao, Z., Cadenas, E., and Brinton, R. D. (2011). 2-Deoxy-D-glucose treatment induces ketogenesis, sustains mitochondrial function, and reduces pathology in female mouse model of Alzheimer's disease. *PLoS ONE* 6:e21788. doi: 10.1371/journal.pone.0021788

Youssef, F. F. (2015). Ketone bodies attenuate excitotoxic cell injury in the rat hippocampal slice under conditions of reduced glucose availability. *Neurol. Res.* 37, 211–216. doi: 10.1179/1743132814Y.0000000430

Yuen, E. Y., Wei, J., Liu, W., Zhong, P., Li, X., and Yan, Z. (2012). Repeated stress causes cognitive impairment by suppressing glutamate receptor expression and function in prefrontal cortex. *Neuron* 73, 962–977. doi: 10.1016/j.neuron.2011.12.033

Zhao, Q., Stafstrom, C. E., Fu, D. D., Hu, Y., and Holmes, G. L. (2004). Detrimental effects of the ketogenic diet on cognitive function in rats. *Pediatr. Res.* 55, 498–506. doi: 10.1203/01.PDR.0000112032.47575.D1

Zhao, W., Varghese, M., Vempati, P., Dzhun, A., Cheng, A., Wang, J., et al. (2012). Caprylic triglyceride as a novel therapeutic approach to effectively improve the performance and attenuate the symptoms due to the motor neuron loss in ALS disease. *PLoS ONE* 7:e49191. doi: 10.1371/journal.pone.0049191

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Personnel (RM, NB) from a government contracting company-Infoscitex a wholly owned subsidiary of DCS Corp. - provided support for this work, by participating in study design (establishing novel procedures, scheduling stressors, and behavioral testing), data collection, analysis, and critically revising the final submitted manuscript. Support from Infoscitex, Inc. pertains to contracting personnel to support research activities at the Air Force Research Laboratory and does not involve financial incentives, products, supplies, or biological applications that would influence our research questions or outcomes.

frontiers in Molecular Neuroscience

REVIEW
published: 21 February 2017
doi: 10.3389/fnmol.2017.00034

Metabolic Dysfunction Underlying Autism Spectrum Disorder and Potential Treatment Approaches

Ning Cheng[1]*, *Jong M. Rho*[1,2,3] *and Susan A. Masino*[4]

[1] Departments of Pediatrics, University of Calgary, Calgary, AB, Canada, [2] Clinical Neurosciences, University of Calgary, Calgary, AB, Canada, [3] Physiology and Pharmacology, Alberta Children's Hospital Research Institute, Cumming School of Medicine, University of Calgary, Calgary, AB, Canada, [4] Neuroscience Program, Department of Psychology, Trinity College, Hartford, CT, USA

Autism spectrum disorder (ASD) is characterized by deficits in sociability and communication, and increased repetitive and/or restrictive behaviors. While the etio-pathogenesis of ASD is unknown, clinical manifestations are diverse and many possible genetic and environmental factors have been implicated. As such, it has been a great challenge to identify key neurobiological mechanisms and to develop effective treatments. Current therapies focus on co-morbid conditions (such as epileptic seizures and sleep disturbances) and there is no cure for the core symptoms. Recent studies have increasingly implicated mitochondrial dysfunction in ASD. The fact that mitochondria are an integral part of diverse cellular functions and are susceptible to many insults could explain how a wide range of factors can contribute to a consistent behavioral phenotype in ASD. Meanwhile, the high-fat, low-carbohydrate ketogenic diet (KD), used for nearly a century to treat medically intractable epilepsy, has been shown to enhance mitochondrial function through a multiplicity of mechanisms and affect additional molecular targets that may address symptoms and comorbidities of ASD. Here, we review the evidence for the use of metabolism-based therapies such as the KD in the treatment of ASD as well as emerging co-morbid models of epilepsy and autism. Future research directions aimed at validating such therapeutic approaches and identifying additional and novel mechanistic targets are also discussed.

Keywords: autism spectrum disorder, ketogenic diet, metabolism, mitochondria, therapeutics, epilepsy, co-morbidity, mechanism

OPEN ACCESS

Edited by:
Kimberly Raab-Graham, Wake Forest School of Medicine, USA

Reviewed by:
Michael J. Schmeisser, Otto-von-Guericke University Magdeburg, Germany
Kasia Radwanska, Nencki Institute of Experimental Biology, Poland

***Correspondence:**
Ning Cheng
ncheng@ucalgary.ca

Received: *20 September 2016*
Accepted: *30 January 2017*
Published: *21 February 2017*

Citation:
Cheng N, Rho JM and Masino SA (2017) Metabolic Dysfunction Underlying Autism Spectrum Disorder and Potential Treatment Approaches. Front. Mol. Neurosci. 10:34. doi: 10.3389/fnmol.2017.00034

AUTISM SPECTRUM DISORDER—COMPLEX ETIOLOGY, LIMITED THERAPIES

Autism spectrum disorder (ASD) is characterized by persistent deficits in sociability and communication, as well as restricted and repetitive patterns of behavior and interests (DiCicco-Bloom et al., 2006; Llaneza et al., 2010; Lai et al., 2014). The term "spectrum" refers to the wide range of symptoms and levels of impairment that can occur in individuals with ASD. Beyond these core behavioral symptoms, ASD is increasingly shown to affect the gastrointestinal, immune, hepatic, and endocrine systems (Goines and Van de Water, 2010; Patterson, 2011; Hsiao, 2013; Frye et al., 2015; Mayer et al., 2015). Common co-morbidities include neurologic, psychiatric and physical conditions: neurologic comorbidities include epilepsy, sleep impairment, sensory abnormalities,

and delays and/or deficits in motor function; psychiatric conditions such as depression, anxiety, irritability and attention deficit hyperactivity disorder; and physical health issues such as chronic gastrointestinal disturbance. The co-occurrence rate of one or more non-ASD developmental diagnoses is as high as 83% (Levy et al., 2010).

ASD occurs in all racial, ethnic, and socioeconomic groups and is highly prevalent. It affects tens of millions individuals worldwide and costs millions of US dollars on average to support an affected individual during his/her lifespan (Buescher et al., 2014). In the U.S., the incidence of ASD is 1 in 68 children (1 in 42 boys and 1 in 189 girls) based on data released by the Centers for Disease Control and Prevention (CDC) in 2014. The prevalence appears to be on the rise (a 10-fold increase in 40 years), and is explained only in part by improved diagnosis and awareness (Hansen et al., 2015). Developmental delay in ASD can be detected as early as 6 months of age, a critical time for the development of higher-order social, emotional, and communications functions (Courchesne et al., 2007); the importance of early intervention is recognized (Orinstein et al., 2014). However, on average, children are not diagnosed until after 4 years of age (CDC, 2014), even though patients can now be reliably diagnosed at 2 years of age (Lord et al., 2006; Kleinman et al., 2008).

Given that ASD has broad and heterogeneous manifestations, and has been associated with a plethora of possible etiological factors (both genetic and environmental), ASD remains a clinical and broad-spectrum diagnosis. In most cases, ASD is diagnosed without any defined etiology. A dearth of knowledge about underlying causes has limited the ability to develop and mobilize effective treatments, and currently only co-morbid manifestations of the disorder can be alleviated. The hope is that reducing co-morbidities such as epileptic seizures, psychiatric disturbances, hyperactivity, sleep problems and digestive issues (DiCicco-Bloom et al., 2006; Llaneza et al., 2010; Lai et al., 2014) will improve overall function and reduce the severity of ASD symptoms (Kohane et al., 2012; Frye and Rossignol, 2016).

Genetic susceptibility factors and environmental influences (and likely often both) contribute to ASD (Chaste and Leboyer, 2012; Sandin et al., 2014; Tordjman et al., 2014; Kim and Leventhal, 2015). Genome screening and sequencing has identified rare chromosomal abnormalities and copy number variations, as well as hundreds of rare gene mutations associated with autism (Devlin and Scherer, 2012; Huguet et al., 2013; Jeste and Geschwind, 2014; Baker and Jeste, 2015). A small number of these genetic changes appear highly penetrant and sufficient to cause autism. However, most genetic factors only increase the risk to varying degrees, and likely combine with additional influences such as advanced parental age at time of conception, adverse metabolic conditions and/or maternal illness during pregnancy, birth complications, and exposure to toxins and/or drugs during early brain development (Stromland et al., 1994; Durkin et al., 2008; Gardener et al., 2011; Krakowiak et al., 2012; Christensen et al., 2013). Not surprisingly, the molecular pathways implicated in ASD are also highly complex and diverse, and include synaptic dysfunction and plasticity of various neurotransmitter systems, transcriptional regulation and chromatin remodeling, protein translation and modification, neuroimmunological modulation, and mitochondrial function (Veenstra-Vanderweele et al., 2004; Bourgeron, 2015; De Rubeis and Buxbaum, 2015; Kopp et al., 2015; Loke et al., 2015; Mahfouz et al., 2015; Nelson and Valakh, 2015; Subramanian et al., 2015; de la Torre-Ubieta et al., 2016; Wen et al., 2016).

METABOLISM, MITOCHONDRIA, AND ASD

Given such extreme etiological diversity, it is reasonable to hypothesize that perturbation of a common nexus can precipitate the behavioral hallmarks of ASD (Geschwind, 2008; Berg and Geschwind, 2012). Identifying such a common factor would provide novel insights into the development of ASD. Further, targeting this pathway could lead to selective therapeutic approaches that might enhance efficacy and address core symptoms. One possibility is mitochondrial function, which is integral to many cellular pathways. In addition to its well-known role as the "powerhouse of the cell," producing the bulk of the cellular energy, mitochondria are also critically involved in cellular metabolism, intracellular calcium signaling, generation of reactive oxygen species (ROS), and apoptosis (Suen et al., 2008; Murphy, 2009; Palmieri et al., 2010; Antico Arciuch et al., 2012; Rizzuto et al., 2012), as well as in the regulation of innate and adaptive immunity (Weinberg et al., 2015). For example, mitochondria carry out both cleavage and synthesis of glycine (Kikuchi et al., 2008), which is the ligand of glycine receptors. These receptors are chloride channels that mediate inhibitory neurotransmission in the adult nervous system. However, they are highly expressed in the embryonic brain and mediate excitatory neurotransmission, and are believed to promote cortical interneuron migration and generation of excitatory projection neurons (Pilorge et al., 2016). Interestingly, recent genetic and functional studies have identified a role of abnormal glycinergic signaling in ASD (Pilorge et al., 2016). Furthermore, mitochondria are known to be affected by many of the same endogenous and exogenous risk factors of ASD, such as toxins, drugs, immune activation, and metabolic disturbances (Frye and Rossignol, 2011). Thus, elucidating the role of mitochondrial dysfunction in ASD may help unify our understanding of this complex disorder.

Mitochondria play a particularly vital role in the central nervous system. The brain has very high energy demands, consuming approximately 20% of calories while accounting for only 2% of total body weight (Raichle and Gusnard, 2002), and demanding a great amount of adenosine triphosphate (ATP) to maintain ionic gradients essential for neurotransmission and plasticity (Harris et al., 2012). In addition, mitochondria are involved in the proliferation, differentiation and maturation of neural stem cells, formation of dendritic processes, developmental and synaptic plasticity, and cell survival and death (Li et al., 2004; Kann and Kovacs, 2007; Mattson et al., 2008; Kimura and Murakami, 2014; Xavier et al., 2016). Thus, it is not surprising that multiple lines of evidence in both human and animal models support a role for mitochondrial dysfunction

in the etiology of ASD (Haas, 2010; Dhillon et al., 2011; Frye and Rossignol, 2011; Rossignol and Frye, 2012; Legido et al., 2013).

The prevalence of mitochondrial disease in the ASD population is estimated to be about 5.0%, 500 times higher than that found in the general population (≈0.01%). The prevalence of abnormal metabolic biomarkers is even higher, suggesting that as many as 30% of children with ASD may experience metabolic abnormalities: almost one-third of autistic children have documented elevations in plasma lactate and/or the lactate-to-pyruvate ratio, and the levels of many other mitochondrial biomarkers (pyruvate, carnitine, and ubiquinone) are significantly different between ASD and controls (Rossignol and Frye, 2012). In addition, several genes known to regulate mitochondrial function are clearly autism-risk genes. These include *SLC25A12* (Ramoz et al., 2004; Segurado et al., 2005; Silverman et al., 2008; Turunen et al., 2008; Kim et al., 2011), which encodes the predominant form of mitochondrial aspartate/glutamate carrier (however, also see Correia et al., 2006; Palmieri et al., 2010). These carriers participate in a wide range of mitochondrial functions, including control of respiration, calcium signaling and antioxidant defense, as well as glutamate-mediated excitotoxicity (Amoedo et al., 2016). Furthermore, *TMLHE*, (trimethyllysine hydroxylase epsilon), which encodes the first enzyme in carnitine biosynthesis, has also been associated with ASD (Celestino-Soper et al., 2012; Nava et al., 2012). It has been well established that carnitines are involved in mitochondrial transport of long-chain fatty acids and play an important role in maintaining normal mitochondrial function (Bremer, 1983). In addition, the gene encoding an inner mitochondrial membrane protease-like protein (*IMMP2L*) may help regulate susceptibility to ASD (Maestrini et al., 2010). It is important to note that metabolic and mitochondrial dysfunction may not exist in all patients with ASD, and biomarkers to identify this impairment would be advantageous in developing personalized treatment.

In parallel with clinical findings, many animal models of ASD also display mitochondrial dysfunction, including those based on susceptibility genes such as *MECP2, UBE3A, TSC,* and *FOXG1*. Mitochondrial dysfunction has also been observed in animal models of ASD induced by environmental risk factors such as maternal immune activation and exposure to propionic acid or valproic acid (VPA). Current evidence linking mitochondrial perturbations to ASD and the corresponding references are summarized in **Tables 1**, **2**.

Common co-morbidities of ASD also suggest metabolic and mitochondrial dysfunction. One of the most significant co-morbidities is epilepsy, with a prevalence of 5–38% in children with ASD—much higher than the 1–2% prevalence in the general population (Frye, 2015). Seizures also occur in 35–60% of individuals with biochemically-confirmed mitochondrial disease (Rahman, 2012), suggesting there may be a common etio-pathology. Similarly, gastrointestinal dysfunction, a frequent comorbidity of ASD (Chaidez et al., 2014), is also common in mitochondrial disease (Frye et al., 2015).

Taken together, we believe that mitochondria act as a central nexus responding to and regulating many domains of cellular biology that have been implicated in ASD. Given the prevalence of metabolic/mitochondrial dysfunction in ASD, options for metabolic therapy should be explored. Below we review some of the emerging clinical and research evidence that metabolic therapy and improved mitochondrial function can ameliorate ASD symptoms and comorbidities.

METABOLIC THERAPY FOR ASD

A metabolic therapy in use for decades is the ketogenic diet (KD), a high-fat, low-carbohydrate diet—a remarkably effective non-pharmacological treatment for medically intractable epilepsy (Neal et al., 2008). Based on historical observations that either fasting or starvation rendered anti-seizure effects, the KD was designed to reproduce the biochemical changes seen in these physiological states (Masino and Rho, 2012). Recently, various dietary and metabolic therapies have been attempted in a wider variety of neurological diseases including ASD, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, sleep disorders, multiple sclerosis, brain trauma, stroke, pain, Huntington's disease and brain cancer (Ruskin et al., 2009; Stafstrom and Rho, 2012; Napoli et al., 2014). Although generally limited in scope, clinical studies thus far showed promising results in conditions such as Alzheimer's disease and ASD and are discussed in more detail below. In addition, research using animal models has pointed to a common mechanism of regulating energy metabolism to afford neuroprotective effects (Stafstrom and Rho, 2012).

Overall, recent clinical and laboratory evidence suggests that the KD may have positive effects in ASD. The complex pathophysiology of ASD and the diversity of mechanisms mobilized by dietary therapy combine to make identifying the key molecular mechanisms challenging, but a number of candidates are emerging. Two hallmark biochemical features after the KD treatment are increased ketone body production by the liver through fatty acid oxidation and reduced blood glucose levels (Stafstrom and Rho, 2012). More specific metabolic effects, such as increases in specific polyunsaturated fatty acids, might regulate neuronal membrane excitability (Voskuyl and Vreugdenhil, 2001), reduce inflammation (Cullingford, 2008; Jeong et al., 2011), or decrease the production of ROS by mitochondria (Kim do and Rho, 2008). Additionally, ketone bodies themselves have been shown to possess neuroprotective properties through improved bioenergetics - raising ATP levels and reducing ROS production through enhancement of NADH oxidation and inhibition of mitochondrial permeability transition (Kim do et al., 2007, 2015); related to this, a KD has also been shown to stimulate mitochondrial biogenesis (Bough et al., 2006; Ahola-Erkkila et al., 2010). In parallel, reduced glycolysis can suppress seizures, improve mitochondrial function, decrease oxidative stress, reduce activity of pro-apoptotic factors, and inhibit inflammatory mediators such as interleukins and tumor necrosis factor alpha (Garriga-Canut et al., 2006; Maalouf et al., 2009). The KD has also been proposed to increase adenosine (a product of extracellular ATP dephosphorylation); ATP and adenosine are purines with pleiotropic neuromodulatory and neuroprotective roles proposed to underlie in part the diet's

TABLE 1 | Studies showing linkage between ASD and mitochondrial dysfunction in ASD patients (only those reporting more than 25 subjects are included in this table).

References	Cases	Evidence of mitochondrial dysfunction
GENETIC LINKAGE BETWEEN MITOCHONDRIA-RELATED GENES AND AUTISM		
Celestino-Soper et al., 2012	909 or 130	Deficiency of the gene *TMLHE* (trimethyl-lysine hydroxylase epsilon), which encodes the first enzyme in carnitine biosynthesis, was more frequent in probands from male-male multiplex ASD families.
Glessner et al., 2009	859	Copy number variations in genes involved in the ubiquitin degradation were implicated in susceptibility for ASD.
Kent et al., 2006	129	The 3243A>G mitochondrial DNA mutation was concluded to be a rare cause of isolated Asperger syndrome.
Silverman et al., 2008; Kim et al., 2011	Multiple families	Polymorphism in *SLC25A12* gene, which encodes a mitochondrial aspartate/glutamate carrier, was found to be associated with restricted repetitive behaviors in autism.
Maestrini et al., 2010	127	A gene encoding an inner mitochondrial membrane protease-like protein (*IMMP2L)* was implicated in susceptibility for ASD.
Nava et al., 2012	501	Mutations in *TMLHE* were identified in patients with ASD and led to an increase in trimethyl-lysine, the precursor of carnitine biosynthesis, in the plasma.
Ramoz et al., 2004; Segurado et al., 2005; Turunen et al., 2008	Multiple families	Polymorphism in *SLC25A12* gene was found to be associated with autism.
INDICATIONS OF IMPAIRED MITOCHONDRIAL FUNCTION IN THE BRAIN		
Goh et al., 2014	75	Lactate doublets detected by brain magnetic resonance spectroscopic imaging were present at a higher rate in autistic patients.
Palmieri et al., 2010	Six or multiple families	Transport rates of mitochondrial aspartate/glutamate carrier (AGC) were higher in temporo-cortical gray matter. In addition, expression of AGC1, cytochrome c oxidase activity, and oxidized mitochondrial proteins were increased. However, variants of the AGC1-encoding *SLC25A12* gene were not correlated with AGC activation or autism phenotype.
Tang et al., 2013	45	Mitochondrial function and intracellular redox status were compromised in the pyramidal neurons of the temporal cortex.
ABNORMAL LEVELS OF MITOCHONDRIA-RELATED METABOLITES IN BLOOD SAMPLES		
Al-Mosalem et al., 2009	30	Increased plasma lactate levels and activity of creatine kinase.
Cohen et al., 1976	25	Increased serum creatine phosphokinase levels.
Correia et al., 2006	241	Increased plasma lactate levels and lactate/pyruvate ratio, but not associated with the variation at the *SLC25A12* gene.
Filipek et al., 2004	100	Reduced levels of carnitine and pyruvate, but increased levels of alanine and ammonia in serum.
Frye et al., 2013	213	Abnormal acyl-carnitine panels and glutathione metabolism in blood samples.
Kuwabara et al., 2013	25	Higher plasma levels of arginine and taurine, and lower levels of 5-oxoproline and lactic acid.
László et al., 1994	30	Increased serum lactate and pyruvate levels.
Moreno et al., 1992	60	Increased lactate and pyruvate levels.
Oliveira et al., 2005	69	20% of ASD patients showed significantly increased lactic acidemia, while 7% were classified with a definite mitochondrial respiratory chain disorder.
Poling et al., 2006	159	Increased blood aspartate aminotransferase and creatine kinase levels.
ABNORMAL MITOCHONDRIAL FUNCTION AND DNA STRUCTURE IN PERIPHERAL CELLS OR CELL LINES		
Boccuto et al., 2013	87	Decreased tryptophan metabolism in lymphoblastoid cell lines.
Chen et al., 2015	78	Mitochondrial DNA copy number in peripheral blood cells was elevated in children with ASD.
Rose et al., 2012	43	Primary immune cells in the blood had a more oxidized intracellular and extracellular microenvironment and a deficit in glutathione-mediated redox/antioxidant capacity.
Rose et al., 2014	25	Mitochondrial dysfunction observed in a subset of autism lymphoblastoid cell lines.
Wong et al., 2016	66	Mitochondrial DNA deletions and higher p53 gene copy ratios in peripheral blood monocytic cells were more common in children with autism and their fathers.

clinical efficacy (Masino and Geiger, 2008; Masino et al., 2009, 2010). Separately, increased adenosine has been proposed to reduce symptoms and comorbidities of ASD (Masino et al., 2013). In addition, the KD has been reported to regulate energy-sensing pathways such as those involving the insulin-like growth factor and the mammalian target of rapamycin (McDaniel et al., 2011; Gano et al., 2014). Epigenetic regulation is a new but potentially important mechanism as well (Boison, 2016).

Given the effects of the KD and its substrates (e.g., ketone bodies, fatty acids) on cognitive and behavioral functioning, it is reasonable to speculate that this diet would induce changes in synaptic morphology and function. Studies have shown that the KD can modulate excitability through actions on potassium

TABLE 2 | Studies showing association between ASD and mitochondrial dysfunction in animal models of ASD.

References	Evidence of mitochondrial dysfunction
LINKAGE BETWEEN MITOCHONDRIA-RELATED GENES AND AUTISTIC PHENOTYPE	
Hullinger et al., 2016	Increased expression of AT-1/SLC33A1 caused an autistic-like phenotype in mice.
Inan et al., 2016	Progressive decline in oxidative phosphorylation led to circuit dysfunction, impaired sensory gating and social disability when the *cox10* gene was selectively deleted in parvalbumin neurons in mouse.
Sakurai et al., 2010	Loss of *SLC25A12* gene resulted in hypomyelination. Myelin deficits in slice cultures from knockout mice were reversed by administration of pyruvate.
Xie et al., 2016	Cell-autonomous insufficiencies in the activity of *TMLHE* reduced neural stem cell pools in the embryonic mouse brain.
Zhao et al., 2010	ASD-like features observed in neuronal glucose transporter isoform 3-deficient mice.
ALTERATIONS IN MITOCHONDRIAL FUNCTION IN ANIMAL OR CELLULAR MODELS OF ASD BASED ON GENETIC FACTORS	
De Filippis et al., 2015	The rate of hydrogen peroxide generation was increased and the function of complex ii impaired in the brain of *MeCP2*-308 heterozygous female mice.
Jin et al., 2015	*Mecp2*, whose mutations cause Rett syndrome, was observed to regulate mitochondrial bioenergetics through a glutamine transporter in microglia.
Kriaucionis et al., 2006	Mitochondrial abnormalities observed in *Mecp2*-null mouse, a model of Rett syndrome.
Nie et al., 2015	Mitochondrial uncoupling protein-2 was highly induced in *Tsc2*-deficient neurons, and also in a neuron-specific *Tsc1* conditional knock-out mouse model.
Norkett et al., 2016	DISC1 protein regulated mitochondrial dynamics in neurites of neurons.
Pancrazi et al., 2015	A fraction of the protein Foxg1, which is implicated in autism, was found to localize to mitochondria and coordinate cell differentiation and bioenergetics.
Santini et al., 2015	A mouse model of Angelman syndrome displayed elevated levels of mitochondria-derived reactive oxygen species in pyramidal neurons in hippocampal CA1 area, and administration of MitoQ, a mitochondria-specific antioxidant, to this model normalized synaptic plasticity and restored memory.
Su et al., 2011	Mitochondrial dysfunction observed in hippocampal neurons of the *UBE3A*-deficient mouse model of Angelman syndrome.
MITOCHONDRIAL DYSFUNCTION IN ANIMAL MODELS OF ASD BASED ON ENVIRONMENTAL FACTORS	
Bhandari and Kuhad, 2015	Propanoic acid exposure induced autism-like behavior in rats and activities of complex I and II were reduced.
Kumar and Sharma, 2016	Prenatal exposure to valproic acid decreased the activity of mitochondrial complex I, II, and IV in rats .
Macfabe, 2012	Mitochondrial dysfunction observed in a rat ASD model in which propionic acid, an enteric bacterial fermentation product, is infused intracerebroventricularly.
TREATMENT RELATED TO METABOLISM AND MITOCHONDRIAL FUNCTION IN ANIMAL MODELS OF ASD*	
Ciarlone et al., 2016	Ketone ester supplementation improved motor coordination, learning and memory, and synaptic plasticity in a mouse model of Angelman syndrome. The treatment also attenuated seizure activity and altered brain amino acid metabolism in this model.
Currais et al., 2016	Dietary glycemic index was found to modulate behavioral and biochemical phenotype of the BTBR mouse model of ASD.
Naviaux et al., 2013, 2014, 2015	Anti-purinergic therapy improved autism-like features in the maternal immune activation mouse model and the Fragile X mouse model.
Park et al., 2014	Dietary therapy with triheptanoin enhanced mitochondrial substrate use and improved metabolism and behaviors of *Mecp2*-null mouse model of Rett syndrome.
Sakurai et al., 2010	Loss of the *SLC25A12* gene resulted in hypomyelination. Myelin deficits in slice cultures from knockout mice are reversed by administration of pyruvate.
Santini et al., 2015	A mouse model of Angelman syndrome displayed elevated levels of mitochondria-derived reactive oxygen species in pyramidal neurons in CA1 hippocampus, and administration of MitoQ, a mitochondria-specific antioxidant, in this model normalized synaptic plasticity and restored memory.

**Studies using the ketogenic diet are described in more detail in the main text.*

ion channels (Tigerholm et al., 2012; Lutas and Yellen, 2013) and glutamatergic synaptic transmission (Xu et al., 2006; Juge et al., 2010; Lutas and Yellen, 2013; Chang et al., 2016), as well as possible regulation of GABA production (Yudkoff et al., 2007). In addition, the KD or its metabolic mediators can induce changes in synaptic vesicular cycling (Hrynevich et al., 2016), hippocampal mossy fiber sprouting (Muller-Schwarze et al., 1999), and both age- and region-dependent changes in synaptic morphology (Balietti et al., 2008, 2009).

Collectively, evidence thus far indicates that the KD affords broad neuroprotective effects, and hence, it is reasonable to hypothesize that this diet could prove to be beneficial for individuals with ASD.

METABOLIC THERAPY AND ASD—CLINICAL EVIDENCE TO DATE

To date, there have been limited clinical trials involving treatment of ASD patients with metabolic therapy using variants of a KD. The first report was a pilot prospective study in autistic children aged between 4 and 10 years carried out by Evangeliou and

colleagues; they applied an intermittent modified medium-chain triglyceride (MCT) diet (Evangeliou et al., 2003). Most of the 18 patients who adhered to the diet improved based on the Childhood Autism Rating Scale (CARS) and several additional clinical parameters. Significant (i.e., >12 units of decrease in CARS) and average (>8–12 units of decrease in CARS) improvement was recorded in two and eight patients, respectively, while minor (2–8 units of decrease in CARS) improvement was reported in the remaining eight patients. More recently, Spilioti and colleagues reported the effects of KD treatment in a group of Greek children with ASD aged between 3.5 and 6 years (Spilioti et al., 2013). Of the 6 patients who implemented the diet successfully, significant and average improvement was recorded in one and two patients, respectively, and minor improvement was reported in the remaining three patients.

The diet is also effective in reducing common comorbidities of ASD such as seizures, not surprisingly, but also improved cognition and behavior. A pilot retrospective study analyzed outcomes in children prescribed the KD to treat epileptic seizures; among these children, some also had autistic symptoms and abnormal behaviors. Children assigned in the KD group were currently on the diet and had been for at least 6 months; children assigned in the non-KD group stopped the diet at least 2 months prior. Fewer abnormal behaviors and significant behavioral improvement were found in the KD group, and behavioral improvement was not correlated with seizure control (Masino et al., 2011). More recently a randomized control trial showed improved cognition, mood and behavior—particularly reduced anxiety—in children prescribed the KD for refractory epilepsy. These behavioral benefits were also unrelated to seizure control (IJff et al., 2016). In a remarkable case study, Herbert and Buckley reported on a 12-year-old child with comorbid autism and epilepsy treated with a gluten- and casein-free KD (fats composed mostly of MCTs) (Herbert and Buckley, 2013). In addition to a significant reduction in seizures, the diet resolved morbid obesity and improved cognitive and behavioral function. Over the course of several years following initial diagnosis, the child's CARS score decreased from 49 to 17, representing a change from severe autism to a non-autistic state, and her intelligence quotient increased by 70 points.

In summary, clinical evidence to date remains limited, but results from the aforementioned studies show promise that metabolic therapy with several different versions of a KD can improve symptoms of ASD and can also improve cognition and behavior—the latter benefits that can facilitate optimal outcomes in ASD. In patients with diagnosed ASD, greater than 50% of autism patients who received this metabolic therapy showed moderate-to-significant clinical improvement, while the remainder displayed minor improvement. At present, more larger-scale clinical studies are required. Meanwhile, as mentioned earlier, metabolic and mitochondrial dysfunction may represent only a subgroup of the ASD population. Thus, it would be important to determine the relation between the effects of the KD and metabolic/genetic profile of ASD patients.

METABOLIC THERAPY AND ASD—EVIDENCE FROM ANIMAL MODELS

Due to the complexity of ASD, investigators have developed and employed numerous animal models. Some have clear metabolic underpinnings, underscoring the link between metabolic dysfunction and symptoms of autism. Metabolic therapy with a KD and/or a restricted diet has already been examined in several models. In agreement with the aforementioned clinical studies, reports in animal models have been positive. The ASD models tested with metabolic therapy discussed here include genetic disorders that mirror clinical conditions, induced ASD that models environmental conditions found to increase ASD risk in humans, and behavioral ASD models with unknown etiologies that recapitulate all or some of the core symptoms, and may or may not have comorbid seizures.

As one genetic example, succinic semialdehyde dehydrogenase (SSADH) deficiency is a rare autosomal recessive condition that results in mild-to-moderate mental retardation, disproportionate language dysfunction, seizures, hypotonia, hyporeflexia, hallucinations, and autistic behaviors (Pearl et al., 2003). In an animal model of SSADH deficiency, the SSADH knockout mouse, Nylen and colleagues found that KD treatment normalized electroencephalogram (EEG) activity and restored miniature inhibitory post-synaptic currents recorded in CA1 pyramidal cells using hippocampal slices. In contrast, there were no significant differences between the groups in terms of miniature excitatory post-synaptic currents. Behaviorally, KD-treated mutant animals experienced significantly fewer seizures compared to mutant animals fed the control diet (Nylen et al., 2008).

Metabolic therapy with dietary restriction (either a standard diet or KD) was tested in another clinically relevant genetic model of Rett syndrome. Rett syndrome is a neurodevelopmental disorder characterized by normal early maturation, followed by a slowing of development, impairment of motor functions, seizure susceptibility, and intellectual disability. In most cases, Rett syndrome is caused by mutations in the methyl-CpG-binding protein 2 (*MECP2*) gene (Amir et al., 1999). Children with Rett syndrome often exhibit autistic-like behaviors in the early stages of the disease (Percy, 2011). Mantis and colleagues found that *Mecp2* mutant mice performed significantly worse in assays of motor function and anxiety compared to wild-type control animals, and restriction of either standard diet or the KD improved motor behavior and reduced anxiety in these mutant animals (Mantis et al., 2009). There is also limited clinical evidence for anti-seizure efficacy and improved behavior after KD treatment in Rett syndrome (Liebhaber et al., 2003).

Most cases of ASD have unknown genetic underpinnings (Gaugler et al., 2014), and models of unidentified etiology have been characterized with behavioral tests assessing autistic symptomatology. The BTBR T+tf/J (BTBR) inbred mouse strain is one of the most clinically relevant animal models of autism; it was identified in an extensive effort to characterize ASD-like behaviors in ten inbred mouse strains (Moy et al., 2007) and displays all the core behavioral features that define the disorder (Moy et al., 2007; McFarlane et al., 2008; Meyza et al., 2013;

Ruskin et al., 2013; Smith et al., 2014; Ellegood and Crawley, 2015). BTBR mice display deficits in social interaction and communication assays and exhibit repetitive and stereotyped behaviors. During the relatively short time since its discovery as an ASD model, the BTBR strain has been increasingly used to study the etiology and to uncover potential interventions for ASD (Moy et al., 2007; McFarlane et al., 2008; Llaneza et al., 2010; Ruskin et al., 2013; Cheng et al., 2016; Mychasiuk and Rho, 2016; Newell et al., 2016). Ruskin and colleagues reported that the BTBR mice showed decreased sociability in the three-chamber test, decreased self-directed repetitive behavior, and improved social communication in a food preference assay after being fed a KD (Ruskin et al., 2013). In addition, the authors showed that the behavioral improvements were probably not related to any anti-seizure effect of the diet, because no spontaneous seizures or abnormal EEG features were observed in the BTBR animals.

Interestingly, a recent study showed that gut microbiota composition of cecal and fecal samples was significantly altered in BTBR mice compared to B6 animals (Newell et al., 2016). In addition, a KD decreased total host bacterial abundance in both sample types, and in the BTBR animals counteracted a low Firmicutes to Bacteroidetes ratio, which is commonly observed in patients with ASD (Finegold et al., 2010; De Angelis et al., 2013). Related to these findings, it has been shown that dietary glycemic index, which is a measure of how much the carbohydrate in a food item affects blood glucose level, modulates behavioral and biochemical phenotype in the BTBR mice (Currais et al., 2016). These data support the idea that in the context of genetic predisposition to ASD, diet could potentially alter the expression of the disorders.

More recently, Ruskin et al. also showed KD-induced behavioral improvements in the EL mouse, a model of comorbid ASD-associated behaviors and progressive spontaneous epilepsy. Mice (of both sexes) were fed a KD for 3 weeks after weaning and prior to the age of onset of the seizure phenotype. Sociability improved and repetititve behaviors decreased; intriguingly, these effects were more pronounced in females. Also, some behavioral benefits were observed in females even when a more liberal dietary formulation was applied (Ruskin et al., 2016).

As mentioned above, environmental factors also contribute to the risk of developing ASD. In this regard, exposure to exogenous chemicals is best exemplified by VPA use during pregnancy. VPA is a pharmacological anticonvulsant used in humans primarily for the treatment of epilepsy and migraine, and epidemiological studies have shown that use of VPA during pregnancy is associated with an increased risk of ASD in the offspring (Bromley et al., 2013; Christensen et al., 2013). The VPA exposure model is one of the most frequently studied models of autism (Chomiak et al., 2013; Roullet et al., 2013) since it exhibits many similar structural and behavioral features seen in ASD individuals. Ahn and colleagues found that KD treatment recovered part of the play behavior of juvenile rats exposed to VPA prenatally (Ahn et al., 2014). Interestingly, the authors also found that prenatal exposure to VPA altered mitochondrial respiration, and the KD was able to partially restore this. A recent study in VPA-treated mice also found improved social behavior (Castro et al., 2016).

Taken together, there is increasing evidence for the beneficial effects of the KD in different animal models of ASD. However, clear evidence for converging mechanistic links remains hypothetical, and few fundamental mechanistic studies have been conducted to date in either animal models or human ASD tissues. Thus, there is a need for further studies utilizing diverse animal models and incorporating comprehensive behavioral assays to elucidate common molecular pathways in ASD and to validate the positive effects of the KD observed thus far in animal models. Equally important, studies aimed at identifying the mechanisms relevant to such models of ASD are required to optimize treatment, discover novel therapeutic targets, and ultimately provide key insights to the neurobiology of ASD. Borrowing from the rich literature on the KD in epilepsy, shifts in energy metabolism, the direct actions of the ketone bodies on the mitochondria, neuromodulatory functions of ATP and adenosine, regulation of excitation/inhibition balance, and epigenetic effects of the diet are among the promising candidate mechanisms.

FUTURE DIRECTIONS: METABOLIC THERAPY AND ASD

At present, there is strong evidence that mitochondrial and metabolic dysfunction may underlie the complex pathophysiology of ASD. Precise mechanisms remain elusive and many questions remain unanswered: Is mitochondrial dysfunction a cause or a consequence of ASD? In which specific organs and cell types is it most relevant? Can addressing mitochondrial and metabolic disturbances directly help at least a subgroup of patients with ASD for more targeted treatments that will ameliorate the diverse symptom complex? Thus far, the KD is a proven metabolic therapy for medically intractable epilepsy, but there are only limited data for its use in ASD and a rudimentary understanding of how the KD may exert positive behavioral effects. The optimum formulation of the KD needs to be established and may be different for ASD compared to epilepsy. Proper efforts to address these fundamental questions and to identify molecular mechanisms and biomarkers will require the collective and collaborative efforts of many, including basic, translational and clinical researchers, as well as investigators with diverse expertise in multi-organ dysfunction, metabolism, and genetic and environmental risk factors. The ultimate reward could be a major breakthrough in understanding its causes and developing much-needed broadly effective therapies for ASD—and in particular, treatments that address core symptoms.

AUTHOR CONTRIBUTIONS

All authors have drafted and revised the manuscript together and approved it for publication.

ACKNOWLEDGMENTS

This work is supported by Alberta Children's Hospital Research Foundation grant to JR, and NIH grant NS066392 to SM.

REFERENCES

Ahn, Y., Narous, M., Tobias, R., Rho, J. M., and Mychasiuk, R. (2014). The ketogenic diet modifies social and metabolic alterations identified in the prenatal valproic acid model of autism spectrum disorder. *Dev. Neurosci.* 36, 371–380. doi: 10.1159/000362645

Ahola-Erkkila, S., Carroll, C. J., Peltola-Mjosund, K., Tulkki, V., Mattila, I., Seppanen-Laakso, T., et al. (2010). Ketogenic diet slows down mitochondrial myopathy progression in mice. *Hum. Mol. Genet.* 19, 1974–1984. doi: 10.1093/hmg/ddq076

Al-Mosalem, O. A., El-Ansary, A., Attas, O., and Al-Ayadhi, L. (2009). Metabolic biomarkers related to energy metabolism in Saudi autistic children. *Clin. Biochem.* 42, 949–957. doi: 10.1016/j.clinbiochem.2009.04.006

Amir, R. E., Van den Veyver, I. B., Wan, M., Tran, C. Q., Francke, U., and Zoghbi, H. Y. (1999). Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. *Nat. Genet.* 23, 185–188. doi: 10.1038/13810

Amoedo, N. D., Punzi, G., Obre, E., Lacombe, D., De Grassi, A., Pierri, C. L., et al. (2016). AGC1/2, the mitochondrial aspartate-glutamate carriers. *Biochim. Biophys. Acta* 1863, 2394–2412. doi: 10.1016/j.bbamcr.2016.04.011

Antico Arciuch, V. G., Elguero, M. E., Poderoso, J. J., and Carreras, M. C. (2012). Mitochondrial regulation of cell cycle and proliferation. *Antioxid. Redox Signal.* 16, 1150–1180. doi: 10.1089/ars.2011.4085

Baker, E., and Jeste, S. S. (2015). Diagnosis and management of autism spectrum disorder in the era of genomics: rare disorders can pave the way for targeted treatments. *Pediatr. Clin. North Am.* 62, 607–618. doi: 10.1016/j.pcl.2015.03.003

Balietti, M., Fattoretti, P., Giorgetti, B., Casoli, T., Di Stefano, G., Platano, D., et al. (2009). Effect of two medium chain triglycerides-supplemented diets on synaptic morphology in the cerebellar cortex of late-adult rats. *Microsc. Res. Tech.* 72, 933–938. doi: 10.1002/jemt.20737

Balietti, M., Giorgetti, B., Fattoretti, P., Grossi, Y., Di Stefano, G., Casoli, T., et al. (2008). Ketogenic diets cause opposing changes in synaptic morphology in CA1 hippocampus and dentate gyrus of late-adult rats. *Rejuvenation Res.* 11, 631–640. doi: 10.1089/rej.2007.0650

Berg, J. M., and Geschwind, D. H. (2012). Autism genetics: searching for specificity and convergence. *Genome Biol.* 13:247. doi: 10.1186/gb-2012-13-7-247

Bhandari, R., and Kuhad, A. (2015). Neuropsychopharmacotherapeutic efficacy of curcumin in experimental paradigm of autism spectrum disorders. *Life Sci.* 141, 156–169. doi: 10.1016/j.lfs.2015.09.012

Boccuto, L., Chen, C.-F., Pittman, A. R., Skinner, C. D., McCartney, H. J., Jones, K., et al. (2013). Decreased tryptophan metabolism in patients with autism spectrum disorders. *Mol. Autism* 4:16. doi: 10.1186/2040-2392-4-16

Boison, D. (2016). The biochemistry and epigenetics of epilepsy: focus on adenosine and glycine. *Front. Mol. Neurosci.* 9:26. doi: 10.3389/fnmol.2016.00026

Bough, K. J., Wetherington, J., Hassel, B., Pare, J. F., Gawryluk, J. W., Greene, J. G., et al. (2006). Mitochondrial biogenesis in the anticonvulsant mechanism of the ketogenic diet. *Ann. Neurol.* 60, 223–235. doi: 10.1002/ana.20899

Bourgeron, T. (2015). From the genetic architecture to synaptic plasticity in autism spectrum disorder. *Nat. Rev. Neurosci.* 16, 551–563. doi: 10.1038/nrn3992

Bremer, J. (1983). Carnitine–metabolism and functions. *Physiol. Rev.* 63, 1420–1480.

Bromley, R. L., Mawer, G. E., Briggs, M., Cheyne, C., Clayton-Smith, J., Garcia-Finana, M., et al. (2013). The prevalence of neurodevelopmental disorders in children prenatally exposed to antiepileptic drugs. *J. Neurol. Neurosurg. Psychiatr.* 84, 637–643. doi: 10.1136/jnnp-2012-304270

Buescher, A. V., Cidav, Z., Knapp, M., and Mandell, D. S. (2014). Costs of autism spectrum disorders in the United Kingdom and the United States. *JAMA Pediatr.* 168, 721–728. doi: 10.1001/jamapediatrics.2014.210

Castro, K., Baronio, D., Perry, I. S., dos Santos Riesgo, R., and Gottfried, C. (2016). The effect of ketogenic diet in an animal model of autism induced by prenatal exposure to valproic acid. *Nutr. Neurosci.* doi: 10.1080/1028415X.2015.1133029. [Epub ahead of print].

CDC (2014). Prevalence of autism spectrum disorder among children aged 8 years—autism and developmental disabilities monitoring network, 11 sites, United States, 2010. *Morb. Mort. Wkly Rep. Surveil. Summ.* 63, 1–21.

Celestino-Soper, P. B. S., Violante, S., Crawford, E. L., Luo, R., Lionel, A. C., Delaby, E., et al. (2012). A common X-linked inborn error of carnitine biosynthesis may be a risk factor for nondysmorphic autism. *Proc. Natl. Acad. Sci. U.S.A.* 109, 7974–7981. doi: 10.1073/pnas.1120210109

Chaidez, V., Hansen, R. L., and Hertz-Picciotto, I. (2014). Gastrointestinal problems in children with autism, developmental delays or typical development. *J. Autism Dev. Disord.* 44, 1117–1127. doi: 10.1007/s10803-013-1973-x

Chang, P., Augustin, K., Boddum, K., Williams, S., Sun, M., Terschak, J. A., et al. (2016). Seizure control by decanoic acid through direct AMPA receptor inhibition. *Brain* 139(Pt 2), 431–443. doi: 10.1093/brain/awv325

Chaste, P., and Leboyer, M. (2012). Autism risk factors: genes, environment, and gene-environment interactions. *Dialogues Clin. Neurosci.* 14, 281–292.

Chen, S., Li, Z., He, Y., Zhang, F., Li, H., Liao, Y., et al. (2015). Elevated mitochondrial DNA copy number in peripheral blood cells is associated with childhood autism. *BMC Psychiatry* 15:50. doi: 10.1186/s12888-015-0432-y

Cheng, N., Khanbabaei, M., Murari, K., and Rho, J. M. (2016). Disruption of visual circuit formation and refinement in a mouse model of autism. *Autism Res.* doi: 10.1002/aur.1687. [Epub ahead of print].

Chomiak, T., Turner, N., and Hu, B. (2013). What we have learned about autism spectrum disorder from valproic acid. *Patholog. Res. Int.* 2013:712758. doi: 10.1155/2013/712758

Christensen, J., Gronborg, T. K., Sorensen, M. J., Schendel, D., Parner, E. T., Pedersen, L. H., et al. (2013). Prenatal valproate exposure and risk of autism spectrum disorders and childhood autism. *JAMA* 309, 1696–1703. doi: 10.1001/jama.2013.2270

Ciarlone, S. L., Grieco, J. C., D'Agostino, D. P., and Weeber, E. J. (2016). Ketone ester supplementation attenuates seizure activity, and improves behavior and hippocampal synaptic plasticity in an Angelman syndrome mouse model. *Neurobiol. Dis.* 96, 38–46. doi: 10.1016/j.nbd.2016.08.002

Cohen, D. J., Johnson, W., Caparulo, B. K., and Young, J. G. (1976). Creatine phosphokinase levels in children with severe developmental disturbances. *Arch. Gen. Psychiatry* 33, 683–686. doi: 10.1001/archpsyc.1976.01770060025004

Correia, C., Coutinho, A. M., Diogo, L., Grazina, M., Marques, C., Miguel, T., et al. (2006). Brief report: high frequency of biochemical markers for mitochondrial dysfunction in autism: no association with the mitochondrial aspartate/glutamate carrier *SLC25A12* gene. *J. Autism Dev. Disord.* 36, 1137–1140. doi: 10.1007/s10803-006-0138-6

Courchesne, E., Pierce, K., Schumann, C. M., Redcay, E., Buckwalter, J. A., Kennedy, D. P., et al. (2007). Mapping early brain development in autism. *Neuron* 56, 399–413. doi: 10.1016/j.neuron.2007.10.016

Cullingford, T. (2008). Peroxisome proliferator-activated receptor alpha and the ketogenic diet. *Epilepsia* 49(Suppl. 8), 70–72. doi: 10.1111/j.1528-1167.2008.01840.x

Currais, A., Farrokhi, C., Dargusch, R., Goujon-Svrzic, M., and Maher, P. (2016). Dietary glycemic index modulates the behavioral and biochemical abnormalities associated with autism spectrum disorder. *Mol. Psychiatry* 21, 426–436. doi: 10.1038/mp.2015.64

De Angelis, M., Piccolo, M., Vannini, L., Siragusa, S., De Giacomo, A., Serrazzanetti, D. I., et al. (2013). Fecal microbiota and metabolome of children with autism and pervasive developmental disorder not otherwise specified. *PLoS ONE* 8:e76993. doi: 10.1371/journal.pone.0076993

De Filippis, B., Valenti, D., de Bari, L., De Rasmo, D., Musto, M., Fabbri, A., et al. (2015). Mitochondrial free radical overproduction due to respiratory chain impairment in the brain of a mouse model of Rett syndrome: protective effect of CNF1. *Free Radic. Biol. Med.* 83, 167–177. doi: 10.1016/j.freeradbiomed.2015.02.014

de la Torre-Ubieta, L., Won, H., Stein, J. L., and Geschwind, D. H. (2016). Advancing the understanding of autism disease mechanisms through genetics. *Nat. Med.* 22, 345–361. doi: 10.1038/nm.4071

De Rubeis, S., and Buxbaum, J. D. (2015). Genetics and genomics of autism spectrum disorder: embracing complexity. *Hum. Mol. Genet.* 24, R24–R31. doi: 10.1093/hmg/ddv273

Devlin, B., and Scherer, S. W. (2012). Genetic architecture in autism spectrum disorder. *Curr. Opin. Genet. Dev.* 22, 229–237. doi: 10.1016/j.gde.2012.03.002

Dhillon, S., Hellings, J. A., and Butler, M. G. (2011). Genetics and mitochondrial abnormalities in autism spectrum disorders: a review. *Curr. Genomics* 12, 322–332. doi: 10.2174/138920211796429745

DiCicco-Bloom, E., Lord, C., Zwaigenbaum, L., Courchesne, E., Dager, S. R., Schmitz, C., et al. (2006). The developmental neurobiology of autism

spectrum disorder. *J. Neurosci.* 26, 6897–6906. doi: 10.1523/JNEUROSCI.1712-06.2006

Durkin, M. S., Maenner, M. J., Newschaffer, C. J., Lee, L. C., Cunniff, C. M., Daniels, J. L., et al. (2008). Advanced parental age and the risk of autism spectrum disorder. *Am. J. Epidemiol.* 168, 1268–1276. doi: 10.1093/aje/kwn250

Ellegood, J., and Crawley, J. N. (2015). Behavioral and neuroanatomical phenotypes in mouse models of autism. *Neurotherapeutics* 12, 521–533. doi: 10.1007/s13311-015-0360-z

Evangeliou, A., Vlachonikolis, I., Mihailidou, H., Spilioti, M., Skarpalezou, A., Makaronas, N., et al. (2003). Application of a ketogenic diet in children with autistic behavior: pilot study. *J. Child Neurol.* 18, 113–118. doi: 10.1177/08830738030180020501

Filipek, P. A., Juranek, J., Nguyen, M. T., Cummings, C., and Gargus, J. J. (2004). Relative carnitine deficiency in autism. *J. Autism Dev. Disord.* 34, 615–623. doi: 10.1007/s10803-004-5283-1

Finegold, S. M., Dowd, S. E., Gontcharova, V., Liu, C., Henley, K. E., Wolcott, R. D., et al. (2010). Pyrosequencing study of fecal microflora of autistic and control children. *Anaerobe* 16, 444–453. doi: 10.1016/j.anaerobe.2010.06.008

Frye, R. E. (2015). Metabolic and mitochondrial disorders associated with epilepsy in children with autism spectrum disorder. *Epilepsy Behav.* 47, 147–157. doi: 10.1016/j.yebeh.2014.08.134

Frye, R. E., Melnyk, S., and Macfabe, D. F. (2013). Unique acyl-carnitine profiles are potential biomarkers for acquired mitochondrial disease in autism spectrum disorder. *Transl. Psychiatry* 3:e220. doi: 10.1038/tp.2012.143

Frye, R. E., Rose, S., Slattery, J., and MacFabe, D. F. (2015). Gastrointestinal dysfunction in autism spectrum disorder: the role of the mitochondria and the enteric microbiome. *Microb. Ecol. Health Dis.* 26:27458. doi: 10.3402/mehd.v26.27458

Frye, R. E., and Rossignol, D. A. (2011). Mitochondrial dysfunction can connect the diverse medical symptoms associated with autism spectrum disorders. *Pediatric Res.* 69(5 Pt 2), 41R–47R. doi: 10.1203/PDR.0b013e318212f16b

Frye, R. E., and Rossignol, D. A. (2016). Identification and treatment of pathophysiological comorbidities of autism spectrum disorder to achieve optimal outcomes. *Clin. Med. Insights Pediatrics* 10, 43–56. doi: 10.4137/CMPed.S38337

Gano, L. B., Patel, M., and Rho, J. M. (2014). Ketogenic diets, mitochondria, and neurological diseases. *J. Lipid Res.* 55, 2211–2228. doi: 10.1194/jlr.R048975

Gardener, H., Spiegelman, D., and Buka, S. L. (2011). Perinatal and neonatal risk factors for autism: a comprehensive meta-analysis. *Pediatrics* 128, 344–355. doi: 10.1542/peds.2010-1036

Garriga-Canut, M., Schoenike, B., Qazi, R., Bergendahl, K., Daley, T. J., Pfender, R. M., et al. (2006). 2-Deoxy-D-glucose reduces epilepsy progression by NRSF-CtBP-dependent metabolic regulation of chromatin structure. *Nat. Neurosci.* 9, 1382–1387. doi: 10.1038/nn1791

Gaugler, T., Klei, L., Sanders, S. J., Bodea, C. A., Goldberg, A. P., Lee, A. B., et al. (2014). Most genetic risk for autism resides with common variation. *Nat. Genet.* 46, 881–885. doi: 10.1038/ng.3039

Geschwind, D. H. (2008). Autism: many genes, common pathways? *Cell* 135, 391–395. doi: 10.1016/j.cell.2008.10.016

Glessner, J. T., Wang, K., Cai, G., Korvatska, O., Kim, C. E., Wood, S., et al. (2009). Autism genome-wide copy number variation reveals ubiquitin and neuronal genes. *Nature* 459, 569–573. doi: 10.1038/nature07953

Goh, S., Dong, Z., Zhang, Y., DiMauro, S., and Peterson, B. S. (2014). Mitochondrial dysfunction as a neurobiological subtype of autism spectrum disorder: evidence from brain imaging. *JAMA Psychiatry* 71, 665–671. doi: 10.1001/jamapsychiatry.2014.179

Goines, P., and Van de Water, J. (2010). The immune system's role in the biology of autism. *Curr. Opin. Neurol.* 23, 111–117. doi: 10.1097/WCO.0b013e3283373514

Haas, R. H. (2010). Autism and mitochondrial disease. *Dev. Disabil. Res. Rev.* 16, 144–153. doi: 10.1002/ddrr.112

Hansen, S. N., Schendel, D. E., and Parner, E. T. (2015). Explaining the increase in the prevalence of autism spectrum disorders: the proportion attributable to changes in reporting practices. *JAMA Pediatr.* 169, 56–62. doi: 10.1001/jamapediatrics.2014.1893

Harris, J. J., Jolivet, R., and Attwell, D. (2012). Synaptic energy use and supply. *Neuron* 75, 762–777. doi: 10.1016/j.neuron.2012.08.019

Herbert, M. R., and Buckley, J. A. (2013). Autism and dietary therapy: case report and review of the literature. *J. Child Neurol.* 28, 975–982. doi: 10.1177/0883073813488668

Hrynevich, S. V., Waseem, T. V., Hebert, A., Pellerin, L., and Fedorovich, S. V. (2016). β-Hydroxybutyrate supports synaptic vesicle cycling but reduces endocytosis and exocytosis in rat brain synaptosomes. *Neurochem. Int.* 93, 73–81. doi: 10.1016/j.neuint.2015.12.014

Hsiao, E. Y. (2013). Immune dysregulation in autism spectrum disorder. *Int. Rev. Neurobiol.* 113, 269–302. doi: 10.1016/B978-0-12-418700-9.00009-5

Huguet, G., Ey, E., and Bourgeron, T. (2013). The genetic landscapes of autism spectrum disorders. *Annu. Rev. Genomics Hum. Genet.* 14, 191–213. doi: 10.1146/annurev-genom-091212-153431

Hullinger, R., Li, M., Wang, J., Peng, Y., Dowell, J. A., Bomba-Warczak, E., et al. (2016). Increased expression of AT-1/SLC33A1 causes an autistic-like phenotype in mice by affecting dendritic branching and spine formation. *J. Exp. Med.* 213, 1267–1284. doi: 10.1084/jem.20151776

IJff, D. M., Postulart, D., Lambrechts, D. A., Majoie, M. H., de Kinderen, R. J., Hendriksen, J. G., et al. (2016). Cognitive and behavioral impact of the ketogenic diet in children and adolescents with refractory epilepsy: a randomized controlled trial. *Epilepsy Behav.* 60, 153–157. doi: 10.1016/j.yebeh.2016.04.033

Inan, M., Zhao, M., Manuszak, M., Karakaya, C., Rajadhyaksha, A. M., Pickel, V. M., et al. (2016). Energy deficit in parvalbumin neurons leads to circuit dysfunction, impaired sensory gating and social disability. *Neurobiol. Dis.* 93, 35–46. doi: 10.1016/j.nbd.2016.04.004

Jeong, E. A., Jeon, B. T., Shin, H. J., Kim, N., Lee, D. H., Kim, H. J., et al. (2011). Ketogenic diet-induced peroxisome proliferator-activated receptor-γ activation decreases neuroinflammation in the mouse hippocampus after kainic acid-induced seizures. *Exp. Neurol.* 232, 195–202. doi: 10.1016/j.expneurol.2011.09.001

Jeste, S. S., and Geschwind, D. H. (2014). Disentangling the heterogeneity of autism spectrum disorder through genetic findings. *Nat. Rev. Neurol.* 10, 74–81. doi: 10.1038/nrneurol.2013.278

Jin, L.-W., Horiuchi, M., Wulff, H., Liu, X.-B., Cortopassi, G. A., Erickson, J. D., et al. (2015). Dysregulation of glutamine transporter SNAT1 in Rett syndrome microglia: a mechanism for mitochondrial dysfunction and neurotoxicity. *J. Neurosci.* 35, 2516–2529. doi: 10.1523/JNEUROSCI.2778-14.2015

Juge, N., Gray, J. A., Omote, H., Miyaji, T., Inoue, T., Hara, C., et al. (2010). Metabolic control of vesicular glutamate transport and release. *Neuron* 68, 99–112. doi: 10.1016/j.neuron.2010.09.002

Kann, O., and Kovacs, R. (2007). Mitochondria and neuronal activity. *Am. J. Physiol. Cell Physiol.* 292, C641–C657. doi: 10.1152/ajpcell.00222.2006

Kent, L., Lambert, C., Pyle, A., Elliott, H., Wheelwright, S., Baron-Cohen, S., et al. (2006). The mitochondrial DNA A3243A>G mutation must be an infrequent cause of Asperger syndrome. *J. Pediatr.* 149, 280–281. doi: 10.1016/j.jpeds.2005.08.060

Kikuchi, G., Motokawa, Y., Yoshida, T., and Hiraga, K. (2008). Glycine cleavage system: reaction mechanism, physiological significance, and hyperglycinemia. *Proc. Jpn Acad. B Phys. Biol. Sci.* 84, 246–263. doi: 10.2183/pjab.84.246

Kim, S. J., Silva, R. M., Flores, C. G., Jacob, S., Guter, S., Valcante, G., et al. (2011). A quantitative association study of SLC25A12 and restricted repetitive behavior traits in autism spectrum disorders. *Mol. Autism* 2:8. doi: 10.1186/2040-2392-2-8

Kim, Y. S., and Leventhal, B. L. (2015). Genetic epidemiology and insights into interactive genetic and environmental effects in autism spectrum disorders. *Biol. Psychiatry* 77, 66–74. doi: 10.1016/j.biopsych.2014.11.001

Kim do, Y., Davis, L. M., Sullivan, P. G., Maalouf, M., Simeone, T. A., van Brederode, J., et al. (2007). Ketone bodies are protective against oxidative stress in neocortical neurons. *J. Neurochem.* 101, 1316–1326. doi: 10.1111/j.1471-4159.2007.04483.x

Kim do, Y., and Rho, J. M. (2008). The ketogenic diet and epilepsy. *Curr. Opin. Clin. Nutr. Metab. Care* 11, 113–120. doi: 10.1097/MCO.0b013e3282f44c06

Kim do, Y., Simeone, K. A., Simeone, T. A., Pandya, J. D., Wilke, J. C., Ahn, Y., et al. (2015). Ketone bodies mediate antiseizure effects through mitochondrial permeability transition. *Ann. Neurol.* 78, 77–87. doi: 10.1002/ana.24424

Kimura, T., and Murakami, F. (2014). Evidence that dendritic mitochondria negatively regulate dendritic branching in pyramidal neurons in the neocortex. *J. Neurosci.* 34, 6938–6951. doi: 10.1523/JNEUROSCI.5095-13.2014

Kleinman, J. M., Ventola, P. E., Pandey, J., Verbalis, A. D., Barton, M., Hodgson, S., et al. (2008). Diagnostic stability in very young children with autism spectrum disorders. *J. Autism Dev. Disord.* 38, 606–615. doi: 10.1007/s10803-007-0427-8

Kohane, I. S., McMurry, A., Weber, G., MacFadden, D., Rappaport, L., Kunkel, L., et al. (2012). The co-morbidity burden of children and young adults with autism spectrum disorders. *PLoS ONE* 7:e33224. doi: 10.1371/journal.pone.0033224

Kopp, N., Climer, S., and Dougherty, J. D. (2015). Moving from capstones toward cornerstones: successes and challenges in applying systems biology to identify mechanisms of autism spectrum disorders. *Front. Genet.* 6:301. doi: 10.3389/fgene.2015.00301

Krakowiak, P., Walker, C. K., Bremer, A. A., Baker, A. S., Ozonoff, S., Hansen, R. L., et al. (2012). Maternal metabolic conditions and risk for autism and other neurodevelopmental disorders. *Pediatrics* 129, e1121–e1128. doi: 10.1542/peds.2011-2583

Kriaucionis, S., Paterson, A., Curtis, J., Guy, J., Macleod, N., and Bird, A. (2006). Gene expression analysis exposes mitochondrial abnormalities in a mouse model of Rett syndrome. *Mol. Cell. Biol.* 26, 5033–5042. doi: 10.1128/MCB.01665-05

Kumar, H., and Sharma, B. (2016). Memantine ameliorates autistic behavior, biochemistry & blood brain barrier impairments in rats. *Brain Res. Bull.* 124, 27–39. doi: 10.1016/j.brainresbull.2016.03.013

Kuwabara, H., Yamasue, H., Koike, S., Inoue, H., Kawakubo, Y., Kuroda, M., et al. (2013). Altered metabolites in the plasma of autism spectrum disorder: a capillary electrophoresis time-of-flight mass spectroscopy study. *PLoS ONE* 8:e73814. doi: 10.1371/journal.pone.0073814

Lai, M. C., Lombardo, M. V., and Baron-Cohen, S. (2014). Autism. *Lancet* 383, 896–910. doi: 10.1016/S0140-6736(13)61539-1

László, A., Horváth, E., Eck, E., and Fekete, M. (1994). Serum serotonin, lactate and pyruvate levels in infantile autistic children. *Clin. Chim. Acta* 229, 205–207. doi: 10.1016/0009-8981(94)90243-7

Legido, A., Jethva, R., and Goldenthal, M. J. (2013). Mitochondrial dysfunction in autism. *Semin. Pediatr. Neurol.* 20, 163–175. doi: 10.1016/j.spen.2013.10.008

Levy, S. E., Giarelli, E., Lee, L. C., Schieve, L. A., Kirby, R. S., Cunniff, C., et al. (2010). Autism spectrum disorder and co-occurring developmental, psychiatric, and medical conditions among children in multiple populations of the United States. *J. Dev. Behav. Pediatr.* 31, 267–275. doi: 10.1097/DBP.0b013e3181d5d03b

Li, Z., Okamoto, K., Hayashi, Y., and Sheng, M. (2004). The importance of dendritic mitochondria in the morphogenesis and plasticity of spines and synapses. *Cell* 119, 873–887. doi: 10.1016/j.cell.2004.11.003

Liebhaber, G. M., Riemann, E., and Baumeister, F. A. (2003). Ketogenic diet in Rett syndrome. *J. Child Neurol.* 18, 74–75. doi: 10.1177/08830738030180011801

Llaneza, D. C., DeLuke, S. V., Batista, M., Crawley, J. N., Christodulu, K. V., and Frye, C. A. (2010). Communication, interventions, and scientific advances in autism: a commentary. *Physiol. Behav.* 100, 268–276. doi: 10.1016/j.physbeh.2010.01.003

Loke, Y. J., Hannan, A. J., and Craig, J. M. (2015). The role of epigenetic change in autism spectrum disorders. *Front. Neurol.* 6:107. doi: 10.3389/fneur.2015.00107

Lord, C., Risi, S., DiLavore, P. S., Shulman, C., Thurm, A., and Pickles, A. (2006). Autism from 2 to 9 years of age. *Arch. Gen. Psychiatry* 63, 694–701. doi: 10.1001/archpsyc.63.6.694

Lutas, A., and Yellen, G. (2013). The ketogenic diet: metabolic influences on brain excitability and epilepsy. *Trends Neurosci.* 36, 32–40. doi: 10.1016/j.tins.2012.11.005

Maalouf, M., Rho, J. M., and Mattson, M. P. (2009). The neuroprotective properties of calorie restriction, the ketogenic diet, and ketone bodies. *Brain Res. Rev.* 59, 293–315. doi: 10.1016/j.brainresrev.2008.09.002

Macfabe, D. F. (2012). Short-chain fatty acid fermentation products of the gut microbiome: implications in autism spectrum disorders. *Microb. Ecol. Health Dis.* 23:19260. doi: 10.3402/mehd.v23i0.19260

Maestrini, E., Pagnamenta, A. T., Lamb, J. A., Bacchelli, E., Sykes, N. H., Sousa, I., et al. (2010). High-density SNP association study and copy number variation analysis of the *AUTS1* and *AUTS5* loci implicate the *IMMP2L-DOCK4* gene region in autism susceptibility. *Mol. Psychiatry* 15, 954–968. doi: 10.1038/mp.2009.34

Mahfouz, A., Ziats, M. N., Rennert, O. M., Lelieveldt, B. P., and Reinders, M. J. (2015). Shared pathways among autism candidate genes determined by co-expression network analysis of the developing human brain transcriptome. *J. Mol. Neurosci.* 57, 580–594. doi: 10.1007/s12031-015-0641-3

Mantis, J. G., Fritz, C. L., Marsh, J., Heinrichs, S. C., and Seyfried, T. N. (2009). Improvement in motor and exploratory behavior in Rett syndrome mice with restricted ketogenic and standard diets. *Epilepsy Behav.* 15, 133–141. doi: 10.1016/j.yebeh.2009.02.038

Masino, S. A., and Geiger, J. D. (2008). Are purines mediators of the anticonvulsant/neuroprotective effects of ketogenic diets? *Trends Neurosci.* 31, 273–278. doi: 10.1016/j.tins.2008.02.009

Masino, S. A., Kawamura, M. Jr., Cote, J. L., Williams, R. B., and Ruskin, D. N. (2013). Adenosine and autism: a spectrum of opportunities. *Neuropharmacology* 68, 116–121. doi: 10.1016/j.neuropharm.2012.08.013

Masino, S. A., Kawamura, M. Jr., Ruskin, D. N., Gawryluk, J., Chen, X., and Geiger, J. D. (2010). Purines and the anti-epileptic actions of ketogenic diets. *Open Neurosci. J.* 4, 58–63. doi: 10.2174/1874082001004010058

Masino, S. A., Kawamura, M., Wasser, C. D., Pomeroy, L. T., and Ruskin, D. N. (2009). Adenosine, ketogenic diet and epilepsy: the emerging therapeutic relationship between metabolism and brain activity. *Curr. Neuropharmacol.* 7, 257–268. doi: 10.2174/157015909789152164

Masino, S. A., and Rho, J. M. (2012). "Mechanisms of ketogenic diet action," in *Jasper's Basic Mechanisms of the Epilepsies, 4th Edn.*, eds J. L. Noebels, M. Avoli, M. A. Rogawski, R. W. Olsen, and A. V. Delgado-Escueta (Bethesda, MD: Oxford University Press).

Masino, S. A., Svedova, J., Kawamura, M., DiMario, F. D., and Eigsti, I. M. (2011). "Adenosine and autism -recent research and a new perspective," in *Autism—a Neurodevelopmental Journey from Genes to Behaviour*, ed V. Eapen (InTech). Available online at: http://www.intechopen.com/books/autism-a-neurodevelopmental-journey-from-genes-to-behaviour/adenosine-and-autism-recent-research-and-a-new-perspective

Mattson, M. P., Gleichmann, M., and Cheng, A. (2008). Mitochondria in neuroplasticity and neurological disorders. *Neuron* 60, 748–766. doi: 10.1016/j.neuron.2008.10.010

Mayer, E. A., Tillisch, K., and Gupta, A. (2015). Gut/brain axis and the microbiota. *J. Clin. Invest.* 125, 926–938. doi: 10.1172/JCI76304

McDaniel, S. S., Rensing, N. R., Thio, L. L., Yamada, K. A., and Wong, M. (2011). The ketogenic diet inhibits the mammalian target of rapamycin (mTOR) pathway. *Epilepsia* 52, e7–e11. doi: 10.1111/j.1528-1167.2011.02981.x

McFarlane, H. G., Kusek, G. K., Yang, M., Phoenix, J. L., Bolivar, V. J., and Crawley, J. N. (2008). Autism-like behavioral phenotypes in BTBR T^{+}tf/J mice. Genes, brain, and behavior 7, 152–163. doi: 10.1111/j.1601-183X.2007.00330.x

Meyza, K. Z., Defensor, E. B., Jensen, A. L., Corley, M. J., Pearson, B. L., Pobbe, R. L., et al. (2013). The BTBR $T^{+}tf$/J mouse model for autism spectrum disorders-in search of biomarkers. *Behav. Brain Res.* 251, 25–34. doi: 10.1016/j.bbr.2012.07.021

Moreno, H., Borjas, L., Arrieta, A., Saez, L., Prassad, A., Estevez, J., et al. (1992). [Clinical heterogeneity of the autistic syndrome: a study of 60 families]. *Invest. Clin.* 33, 13–31.

Moy, S. S., Nadler, J. J., Young, N. B., Perez, A., Holloway, L. P., Barbaro, R. P., et al. (2007). Mouse behavioral tasks relevant to autism: phenotypes of 10 inbred strains. *Behav. Brain Res.* 176, 4–20. doi: 10.1016/j.bbr.2006.07.030

Muller-Schwarze, A. B., Tandon, P., Liu, Z., Yang, Y., Holmes, G. L., and Stafstrom, C. E. (1999). Ketogenic diet reduces spontaneous seizures and mossy fiber sprouting in the kainic acid model. *Neuroreport* 10, 1517–1522. doi: 10.1097/00001756-199905140-00023

Murphy, M. P. (2009). How mitochondria produce reactive oxygen species. *Biochem. J.* 417, 1–13. doi: 10.1042/BJ20081386

Mychasiuk, R., and Rho, J. M. (2016). Genetic modifications associated with ketogenic diet treatment in the $BTBR^{T+Tf/J}$ mouse model of autism spectrum disorder. *Autism Res.* doi: 10.1002/aur.1682. [Epub ahead of print].

Napoli, E., Duenas, N., and Giulivi, C. (2014). Potential therapeutic use of the ketogenic diet in autism spectrum disorders. *Front. Pediatrics* 2:69. doi: 10.3389/fped.2014.00069

Nava, C., Lamari, F., Héron, D., Mignot, C., Rastetter, A., Keren, B., et al. (2012). Analysis of the chromosome X exome in patients with autism spectrum disorders identified novel candidate genes, including *TMLHE*. *Transl. Psychiatry* 2, e179. doi: 10.1038/tp.2012.102

Naviaux, R. K., Zolkipli, Z., Wang, L., Nakayama, T., Naviaux, J. C., Le, T. P., et al. (2013). Antipurinergic therapy corrects the autism-like features in

the poly(IC) mouse model. *PLoS ONE* 8:e57380. doi: 10.1371/journal.pone.0057380

Naviaux, J. C., Schuchbauer, M. A., Li, K., Wang, L., Risbrough, V. B., Powell, S. B., et al. (2014). Reversal of autism-like behaviors and metabolism in adult mice with single-dose antipurinergic therapy. *Transl. Psychiatry* 4, e400. doi: 10.1038/tp.2014.33

Naviaux, J. C., Wang, L., Li, K., Bright, A. T., Alaynick, W. A., Williams, K. R., et al. (2015). Antipurinergic therapy corrects the autism-like features in the Fragile X (*Fmr1* knockout) mouse model. *Mol. Autism* 6:1. doi: 10.1186/2040-2392-6-1

Neal, E. G., Chaffe, H., Schwartz, R. H., Lawson, M. S., Edwards, N., Fitzsimmons, G., et al. (2008). The ketogenic diet for the treatment of childhood epilepsy: a randomised controlled trial. *Lancet Neurol.* 7, 500–506. doi: 10.1016/S1474-4422(08)70092-9

Nelson, S. B., and Valakh, V. (2015). Excitatory/inhibitory balance and circuit homeostasis in autism spectrum disorders. *Neuron* 87, 684–698. doi: 10.1016/j.neuron.2015.07.033

Newell, C., Bomhof, M. R., Reimer, R. A., Hittel, D. S., Rho, J. M., and Shearer, J. (2016). Ketogenic diet modifies the gut microbiota in a murine model of autism spectrum disorder. *Mol. Autism* 7:37. doi: 10.1186/s13229-016-0099-3

Nie, D., Chen, Z., Ebrahimi-Fakhari, D., Di Nardo, A., Julich, K., Robson, V. K., et al. (2015). The stress-induced Atf3-gelsolin cascade underlies dendritic spine deficits in neuronal models of tuberous sclerosis complex. *J. Neurosci.* 35, 10762–10772. doi: 10.1523/JNEUROSCI.4796-14.2015

Norkett, R., Modi, S., Birsa, N., Atkin, T. A., Ivankovic, D., Pathania, M., et al. (2016). DISC1-dependent regulation of mitochondrial dynamics controls the morphogenesis of complex neuronal dendrites. *J. Biol. Chem.* 291, 613–629. doi: 10.1074/jbc.M115.699447

Nylen, K., Velazquez, J. L., Likhodii, S. S., Cortez, M. A., Shen, L., Leshchenko, Y., et al. (2008). A ketogenic diet rescues the murine succinic semialdehyde dehydrogenase deficient phenotype. *Exp. Neurol.* 210, 449–457. doi: 10.1016/j.expneurol.2007.11.015

Oliveira, G., Diogo, L., Grazina, M., Garcia, P., Ataíde, A., Marques, C., et al. (2005). Mitochondrial dysfunction in autism spectrum disorders: a population-based study. *Dev. Med. Child Neurol.* 47, 185–189. doi: 10.1017/S0012162205000332

Orinstein, A. J., Helt, M., Troyb, E., Tyson, K. E., Barton, M. L., Eigsti, I. M., et al. (2014). Intervention for optimal outcome in children and adolescents with a history of autism. *J. Dev. Behav. Pediatrics* 35, 247–256. doi: 10.1097/DBP.0000000000000037

Palmieri, L., Papaleo, V., Porcelli, V., Scarcia, P., Gaita, L., Sacco, R., et al. (2010). Altered calcium homeostasis in autism-spectrum disorders: evidence from biochemical and genetic studies of the mitochondrial aspartate/glutamate carrier AGC1. *Mol. Psychiatry* 15, 38–52. doi: 10.1038/mp.2008.63

Pancrazi, L., Di Benedetto, G., Colombaioni, L., Della Sala, G., Testa, G., Olimpico, F., et al. (2015). Foxg1 localizes to mitochondria and coordinates cell differentiation and bioenergetics. *Proc. Natl. Acad. Sci. U.S.A.* 112, 13910–13915. doi: 10.1073/pnas.1515190112

Park, M. J., Aja, S., Li, Q., Degano, A. L., Penati, J., Zhuo, J., et al. (2014). Anaplerotic triheptanoin diet enhances mitochondrial substrate use to remodel the metabolome and improve lifespan, motor function, and sociability in MeCP2-null mice. *PLoS ONE* 9:e109527. doi: 10.1371/journal.pone.0109527

Patterson, P. H. (2011). Maternal infection and immune involvement in autism. *Trends Mol. Med.* 17, 389–394. doi: 10.1016/j.molmed.2011.03.001

Pearl, P. L., Gibson, K. M., Acosta, M. T., Vezina, L. G., Theodore, W. H., Rogawski, M. A., et al. (2003). Clinical spectrum of succinic semialdehyde dehydrogenase deficiency. *Neurology* 60, 1413–1417. doi: 10.1212/01.WNL.0000059549.70717.80

Percy, A. K. (2011). Rett syndrome: exploring the autism link. *Arch. Neurol.* 68, 985–989. doi: 10.1001/archneurol.2011.149

Pilorge, M., Fassier, C., Le Corronc, H., Potey, A., Bai, J., De Gois, S., et al. (2016). Genetic and functional analyses demonstrate a role for abnormal glycinergic signaling in autism. *Mol. Psychiatry* 21, 936–945. doi: 10.1038/mp.2015.139

Poling, J. S., Frye, R. E., Shoffner, J., and Zimmerman, A. W. (2006). Developmental regression and mitochondrial dysfunction in a child with autism. *J. Child Neurol.* 21, 170–172. doi: 10.1177/08830738060210021401

Rahman, S. (2012). Mitochondrial disease and epilepsy. *Dev. Med. Child Neurol.* 54, 397–406. doi: 10.1111/j.1469-8749.2011.04214.x

Raichle, M. E., and Gusnard, D. A. (2002). Appraising the brain's energy budget. *Proc. Natl. Acad. Sci. U.S.A.* 99, 10237–10239. doi: 10.1073/pnas.172399499

Ramoz, N., Reichert, J. G., Smith, C. J., Silverman, J. M., Bespalova, I. N., Davis, K. L., et al. (2004). Linkage and association of the mitochondrial aspartate/glutamate carrier *SLC25A12* gene with autism. *Am. J. Psychiatry* 161, 662–669. doi: 10.1176/appi.ajp.161.4.662

Rizzuto, R., De Stefani, D., Raffaello, A., and Mammucari, C. (2012). Mitochondria as sensors and regulators of calcium signalling. *Nat. Rev. Mol. Cell Biol.* 13, 566–578. doi: 10.1038/nrm3412

Rose, S., Melnyk, S., Trusty, T. A., Pavliv, O., Seidel, L., Li, J., et al. (2012). Intracellular and extracellular redox status and free radical generation in primary immune cells from children with autism. *Autism Res. Treat.* 2012:986519. doi: 10.1155/2012/986519

Rose, S., Frye, R. E., Slattery, J., Wynne, R., Tippett, M., Pavliv, O., et al. (2014). Oxidative stress induces mitochondrial dysfunction in a subset of autism lymphoblastoid cell lines in a well-matched case control cohort. *PLoS ONE* 9:e85436. doi: 10.1371/journal.pone.0085436

Rossignol, D. A., and Frye, R. E. (2012). Mitochondrial dysfunction in autism spectrum disorders: a systematic review and meta-analysis. *Mol. Psychiatry* 17, 290–314. doi: 10.1038/mp.2010.136

Roullet, F. I., Lai, J. K., and Foster, J. A. (2013). *In utero* exposure to valproic acid and autism–a current review of clinical and animal studies. *Neurotoxicol. Teratol.* 36, 47–56. doi: 10.1016/j.ntt.2013.01.004

Ruskin, D. N., Fortin, J. A., Bisnauth, S. N., and Masino, S. A. (2016). Ketogenic diets improve behaviors associated with autism spectrum disorder in a sex-specific manner in the EL mouse. *Physiol. Behav.* 168, 138–145. doi: 10.1016/j.physbeh.2016.10.023

Ruskin, D. N., Kawamura, M., and Masino, S. A. (2009). Reduced pain and inflammation in juvenile and adult rats fed a ketogenic diet. *PLoS ONE* 4:e8349. doi: 10.1371/journal.pone.0008349

Ruskin, D. N., Svedova, J., Cote, J. L., Sandau, U., Rho, J. M., Kawamura, M. Jr., et al. (2013). Ketogenic diet improves core symptoms of autism in BTBR mice. *PLoS ONE* 8:e65021. doi: 10.1371/journal.pone.0065021

Sakurai, T., Ramoz, N., Barreto, M., Gazdoiu, M., Takahashi, N., Gertner, M., et al. (2010). *Slc25a12* disruption alters myelination and neurofilaments: a model for a hypomyelination syndrome and childhood neurodevelopmental disorders. *Biol. Psychiatry* 67, 887–894. doi: 10.1016/j.biopsych.2009.08.042

Sandin, S., Lichtenstein, P., Kuja-Halkola, R., Larsson, H., Hultman, C. M., and Reichenberg, A. (2014). The familial risk of autism. *JAMA* 311, 1770–1777. doi: 10.1001/jama.2014.4144

Santini, E., Turner, K. L., Ramaraj, A. B., Murphy, M. P., Klann, E., and Kaphzan, H. (2015). Mitochondrial superoxide contributes to hippocampal synaptic dysfunction and memory deficits in Angelman syndrome model mice. *J. Neurosci.* 35, 16213–16220. doi: 10.1523/JNEUROSCI.2246-15.2015

Segurado, R., Conroy, J., Meally, E., Fitzgerald, M., Gill, M., and Gallagher, L. (2005). Confirmation of association between autism and the mitochondrial aspartate/glutamate carrier *SLC25A12* gene on chromosome 2q31. *Am. J. Psychiatry* 162, 2182–2184. doi: 10.1176/appi.ajp.162.11.2182

Silverman, J. M., Buxbaum, J. D., Ramoz, N., Schmeidler, J., Reichenberg, A., Hollander, E., et al. (2008). Autism-related routines and rituals associated with a mitochondrial aspartate/glutamate carrier SLC25A12 polymorphism. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 147, 408–410. doi: 10.1002/ajmg.b.30614

Smith, J. D., Rho, J. M., Masino, S. A., and Mychasiuk, R. (2014). Inchworming: a novel motor stereotypy in the BTBR T^{+} $Itpr3^{tf}$/J mouse model of autism. *J. Visual. Exp.* 50791. doi: 10.3791/50791

Spilioti, M., Evangeliou, A. E., Tramma, D., Theodoridou, Z., Metaxas, S., Michailidi, E., et al. (2013). Evidence for treatable inborn errors of metabolism in a cohort of 187 Greek patients with autism spectrum disorder (ASD). *Front. Hum. Neurosci.* 7:858. doi: 10.3389/fnhum.2013.00858

Stafstrom, C. E., and Rho, J. M. (2012). The ketogenic diet as a treatment paradigm for diverse neurological disorders. *Front. Pharmacol.* 3:59. doi: 10.3389/fphar.2012.00059

Stromland, K., Nordin, V., Miller, M., Akerstrom, B., and Gillberg, C. (1994). Autism in thalidomide embryopathy: a population study. *Dev. Med. Child Neurol.* 36, 351–356. doi: 10.1111/j.1469-8749.1994.tb11856.x

Su, H., Fan, W., Coskun, P. E., Vesa, J., Gold, J.-A., Jiang, Y.-H., et al. (2011). Mitochondrial dysfunction in CA1 hippocampal neurons of the *UBE3A* deficient mouse model for Angelman syndrome. *Neurosci. Lett.* 487, 129–133. doi: 10.1016/j.neulet.2009.06.079

Subramanian, M., Timmerman, C. K., Schwartz, J. L., Pham, D. L., and Meffert, M. K. (2015). Characterizing autism spectrum disorders by key biochemical pathways. *Front. Neurosci.* 9:313. doi: 10.3389/fnins.2015.00313

Suen, D. F., Norris, K. L., and Youle, R. J. (2008). Mitochondrial dynamics and apoptosis. *Genes Dev.* 22, 1577–1590. doi: 10.1101/gad.1658508

Tang, G., Gutierrez Rios, P., Kuo, S.-H., Akman, H. O., Rosoklija, G., Tanji, K., et al. (2013). Mitochondrial abnormalities in temporal lobe of autistic brain. *Neurobiol. Dis.* 54, 349–361. doi: 10.1016/j.nbd.2013.01.006

Tigerholm, J., Borjesson, S. I., Lundberg, L., Elinder, F., and Fransen, E. (2012). Dampening of hyperexcitability in CA1 pyramidal neurons by polyunsaturated fatty acids acting on voltage-gated ion channels. *PLoS ONE* 7:e44388. doi: 10.1371/journal.pone.0044388

Tordjman, S., Somogyi, E., Coulon, N., Kermarrec, S., Cohen, D., Bronsard, G., et al. (2014). Gene x Environment interactions in autism spectrum disorders: role of epigenetic mechanisms. *Front. Psychiatry* 5:53. doi: 10.3389/fpsyt.2014.00053

Turunen, J. A., Rehnstrom, K., Kilpinen, H., Kuokkanen, M., Kempas, E., and Ylisaukko-Oja, T. (2008). Mitochondrial aspartate/glutamate carrier SL*C25A12* gene is associated with autism. *Autism Res.* 1, 189–192. doi: 10.1002/aur.25

Veenstra-Vanderweele, J., Christian, S. L., and Cook, E. H. Jr. (2004). Autism as a paradigmatic complex genetic disorder. *Annu. Rev. Genomics Hum. Genet.* 5, 379–405. doi: 10.1146/annurev.genom.5.061903.180050

Voskuyl, R. A., and Vreugdenhil, M. (2001). "Effects of essential fatty acids on voltage-regulated ionic channels and seizure thresholds in animals," in *Fatty Acids: Physiological and Behavioral Functions*, eds D. Mostofsky, S. Yehuda Jr., and N. Salem (Totowa, NJ: Humana Press), 63–78.

Weinberg, S. E., Sena, L. A., and Chandel, N. S. (2015). Mitochondria in the regulation of innate and adaptive immunity. *Immunity* 42, 406–417. doi: 10.1016/j.immuni.2015.02.002

Wen, Y., Alshikho, M. J., and Herbert, M. R. (2016). Pathway network analyses for autism reveal multisystem involvement, major overlaps with other diseases and convergence upon MAPK and calcium signaling. *PLoS ONE* 11:e0153329. doi: 10.1371/journal.pone.0153329

Wong, S., Napoli, E., Krakowiak, P., Tassone, F., Hertz-Picciotto, I., and Giulivi, C. (2016). Role of *p53*, mitochondrial DNA deletions, and paternal age in autism: a case-control study. *Pediatrics* 137:e20151888. doi: 10.1542/peds.2015-1888

Xavier, J. M., Rodrigues, C. M. P., and Solá, S. (2016). Mitochondria: major regulators of neural development. *Neuroscientist* 22, 346–358. doi: 10.1177/1073858415585472

Xie, Z., Jones, A., Deeney, J. T., Hur, S. K., and Bankaitis, V. A. (2016). Inborn Errors of Long-Chain fatty acid β-oxidation link neural stem cell self-renewal to autism. *Cell Rep.* 14, 991–999. doi: 10.1016/j.celrep.2016.01.004

Xu, X. P., Sun, R. P., and Jin, R. F. (2006). Effect of ketogenic diet on hippocampus mossy fiber sprouting and GluR5 expression in kainic acid induced rat model. *Chinese Med. J.* 119, 1925–1929.

Yudkoff, M., Daikhin, Y., Melo, T. M., Nissim, I., Sonnewald, U., and Nissim, I. (2007). The ketogenic diet and brain metabolism of amino acids: relationship to the anticonvulsant effect. *Annu. Rev. Nutr.* 27, 415–430. doi: 10.1146/annurev.nutr.27.061406.093722

Zhao, Y., Fung, C., Shin, D., Shin, B.-C., Thamotharan, S., Sankar, R., et al. (2010). Neuronal glucose transporter isoform 3 deficient mice demonstrate features of autism spectrum disorders. *Mol. Psychiatry* 15, 286–299. doi: 10.1038/mp.2009.51

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Molecular Neuroscience

ORIGINAL RESEARCH
published: 03 June 2016
doi: 10.3389/fnmol.2016.00042

Adenosine Kinase Inhibition Protects against Cranial Radiation-Induced Cognitive Dysfunction

Munjal M. Acharya[1], Janet E. Baulch[1], Theresa A. Lusardi[2], Barrett. D. Allen[1], Nicole N. Chmielewski[1], Al Anoud D. Baddour[1], Charles L. Limoli[1] and Detlev Boison[2]*

[1] Department of Radiation Oncology, University of California, Irvine, CA, USA, [2] R. S. Dow Neurobiology Laboratories, Legacy Research Institute, Portland, OR, USA

Clinical radiation therapy for the treatment of CNS cancers leads to unintended and debilitating impairments in cognition. Radiation-induced cognitive dysfunction is long lasting; however, the underlying molecular and cellular mechanisms are still not well established. Since ionizing radiation causes microglial and astroglial activation, we hypothesized that maladaptive changes in astrocyte function might be implicated in radiation-induced cognitive dysfunction. Among other gliotransmitters, astrocytes control the availability of adenosine, an endogenous neuroprotectant and modulator of cognition, via metabolic clearance through adenosine kinase (ADK). Adult rats exposed to cranial irradiation (10 Gy) showed significant declines in performance of hippocampal-dependent cognitive function tasks [novel place recognition, novel object recognition (NOR), and contextual fear conditioning (FC)] 1 month after exposure to ionizing radiation using a clinically relevant regimen. Irradiated rats spent less time exploring a novel place or object. Cranial irradiation also led to reduction in freezing behavior compared to controls in the FC task. Importantly, immunohistochemical analyses of irradiated brains showed significant elevation of ADK immunoreactivity in the hippocampus that was related to astrogliosis and increased expression of glial fibrillary acidic protein (GFAP). Conversely, rats treated with the ADK inhibitor 5-iodotubercidin (5-ITU, 3.1 mg/kg, i.p., for 6 days) prior to cranial irradiation showed significantly improved behavioral performance in all cognitive tasks 1 month post exposure. Treatment with 5-ITU attenuated radiation-induced astrogliosis and elevated ADK immunoreactivity in the hippocampus. These results confirm an astrocyte-mediated mechanism where preservation of extracellular adenosine can exert neuroprotection against radiation-induced pathology. These innovative findings link radiation-induced changes in cognition and CNS functionality to altered purine metabolism and astrogliosis, thereby linking the importance of adenosine homeostasis in the brain to radiation injury.

Keywords: adenosine, adenosine kinase, astrogliosis, radiation, cancer therapy, cognition, neuroprotection

OPEN ACCESS

Edited by:
Marina Guizzetti, Oregon Health & Science University, USA

Reviewed by:
Samaneh Maysami, The University of Manchester, UK
Gary D. Housley, The University of New South Wales, Australia

****Correspondence:***
Munjal M. Acharya
macharya@uci.edu

Received: *01 March 2016*
Accepted: *20 May 2016*
Published: *03 June 2016*

Citation:
Acharya MM, Baulch JE, Lusardi TA, Allen BD, Chmielewski NN, Baddour AD, Limoli CL and Boison D (2016) Adenosine Kinase Inhibition Protects against Cranial Radiation-Induced Cognitive Dysfunction. Front. Mol. Neurosci. 9:42. doi: 10.3389/fnmol.2016.00042

INTRODUCTION

The adverse neurocognitive side effects of radiotherapy used to treat CNS cancers are unintended and largely unavoidable. It is now well-documented that major changes occur in the brain following exposure to clinical radiotherapy protocols, including severe morphologic and physiological damage that coincides with substantial depletion of CNS stem cell populations

(Monje et al., 2002; Mizumatsu et al., 2003; Rola et al., 2004; Limoli et al., 2007; Parihar et al., 2014a). These changes occur along with reductions in dendritic complexity and synaptic density of more mature neurons (Parihar and Limoli, 2013; Parihar et al., 2014b). Consequently, cranial radiotherapy causes substantial decrements in short- and long-term learning and memory function that persist well after exposure (Greene-Schloesser and Robbins, 2012; Greene-Schloesser et al., 2013). We have previously shown in rodent models that exposure to radiation leads to long lasting reductions in neural stem cell (NSC) proliferation, prolonged oxidative stress, inhibition of neurogenesis, elevated CNS inflammation and cognitive dysfunction (Acharya et al., 2009, 2010, 2011, 2014a,c; Lan et al., 2012; Parihar et al., 2014a). While these factors are likely to contribute to the disruption of CNS function, our current understanding of the molecular and cellular mechanisms underlying radiation-induced damage in the brain, and how they impact neurocognition, are limited.

Astroglial activation is known to be a major consequence of radiation-induced chronic injury (Zhou et al., 2011; Ballesteros-Zebadua et al., 2012; Osman et al., 2014). Astrocytes form complex networks by contacting thousands of synapses and any disruption of astrocytic function following exposure to radiation will disrupt the global homeostasis of the brain (Giaume et al., 2010; Pannasch et al., 2011). Astrocytes also constitute a 'sink' for the metabolic clearance of neurotransmitters and the signaling molecule adenosine (Boison, 2007, 2008, 2009, 2013; Halassa et al., 2007). Adenosine is a ubiquitous modulator of synaptic transmission and neuronal activity, exerting its functions via activation of $G_{i/o}$ protein coupled- A_1 and A_3 and G_s coupled A_{2A} and A_{2B} receptors (Boison, 2009, 2013; Boison et al., 2010; Diogenes et al., 2014). Mechanisms and physiologic functions of adenosine receptors have extensively been studied (for details see: Boison, 2013). A shift in the A_1/A_{2A} receptor ratio/activation during radiation-induced CNS injury may reinforce the excitatory tone at synapses and contribute to cognitive dysfunction. Therefore, adenosine regulates global brain function under normal physiological settings and under pathophysiological conditions to provide neuroprotection.

Due to the widespread distribution of adenosine receptors in the brain, a tight regulation of endogenous levels of adenosine is a necessity (Boison, 2007, 2008, 2009, 2013). Astrocytes play a key role in regulating the levels of extracellular adenosine through cytosolic adenosine kinase (ADK) (Boison, 2007, 2008, 2009, 2013). ADK, which phosphorylates adenosine to 5′-AMP, is considered to be the key metabolic enzyme for the regulation of extracellular adenosine in the brain (Lloyd and Fredholm, 1995). Thus, inhibition or knockout of ADK leads to rapid increases in extracellular adenosine, while overexpression of ADK leads to a reduction of the synaptic adenosine tone. During astrogliosis, ADK is overexpressed, thereby limiting the availability of synaptic adenosine that fosters neurodegeneration (Boison, 2007, 2008, 2009, 2013). Thus, metabolic regulation of adenosine by astroglial-synaptic compartments directly impacts neuronal plasticity. Importantly, relatively little is known about the impact of radiation-exposure on adenosine metabolism, astroglial and synaptic function and its correlation with cognitive function. We hypothesized that adenosine-dependent metabolic regulation is a key mechanism in ionizing radiation-induced neurodegeneration and cognitive dysfunction. Using a specific ADK inhibitor, this *proof-of-principle* study delineates the protective role of adenosine to attenuate radiation-induced cognitive decline.

MATERIALS AND METHODS

Animals, Irradiation and 5-ITU Treatment

All animal procedures described are in accordance with NIH guidelines and approved by the University of California Institutional Animal Care and Use Committee. Four month old male athymic nude (ATN) rats (Cr:NIH-Foxn1rnu, strain 316; Charles River, San Diego) were maintained in sterile housing conditions (20°C ± 1°C; 70% ± 10% humidity; 12 h:12 h light and dark cycle) and had free access to sterilized diet and water. Rats were divided into 4 experimental groups (8–10 animals per group): 0 Gy receiving vehicle (Con), 0 Gy receiving 5-iodotubercidin, 5-ITU (Con+5-ITU), 10 Gy head-only irradiation receiving vehicle (IRR) and 10 Gy head only irradiation receiving 5-ITU (IRR + 5-ITU). Animals showing signs of eye infection and/or neophobic behavior were excluded from the study. In order to augment adenosine signaling in the brain we decided to use a pharmacological approach using the well-characterized ADK inhibitor 5-iodotubercidin (5-ITU), which induces mild and transient sedation after brain penetration. The use of a pharmacological agent allows us to test a possible clinical route of therapeutic adenosine augmentation and to prepare for future cell transplantation approaches, which will require the use of immunocompromised ATN rats, used in the present study. The ADK inhibitor 5-ITU (HY-15424, NSC 113939, MedChem Express, Princeton, NJ, USA) was made up fresh daily by dissolving in saline with 2% ethanol (v/v, Sigma, St. Louis, MO, USA). Animals received either vehicle (2% ethanol in saline, i.p.) or 5-ITU daily (3.1 mg/kg, i.p.) for 6 days in order to precondition the brain with neuroprotective adenosine. One hour after the last 5-ITU injection, animals received 0 or 10 Gy head-only X-rays. For cranial irradiation, animals were anesthetized with isoflurane (5% for induction and 2% for maintenance of anesthesia), placed ventrally on the treatment table (XRAD 320 irradiator, Precision X-ray, North Branford, CT, USA) without restraint, and positioned under a collimated (1.0 cm^2 diameter) beam for head-only irradiation delivered at a dose rate of 1.10 Gy/min. 5-ITU dosing was based on our previous studies (Fedele et al., 2005; Williams-Karnesky et al., 2013). Neither irradiation nor 5-ITU treatment resulted in a change in the body weight of animals. All behavioral and immunohistochemical analyses were carried out at 1 month post-irradiation.

Behavior Testing

To determine the effect of 5-ITU treatment on radiation-induced alteration in hippocampal- and frontal cortex-dependent cognition, rats from each group were subjected to cognitive testing 1 month after irradiation. Behavioral testing was

conducted over 3 weeks and included two open arena, spontaneous exploration tasks (novel place recognition, NPR and NOR) followed by a fear conditioning (FC) task. Behavioral testing closely followed our previously described protocols (Acharya et al., 2009, 2015a; Christie et al., 2012) in immunocompromised animals. To avoid infections in our strain of rats, we avoided water-based test paradigms such as the Morris water maze and opted for NOR and NPR as open arena tests. All behavioral testing data were collected by independent, blinded observers and the average of these data was used to compute the results for each task. Animals were first subjected to the NPR task followed by the NOR task. For the NOR and NPR tasks, the 'head direction to zone' function in Ethovision XT (Noldus) was used to track object exploration. An animal was considered to be exploring an object when its head was oriented toward it and its nose was within a 1-cm radius. All experimenters were blinded to the experimental condition and animal identification. Furthermore, an additional observer blinded to all experimental conditions re-scored the behavioral data (video files) thereby confirming the automated tracking results of Ethovision XT independently. The average of both scores was used to compute all behavioral data. We did not observe animals climbing on the object or any neophobic behavior. NPR and NOR data are presented as a discrimination index (DI) and calculated as ([Novel location exploration time/Total exploration time] – [Familiar location exploration time/Total exploration time]) × 100. A positive index indicates that rats spent more time exploring novelty (i.e., switched objects or locations), while a negative score indicates that rats exhibited little or no preference for exploring novelty. The FC task was administered in three sequential phases over 3 days including a training phase, a context test and a cue test as described previously (Christie et al., 2012; Acharya et al., 2015a).

Immunohistochemistry

Following completion of behavioral testing, animals were euthanized and perfused (intracardiac) with 4% paraformaldehyde (Acros Organics) made in phosphate buffered saline (100 mM, pH 7.4, Gibco), brains were cryoprotected (10–30% sucrose gradient) and sectioned coronally (30 μm thick) using a cryostat (Leica Microsystems, Germany). For the dual-immunofluorescence analysis of ADK and glial fibrillary acidic protein (GFAP), the following antibodies were used: rabbit anti-ADK (from the same batch that was previously characterized and validated on knockout tissue; Gouder et al., 2004), mouse anti-GFAP (EMD Millipore), goat anti-rabbit or anti-mouse conjugated with Alexa Fluor 488 or 594 (Life Technologies/Invitrogen) and DAPI (Sigma–Aldrich). Representative sections (3–4 sections/animal, four animals/group) through the middle of the hippocampus were selected for staining and stored in Tris-buffered saline (TBS, 100 mM, pH 7.4, Sigma–Aldrich) overnight. Free floating sections were first rinsed in TBS followed by Tris-A (TBS with 0.1% Triton-X-100, Sigma–Aldrich), blocked with 10% normal goat serum (NGS with Tris-A, Sigma–Aldrich) and incubated overnight in a mixture of rabbit anti-ADK (1:3000) and mouse anti-GFAP (1:500) antibodies prepared in 3% NGS and Tris-A. The next day, the sections were treated with a mixture of goat anti-rabbit Alexa Fluor 488 (1:750) and goat anti-mouse Alexa Fluor 594 (1:500 dilution each) made with Tris-A and 3% NGS for 1 h. The sections were light protected, washed with Tris-A, and counterstained with DAPI nuclear dye (1 μmol/L in TBS, 15 min) for visualization of hippocampal morphology. Immunostained sections were rinsed in TBS and mounted on clean gelatin coated slides using SlowFade Anti-fade Gold mounting medium (Life Technologies/Invitrogen). ADK positive cells were visualized under fluorescence as green and GFAP as red fluorescence.

Confocal Microscopy, Image Processing and 3D Quantification of Immunoreactivity

Immunostained sections were imaged using a laser-scanning confocal microscope (Nikon Eclipse Ti C2) equipped with a 40× PlanApo oil-immersion lens (1.3 NA) and an NIS-Elements AR module (v4.30, Nikon). 30 z stacks (1024 bit depth) at 1 μm from three different fields (318 μm × 318 μm area) in each section were imaged from the dentate gyrus. ADK immunofluorescence was imaged with 493 nm excitation and 518 nm emissions and GFAP was imaged with 592 nm excitation and 617 nm emissions. Images were deconvoluted using the AutoQuant software (version X3.0.4, Media Cybernetics, Rockville, MD, USA) with 1.26867 × 1.26867 × 1 μm spacing, and wavelengths set at 447 nm (DAPI), 510 (ADK) and 594 nm (GFAP). An adaptive, 3D blinded deconvolution method was used (**Figure 3**). AutoQuant automatically creates and stores deconvoluted images for direct import into the Imaris module (version 8.1.2, Bitplane, Inc., Zurich, Switzerland). The 3D algorithm-based surface rendering and quantification of fluorescence intensity for ADK and GFAP were carried out in Imaris at 100% rendering quality. Each channel was analyzed separately. 3D surface rendering detects immunostained puncta (ADK) or cell processes [GFAP, satisfying pre-defined criteria, verified visually for accuracy] (**Figure 3**). A channel mean intensity filter was applied and minimum thresholds were used for all the experimental groups. The pre-set parameters were kept constant throughout the subsequent analysis of ADK and GFAP immunoreactivity. The quantification of astrocyte number (GFAP co-labeled with DAPI) was facilitated using the Co-localization and the Spot tools of Imaris module. ADK and GFAP data was expressed as a mean immunoreactivity (percentage) relative to unirradiated controls. The method is summarized in **Figure 3**.

Statistical Analysis

Statistical analyses were carried out using GraphPad Prism (v6). One-way ANOVA was used to assess the normal distribution of data and significance between control and irradiated groups receiving either vehicle or 5-ITU treatment. When overall group effects were found to be statistically significant, a Bonferroni multiple comparisons test was used to compare the IRR with individual experimental groups. For analysis of FC data, repeated measures two-way ANOVA were performed. All analyses considered a value of $P \leq 0.05$ to be statistically significant.

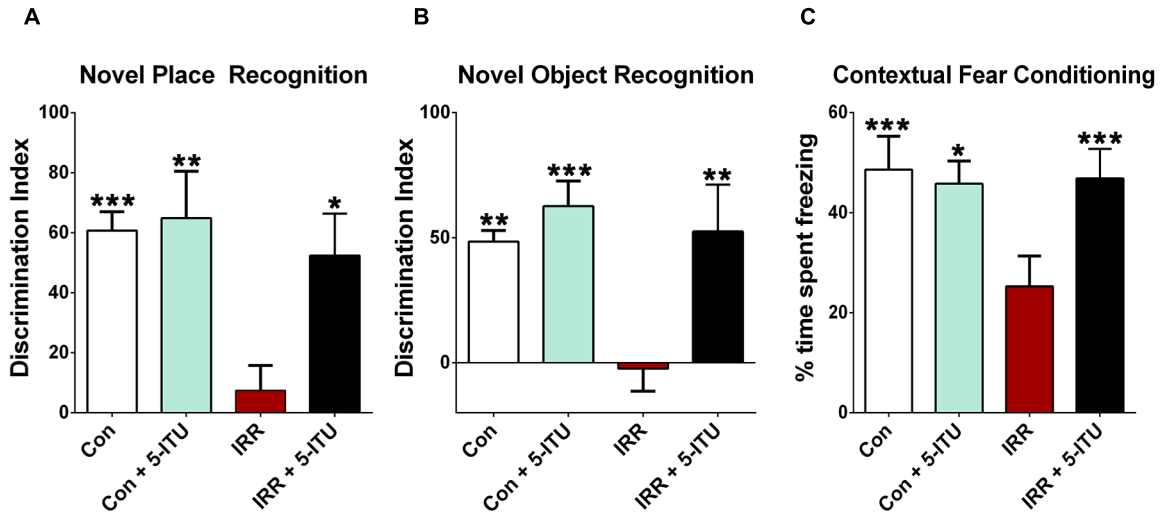


FIGURE 1 | Adenosine kinase (ADK) inhibition by systemic 5-iodotubercidin (5-ITU) treatment protects against radiation-induced cognitive dysfunction. Adult rats received 5-ITU (3.1 mg/kg, i.p., daily for 6 days) and were irradiated (0 or 10 Gy, head only) 1 h after the last injection. Animals were divided into four experimental groups: 0 or 10 Gy whole brain irradiated receiving either vehicle or 5-ITU (Con, Con + 5-ITU, IRR, IRR+5-ITU). **(A,B)** 1 month post-irradiation, animals were tested on spatial and episodic memory retention using the NPR and NOR tasks followed by fear conditioning (FC). The tendency to explore a novel place (NPR) or object (NOR) was derived from the Discrimination Index (DI). **(A,B)** Whole brain irradiation (IRR) shows significant behavioral deficits on NPR and NOR tasks compared to controls (Con and Con + 5-ITU) as indicated by impaired preference to a novel place or object. Irradiated animals treated with 5-ITU (IRR + 5-ITU) show significant preference for the novelty when compared with irradiated (IRR) animals receiving vehicle. **(C)** 5-ITU treatment also improves behavior on the hippocampal-dependent contextual FC task. The baseline freezing levels were comparable across groups, and all groups showed elevated freezing behavior following a series of 5 tone-shock pairings. The context test was administered 24 h later, and IRR animals showed significantly decreased freezing compared to controls (Con and Con + 5-ITU). Irradiated animals receiving 5-ITU showed a significant elevation in freezing behavior that was indistinguishable from the Con group. Data are presented as mean ± SEM. (N = 8–10 animals/group). P-values are derived from ANOVA and Bonferroni's multiple comparisons test. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ compared with the IRR group.

RESULTS

ADK Inhibition and Cognitive Function

Novel Place Recognition (NPR)

One month post-IRR, rats were habituated in an open field arena and then tested on the NPR task (**Figure 1A**). The ability to explore a novel spatial location on the NPR task is dependent on intact hippocampal function (Save et al., 1992; Mumby et al., 2002; Barker et al., 2007; Barker and Warburton, 2011). The total exploration of both objects during the familiarization and test phases were comparable between all groups for this task. The DI was calculated to measure preference or indifference for exploring novelty. A positive DI indicates a preference, or more time exploring the novel place, while a negative DI indicates indifference, or more time exploring the familiar object. Following a 1 h retention interval between the familiarization and test phases, a significant overall group effect was found for the DI [$F_{(3,28)} = 5.88$, $P = 0.003$] that differed between the groups. In the test phase, IRR animals spent significantly less time exploring the novel place compared to Con ($P = 0.001$), Con + 5-ITU ($P = 0.01$) and IRR + 5-ITU groups ($P = 0.05$, **Figure 1A**). Unirradiated animals receiving vehicle (Con) or 5-ITU (Con + 5-ITU) treatment showed comparable novel place exploration. Furthermore, after the 1 h retention interval, irradiated animals treated with 5-ITU (IRR + 5-ITU) did not differ from either Con or Con + 5-ITU animals. These data indicate that ADK inhibition by 5-ITU treatment prior to cranial IRR improved object location exploration on the NPR task as compared to irradiated animals receiving vehicle.

Novel Object Recognition

After NPR testing, rats were habituated and then tested on the NOR task 1 month post-IRR (**Figure 1B**). Impairment in prefrontal cortex and hippocampal function manifests as an inability to discriminate a novel from a familiar object in the NOR task (Barker et al., 2007; Barker and Warburton, 2011). The total exploration times for both objects were not different between all experimental groups for this task. In the test phase, a significant overall group difference was found between the four cohorts for the DI [$F_{(3,28)} = 8.95$, $P = 0.001$]. After a 5 min retention interval between the familiarization and test phases, Con and Con + 5-ITU rats showed a preference for the novel object (**Figure 1B**). However, irradiated rats receiving vehicle (IRR) showed a significantly diminished preference to explore novel object compared to either Con or Con + 5-ITU animals ($P < 0.01$). The novel object exploration for the Con and Con + 5-ITU animals did not differ. 5-ITU treated irradiated animals (IRR + 5-ITU) exhibited significantly improved performance on the NOR task compared to the IRR group ($P = 0.01$). The DIs for Con, Con + 5-ITU and IRR + 5-ITU groups

were statistically indistinguishable. Thus, 5-ITU treatment improved novel object exploration behavior in irradiated animals.

In summary, for each of the open arenas, episodic memory tasks (NPR and NOR), a preference toward novelty (as indicated by DI) was found to be significantly greater for Con, Con + 5-ITU and IRR + 5-ITU groups in comparison with IRR group (**Figures 1A,B**), demonstrating the protective effect of ADK inhibition. These spontaneous exploration tasks (NPR, NOR) rely on the innate curiosity of an animal to explore a 'new object placement' or a 'new object.' Other factors such as fatigue, depression and/or anxiety may also affect the overall performance on these tasks, although differences in exploration during either the habituation or familiarization phases of these tasks were not found. To account for these possible confounds, animals were subsequently tested in the FC task to interrogate hippocampal function using a task not reliant on spontaneous exploration.

Contextual Fear Conditioning (FC)

Three distinct phases of the FC task – training, context and cue – were administered over 3 days. Group means and 95% CIs for the post-training and context phases freezing (percent) were as follows: Post-training Con (mean = 97.6, 95% CI = 95.0–100.2); IRR (mean = 94.56, 95% CI = 88.66–100.5); Con + 5-ITU (mean = 80.32; 95% CI = 69.10–91.54); IRR + 5-ITU (mean = 82.17; 95% CI = 66.35–97.98); Context Con (mean = 50.71, 95% CI = 32.88–68.54); IRR (mean = 20.68, 95% CI = 9.34–32.02); Con + 5-ITU (mean = 45.81, 95% CI = 34.78–56.83); IRR + 5-ITU (mean = 51.11, 95% CI = 39.47–62.75). Repeated measure (RM) ANOVA for the context phase (**Figure 1C**) revealed significant differences between IRR and Con groups ($P = 0.001$); between IRR and Con + 5-ITU groups ($P = 0.001$) and between IRR and IRR+5-ITU groups ($P = 0.001$). Groups did not differ significantly in the freezing behavior across baseline, post-training, pre-cue and post-cue phases (data not shown), indicating a selective deficit on the hippocampal-dependent contextual memory phase of the task (Phillips and LeDoux, 1992; Winocur et al., 2006). Irradiation did not impair motor or sensory function, since all groups demonstrated significant increases in freezing behavior after the tone-shock pairings (post-training phase). Group means and 95% CIs for the cue phase (percent time freezing) were: Con (mean = 91.53, 95% CI = 82.36–100.7); IRR (mean = 85.71, 95% CI = 66.72–104.7); Con + 5-ITU (mean = 84.48; 95% CI = 73.56–95.41); IRR+5-ITU (mean = 93.86; 95% CI = 86.02-101.7). Thus, intact amygdala-dependent cued memory acquisition of the tone-shock pairing was not impaired, and that the deficit was specific to the hippocampal-dependent contextual memory (**Figure 1C**) in which the pairing was learned.

Radiation-Induced Elevation in ADK and Astrogliosis

To assess the impact of cranial irradiation on the status of ADK and astrocytes, immunoreactivity for ADK and GFAP was assessed via dual-immunofluorescence confocal microscopy (**Figure 2**). Representative confocal micrographs revealed a marked impact of cranial irradiation on ADK and GFAP immunoreactivity. Compared to Con and Con + 5-ITU groups, irradiated animals (IRR) showed increased expression of ADK in the hippocampal granule cell layer (GCL), sub-granular zone (SGZ) and dentate hilus (DH) at 1 month post-IRR (**Figure 2B**). Concurrently, GFAP staining in unirradiated controls (Con and Con + 5-ITU) show morphological characteristics consistent with resting astrocytes (**Figure 2C**). Astrocytes in the IRR group display enlarged cell bodies with thicker and longer processes; this is consistent with hypertrophic, reactive astrocytes, or astrogliosis (**Figure 2C**).

High resolution, 3D algorithm-based quantification of ADK, GFAP and astrocyte number from the confocal z stacks was facilitated by blinded deconvolution (AutoQuant) and subsequent analysis using Imaris module (**Figure 3**). ADK and GFAP quantitative immunofluorescence revealed a significant increase in the immunoreactivity of the IRR group compared to controls (Con and Con + 5-ITU, **Figure 4**). Cranial radiation exposure (IRR group) significantly elevated ADK levels by 1.5 fold ($P = 0.01$ vs. Con and $P = 0.02$ vs. Con + 5-ITU group) in the hippocampus 1 month after exposure (**Figure 4A**). In parallel, hippocampal GFAP immunoreactivity was elevated by ∼2 fold in the IRR group ($P = 0.001$, **Figure 4B**) without a significant change in the total number of astrocytes (**Figure 4C**) at 1 month. However, irradiated animals receiving 5-ITU treatment (IRR + 5-ITU) showed a significant reduction in ADK immunoreactivity ($P = 0.02$) and astrogliosis ($P = 0.001$) throughout the hippocampus compared to IRR animals. These qualitative (**Figure 2**) and quantitative (**Figure 4**) data demonstrate that pharmacological ADK inhibition could substantially protect against radiation-induced neuropathology.

DISCUSSION

Our findings demonstrate that adenosine's well-known protective role also extends to the attenuation of radiation-induced cognitive impairments. Our previous data demonstrated persistent, long-term cognitive impairments from 1 to 8 months following a single IRR exposure (Acharya et al., 2014b, 2015b; Parihar et al., 2014b, 2015), and suggest that global disruption of homeostatic functions in the brain combine to compromise cognitive performance over protracted post-IRR intervals. Several neurodegenerative conditions (Alzheimer's Parkinson's, ALS, epilepsy) share two key features with radiation-induced neuropathology: (i) astrogliosis as a histopathological hallmark and (ii) the onset of cognitive impairment (Bell and Zlokovic, 2009; Palop and Mucke, 2009; Aarsland and Kurz, 2010; Rusina et al., 2010). Astrocytes play a key role in regulating the levels of extracellular adenosine via cytosolic ADK to form a metabolic reuptake system. Our data show that cranial irradiation triggers astrogliosis and ADK overexpression 1 month after exposure that could lead to enhanced metabolic clearance of adenosine and resulting adenosine deficiency. Radiation-induced synaptotoxicity, astrogliosis, and adenosine deficiency in turn influence cognitive function (Boison and Aronica, 2015).

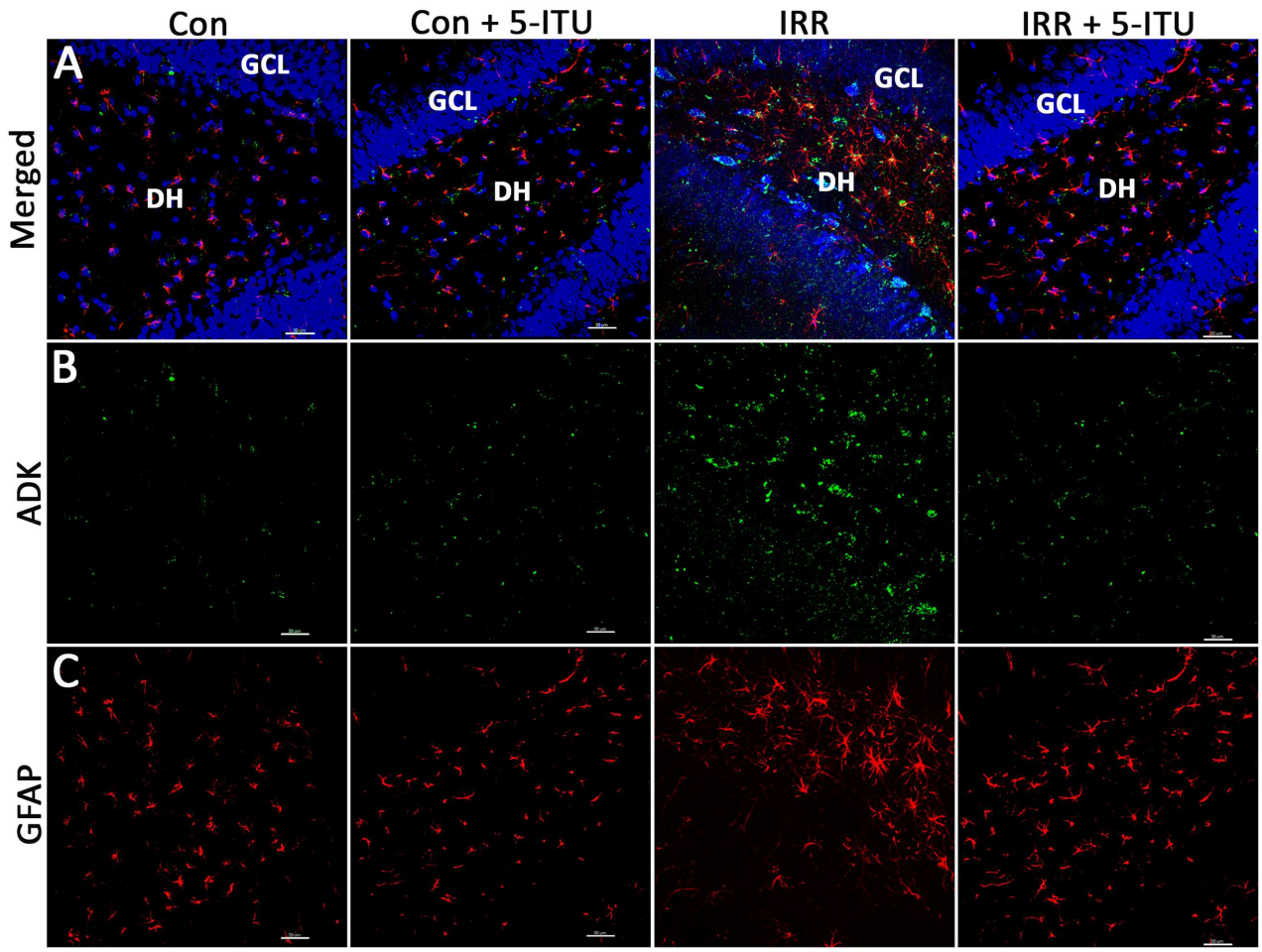


FIGURE 2 | Cranial irradiation elevates adenosine kinase (ADK) immunoreactivity and astrogliosis. Immunofluorescence analysis demonstrates that at 1 month post-treatment, compared to controls (Con and Con + 5-ITU), exposure to cranial irradiation (10 Gy) leads to elevated ADK immunoreactivity (**A,B**; IRR group; ADK, green; DAPI nuclear counterstain, blue) that is reduced to control levels in irradiated animals treated with 5-ITU (IRR + 5-ITU). Representative confocal micrographs show the presence reactive astrocytic cell bodies (**A,C**; glial fibrillary acidic protein; GFAP, red) in the hippocampal dentate hilus (DH), sub-granular zone and granule cell layer (GCL) indicating astrogliosis. IRR + 5-ITU animals showed reduced ADK and GFAP immunoreactivity compared to IRR animals. Scale bar: 30 μm.

Our data critically test our hypothesis that adenosine augmentation prior to irradiation is protective against radiation-induced neuropathology. Animals receiving the ADK-inhibitor prior to cranial IRR were characterized by improved behavioral performance as characterized in three distinct tasks to assess cognitive function (**Figure 1**). Treatment with 5-ITU prior to irradiation (IRR + 5-ITU) prevented development of radiation-induced memory impairments on the NPR and NOR tasks at 1 month post-exposure. In contrast to irradiated rats receiving vehicle (IRR), DIs of irradiated animals with 5-ITU treatment (IRR + 5-ITU) were indistinguishable from unirradiated controls, where both controls (Con) and 5-ITU injected animals (Con + 5-ITU) showed significant preference for exploring the novel place or object. Moreover, unirradiated animals receiving 5-ITU were statistically indistinguishable from the controls receiving vehicle. In these preventative studies we chose a time point of analysis (4 weeks after irradiation), which reflects the delayed onset of cognitive dysfunction after radiation therapy (Tofilon and Fike, 2000) in combination with the prophylactic use of an ADK inhibitor. Whether, prophylactic ADK inhibition affects cognitive function at different time points post irradiation, or whether post-irradiation treatment with an ADK inhibitor might be of therapeutic benefit has not been addressed here, but might be interesting to investigate in future work.

The effectiveness of ADK inhibition to prevent radiation-induced behavioral deficits was further confirmed using the contextual FC task (**Figure 1C**) that engages the hippocampus and does not rely on spontaneous exploration (Phillips and LeDoux, 1992; Winocur et al., 2006). Irradiated animals (IRR) spent significantly less time in freezing than Con and Con + 5-ITU cohorts during the context phase of the FC task. These

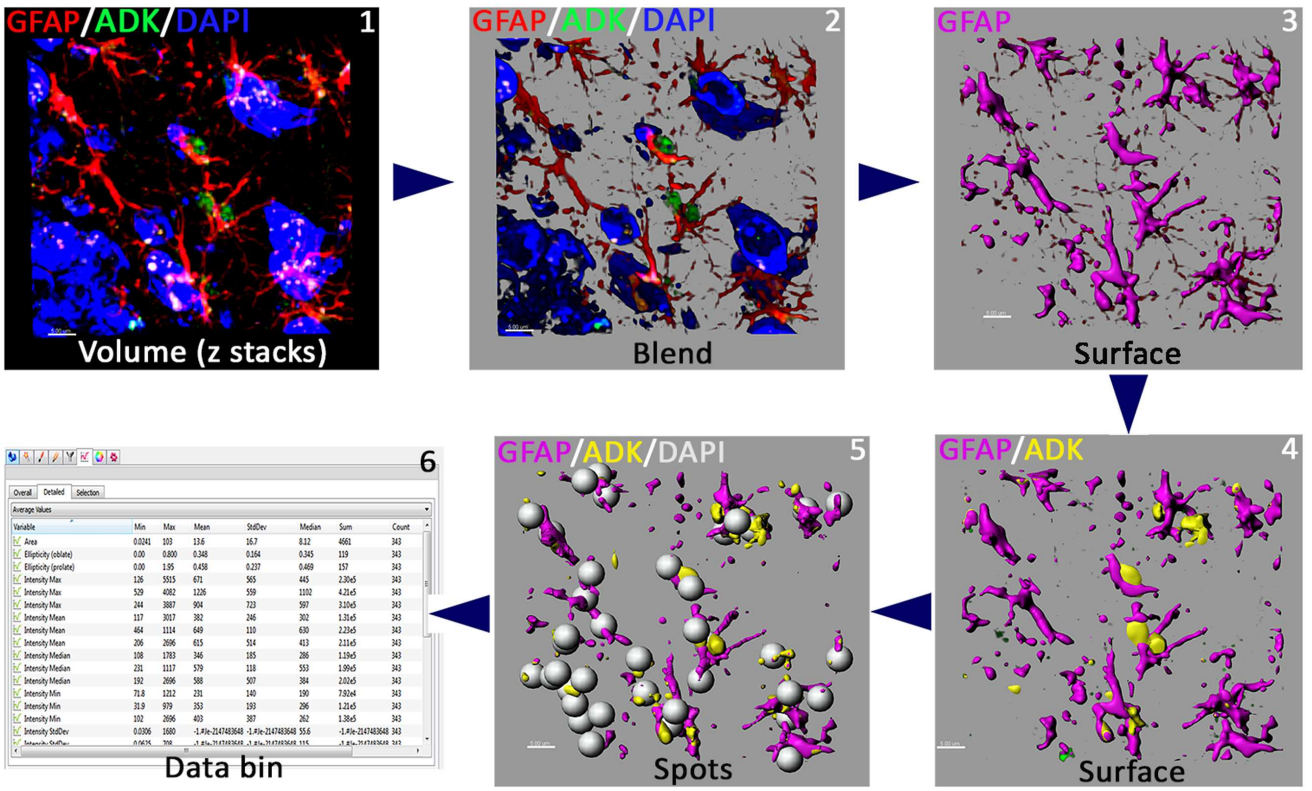


FIGURE 3 | Work flow of quantitative immunofluorescence. Blinded deconvoluted volume of z stacks (step 1) were uploaded into Imaris (v8.1.2) for quantification of GFAP and ADK immunoreactivity (step 2). 3D algorithm-based Surface rendering of individual channels (pseudo-colored, steps 3–4) provide quantitative analyses of fluorescence intensity (GFAP, ADK) and Spot tool and Co-localization modules provide quantification of the number of astrocytes (steps 4–5). Data bin (step 6) provide quantitative immunofluorescence data for the individual channels. Scale bars: 5 μm.

data suggest that irradiation disrupted long-term (24 h) memory for the tone-shock (context) association that has been shown to rely on intact hippocampal function (Phillips and LeDoux, 1992; Winocur et al., 2006). Importantly, animals treated with 5-ITU prior to irradiation (IRR + 5-ITU) exhibited intact freezing behavior, and were statistically indistinguishable from Con and Con + 5-ITU animals in their contextual fear memory. This finding indicates that radiation-induced deficits in hippocampal-dependent long-term memory function may be prevented by ADK inhibitor-induced adenosine augmentation at the time of irradiation. The amount of post-training freezing observed was comparable between all experimental cohorts, suggesting that experimental procedures did not affect initial acquisition of the conditioned freezing response and memory consolidation. Similarly, irradiation or 5-ITU treatments did not affect freezing behavior during the cue test phase, indicating intact amygdala-dependent acquisition and memory formation (Phillips and LeDoux, 1992; Winocur et al., 2006). The specific deficits observed in contextual fear memory tasks are consistent with the impairments in the NPR and NOR tasks and suggest that cranial irradiation disrupts hippocampal and frontal cortex function, and pre-IRR treatment with an ADK inhibitor prevents radiation-induced cognitive deficits.

Our data clearly show that pharmacological inhibition of ADK can prevent a decline in cognition following cranial irradiation. In the present study, we demonstrate that in the irradiated brain (IRR), ADK is co-expressed in GFAP-positive reactive astrocytes, which are characterized by a hypertrophic morphology with larger soma and increased length and width of astrocytic stellae compared to unirradiated controls (**Figures 2A,B**). The total number of $GFAP^+$ astrocytes did not differ between control and irradiated groups (**Figure 4C**). These findings indicate that astrogliosis is accompanied by ADK overexpression. Quantification of ADK and GFAP immunoreactivity by high resolution confocal microscopy showed a marked rise of fluorescence intensity in the irradiated hippocampus (**Figures 4A,B**) whereas, pre-treatment with the ADK inhibitor, 5-ITU, prevented increases in ADK and GFAP immunoreactivity in the irradiated brain. It is likely that pre-treatment with 5-ITU attenuated the initial radiation-induced injury and therefore the very processes that eventually cause increases in GFAP and ADK immunoreactivity, although epigenetic mechanisms (Williams-Karnesky et al., 2013; Boison, 2016) might also be implicated. At the doses used, 5-ITU is not known to exert any cytotoxic or apoptotic effects on astrocytes (Ugarkar et al., 2000).

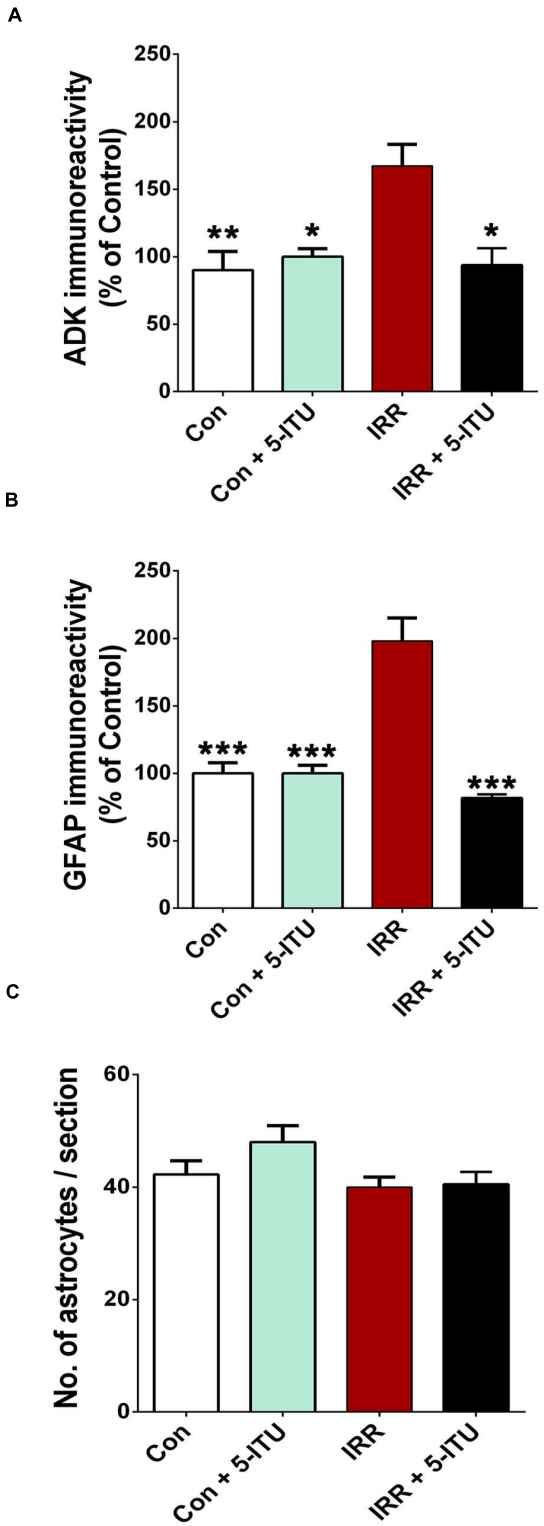


FIGURE 4 | Treatment with 5-iodotubercidin (5-ITU) attenuated radiation-induced elevation in adenosine kinase (ADK) and astrogliosis. 3D algorithm-based deconvolution and quantification (Imaris, Bitplane, Inc.) of ADK and glial fibrillary acidic protein (GFAP)

(Continued)

FIGURE 4 | Continued

immunoreactivity show that compared to controls (Con and Con + 5-ITU), irradiation significantly increased the ADK **(A)** and astrogliotic cell bodies **(B)** in the hippocampal dentate hilus, granule cell layer, sub-granular zone and CA3/CA1 subfields. Compared with the irradiated cohort (IRR), animals receiving 5-ITU (IRR + 5-ITU) had significantly lower ADK and GFAP immunoreactivity in all hippocampal subfields. The reduced ADK and GFAP immunofluorescence was comparable to controls (Con). The number of astrocytes per hippocampal section did not change after irradiation or 5-ITU treatment **(C)**. All data are presented as mean ± SEM. ($N = 4$ animals per group). $^{*}P < 0.02$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with the IRR group (ANOVA and Bonferroni multiple comparisons test).

Chronic inflammation is a hallmark of the irradiated brain that is linked with cognitive decline (Zhao and Robbins, 2009; Moravan et al., 2011; Belarbi et al., 2013; Acharya et al., 2014c; Parihar et al., 2014a). Our results suggest that: (1) increased ADK expression is associated with astrogliosis in the irradiated brain; ADK-induced adenosine deficiency in turn may contribute to the radiation-induced neuropathology, and (2) chronic upregulation of ADK in the irradiated brain can be prevented by the transient prophylactic administration of an ADK inhibitor; this finding is in line with a lack of radiation-induced cognitive impairments. Past studies have shown that 5-ITU was effective at increasing extracellular adenosine levels in the brain (Pazzagli et al., 1995; Boison and Stewart, 2009; Boison, 2013), and supports our current findings suggesting that inhibition of ADK prior to irradiation is neuroprotective through a similar mechanism. Inhibition of ADK reduced synaptotoxicity in the hippocampus by modulating adenosine receptors, indicating an important role of ADK in the regulation of basal extracellular adenosine (Pazzagli et al., 1995; Gouder et al., 2004; Boison and Stewart, 2009; Boison, 2013). (3) Lastly, the contribution of ADK expression to radiomimetic neuropathology would favor the development of adenosine-based therapeutic interventions such as stem cell therapies to augment adenosine signaling locally via transplanted adenosine releasing cells.

CONCLUSION

Our experimental data support the overall concept that a combination of neurotoxicity, astrogliosis, and elevation of ADK, resulting in a deficiency of extracellular adenosine can directly cause a broad spectrum of comorbid symptoms that are collectively present across several neurological conditions (Aronica et al., 2013; Boison and Aronica, 2015). If radiation-induced adenosine deficiency, triggered by ADK upregulation, is sufficient to precipitate neurocognitive impairments, then therapeutic adenosine augmentation (molecular, cellular or pharmacological) should ameliorate those symptoms. More work is needed to assess whether a neuroprotective treatment interferes with the therapeutic efficacy of radiotherapy or if transient treatment with ADK inhibitors post-irradiation are as effective as pre-irradiation treatments against radiation-induced CNS dysfunction. ADK inhibitors represent some of the most

promising adenosine elevating agents (Kowaluk et al., 2000; McGaraughty et al., 2005; Boison, 2013). Our experimental data support the concept that such therapeutic approaches might be useful as prophylactic pre-treatment to avoid radiation-induced cognitive impairment.

AUTHORS CONTRIBUTIONS

Conception and Design: JB, CL, DB, MA; Development of methodology: TL, BA, NC, AB, MA; Acquisition of data: BA, NC, AB, MA; Analysis and interpretation of data: JB, BA, CL, DB, MA; Writing, review and/or revision of manuscript: JB, TL, CL, DB, MA; Administrative, technical or material support: JB, CL, MA.

FUNDING

This work was supported by National Institutes of Health grant R01 MH083973 (DB) and 1R21 NS088024 (DB), UC Irvine School of Medicine 19900 (MA) and UC Irvine Institute for Clinical and Translational Science (ICTS) KL2 award, KL2TR001416 (MA).

ACKNOWLEDGMENTS

We thank Dr. Vipan K. Parihar for critical inputs in behavior data interpretation and Vahan Martirosian for behavior protocol optimization.

REFERENCES

Aarsland, D., and Kurz, M. W. (2010). The epidemiology of dementia associated with Parkinson disease. *J. Neurol. Sci.* 289, 18–22. doi: 10.1016/j.jns.2009.08.034

Acharya, M. M., Christie, L. A., Hazel, T. G., Johe, K. K., and Limoli, C. L. (2014a). Transplantation of human fetal-derived neural stem cells improves cognitive function following cranial irradiation. *Cell Transplant.* 23, 1255–1266. doi: 10.3727/096368913X670200

Acharya, M. M., Christie, L. A., Lan, M. L., Donovan, P. J., Cotman, C. W., Fike, J. R., et al. (2009). Rescue of radiation-induced cognitive impairment through cranial transplantation of human embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 106, 19150–19155. doi: 10.1073/pnas.0909293106

Acharya, M. M., Christie, L. A., Lan, M. L., Giedzinski, E., Fike, J. R., Rosi, S., et al. (2011). Human neural stem cell transplantation ameliorates radiation-induced cognitive dysfunction. *Cancer Res.* 71, 4834–4845. doi: 10.1158/0008-5472.CAN-11-0027

Acharya, M. M., Lan, M. L., Kan, V. H., Patel, N. H., Giedzinski, E., Tseng, B. P., et al. (2010). Consequences of ionizing radiation-induced damage in human neural stem cells. *Free Radic. Biol. Med.* 49, 1846–1855. doi: 10.1016/j.freeradbiomed.2010.08.021

Acharya, M. M., Martirosian, V., Chmielewski, N. N., Hanna, N., Tran, K. K., Liao, A. C., et al. (2015a). Stem cell transplantation reverses chemotherapy-induced cognitive dysfunction. *Cancer Res.* 75, 676–686. doi: 10.1158/0008-5472.CAN-14-2237

Acharya, M. M., Martirosian, V., Christie, L. A., and Limoli, C. L. (2014b). Long-term cognitive effects of human stem cell transplantation in the irradiated brain. *Int. J. Radiat. Biol.* 90, 816–820. doi: 10.3109/09553002.2014.927934

Acharya, M. M., Martirosian, V., Christie, L. A., Riparip, L., Strnadel, J., Parihar, V. K., et al. (2015b). Defining the optimal window for cranial transplantation of human induced pluripotent stem cell-derived cells to ameliorate radiation-induced cognitive impairment. *Stem Cells Transl. Med.* 4, 74–83. doi: 10.5966/sctm.2014-0063

Acharya, M. M., Rosi, S., Jopson, T., and Limoli, C. L. (2014c). Human neural stem cell transplantation provides long-term restoration of neuronal plasticity in the irradiated hippocampus. *Cell Transplant* 24, 691–702. doi: 10.3727/096368914X684600

Aronica, E., Sandau, U. S., Iyer, A., and Boison, D. (2013). Glial adenosine kinase–a neuropathological marker of the epileptic brain. *Neurochem. Int.* 63, 688–695. doi: 10.1016/j.neuint.2013.01.028

Ballesteros-Zebadua, P., Chavarria, A., Celis, M. A., Paz, C., and Franco-Perez, J. (2012). Radiation-induced neuroinflammation and radiation somnolence syndrome. *CNS Neurol. Disord. Drug Targets* 11, 937–949. doi: 10.2174/1871527311201070937

Barker, G. R., Bird, F., Alexander, V., and Warburton, E. C. (2007). Recognition memory for objects, place, and temporal order: a disconnection analysis of the role of the medial prefrontal cortex and perirhinal cortex. *J. Neurosci.* 27, 2948–2957. doi: 10.1523/JNEUROSCI.5289-06.2007

Barker, G. R., and Warburton, E. C. (2011). When is the hippocampus involved in recognition memory? *J. Neurosci.* 31, 10721–10731. doi: 10.1523/JNEUROSCI.6413-10.2011

Belarbi, K., Jopson, T., Arellano, C., Fike, J. R., and Rosi, S. (2013). CCR2 deficiency prevents neuronal dysfunction and cognitive impairments induced by cranial irradiation. *Cancer Res.* 73, 1201–1210. doi: 10.1158/0008-5472.CAN-12-2989

Bell, R. D., and Zlokovic, B. V. (2009). Neurovascular mechanisms and blood-brain barrier disorder in Alzheimer's disease. *Acta Neuropathol.* 118, 103–113. doi: 10.1007/s00401-009-0522-3

Boison, D. (2007). Adenosine as a modulator of brain activity. *Drug News Perspect.* 20, 607–611. doi: 10.1358/dnp.2007.20.10.1181353

Boison, D. (2008). Adenosine as a neuromodulator in neurological diseases. *Curr. Opin. Pharmacol.* 8, 2–7. doi: 10.1016/j.coph.2007.09.002

Boison, D. (2009). Adenosine-based modulation of brain activity. *Curr. Neuropharmacol.* 7, 158–159. doi: 10.2174/157015909789152173

Boison, D. (2013). Adenosine kinase: exploitation for therapeutic gain. *Pharmacol. Rev.* 65, 906–943. doi: 10.1124/pr.112.006361

Boison, D. (2016). The Biochemistry and epigenetics of epilepsy: focus on adenosine and glycine. *Front. Mol. Neurosci.* 9:26. doi: 10.3389/fnmol.2016.00026

Boison, D., and Aronica, E. (2015). Comorbidities in neurology: is adenosine the common link? *Neuropharmacology* 97, 18–34. doi: 10.1016/j.neuropharm.2015.04.031

Boison, D., Chen, J. F., and Fredholm, B. B. (2010). Adenosine signaling and function in glial cells. *Cell Death Differ.* 17, 1071–1082. doi: 10.1038/cdd.2009.131

Boison, D., and Stewart, K. A. (2009). Therapeutic epilepsy research: from pharmacological rationale to focal adenosine augmentation. *Biochem. Pharmacol.* 78, 1428–1437. doi: 10.1016/j.bcp.2009.08.005

Christie, L. A., Acharya, M. M., Parihar, V. K., Nguyen, A., Martirosian, V., and Limoli, C. L. (2012). Impaired cognitive function and hippocampal neurogenesis following cancer chemotherapy. *Clin. Cancer Res.* 18, 1954–1965. doi: 10.1158/1078-0432.CCR-11-2000

Diogenes, M. J., Neves-Tome, R., Fucile, S., Martinello, K., Scianni, M., Theofilas, P., et al. (2014). Homeostatic control of synaptic activity by endogenous adenosine is mediated by adenosine kinase. *Cereb. Cortex* 24, 67–80. doi: 10.1093/cercor/bhs284

Fedele, D. E., Gouder, N., Guttinger, M., Gabernet, L., Scheurer, L., Rulicke, T., et al. (2005). Astrogliosis in epilepsy leads to overexpression of adenosine kinase, resulting in seizure aggravation. *Brain* 128, 2383–2395. doi: 10.1093/brain/awh555

Giaume, C., Koulakoff, A., Roux, L., Holcman, D., and Rouach, N. (2010). Astroglial networks: a step further in neuroglial and gliovascular interactions. *Nat. Rev. Neurosci.* 11, 87–99. doi: 10.1038/nrn2757

Gouder, N., Scheurer, L., Fritschy, J. M., and Boison, D. (2004). Overexpression of adenosine kinase in epileptic hippocampus contributes to epileptogenesis. *J. Neurosci.* 24, 692–701. doi: 10.1523/JNEUROSCI.4781-03.2004

Greene-Schloesser, D., Moore, E., and Robbins, M. E. (2013). Molecular pathways: radiation-induced cognitive impairment. *Clin. Cancer Res.* 19, 2294–2300. doi: 10.1158/1078-0432.CCR-11-2903

Greene-Schloesser, D., and Robbins, M. E. (2012). Radiation-induced cognitive impairment–from bench to bedside. *Neuro Oncol.* 14, iv37–iv44. doi: 10.1093/neuonc/nos196

Halassa, M. M., Fellin, T., and Haydon, P. G. (2007). The tripartite synapse: roles for gliotransmission in health and disease. *Trends Mol. Med.* 13, 54–63. doi: 10.1016/j.molmed.2006.12.005

Kowaluk, E. A., Mikusa, J., Wismer, C. T., Zhu, C. Z., Schweitzer, E., Lynch, J. J., et al. (2000). ABT-702 (4-amino-5-(3-bromophenyl)-7-(6-morpholino-pyridin- 3-yl)pyrido[2,3-d]pyrimidine), a novel orally effective adenosine kinase inhibitor with analgesic and anti-inflammatory properties. II. In vivo characterization in the rat. *J. Pharmacol. Exp. Ther.* 295, 1165–1174.

Lan, M. L., Acharya, M. M., Tran, K. K., Bahari-Kashani, J., Patel, N. H., Strnadel, J., et al. (2012). Characterizing the radioresponse of pluripotent and multipotent human stem cells. *PLoS ONE* 7:e50048. doi: 10.1371/journal.pone.0050048

Limoli, C. L., Giedzinski, E., Baure, J., Rola, R., and Fike, J. R. (2007). Redox changes induced in hippocampal precursor cells by heavy ion irradiation. *Radiat. Environ. Biophys.* 46, 167–172. doi: 10.1007/s00411-006-0077-9

Lloyd, H. G., and Fredholm, B. B. (1995). Involvement of adenosine deaminase and adenosine kinase in regulating extracellular adenosine concentration in rat hippocampal slices. *Neurochem. Int.* 26, 387–395. doi: 10.1016/0197-0186(94)00144-J

McGaraughty, S., Cowart, M., Jarvis, M. F., and Berman, R. F. (2005). Anticonvulsant and antinociceptive actions of novel adenosine kinase inhibitors. *Curr. Top. Med. Chem.* 5, 43–58. doi: 10.2174/1568026053386845

Mizumatsu, S., Monje, M., Morhardt, D., Rola, R., Palmer, T., and Fike, J. (2003). Extreme sensitivity of adult neurogenesis to low doses of X-irradiation. *Cancer Res.* 63, 4021–4027.

Monje, M. L., Mizumatsu, S., Fike, J. R., and Palmer, T. D. (2002). Irradiation induces neural precursor-cell dysfunction. *Nat. Med.* 8, 955–962. doi: 10.1038/nm749

Moravan, M. J., Olschowka, J. A., Williams, J. P., and O'banion, M. K. (2011). Cranial irradiation leads to acute and persistent neuroinflammation with delayed increases in T-cell infiltration and CD11c expression in C57BL/6 mouse brain. *Radiat. Res.* 176, 459–473. doi: 10.1667/RR2587.1

Mumby, D. G., Gaskin, S., Glenn, M. J., Schramek, T. E., and Lehmann, H. (2002). Hippocampal damage and exploratory preferences in rats: memory for objects, places, and contexts. *Learn. Mem.* 9, 49–57. doi: 10.1101/lm.41302

Osman, A. M., Zhou, K., Zhu, C., and Blomgren, K. (2014). Transplantation of enteric neural stem/progenitor cells into the irradiated young mouse hippocampus. *Cell Transplant.* 23, 1657–1671. doi: 10.3727/096368913X674648

Palop, J. J., and Mucke, L. (2009). Epilepsy and cognitive impairments in Alzheimer disease. *Arch. Neurol.* 66, 435–440. doi: 10.1001/archneurol.2009.15

Pannasch, U., Vargova, L., Reingruber, J., Ezan, P., Holcman, D., Giaume, C., et al. (2011). Astroglial networks scale synaptic activity and plasticity. *Proc. Natl. Acad. Sci. U.S.A.* 108, 8467–8472. doi: 10.1073/pnas.1016650108

Parihar, V. K., Acharya, M. M., Roa, D. E., Bosch, O., Christie, L. A., and Limoli, C. L. (2014a). Defining functional changes in the brain caused by targeted stereotaxic radiosurgery. *Transl. Cancer Res.* 3, 124–137.

Parihar, V. K., Allen, B. D., Tran, K. K., Chmielewski, N. N., Craver, B. M., Martirosian, V., et al. (2015). Targeted overexpression of mitochondrial catalase prevents radiation-induced cognitive dysfunction. *Antioxid. Redox. Signal.* 22, 78–91. doi: 10.1089/ars.2014.5929

Parihar, V. K., and Limoli, C. L. (2013). Cranial irradiation compromises neuronal architecture in the hippocampus. *Proc. Natl. Acad. Sci. U.S.A.* 110, 12822–12827. doi: 10.1073/pnas.1307301110

Parihar, V. K., Pasha, J., Tran, K. K., Craver, B. M., Acharya, M. M., and Limoli, C. L. (2014b). Persistent changes in neuronal structure and synaptic plasticity caused by proton irradiation. *Brain Struct. Funct.* 220, 1161–1171. doi: 10.1007/s00429-014-0709-9

Pazzagli, M., Corsi, C., Fratti, S., Pedata, F., and Pepeu, G. (1995). Regulation of extracellular adenosine levels in the striatum of aging rats. *Brain Res.* 684, 103–106. doi: 10.1016/0006-8993(95)00471-2

Phillips, R. G., and LeDoux, J. E. (1992). Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behav. Neurosci.* 106, 274–285. doi: 10.1037/0735-7044.106.2.274

Rola, R., Raber, J., Rizk, A., Otsuka, S., Vandenberg, S. R., Morhardt, D. R., et al. (2004). Radiation-induced impairment of hippocampal neurogenesis is associated with cognitive deficits in young mice. *Exp. Neurol.* 188, 316–330. doi: 10.1016/j.expneurol.2004.05.005

Rusina, R., Ridzon, P., Kulist'ak, P., Keller, O., Bartos, A., Buncova, M., et al. (2010). Relationship between ALS and the degree of cognitive impairment, markers of neurodegeneration and predictors for poor outcome. A prospective study. *Eur. J. Neurol.* 17, 23–30. doi: 10.1111/j.1468-1331.2009.02717.x

Save, E., Buhot, M. C., Foreman, N., and Thinus-Blanc, C. (1992). Exploratory activity and response to a spatial change in rats with hippocampal or posterior parietal cortical lesions. *Behav. Brain Res.* 47, 113–127. doi: 10.1016/S0166-4328(05)80118-4

Tofilon, P. J., and Fike, J. R. (2000). The radioresponse of the central nervous system: a dynamic process. *Radiat. Res.* 153, 354–370. doi: 10.1667/0033-7587(2000)153[0357:TROTCN]2.0.CO;2

Ugarkar, B. G., Dare, J. M., Kopcho, J. J., Browne, C. E. III, Schanzer, J. M., Wiesner, J. B., et al. (2000). Adenosine kinase inhibitors. 1. Synthesis, enzyme inhibition, and antiseizure activity of 5-iodotubercidin analogues. *J. Med. Chem.* 43, 2883–2893. doi: 10.1021/jm000024g

Williams-Karnesky, R. L., Sandau, U. S., Lusardi, T. A., Lytle, N. K., Farrell, J. M., Pritchard, E. M., et al. (2013). Epigenetic changes induced by adenosine augmentation therapy prevent epileptogenesis. *J. Clin. Invest.* 123, 3552–3563. doi: 10.1172/JCI65636

Winocur, G., Vardy, J., Binns, M. A., Kerr, L., and Tannock, I. (2006). The effects of the anti-cancer drugs, methotrexate and 5-fluorouracil, on cognitive function in mice. *Pharmacol. Biochem. Behav.* 85, 66–75. doi: 10.1016/j.pbb.2006.07.010

Zhao, W., and Robbins, M. E. (2009). Inflammation and chronic oxidative stress in radiation-induced late normal tissue injury: therapeutic implications. *Curr. Med. Chem.* 16, 130–143. doi: 10.2174/092986709787002790

Zhou, H., Liu, Z., Liu, J., Wang, J., Zhou, D., Zhao, Z., et al. (2011). Fractionated radiation-induced acute encephalopathy in a young rat model: cognitive dysfunction and histologic findings. *AJNR Am. J. Neuroradiol.* 32, 1795–1800. doi: 10.3174/ajnr.A2643

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Molecular Neuroscience

ORIGINAL RESEARCH
published: 21 December 2016
doi: 10.3389/fnmol.2016.00156

Achaete-Scute Homolog 1 Expression Controls Cellular Differentiation of Neuroblastoma

Mumtaz Kasim, Vicky Heß, Holger Scholz, Pontus B. Persson and Michael Fähling**

Institut für Vegetative Physiologie, Charité-Universitätsmedizin Berlin, Berlin, Germany

Neuroblastoma, the major cause of infant cancer deaths, results from fast proliferation of undifferentiated neuroblasts. Treatment of high-risk neuroblastoma includes differentiation with retinoic acid (RA); however, the resistance of many of these tumors to RA-induced differentiation poses a considerable challenge. Human achaete-scute homolog 1 (hASH1) is a proneural basic helix-loop-helix transcription factor essential for neurogenesis and is often upregulated in neuroblastoma. Here, we identified a novel function for hASH1 in regulating the differentiation phenotype of neuroblastoma cells. Global analysis of 986 human neuroblastoma datasets revealed a negative correlation between hASH1 and neuron differentiation that was independent of the N-myc (MYCN) oncogene. Using RA to induce neuron differentiation in two neuroblastoma cell lines displaying high and low levels of hASH1 expression, we confirmed the link between hASH1 expression and the differentiation defective phenotype, which was reversed by silencing hASH1 or by hypoxic preconditioning. We further show that hASH1 suppresses neuronal differentiation by inhibiting transcription at the RA receptor element. Collectively, our data indicate hASH1 to be key for understanding neuroblastoma resistance to differentiation therapy and pave the way for hASH1-targeted therapies for augmenting the response of neuroblastoma to differentiation therapy.

Keywords: differentiation therapy, retinoic acid, ASCL1, hASH1, hypoxia, MYCN

OPEN ACCESS

Edited by:
Jochen C. Meier, Braunschweig University of Technology, Germany

Reviewed by:
Giovanni Piccoli, University of Trento, Italy
Krishanu Ray, Tata Institute of Fundamental Research, India

****Correspondence:***
Mumtaz Kasim
mumtaz.kasim@charite.de
Michael Fähling
michael.faehling@charite.de

Received: *19 September 2016*
Accepted: *07 December 2016*
Published: *21 December 2016*

Citation:
Kasim M, Heß V, Scholz H, Persson PB and Fähling M (2016) Achaete-Scute Homolog 1 Expression Controls Cellular Differentiation of Neuroblastoma. Front. Mol. Neurosci. 9:156. doi: 10.3389/fnmol.2016.00156

INTRODUCTION

Neuroblastoma, a malignant tumor derived from the sympathetic nervous system, represents one of the most common solid childhood tumors. They are classified into different stages based on genetic profile, age of onset, and tumor stage, with amplification of the N-myc (MYCN) gene seen in 22% of primary tumors and associated with worse clinical outcome (Cohn et al., 2009; Brodeur and Bagatell, 2014). Gene expression profiles suggest neuroblastomas to be locked in development at an early stage, being irresponsive to the normal cues that trigger differentiation. They, however, have the capacity to differentiate into mature cells in response to a variety of physiological and pharmacological agents. Thus, retinoic acid (RA), by its ability to induce differentiation, remains the first line of therapy for high-risk neuroblastoma (Reynolds et al., 2003). The greatest obstacle to differentiation therapy lies in their refractiveness to RA and it is therefore desirable to understand the mechanisms of resistance and to identify means by which to improve RA effectiveness.

The transcription factor achaete-scute homolog 1 protein (hASH1 for human and Mash1 for mammalian), encoded by the achaete-scute complex-like 1 (*ASCL1*) gene and hereafter referred to as hASH1, is expressed in neural crest cells and neural crest derived progenitor cells of the sympathoadrenal system and is required for neurogenesis (Castro et al., 2011; Imayoshi et al., 2013;

Jacob et al., 2013). The hASH1 transcriptional program and regulation of its activity have been well documented (Castro et al., 2011; Imayoshi et al., 2013; Huang et al., 2014; Raposo et al., 2015; Wylie et al., 2015). Detailed characterization of the transcription factor activity has previously revealed that it activates target genes involved in both proliferation and differentiation that are associated with its oscillatory or sustained mode of expression, respectively (Castro et al., 2011; Imayoshi et al., 2013). During development, hASH1 expression is mostly restricted to the embryonic state, being regulated spatially and temporally primarily by transcriptional inhibition by the Notch pathway (Axelson, 2004; Huang et al., 2014). hASH1 is also regulated by post-transcriptional mechanisms involving both mRNA stability and translation (Fähling et al., 2009; Benko et al., 2011). One such important post-transcriptional regulator of hASH1 expression is the heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1) and its role has been described in neuroblastoma cells exposed to hypoxia, indicating that low oxygen tension could be an important determinant of neuroendocrine development and tumor development (Kasim et al., 2014). Surprisingly, aberrantly high levels of hASH1 have been detected only in neuroblastoma cell lines; these being mostly derived from highly malignant tumors and high hASH1 levels thus associated with poor clinical outcome (Isogai et al., 2011). Gaining insight into the regulatory cues leading to hASH1 down-regulation during development could therefore be important for understanding neuroblastoma. Consistently, expression of hASH1 is down-regulated during differentiation, independent of differentiation agent. Whether the down-regulation of hASH1 is essential for differentiation or a consequence thereof could not be clearly established as stable cell clones of the SH-SY5Y cell line expressing high levels of hASH1 were not tolerated (Söderholm et al., 1999). Thus, a functional link between hASH1 expression and the responsiveness of neuroblastoma to differentiation remains elusive (Söderholm et al., 1999; Grynfeld et al., 2000).

Here, we exploit two neuroblastoma cell lines, Kelly and SH-SY5Y as paradigms of malignant tumors expressing endogenously high and low levels of hASH1, respectively, to investigate the functional importance of hASH1 in neuroblastoma. By analysis of three publicly available microarray datasets of human neuroblastoma patients (n = 986) and by experiments performed in the above mentioned cell lines, we identify a crucial role for hASH1 in regulating the differentiation potential of neuronal cells via its ability to repress RA-mediated transcription. These results not only expand the repertoire of hASH1 functions but also help to explain and eventually overcome the refractiveness of many neuroblastomas to differentiation therapy. Our data further reveal the functionality of hASH1-targeted therapies for augmenting the response of neuroblastoma to differentiation therapy.

MATERIALS AND METHODS

Microarray Data Analysis

We used the R2: Genomics Analysis and Visualization web tool to find genes correlated with hASH1 (ASCL1). We included the following three public datasets for analysis: the Versteeg dataset (GSE16476) which included 88 human neuroblastoma samples (Valentijn et al., 2012), the Asgharzadeh dataset (Therapeutically Applicable Research to Generate Effective Treatments initiative[1]) included 249 human neuroblastoma samples (Russell et al., 2015) and the Kocak dataset (GSE45547) with 649 human neuroblastoma samples (Kocak et al., 2013), all with different clinical characteristics. We applied a p-value cut-off of 0.01 for the correlation coefficient (R value) to obtain significantly correlated genes. The p values were corrected for multiple testing according to the false discovery rate. Only for the Kocak dataset that yielded >4500 correlated genes, we applied an additional R-value cutoff of ≤ -0.3 or $\geq +0.3$. Gene ontology (GO) analysis (database release date 2016-04-23), using the Bonferroni correction for multiple testing and a cutoff p value < 0.05, was independently performed on the list of significantly correlated genes obtained from each of the three datasets.

Cell Culture and Treatments

The human neuroblastoma cell lines, Kelly (ACC 355) and SH-SY5Y (CRL-2266), were grown at 37°C, 5% CO_2 in RPMI medium supplemented with 10% Fetal Bovine Serum (FBS). Cells were differentiated with 1 μM and 10 μM all-trans RA for the indicated times. For shorter treatment times (up to 24 h), cells that had outgrowths greater than the length of the cell body were considered to be expressing neurites. For longer treatment times (4 days), cells that had one or more outgrowths that reached at least double the diameter of the cell body were considered to be differentiated. For hypoxia, a hypoxic chamber set at 1% O_2, 37°C and 5% CO_2 was used.

Antibodies

The following antibodies were used: mouse monoclonal anti-Mash1 (murine homolog of hASH1; BD Pharmingen), mouse monoclonal anti-hnRNP-A2/B1 (Acris Antibodies, Rockville, MD, USA), rabbit polyclonal anti-tubulin (Proteintech), rabbit polyclonal anti-neurofilament L (Proteintech). Secondary antibodies used were donkey anti-rabbit and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The Cy3-coupled secondary antibody for immunofluorescence was from Jackson ImmunoResearch Laboratories.

Protein Isolation and Western Blot

Total cellular extracts were prepared by direct lysis of cells in 50 mM Tris (pH 6.8) buffer containing 4 M Urea and 1% SDS. Western blotting was performed as previously described (Fähling et al., 2006).

Real Time PCR Analysis

Total RNA was prepared using RNA-Bee (Biozol Diagnostica Vertrieb GmbH) and real-time PCR experiments performed

[1]http://target.nci.nih.gov/dataMatrix/TARGET_DataMatrix.html

as previously described (Kasim et al., 2014). Each sample was measured in triplicate. mRNA expression levels were normalized to 18S rRNA using the $\Delta\Delta C_t$ method. Primer sequences used are listed in Supplementary Table S1.

RNA Interference

Control siRNA and siRNA targeting hASH1 and hnRNP-A2/B1 was transfected into Kelly cells using SilenceMag (Oz Biosciences) or DharmaFECT 2 (Thermo Scientific, Waltham, MA, USA) as specified by the manufacturer. All siRNAs were purchased as SMARTpool siRNAs from Thermo Scientific Dharmacon. Cells were analyzed 48 h post-transfection for knock-down efficiency or used for further experiments at 24 h or 48 h post-transfection.

Cell Transfection and Luciferase Reporter Assays

Control and backbone vectors were purchased directly from Clontech and Promega. For knock-down and overexpression experiments, cells were grown in 24-well plates. Following 24 h knockdown in Kelly cells, a control luciferase (CONTROL-LUC) plasmid or the RA receptor element containing luciferase plasmid along with the renilla luciferase phRL-TK vector (Promega) was co-transfected using PolyMag (Oz Biosciences). For over-expression of hASH1 in SH-SY5Y, a pCMV-Sport6-hASH1 plasmid or control plasmid was co-transfected with the reporter constructs. After an overnight incubation, the medium was changed and RA was supplemented at a final concentration of 1 μM. Cells were grown for an additional 18 h. Luciferase activity was measured using the Dual-Glo^{TM} luciferase assay system and the data collected using a luminometer (Berthold Detection Systems). Co-transfection of hASH1 with EGFP in SH-SY5Y was performed in 6-well plates with a final concentration of 2 μg DNA. The ratio of hASH1 to EGFP plasmid was maintained at 9:1.

Microscopy

The cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Immunofluorescence was performed as recommended in the antibody data sheet. An epifluorescence microscope (Axiovert100, Carl Zeiss, Berlin, Germany) connected to a digital camera (SPOT RT Slider, Diagnostic Instruments, Sterling Heights, MI, USA) with SPOT software (Universal Imaging Corp., Marlow Buckinghamshire, UK) was used for image acquisition. Where indicated, image analysis was performed using the ImageJ software with the NeuronJ plugin.

Statistics

If not indicated otherwise, all values are presented as mean ± SD of at least three independent experiments, each with three biological replicates. Student's paired *t* test was applied to reveal statistical significances between two samples. To compare means of three or more groups, a one-way ANOVA was used. $p < 0.05$ was considered significant.

TABLE 1 | Number of genes correlated with human achaete-scute homolog 1 (hASH1) and N-myc (MYCN) in human neuroblastoma.

DATASET	Number of samples	Number of correlated genes*			
		hASH1		MYCN	
		Negative	Positive	Negative	Positive
Versteeg	88	103	366	1050	1148
Asgharzadeh	249	574	1355	3456	2405
Kocak	649	4528	4655	5896	5732

*A cutoff of $p < 0.01$ was used.

RESULTS

hASH1 Is Negatively Correlated with Genes Involved in Neuron Differentiation in Neuroblastoma Patients

We first identified genes correlated with either hASH1 or MYCN, the latter being a well-known proto-oncogene with clearly established prognostic value in neuroblastoma patients. We analyzed three datasets that are deposited in the R2 Genomics Analysis and Visualization platform[2]: the Versteeg dataset included 88 patient samples (Valentijn et al., 2012), the Asgharzadeh dataset contained 249 patient samples (Russell et al., 2015) and the Kocak dataset contained 649 patient samples (Kocak et al., 2013) (**Table 1**). The overall small number of genes correlated with hASH1 relative to MYCN highlights the specificity of its putative target group (**Table 1**). With focus on hASH1, we further narrowed our list for the Kocak dataset to the top most correlated genes by setting a cut off for the correlation coefficient (*R* value) of ≤ -0.3 or $\geq +0.3$, which reduced the number of negatively and positively correlated genes to 419 and 809, respectively. The list of identified genes correlated with hASH1 for all three datasets along with their associated *R* and *p* values is given in Supplementary Tables S2–S4. We next performed gene ontology analysis[3] in order to determine the significantly enriched biological processes ($p \leq 0.05$) in these gene lists. Notably, the enriched biological processes were consistent with the known functions of hASH1 in cell proliferation and differentiation (Castro et al., 2011). This is exemplarily shown in **Figure 1A** for the negatively correlated genes identified in the Kocak dataset, which contained the largest number of patient samples. We observed a striking association of the negatively correlated genes predominantly with processes involved in neuron projection development (e.g., regulation of neuron differentiation, regulation of neurogenesis, regulation of cell projection organization) and neuron projection morphogenesis (e.g., neuron development, neuron differentiation, generation of neurons). These findings were independently validated in the Asgharzadeh dataset (Supplementary Table S5). Despite the smaller number of negatively correlated genes in the Versteeg dataset, the only significantly enriched biological processes belonged to the category "regulation of neuron projection

[2]http://hgserver1.amc.nl/cgi-bin/r2/main.cgi
[3]http://geneontology.org/

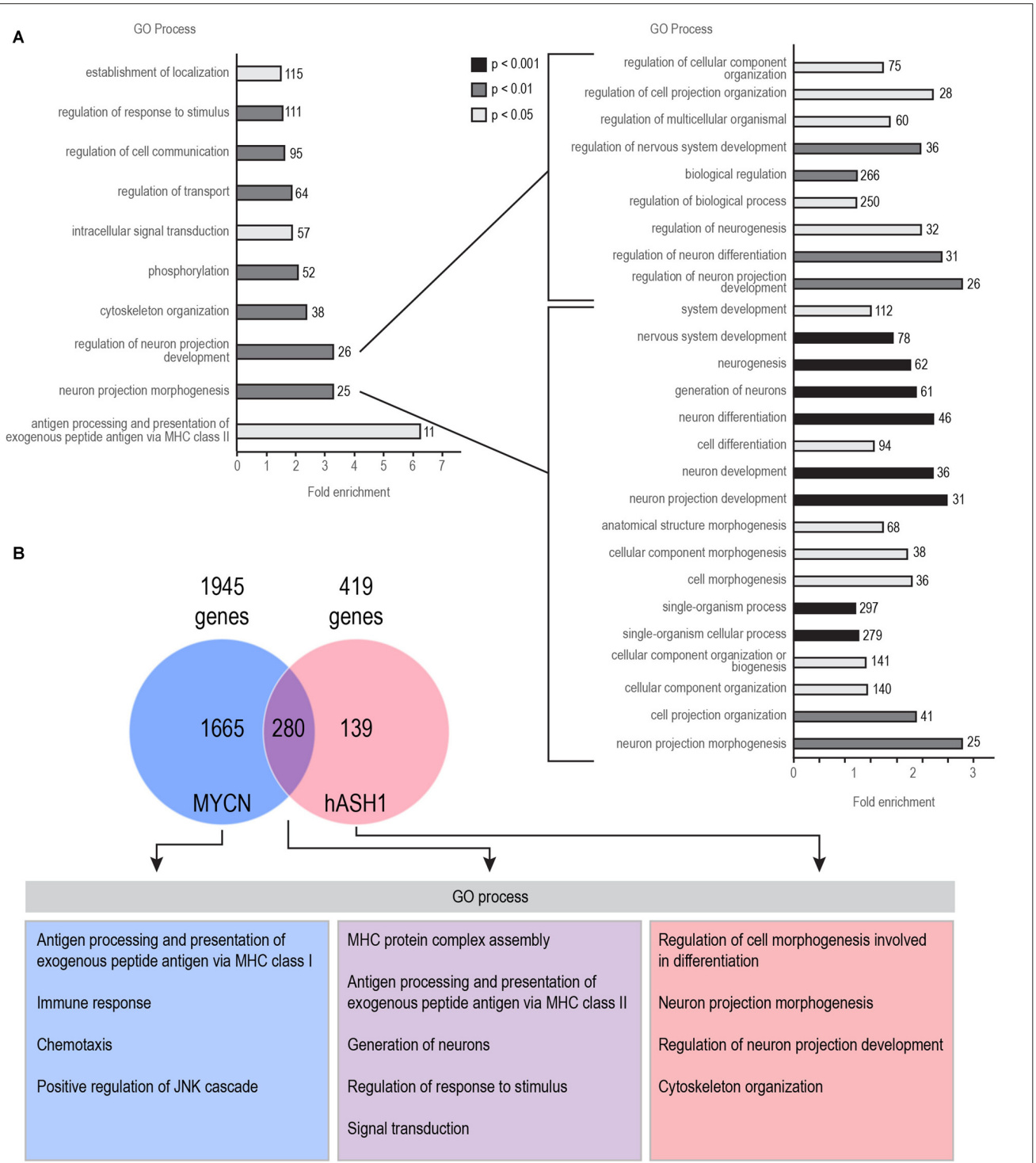


FIGURE 1 | Human achaete-scute homolog 1 (hASH1) is negatively correlated with genes associated with neurite outgrowth in neuroblastoma. **(A)** Enrichment of Gene ontology (GO) biological processes in the 419 genes negatively correlated with hASH1 (R value < -0.3 and with a p value < 0.01) using the Kocak dataset (Kocak et al., 2013). This corresponds to the top 10% of the strongest correlated genes. A detailed view of all the categories related to regulation of neuron projection development and neuron projection morphogenesis is shown on the right. Each biological process is color coded according to its GO analysis p value as shown and the number of genes identified is also indicated. **(B)** Genes negatively correlated with hASH1 and N-myc (MYCN) were compared. GO analysis was performed separately for genes correlated with either MYCN or hASH1 and for the genes associated with both. A detailed list of all the GO processes and associated p values is provided in Supplementary Table S6. Note that the genes correlated only with hASH1 belong to the GO categories of neuron projection morphogenesis and development.

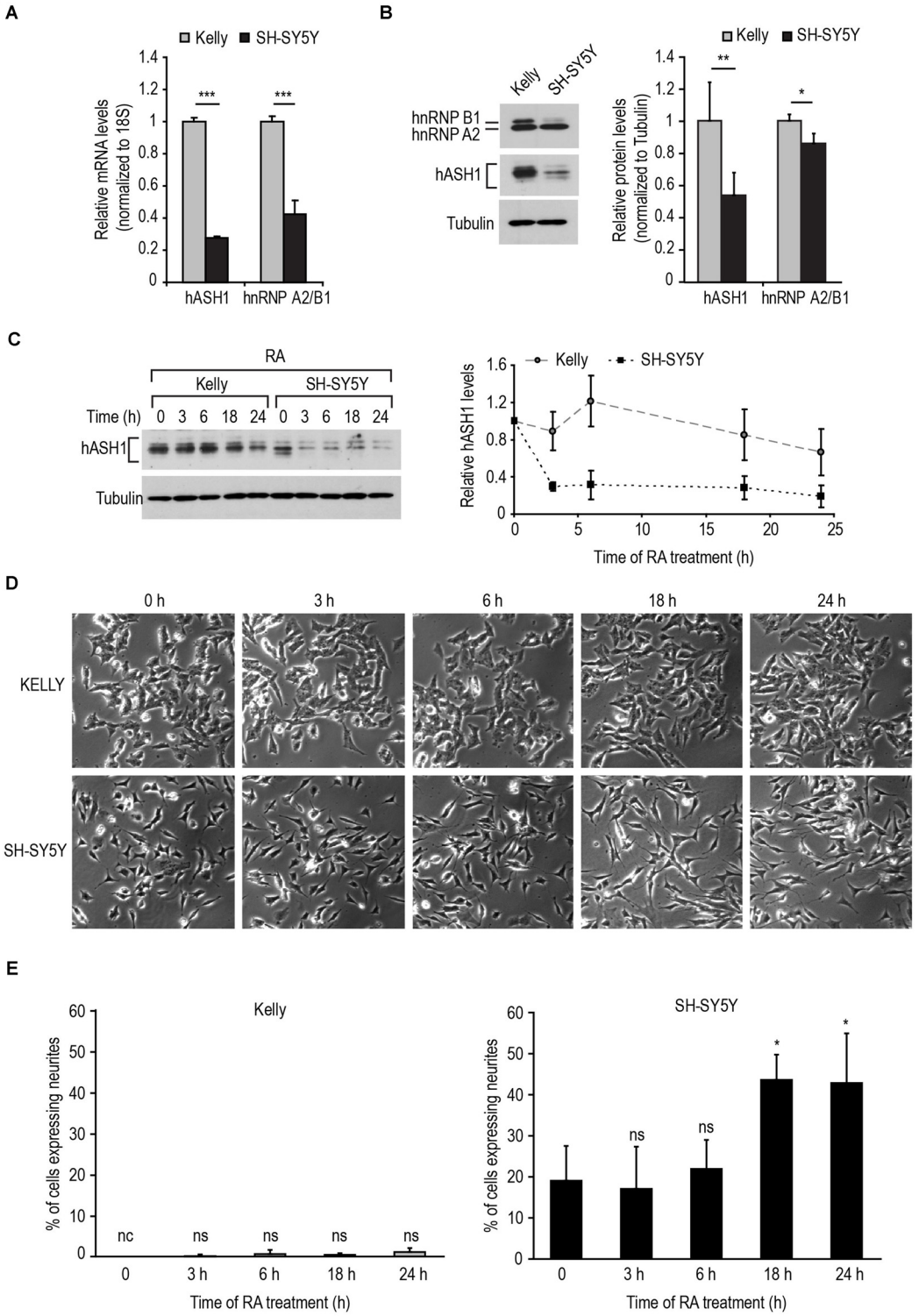


FIGURE 2 | Response of neuroblastoma cell lines to retinoic acid (RA)-induced differentiation is dependent on their hASH1 levels. (A) hASH1 and heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1) mRNA as determined in Kelly and SH-SY5Y cells by real-time PCR. Values were normalized to 18S ribosomal RNA and significant differences between the cell lines indicated with asterisks (***$p < 0.001$, $n = 6$). **(B)** Western blot analysis of hASH1 and hnRNP A2/B1 protein levels in Kelly and SH-SY5Y cells. Tubulin served as a loading control. **$p < 0.01$ and *$p < 0.05$. Error bars indicate standard deviation ($n = 6$). **(C)** Quantitation by Western blot of the time-dependent decrease in hASH1 levels in Kelly and SH-SY5Y cells upon 1 μM RA treatment. **(D)** Phase contrast images

(*Continued*)

FIGURE 2 | Continued
showing the time-dependent phenotypic response to 1 μM RA. Note that RA treatment causes outgrowth of neurite-like processes in SH-SY5Y cells, but not in the Kelly cells. **(E)** Percentage of neurite containing cells were defined as cells with at least one neurite extension greater than the length of the cell body. More than 100 cells for each cell type and treatment were counted using ImageJ software, and the percentage of cells with neurite extensions are indicated. $^*p < 0.05$, nc: not counted as no cells matching the above criteria were observed, and ns = not significant. Error bars indicate standard deviation ($n = 3$).

development", thus further supporting a negative influence of hASH1 on neuron differentiation (Supplementary Table S5). While a myriad of biological processes were highly represented in the positively correlated genes in all three datasets, we found that they were enriched for GO biological processes corresponding to various aspects of the cell cycle (e.g., G1/S phase transition, G2/M transition of the mitotic cell cycle, mitotic interphase, spindle checkpoint), DNA replication (e.g., DNA strand elongation, DNA metabolic processes), telomere maintenance and DNA damage response and repair (e.g., double-strand break repair, cellular response to DNA damage stimulus; Supplementary Table S5). Compared to the positively correlated genes, the negatively correlated genes had fewer biological processes associated with them, and the level of significance was lower ($p \geq 10^{-16}$ vs. $p \geq 10^{-96}$ for processes associated with the positively correlated genes). However, processes involving neurogenesis and neuron differentiation were the only significant associations among the negatively correlated genes that were consistently seen across all three datasets (Supplementary Table S5). We also identified a significant association of hASH1 with neuroblastoma stage and MYCN amplification status, pointing to its functional importance in neuroblastoma (Supplementary Figures S1, S2).

To exclude MYCN from mediating this effect, we compared the profile of genes correlated with both hASH1 and MYCN (**Figure 1B**). Despite the high overlap of genes (280/419 genes) between the two, the GO processes neuron projection morphogenesis and regulation of neuron projection development were significantly over-represented in the 139 genes not correlated with MYCN, highlighting hASH1 function in regulating these processes (**Figure 1B**). Interestingly, GO analysis of the MYCN correlated genes identified primarily categories associated with the immune response (e.g., T-helper 1 type immune response, regulation of T cell proliferation, T cell selection, interferon gamma mediated signaling pathway; Supplementary Table S6). These findings were corroborated in both of the other datasets (data not shown). Together, these gene expression data from three independent experiments containing a total of 986 human neuroblastoma samples reveal a significant negative correlation between hASH1 and genes involved in neuron differentiation in neuroblastoma that is independent of MYCN function.

Characterization of RA-Induced Differentiation in Neuroblastoma Cell Lines

To reconcile these findings with the known function of hASH1 in neurogenesis, we postulated that an increase in hASH1 expression would have an inhibitory influence on neuron differentiation. Therefore, to investigate the effect of hASH1 levels on neuron differentiation, we selected two neuroblastoma cell lines derived from metastatic bone tumor biopsies, Kelly and SH-SY5Y, expressing endogenous high and low levels of hASH1, respectively. As neuroblastomas are also genetically characterized based on their genomic MYCN amplification status and given the observed correlation with hASH1 (Supplementary Figure S2), the Kelly cells with the MYCN-amplified genome and the MYCN-non-amplified SH-SY5Y cells also enabled us to dissect the contribution of hASH1 independent of MYCN amplification. Since we have previously shown that hASH1 is regulated by hnRNP A2/B1 (Kasim et al., 2014), we monitored the levels of both hASH1 and hnRNP A2/B1 in these cells. Real-time PCR analysis revealed higher levels of both hASH1 and hnRNP A2/B1 mRNA in Kelly vs. SH-SY5Y cells (**Figure 2A**). Western blot analysis confirmed the high levels of hASH1 protein in Kelly cells and the relatively low levels in SH-SY5Y cells (**Figure 2B**), the multiple protein bands likely reflecting post-translational modifications (Wylie et al., 2015). We detected overall lower hnRNP A2/B1 protein levels in SH-SY5Y than in Kelly cells, which was primarily due to the decreased levels of the hnRNP B1 isoform (**Figure 2B**). Both cell lines expressed neuropeptide Y and neuronal marker genes, i.e., growth associated protein 43 and microtubule-associated protein 2, confirming their neuronal character (Supplementary Figure S3).

We then used RA to differentiate the cells toward a neuronal phenotype. We first tested the response of the two cell lines to 1 μM RA (**Figure 2C**). As a rapid reduction in hASH1 levels upon RA treatment of SH-SY5Y cells has been reported (Söderholm et al., 1999), we monitored the decrease in hASH1 levels by Western blot analysis in both cell lines in a time course of up to 24 h. As expected, SH-SY5Y cells displayed a rapid decrease in hASH1 levels that was noticeable as early as 3 h (**Figure 2C**). Surprisingly, the Kelly cells maintained stable hASH1 expression at early time points with a decline in hASH1 levels becoming evident only at 18–24 h, which did not reach the low levels observed in the SH-SY5Y cells (**Figure 2C**). More interestingly, phase contrast microscopy revealed that these changes in hASH1 levels correlated with the cells ability to undergo the morphological changes associated with neuronal differentiation (**Figures 2D,E**). Although the SH-SY5Y cells contain small neurites even in the absence of retinoids, RA treatment enhanced neurite outgrowth in these cells. However, no similar morphological change was observed in the Kelly cells. Both cell lines responded to RA by down-regulating hASH1 mRNA and protein confirming that RA was indeed active in these cells (**Figures 3A,B**). Consistently, both cell lines also responded to RA by decreasing neuropeptide Y levels (**Figure 3C**); a decrease in levels of neuropeptide Y in RA-treated SH-SY5Y has already been documented (Edsjö et al., 2004). Although a higher concentration of RA was more effective at stimulating differentiation as assessed by the development of neurite-like processes in the SH-SY5Y cells (**Figure 3D**), the Kelly cells remained morphologically resistant to RA and lacked

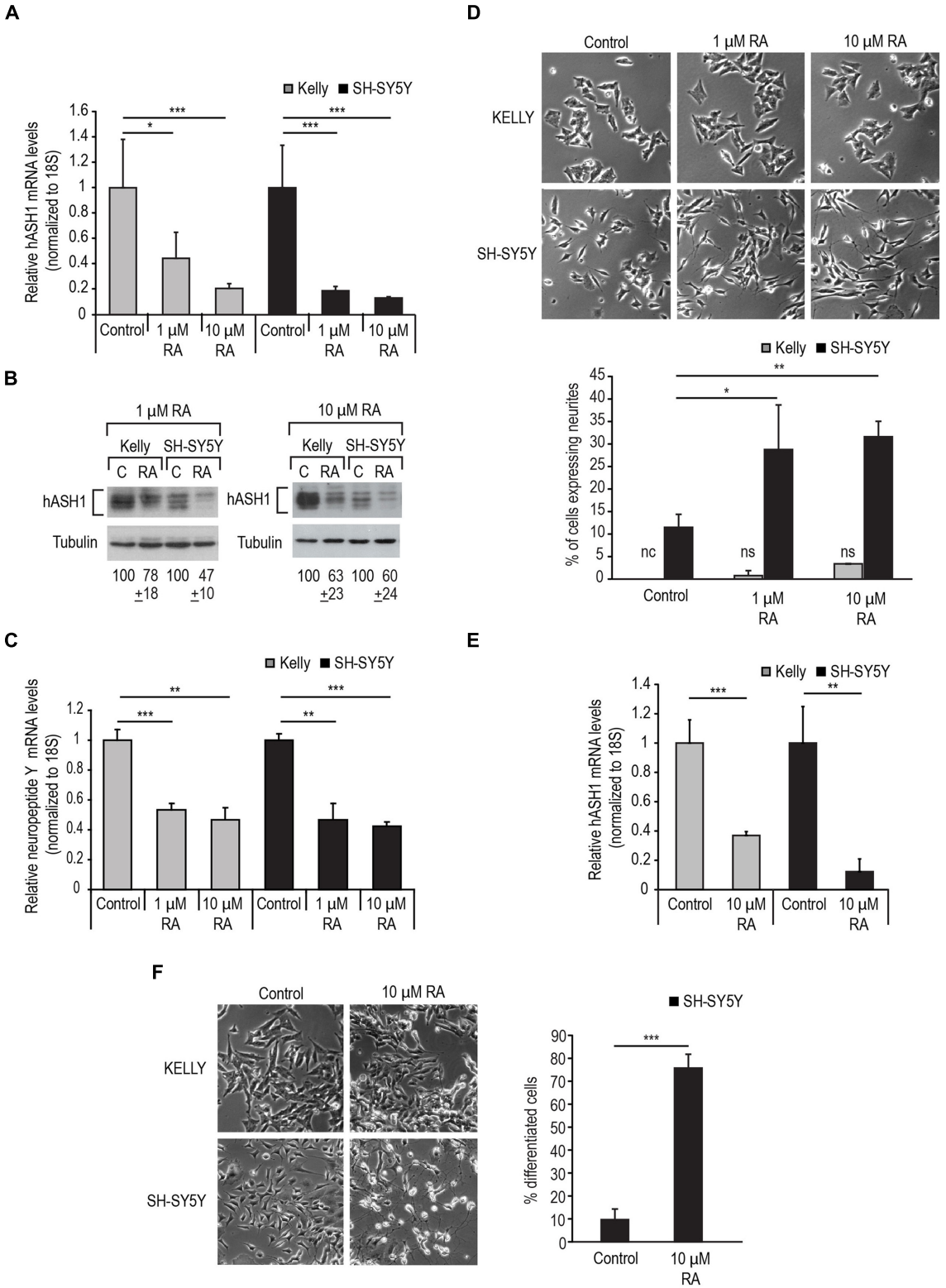


FIGURE 3 | Kelly cells are responsive to RA but resistant to RA-induced differentiation. (A) Decrease in hASH1 levels upon 1 and 10 μM RA treatment at 24 h monitored by real-time PCR and **(B)** Western blot analysis.***$p < 0.001$ and *$p < 0.05$. Error bars indicate standard deviation ($n = 6$). **(C)** Real-time PCR analysis of NPY levels upon RA treatment as in **(A)**. Significant differences are indicated with asterisks ***$p < 0.001$ and **$p < 0.01$. Error bars indicate standard

(*Continued*)

FIGURE 3 | Continued
deviation ($n = 3$). **(D)** Phase contrast images showing concentration dependent effect of RA on the morphology of Kelly and SH-SY5Y cells. Note the formation of neurite-like processes in the RA-treated SH-SY5Y cells, whereas Kelly cells did not respond morphologically to RA. Percentage of neurite containing cells were counted as in **Figure 2E**. $^{**}p < 0.01$, $^{*}p < 0.05$, nc: not counted as no cells matching the above criteria were observed, and ns: not significant. Error bars indicate standard deviation ($n = 3$). **(E)** Kelly and SH-SY5Y cells were treated with 10 μM RA for 4 days and hASH1 mRNA levels monitored by real time PCR analysis. Values were normalized to 18S ribosomal RNA and significant differences indicated with asterisks ($^{**}p < 0.01$ and $^{***}p < 0.001$). Error bars indicate standard deviation ($n = 3$). **(F)** Kelly and SH-SY5Y cells were treated with 10 μM RA and imaged at day 4. Percentage of differentiated cells were defined as those with neurite extensions at least two times the length of the cell body. The total number of cells were counted for each cell type and treatment and the percentage of differentiated cells for the SH-SY5Y cells indicated. $^{***}p < 0.001$. Error bars indicate standard deviation ($n = 3$). Note that for the Kelly cells, the neurite extensions did not fit our criteria to be counted as differentiated.

the neurite-like processes despite a similar down-regulation of hASH1 mRNA and protein levels (**Figures 3A–C**). Longer RA treatment times up to 4 days confirmed the resistance of majority of the Kelly cells to RA-induced differentiation (**Figures 3E,F**). The SH-SY5Y cells, on the other hand, showed an intense neurite network after a 4 day exposure to RA and a concomitant strong down-regulation in hASH1 (**Figures 3E,F**). Although the variability inherent to different cell lines raises the possibility of cell-type specific effects in the regulation of RA-induced differentiation, these data suggest that high hASH1 expression during the early phase of RA treatment inhibits the phenotypic changes associated with retinoid induced differentiation.

hASH1 Suppresses RA-Induced Differentiation

To determine if indeed hASH1 is responsible for the observed lack of responsiveness of the Kelly cells to RA, we silenced hASH1 in these cells by RNA interference. The efficiency of silencing was determined by Western blot analysis at 2 days post-transfection (**Figure 4A**). Interestingly, knock-down of hASH1 had no effect on hnRNP A2/B1 that we have shown previously to regulate hASH1 levels (Kasim et al., 2014). We next investigated the response of these Kelly cells with high (siControl) and with reduced hASH1 levels (sihASH1) to RA treatment for 4 days. We found that both siControl and sihASH1 cells responded effectively to RA by down-regulating hASH1 (**Figure 4B**). The sihASH1 cells retained their lower level of hASH1 for the course of the experiment (**Figure 4B**, compare siControl to sihASH1) and their lower hASH1 level is also reflected in their significantly lower levels of hASH1 at the end of RA treatment (**Figure 4B**, compare siControl + RA to sihASH1 + RA). Morphological examination by phase contrast microscopy revealed that in comparison to siControl cells, more RA-treated si-hASH1 Kelly cells developed neurite-like processes (**Figures 4C,D**). There was no significant difference in neurite length between differentiated cells obtained from the high and low hASH1 expressing cells (**Figure 4E**). Qualitatively similar results were obtained by silencing hnRNP A2/B1 that has previously been documented to decrease hASH1 protein levels (Supplementary Figure S4; Kasim et al., 2014), thus providing additional evidence for the inhibitory effect of high hASH1 on neuronal differentiation.

The data described above indicate that hASH1 prevents RA-induced differentiation of neuroblastoma cells. To further prove the specificity of hASH1 in regulating this effect, we examined the hASH1-dependent RA-differentiation response in SH-SY5Y cells that have a lower basal expression of hASH1. We transiently overexpressed hASH1 in these cells (**Figure 4F**). Interestingly, ectopically expressed hASH1 had a lower molecular mass indicative of the non-phosphorylated form of hASH1 and unlike endogenous hASH1 that is phosphorylated in neuroblastoma cell lines (Wylie et al., 2015). Co-transfection with EGFP allowed for visualization of hASH1 transfected cells by fluorescence microscopy and allowed for live-cell imaging to monitor cell morphology following RA treatment. Since the hASH1 and EGFP plasmids were transfected at a 9:1 ratio, fluorescent cells are expected to overexpress hASH1. We confirmed the overexpression of hASH1 in the co-transfected cells by immunofluorescence microscopy and by co-localization of hASH1 and EGFP (Supplementary Figure S5). The EGFP + hASH1 co-transfected cells expressed up to 9.6-fold higher levels of hASH1 compared to the control EGFP transfected cells (Supplementary Figure S5). These transfected cells were then allowed to differentiate with RA for 4 days. The EGFP alone expressing cells also served as a transfection control and developed long neurites by day 4 of differentiation (**Figure 4G**, left upper panel, white arrowheads), while hASH1 overexpression strikingly diminished neurite extension in the majority of the cells (**Figure 4G**, right upper panels, red arrowheads). Notably, hASH1 overexpression resulted in a significant decrease in the number of differentiated cells (**Figure 4H**). There was no significant difference in neurite length between the differentiated cells from the control or high hASH1 overexpressing cell line (**Figure 4I**). Overlay of the fluorescent images on the phase contrast images confirmed the efficacy of RA to induce differentiation in both cell populations as the non-transfected cells in each showed a similar degree of neurite outgrowth (**Figure 4G**, lower panels). As EGFP alone had no influence on neurite extension, we conclude that overexpression of hASH1 inhibits neurite extension. Thus, our findings in the two cell lines reveal a universal role for hASH1 in regulating RA differentiation.

hASH1 Represses Retinoic Acid Receptor Element-Dependent Transcription

To gain insight into the molecular mechanism behind hASH1 repression of RA-induced differentiation, we examined the effect of hASH1 on RA-dependent transcription at a retinoic acid response element (RARE) in the context of a luciferase reporter construct. We first reduced hASH1 levels by siRNA-mediated knockdown in Kelly cells prior to RA treatment. As shown in **Figure 5A**, a 1.8 fold increase in RA-dependent luciferase activity upon hASH1 knockdown was observed, indicating a hASH1-dependent repression

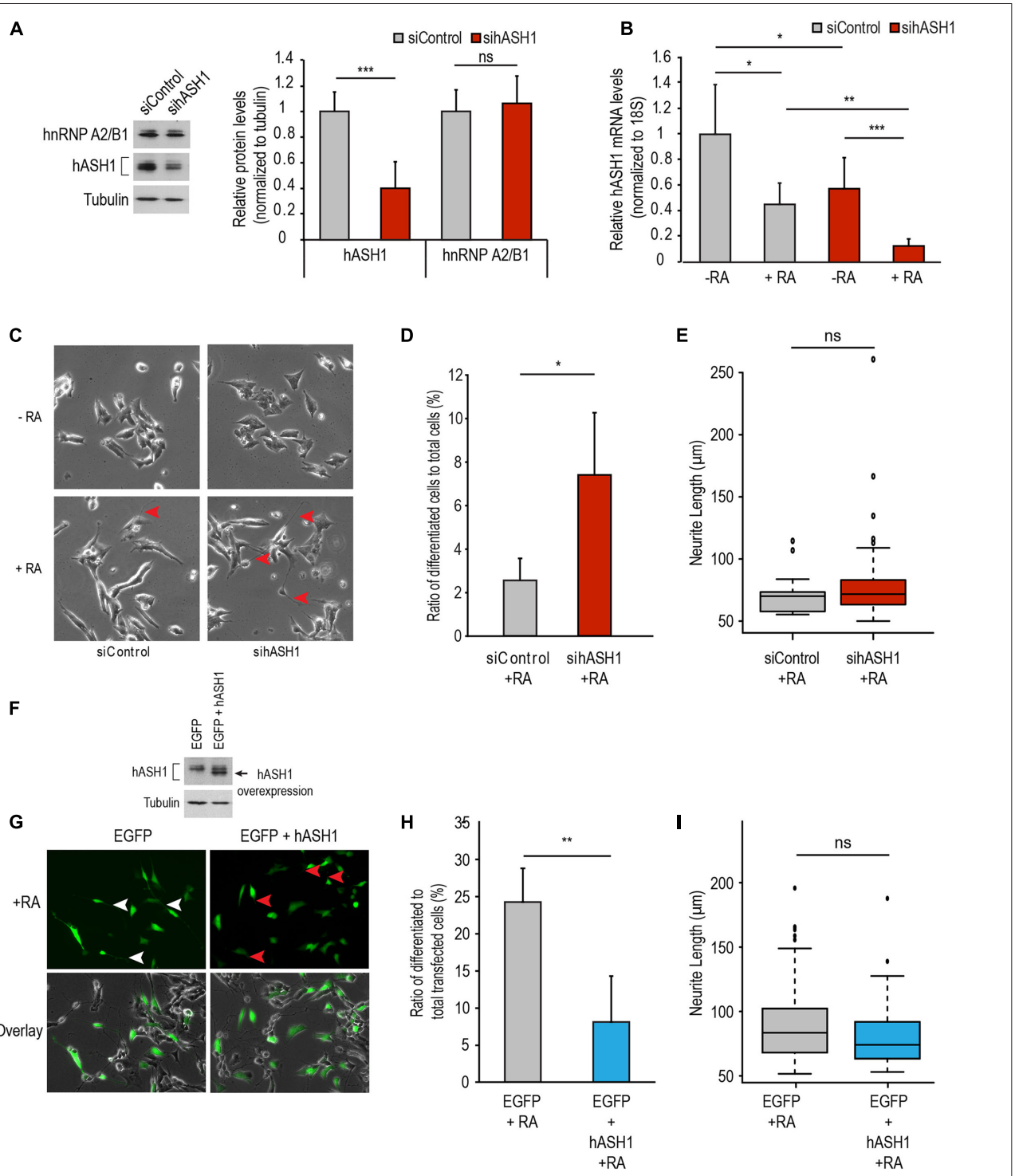


FIGURE 4 | hASH1 levels affect RA-induced differentiation in Kelly and SH-SY5Y cells. (A) siRNA mediated knock-down of hASH1 was performed in Kelly cells and cells were harvested following 2 days of siRNA transfection (10 nM final concentration). A representative Western blot experiment is shown demonstrating knock-down efficiency at the protein level (left panel) and quantitation of the data from $n = 9$ (right panel). **(B)** Kelly cells from **(A)** were allowed to differentiate for 4 days and the decrease in hASH1 levels monitored by real time PCR. Values were normalized to 18S ribosomal RNA and significant differences indicated with asterisks (***$p < 0.001$, **$p < -0.01$ and *$p < 0.05$). $n = 8$. **(C)** Kelly cells were imaged at day 4 of differentiation and the neurite-like processes indicated by red arrowheads. **(D)** Cells from RA treated siControl and sihASH1 were counted and neurites measured using NeuronJ software (*$p < 0.05$; total number of cells counted for siControl + RA = 680 and sihASH1 + RA = 776). Data is expressed as the ratio of differentiated cells to total cells counted. **(E)** Box plot analysis of

(*Continued*)

FIGURE 4 | (Continued
neurite length of the differentiated cells from siControl and sihASH1. ns = not significant. **(F)** Western blot analysis of total cell populations of SH-SY5Y cells over-expressing EGFP or EGFP + hASH1. Tubulin served as a loading control. **(G)** SH-SY5Y cells overexpressing EGFP or EGFP + hASH1 were enabled to differentiate with RA and imaged at day 4. Long neurite-like processes are indicated by white arrowheads in the EGFP overexpressing cells and the shorter processes with red arrowheads in the EGFP + hASH1 overexpressing cells. **(H)** RA treated transfected cells were counted and neurites measured using NeuronJ software (total number of cells counted for EGFP + RA = 437 and EGFP + hASH1 + RA = 446). Data are expressed as the ratio of differentiated cells to total transfected cells counted. **(I)** Box plot analysis of neurite length of the differentiated cells from control and hASH1 transfected cells. ns = not significant.

of retinoid activity. We then compared the retinoid activity upon hASH1 overexpression in SH-SY5Y cells and found that luciferase activity was significantly lower upon hASH1 overexpression, again indicating a hASH1-dependent repression of retinoid activity (**Figure 5B**). Collectively, these data show that hASH1 represses RA-dependent transcription at the RA receptor element.

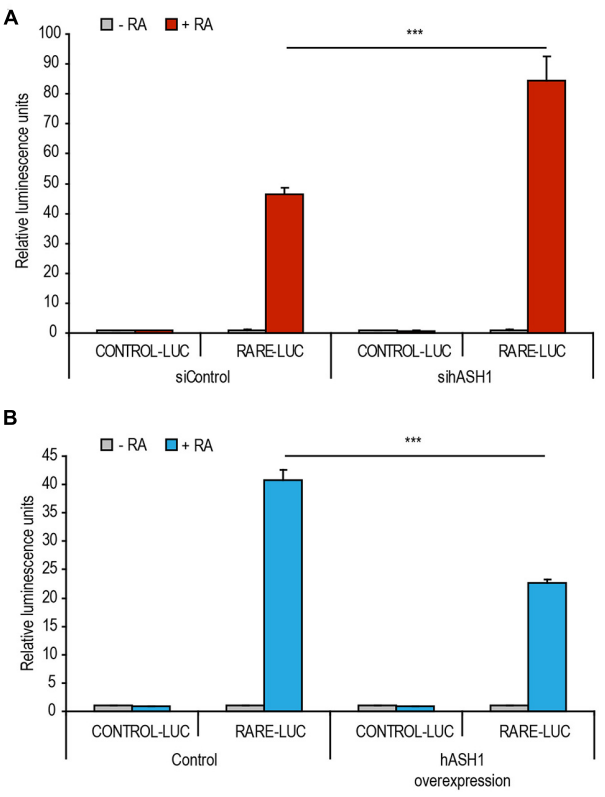


FIGURE 5 | hASH1 represses RA-dependent transcription at the retinoic acid response element (RARE). (A) Kelly cells transfected with either control siRNA or siRNA targeting hASH1 or **(B)** SH-SY5Y cells transfected with control plasmid or a plasmid expressing hASH1 were also transfected with a control luciferase (CONTROL-LUC) reporter construct or a luciferase plasmid containing the RARE-LUC. Transcription of RARE-LUC is thus dependent on RA. Luminescence values were normalized to renilla activity. Asterisks indicate significant changes with $^{***}p < 0.001$, $n = 6$.

Hypoxic Preconditioning Promotes RA-Differentiation of High hASH1-Expressing Cells

To explore the possibility of promoting differentiation of the otherwise differentiation resistant Kelly cell line under physiological circumstances, we used hypoxia as a tool to first manipulate hASH1 levels (Kasim et al., 2014). To this end, Kelly cells were preconditioned to hypoxia for 24 h, a time point at which a strong reduction in hASH1 levels was observed (**Figure 6A**). The efficacy of hypoxia treatment was confirmed by up-regulation of hypoxia marker genes (aldolase C and prolyl-4-hydroxylase) as well as the down-regulation in NPY that has been previously documented (**Figure 6A**) (Jögi et al., 2002). Immediately after 24 h of hypoxic preconditioning, when these cells have lowered levels of hASH1, cells were induced to differentiate with RA under normoxic conditions. Control cells grown at normoxia for 24 h were processed similarly. Western blot analysis confirmed the down-regulation in hASH1 protein levels indicative of effective RA treatment after both normoxia and hypoxia growth conditions (**Figure 6B**). It is worth mentioning that no significant difference was observed in hASH1 levels between the control and hypoxia preconditioned cells when placed in normoxia for 24 h (**Figure 6B**). However, only the hypoxia preconditioned cells, with lower hASH1 levels at the time of RA treatment, displayed retinoid-induced neurite-like processes that stained positive for neurofilament L, which is expressed during neuronal differentiation (**Figures 6C–E**). Taken together, our data show that manipulation of hASH1 expression by hypoxia influences the sensitivity of neuroblastoma cells to RA-induced differentiation.

DISCUSSION

Neuroblastoma is one of the few malignancies capable of spontaneous differentiation and regression (Maris et al., 2007). Increasing the ability to induce neuroblastoma differentiation is thus key to disease therapeutics; however, this is often impeded by the acquired refractiveness of neuroblastoma to the differentiation agent. Our data here revealed hASH1 to play a decisive role in inhibiting the differentiation of neuroblastoma in response to RA, a finding that may have important implications in differentiation therapy.

We discovered a common function of hASH1 in inducing genes involved in cell cycle and DNA repair while repressing genes involved in neuron differentiation in neuroblastoma. This was evident across a large number of neuroblastoma tumors from three different datasets that were obtained using different microarray platforms, thus supporting the clinical significance of our findings. Interestingly, this function of hASH1 in neuroblastoma mirrors that of MYCN (Valentijn et al., 2012). The ability of MYCN to repress neuron differentiation was inferred from the neuronal expression of its negatively regulated genes, most of which were not identified to be correlated with hASH1 (data not shown; Valentijn et al., 2012). It is possible that MYCN cooperates with hASH1 to exert a

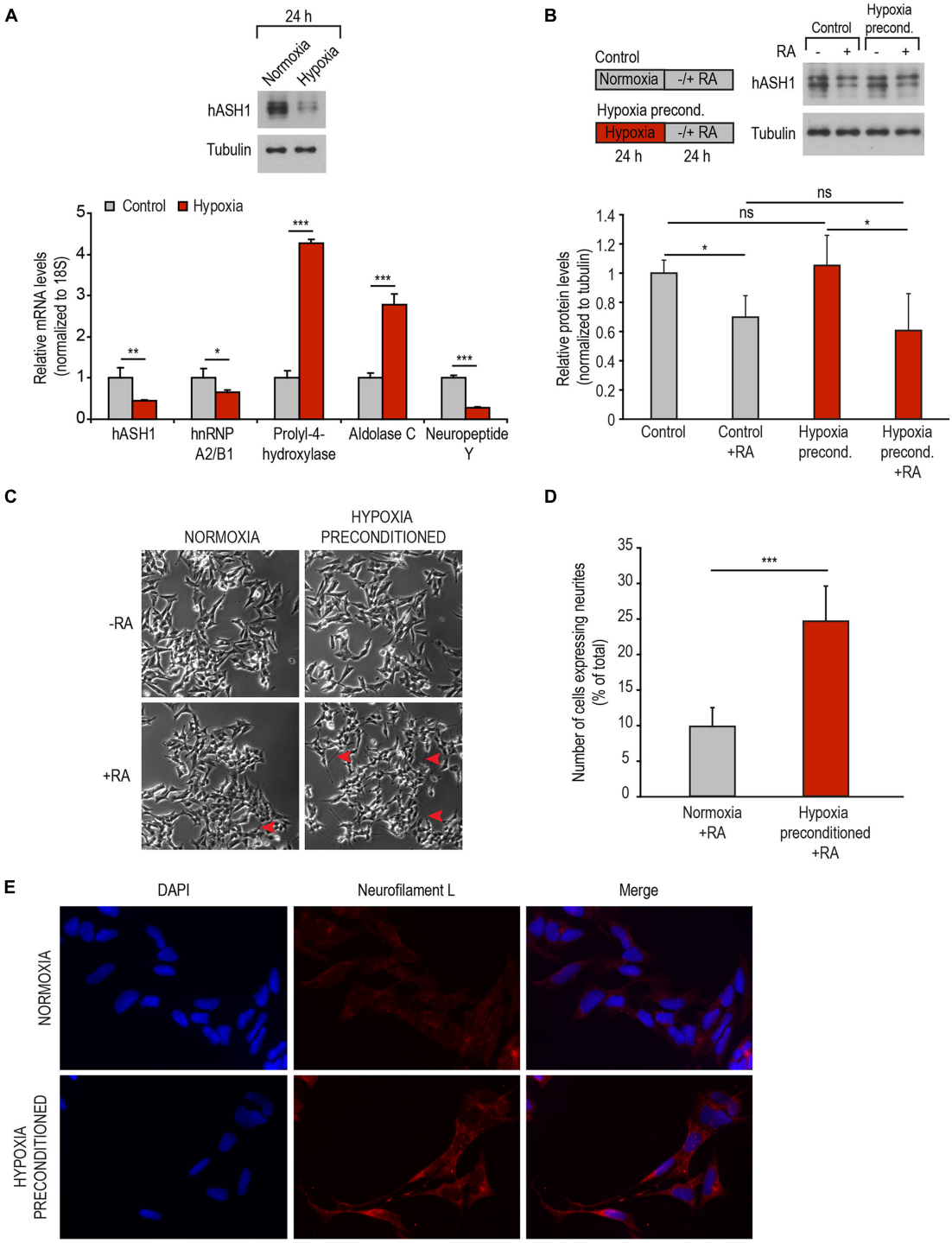


FIGURE 6 | Hypoxic preconditioning promotes RA-induced differentiation. (A) Representative Western blot showing decreased levels of hASH1 following 24 h of hypoxia (top panel). Tubulin served as a loading control. Efficacy of hypoxia treatment was monitored by real-time PCR analysis of hypoxia marker genes prolyl-4-hydroxylase and aldolase C. hASH1, hnRNP A2/B1 and neuropeptide Y levels are shown in addition (bottom panel). Values were normalized to 18S ribosomal RNA. Significant differences are indicated with asterisks ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; $n = 6$. **(B)** Representative Western blot showing hASH1 levels of control cells and cells preconditioned to hypoxia following RA treatment. Schematic of the experimental design is shown in the left panel. Values were normalized to tubulin. Significant differences are indicated with an asterisk *$p < 0.05$ and ns: not significant; $n = 6$. **(C)** Phase contrast images of cells as described in **(B)**. Neurite-like processes in the hypoxia preconditioned cells are indicated by red arrows. **(D)** The number of RA treated cells expressing neurites and the total number of cells were counted using NeuronJ software (***$p < 0.001$; total number of cells counted for normoxia + RA = 2224 and hypoxia preconditioned + RA = 2139). **(E)** Immunofluorescence assays showing neurofilament L staining of normoxia and hypoxia preconditioned cells treated with RA for 24 h.

concerted effect, which is reflected in the observed correlation of hASH1 expression with MYCN amplification. Nevertheless, the over-representation of MYCN non-amplified tumors in the neuroblastoma datasets that we used and the regulation of the MYCN non-amplified SH-SY5Y cell differentiation by hASH1 indicates that hASH1 function in neuron differentiation in neuroblastoma is likely to be independent of MYCN amplification status. The importance of this being underlined by the fact that MYCN amplification, although the most well-known prognostic marker, is observed in only 22% of primary tumors (Brodeur and Bagatell, 2014).

Given the lack of responsiveness of neuroblastomas to RA, the negative association of hASH1 with neuron differentiation was of particular interest as it provided the first clue to the inhibitory role of hASH1 on differentiation. The two neuroblastoma cell lines we used to confirm this are ideal examples of neuroblastoma that are either responsive or non-responsive to differentiation by RA. Interestingly, it was the low hASH1 expressing SH-SY5Y cells that were capable of undergoing the morphological changes associated with differentiation. In these cells, hASH1 levels decreased more or less concomitantly with RA treatment, evident as early as after 3 h. In contrast, majority of the high hASH1 expressing Kelly cells failed to express neurite-like processes even upon prolonged RA treatment. Here, it is important to emphasize that RA was indeed active in the Kelly cells, as evidenced by the down-regulation of NPY and also hASH1; yet, despite the decreased hASH1 levels, the Kelly cells failed to differentiate in response to RA. We assume that an initial threshold level for hASH1 expression exists beyond which the inhibitory effects on differentiation are observed. Therefore, it is the basal hASH1 level at the time of RA exposure (early phase) that is crucial in determining the differentiation fate of the cells. This is particularly true for Kelly cells that do not show a rapid decrease in hASH1 protein levels upon retinoid treatment. The hASH1 levels in both cell lines correlated with hnRNP B1 that is known to have a stabilizing effect on hASH1 mRNA and protein and could also play a role in their different basal hASH1 expression and their response to RA (Kasim et al., 2014).

Several lines of evidence strongly suggest hASH1 to be involved in modulating the cellular differentiation in response to RA. First, down-regulation of hASH1 by directly targeting the hASH1 gene by RNA interference in high-hASH1 expressing Kelly neuroblastoma cells promoted RA-induced differentiation. Conversely, over-expression of hASH1 in low-hASH1 expressing SH-SY5Y neuroblastoma cells conferred resistance to RA-induced differentiation. Second, down-regulation of hASH1 by targeting a modulator of hASH1, namely hnRNP A2/B1, also promoted RA-induced differentiation. Third, hypoxic preconditioning that significantly decreases hASH1 levels prior to RA treatment also enhanced differentiation. Finally, the ability of hASH1 to modulate RA-induced differentiation correlated with its influence on transcription at the RA receptor element. The mechanistic basis behind hASH1-dependent transcription repression is not known. To date, a myriad of transcription regulatory proteins have been described to influence retinoid signaling (Gudas and Wagner, 2011; Cunningham and Duester, 2015). For instance, Zinc Finger Protein 423 was identified by a large-scale RNA interference screen to be critically required for RA-induced differentiation of neuroblastoma cells (Huang et al., 2009). Here, we identified hASH1 to inhibit RA-induced transcription at the RA receptor element, thus, acting as a possible co-repressor of retinoid signaling. In this regard, binding of several proneural proteins including Neurogenin1, Neurogenin2 and hASH1 to RA receptor in the absence of ligand has already been described (Lee et al., 2009). We cannot, however, exclude the involvement of other factors that are targets of hASH1 in mediating this response. The known function of hASH1, a proneural transcription factor belonging to the achaete-scute gene family, is to promote neurogenesis by regulating the expression of differentiation genes; a function that is conserved in all organisms (Huang et al., 2014). In *Drosophila*, achaete-scute expression confers neural identity to the developing neuroblast, whereas in mammalian systems, expression is restricted to cells that already have neural identity (Lo et al., 1991; Skeath and Carroll, 1992). Regulation of hASH1 expression is critical to the development program (Imayoshi et al., 2013). Our data suggest that aberrant regulation of hASH1, leading to high hASH1 expression, would inhibit differentiation and maintain neuronal cells in a proliferative state. It is important to keep in mind that retinoid activity *in vivo* reciprocally maintains hASH1 at carefully regulated levels that in turn determines neuronal identities (Jacob et al., 2013). Thus, the dysregulation of hASH1 in neuroblastoma could in turn be linked to defects in the RA synthesis and metabolism

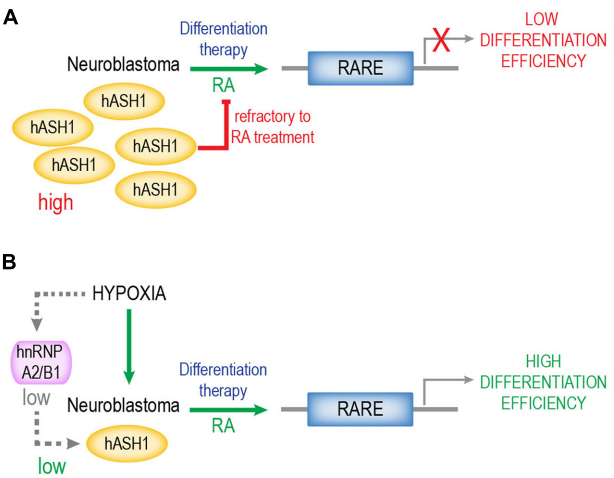


FIGURE 7 | Proposed model of hASH1 function in neuroblastoma. High hASH1 levels in neuroblastoma inhibit RA-mediated transcription at the RARE resulting in low differentiation efficiency **(A)**. Exposure to hypoxia causes an hnRNP A2/B1-dependent decrease in hASH1 levels (Kasim et al., 2014) and thus promotes RA-induced differentiation and neurite outgrowth, resulting in higher differentiation efficiency **(B)**. Thus, hypoxic preconditioning could serve to enhance retinoid efficacy during differentiation therapy, especially for tumors refractive to RA. We propose that hASH1 levels in neuroblastoma is indicative of their differentiation potential and thus their response to differentiation therapy.

pathway as has been described in glioblastoma (Campos et al., 2015).

A summary of our findings is depicted in **Figure 7**. RA remains one of the most potent inducers of differentiation for neuroblastomas and several derivatives are used as therapy for high-risk neuroblastomas (Reynolds et al., 2003). A high tumor differentiation grade is associated with a positive outcome. Since efficacy of RA *in vivo* is limited by the refractiveness of many neuroblastomas, combination therapy with, for instance, histone deacetylase inhibitors has been applied (Hahn et al., 2008; Brodeur and Bagatell, 2014). We propose that refractiveness to RA is a function of the hASH1 levels in these tumors. One of the surprising observations that we describe is that hypoxic preconditioning remarkably enhanced RA-differentiation efficacy. These data suggest that the local oxygen environment can serve as a tool to manipulate hASH1 levels in neuroblastomas that could effectively facilitate differentiation therapy. Here, we would like to add a cautionary note and stress the point that these data were obtained from cell lines *in vitro*. The Kelly and SH-SY5Y are two different cell lines, and additional cell type-specific effects of hASH1 on the regulation of RA-induced differentiation cannot be ruled out. Despite the negative correlation between hASH1 and neuron differentiation observed in neuroblastoma patients, future *in vivo* studies aimed at evaluating the potential prognostic usefulness of hASH1 in predicting the responsiveness of these tumors to RA-based therapies are needed. Although other factors have been described to modulate RA differentiation in neuroblastoma, hASH1 represents a unique target and a possible biomarker for all neuroblastoma, independent of MYCN amplification status (Shah et al., 2014; Cimmino et al., 2015; Heynen et al., 2016). Future studies will focus on defining the mechanism of action of hASH1 during the early phase of RA-differentiation, which may reveal novel cellular targets of importance to retinoid therapy. Taken together, this study highlights hASH1 to be a modulator of retinoid-induced differentiation that is of potential clinical significance to neuroblastoma.

AUTHOR CONTRIBUTIONS

MK, HS and MF designed experiments; MK, VH and MF performed experiments; MK, VH, HS, PBP and MF analyzed and interpreted the data, contributed to discussion; MK and MF wrote the manuscript.

FUNDING

This work was supported by the Deutsche Forschungsgemeinschaft (Grant FA 845/2-2 to MF).

ACKNOWLEDGMENTS

We thank Dr. Michael Schupp for his gifts of retinoic acid and the retinoic acid receptor element containing luciferase construct. We also thank Jeannette Schmidt, Ulrike Neumann and Ursula Kastner for excellent technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnmol.2016.00156/full#supplementary-material

REFERENCES

Axelson, H. (2004). The Notch signaling cascade in neuroblastoma: role of the basic helix-loop-helix proteins HASH-1 and HES-1. *Cancer Lett.* 204, 171–178. doi: 10.1016/s0304-3835(03)00453-1

Benko, E., Winkelmann, A., Meier, J. C., Persson, P. B., Scholz, H., and Fähling, M. (2011). Phorbol-ester mediated suppression of hASH1 synthesis: multiple ways to keep the level down. *Front. Mol. Neurosci.* 4:1. doi: 10.3389/fnmol.2011.00001

Brodeur, G. M., and Bagatell, R. (2014). Mechanisms of neuroblastoma regression. *Nat. Rev. Clin. Oncol.* 11, 704–713. doi: 10.1038/nrclinonc.2014.168

Campos, B., Weisang, S., Osswald, F., Ali, R., Sedlmeier, G., Bageritz, J., et al. (2015). Retinoid resistance and multifaceted impairment of retinoic acid synthesis in glioblastoma. *Glia* 63, 1850–1859. doi: 10.1002/glia.22849

Castro, D. S., Martynoga, B., Parras, C., Ramesh, V., Pacary, E., Johnston, C., et al. (2011). A novel function of the proneural factor Ascl1 in progenitor proliferation identified by genome-wide characterization of its targets. *Genes Dev.* 25, 930–945. doi: 10.1101/gad.627811

Cimmino, F., Pezone, L., Avitabile, M., Acierno, G., Andolfo, I., Capasso, M., et al. (2015). Inhibition of hypoxia inducible factors combined with all-trans retinoic acid treatment enhances glial transdifferentiation of neuroblastoma cells. *Sci. Rep.* 5:11158. doi: 10.1038/srep11158

Cohn, S. L., Pearson, A. D., London, W. B., Monclair, T., Ambros, P. F., Brodeur, G. M., et al. (2009). The International Neuroblastoma Risk Group (INRG) classification system: an INRG task force report. *J. Clin. Oncol.* 27, 289–297. doi: 10.1200/JCO.2008.16.6785

Cunningham, T. J., and Duester, G. (2015). Mechanisms of retinoic acid signalling and its roles in organ and limb development. *Nat. Rev. Mol. Cell Biol.* 16, 110–123. doi: 10.1038/nrm3932

Edsjö, A., Nilsson, H., Vandesompele, J., Karlsson, J., Pattyn, F., Culp, L. A., et al. (2004). Neuroblastoma cells with overexpressed MYCN retain their capacity to undergo neuronal differentiation. *Lab. Invest.* 84, 406–417. doi: 10.1038/labinvest.3700061

Fähling, M., Mrowka, R., Steege, A., Kirschner, K. M., Benko, E., Forstera, B., et al. (2009). Translational regulation of the human achaete-scute homologue-1 by fragile X mental retardation protein. *J. Biol. Chem.* 284, 4255–4266. doi: 10.1074/jbc.M807354200

Fähling, M., Mrowka, R., Steege, A., Martinka, P., Persson, P. B., and Thiele, B. J. (2006). Heterogeneous nuclear ribonucleoprotein-A2/B1 modulate collagen prolyl 4-hydroxylase, alpha (I) mRNA stability. *J. Biol. Chem.* 281, 9279–9286. doi: 10.1074/jbc.m510925200

Grynfeld, A., Påhlman, S., and Axelson, H. (2000). Induced neuroblastoma cell differentiation, associated with transient HES-1 activity and reduced HASH-1 expression, is inhibited by Notch1. *Int. J. Cancer* 88, 401–410. doi: 10.1002/1097-0215(20001101)88:3<401::AID-IJC12>3.3.CO;2-M

Gudas, L. J., and Wagner, J. A. (2011). Retinoids regulate stem cell differentiation. *J. Cell Physiol.* 226, 322–330. doi: 10.1002/jcp.22417

Hahn, C. K., Ross, K. N., Warrington, I. M., Mazitschek, R., Kanegai, C. M., Wright, R. D., et al. (2008). Expression-based screening identifies the combination of histone deacetylase inhibitors and retinoids for neuroblastoma differentiation. *Proc. Natl. Acad. Sci. U S A* 105, 9751–9756. doi: 10.1073/pnas.0710413105

Heynen, G. J., Nevedomskaya, E., Palit, S., Jagalur Basheer, N., Lieftink, C., Schlicker, A., et al. (2016). Mastermind-like 3 controls proliferation and differentiation in neuroblastoma. *Mol. Cancer Res.* 14, 411–422. doi: 10.1158/1541-7786.MCR-15-0291-t

Huang, C., Chan, J. A., and Schuurmans, C. (2014). Proneural bHLH genes in development and disease. *Curr. Top. Dev. Biol.* 110, 75–127. doi: 10.1016/B978-0-12-405943-6.00002-6

Huang, S., Laoukili, J., Epping, M. T., Koster, J., Hölzel, M., Westerman, B. A., et al. (2009). ZNF423 is critically required for retinoic acid-induced differentiation and is a marker of neuroblastoma outcome. *Cancer Cell* 15, 328–340. doi: 10.1016/j.ccr.2009.02.023

Imayoshi, I., Isomura, A., Harima, Y., Kawaguchi, K., Kori, H., Miyachi, H., et al. (2013). Oscillatory control of factors determining multipotency and fate in mouse neural progenitors. *Science* 342, 1203–1208. doi: 10.1126/science.1242366

Isogai, E., Ohira, M., Ozaki, T., Oba, S., Nakamura, Y., and Nakagawara, A. (2011). Oncogenic LMO3 collaborates with HEN2 to enhance neuroblastoma cell growth through transactivation of Mash1. *PLoS One* 6:e19297. doi: 10.1371/journal.pone.0019297

Jacob, J., Kong, J., Moore, S., Milton, C., Sasai, N., Gonzalez-Quevedo, R., et al. (2013). Retinoid acid specifies neuronal identity through graded expression of Ascl1. *Curr. Biol.* 23, 412–418. doi: 10.1016/j.cub.2013.01.046

Jögi, A., Øra, I., Nilsson, H., Lindeheim, A., Makino, Y., Poellinger, L., et al. (2002). Hypoxia alters gene expression in human neuroblastoma cells toward an immature and neural crest-like phenotype. *Proc. Natl. Acad. Sci. U S A* 99, 7021–7026. doi: 10.1073/pnas.102660199

Kasim, M., Benko, E., Winkelmann, A., Mrowka, R., Staudacher, J. J., Persson, P. B., et al. (2014). Shutdown of achaete-scute homolog-1 expression by heterogeneous nuclear ribonucleoprotein (hnRNP)-A2/B1 in hypoxia. *J. Biol. Chem.* 289, 26973–26988. doi: 10.1074/jbc.M114.579391

Kocak, H., Ackermann, S., Hero, B., Kahlert, Y., Oberthuer, A., Juraeva, D., et al. (2013). Hox-C9 activates the intrinsic pathway of apoptosis and is associated with spontaneous regression in neuroblastoma. *Cell Death Dis.* 4:e586. doi: 10.1038/cddis.2013.84

Lee, S., Lee, B., Lee, J. W., and Lee, S. K. (2009). Retinoid signaling and neurogenin2 function are coupled for the specification of spinal motor neurons through a chromatin modifier CBP. *Neuron* 62, 641–654. doi: 10.1016/j.neuron.2009.04.025

Lo, L. C., Johnson, J. E., Wuenschell, C. W., Saito, T., and Anderson, D. J. (1991). Mammalian achaete-scute homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells. *Genes Dev.* 5, 1524–1537. doi: 10.1101/gad.5.9.1524

Maris, J. M., Hogarty, M. D., Bagatell, R., and Cohn, S. L. (2007). Neuroblastoma. *Lancet* 369, 2106–2120. doi: 10.1016/S0140-6736(07)60983-0

Raposo, A. A., Vasconcelos, F. F., Drechsel, D., Marie, C., Johnston, C., Dolle, D., et al. (2015). Ascl1 coordinately regulates gene expression and the chromatin landscape during neurogenesis. *Cell Rep.* 10, 1544–1556. doi: 10.1016/j.celrep.2015.02.025

Reynolds, C. P., Matthay, K. K., Villablanca, J. G., and Maurer, B. J. (2003). Retinoid therapy of high-risk neuroblastoma. *Cancer Lett.* 197, 185–192. doi: 10.1016/s0304-3835(03)00108-3

Russell, M. R., Penikis, A., Oldridge, D. A., Alvarez-Dominguez, J. R., McDaniel, L., Diamond, M., et al. (2015). CASC15-S is a tumor suppressor lncRNA at the 6p22 neuroblastoma susceptibility locus. *Cancer Res.* 75, 3155–3166. doi: 10.1158/0008-5472.CAN-14-3613

Shah, N., Wang, J., Selich-Anderson, J., Graham, G., Siddiqui, H., Li, X., et al. (2014). PBX1 is a favorable prognostic biomarker as it modulates 13-cis retinoic acid-mediated differentiation in neuroblastoma. *Clin. Cancer Res.* 20, 4400–4412. doi: 10.1158/1078-0432.ccr-13-1486

Skeath, J. B., and Carroll, S. B. (1992). Regulation of proneural gene expression and cell fate during neuroblast segregation in the *Drosophila* embryo. *Development* 114, 939–946.

Söderholm, H., Ortoft, E., Johansson, I., Ljungberg, J., Larsson, C., Axelson, H., et al. (1999). Human achaete-scute homologue 1 (HASH-1) is downregulated in differentiating neuroblastoma cells. *Biochem. Biophys. Res. Commun.* 256, 557–563. doi: 10.1006/bbrc.1999.0314

Valentijn, L. J., Koster, J., Haneveld, F., Aissa, R. A., van Sluis, P., Broekmans, M. E., et al. (2012). Functional MYCN signature predicts outcome of neuroblastoma irrespective of MYCN amplification. *Proc. Natl. Acad. Sci. U S A* 109, 19190–19195. doi: 10.1073/pnas.1208215109

Wylie, L. A., Hardwick, L. J., Papkovskaia, T. D., Thiele, C. J., and Philpott, A. (2015). Ascl1 phospho-status regulates neuronal differentiation in a *Xenopus* developmental model of neuroblastoma. *Dis. Model. Mech.* 8, 429–441. doi: 10.1242/dev.125153

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Molecular Neuroscience

REVIEW
published: 16 November 2016
doi: 10.3389/fnmol.2016.00122

Tumor Metabolism, the Ketogenic Diet and β-Hydroxybutyrate: Novel Approaches to Adjuvant Brain Tumor Therapy

Eric C. Woolf[1,2], *Nelofer Syed*[3] *and Adrienne C. Scheck*[1,2]*

[1] Neuro-Oncology Research, Barrow Brain Tumor Research Center, Barrow Neurological Institute, St. Joseph's Hospital and Medical Center, Phoenix, AZ, USA, [2] School of Life Sciences, Arizona State University, Tempe, AZ, USA, [3] The John Fulcher Molecular Neuro-Oncology Laboratory, Division of Brain Sciences, Imperial College London, London, UK

Malignant brain tumors are devastating despite aggressive treatments such as surgical resection, chemotherapy and radiation therapy. The average life expectancy of patients with newly diagnosed glioblastoma is approximately ~18 months. It is clear that increased survival of brain tumor patients requires the design of new therapeutic modalities, especially those that enhance currently available treatments and/or limit tumor growth. One novel therapeutic arena is the metabolic dysregulation that results in an increased need for glucose in tumor cells. This phenomenon suggests that a reduction in tumor growth could be achieved by decreasing glucose availability, which can be accomplished through pharmacological means or through the use of a high-fat, low-carbohydrate ketogenic diet (KD). The KD, as the name implies, also provides increased blood ketones to support the energy needs of normal tissues. Preclinical work from a number of laboratories has shown that the KD does indeed reduce tumor growth *in vivo*. In addition, the KD has been shown to reduce angiogenesis, inflammation, peri-tumoral edema, migration and invasion. Furthermore, this diet can enhance the activity of radiation and chemotherapy in a mouse model of glioma, thus increasing survival. Additional studies *in vitro* have indicated that increasing ketones such as β-hydroxybutyrate (βHB) in the absence of glucose reduction can also inhibit cell growth and potentiate the effects of chemotherapy and radiation. Thus, while we are only beginning to understand the pluripotent mechanisms through which the KD affects tumor growth and response to conventional therapies, the emerging data provide strong support for the use of a KD in the treatment of malignant gliomas. This has led to a limited number of clinical trials investigating the use of a KD in patients with primary and recurrent glioma.

Keywords: glioblastoma, glioma, cancer, metabolism, ketogenic diet, beta-hydroxybutyrate, ketones

OPEN ACCESS

Edited by:
Susan A. Masino,
Trinity College, USA

Reviewed by:
Katja Kobow,
Universitätsklinikum Erlangen, Germany
Undurti Narasimha Das,
UND Life Sciences, USA
Dominic D'Agostino,
University of South Florida, USA

***Correspondence:**
Adrienne C. Scheck
Adrienne.scheck@dignityhealth.org

Received: *19 August 2016*
Accepted: *31 October 2016*
Published: *16 November 2016*

Citation:
Woolf EC, Syed N and Scheck AC (2016) Tumor Metabolism, the Ketogenic Diet and β-Hydroxybutyrate: Novel Approaches to Adjuvant Brain Tumor Therapy.
Front. Mol. Neurosci. 9:122.
doi: 10.3389/fnmol.2016.00122

INTRODUCTION

Human malignant glioma is a uniformly fatal disease due, in part, to the limitations of currently available treatments which include surgery, chemotherapy and radiation therapy. Average survival of patients with glioblastoma multiforme (GBM) is 1.5 years, and tumors of the central nervous system are the most common solid tumor in the pediatric population. It is therefore of paramount

importance that new therapeutic strategies for brain cancer patients be developed, especially those that can enhance the efficacy of current treatment options without damaging normal brain tissue. Advances in our understanding of the biology of these tumors have led to an increase in the number of targeted therapies in preclinical and clinical trials (Roesler et al., 2010; Nicholas et al., 2011; Niyazi et al., 2011). While these therapies may prove somewhat effective, the heterogeneity of this tumor often precludes the targeted molecules from being found on all cells in the tumor thus reducing the efficacy of these treatments. In contrast, one trait shared by virtually all tumor cells is altered metabolism.

TUMOR METABOLISM

Alterations in the metabolism of cancer cells, what we now call the "Warburg effect" or aerobic glycolysis, was first described by Warburg et al. (1927). Cancer cells use glycolysis to provide energy and biomolecules regardless of the availability of oxygen. This results in the production of fewer ATP molecules per molecule of glucose, and thus tumor cells require large amounts of glucose. This shift towards increased glycolytic flux in the cytosol and away from the tricarboxylic acid cycle and oxidative phosphorylation in the mitochondria occurs very early in tumorigenesis. This allows for a rapid cell proliferation even under conditions of hypoxia and in the presence of dysfunctional mitochondria. Since Warburg's discovery, metabolism has been of interest in the cancer field but it was often overshadowed by discoveries of oncogenes, tumor suppressor genes, growth factor pathways, molecular subtypes of cancers, etc. There is a resurgence of interest in metabolism as a central theme in cancer, and we continue to find that metabolic pathways intersect and often regulate key components of tumor initiation, progression and therapy response (Clark et al., 2016; Pavlova and Thompson, 2016). In fact, altered metabolism itself has been referred to as a hallmark of cancer (Hanahan and Weinberg, 2011; Cantor and Sabatini, 2012; Ward and Thompson, 2012) in addition to being involved in virtually all of the cancer hallmarks described in the seminal article by Hanahan and Weinberg (2011) (**Figure 1**; Lewis and Abdel-Haleem, 2013).

The term "metabolic remodeling" has been used to describe metabolic changes that can occur in cancer cells (Obre and Rossignol, 2015), and oncogene associated pathways are now known to intersect with, and alter metabolic pathways. For example, the tumor suppressor protein p53 which plays a pivotal role in the cellular responses to hypoxia, DNA damage and oncogene activation is now known to regulate glycolysis and assist in maintaining mitochondrial integrity (Olovnikov et al., 2009; Madan et al., 2011; Puzio-Kuter, 2011; Kim and Kim, 2013; Iurlaro et al., 2014; Barron et al., 2016). Another important connection between metabolism and tumor growth is through regulation of c-MYC. Over-expression of c-MYC occurs in a wide variety of cancers including gliomas. c-MYC is a multi-functional transcription factor and the list of its target genes include those involved in both cell proliferation and cell metabolism (Miller et al., 2012; Zwaans and Lombard, 2014; Altman et al., 2015; Hsieh et al., 2015; Stine et al., 2015). In addition to stimulating glycolysis, c-MYC has been found to activate glutaminolysis and lipid synthesis from citrate (Obre and Rossignol, 2015).

With the advent of molecular analyses, studies of growth factor pathways seemed to overshadow the influence of metabolism on cancer growth. Over-activation of the stress responsive PI3K/AKT signaling pathway is typical in many cancers and often due to activation of growth factor signaling pathways involved in glioma growth such as platelet-derived growth factor, epidermal growth factor and insulin growth factor. We now know that these growth factor pathways are intertwined with metabolic signally pathways (Iurlaro et al., 2014; Martini et al., 2014; Courtnay et al., 2015; Dibble and Cantley, 2015; Roberts and Miyamoto, 2015). PI3K/AKT signaling has been closely linked to metabolism and under low glucose conditions results in rapid tumor cell death (Robey and Hay, 2009; Yang et al., 2009; Marie and Shinjo, 2011).

Another important "hub" linking metabolism and cancer is hypoxia-inducible factor 1 (HIF-1). HIF-1 expression is activated by hypoxia, which is typically found in high grade gliomas and other cancers. HIF-1 is a heterodimeric transcription factor that induces the transcription of a variety of genes involved in angiogenesis (vascular endothelial growth factor (VEGF) and other cytokines) in an attempt to improve tissue perfusion. This results in the formation of abnormal blood vessels that can increase inflammation and edema in brain tumors, as well as induction of the transcription of a variety of genes that promote invasion, migration and tumor growth (Fischer et al., 2002; Kaur et al., 2005; Fujiwara et al., 2007; Hayashi et al., 2007; Mou et al., 2010; Horing et al., 2012; Proescholdt et al., 2012; Yang et al., 2012; Masson and Ratcliffe, 2014; Justus et al., 2015). In addition to specific actions that relate to the tumor cell's response to oxygen availability, HIF-1 interacts with the PI3K/AKT signaling path to act as a regulator of cancer metabolism, proliferation and glycolysis (Pore et al., 2006; Wei et al., 2013; Courtnay et al., 2015; Justus et al., 2015). It also affects the activation of nuclear factor-kappa B (NF-κB), a transcriptional activator that is central to the regulation of various signal transduction pathways and to transcriptional activation events that mediate inflammation, cell proliferation, cell migration and angiogenesis. HIF-1 may, at least in part, provide the molecular basis for the Warburg effect by "reprograming" cellular metabolism in response to oxygen availability (Corbet and Feron, 2015; Courtnay et al., 2015). HIF-1 also is a central figure in alterations to the tumor microenvironment which not only affects tumor cell growth, but also response to therapy (Yamada et al., 1999; Joon et al., 2004; Amberger-Murphy, 2009; Dewhirst, 2009; Hattingen et al., 2011; Metallo et al., 2011; Yang et al., 2012; Danhier et al., 2013; Justus et al., 2015).

It is clear that cancer cell metabolism is far more complex than originally thought. A number of cancer associated mutations affect metabolism and defects in mitochondria are seen in cancer that also link metabolism with cancer initiation and progression. Recent studies have shown that changes in cellular

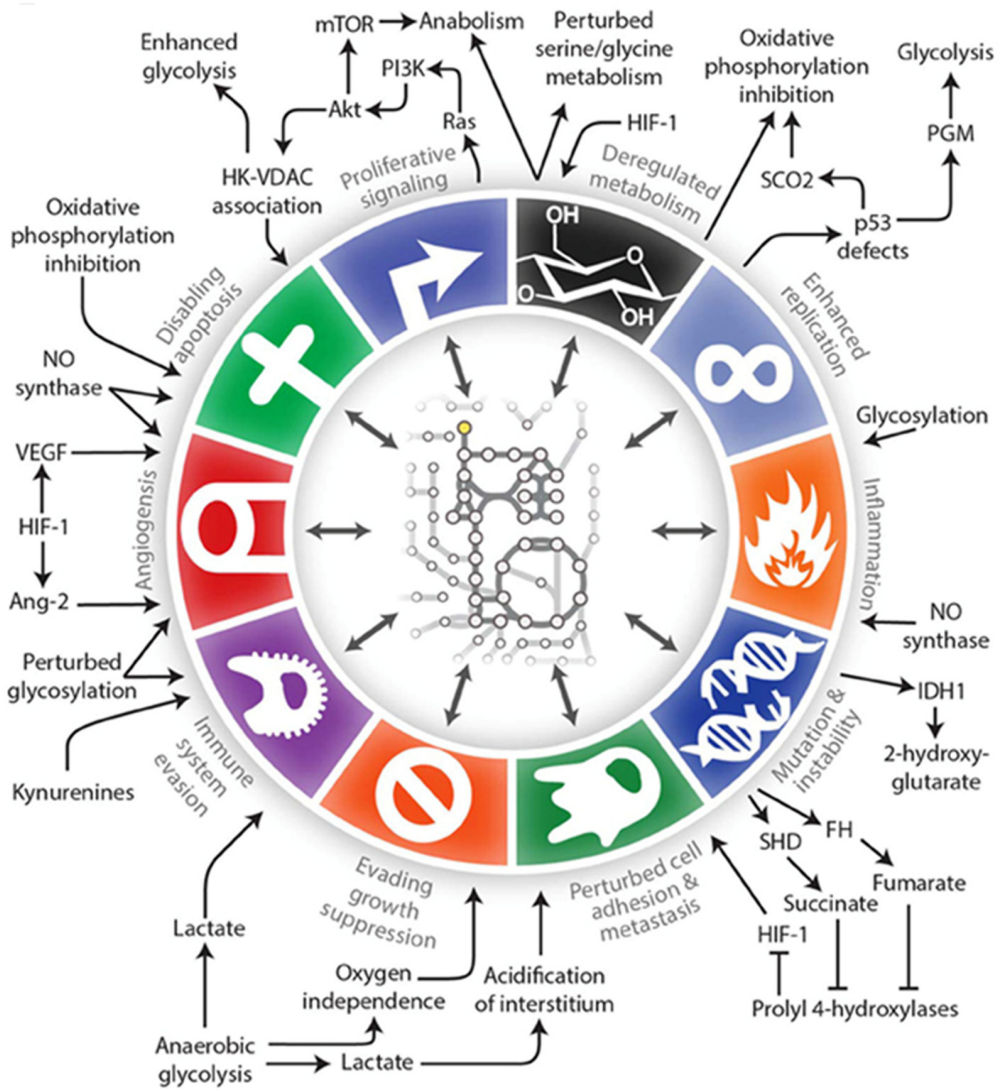


FIGURE 1 | An illustration of the interconnections between tumor metabolism with Hanahan and Weinberg's Hallmarks of Cancer (Lewis and Abdel-Haleem, 2013).

metabolism can alter the expression of specific microRNAs and promote epigenetic changes in tumor cells (Arora et al., 2015; Bishop and Ferguson, 2015; Chan et al., 2015). Although some of these interactions are mentioned above, in-depth discussions of all of the interactions that occur between cancer and metabolism are beyond the scope of this review and the reader is referred to a number of reviews on these subjects (Gatenby and Gillies, 2004; Vander Heiden et al., 2009; Cantor and Sabatini, 2012; Ward and Thompson, 2012; Semenza, 2013; Gaude and Frezza, 2014; Masson and Ratcliffe, 2014; Boroughs and DeBerardinis, 2015; Casey et al., 2015; Robey et al., 2015; Asati et al., 2016; Barron et al., 2016; Bost et al., 2016; Molon et al., 2016; Pavlova and Thompson, 2016; Pérez-Escuredo et al., 2016). The fact that metabolic dysregulation is seen in virtually all tumor cells has led to suggestions that a promising therapeutic strategy may be to exploit this feature. One potential way to achieve this goal is through the use of the therapeutic ketogenic diet (KD) or physiologically similar methods, such as caloric restriction (CR) or intermittent fasting.

THE KETOGENIC DIET: OVERVIEW AND PRECLINICAL EVIDENCE

The KD is a high-fat low protein/carbohydrate diet used to treat refractory epilepsy (Kim and Rho, 2008; Cross, 2013). It has been shown to have neuroprotective effects and there are now studies to determine its efficacy for a number of neurological disorders, including epilepsy, Alzheimer's disease, Parkinson's disease, sleep disorders, headache, traumatic brain injury, amyotrophic lateral sclerosis, pain and autism (Masino and Ruskin, 2013; Gano et al., 2014). The KD increases

blood ketones and decreases blood glucose by simulating the physiological response to fasting, thus leading to high rates of fatty acid oxidation and an increase in the production of acetyl coenzyme A (acetyl-CoA). When the amount of acetyl-CoA exceeds the capacity of the tricarboxylic acid cycle to utilize it, there is an increase in the production of the ketone bodies β-hydroxybutyrate (βHB) and acetoacetate (ACA), which can be used as an energy source in the normal brain (Veech et al., 2001; Cahill and Veech, 2003; Vanitallie and Nufert, 2003; Morris, 2005; Gasior et al., 2006). Since normal cells readily use ketones as an alternate energy source, they are unlikely to be adversely affected by reduced glucose. In contrast, the metabolic alterations found in cancer cells are generally thought to reduce their ability to be "flexible" regarding their primary energy source, and thus they require glucose (Tisdale and Brennan, 1983; Seyfried and Mukherjee, 2005; Zhou et al., 2007; Maurer et al., 2011; Seyfried et al., 2011; Seyfried, 2012). By reducing the glucose availability to cancer cells and providing ketones as an alternative energy source for normal cells, the KD may target the Warburg Effect in highly glycolytic tumors, such as malignant gliomas.

The use of metabolic alteration for the therapy of brain tumors has been championed by Seyfried et al. (2011). They used the VM (Shelton et al., 2010) and CT-2A (Marsh et al., 2008) mouse tumor models to show that a KD, especially when given in restricted amounts, extends survival. D'Agostino and co-workers have added hyperbaric oxygen and ketone supplementation to demonstrate reduced tumor cell growth and metastatic spread in the VM metastatic tumor model (Poff et al., 2014, 2015). We used the syngeneic intracranial GL261-luc/albino C57/Bl6 model to demonstrate that CR was not necessary for the anti-tumor effects of the KD (Stafford et al., 2010), particularly when a 4:1 fat:carbohydrate plus protein formulation is used (Scheck et al., 2012; Woolf et al., 2015; Lussier et al., 2016). Recently Martuscello et al. (2016) demonstrated inhibition of glioma stem cell growth *in vitro* and *in vivo* through the use of a supplemented high fat low carbohydrate diet.

The KD and similar diets used as a monotherapy have a pluripotent effect on the growth on tumors both *in vitro* and *in vivo* which may depend, at least in part, on the model system, the specific metabolic intervention and the molecular underpinnings of the tumor itself (Freedland et al., 2008; Otto et al., 2008; Mavropoulos et al., 2009; Stafford et al., 2010; Kim H. S. et al., 2012; Caso et al., 2013; Poff et al., 2013, 2015; Simone et al., 2013; Lv et al., 2014; Shukla et al., 2014; Hao et al., 2015; Woolf et al., 2015). In addition, the exact composition of the diet may also alter its effects, and there are studies in some cancers looking specifically at polyunsaturated fatty acids (PUFAs), particularly the omega-3 class, for their anti-cancer properties (Sauer et al., 2007; Pifferi et al., 2008; Wang et al., 2012, 2016; Hofmanova et al., 2013; Abel et al., 2014). The striking feature of the work done to date in a number of model systems using different dietary interventions is that alterations in metabolism have a far reaching effect on tumor cells, tumors and the tumor microenvironment. Studies have shown reductions in growth rate as one might expect however, there are also changes in the formation of reactive oxygen species and oxidative stress (Stafford et al., 2010; Milder and Patel, 2012; Allen et al., 2013), angiogeneisis (Zhou et al., 2007; Jiang and Wang, 2013; Woolf et al., 2015), hypoxia (Maurer et al., 2011; Poff et al., 2015; Woolf et al., 2015), inflammation and peri-tumoral edema (Mavropoulos et al., 2009; Woolf et al., 2015), metastasis and invasion (Gluschnaider et al., 2014; Lv et al., 2014; Hao et al., 2015; Poff et al., 2015) and the expression of various transcriptional and post-transcriptional modulators such as NF-κB (Woolf et al., 2015) and microRNAs (Pazmandi et al., 2015).

KD IN COMBINATION WITH STANDARD THERAPIES

Although evidence suggests that the KD provides anti-tumor benefits on its own, perhaps the most effective use of the KD is in combination with standard cancer therapies such as radiation and chemotherapy (Allen et al., 2014). The KD greatly enhanced survival in a mouse model of malignant glioma when combined with temozolomide (TMZ) when compared to either treatment alone (**Figure 2**; Scheck et al., 2011). Using a bioluminescent, syngeneic intracranial model of malignant glioma, the KD was shown to significantly potentiate the anti-tumor effect of radiotherapy. In fact, 9 out of 11 animals maintained on the KD and treated with radiation had complete and sustained remission of their implanted tumors, even after being switched back to a standard rodent diet (**Figure 2**; Abdelwahab et al., 2012). Allen et al. (2013) reported similar results when the KD is combined with radiation and chemotherapy in a lung cancer xenograft model. That is, they found decreased tumor growth rate and increased survival. CR and short-term fasting have also been found to be synergistic with radiation and other anti-cancer therapeutics in both preclinical and clinical studies (Raffaghello et al., 2008, 2010; Lee et al., 2010, 2012; Safdie et al., 2012; Champ et al., 2013, 2014; Saleh et al., 2013; Klement and Champ, 2014).

The effectiveness of radiation therapy is due to a number of factors including relative damage done to tumor cells vs. normal tissue and the ability of normal cells and tumor cells to repair the damage (Klement and Champ, 2014; Santivasi and Xia, 2014). KD or CR may modulate the ability of tumor and normal cells to repair radiation-induced damage (Klement and Champ, 2014). Studies have shown that CR can enhance DNA repair in normal cells (Heydari et al., 2007); however, this may not be the case in tumor cells, and the differential response of tumor cells and normal cells to genotoxic stress may be mediated by reduced Insulin-like growth factor 1 (IGF1) and glucose in the tumor cells. We and others have shown that insulin growth factor is reduced in animals maintained on a KD (Freedland et al., 2008; Mavropoulos et al., 2009; Scheck et al., 2012; Klement and Champ, 2014).

Finally, ketones and the KD have been shown to affect the immune system (Kim D. Y. et al., 2012; Husain et al., 2013; Rahman et al., 2014; Youm et al., 2015), and we have shown that the KD also reverses tumor-mediated immune suppression in a mouse model of malignant glioma (Lussier et al., 2016). As radiation-induced tumor killing is known to expose the immune

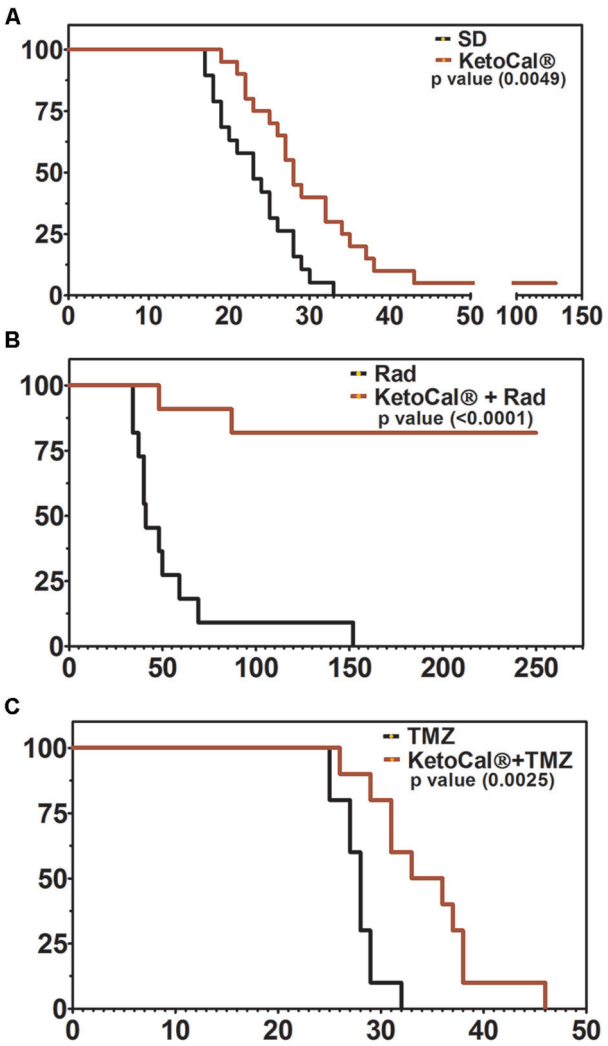


FIGURE 2 | Kaplan-Meier survival plot of animals implanted intracranially with GL261-luc2 malignant glioma cells and (A) maintained on KetoCal® [KC, the 4:1 fat:carbohydrate plus protein formulation of the ketogenic diet (KD)] vs. standard diet (SD); **(B)** treated with 2x4Gy radiation vs. KC plus radiation, and **(C)** treated with 50 mg/kg temozolomide (TMZ) vs. KC plus TMZ. Animals on KC survived significantly longer when treated with KC alone, when KC was combined with radiation, and when KC was combined with TMZ (Scheck et al., 2011; Abdelwahab et al., 2012).

system to a greater diversity of tumor antigens, it is possible that the KD as an adjuvant works to augment the effect of radiation in part by enhancing immunity against GBM.

The variety of effects seen when glucose in lowered and/or ketones are increased suggests that this may also potentiate other therapies, including newer immune- and targeted therapies. Concerns that potentiation of the anti-tumor effect of a particular therapy may also increase its effect on normal brain are valid; however, we and others have shown that the gene expression changes seen in tumor are different than those seen in normal brain (Stafford et al., 2010; Maurer et al., 2011). Further, the KD is known to have neuroprotective effects (Puchowicz et al., 2008; Lund et al., 2009; Maalouf et al., 2009; Hartman, 2012) and thus it has been postulated that this may actually help to protect the normal brain from the deleterious effects of radio and chemotherapy. Taken together, the preclinical data provides strong support for the clinical use of the KD or CR as an adjuvant therapy for the treatment of gliomas and other cancers.

β-HYDROXYBUTYRATE AS AN ANTI-CANCER AGENT

The ketone body βHB has traditionally been thought of as simply a metabolic substrate that replaces glucose during the KD, fasting or exercise; however, the effects of increased ketones go beyond simple considerations of energy availability (Newman and Verdin, 2014; Jaworski et al., 2016). *In vitro* investigations demonstrated that βHB is able to recapitulate, in part, the *in vivo* effects of the full KD (Skinner et al., 2009; Rossi et al., 2015). This suggests that the ketone bodies themselves possess antitumor effects, and that perhaps the effects of the KD are mediated, at least in part, by the ketone bodies. Additional evidence for this comes from data showing that the use of ketone supplementation can enhance the effects of the KD and may even be effective in some diseases when used alone (Veech, 2004, 2014; Kashiwaya et al., 2013; Poff et al., 2014; Shukla et al., 2014; Newport et al., 2015; Youm et al., 2015). Though the mechanisms are still under investigation, it is known that βHB is an endogenous Class I and IIa histone deacetylase (HDAC) inhibitor (Shimazu et al., 2013). HDACs primarily functions by deacetylating lysine residues on both histone and non-histone proteins, resulting in increased global acetylation and regulation of gene expression. In this way, βHB has the capacity to modulate the epigenetic environment within cells, which may contribute to the beneficial effect of the KD and CR.

Our own investigations into the interactions between βHB and glioblastoma cells have revealed insights into the molecular basis for some of the KD's effects, most notably its radio- and chemo-sensitizing effects. *In vitro* studies using βHB demonstrated that, even in the presence of high glucose, physiologically relevant doses of βHB reduced proliferation of several human glioblastoma cell lines, two human cancer stem cell lines, and a murine glioma cell line. Additionally, similar treatment with βHB resulted in potentiation of low doses of ionizing radiation therapy in both sensitive and resistant populations (Rossi et al., 2015; Silva-Nichols et al., 2015). Further, in a separate study βHB potentiated the chemotherapeutic agent 1,3-bis(2-chloroethyl)-1 nitrosourea (BCNU, carmustine) in a cell line derived from a recurrent human glioblastoma (Scheck et al., 2012). Taken together, these results suggest that ketone supplementation may provide an effective, less stringent alternative to the rigors of the KD; yet additional studies are needed to further develop this approach.

KD IN HUMANS

Studies of glucose utilization in cancer go back prior to the 1980s, including studies of metabolism and cancer cachexia (Tisdale et al., 1987; Fearon et al., 1988). These and other studies

TABLE 1 | Active clinical trials: ketogenic diet and gliomas.

ClinicalTrials.gov Identifier	Dates	Title	Location	Data (Enrollment)
01716468	First received: 9/18/12 Last updated: 4/9/16 Last verified: April 2016	Ketogenic Diet in Advanced Cancer PI: Jocelyn Tan, MD	VA Pittsburgh Healthcare System	Safety; long term tolerability; quality of life; tumor growth/spread; overall and progression free survival (17 patients)
02046187	First received: 1/17/14 Last updated: 3/5/15 Last verified: March 2015	Ketogenic Diet With Radiation and Chemotherapy for Newly Diagnosed Glioblastoma PI: Adrienne C Scheck, PhD Christopher Dardis, MD	St. Joseph's Hospital and Medical Center, Phoenix	Tolerability; overall survival; time to progression; patient quality of life (QOL); caregiver quality of life; cognitive changes; seizure activity (40 patients)
01754350	First received: 12/14/12 Last updated: 3/31/15 Last verified: March 2015	Calorie-restricted, Ketogenic Diet and Transient Fasting During Reirradiation for Patients With Recurrent Glioblastoma (ERGO2) PI: Johannes Rieger, PD Dr. med.	Johann Wolfgang Goethe University Hospitals TAVARLIN (Darmstadt, Germany)	Tolerability; progression free survival (6 months after re-irradiation); overall survival; seizure frequency; QOL; depression; attention (50 patients)
02286167	First received: 11/5/14 Last updated: 11/17/14 Last verified: October 2014	Glioma Modified Atkins-based Diet in Patients With Glioblastoma PI: Jaishri O. Blakeley, MD	Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins	Feasibility of MAD; cerebral glutamate and glutamine concentrations (MRS); dietary compliance (25 patients)
01535911	First received: 2/3/12 Last updated: 9/8/15 Last verified: September 2015	Pilot Study of a Metabolic Nutritional Therapy for the Management of Primary Brain Tumors (Ketones)	Michigan State University	Safety/Efficacy Study CT-PET scan will be used to measure changes in brain tumor size. Energy restricted ketogenic diet (ERKD) newly diagnosed GBM subjects.
01865162	First received: 5/24/13 Last updated: 11/25/14 Last verified: May 2013	Ketogenic Diet as Adjunctive Treatment in Refractory/End-stage Glioblastoma Multiforme: a Pilot Study PI: Pavel Klein, M.D.	Mid-Atlantic Epilepsy and Sleep Center, LLC Collaborator University of Pittsburgh	Safety; compliance (tolerability); survival; time to edema requiring steroids (6 patients)
02302235	First received: 11/24/14 Last updated: 11/25/14 Last verified: November 2014	Ketogenic Diet Treatment Adjunctive to Radiation and Chemotherapy in Glioblastoma Multiforme: a Pilot Study (GBMXRT) PI: Pavel Klein, M.D.	Mid-Atlantic Epilepsy and Sleep Center, LLC Collaborator Neuroscience Research Foundation	Survival; time to recurrence; time to radiological progression; tolerability (42 patients)

suggested that the KD consisting of a high percentage of medium chain triglycerides (MCT) along with various supplements resulted in weight gain and improved nitrogen balance in both animals and humans. Nebeling et al. (1995) published a case report in which they used a similar KD based on MCT oil to treat two female pediatric patients with advanced stage malignant brain tumors (Nebeling and Lerner, 1995). They demonstrated that dietary induced ketosis decreased the availability of glucose to the tumor without causing a decrease in patient weight or overall nutritional status. Furthermore, both children had long-term tumor management (Nebeling et al., 1995).

The 2nd case report was published by Zuccoli et al. (2010). This patient was a 65-year-old female with a multicentric glioblastoma. She was put on a 4:1 (ratio of fats:carbohydrate plus protein) calorie restricted (600 kcal/day) KD during radiation and chemotherapy. During this time her body weight dropped by 20%, she had reduced blood glucose, increased urinary ketones and, most importantly, no observable brain tumor by either fluorodeoxyglucose Positron Emission Tomography (FDG-PET) or magnetic resonance imaging (MRI). The tumor recurred 10 weeks after the patient resumed her normal eating habits and she succumbed to her disease less than 2 years after diagnosis. While this patient did not experience long-term tumor control after cessation of the diet, this report demonstrated that the diet could be tolerated, even when used in a calorie-restricted setting. Results of a phase 1 clinical trial were reported in 2011 by a German group (Schmidt et al., 2011). Tolerability of a restricted calorie KD was tested in 16 patients with a variety of advanced (end-stage) cancers. There were no severe side effects and 5 of the 16 patients were able to complete the 3 months treatment. These five patients had stable disease while on the diet. Two of the 11 remaining patients died early following the beginning of the trial, one was unable to tolerate the diet and dropped out immediately, two patients dropped out for personal reasons, one was unable to continue the diet for more than a month and three had disease progression

within less than 2 months of starting the diet and one dropped out to resume chemotherapy. While this trial demonstrated tolerability and favorable side effect profile, the antitumor efficacy could not be assessed due to the variety and severity of disease in the patients. Recently, Schwartz et al. (2015) reported on two patients with recurrent GBM treated with a calorie restricted KD as a monotherapy and although the diet was tolerated, both patients showed tumor progression—The first within 4 weeks and the second within 12 weeks of beginning the protocol. This group also hypothesized that an analysis of ketolytic and glycolytic enzyme levels in tumor tissue may help identify patients that are more likely to respond to a KD, although this has not yet been proven. More recently, a number of prospective clinical trials have been initiated which have been summarized in **Table 1**. These trials include studies of up-front treatment using the KD in addition to standard radiation and chemotherapy in patients diagnosed with GBM.

The case reports described above along with numerous anecdotal reports suggest that the KD may be a promising anti-cancer therapy; however, more work is needed to determine how to best utilize this, and other metabolic therapies for the treatment of tumors. Most of the information regarding the best way to use the KD comes from the epilepsy literature. Further research is needed to determine optimum blood ketone and glucose levels for anticancer effects. In addition, a variety of KDs are used for seizure control and it is not clear if one or more of the different formulations will provide the best results for cancer patients. Finally, while the KD has a long record of safety in the epilepsy community, side effects that occur when used in combination with cancer therapies may differ in type or severity. This data will come from carefully controlled clinical trials that include input from registered dietitians well-versed in the use of the KD. Patient enrollment into clinical trials requires "buy-in" from the medical community. Physicians must be educated on the therapeutic benefits of metabolic alteration as an adjuvant therapy. As with any decision regarding therapy, the patient's overall condition, including nutritional status, must be taken into account. As suggested by Klement and Champ (2014), cancer patients should be assessed for nutritional needs and tolerability of such interventions.

Concern about patients' quality of life is sometimes given as a reason not to employ KD. Compliance can be made more difficult by the use of steroids (prescribed for peritumoral edema) that often increase hunger and raise blood glucose levels. To address this, at least one clinical trial (NCT02046187) includes an analysis of both patient and caregiver quality of life. Quality of life measurements are being added to more clinical trials, as the importance of this has become recognized at the national level (van den Bent et al., 2011; Boele et al., 2013; Dirven et al., 2014). While some clinicians are concerned compliance will reduce quality of life, the patient's that do remain on the KD often comment that this allows them to participate in their own therapy. Despite these caveats, the existing preclinical data suggesting anti-tumor efficacy and a synergistic effect with standard therapies provides a strong impetus to conduct controlled clinical trials, particularly those that will shed light on the interactions between the KD and other therapies.

CONCLUSION

Improvements in the survival and quality of life for patients with malignant brain tumors require the implementation of new therapeutic modalities, especially those that increase the efficacy of current therapies without increasing toxic side effects. While the rapid accumulation of data defining the molecular and genetic aberrations present in these tumors has suggested a host of targets for the development of new therapies, targeted therapies tried to date have met with limited success. This is at least in part due to the molecular heterogeneity of these tumors that prevents any one target from being present on all cells. In contrast, metabolic dysregulation is present in virtually all tumor cells and there is increased interest in using metabolic therapies such as the KD and ketone supplementation for the treatment of various cancers, especially brain tumors. Preclinical data has demonstrated that the anti-tumor effects of the KD and CR are multi-faceted, and alterations in energy metabolism can inhibit cancer cell growth and increase the tumor's response to therapy. This provides a strong impetus to continue work designed to elucidate the mechanisms through which the KD exerts its anticancer effects, as well as suggesting the need for the design of controlled clinical trials that will shed light on the most effective way to implement metabolic therapies in combination with standard therapies for the treatment of malignant disease. This is a novel therapeutic paradigm, and we have only begun to scratch the surface of its potential.

AUTHOR CONTRIBUTIONS

ECW, NS and ACS contributed to the writing of this review article.

FUNDING

This work was funded in part by grants to ACS from Students Supporting Brain Tumor Research and support for ECW from the School of Life Sciences, Arizona State University. Brain Tumour Research Campaign helps to fund the work done in Dr. Nelofer Syed's laboratory in the United Kingdom.

REFERENCES

Abdelwahab, M. G., Fenton, K. E., Preul, M. C., Rho, J. M., Lynch, A., Stafford, P., et al. (2012). The ketogenic diet is an effective adjuvant to radiation therapy for the treatment of malignant glioma. *PLoS One* 7:e36197. doi: 10.1371/journal.pone.0036197

Abel, S., Riedel, S., and Gelderblom, W. C. A. (2014). Dietary PUFA and cancer. *Proc. Nutr. Soc.* 73, 361–367. doi: 10.1017/s0029665114000585

Allen, B. G., Bhatia, S. K., Anderson, C. M., Eichenberger-Gilmore, J. M., Sibenaller, Z. A., Mapuskar, K. A., et al. (2014). Ketogenic diets as an adjuvant cancer therapy: history and potential mechanism. *Redox Biol.* 2, 963–970. doi: 10.1016/j.redox.2014.08.002

Allen, B. G., Bhatia, S. K., Buatti, J. M., Brandt, K. E., Lindholm, K. E., Button, A. M., et al. (2013). Ketogenic diets enhance oxidative stress and radio-chemo-therapy responses in lung cancer xenografts. *Clin. Cancer Res.* 19, 3905–3913. doi: 10.1158/1078-0432.CCR-12-0287

Altman, B. J., Hsieh, A. L., Sengupta, A., Krishnanaiah, S. Y., Stine, Z. E., Walton, Z. E., et al. (2015). MYC disrupts the circadian clock and metabolism in cancer cells. *Cell Metab.* 22, 1009–1019. doi: 10.1016/j.cmet.2015.09.003

Amberger-Murphy, V. (2009). Hypoxia helps glioma to fight therapy. *Curr. Cancer Drug Targets* 9, 381–390. doi: 10.2174/156800909788166637

Arora, A., Singh, S., Bhatt, A. N., Pandey, S., Sandhir, R., and Dwarakanath, B. S. (2015). Interplay between metabolism and oncogenic process: role of microRNAs. *Transl. Oncogenomics* 7, 11–27. doi: 10.4137/TOG.S29652

Asati, V., Mahapatra, D. K., and Bharti, S. K. (2016). PI3K/Akt/mTOR and Ras/Raf/MEK/ERK signaling pathways inhibitors as anticancer agents: structural and pharmacological perspectives. *Eur. J. Med. Chem.* 109, 314–341. doi: 10.1016/j.ejmech.2016.01.012

Barron, C. C., Bilan, P. J., Tsakiridis, T., and Tsiani, E. (2016). Facilitative glucose transporters: implications for cancer detection, prognosis and treatment. *Metabolism* 65, 124–139. doi: 10.1016/j.metabol.2015.10.007

Bishop, K. S., and Ferguson, L. R. (2015). The interaction between epigenetics, nutrition and the development of cancer. *Nutrients* 7, 922–947. doi: 10.3390/nu7020922

Boele, F. W., Heimans, J. J., Aaronson, N. K., Taphoorn, M. J., Postma, T. J., Reijneveld, J. C., et al. (2013). Health-related quality of life of significant others of patients with malignant CNS versus non-CNS tumors: a comparative study. *J. Neurooncol.* 115, 87–94. doi: 10.1007/s11060-013-1198-z

Boroughs, L. K., and DeBerardinis, R. J. (2015). Metabolic pathways promoting cancer cell survival and growth. *Nat. Cell Biol.* 17, 351–359. doi: 10.1038/ncb3124

Bost, F., Decoux-Poullot, A. G., Tanti, J. F., and Clavel, S. (2016). Energy disruptors: rising stars in anticancer therapy? *Oncogenesis* 5:e188. doi: 10.1038/oncsis.2015.46

Cahill, G. F. Jr., and Veech, R. L. (2003). Ketoacids? Good medicine? *Trans. Am. Clin. Climatol. Assoc.* 114, 149–161.

Cantor, J. R., and Sabatini, D. M. (2012). Cancer cell metabolism: one hallmark, many faces. *Cancer Discov.* 2, 881–898. doi: 10.1158/2159-8290.CD-12-0345

Casey, S. C., Amedei, A., Aquilano, K., Azmi, A. S., Benencia, F., Bhakta, D., et al. (2015). Cancer prevention and therapy through the modulation of the tumor microenvironment. *Semin. Cancer Biol.* 35, S199–S223. doi: 10.1016/j.semcancer.2015.02.007

Caso, J., Masko, E. M., Ii, J. A., Poulton, S. H., Dewhirst, M., Pizzo, S. V., et al. (2013). The effect of carbohydrate restriction on prostate cancer tumor growth in a castrate mouse xenograft model. *Prostate* 73, 449–454. doi: 10.1002/pros.22586

Champ, C. E., Baserga, R., Mishra, M. V., Jin, L., Sotgia, F., Lisanti, M. P., et al. (2013). Nutrient restriction and radiation therapy for cancer treatment: when less is more. *Oncologist* 18, 97–103. doi: 10.1634/theoncologist.2012-0164

Champ, C. E., Palmer, J. D., Volek, J. S., Werner-Wasik, M., Andrews, D. W., Evans, J. J., et al. (2014). Targeting metabolism with a ketogenic diet during the treatment of glioblastoma multiforme. *J. Neurooncol.* 117, 125–131. doi: 10.1007/s11060-014-1362-0

Chan, B., Manley, J., Lee, J., and Singh, S. R. (2015). The emerging roles of microRNAs in cancer metabolism. *Cancer Lett.* 356, 301–308. doi: 10.1016/j.canlet.2014.10.011

Clark, P. M., Mai, W. X., Cloughesy, T. F., and Nathanson, D. A. (2016). Emerging approaches for targeting metabolic vulnerabilities in malignant glioma. *Curr. Neurol. Neurosci. Rep.* 16:17. doi: 10.1007/s11910-015-0613-6

Corbet, C., and Feron, O. (2015). Metabolic and mind shifts: from glucose to glutamine and acetate addictions in cancer. *Curr. Opin. Clin. Nutr. Metab. Care* 18, 346–353. doi: 10.1097/MCO.0000000000000178

Courtnay, R., Ngo, D. C., Malik, N., Ververis, K., Tortorella, S. M., and Karagiannis, T. C. (2015). Cancer metabolism and the Warburg effect: the role of HIF-1 and PI3K. *Mol. Biol. Rep.* 42, 841–851. doi: 10.1007/s11033-015-3858-x

Cross, J. H. (2013). New research with diets and epilepsy. *J. Child Neurol.* 28, 970–974. doi: 10.1177/0883073813487593

Danhier, P., De Saedeleer, C. J., Karroum, O., De Preter, G., Porporato, P. E., Jordan, B. F., et al. (2013). Optimization of tumor radiotherapy with modulators of cell metabolism: toward clinical applications. *Semin. Radiat. Oncol.* 23, 262–272. doi: 10.1016/j.semradonc.2013.05.008

Dewhirst, M. W. (2009). Relationships between cycling hypoxia, HIF-1, angiogenesis and oxidative stress. *Radiat. Res.* 172, 653–665. doi: 10.1667/RR1926.1

Dibble, C. C., and Cantley, L. C. (2015). Regulation of mTORC1 by PI3K signaling. *Trends Cell Biol.* 25, 545–555. doi: 10.1016/j.tcb.2015.06.002

Dirven, L., Taphoorn, M. J., Reijneveld, J. C., Blazeby, J., Jacobs, M., Pusic, A., et al. (2014). The level of patient-reported outcome reporting in randomised controlled trials of brain tumour patients: a systematic review. *Eur. J. Cancer* 50, 2432–2448. doi: 10.1016/j.ejca.2014.06.016

Fearon, K. C., Borland, W., Preston, T., Tisdale, M. J., Shenkin, A., and Calman, K. C. (1988). Cancer cachexia: influence of systemic ketosis on substrate levels and nitrogen metabolism. *Am. J. Clin. Nutr.* 47, 42–48.

Fischer, S., Wobben, M., Marti, H. H., Renz, D., and Schaper, W. (2002). Hypoxia-induced hyperpermeability in brain microvessel endothelial cells involves VEGF-mediated changes in the expression of zonula occludens-1. *Microvasc. Res.* 63, 70–80. doi: 10.1006/mvre.2001.2367

Freedland, S. J., Mavropoulos, J., Wang, A., Darshan, M., Demark-Wahnefried, W., Aronson, W. J., et al. (2008). Carbohydrate restriction, prostate cancer growth and the insulin-like growth factor axis. *Prostate* 68, 11–19. doi: 10.1002/pros.20683

Fujiwara, S., Nakagawa, K., Harada, H., Nagato, S., Furukawa, K., Teraoka, M., et al. (2007). Silencing hypoxia-inducible factor-1α inhibits cell migration and invasion under hypoxic environment in malignant gliomas. *Int. J. Oncol.* 30, 793–802. doi: 10.3892/ijo.30.4.793

Gano, L. B., Patel, M., and Rho, J. M. (2014). Ketogenic diets, mitochondria and neurological diseases. *J. Lipid Res.* 55, 2211–2228. doi: 10.1194/jlr.r048975

Gasior, M., Rogawski, M. A., and Hartman, A. L. (2006). Neuroprotective and disease-modifying effects of the ketogenic diet. *Behav. Pharmacol.* 17, 431–439. doi: 10.1097/00008877-200609000-00009

Gatenby, R. A., and Gillies, R. J. (2004). Why do cancers have high aerobic glycolysis? *Nat. Rev. Cancer* 4, 891–899. doi: 10.1038/nrc1478

Gaude, E., and Frezza, C. (2014). Defects in mitochondrial metabolism and cancer. *Cancer Metab.* 2:10. doi: 10.1186/2049-3002-2-10

Gluschnaider, U., Hertz, R., Ohayon, S., Smeir, E., Smets, M., Pikarsky, E., et al. (2014). Long-chain Fatty Acid analogues suppress breast tumorigenesis and progression. *Cancer Res.* 74, 6991–7002. doi: 10.1158/0008-5472.CAN-14-0385

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell* 144, 646–674. doi: 10.1016/j.cell.2011.02.013

Hao, G. W., Chen, Y. S., He, D. M., Wang, H. Y., Wu, G. H., and Zhang, B. (2015). Growth of human colon cancer cells in nude mice is delayed by ketogenic diet with or without omega-3 fatty acids and medium-chain triglycerides. *Asian Pac. J. Cancer Prev.* 16, 2061–2068. doi: 10.7314/apjcp.2015.16.5.2061

Hartman, A. L. (2012). Neuroprotection in metabolism-based therapy. *Epilepsy Res.* 100, 286–294. doi: 10.1016/j.eplepsyres.2011.04.016

Hattingen, E., Jurcoane, A., Bähr, O., Rieger, J., Magerkurth, J., Anti, S., et al. (2011). Bevacizumab impairs oxidative energy metabolism and shows antitumoral effects in recurrent glioblastomas: a 31P/1H MRSI and quantitative magnetic resonance imaging study. *Neuro Oncol.* 13, 1349–1363. doi: 10.1093/neuonc/nor132

Hayashi, Y., Edwards, N. A., Proescholdt, M. A., Oldfield, E. H., and Merrill, M. J. (2007). Regulation and function of aquaporin-1 in glioma cells. *Neoplasia* 9, 777–787. doi: 10.1593/neo.07454

Heydari, A. R., Unnikrishnan, A., Lucente, L. V., and Richardson, A. (2007). Caloric restriction and genomic stability. *Nucleic Acids Res.* 35, 7485–7496. doi: 10.1093/nar/gkm860

Hofmanova, J., Hyrslova Vaculova, A., and Kozubik, A. (2013). Regulation of the metabolism of polyunsaturated Fatty acids and butyrate in colon cancer cells. *Curr. Pharm. Biotechnol.* 14, 274–288. doi: 10.2174/13892010113140030004

Horing, E., Harter, P. N., Seznec, J., Schittenhelm, J., Buhring, H. J., Bhattacharyya, S., et al. (2012). The "go or grow" potential of gliomas is linked to the neuropeptide processing enzyme carboxypeptidase E and mediated

by metabolic stress. *Acta Neuropathol.* 124, 83–97. doi: 10.1007/s00401-011-0940-x

Hsieh, A. L., Walton, Z. E., Altman, B. J., Stine, Z. E., and Dang, C. V. (2015). MYC and metabolism on the path to cancer. *Semin. Cell Dev. Biol.* 43, 11–21. doi: 10.1016/j.semcdb.2015.08.003

Husain, Z., Huang, Y., Seth, P., and Sukhatme, V. P. (2013). Tumor-derived lactate modifies antitumor immune response: effect on myeloid-derived suppressor cells and NK cells. *J. Immunol.* 191, 1486–1495. doi: 10.4049/jimmunol.1202702

Iurlaro, R., León-Annicchiarico, C. L., and Muñoz-Pinedo, C. (2014). Regulation of cancer metabolism by oncogenes and tumor suppressors. *Methods Enzymol.* 542, 59–80. doi: 10.1016/B978-0-12-416618-9.00003-0

Jaworski, D. M., Namboodiri, A. M., and Moffett, J. R. (2016). Acetate as a metabolic and epigenetic modifier of cancer therapy. *J. Cell. Biochem.* 117, 574–588. doi: 10.1002/jcb.25305

Jiang, Y.-S., and Wang, F.-R. (2013). Caloric restriction reduces edema and prolongs survival in a mouse glioma model. *J. Neurooncol.* 114, 25–32. doi: 10.1007/s11060-013-1154-y

Joon, Y. A., Bazar, K. A., and Lee, P. Y. (2004). Tumors may modulate host immunity partly through hypoxia-induced sympathetic bias. *Med. Hypotheses* 63, 352–356. doi: 10.1016/j.mehy.2004.02.026

Justus, C. R., Sanderlin, E. J., and Yang, L. V. (2015). Molecular connections between cancer cell metabolism and the tumor microenvironment. *Int. J. Mol. Sci.* 16, 11055–11086. doi: 10.3390/ijms160511055

Kashiwaya, Y., Bergman, C., Lee, J. H., Wan, R., King, M. T., Mughal, M. R., et al. (2013). A ketone ester diet exhibits anxiolytic and cognition-sparing properties and lessens amyloid and tau pathologies in a mouse model of Alzheimer's disease. *Neurobiol. Aging* 34, 1530–1539. doi: 10.1016/j.neurobiolaging.2012.11.023

Kaur, B., Khwaja, F. W., Severson, E. A., Matheny, S. L., Brat, D. J., and Van Meir, E. G. (2005). Hypoxia and the hypoxia-inducible-factor pathway in glioma growth and angiogenesis. *Neuro Oncol.* 7, 134–153. doi: 10.1215/s1152851704001115

Kim, D. Y., Hao, J., Liu, R., Turner, G., Shi, F.-D., and Rho, J. M. (2012). Inflammation-mediated memory dysfunction and effects of a ketogenic diet in a murine model of multiple sclerosis. *PLoS One* 7:e35476. doi: 10.1371/journal.pone.0035476

Kim, H. S., Masko, E. M., Poulton, S. L., Kennedy, K. M., Pizzo, S. V., Dewhirst, M. W., et al. (2012). Carbohydrate restriction and lactate transporter inhibition in a mouse xenograft model of human prostate cancer. *BJU Int.* 110, 1062–1069. doi: 10.1111/j.1464-410x.2012.10971.x

Kim, M. H., and Kim, H. (2013). Oncogenes and tumor suppressors regulate glutamine metabolism in cancer cells. *J. Cancer Prev.* 18, 221–226. doi: 10.15430/jcp.2013.18.3.221

Kim, D. Y., and Rho, J. M. (2008). The ketogenic diet and epilepsy. *Curr. Opin. Clin. Nutr. Metab. Care* 11, 113–120. doi: 10.1097/MCO.0b013e3282f44c06

Klement, R. J., and Champ, C. E. (2014). Calories, carbohydrates and cancer therapy with radiation: exploiting the five R's through dietary manipulation. *Cancer Metastasis Rev.* 33, 217–229. doi: 10.1007/s10555-014-9495-3

Lee, C., Raffaghello, L., Brandhorst, S., Safdie, F. M., Bianchi, G., Martin-Montalvo, A., et al. (2012). Fasting cycles retard growth of tumors and sensitize a range of cancer cell types to chemotherapy. *Sci. Transl. Med.* 4:124ra127. doi: 10.1126/scitranslmed.3003293

Lee, C., Safdie, F. M., Raffaghello, L., Wei, M., Madia, F., Parrella, E., et al. (2010). Reduced levels of IGF-I mediate differential protection of normal and cancer cells in response to fasting and improve chemotherapeutic index. *Cancer Res.* 70, 1564–1572. doi: 10.1158/0008-5472.CAN-09-3228

Lewis, N. E., and Abdel-Haleem, A. M. (2013). The evolution of genome-scale models of cancer metabolism. *Front. Physiol.* 4:237. doi: 10.3389/fphys.2013.00237

Lund, T. M., Risa, O., Sonnewald, U., Schousboe, A., and Waagepetersen, H. S. (2009). Availability of neurotransmitter glutamate is diminished when β-hydroxybutyrate replaces glucose in cultured neurons. *J. Neurochem.* 110, 80–91. doi: 10.1111/j.1471-4159.2009.06115.x

Lussier, D. M., Woolf, E. C., Johnson, J. L., Brooks, K. S., Blattman, J. N., and Scheck, A. C. (2016). Enhanced immunity in a mouse model of malignant glioma is mediated by a therapeutic ketogenic diet. *BMC Cancer* 16:310. doi: 10.1186/s12885-016-2337-7

Lv, M., Zhu, X., Wang, H., Wang, F., and Guan, W. (2014). Roles of caloric restriction, ketogenic diet and intermittent fasting during initiation, progression and metastasis of cancer in animal models: a systematic review and meta-analysis. *PLoS One* 9:e115147. doi: 10.1371/journal.pone.0115147

Maalouf, M., Rho, J. M., and Mattson, M. P. (2009). The neuroprotective properties of calorie restriction, the ketogenic diet and ketone bodies. *Brain Res. Rev.* 59, 293–315. doi: 10.1016/j.brainresrev.2008.09.002

Madan, E., Gogna, R., Bhatt, M., Pati, U., Kuppusamy, P., and Mahdi, A. A. (2011). Regulation of glucose metabolism by p53: emerging new roles for the tumor suppressor. *Oncotarget* 2, 948–957. doi: 10.18632/oncotarget.389

Marie, S. K., and Shinjo, S. M. (2011). Metabolism and brain cancer. *Clinics* 66, 33–43. doi: 10.1590/s1807-59322011001300005

Marsh, J., Mukherjee, P., and Seyfried, T. N. (2008). Drug/diet synergy for managing malignant astrocytoma in mice: 2-deoxy-D-glucose and the restricted ketogenic diet. *Nutr. Metab. (Lond)* 5:33. doi: 10.1186/1743-7075-5-33

Martini, M., De Santis, M. C., Braccini, L., Gulluni, F., and Hirsch, E. (2014). PI3K/AKT signaling pathway and cancer: an updated review. *Ann. Med.* 46, 372–383. doi: 10.3109/07853890.2014.912836

Martuscello, R. T., Vedam-Mai, V., McCarthy, D. J., Schmoll, M. E., Jundi, M. A., Louviere, C. D., et al. (2016). A supplemented high-fat low-carbohydrate diet for the treatment of glioblastoma. *Clin. Cancer Res.* 22, 2482–2495. doi: 10.1158/1078-0432.CCR-15-0916

Masino, S. A., and Ruskin, D. N. (2013). Ketogenic diets and pain. *J. Child Neurol.* 28, 993–1001. doi: 10.1177/0883073813487595

Masson, N., and Ratcliffe, P. J. (2014). Hypoxia signaling pathways in cancer metabolism: the importance of co-selecting interconnected physiological pathways. *Cancer Metab.* 2:3. doi: 10.1186/2049-3002-2-3

Maurer, G. D., Brucker, D. P., Bähr, O., Harter, P. N., Hattingen, E., Walenta, S., et al. (2011). Differential utilization of ketone bodies by neurons and glioma cell lines: a rationale for ketogenic diet as experimental glioma therapy. *BMC Cancer* 11:315. doi: 10.1186/1471-2407-11-315

Mavropoulos, J. C., Buschemeyer, W. C. III, Tewari, A. K., Rokhfeld, D., Pollak, M., Zhao, Y., et al. (2009). The effects of varying dietary carbohydrate and fat content on survival in a murine LNCaP prostate cancer xenograft model. *Cancer Prev. Res. (Phila)* 2, 557–565. doi: 10.1158/1940-6207.capr-08-0188

Metallo, C. M., Gameiro, P. A., Bell, E. L., Mattaini, K. R., Yang, J., Hiller, K., et al. (2011). Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. *Nature* 481, 380–384. doi: 10.1038/nature10602

Milder, J., and Patel, M. (2012). Modulation of oxidative stress and mitochondrial function by the ketogenic diet. *Epilepsy Res.* 100, 295–303. doi: 10.1016/j.eplepsyres.2011.09.021

Miller, D. M., Thomas, S. D., Islam, A., Muench, D., and Sedoris, K. (2012). c-Myc and cancer metabolism. *Clin. Cancer Res.* 18, 5546–5553. doi: 10.1158/1078-0432.CCR-12-0977

Molon, B., Cali, B., and Viola, A. (2016). T cells and cancer: how metabolism shapes immunity. *Front. Immunol.* 7:20. doi: 10.3389/fimmu.2016.00020

Morris, A. A. M. (2005). Cerebral ketone body metabolism. *J. Inherit. Metab. Dis.* 28, 109–121. doi: 10.1007/s10545-005-5518-0

Mou, K., Chen, M., Mao, Q., Wang, P., Ni, R., Xia, X., et al. (2010). AQP-4 in peritumoral edematous tissue is correlated with the degree of glioma and with expression of VEGF and HIF-alpha. *J. Neurooncol.* 100, 375–383. doi: 10.1007/s11060-010-0205-x

Nebeling, L. C., and Lerner, E. (1995). Implementing a ketogenic diet based on medium-chain triglyceride oil in pediatric patients with cancer. *J. Am. Diet. Assoc.* 95, 693–697. doi: 10.1016/s0002-8223(95)00189-1

Nebeling, L. C., Miraldi, F., Shurin, S. B., and Lerner, E. (1995). Effects of a ketogenic diet on tumor metabolism and nutritional status in pediatric oncology patients: two case reports. *J. Am. Coll. Nutr.* 14, 202–208. doi: 10.1080/07315724.1995.10718495

Newman, J. C., and Verdin, E. (2014). Ketone bodies as signaling metabolites. *Trends Endocrinol. Metab.* 25, 42–52. doi: 10.1016/j.tem.2013.09.002

Newport, M. T., Vanitallie, T. B., Kashiwaya, Y., King, M. T., and Veech, R. L. (2015). A new way to produce hyperketonemia: use of ketone ester in a case of Alzheimer's disease. *Alzheimers Dement.* 11, 99–103. doi: 10.1016/j.jalz.2014.01.006

Nicholas, M. K., Lukas, R. V., Chmura, S., Yamini, B., Lesniak, M., and Pytel, P. (2011). Molecular heterogeneity in glioblastoma: therapeutic opportunities and challenges. *Semin. Oncol.* 38, 243–253. doi: 10.1053/j.seminoncol.2011.01.009

Niyazi, M., Siefert, A., Schwarz, S. B., Ganswindt, U., Kreth, F. W., Tonn, J. C., et al. (2011). Therapeutic options for recurrent malignant glioma. *Radiother. Oncol.* 98, 1–14. doi: 10.1016/j.radonc.2010.11.006

Obre, E., and Rossignol, R. (2015). Emerging concepts in bioenergetics and cancer research: metabolic flexibility, coupling, symbiosis, switch, oxidative tumors, metabolic remodeling, signaling and bioenergetic therapy. *Int. J. Biochem. Cell Biol.* 59C, 167–181. doi: 10.1016/j.biocel.2014.12.008

Olovnikov, I. A., Kravchenko, J. E., and Chumakov, P. M. (2009). Homeostatic functions of the p53 tumor suppressor: regulation of energy metabolism and antioxidant defense. *Semin. Cancer Biol.* 19, 32–41. doi: 10.1016/j.semcancer.2008.11.005

Otto, C., Kaemmerer, U., Illert, B., Muehling, B., Pfetzer, N., Wittig, R., et al. (2008). Growth of human gastric cancer cells in nude mice is delayed by a ketogenic diet supplemented with omega-3 fatty acids and medium-chain triglycerides. *BMC Cancer* 8:122. doi: 10.1186/1471-2407-8-122

Pavlova, N. N., and Thompson, C. B. (2016). The emerging hallmarks of cancer metabolism. *Cell Metab.* 23, 27–47. doi: 10.1016/j.cmet.2015.12.006

Pazmandi, J., O'Neill, K. S., Scheck, A. C., Szlosarek, P. W., Woolf, E. C., Brooks, K. S., et al. (2015). Abstract 240: the ketogenic diet alters the expression of microRNAs that play key roles in tumor development. *Cancer Res.* 75, 240–240. doi: 10.1158/1538-7445.am2015-240

Pérez-Escuredo, J., Dadhich, R. K., Dhup, S., Cacace, A., Van Hée, V. F., De Saedeleer, C. J., et al. (2016). Lactate promotes glutamine uptake and metabolism in oxidative cancer cells. *Cell Cycle* 15, 72–83. doi: 10.1080/15384101.2015.1120930

Pifferi, F., Tremblay, S., Plourde, M., Tremblay-Mercier, J., Bentourkia, M., and Cunnane, S. C. (2008). Ketones and brain function: possible link to polyunsaturated fatty acids and availability of a new brain PET tracer, 11C-acetoacetate. *Epilepsia* 49, 76–79. doi: 10.1111/j.1528-1167.2008.01842.x

Poff, A. M., Ari, C., Arnold, P., Seyfried, T. N., and D'Agostino, D. P. (2014). Ketone supplementation decreases tumor cell viability and prolongs survival of mice with metastatic cancer. *Int. J. Cancer* 135, 1711–1720. doi: 10.1002/ijc.28809

Poff, A. M., Ari, C., Seyfried, T. N., and D'Agostino, D. P. (2013). The ketogenic diet and hyperbaric oxygen therapy prolong survival in mice with systemic metastatic cancer. *PLoS One* 8:e65522. doi: 10.1371/journal.pone.0065522

Poff, A. M., Ward, N., Seyfried, T. N., Arnold, P., and D'Agostino, D. P. (2015). Non-toxic metabolic management of metastatic cancer in vm mice: novel combination of ketogenic diet, ketone supplementation and hyperbaric oxygen therapy. *PLoS One* 10:e0127407. doi: 10.1371/journal.pone.0127407

Pore, N., Jiang, Z., Shu, H. K., Bernhard, E., Kao, G. D., and Maity, A. (2006). Akt1 activation can augment hypoxia-inducible factor-1α expression by increasing protein translation through a mammalian target of rapamycin-independent pathway. *Mol. Cancer Res.* 4, 471–479. doi: 10.1158/1541-7786.mcr-05-0234

Proescholdt, M. A., Merrill, M. J., Stoerr, E. M., Lohmeier, A., Pohl, F., and Brawanski, A. (2012). Function of carbonic anhydrase IX in glioblastoma multiforme. *Neuro Oncol.* 14, 1357–1366. doi: 10.1093/neuonc/nos216

Puchowicz, M. A., Zechel, J. L., Valerio, J., Emancipator, D. S., Xu, K., Pundik, S., et al. (2008). Neuroprotection in diet-induced ketotic rat brain after focal ischemia. *J. Cereb. Blood Flow Metab.* 28, 1907–1916. doi: 10.1038/jcbfm.2008.79

Puzio-Kuter, A. M. (2011). The role of p53 in metabolic regulation. *Genes Cancer* 2, 385–391. doi: 10.1177/1947601911409738

Raffaghello, L., Lee, C., Safdie, F. M., Wei, M., Madia, F., Bianchi, G., et al. (2008). Starvation-dependent differential stress resistance protects normal but not cancer cells against high-dose chemotherapy. *Proc. Natl. Acad. Sci. U S A* 105, 8215–8220. doi: 10.1073/pnas.0708100105

Raffaghello, L., Safdie, F., Bianchi, G., Dorff, T., Fontana, L., and Longo, V. D. (2010). Fasting and differential chemotherapy protection in patients. *Cell Cycle* 9, 4474–4476. doi: 10.4161/cc.9.22.13954

Rahman, M., Muhammad, S., Khan, M. A., Chen, H., Ridder, D. A., Müller-Fielitz, H., et al. (2014). The β-hydroxybutyrate receptor HCA2 activates a neuroprotective subset of macrophages. *Nat. Commun.* 5:3944. doi: 10.1038/ncomms4944

Roberts, D. J., and Miyamoto, S. (2015). Hexokinase II integrates energy metabolism and cellular protection: akting on mitochondria and TORCing to autophagy. *Cell Death Differ.* 22, 248–257. doi: 10.1038/cdd.2014.173

Robey, R. B., and Hay, N. (2009). Is Akt the "Warburg kinase"?-Akt-energy metabolism interactions and oncogenesis. *Semin. Cancer Biol.* 19, 25–31. doi: 10.1016/j.semcancer.2008.11.010

Robey, R. B., Weisz, J., Kuemmerle, N. B., Salzberg, A. C., Berg, A., Brown, D. G., et al. (2015). Metabolic reprogramming and dysregulated metabolism: cause, consequence and/or enabler of environmental carcinogenesis? *Carcinogenesis* 36, S203–S231. doi: 10.1093/carcin/bgv037

Roesler, R., Brunetto, A. T., Abujamra, A. L., de Farias, C. B., Brunetto, A. L., and Schwartsmann, G. (2010). Current and emerging molecular targets in glioma. *Expert. Rev Anticancer Ther.* 10, 1735–1751. doi: 10.1586/era.10.167

Rossi, A. P., Woolf, E. C., Brooks, K. S., Fairres, M. J., and Scheck, A. C. (2015). Abstract 3346: the ketone body β-hydroxybutyrate increases radiosensitivity in glioma cell lines *in vitro*. *Cancer Res.* 75:3346. doi: 10.1158/1538-7445.am2015-3346

Safdie, F., Brandhorst, S., Wei, M., Wang, W., Lee, C., Hwang, S., et al. (2012). Fasting enhances the response of glioma to chemo- and radiotherapy. *PLoS One* 7:e44603. doi: 10.1371/journal.pone.0044603

Saleh, A. D., Simone, B. A., Palazzo, J., Savage, J. E., Sano, Y., Dan, T., et al. (2013). Caloric restriction augments radiation efficacy in breast cancer. *Cell Cycle* 12, 1955–1963. doi: 10.4161/cc.25016

Santivasi, W. L., and Xia, F. (2014). Ionizing radiation-induced DNA damage, response and repair. *Antioxid. Redox Signal.* 21, 251–259. doi: 10.1089/ars.2013.5668

Sauer, L. A., Blask, D. E., and Dauchy, R. T. (2007). Dietary factors and growth and metabolism in experimental tumors. *J. Nutr. Biochem.* 18, 637–649. doi: 10.1016/j.jnutbio.2006.12.009

Scheck, A. C., Abdelwahab, M. G., Fenton, K., and Stafford, P. (2012). The ketogenic diet for the treatment of glioma: insights from genetic profiling. *Epilepsy Res.* 100, 327–337. doi: 10.1016/j.eplepsyres.2011.09.022

Scheck, A. C., Abdelwahab, M. G., Stafford, P., Kim, D. Y., Iwai, S., Preul, M. C., et al. (2011). Abstract 638: mechanistic studies of the ketogenic diet as an adjuvant therapy for malignant gliomas. *Cancer Res.* 70, 638–638. doi: 10.1158/1538-7445.am10-638

Schmidt, M., Pfetzer, N., Schwab, M., Strauss, I., and Kammerer, U. (2011). Effects of a ketogenic diet on the quality of life in 16 patients with advanced cancer: a pilot trial. *Nutr. Metab. (Lond)* 8:54. doi: 10.1186/1743-7075-8-54

Schwartz, K., Chang, H. T., Nikolai, M., Pernicone, J., Rhee, S., Olson, K., et al. (2015). Treatment of glioma patients with ketogenic diets: report of two cases treated with an IRB-approved energy-restricted ketogenic diet protocol and review of the literature. *Cancer Metab.* 3:3. doi: 10.1186/s40170-015-0129-1

Semenza, G. L. (2013). HIF-1 mediates metabolic responses to intratumoral hypoxia and oncogenic mutations. *J. Clin. Invest* 123, 3664–3671. doi: 10.1172/JCI67230

Seyfried, T. N. (2012). *Cancer as a Metabolic Disease: On the Origin, Management and Prevention of Cancer.* Hoboken, NJ: John Wiley and Sons, Inc.

Seyfried, T. N., Kiebish, M. A., Marsh, J., Shelton, L. M., Huysentruyt, L. C., and Mukherjee, P. (2011). Metabolic management of brain cancer. *Biochim. Biophys. Acta* 1807, 577–594. doi: 10.1016/j.bbabio.2010.08.009

Seyfried, T. N., and Mukherjee, P. (2005). Targeting energy metabolism in brain cancer: review and hypothesis. *Nutr. Metab. (Lond)* 2:30. doi: 10.1186/1743-7075-2-30

Shelton, L. M., Huysentruyt, L. C., Mukherjee, P., and Seyfried, T. N. (2010). Calorie restriction as an anti-invasive therapy for malignant brain cancer in the VM mouse. *ASN Neuro* 2:e00038. doi: 10.1042/AN20100002

Shimazu, T., Hirschey, M. D., Newman, J., He, W., Shirakawa, K., Le Moan, N., et al. (2013). Suppression of oxidative stress by β-hydroxybutyrate, an endogenous histone deacetylase inhibitor. *Science* 339, 211–214. doi: 10.1126/science.1227166

Shukla, S. K., Gebregiworgis, T., Purohit, V., Chaika, N. V., Gunda, V., Radhakrishnan, P., et al. (2014). Metabolic reprogramming induced by ketone bodies diminishes pancreatic cancer cachexia. *Cancer Metab.* 2:18. doi: 10.1186/2049-3002-2-18

Silva-Nichols, H. B., Woolf, E. C., Deleyrolle, L. P., Reynolds, B. A., and Scheck, A. C. (2015). The ketone body β-hydroxybutyrate radiosensitizes glioblastoma multiforme stem cells. *Neuro Oncol.* 17:v35. doi: 10.1093/neuonc/nov204.77

Simone, B. A., Champ, C. E., Rosenberg, A. L., Berger, A. C., Monti, D. A., Dicker, A. P., et al. (2013). Selectively starving cancer cells through dietary manipulation: methods and clinical implications. *Future Oncol.* 9, 959–976. doi: 10.2217/fon.13.31

Skinner, R., Trujillo, A., Ma, X., and Beierle, E. A. (2009). Ketone bodies inhibit the viability of human neuroblastoma cells. *J. Pediatr. Surg.* 44, 212–216. doi: 10.1016/j.jpedsurg.2008.10.042

Stafford, P., Abdelwahab, M. G., Kim, D. Y., Preul, M. C., Rho, J. M., and Scheck, A. C. (2010). The ketogenic diet reverses gene expression patterns and reduces reactive oxygen species levels when used as an adjuvant therapy for glioma. *Nutr. Metab. (Lond)* 7:74. doi: 10.1186/1743-7075-7-74

Stine, Z. E., Walton, Z. E., Altman, B. J., Hsieh, A. L., and Dang, C. V. (2015). MYC, metabolism and cancer. *Cancer Discov.* 5, 1024–1039. doi: 10.1158/2159-8290.CD-15-0507

Tisdale, M. J., and Brennan, R. A. (1983). Loss of acetoacetate coenzyme A transferase activity in tumours of peripheral tissues. *Br. J. Cancer* 47, 293–297. doi: 10.1038/bjc.1983.38

Tisdale, M. J., Brennan, R. A., and Fearon, K. C. (1987). Reduction of weight loss and tumour size in a cachexia model by a high fat diet. *Br. J. Cancer* 56, 39–43. doi: 10.1038/bjc.1987.149

van den Bent, M. J., Wefel, J. S., Schiff, D., Taphoorn, M. J., Jaeckle, K., Junck, L., et al. (2011). Response assessment in neuro-oncology (a report of the RANO group): assessment of outcome in trials of diffuse low-grade gliomas. *Lancet Oncol.* 12, 583–593. doi: 10.1016/s1470-2045(11)70057-2

Vander Heiden, M. G., Cantley, L. C., and Thompson, C. B. (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 324, 1029–1033. doi: 10.1126/science.1160809

Vanitallie, T. B., and Nufert, T. H. (2003). Ketones: metabolism's ugly duckling. *Nutr. Rev.* 61, 327–341. doi: 10.1301/nr.2003.oct.327-341

Veech, R. L. (2004). The therapeutic implications of ketone bodies: the effects of ketone bodies in pathological conditions: ketosis, ketogenic diet, redox states, insulin resistance and mitochondrial metabolism. *Prostaglandins Leukot. Essent. Fatty Acids* 70, 309–319. doi: 10.1016/j.plefa.2003.09.007

Veech, R. L. (2014). Ketone ester effects on metabolism and transcription. *J. Lipid Res.* 55, 2004–2006. doi: 10.1194/jlr.r046292

Veech, R. L., Chance, B., Kashiwaya, Y., Lardy, H. A., and Cahill, G. F. Jr. (2001). Ketone bodies, potential therapeutic uses. *IUBMB Life* 51, 241–247. doi: 10.1080/152165401753311780

Wang, Q., Hu, M., Xu, H., and Yang, X. (2016). Anti-inflammatory and pro-resolving effects of N-3 PUFA in cancers: structures and mechanisms. *Curr. Top. Med. Chem.* 16, 888–894. doi: 10.2174/1568026615666150827101602

Wang, J., Luo, T., Li, S., and Zhao, J. (2012). The powerful applications of polyunsaturated fatty acids in improving the therapeutic efficacy of anticancer drugs. *Expert Opin. Drug Deliv.* 9, 1–7. doi: 10.1517/17425247.2011.618183

Warburg, O., Wind, F., and Negelein, E. (1927). The metabolism of tumors in the body. *J. Gen. Physiol.* 8, 519–530. doi: 10.1085/jgp.8.6.519

Ward, P. S., and Thompson, C. B. (2012). Metabolic reprogramming: a cancer hallmark even warburg did not anticipate. *Cancer Cell* 21, 297–308. doi: 10.1016/j.ccr.2012.02.014

Wei, W., Shi, Q., Remacle, F., Qin, L., Shackelford, D. B., Shin, Y. S., et al. (2013). Hypoxia induces a phase transition within a kinase signaling network in cancer cells. *Proc. Natl. Acad. Sci. U S A* 110, E1352–E1360. doi: 10.1073/pnas.1303060110

Woolf, E. C., Curley, K. L., Liu, Q., Turner, G. H., Charlton, J. A., Preul, M. C., et al. (2015). The ketogenic diet alters the hypoxic response and affects expression of proteins associated with angiogenesis, invasive potential and vascular permeability in a mouse glioma model. *PLoS One* 10:e0130357. doi: 10.1371/journal.pone.0130357

Yamada, M., Tomida, A., Yun, J., Cai, B., Yoshikawa, H., Taketani, Y., et al. (1999). Cellular sensitization to cisplatin and carboplatin with decreased removal of platinum-DNA adduct by glucose-regulated stress. *Cancer Chemother. Pharmacol.* 44, 59–64. doi: 10.1007/s002800050945

Yang, L., Lin, C., Wang, L., Guo, H., and Wang, X. (2012). Hypoxia and hypoxia-inducible factors in glioblastoma multiforme progression and therapeutic implications. *Exp. Cell Res.* 318, 2417–2426. doi: 10.1016/j.yexcr.2012.07.017

Yang, C., Sudderth, J., Dang, T., Bachoo, R. G., McDonald, J. G., and DeBerardinis, R. J. (2009). Glioblastoma cells require glutamate dehydrogenase to survive impairments of glucose metabolism or Akt signaling. *Cancer Res.* 69, 7986–7993. doi: 10.1158/0008-5472.CAN-09-2266

Youm, Y. H., Nguyen, K. Y., Grant, R. W., Goldberg, E. L., Bodogai, M., Kim, D., et al. (2015). The ketone metabolite β-hydroxybutyrate blocks NLRP3 inflammasome-mediated inflammatory disease. *Nat. Med.* 21, 263–269. doi: 10.1038/nm.3804

Zhou, W., Mukherjee, P., Kiebish, M. A., Markis, W. T., Mantis, J. G., and Seyfried, T. N. (2007). The calorically restricted ketogenic diet, an effective alternative therapy for malignant brain cancer. *Nutr. Metab. (Lond)* 4:5. doi: 10.1186/1743-7075-4-5

Zuccoli, G., Marcello, N., Pisanello, A., Servadei, F., Vaccaro, S., Mukherjee, P., et al. (2010). Metabolic management of glioblastoma multiforme using standard therapy together with a restricted ketogenic diet: case report. *Nutr. Metab. (Lond)* 7:33. doi: 10.1186/1743-7075-7-33

Zwaans, B. M., and Lombard, D. B. (2014). Interplay between sirtuins, MYC and hypoxia-inducible factor in cancer-associated metabolic reprogramming. *Dis. Model. Mech.* 7, 1023–1032. doi: 10.1242/dmm.016287

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Molecular Neuroscience

HYPOTHESIS AND THEORY
published: 08 July 2016
doi: 10.3389/fnmol.2016.00053

Can Ketones Help Rescue Brain Fuel Supply in Later Life? Implications for Cognitive Health during Aging and the Treatment of Alzheimer's Disease

Stephen C. Cunnane[1,2,3], Alexandre Courchesne-Loyer[1,3], Camille Vandenberghe[1,3], Valérie St-Pierre[1,3], Mélanie Fortier[1], Marie Hennebelle[1], Etienne Croteau[1], Christian Bocti[1,2], Tamas Fulop[1,2] and Christian-Alexandre Castellano[1]*

[1] Research Center on Aging, Sherbrooke, QC, Canada, [2] Department of Medicine, Université de Sherbrooke, Sherbrooke, QC, Canada, [3] Department of Pharmacology and Physiology, Université de Sherbrooke, Sherbrooke, QC, Canada

We propose that brain energy deficit is an important pre-symptomatic feature of Alzheimer's disease (AD) that requires closer attention in the development of AD therapeutics. Our rationale is fourfold: (i) Glucose uptake is lower in the frontal cortex of people >65 years-old despite cognitive scores that are normal for age. (ii) The regional deficit in brain glucose uptake is present in adults <40 years-old who have genetic or lifestyle risk factors for AD but in whom cognitive decline has not yet started. Examples include young adult carriers of presenilin-1 or apolipoprotein E4, and young adults with mild insulin resistance or with a maternal family history of AD. (iii) Regional brain glucose uptake is impaired in AD and mild cognitive impairment (MCI), but brain uptake of ketones (beta-hydroxybutyrate and acetoacetate), remains the same in AD and MCI as in cognitively healthy age-matched controls. These observations point to a brain fuel deficit which appears to be specific to glucose, precedes cognitive decline associated with AD, and becomes more severe as MCI progresses toward AD. Since glucose is the brain's main fuel, we suggest that gradual brain glucose exhaustion is contributing significantly to the onset or progression of AD. (iv) Interventions that raise ketone availability to the brain improve cognitive outcomes in both MCI and AD as well as in acute experimental hypoglycemia. Ketones are the brain's main alternative fuel to glucose and brain ketone uptake is still normal in MCI and in early AD, which would help explain why ketogenic interventions improve some cognitive outcomes in MCI and AD. We suggest that the brain energy deficit needs to be overcome in order to successfully develop more effective therapeutics for AD. At present, oral ketogenic supplements are the most promising means of achieving this goal.

Keywords: ketone, Alzheimer's disease, acetoacetate, beta-hydroxybutyrate, glucose, mild cognitive impairment, aging, medium chain fatty acid

OPEN ACCESS

Edited by:
Susan A. Masino, Trinity College, USA

Reviewed by:
David Ruskin, Trinity College, USA
Yuri Zilberter, INSERM – UMR 1106 – Institut de Neurosciences des Systèmes, France

****Correspondence:***
Stephen C. Cunnane
stephen.cunnane@usherbrooke.ca

Received: *23 March 2016*
Accepted: *21 June 2016*
Published: *08 July 2016*

Citation:
Cunnane SC, Courchesne-Loyer A, Vandenberghe C, St-Pierre V, Fortier M, Hennebelle M, Croteau E, Bocti C, Fulop T and Castellano C-A (2016) Can Ketones Help Rescue Brain Fuel Supply in Later Life? Implications for Cognitive Health during Aging and the Treatment of Alzheimer's Disease. Front. Mol. Neurosci. 9:53. doi: 10.3389/fnmol.2016.00053

Abbreviations: AcAc, acetoacetate; AD, Alzheimer's disease; CMRa, cerebral metabolic rate of acetoacetate; CMRg, cerebral metabolic rate of glucose; FDG, ^{18}F-fluorodeoxyglucose; GLUT, glucose transporter; β-HBA, beta-hydroxybutyrate; HOMA2-IR, homeostatic model of insulin resistance (version 2); MCFA, medium chain fatty acid; MCT, medium chain triglyceride; PCOS, polycystic ovary syndrome; PET, positron emission tomography.

INTRODUCTION

Compensating for deteriorating brain energy metabolism is the core feature of an emerging strategy aimed at delaying the onset and/or progression of AD. Relative to its size, the adult human brain requires a disproportionately large amount of energy that is provided principally by glucose. In those at risk of AD including cognitively healthy older people, regional brain glucose hypometabolism can be present long *before* the clinical diagnosis of AD. The brain's alternative energy supply to glucose is unique compared to other organs in that it specifically requires ketones (also known as ketone bodies) to compensate for occasions when glucose supply to the brain is inadequate. In contrast to glucose, brain uptake of ketones appears to still be normal in AD. Hence, ketogenic interventions may help delay AD.

An AD treatment strategy focused on preventing brain energy starvation during aging is based on research that started at least 40 years ago. For instance, the unique dependence of the brain on ketones to replace low glucose supply has been known since the 1960s (Cahill, 2006). It has been proposed since the early 1980s that failing brain glucose supply to or metabolism by the brain could be contributing to AD risk or progression (Hoyer et al., 1988; Veech et al., 2001; Cunnane et al., 2011, 2016). Concrete clinical efforts to develop a 'keto-neurotherapeutic' strategy to bypass the problem with brain glucose metabolism in AD were first reported a decade ago (Reger et al., 2004; Henderson, 2008; Krikorian et al., 2012; Newport et al., 2015). Despite these pioneering studies, this approach to combat AD is still very much a novel area of research.

We see the challenge facing brain energy metabolism during aging through the lens of the similar challenge of assuring sufficient energy supply to the rapidly growing brain of the infant. At no time in our life cycle is the challenge of supplying the brain with sufficient energy more acute than in early human brain development. Indeed, the human species must have confronted this energy constraint for normal brain development when the brain started to triple in size more than 2 million years ago (Cunnane and Crawford, 2014). We suggest that the energetic (glucose) deficit confronted by the aging brain today is essentially the same as the challenge faced during brain expansion at the dawn of our species and that ketones were part of the solution then as now. Hence, it makes physiological sense to apply what we know about the importance of ketones in early brain development to the challenge of maintaining brain energy supply and brain function during aging.

This review will therefore focus on providing the rationale for proposing that deteriorating brain energy metabolism is a constraint for healthy cognitive aging that will have to be overcome in order to successfully limit the impact of AD no matter what therapeutic strategy is used (neurotransmitter-based, anti-amyloid, exercise, etc.). We will describe here our brain ketone PET studies in aging and AD with the ketone tracer, ^{11}C-acetoacetate, because this tracer provides a valuable window on brain energy metabolism to compare with glucose. As we and others have previously proposed, the pre-symptomatic presence of brain glucose hypometabolism in people at risk of AD has clear implications for potential therapeutic strategies (Gibson et al., 2000; Veech et al., 2001; Blass, 2008; Henderson et al., 2009; Cunnane et al., 2011, 2016). We will also refer to a number of issues that will need to be addressed as this field matures.

DETERIORATING BRAIN GLUCOSE SUPPLY IN THOSE AT RISK OF AD

The concept that ketones could be of therapeutic value during brain aging hinges on demonstrating that there is a *pre-symptomatic* problem with brain glucose metabolism in people at risk of AD but who are still cognitively normal. Older people are a key focus of this work because they are at the highest risk of AD but this concept applies to any genetic, lifestyle, or demographic risk factor for AD. PET imaging with the glucose tracer, FDG, and oxygen tracer, ^{15}O-oxygen, was first used to image brain energy metabolism in the late 1970s with reports on AD first appearing in the early 1980s (Benson et al., 1983). Since then, PET-FDG has been a cornerstone of human and animal studies on brain energy metabolism in aging and AD (Cunnane et al., 2011). Indeed, without PET-FDG, it is doubtful that brain glucose hypometabolism in AD would have become so widely studied because the only option besides PET, the arterio-venous difference method, is highly invasive and is used less and less in research today. Nevertheless, the arterio-venous difference method was the first to be used to assess brain oxygen and glucose uptake during aging (Dastur, 1985) and the first to show that specifically brain glucose uptake and not brain ketone uptake was significantly impaired in AD (Lying-Tunell et al., 1981; **Table 1** and **Figure 1**). The arterio-venous difference method also produced several ground-breaking reports comparing brain ketone and glucose uptake. These studies laid the foundation for our current understanding that ketones are an essential physiological brain fuel working in tandem with glucose to assure that brain energy requirements are being met on a daily basis, not just in infants or during starvation (Cunnane et al., 2016). The advantage of the arterio-venous difference method is that it permits quantification of global brain energy metabolism. Aside from its invasiveness, its disadvantage is that it provides no information about brain regions that are most or least affected in AD.

Decades of research with PET-FDG have made it abundantly clear that regional brain glucose uptake is defective in AD (Cunnane et al., 2011, 2016). This AD-type pattern is particularly evident in the parietal, posterior cingulate and temporal cortex and much less present in the occipital cortex and cerebellum. Indeed, the parieto-temporal pattern is relatively specific to AD itself and distinct from the pattern in other forms of dementia and from normal aging itself. This regional pattern of brain glucose hypometabolism in AD has generally been interpreted as a consequence of neuronal failure and death because, logically, brain regions with dysfunctional or dead brain cells need less fuel. However, this interpretation is insufficient because it does not take into account multiple examples of conditions in which the risk of AD is elevated and in which the regional brain glucose hypometabolism is present *before* the clinical (cognitive) onset of the disease. Examples of conditions in which regional brain

TABLE 1 | Lower glucose consumption but not brain blood flow or oxygen consumption at the start of early-onset AD compared to healthy young adults or cognitively normal older adults (Hoyer et al., 1988; Hoyer, 1992).

	Young ($n = 15$)	Older ($n = 11$)	Start of early onset AD* ($n = 20$)
Cerebral blood flow (ml/100 g/min)	53 ± 5¤	56 ± 3	54 ± 3
Cerebral metabolic rate of O_2 (ml/100 g/min)	3.5 ± 0.4	3.7 ± 0.5	3.4 ± 0.3
Cerebral metabolic rate of glucose (mg/100 g/min)	5.0 ± 0.8	5.0 ± 0.3	2.8 ± 0.3**

¤*Data are mean ± SD. *No brain atrophy on CT (no other measures of neuropathology available). **$p < 0.05$ vs. Older and Young groups.*

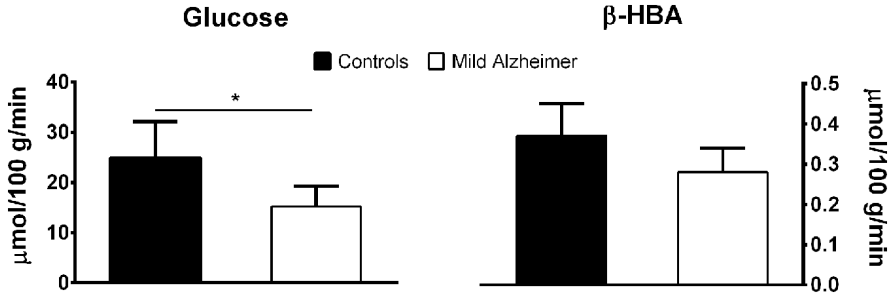


FIGURE 1 | Lower brain glucose uptake (mean ± SD; *$p < 0.05$) but normal brain β-HBA uptake measured by arterio-venous difference in mild AD compared to age-matched healthy controls. These results are averaged from two previous publications (Lying-Tunell et al., 1981; Ogawa et al., 1996).

glucose hypometabolism is present pre-symptomatically include carriers of the Pre-senilin-1 mutation, carriers of apolipoprotein E4, maternal family history of AD, cognitively healthy aging, and insulin resistance in both young and older persons (**Table 2**). Since regional brain glucose hypometabolism is present before measurable cognitive deficit in these conditions, it is clearly pre-symptomatic and therefore cannot only be a consequence of neuronal loss caused by AD. As such, it is plausible that brain glucose hypometabolism could increase the risk of developing AD. This argument has previously been made in detail (Cunnane

TABLE 2 | Brain glucose hypometabolism in persons at risk of AD but in whom cognitive performance is normal.

	Mean age (y)	Brain region	Brain glucose hypometabolism (% difference from control)	Reference
Insulin resistant young women with PCOS	25	Frontal cortex Middle temporal cortex	−9 to −14	Castellano et al., 2015a
Young adult carriers of Presenilin-1	30	Posterior cingulate Parietal cortex Temporal cortex	−14 to −25	Scholl et al., 2011
Young adult carriers of Apolipoprotein-E4	31	Parietal cortex Temporal cortex Posterior cingulate Prefrontal cortex	−9 to −11	Reiman et al., 2004
Maternal family history of AD	43	Parietal cortex Temporal cortex Hippocampus Entorhinal cortex Posterior cingulate	−12 to −21	Mosconi et al., 2006
Cognitively healthy older adults	72	Frontal cortex Temporal cortex Anterior cingulate Putamen Thalamus	−10 to −18	Nugent et al., 2016
Pre-diabetic older persons	74	Temporal cortex Parietal cortex Posterior cingulate Precuneus Prefrontal cortex	N/A	Baker et al., 2011

N/A, not available.

et al., 2011, 2016) so we will focus here on two examples that collectively represent the populations at highest overall risk for AD – older people and those with insulin resistance (Ronnemaa et al., 2008; Craft, 2009, 2012; Matsuzaki et al., 2010; Schrijvers et al., 2010; Baker et al., 2011).

DETERIORATING BRAIN GLUCOSE UPTAKE DURING AGING

The risk of AD increases with advancing age but it has not been clear until recently whether healthy aging *per se*, i.e., cognitively normal and relatively free of overt risk factors for AD, is associated with deteriorating brain glucose uptake. This uncertainty has been due to a lack of a standard definition of healthy aging, a lack of sufficient verification in several reports as to whether or not cognition was normal in the older group, as well as infrequent quantification of brain glucose uptake to determine the *actual magnitude* of the problem (Nugent et al., 2014a). Knowing that there is a statistical difference in brain glucose across brain regions or between two groups is not sufficient; being able to assess the magnitude of the problem is essential in order to set a therapeutic target to counteract the problem.

We have therefore developed a database on brain ketone and glucose uptake in cohorts of cognitively normal young and older people. The older group had a minimum age of 65 years and was relatively free of overt disease. A detailed neuropsychological assessment showed that they were cognitively normal and had a metabolic profile as closely matched to healthy young adults as possible (**Tables 3** and **4**). Brain glucose uptake was quantified as CMRg with the units, μmol/100 g/min, both globally and regionally in each participant. This cognitively healthy older cohort had 9% lower global brain glucose uptake compared to our younger controls, a deficit that was mostly though not exclusively limited to the frontal cortex (−14%) and the caudate (−18%; Nugent et al., 2014a, 2016; **Figure 2**). This pattern is regionally different from the situation in AD where glucose hypometabolism is not only in the frontal cortex but includes glucose uptake that was as much as 33% lower in parts of the temporal and parietal cortex, 17% lower in the thalamus and 26% lower in the posterior cingulate cortex (Castellano et al., 2015b). Significantly lower glucose uptake in the frontal cortex is therefore commonly present in *cognitively healthy* older persons despite the absence of any clinical sign of AD.

TABLE 3 | Demographics of our cognitively normal young and older adults (mean ± SD).

	Young	Older	*p*-value
Number	30	41	
Male/female	14/16	15/26	
Age (years)	26 ± 4	71 ± 5	≤0.001
Body Mass Index	23 ± 3	26 ± 4	0.001
Blood pressure (mm Hg)	114/69	133/79	0.001
Homocysteine (μM)	7.9 ± 1.5	10.1 ± 2.4	≤0.001
Hemoglobin A1c (%)	5.2 ± 0.2	5.8 ± 0.3	≤0.001
Glucose (mM)	4.9 ± 0.4	5.0 ± 0.5	
Acetoacetate (μM)	162 ± 129	129 ± 107	
β-Hydroxybutyrate (μM)	351 ± 298	272 ± 259	

*Their cognitive scores are reported in **Table 4** and their brain glucose and ketone uptake in **Figure 2**.*

TABLE 4 | Cognitive scores (mean ± SD) of healthy young and older adults reported in Figure 2 and Table 3*.

	Young	Older	*p*-value
Global cognition			
MMSE	29.9 ± 0.3	29.4 ± 0.9	0.051
Speed processing and attention			
Digit symbol substitution	11.4 ± 2.5	10.9 ± 2.3	0.875
Executive function			
Trail making number sequencing	12.5 ± 1.7	11.0 ± 3.3	0.247
Trail making number-letter switching	12.0 ± 1.4	10.4 ± 3.0	0.226
Stroop-inhibition	12.1 ± 2.5	10.6 ± 2.7	0.096
Stoop-inhibition/switching	10.5 ± 2.9	10.4 ± 2.3	0.999
Verbal fluency-letter	10.1 ± 2.9	9.9 ± 3.3	0.968
Verbal fluency-category	12.7 ± 3.1	11.5 ± 2.9	0.397
Working memory			
Digit span	9.2 ± 2.6	7.9 ± 3.0	0.555
Spatial span	11.8 ± 3.3	11.4 ± 2.8	0.666
Episodic memory			
RCFT-Immediate recall	61.3 ± 15.4	67.3 ± 12.8	0.005
RCFT-Delayed recall	62.2 ± 10.7	69.0 ± 11.8	≤0.001
VPA-Immediate recall	12.3 ± 2.5	12.4 ± 3.3	0.437
VPA-Delayed recall	12.1 ± 1.0	12.7 ± 2.6	0.017
LM-Immediate recall	14.9 ± 2.0	13.2 ± 3.0	0.289
LM-Delayed recall	15.9 ± 1.8	14.0 ± 2.7	0.170

**Results corrected for age and education. MMSE, mini-mental state examination; RCFT, Rey complex figure test; VPA, verbal paired associates; LM, logical memory.*

DETERIORATING BRAIN GLUCOSE UPTAKE IN INSULIN RESISTANCE

The metabolic profile of the older cohort described above was moderately well matched to the younger group but some parameters did differ significantly, i.e., body-mass index, blood pressure, homocysteine, and some measures of glucose homeostasis were not identical and could have adversely influenced brain glucose metabolism (Nugent et al., 2016; **Table 3**). However, it is difficult to find older people in whom the metabolic profile is strictly within the same limits as adults <35 years-old. Mild insulin resistance seems to be a common feature of aging, but it is by no means limited to the older population. The importance of this point became clear to us when we studied young women with mild insulin resistance due to PCOS. They had a brain glucose uptake deficit in the superior and middle frontal cortex of about 14%, i.e., a profile resembling that of people in their 70 and 80s (Castellano et al., 2015a). PCOS is a multi-factorial endocrine disease involving not only mild-moderate insulin resistance but also infertility and hyperandrogenism. The mild insulin resistance was of particular interest to us because it is associated with increased risk of AD

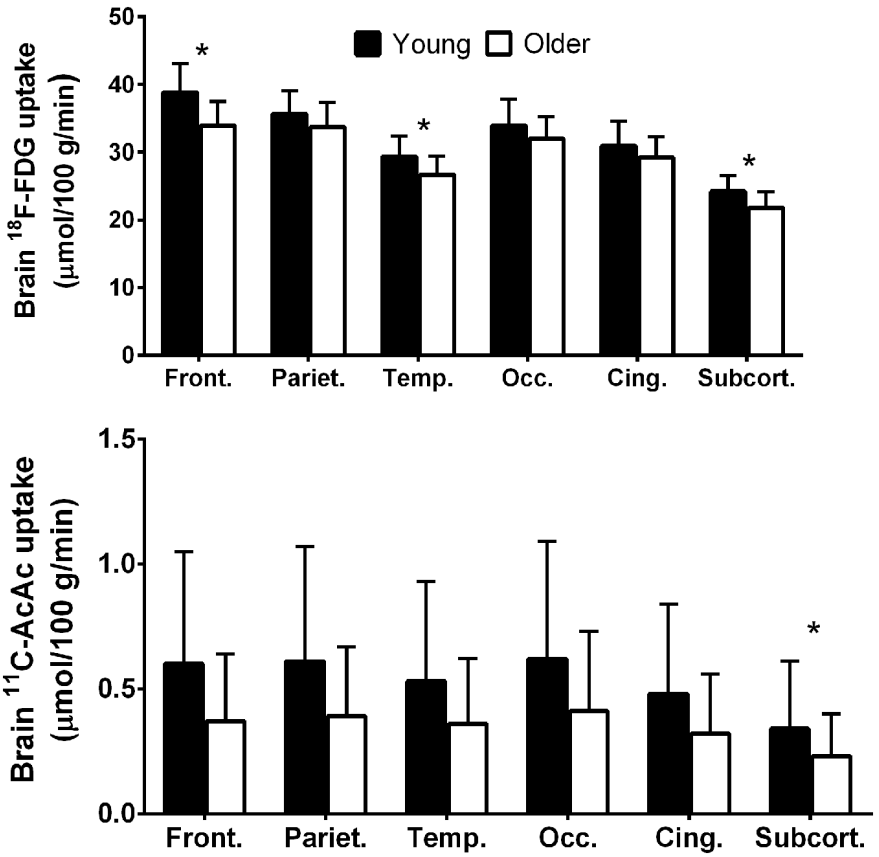


FIGURE 2 | Regional ^{18}F-FDG (upper panel) and ^{11}carbon-acetoacetate (^{11}C-AcAc; lower panel) uptake in young adults (black bars; $n = 30$) compared to cognitively healthy older adults (white bars; $n = 42$; Nugent et al., 2014b, 2016). Front., frontal lobe; Pariet., parietal lobe; Temp., temporal lobe; Occ., occipital lobe; Cing., cingulate gyrus; Subcort., subcortical regions. *$p < 0.05$.

in middle-aged and older adults (Ronnemaa et al., 2008; Craft, 2009, 2012; Matsuzaki et al., 2010; Schrijvers et al., 2010; Baker et al., 2011).

In our women with PCOS, glucose uptake in several brain regions was significantly inversely correlated to both the mild insulin resistance and to fasting plasma glucose. Recent studies in young women with PCOS demonstrate that insulin resistance is associated not only with impaired brain glucose metabolism but also with altered white matter microstructure and cognitive performance (especially working memory) in young adults (Castellano et al., 2015a; Rees et al., 2016). These results suggest that glucose dysregulation and the development of a pattern of deteriorating brain glucose in older people can start in the second to third decade of life (Burns et al., 2013; Ishibashi et al., 2015). Whether women with PCOS are predisposed to a higher risk of cognitive decline as they age and whether this apparently increased risk of cognitive decline can be prevented or reversed requires further attention.

Thus, regional brain glucose hypometabolism can be present in those at risk of AD due to old age, or to insulin resistance regardless of age. Multiple mechanisms are undoubtedly involved in the mechanism by which insulin resistance affects the onset and/or progression of AD (Schioth et al., 2012). This pre-symptomatic glucose uptake deficit is commonly but not exclusively in the frontal cortex and its magnitude is of the order of 12–15% (Cunnane et al., 2011; Castellano et al., 2015b; Nugent et al., 2016). We interpret these findings to mean that a vicious cycle can develop in which chronic pre-symptomatic brain glucose hypometabolism develops and then contributes to deteriorating neuronal function, further decline in demand for glucose, and the emergence of cognitive decline which then further decreases brain glucose consumption (**Figure 3**; Cunnane et al., 2011, 2016). Chronic sedentarity commonly contributes to chronic hyperinsulinemia and insulin resistance which not only compromise tissue glucose uptake but also decreased ketogenesis and ketone metabolism (Fukao et al., 2004; Bickerton et al., 2008). In effect, this puts the aging brain at risk of exhaustion because now it is not only getting insufficient glucose but is also less ketones (Cunnane et al., 2011, 2016; Mamelak, 2012).

KETONES: ESSENTIAL IN EARLY HUMAN BRAIN DEVELOPMENT

The adult brain accounts for about 2% of adult body weight but it consumes about 20–23% of the energy needs of the

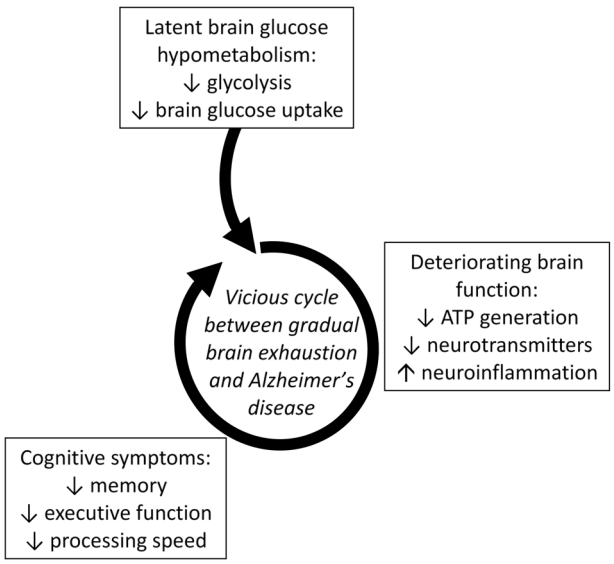


FIGURE 3 | Latent brain glucose hypometabolism leading to a vicious cycle of accelerating metabolic deterioration, neuroinflammation and neuronal dysfunction that increases the risk of developing AD (Cunnane et al., 2016). Lower brain glucose uptake is a direct reflection of lower glycolysis in brain cells. Lower glycolysis contributes to impaired brain function (ATP generation and neurotransmitter production) which in turn contributes to cognitive symptoms. Deteriorating cognitive function then reduces demand for glucose turning this sequence into a vicious cycle.

whole body. Like other organs, the brain has a back-up fuel for occasions when glucose supply is insufficient, i.e., during fasting, starvation, strenuous exercise, or malnutrition. Ketones are the only significant alternative fuel to glucose for the brain, which is a unique situation because as far as is known all other organs use free fatty acids to replace insufficient availability of glucose (Cunnane et al., 2011). β-HBA and AcAc are the two ketones that replace glucose for the brain. The third ketone, acetone, is a decarboxylation product of AcAc that is mainly excreted on breath but can also potentially enter intermediary metabolism. The focus here will be on β-HBA and AcAc; neither acetone nor the less common five carbon ketones derived from odd-chain fatty acids will be discussed further.

Unlike in the human adult in whom the brain appears to use ketones only to compensate for periodic insufficiency in glucose supply, in the developing infant, ketones are *essential* both as a major fuel and also as the main substrate for brain lipid synthesis (Cunnane et al., 2003, 2016). Ketones are an essential fuel for the human neonatal brain because there is insufficient glucose available to meet its brain energy requirements (Settergren et al., 1976; Robinson and Williamson, 1980; Bougneres et al., 1986). This important role of ketones in infant brain development and energy metabolism starts to develop in the fetus (Adam et al., 1975). Postnatally, the brain's dependence on ketones is made possible because infants are normally in a *sustained state of mild ketosis* (0.2–0.5 mM β-HBA). This neonatal ketosis is present regardless of whether the infant has just been fed or is in a post-prandial state, i.e., the ketosis is not a function of food restriction or hypoglycemia (Settergren et al., 1976). This contrasts with the adult human in whom 0.5 mM β-HBA in plasma is normally only achieved after 24–48 h fasting accompanied by hypoglycemia and hypoinsulinemia.

The constant state of ketosis in infants is due mostly to MCFAs supplied in breast milk; indeed, the milk of most (probably all) mammalian species contains 10–20% of all fatty acids as MCFA (Hilditch and Meara, 1944; Insull and Ahrens, 1959; Breckenridge and Kuksis, 1967). Some of the MCFA in breast milk end up in the adipose stores of the infant and can be used days or weeks later, thereby in effect extending lactation for some period of time with respect to the availability of ready-made ketone substrates (Sarda et al., 1987). However, unlike in humans, the offspring of other terrestrial mammals have virtually no adipose tissue so they have very limited ability to store MCFA and, hence, poor ability to generate ketones post-weaning (Robinson and Williamson, 1980). Human babies on the other hand have significant subcutaneous fat stores, i.e., 500–600 g if they are born at term, but markedly less if they are born pre-term. After lactation ends, the long chain fatty acids and the small amount of MCFA stored in adipose tissue provide the substrate to prolong mild ketonemia for many months. Incidentally, in addition to the liver, the infant gut can also synthesize ketones (Bekesi and Williamson, 1990).

KETONES: THE BRAIN'S PREFERRED FUEL

That ketones are the main reserve fuel for the adult human brain when glucose supply is compromised by starvation was convincingly demonstrated in the now classic studies of medically supervised long-term starvation reported by Owen et al. (1967) and Drenick et al. (1972). The brain's need for energy during prolonged starvation can be met by the high ketogenic capacity of the liver which can produce up to 150 g ketones/day (Flatt, 1972; Reichard et al., 1974). Despite the liver's high energy consumption, it cannot catabolize ketones, so they diffuse into the circulation where they become available to all organs. However, as starvation progresses, other organs, particularly skeletal muscle, come to use free fatty acids more efficiently so ketones therefore become increasingly available for the brain which has no other energy substrate to replace low glucose (Owen and Reichard, 1971; Drenick et al., 1972).

In adults, long chain fatty acids stored in adipose tissue are the main substrate for ketogenesis. They are released as free fatty acids when low blood glucose in turn causes hypoinsulinemia (Mitchell et al., 1995). Free fatty acids entering the liver are beta-oxidized, generating acetyl-CoA. As hypoinsulinemia continues, free fatty acid delivery to the liver continues and acetyl-CoA starts to accumulate because its concentration exceeds the capacity of the citric acid cycle to metabolize it. Acetyl-CoA accumulation in the liver leads to condensation of two acetyl-CoAs to ketones via hydroxyl-methyl-glutaryl CoA. During short-term fasting, ketone metabolism generally matches ketone synthesis so plasma ketones usually do not rise much above ≤0.3 mM (Hall et al., 1984; Balasse and Fery, 1989; Avogaro et al.,

TABLE 5 | Overview of ketones (β-hydroxybutyrate + acetoacetate) kinetics in humans.

	Fasting period	Plasma ketones (mM)	Utilization (μmol/kg/min)	Synthesis (μmol/kg/min)	Metabolic clearance (ml/kg/min)	Urinary excretion (μmol/min)
Healthy adults	12–16 h[A,B,C]	0.1–0.3*	3–5	2–5	18	ND
	3 days[D]	2.5	ND	10	ND	4
Healthy adults + 30 min exercise	16 h[B,E]	0.2–0.4	6	6	21	ND
	3–5 days[B,E,F]	4–5	20	22	4–6	ND

Mean values extracted from the references indicated. ND, no data available. [A]Hall et al., 1984; [B]Fery and Balasse, 1983; [C]Avogaro et al., 1990; [D]Garber et al., 1974; [E]Fery and Balasse, 1986; [F]Balasse et al., 1978.

1990; **Table 5**). Greatly increased ketogenesis relative to ketone clearance after 3–5 days fasting causes plasma ketones to rise about 10-fold. In the presence of hypoglycemia, the liver depends on gluconeogenesis to support the energy costs of ketogenesis (Flatt, 1972; Garber et al., 1974).

Ketone transport into tissues including the brain occurs via monocarboxylic acid transporters of which there are several subtypes (Simpson et al., 2007). Monocarboxylic acid transporter expression in the brain responds rapidly to hyperketonemia (Halestrap and Price, 1999; Pan et al., 2001). Thus, brain uptake of ketones is normally directly proportional to their plasma concentration over at least the range of 0.02–12 mM (Cunnane et al., 2011, 2016; Courchesne-Loyer et al., 2013). In contrast to ketones which are 'pushed' into the brain in proportion to their plasma concentration, glucose is 'pulled' into the brain in proportion to its utilization by astrocytes and neurons (**Figure 4**). The 'push-pull' strategy assures that ketones will enter the brain under conditions in which glucose availability is decreased and ketone synthesis is stimulated.

Both short-term PET and arterio-venous difference studies in humans show that brain glucose consumption *decreases* as ketone availability to the brain increases (Hasselbalch et al., 1995). These results suggest that ketones are actually the preferred energy substrate for the brain because they enter the brain in proportion to their plasma concentration irrespective of glucose availability; if the energy needs of the brain are being increasingly met by ketones, glucose uptake decreases accordingly. This decrease in brain glucose uptake when both ketones and glucose are available supports the notion that ketones are the brain's preferred fuel. Nevertheless, it is uncommon for both ketones and glucose to be available; normally, when one is increased in the blood the other is decreased. Under conditions of normal energy sufficiency and three meals per day, ketogenesis is supressed and glucose supplies >95% of the brain's energy requirements; hence, glucose (or fuels derived from glucose, i.e., lactate or pyruvate) is the brain's dominant but not actually its preferred fuel.

The problem for the aging brain is that low glucose *supply* in the blood is not the same as low brain glucose *utilization*. When blood glucose decreases, ketogenesis normally occurs rapidly in response to decreased insulin. However, when brain glucose utilization is decreased, plasma insulin does not necessarily decrease; indeed, during aging, plasma insulin and glucose are commonly mildly elevated and there is a state of mild-moderate insulin resistance. Hyperinsulinemia inhibits the normal ketogenic response (Bickerton et al., 2008), thereby putting the aging brain in double jeopardy of being deprived of both its primary fuels. We believe that this problem is at the root of the vicious cycle between deteriorating brain fuel uptake/availability and deteriorating brain function that leads to AD.

KETOSIS, MCT, AND BRAIN FUNCTION

In discussing keto-neurotherapeutics, it is essential to distinguish between nutritional or dietary ketosis and pathological ketosis; they differ in origin, in severity of ketosis and in medical consequences. Nutritional ketosis is a physiological response to sustained low carbohydrate intake resulting in low plasma glucose and insulin, and plasma ketones of 2–5 mM after a week or so. Nutritional ketosis has never been shown to induce ketoacidosis, i.e., to alter acid-base balance or to lower blood pH whether after experimental ketone infusion (Hasselbalch et al., 1995) or during medically supervised starvation lasting as long as 60 days (Drenick et al., 1972). Nutritional ketosis can be sustained for weeks, months, or even years; indeed, there are numerous metabolic and cardiovascular benefits of nutritional ketosis in addition to the clinical benefits that are well-documented for intractable epilepsy. Experimental ketone infusion shows that nutritional ketosis is usually self-limiting because raising ketones to about 3–5 mM by sodium-AcAc infusion stimulates insulin secretion which in turn rapidly reduces plasma ketones (Owen et al., 1973). Experimental insulin infusion can be used to induce severe hypoglycemia but in the presence of prolonged starvation does not induce ketoacidosis (Drenick et al., 1972).

Pathological ketoacidosis on the other hand is a medical emergency arising in type 1 diabetes because of acute severe insulin deficiency due usually to interruption of insulin injection. Plasma ketones generally exceed 10–15 mM and blood pH may decrease to 7. Comorbidities such as alcoholism, malnutrition and/or serious infection often contribute to exacerbating insulin deficiency and increase the severity of the ketoacidosis. Hence, unlike pathological ketosis, nutritional ketosis is a safe and sustainable condition in which insulin decreases due to low carbohydrate intake not because of a disease process. It can be undertaken with medical supervision and usually involves low to negligible risk to the individual. Nutritional ketosis can also be rapidly reversed by consuming carbohydrate.

Long chain fatty acids (14–22 carbons) stored in adipose tissue are normally the main substrate for ketogenesis in adults because the diet rarely contains MCFA. However, breast-feeding infants are in mild ketosis principally because of the MCFA

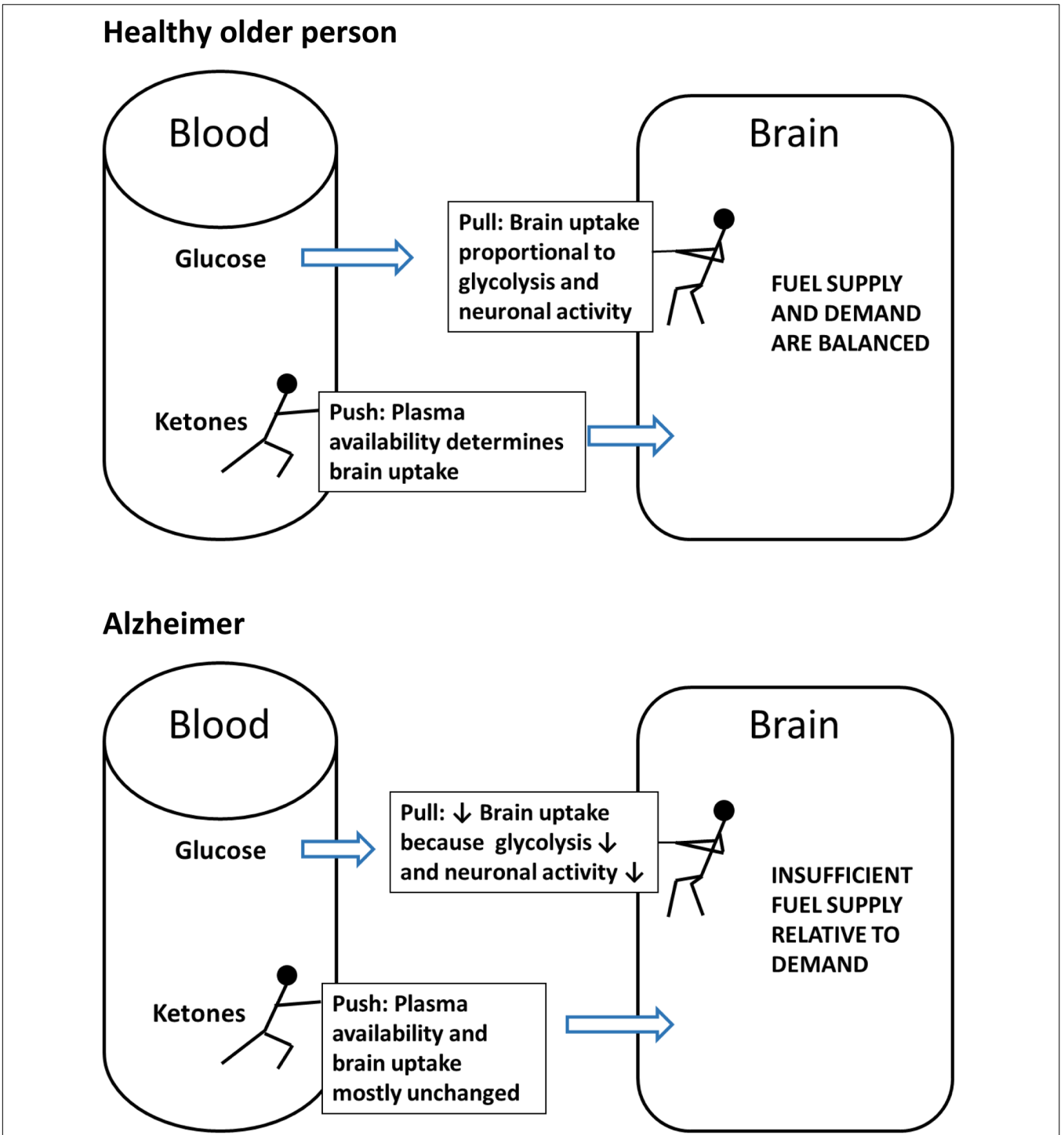


FIGURE 4 | The contrasting 'Push-Pull' mechanism of brain fuel supply. Glucose is pulled from the blood into the brain as a function of the brain's metabolic demand during neuronal activation. Under normal conditions (excluding insulin resistance), ketones are pushed from the blood into the brain in direct proportion to their plasma concentration and irrespective of plasma glucose level.

they are consuming from breast milk (Cunnane and Crawford, 2014). There are two reasons why MCFA are particularly effective ketogenic substrates (**Figure 5**): first, an oral dose of MCFA is mostly absorbed from the gut directly into the portal vein. This is a more rapid route to the liver than for dietary long chain fatty acids which are absorbed as chylomicrons via the lymphatic system and pass into the peripheral circulation before reaching the liver. Second, unlike a long chain fatty acid which

requires carnitine-dependant activation to a Coenzyme A before accessing the mitochondria, beta-oxidation of MCFA occurs without activation by carnitine. The net result is more rapid beta-oxidation and ketogenesis of MCFA than from long chain fatty acids (Guillot et al., 1993).

The potential clinical benefit of MCTs as a rapid energy source was initially reported for surgically stressed and/or malnourished patients (Bach and Babayan, 1982; De Gaetano et al., 1994; St-Onge and Jones, 2002). The ketogenic effect of MCT was already well-known (Freund and Weinsier, 1966; Bach and Babayan, 1982) but was not clinically exploited until they were tried as an alternative ketogenic approach to the ketogenic diet in refractory childhood epilepsy (Huttenlocher, 1976). At about the same time, MCT started to be introduced into formula milk for infants. Owing to the interest in ketones as possible brain fuels to bypass deteriorating brain glucose, the effects of MCT supplementation on cognitive outcomes has been investigated in mild-moderate AD in studies lasting several months (Henderson et al., 2009) but also after just a single dose of MCT (Reger et al., 2004). Very high fat diets are also ketogenic by virtue of their low carbohydrate content and have been reported to have beneficial effects on cognitive and cardiovascular outcomes in mild cognitive impairment (MCI), the prodromal state to AD (Krikorian et al., 2012). In Type 1 diabetics undergoing controlled experimental hypoglycemia caused by insulin infusion, MCT improve several cognitive outcomes (Page et al., 2009). Collectively, these reports suggest that beyond the well-established role of MCFA in infant development and the use of MCT in parenteral and enteral nutrition and intractable epilepsy, several aspects of cognitive function that deteriorate during acute hyperinsulinemia or with cognitive decline associated with AD can be partially or completely normalized when ketones contribute to fuelling the brain (**Table 6**).

Salts or esters of AcAc and β-HBA can also be directly administered orally or by intra-venous infusion (Hasselbalch et al., 1996; Plecko et al., 2002; Clarke et al., 2012), and inhibit the autonomic and neurological symptoms of acute severe experimental hypoglycemia in humans (Amiel et al., 1991; Veneman et al., 1994). The safety of sustained oral use of a β-HBA-monoester and its anecdotal utility in improving some aspects of cognitive function in an advanced case of early onset AD have recently been reported (Newport et al., 2015) and are being increasingly investigated in animal models (D'Agostino et al., 2013; Viggiano et al., 2015).

MCT: A SAFE, EFFICIENT KETOGENIC SUBSTRATE ACROSS THE LIFESPAN

There is normally no further opportunity to consume MCFA once breast-feeding is terminated. However, coconut and palm kernel oils contain MCFA. The MCFA-enriched fraction of these 'tropical' oils can be concentrated resulting in a generic MCT product containing mostly fatty acids of eight (octanoic or caprylic acid) and 10 carbons (decanoic or capric acid). The ratio of caprylic and capric acids and their proportion of the total can vary widely from one MCT product to another. Notwithstanding the generic nature of MCT and different study

TABLE 6 | Clinical studies in which hormonal and cognitive responses indicate that ketones maintain brain function by compensating for hypoglycemia.

	Treatment	Reference
Acute studies in healthy adults		
Controlled insulin-induced hypoglycemia ± fasting in obesity ($n = 9$)	Treatment: 2 h insulin infusion ± 60 days fast Outcomes: ↓ effect of acute severe hypoglycemia (0.5 mM in one case), including ↓ mental confusion, anxiety, sweating, tachycardia, blood pressure if fasted for 60 days before the insulin infusion	Drenick et al., 1972
Controlled insulin-induced hypoglycemia in healthy adults ($n = 6$)	Treatment: 4 h i.v. β-HBA infusion Dose: 30 μmol/min/kg body weight Outcomes: ↓ hormonal response to hypoglycemia	Amiel et al., 1991
Controlled insulin-induced hypoglycemia in healthy adults ($n = 13$)	Treatment: 6 h i.v. β-HBA infusion Dose: 20 μmol/min/kg body weight Outcomes: ↓ hormonal and cognitive symptoms of acute hypoglycemia	Veneman et al., 1994
Controlled insulin-induced hypoglycemia in type 1 diabetes ($n = 11$)	Treatment: oral MCT Dose: 40 g in three stages (20, 10, 10 g) Outcomes: ↓ cognitive symptoms of acute hypoglycemia	Page et al., 2009
Age-associated cognitive decline		
Mild cognitive impairment ($n = 23$)	Treatment: 6 weeks high fat ketogenic diet Outcomes: ↑ secondary memory performance	Krikorian et al., 2012
Mild-moderate AD ($n = 20$)	Treatment: single dose of 95% octanoate Dose: 40 g orally Outcomes: ↑ cognitive score in Apolipoprotein E4(-) patients	Reger et al., 2004
Mild-moderate AD ($n = 77$)	Treatment: 90 days 95% octanoate Dose: 20 g/d orally Outcomes: ↑ cognitive score in Apolipoprotein E4(-) patients	Henderson et al., 2009
Severe AD ($n = 1$)	Treatment: 20 months MCT + coconut oil (4:3), including ketone ester Dose: 165 ml/d orally Outcomes: ↑ mood, affect, self-care, and cognitive and daily activities	Newport et al., 2015

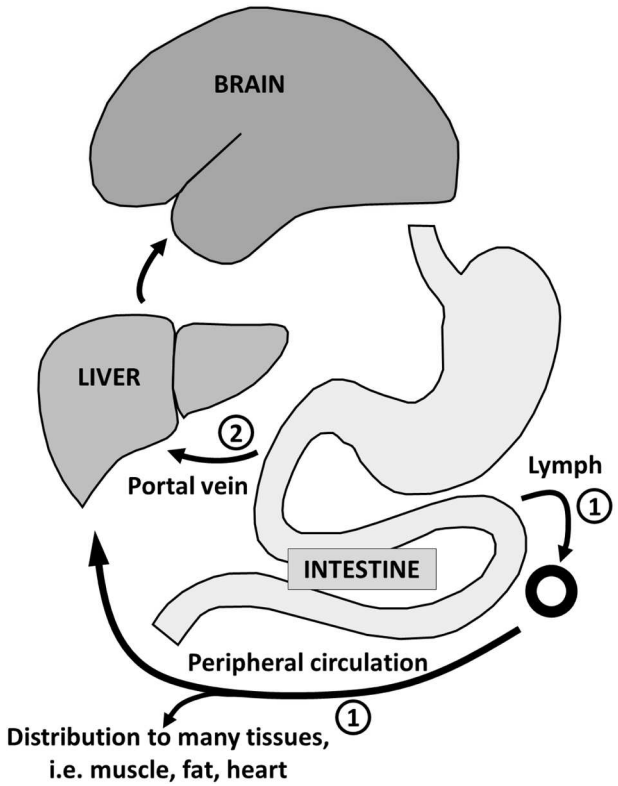


FIGURE 5 | Absorption of long-chain fatty acids ① takes place in the intestine with transport *via* chylomicrons and the lymphatic system to the peripheral circulation with distribution to adipose tissue and other organs (except the brain). The route for medium-chain fatty acid absorption ② is via the portal vein directly to the liver where they are almost entirely beta-oxidized. Some of the resulting acetyl-CoA is transformed into ketones which reenter the circulation for use as energy substrate principally by the brain.

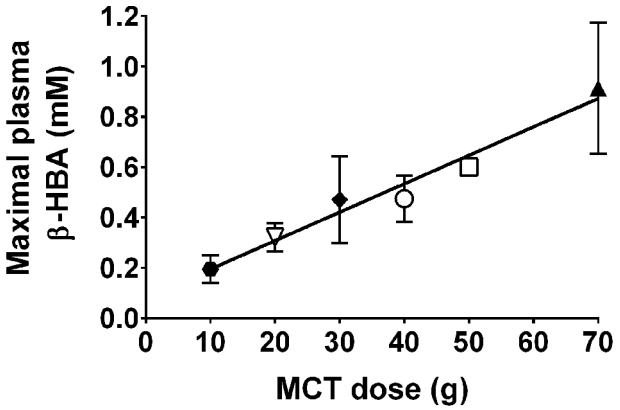


FIGURE 6 | Direct relationship between oral dose of MCTs and maximal observed plasma β-HBA ($r = 0.95$; $p < 0.0001$). MCT were given as a single dose (Seaton et al., 1986; Pi-Sunyer et al., 1969; Reger et al., 2004; Page et al., 2009) or multiple doses (Henderson et al., 2009) for days to weeks. Data for the same dose are pooled from multiple sources: ● 10 g MCT; (Henderson et al., 2009). ▽ 20 g MCT; (Henderson et al., 2009). ◆ 30 g MCT. ○ 40 g MCT; (Reger et al., 2004; Page et al., 2009). □ 50 g MCT; (Seaton et al., 1986). ▲ 70 g MCT; (Pi-Sunyer et al., 1969; Freemantle et al., 2009).

designs to assess their metabolism, there is a significant positive correlation between the oral dose of MCT taken and the maximal plasma β-HBA level achieved (**Figure 6**). This dose-response relationship can be used to estimate a therapeutic dose of MCT needed to achieve particular plasma ketone level. Octanoic acid can be taken up by the brain (Kuge et al., 1995; Ebert et al., 2003) so it may have direct effects on brain function including but not limited to conversion to ketones by astrocytes (Auestad et al., 1991).

Healthy older people have the same plasma ketone response and beta-oxidize ^{13}C-β-HBA to ^{13}C-CO_2 to the same extent after a standard high fat ketogenic breakfast containing 70 g MCT as middle aged or young adults (76 years-old vs. 50 or 23 years-old, respectively; Freemantle et al., 2009). Another report suggests that the plasma ketone response to 18 h fasting is somewhat higher in older compared to younger adults (London et al., 1986). However, in our experience, plasma β-HBA and AcAc tend to be lower after an overnight fast in cognitively healthy older vs. young adults but so far the trend is not significant owing to wide inter-individual variability in plasma ketone data (Nugent et al., 2014b, 2016; **Table 2**). Hence, it seems likely that the capacity to produce and utilize ketones does not change appreciably during healthy aging but this still requires further work.

Medium chain triglyceride are saturated fats and, as such, their consumption is commonly associated with increased cardiovascular risk. However, consuming 30 g/d of MCT for 30 days does not adversely affect serum glucose, insulin, triglycerides, cholesterol, free fatty acids, body weight, or body-mass index (Courchesne-Loyer et al., 2013). In extensive tests, the safety of oral MCT at up to 1 g/kg/day is well-established in all species including humans (Bach and Babayan, 1982; Traul et al., 2000). MCT have important uses in parenteral nutrition and are widely present in infant formula. Nevertheless, they can have secondary side effects involving gastrointestinal distress, gastric reflux, and possible diarrhea, issues that can usually be mitigated by gradual dose titration.

REGIONAL BRAIN KETONE UPTAKE IN AD

The aforementioned clinical reports of a cognitive benefit of MCT in AD (Reger et al., 2004; Henderson, 2008; Krikorian et al., 2012; Newport et al., 2015) are still preliminary and require replication on a larger scale. Nevertheless, they provisionally support the hypothesis that a regional brain glucose deficit contributes to impaired cognition associated with aging and that this deficit can at least in part be bypassed by ketogenic treatments. A core element of this interpretation is that brain cells and/or networks that were previously dysfunctional can start to function more normally again once they are provided with more fuel, i.e., they were starving or exhausting but not dead; otherwise this cognitive improvement would not be possible.

Apart from needing further cognitive studies, a crucial step in building the case that ketones could have beneficial

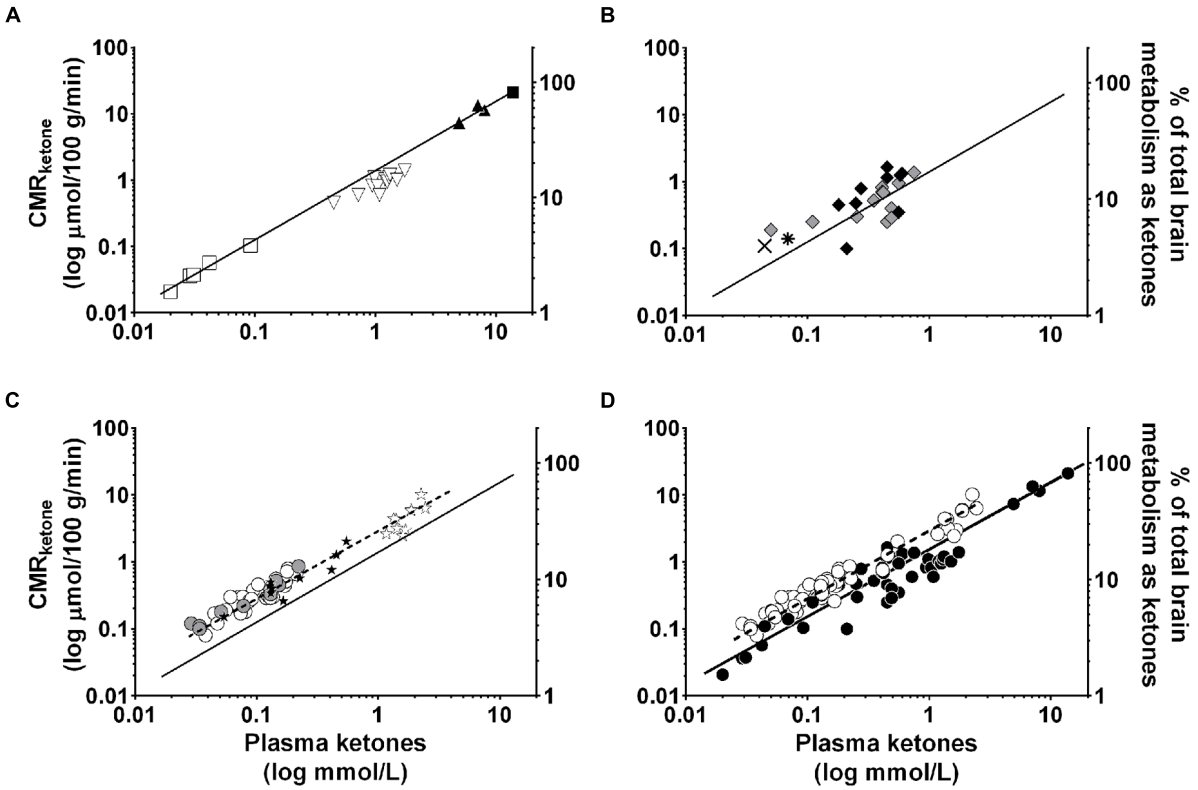


FIGURE 7 | Direct, linear relation between plasma ketone concentration (X axis), brain ketone uptake (left-hand Y axis), and percent contribution by ketones to total brain energy requirement (right-hand Y axis). Data are for adults. Two relationships are shown, one for plasma β-HBA vs. the rate of brain β-HBA uptake (solid line, $R^2 = 0.97$; $Y = 1.57X - 0.20$; $p < 0.0001$; **A–D**), and the other for plasma acetoacetate vs. the rate of brain acetoacetate uptake (Castellano et al., 2015b; dotted line, $R^2 = 0.83$; $Y = 3.46X - 0.03$; $p < 0.0001$; **C,D**). Units are the same for both ketones – CMR (μmol/100 g/min). The data have been combined from several sources. **(A)** Plasma β-HBA vs. brain uptake in post-prandial state (□) (Blomqvist et al., 1995), after β-HBA infusion (▽) (Blomqvist et al., 2002), as well as after a 40 day fast (▲) (Owen et al., 1967), or 60 day fast (■) (Drenick et al., 1972). **(B)** Two studies of plasma β-HBA vs. brain uptake in AD [⧫] and healthy older controls (⧫) (Lying-Tunell et al., 1981), and AD (✱) and healthy older controls (✖) (Ogawa et al., 1996). **(C)** Plasma acetoacetate vs. brain uptake in AD (●) and cognitively healthy age-matched controls (○) (Castellano et al., 2015b), as well as before (★) and 4 days after (☆) a very high fat ketogenic diet in healthy adults (Courchesne-Loyer et al., unpublished). **(D)** Pooled data from **(A–C)**. All the brain β-HBA uptake data are from arteriovenous difference studies except for the one report which used ^{11}C-β-HBA PET (Blomqvist et al., 1995). The brain acetoacetate uptake data were obtained using ^{11}C-acetoacetate PET (Castellano et al., 2015b). Each symbol represents whole brain ketone uptake in a single individual except when not available in the original publication, i.e., Drenick et al. (1972) for which ■ represents the mean of $n = 5$ participants, and Ogawa et al. (1996) for which ✱ and ✖ both represent the mean of $n = 7$. The relationship between plasma β-HBA and the percent of brain energy consumption supplied by β-HBA in adults is broadly as follows: at plasma β-HBA values around 0.1 mM, ketones supply >5% of brain energy; at 1 mM β-HBA, they supply about 10–15%; at 5–7 mM β-HBA, 50–65% and over 7–8 mM β-HBA, >75% of brain energy consumption. For a given plasma acetoacetate concentration, acetoacetate is taken up by the brain more rapidly than β-HBA which explains why the dotted regression line for acetoacetate lies above that of the solid line for β-HBA.

neurotherapeutic properties is to be able to measure brain uptake of ketones in disease and before and after ketogenic interventions. We developed a PET research program using the ketone tracer, ^{11}C-AcAc, to better understand the relation between brain fuel uptake and brain function in people with or at risk of AD. We use this dual tracer PET protocol to compare the brain uptake of ^{11}C-AcAc to that of FDG in each individual studied. We quantify the magnitude of FDG and ^{11}C-AcAc uptake regionally throughout the brain. The kinetics of brain ketone metabolism assessed using PET or arterio-venous difference suggest a one-tissue compartment model in which brain utilization essentially matches brain uptake (Lying-Tunell et al., 1981; Blomqvist et al., 1995; Ogawa et al., 1996). PET studies of brain ^{11}C-β-HBA uptake in humans have already confirmed the earlier arterio-venous difference studies showing that the brain uptake of β-HBA is directly proportional to plasma β-HBA over a wide range of plasma ketone concentrations 20 μM to >10 mM (Lying-Tunell et al., 1981; Blomqvist et al., 1995, 2002; **Figure 7**). However, prior to our work no one had established whether the slope of the brain/plasma ketone relationship changed during aging or AD, or after taking a ketogenic supplement.

Acetoacetate is the ketone that actually enters the mitochondria and is catabolized to acetyl CoA. Since synthesis of ^{11}C-AcAc is easier than for ^{11}C-β-HBA (Tremblay et al., 2007), we chose ^{11}C-AcAc as our brain ketone PET tracer. In our PET protocol, ^{11}C-AcAc is infused first followed by a wash-out period and then FDG is infused (**Figure 8**). A period of time equivalent to four half-lives of ^{11}C occurs between the ^{11}C-AcAc

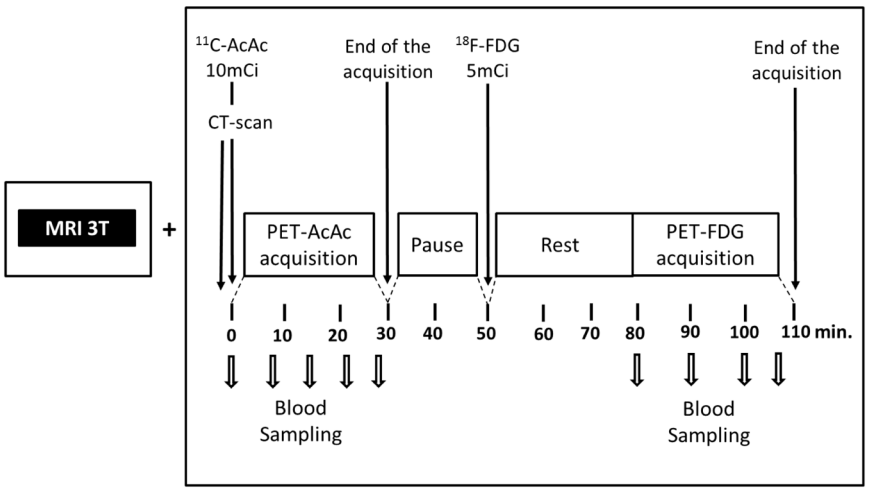


FIGURE 8 | Our ^{11}carbon-acetoacetate (^{11}C-AcAc) and ^{18}F-FDG PET protocol (Pifferi et al., 2011; Roy et al., 2012; Nugent et al., 2014b; Castellano et al., 2015b). The MRI is used for regional segmentation during the PET image analysis.

infusion and the acquisition of the FDG image. This dual tracer technique allows for a quantitative same-day comparison of brain uptake of glucose and ketones thereby avoiding the unnecessary inconvenience to the participant of returning a second time, as well as reducing the biological variability between PET scans done on different days. This dual tracer protocol has been applied in human (Nugent et al., 2014b; Castellano et al., 2015b) and animal studies (Pifferi et al., 2011; Roy et al., 2012).

We have compared brain uptake of FDG and ^{11}C-AcAc in early AD (Castellano et al., 2015b; **Figure 9**). This study had three aims: first, to confirm for the first time using PET methodology the arterio-venous difference reports of normal ketone but low brain glucose uptake early in AD (Ogawa et al., 1996). Second, to assess brain fuel metabolism early in AD rather than the more advanced stages previously reported (Lying-Tunell et al., 1981). Third, to quantify the *regional* pattern of brain uptake

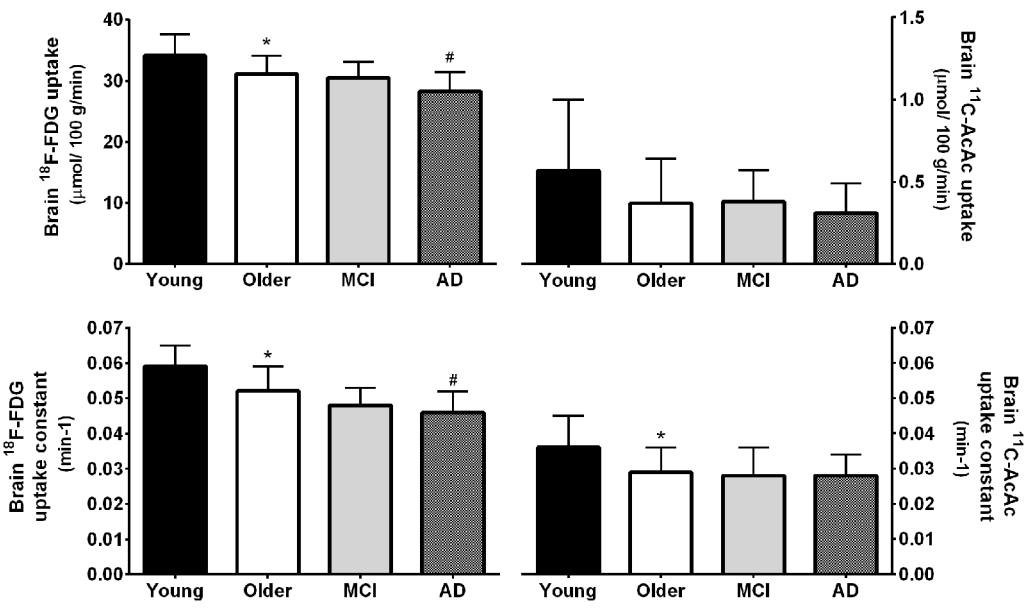


FIGURE 9 | (Upper) Whole brain ^{18}F-FDG uptake was 9% lower in mild AD ($n = 12$) compared to cognitively normal age-matched controls (Older; $n = 42$; #$p < 0.05$ corrected for false discovery rate). ^{18}F-FDG uptake was also 9% lower in healthy older adults compared to younger adults (Young; $n = 30$; *$p < 0.05$ corrected for false discovery rate). Values for mild cognitive impairment (MCI; $n = 9$) were not statistically different between the Older and AD groups. In contrast to FDG, whole brain ^{11}carbon-acetoacetate (^{11}C-AcAc) uptake did not differ significantly between any groups. **(Lower)** statistical differences were present for the ^{18}F-FDG and ^{11}C-AcAc uptake constants (Nugent et al., 2014b; Castellano et al., 2015b).

of both fuels under post-prandial conditions, information that arterio-venous difference studies cannot provide. Our FDG and ^{11}C-AcAc PET studies have so far confirmed several important points: first, as reported previously by arterio-venous difference in the brain taken as a whole (**Figure 1**), global CMRg was 14% lower in early AD vs. cognitively normal, age-matched controls. Second, and as also previously shown by PET, this global CMRg deficit in AD was primarily confined to the parietal cortex, posterior cingulate and thalamus. Third, neither ^{11}C-AcAc uptake (CMRa) nor the AcAc uptake constant (*Ka*) were significantly different in the brain as a whole or in any brain region in AD or MCI vs. the age-matched cognitively healthy controls (**Figure 7**). Fourth, plasma AcAc and CMRa were significantly positively correlated, the slope of which did not differ between early AD and cognitively healthy age-matched controls or young adults (Castellano et al., 2015b; **Figure 5**). Since brain ketone utilization in AD was proportional to plasma concentration and this relationship had the same slope as in age-matched controls (**Figure 7**), we conclude that brain ketone uptake is not significantly disrupted early in AD.

As our database grows, we see a trend emerging toward a lower AcAc uptake constant for the brain *as a whole* in cognitively normal older people vs. young adults. However, when analyzed region by region, the results are not significant but pooled for the whole brain they are (**Figure 9**). So far, there is no trend toward lower brain ^{11}C-AcAc uptake or a lower AcAc uptake constant in MCI or AD compared to our healthy older controls. Hence, caution is needed in comparing either brain ketone uptake results across age groups or in aging-associated cognitive decline for the whole brain vs. measurements focused more on major brain regions, specific nuclei or parts of the cortex.

COMPLIMENTARY APPROACHES TO INCREASE KETOGENESIS?

We have investigated whether substances that increase fatty acid availability to or oxidation by the liver might also increase ketogenesis. If so, this would have the potential benefit of either reducing the dose of MCT needed thereby reducing side-effects, or increasing the ketogenic effect of the same dose of MCT. Three such substances we have tested are alpha-linolenic acid (18:3n-3) and bezafibrate, two PPAR-alpha stimulators, and caffeine, which stimulates lipolysis thereby raising plasma free fatty acids (Acheson et al., 2004). Alpha-linolenic acid is also potentially ketogenic because it is the most beta-oxidized of the common dietary long chain fatty acids (Mamelak, 2012). Consuming 2 g of alpha-linolenic acid daily in the form of flaxseed over 4 weeks didn't change overnight fasting plasma ketones. However, it did raise post-prandial ketone production by 26% but only in young adults; there was no significant effect in older adults (Hennebelle et al., 2016). Treatment with 400 mg of bezafibrate daily for 12 weeks was mildly ketogenic and increased fatty acid oxidation. Bezafibrate reduced plasma insulin and glucose suggesting that it may have a mild insulin-sensitizing effect. Plasma long chain fatty acids were also significantly lower after bezafibrate (Tremblay-Mercier et al., 2010). Co-treatment with bezafibrate (400 mg/d for 8 weeks) and 60 g/day of MCT transiently increased AcAc/β-HBA more than MCT alone (Courchesne-Loyer et al., 2015). A 2.5 or 5.0 mg/kg dose of caffeine taken with a small breakfast was moderately ketogenic 2–4 h post-dose. During the 4 h test period, plasma ketones and free fatty acids rose significantly more on caffeine than in the control test and the rise was broadly proportional to the dose of caffeine (**Figure 10**). We have not yet explored whether caffeine affects the ketogenic effect of MCT. Overall, these effects are modest but may merit further investigation in a clinical population.

NEURONAL HYPEREXCITABILITY, EPILEPTIC SEIZURES AND AD

Epileptic seizures are more prevalent in the aging population that at any other age, a situation that seems to be related to a higher incidence of cerebrovascular disease in older people. Sporadic or late-onset AD is associated with higher risk of seizures

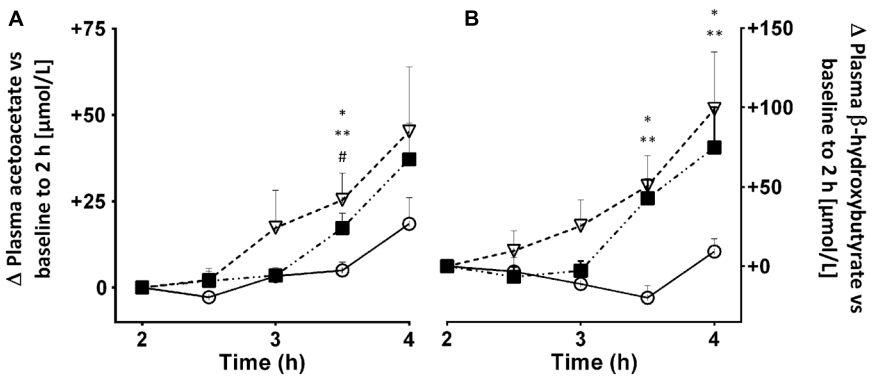


FIGURE 10 | Plasma acetoacetate (A) and β-HBA (B) 2–4 h after an oral dose of 2.5 (■) or 5.0 mg/kg (▽) of caffeine vs. no treatment control (○). Plasma caffeine reached a plateau 1–2 h after being consumed (data not shown), hence the reason for starting the ketone measurements at 2 h post-dose. Values are mean ± SEM ($n = 10$/point, with each participant undertaking each of the three treatments). Ketone data were normalized to zero at 2 h. * Control vs. 2.5 mg/kg (*$p < 0.05$) vs. 5.0 mg/kg (**$p < 0.05$), and caffeine 2.5 vs. 5.0 mg/kg ($^{\#}p < 0.05$).

than in the general population (Amatniek et al., 2006; Vossel et al., 2013; Zilberter et al., 2013). Higher genetic or familial risk of AD is associated with hippocampal hyperexcitability and may be linked to neuronal loss and reorganization and to greater medial temporal lobe atrophy than in controls (Lapointe et al., 2016). Other factors that may contribute to this hyperexcitability include weakening of the inhibitory effect of GABA, and neuronal hypometabolism in the brain (Zilberter et al., 2013). Hyperexcitability in turn can further disrupt brain energy metabolism thereby contributing to a vicious cycle of deteriorating brain function and energy exhaustion. Given the efficacy of ketogenic treatments in controlling refractory epileptic seizures, especially in children, and their emerging utility for cognitive decline in AD, it would therefore seem to be worth assessing the efficacy of ketogenic treatments for seizures in the geriatric population.

ISSUE – IS MITOCHONDRIAL FUNCTION IMPAIRED EARLY IN AD?

The bulk of the ATP needed during energy metabolism is produced by oxidative phosphorylation in mitochondria. Glucose can also produce some ATP via glycolysis, a process occurring outside mitochondria, whereas ketones produce ATP uniquely via oxidative phosphorylation. Mitochondrial damage and increased production of reactive oxygen species have been proposed to underlie beta-amyloid accumulation and cognitive deterioration in AD (Gibson et al., 2000; Swerdlow and Khan, 2004; Yao et al., 2009; Swerdlow et al., 2014). Ketone catabolism is entirely mitochondrial, so normal whole body ketone oxidation in older humans (Freemantle et al., 2009) argues for largely intact mitochondrial function in reasonably healthy older people. Normal brain ketone metabolism in AD (**Figure 7**) also suggests that the enzymes of mitochondrial oxidative phosphorylation in the brain continue to function relatively normally, at least early in AD. Hence, early in AD, the problem with brain glucose metabolism is not necessarily at the mitochondrial level but possibly more because of lower glycolysis to acetyl CoA (see Issue – Brain Glucose Uptake or Glycolysis or Both?). Lower production of acetyl CoA would impair neuronal function thereby accounting for the well-known observation of lower glucose transport into the brain in AD. Oxidative damage to mitochondria and mitochondrial dysfunction would also tend to increase.

The ^{11}C-AcAc used in our PET studies is chemically identical to AcAc produced by the body and is metabolized to $^{11}C\text{-}CO_2$. Since the combined process of both brain uptake and metabolism of ^{11}C-AcAc is still normal in early AD, and since ketone metabolism is uniquely mitochondrial, these results indirectly support the speculation nearly 30 years ago by Hoyer et al. (1988) that oxidative phosphorylation and therefore mitochondrial function is relatively normal early in AD. Such an interpretation is also supported by the clinical studies showing better cognitive scores in AD when more ketones are provided to the brain (**Table 6**) because otherwise ketones would not have clinical benefit.

The very high fat ketogenic diet increases various parameters that represent the overall energy status of the brain, including ATP and brain/blood glucose (DeVivo et al., 1978; Veech et al., 2001; Cahill, 2006), as well as citric acid cycle activity (Roy et al., 2015). Neural protection by ketones may also be related to improved mitochondrial biogenesis and improved respiratory function (Bough et al., 2006), as well as reduced mitochondrial production of reactive oxygen species in response to glutamate (Maalouf et al., 2009). Since oxidative phosphorylation in mitochondria generates free radicals and ketone metabolism is uniquely oxidative, it could also be argued that ketogenic supplements should actually make mitochondrial dysfunction *worse,* which should in turn cause cognitive deterioration. This doesn't happen so, again, mitochondrial function can apparently cope with the increased oxidative load caused by metabolizing ketones. Nevertheless, this topic definitely needs further investigation.

ISSUE – BRAIN GLUCOSE UPTAKE OR GLYCOLYSIS OR BOTH?

The magnitude of the lower glucose metabolism by the AD brain was well-established by arterio-venous difference studies done 20–30 years ago (Lying-Tunell et al., 1981; Hoyer et al., 1988; Ogawa et al., 1996). However, these studies could not establish whether the glucose problem was with glucose transport into the brain via GLUT, or with glycolysis, i.e., the metabolism of glucose to pyruvate within the brain, or both. PET studies clearly show that glucose (FDG) uptake into the brain and its conversion to glucose-6-phosphate by hexokinase is lower in AD. However, FDG-PET cannot establish whether the glycolytic steps are also impaired. Since mitochondrial function seems to be normal in early AD and glucose transport in the brain is dependent on neuronal activity, the brain glucose hypometabolism problem in AD seems at least initially to be with glycolysis because several glycolytic enzymes are impaired in AD, including phosphofructokinase (Bowen et al., 1979; Iwangoff et al., 1980), alpha-ketoglutarate dehydrogenase complex (Gibson et al., 2000), and pyruvate dehydrogenase (Perry et al., 1980; Sorbi et al., 1983; Cunnane et al., 2011). If the problem in AD starts with deteriorating glycolysis, neural viability would eventually decrease, which would in turn decrease glucose transport into the brain because it depends on neural activity (Cunnane et al., 2011). As proposed many years ago by Hoyer et al. (1988), lower glycolysis to acetyl CoA would increase the brain's dependence on other routes to generate ATP, including lactate and possibly even gluconeogenesis. The brain's dependence on these routes to generate ATP could help account for the adverse changes in brain amino acid metabolism and neurotransmitter production including acetylcholine (Hoyer et al., 1988). Hence, our perspective is that the AD brain must be gradually pushed toward starvation mostly due to deteriorating glycolysis.

ISSUE – IS EXCESSIVE CATAPLEROSIS AN IMPEDIMENT TO SUCCESSFUL KETO-NEUROTHERAPEUTICS?

In addition to generating ATP, the citric acid cycle also has a key role in providing intermediates for several brain molecules including the neurotransmitters, gamma-aminobutyric acid and acetylcholine. The use of intermediates in the citric acid cycle to make molecules other than ATP is known as *cataplerosis* (Owen et al., 2002). Cataplerosis is usually balanced by *anaplerosis*, which is the net contribution of carbon from various sources to synthesize molecules derived from intermediates in the citric acid cycle. Glucose and oxaloacetate are anaplerotic so when both are insufficiently available, cataplerosis rapidly depletes the citric acid cycle (Wilkins et al., 2016). Unlike glucose, glutamine, pyruvate and precursors to propionyl CoA, the four carbon ketones (AcAc and β-HBA) do not contribute any carbon to anaplerosis (Brunengraber and Roe, 2006). Indeed, ketones are probably cataplerotic in part because they increase citric acid cycle activity (Roy et al., 2015). Hence, glucose itself or an alternative anaplerotic substrate is essential in order to metabolize ketones, especially as ketosis becomes more extreme.

When glucose supply to the brain is severely limited, such as in inherited GLUT-1 deficiency, there is insufficient glucose entering tissues to support energy production. Providing a ketogenic supplement is clinically beneficial but without anaplerotic input, chronic ketosis could potentially exhaust the citric acid cycle (Mochel et al., 2005; Brunengraber and Roe, 2006; Roe and Mochel, 2006). Triheptanoin (triglyceride with three heptanoic acids) is an odd-carbon MCT that is both ketogenic and anaplerotic and has clinically significant beneficial effects in GLUT-1 transporter deficiency and in Huntington's disease (Mochel et al., 2005, 2010; Pascual et al., 2014). Furthermore, ketogenesis in the liver requires about 150 g/day of glucose that needs to be supplied by gluconeogenesis (Garber et al., 1974; Fukao et al., 2004). An alternative route of gluconeogenesis of unknown importance during nutritional ketosis involves increased acetone production which can be converted to glucose (Owen and Reichard, 1971; Reichard et al., 1974), thereby potentially contributing to sustaining both ketogenesis and anaplerosis.

This question of ketogenesis and anaplerosis is relevant to brain hypometabolism in AD because as AD becomes more severe, brain glucose uptake and/or utilization continue to deteriorate thereby further compromising both energy production and anaplerosis which are both needed for neurotransmitter synthesis. This adverse situation could potentially improve or worsen with sustained ketosis; it all depends on the trade-off between supplying more ketones to compensate for the glucose deficit and generate ATP, vs. burning out the citric acid cycle and depleting acetylcholine and GABA if cataplerosis exceed anaplerosis. Nevertheless, ketones are not the only fuel that bypasses impaired glycolysis: recent animal studies suggest that direct administration of pyruvate could be beneficial component of a ketogenic intervention for AD (Zilberter et al., 2013). *In vitro* studies show that exogenous oxaloacetate may also help bypass issues with glycolysis and maintain mitochondrial respiration (Wilkins et al., 2016).

ISSUE – IS THE KETOGENIC RESPONSE TO ALL MCT THE SAME?

Medium chain triglyceride are a generic product that varies widely in composition. They are usually concentrated in caprylic and capric acids but the ratio of these two fatty acids can vary from 70:30 to 30:70. Despite this variability, a compilation of various studies shows a significant positive correlation between the oral dose of MCT given and the resulting maximal plasma ketone response (**Figure 6**). Although MCT are most commonly a combination of caprylic and capric acids, these two fatty acids are also available separately. In unpublished work, we have observed that the net plasma ketone response to an equal dose of essentially pure caprylic acid exceeds by 15–20% that of an MCT containing caprylic:capric acid at 60:40 which in turn exceeded that of coconut oil by a wide margin (manuscript in preparation). At the same time, the change in plasma AcAc/β-HBA was actually significantly higher for coconut oil than for caprylic acid. This was an acute metabolic study conducted during an 8 h period with a 20 ml dose of the test ketogenic substance taken at breakfast and a second 20 ml dose taken at mid-day without a meal; whether the same changes occur in the long-term still needs to be evaluated.

The formulation of the oral dose of MCT may also impact on the ketone response. We have observed that a 30 g dose of MCT in one formulation can generate a 40% higher plasma ketone response over 4 h than a different formulation containing 50 g of MCT (**Figure 11**). These results raise the questions of whether it matters which MCT is consumed, the bioequivalence of coconut oil vs. MCT, and what metric is most important in assessing the metabolic response to ketogenic supplements; β-HBA, AcAc, both combined, their ratio, or something else.

ISSUE – METABOLIC PHENOTYPE OF THE COGNITIVELY HEALTHY OLDER PERSON OR RED FLAG?

Age-normalization is a standard procedure with cognitive scores for older people and in MCI and AD. Hence, a lower raw cognitive score in an older person is not necessarily indicative of progression toward AD; rather, it depends on the type of test and the degree that the score is lower relative to age-normalized values. In the same vein, should regional brain glucose uptake (CMRg) also be normalized for age because CMRg too declines with age even in cognitively healthy older persons? In order to determine whether a lower CMRg value with age is normal or represents a risk for AD, age-normalized reference values need to be established so as to be able to

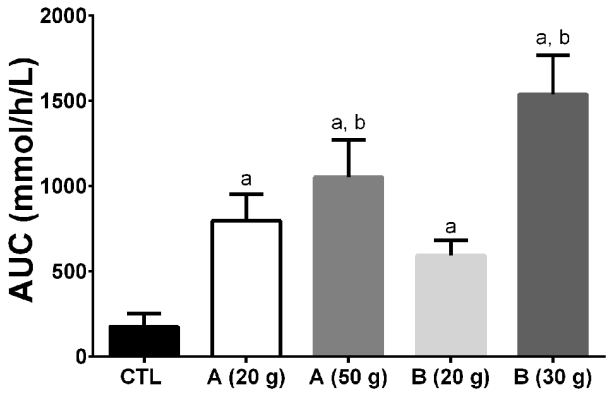


FIGURE 11 | Area under the curve (AUC) of plasma ketone response (beta-hydroxybutyrate + acetoacetate) after ingestion of two different MCT emulsions (A,B) during a 4 h metabolic study day. All the treatments differed from the Control (a – $p \leq 0.05$). The 30 g dose of product B and the 50 g dose of product A differed from the 20 g dose of product B (b – $p \leq 0.05$). Interestingly, the 50 g dose of product A gave a 40% lower AUC than the 30 g dose of product B demonstrating that different homogenates of MCTs can induce widely differing plasma ketone responses, at least in an acute study design. Proprietary considerations prevent providing a full description of products A and B.

distinguish a physiologically normal from a pathological change in CMRg.

Changes in peripheral glucose metabolism leading toward insulin resistance and type 2 diabetes increase the risk of AD (Craft, 2009, 2012; Baker et al., 2011). As with low cognitive scores, should metabolic parameters be considered in including or excluding older persons from a reference group of cognitively healthy older people? In other words, from an energy metabolism perspective, how should we define 'normal' or 'healthy' brain aging? The answer affects not only how data on brain energy metabolism in MCI and AD are interpreted but whether lower brain energy metabolism during 'normal' aging is truly physiological or imminently pathological. Such age-normalization would have two goals – to facilitate the early diagnosis of AD and, if such age-normalized brain glucose hypometabolism were present, to implement a pre-emptive intervention, whether ketone-based or other. One of the challenges with age-normalization of cognitive or metabolic data is that those in whom the onset of AD is imminent cannot presently be distinguished from those who may not get it for 10–20 years (or at all).

Our work shows that older people classified as cognitively normal by conventional neuropsychological tests corrected for age and education have significant brain atrophy, cortical thinning and lower brain glucose metabolism compared to cognitively normal younger adults (Nugent et al., 2014b, 2016). The differences were region-specific and more widespread for regional gray matter atrophy and cortical thinning than for CMRg. The age-related difference in CMRg was similar to the decline in volume and cortical thickness, and represents a decline of 0.3–0.4%/year (Nugent et al., 2016). Our work confirms several previous reports and suggests that the most consistent finding for CMRg during normal aging is glucose hypometabolism in the frontal cortex (De Santi et al., 1995; Moeller et al., 1996; Petit-Taboue et al., 1998; Garraux et al., 1999; Herholz et al., 2002; Zuendorf et al., 2003; Kalpouzos et al., 2009; Nugent et al., 2014a,b). Lower CMRg in the frontal cortex may be contributing to less efficient executive function in older people, i.e., greater recruitment or less inhibition of frontal regions for the same task than in younger adults (Hedden and Gabrieli, 2004). However, our older group still had cognitive test scores that were normal-for-age.

Quite a few neurometabolic differences can be expected between cognitively healthy older adults (**Table 3**). There were no diabetics or pre-diabetics in either group but our measure of insulin resistance, the HOMA2-IR, tended toward the high end of the normal range and was positively associated with higher CMRg, but only in the older group (Nugent et al., 2016). A HOMA2-IR toward the high end of the normal range and body fat content of at least 30% were both associated with normal cognitive function in older adults in this particular population (Nugent et al., 2016). Do these metabolic differences with age suggest that higher plasma insulin and a higher metabolic rheostat are necessary to maintain normal cognition in older people? Alternatively, do declining (though normal-for-age) cognitive scores with age represent a red flag because they drive up the metabolic rheostat? Either way, more work needs to be done evaluating the relation between changing metabolic-endocrine status and cognition regardless of age.

PERSPECTIVE

We make the case here that regional brain glucose hypometabolism can definitely be present in those at risk of AD but decades before the onset of cognitive decline associated with AD, i.e., that it is a pre-symptomatic problem (**Figure 3**). Hence, it is incorrect to perceive of brain glucose hypometabolism in AD as being uniquely a consequence of irreversible neuronal failure or death. Pre-symptomatic brain glucose hypometabolism isn't necessarily the cause of AD or even the first step in the pathogenesis of AD. However, two points are clear – (i) AD is at least in part exacerbated by (if not actually caused by) chronic, progressive brain fuel starvation due specifically to brain glucose deficit, and (ii) attempting to treat the cognitive deficit early in AD using ketogenic interventions in clinical trials is safe, ethical, and scientifically well-founded (Henderson et al., 2009; Rebello et al., 2015; see also ClinicalTrials.gov).

A number of issues have been flagged here that will require further work in order to optimize keto-neurotherapeutics in AD. They include the state of brain mitochondrial respiration, whether the problem with brain glucose utilization starts with impaired glycolysis, the importance of balancing anaplerosis and cataplerosis, differential ketone responses to MCT mixtures depending on formulation, and the significance of lower CMRg in the frontal cortex during cognitively normal aging. The elephant in the room that we have intentionally not discussed till now is the beta-amyloid hypothesis of AD. Suffice it to note here that beta-amyloid accumulation occurs as a result of either

impaired brain glucose availability to the brain (Velliquette et al., 2005) or impaired glycolysis within the brain (Meier-Ruge et al., 1994). Beta-amyloid also contributes to impaired glycolysis in the AD brain (Meier-Ruge and Bertoni-Freddari, 1997). Ketogenic treatments reduce amyloid burden in animal models of AD (Kashiwaya et al., 2000; Hertz et al., 2015; Yin et al., 2016) but this extremely encouraging effect remains to be verified in humans. Clinical trials with ketogenic interventions in AD, MCI or insulin-induced hypoglycemia start to improve some cognitive outcomes within hours to days (Hasselbalch et al., 1996; Reger et al., 2004; Henderson, 2008; Newport et al., 2015), arguing that if they also reduce amyloid burden, it is a secondary effect. Hence, we feel that dealing with the brain energy deficit would help the brain metabolize beta-amyloid as it normally should.

The challenge that immune function and neuroinflammation may pose with respect to providing the brain with sufficient energy during aging also deserves comment. We and others have previously proposed that immunosenescence in older people can tip toward a pro-inflammatory condition. Since the activated immune system consumes a lot of energy, heightened immune surveillance during aging constitutes a potentially important energy sink in the body which can compete with the brain for a fixed (or declining) energy intake in older people. This situation is probably exacerbated by infection, inflammation, or other chronic response of the immune system. Under such circumstances, it takes little to tip the body's energy intake more toward supporting the immune response and away from the brain, which then contributes to brain energy starvation and increased risk of cognitive decline during aging (Fulop et al., 2013, 2015). Apart from the greatly increased need for energy by activated immune cells, pro-inflammatory cytokines impair ketone production (Pailla et al., 2001), making the brain's energy supply even more vulnerable. Hence, brain energy deficit, neuropathology, neuroinflammation and cognitive decline all feed off each other in a vicious cycle that can lead to rapid progression of AD (**Figure 3**). Ketogenic treatments help elicit a protective response to neuroinflammation (Rahman et al., 2014), an encouraging observation that needs confirmation and further work.

Several different ketogenic interventions including prolonged fasting, a very high fat ketogenic diet containing no MCT, or a regular diet to which MCT or ketone esters all have a broadly similar neurological/cognitive benefit (**Table 6**). The cognitive benefit of ketones is observed under different conditions including in the presence of chronically impaired brain glucose availability or utilization due to disease (i.e., MCI or AD), or when the brain glucose deficit is acute, i.e., severe hypoglycemia experimentally induced by insulin infusion and starvation (Owen et al., 1967). The wide range of ketogenic conditions that protect cognition suggests that ketones themselves (rather than MCFAs) are central to their beneficial effect but this needs to be confirmed along with determining the actual mechanism. At present, a keto-neurotherapeutic approach is a safe and scientifically well-supported strategy to bypass deteriorating brain energy metabolism arising during aging; time will tell whether it is truly clinically effective to limit the onset or progression of cognitive decline in AD.

AUTHOR CONTRIBUTIONS

SC, AC-L, C-AC participated in the conception and drafting of the work. All authors participated in the revising and final approval of the content of this review.

FUNDING

Financial support was provided by a Canada Research Chair and University Research Chair (SC), CFI, CIHR, NSERC, FRQS, FQRNT, Sojecci II, The Alzheimer Association (USA), and the Université de Sherbrooke.

ACKNOWLEDGMENTS

Invaluable help and clinical collaboration were provided by Christine Brodeur-Dubreuil, Odette Baril, Marilyn Tousignant, Eric Lavallée, Sébastien Tremblay, Francis Langlois, Guy Lacombe, Daniel Tessier, Hélène Imbeault, Jean-Patrice Baillargeon, and Jacques Matte (Agriculture and Agri-foods Canada). Some MCT used in our more recent studies was provided by Abitec Corporation. Our clinical trials on this topic can be found on ClinicalTrials.gov.

REFERENCES

Acheson, K. J., Gremaud, G., Meirim, I., Montigon, F., Krebs, Y., Fay, L. B., et al. (2004). Metabolic effects of caffeine in humans: lipid oxidation or futile cycling? *Am. J. Clin. Nutr.* 79, 40–46.

Adam, P. A., Raiha, N., Rahiala, E. L., and Kekomaki, M. (1975). Oxidation of glucose and D-B-OH-butyrate by the early human fetal brain. *Acta Paediatr. Scand.* 64, 17–24. doi: 10.1111/j.1651-2227.1975.tb04375.x

Amatniek, J. C., Hauser, W. A., DelCastillo-Castaneda, C., Jacobs, D. M., Marder, K., Bell, K., et al. (2006). Incidence and predictors of seizures in patients with Alzheimer's disease. *Epilepsia* 47, 867–872. doi: 10.1111/j.1528-1167.2006.00554.x

Amiel, S. A., Archibald, H. R., Chusney, G., Williams, A. J., and Gale, E. A. (1991). Ketone infusion lowers hormonal responses to hypoglycaemia: evidence for acute cerebral utilization of a non-glucose fuel. *Clin. Sci. (Lond).* 81, 189–194. doi: 10.1042/cs0810189

Auestad, N., Korsak, R. A., Morrow, J. W., and Edmond, J. (1991). Fatty acid oxidation and ketogenesis by astrocytes in primary culture. *J. Neurochem.* 56, 1376–1386. doi: 10.1111/j.1471-4159.1991.tb11435.x

Avogaro, A., Nosadini, R., Bier, D. M., Cobelli, C., Toffolo, G., Doria, A., et al. (1990). Ketone body kinetics in vivo using simultaneous administration of acetoacetate and 3-hydroxybutyrate labelled with stable isotopes. *Acta Diabetol Lat.* 27, 41–51. doi: 10.1007/BF02624721

Bach, A. C., and Babayan, V. K. (1982). Medium-chain triglycerides: an update. *Am. J. Clin. Nutr.* 36, 950–962.

Baker, L. D., Cross, D. J., Minoshima, S., Belongia, D., Watson, G. S., and Craft, S. (2011). Insulin resistance and Alzheimer-like reductions in regional cerebral glucose metabolism for cognitively normal adults with prediabetes or early type 2 diabetes. *Arch. Neurol.* 68, 51–57. doi: 10.1001/archneurol.2010.225

Balasse, E. O., and Fery, F. (1989). Ketone body production and disposal: effects of fasting, diabetes, and exercise. *Diabetes Metab. Rev.* 5, 247–270. doi: 10.1002/dmr.5610050304

Balasse, E. O., Fery, F., and Neef, M. A. (1978). Changes induced by exercise in rates of turnover and oxidation of ketone bodies in fasting man. *J. Appl. Physiol. Respir. Environ. Exerc. Physiol.* 44, 5–11.

Bekesi, A., and Williamson, D. H. (1990). An explanation for ketogenesis by the intestine of the suckling rat: the presence of an active hydroxymethylglutaryl-coenzyme A pathway. *Biol. Neonate* 58, 160–165. doi: 10.1159/000243256

Benson, D. F., Kuhl, D. E., Hawkins, R. A., Phelps, M. E., Cummings, J. L., and Tsai, S. Y. (1983). The fluorodeoxyglucose 18F scan in Alzheimer's disease and multi-infarct dementia. *Arch. Neurol.* 40, 711–714. doi: 10.1001/archneur.1983.04050110029003

Bickerton, A. S., Roberts, R., Fielding, B. A., Tornqvist, H., Blaak, E. E., Wagenmakers, A. J., et al. (2008). Adipose tissue fatty acid metabolism in insulin-resistant men. *Diabetologia* 51, 1466–1474. doi: 10.1007/s00125-008-1040-x

Blass, J. P. (2008). A new approach to treating Alzheimer's disease. *Ann. N. Y. Acad. Sci.* 1147, 122–128. doi: 10.1196/annals.1427.022

Blomqvist, G., Alvarsson, M., Grill, V., Von Heijne, G., Ingvar, M., Thorell, J. O., et al. (2002). Effect of acute hyperketonemia on the cerebral uptake of ketone bodies in nondiabetic subjects and IDDM patients. *Am. J. Physiol. Endocrinol. Metab.* 283, E20–E28. doi: 10.1152/ajpendo.00294.2001

Blomqvist, G., Thorell, J. O., Ingvar, M., Grill, V., Widen, L., and Stone-Elander, S. (1995). Use of R-beta-[1-11C]hydroxybutyrate in PET studies of regional cerebral uptake of ketone bodies in humans. *Am. J. Physiol.* 269(5 Pt 1), E948–E959.

Bough, K. J., Wetherington, J., Hassel, B., Pare, J. F., Gawryluk, J. W., Greene, J. G., et al. (2006). Mitochondrial biogenesis in the anticonvulsant mechanism of the ketogenic diet. *Ann. Neurol.* 60, 223–235. doi: 10.1002/ana.20899

Bougneres, P. F., Lemmel, C., Ferre, P., and Bier, D. M. (1986). Ketone body transport in the human neonate and infant. *J. Clin. Invest.* 77, 42–48. doi: 10.1172/JCI112299

Bowen, D. M., White, P., Spillane, J. A., Goodhardt, M. J., Curzon, G., Iwangoff, P., et al. (1979). Accelerated ageing or selective neuronal loss as an important cause of dementia? *Lancet* 1, 11–14.

Breckenridge, W. C., and Kuksis, A. (1967). Molecular weight distributions of milk fat triglycerides from seven species. *J. Lipid Res.* 8, 473–478.

Brunengraber, H., and Roe, C. R. (2006). Anaplerotic molecules: current and future. *J. Inherit. Metab. Dis.* 29, 327–331. doi: 10.1007/s10545-006-0320-1

Burns, C. M., Chen, K., Kaszniak, A. W., Lee, W., Alexander, G. E., Bandy, D., et al. (2013). Higher serum glucose levels are associated with cerebral hypometabolism in Alzheimer regions. *Neurology* 80, 1557–1564. doi: 10.1212/WNL.0b013e31828f17de

Cahill, G. F. Jr. (2006). Fuel metabolism in starvation. *Annu. Rev. Nutr.* 26, 1–22. doi: 10.1146/annurev.nutr.26.061505.111258

Castellano, C. A., Baillargeon, J. P., Nugent, S., Tremblay, S., Fortier, M., Imbeault, H., et al. (2015a). Regional brain glucose hypometabolism in young women with polycystic ovary syndrom: possible link to mild insulin resistance. *PLoS ONE* 10:e0144116. doi: 10.1371/journal.pone.0144116

Castellano, C. A., Nugent, S., Paquet, N., Tremblay, S., Bocti, C., Lacombe, G., et al. (2015b). Lower brain 18F-Fluorodeoxyglucose uptake but normal 11C-Acetoacetate metabolism in mild Alzheimer's Disease dementia. *J. Alzheimers Dis.* 43, 1343–1353. doi: 10.3233/JAD-141074

Clarke, K., Tchabanenko, K., Pawlosky, R., Carter, E., Todd King, M., Musa-Veloso, K., et al. (2012). Kinetics, safety and tolerability of (R)-3-hydroxybutyl (R)-3-hydroxybutyrate in healthy adult subjects. *Regul. Toxicol. Pharmacol.* 63, 401–408. doi: 10.1016/j.yrtph.2012.04.008

Courchesne-Loyer, A., Fortier, M., Tremblay-Mercier, J., Chouinard-Watkins, R., Roy, M., Nugent, S., et al. (2013). Stimulation of mild, sustained ketonemia by medium-chain triacylglycerols in healthy humans: estimated potential contribution to brain energy metabolism. *Nutrition* 29, 635–640. doi: 10.1016/j.nut.2012.09.009

Courchesne-Loyer, A., St-Pierre, V., Hennebelle, M., Castellano, C. A., Fortier, M., Tessier, D., et al. (2015). Ketogenic response to cotreatment with bezafibrate and medium chain triacylglycerols in healthy humans. *Nutrition* 31, 1255–1259. doi: 10.1016/j.nut.2015.05.015

Craft, S. (2009). The role of metabolic disorders in Alzheimer disease and vascular dementia: two roads converged. *Arch. Neurol.* 66, 300–305. doi: 10.1001/archneurol.2009.27

Craft, S. (2012). Alzheimer disease: insulin resistance and AD–extending the translational path. *Nat. Rev. Neurol.* 8, 360–362. doi: 10.1038/nrneurol.2012.112

Cunnane, S., Nugent, S., Roy, M., Courchesne-Loyer, A., Croteau, E., Tremblay, S., et al. (2011). Brain fuel metabolism, aging, and Alzheimer's disease. *Nutrition* 27, 3–20. doi: 10.1016/j.nut.2010.07.021

Cunnane, S. C., Courchesne-Loyer, A., St-Pierre, V., Vandenberghe, C., Pierotti, T., Fortier, M., et al. (2016). Can ketones compensate for deteriorating brain glucose uptake during aging? Implications for the risk and treatment of Alzheimer's disease. *Ann. N. Y. Acad. Sci.* 1367, 12–20. doi: 10.1111/nyas.12999

Cunnane, S. C., and Crawford, M. A. (2014). Energetic and nutritional constraints on infant brain development: implications for brain expansion during human evolution. *J. Hum. Evol.* 77, 88–98. doi: 10.1016/j.jhevol.2014.05.001

Cunnane, S. C., Ryan, M. A., Nadeau, C. R., Bazinet, R. P., Musa-Veloso, K., and McCloy, U. (2003). Why is carbon from some polyunsaturates extensively recycled into lipid synthesis? *Lipids* 38, 477–484. doi: 10.1007/s11745-003-1087-8

D'Agostino, D. P., Pilla, R., Held, H. E., Landon, C. S., Puchowicz, M., Brunengraber, H., et al. (2013). Therapeutic ketosis with ketone ester delays central nervous system oxygen toxicity seizures in rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 304, R829–R836. doi: 10.1152/ajpregu.00506.2012

Dastur, D. K. (1985). Cerebral blood flow and metabolism in normal human aging, pathological aging, and senile dementia. *J. Cereb. Blood Flow Metab.* 5, 1–9. doi: 10.1038/jcbfm.1985.1

De Gaetano, A., Castagneto, M., Mingrone, G., Gangeri, G., Sganga, G., Tataranni, P. A., et al. (1994). Kinetics of medium-chain triglycerides and free fatty acids in healthy volunteers and surgically stressed patients. *JPEN J. Parenter. Enteral. Nutr.* 18, 134–140. doi: 10.1177/0148607194018002134

De Santi, S., de Leon, M. J., Convit, A., Tarshish, C., Rusinek, H., Tsui, W. H., et al. (1995). Age-related changes in brain: II. Positron emission tomography of frontal and temporal lobe glucose metabolism in normal subjects. *Psychiatr. Q. Winter* 66, 357–370. doi: 10.1007/BF02238755

DeVivo, D. C., Leckie, M. P., Ferrendelli, J. S., and McDougal, D. B. Jr. (1978). Chronic ketosis and cerebral metabolism. *Ann. Neurol.* 3, 331–337. doi: 10.1002/ana.410030410

Drenick, E. J., Alvarez, L. C., Tamasi, G. C., and Brickman, A. S. (1972). Resistance to symptomatic insulin reactions after fasting. *J. Clin. Invest.* 51, 2757–2762. doi: 10.1172/JCI107095

Ebert, D., Haller, R. G., and Walton, M. E. (2003). Energy contribution of octanoate to intact rat brain metabolism measured by 13C nuclear magnetic resonance spectroscopy. *J. Neurosci.* 23, 5928–5935.

Fery, F., and Balasse, E. O. (1983). Ketone body turnover during and after exercise in overnight-fasted and starved humans. *Am. J. Physiol.* 245, E318–E325.

Fery, F., and Balasse, E. O. (1986). Response of ketone body metabolism to exercise during transition from postabsorptive to fasted state. *Am. J. Physiol.* 250(5 Pt 1), E495–E501.

Flatt, J. P. (1972). On the maximal possible rate of ketogenesis. *Diabetes Metab. Res. Rev.* 21, 50–53. doi: 10.2337/diab.21.1.50

Freemantle, E., Vandal, M., Tremblay Mercier, J., Plourde, M., Poirier, J., and Cunnane, S. C. (2009). Metabolic response to a ketogenic breakfast in the healthy elderly. *J. Nutr. Health Aging* 13, 293–298. doi: 10.1007/s12603-009-0026-9

Freund, G., and Weinsier, R. L. (1966). Standardized ketosis in man following medium chain triglyceride ingestion. *Metabolism* 15, 980–991. doi: 10.1016/0026-0495(66)90046-1

Fukao, T., Lopaschuk, G. D., and Mitchell, G. A. (2004). Pathways and control of ketone body metabolism: on the fringe of lipid biochemistry. *Prostaglandins Leukot Essent Fatty Acids* 70, 243–251. doi: 10.1016/j.plefa.2003.11.001

Fulop, T., Dupuis, G., Baehl, S., Le Page, A., Bourgade, K., Frost, E., et al. (2015). From inflamm-aging to immune-paralysis: a slippery slope during aging for immune-adaptation. *Biogerontology* 17, 147–157. doi: 10.1007/s10522-015-9615-7

Fulop, T., Lacombe, G., Cunnane, S., Le Page, A., Dupuis, G., Frost, E. H., et al. (2013). Elusive Alzheimer's disease: can immune signatures help our understanding of this challenging disease? Part 2: new immune paradigm. *Discov. Med.* 15, 33–42.

Garber, A. J., Menzel, P. H., Boden, G., and Owen, O. E. (1974). Hepatic ketogenesis and gluconeogenesis in humans. *J. Clin. Invest.* 54, 981–989. doi: 10.1172/JCI107839

Garraux, G., Salmon, E., Degueldre, C., Lemaire, C., Laureys, S., and Franck, G. (1999). Comparison of impaired subcortico-frontal metabolic networks in normal aging, subcortico-frontal dementia, and cortical frontal dementia. *Neuroimage* 10, 149–162. doi: 10.1006/nimg.1999.0463

Gibson, R. E., Burns, H. D., Hamill, T. G., Eng, W. S., Francis, B. E., and Ryan, C. (2000). Non-invasive radiotracer imaging as a tool for drug development. *Curr. Pharm. Des.* 6, 973–989. doi: 10.2174/1381612003399987

Guillot, E., Vaugelade, P., Lemarchal, P., and Rerat, A. (1993). Intestinal absorption and liver uptake of medium-chain fatty acids in non-anaesthetized pigs. *Br. J. Nutr.* 69, 431–442. doi: 10.1079/BJN19930045

Halestrap, A. P., and Price, N. T. (1999). The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. *Biochem. J.* 343(Pt 2), 281–299. doi: 10.1042/0264-6021:3430281

Hall, S. E., Wastney, M. E., Bolton, T. M., Braaten, J. T., and Berman, M. (1984). Ketone body kinetics in humans: the effects of insulin-dependent diabetes, obesity, and starvation. *J. Lipid Res.* 25, 1184–1194.

Hasselbalch, S. G., Knudsen, G. M., Jakobsen, J., Hageman, L. P., Holm, S., and Paulson, O. B. (1995). Blood-brain barrier permeability of glucose and ketone bodies during short-term starvation in humans. *Am. J. Physiol.* 268(6 Pt 1), E1161–E1166.

Hasselbalch, S. G., Madsen, P. L., Hageman, L. P., Olsen, K. S., Justesen, N., Holm, S., et al. (1996). Changes in cerebral blood flow and carbohydrate metabolism during acute hyperketonemia. *Am. J. Physiol.* 270(5 Pt 1), E746–E751.

Hedden, T., and Gabrieli, J. D. (2004). Insights into the ageing mind: a view from cognitive neuroscience. *Nat. Rev. Neurosci.* 5, 87–96. doi: 10.1038/nrn1323

Henderson, S. T. (2008). Ketone bodies as a therapeutic for Alzheimer's disease. *Neurotherapeutics* 5, 470–480. doi: 10.1016/j.nurt.2008.05.004

Henderson, S. T., Vogel, J. L., Barr, L. J., Garvin, F., Jones, J. J., and Costantini, L. C. (2009). Study of the ketogenic agent AC-1202 in mild to moderate Alzheimer's disease: a randomized, double-blind, placebo-controlled, multicenter trial. *Nutr. Metab. (Lond.)* 6:31. doi: 10.1186/1743-7075-6-31

Hennebelle, M., Courchesne-Loyer, A., St-Pierre, V., Vandenberghe, C., Castellano, C. A., Fortier, M., et al. (2016). Preliminary evaluation of a differential effect of an alpha-linolenate-rich supplement on ketogenesis and plasma omega-3 fatty acids in young compared to older adults. *Nutrition* 16, 30040–30045. doi: 10.1016/j.nut.2016.03.025

Herholz, K., Salmon, E., Perani, D., Baron, J. C., Holthoff, V., Frolich, L., et al. (2002). Discrimination between Alzheimer dementia and controls by automated analysis of multicenter FDG PET. *Neuroimage* 17, 302–316. doi: 10.1006/nimg.2002.1208

Hertz, L., Chen, Y., and Waagepetersen, H. S. (2015). Effects of ketone bodies in Alzheimer's disease in relation to neural hypometabolism, beta-amyloid toxicity, and astrocyte function. *J. Neurochem.* 134, 7–20. doi: 10.1111/jnc.13107

Hilditch, T. P., and Meara, M. L. (1944). Human-milk fat: 1. Component fatty acids. *Biochem. J.* 38, 29–34.

Hoyer, S. (1992). Oxidative energy metabolism in Alzheimer brain. Studies in early-onset and late-onset cases. *Mol. Chem. Neuropathol.* 16, 207–224. doi: 10.1007/BF03159971

Hoyer, S., Oesterreich, K., and Wagner, O. (1988). Glucose metabolism as the site of the primary abnormality in early-onset dementia of Alzheimer type? *J. Neurol.* 235, 143–148. doi: 10.1007/BF00314304

Huttenlocher, P. R. (1976). Ketonemia and seizures: metabolic and anticonvulsant effects of two ketogenic diets in childhood epilepsy. *Pediatr. Res.* 10, 536–540. doi: 10.1203/00006450-197605000-00006

Insull, W. Jr., and Ahrens, E. H. Jr. (1959). The fatty acids of human milk from mothers on diets taken ad libitum. *Biochem. J.* 72, 27–33. doi: 10.1042/bj0720027

Ishibashi, K., Onishi, A., Fujiwara, Y., Ishiwata, K., and Ishii, K. (2015). Relationship between Alzheimer disease-like pattern of 18F-FDG and fasting plasma glucose levels in cognitively normal volunteers. *J. Nucl. Med.* 56, 229–233. doi: 10.2967/jnumed.114.150045

Iwangoff, P., Armbruster, R., Enz, A., and Meier-Ruge, W. (1980). Glycolytic enzymes from human autoptic brain cortex: normal aged and demented cases. *Mech. Ageing Dev.* 14, 203–209. doi: 10.1016/0047-6374(80)90120-7

Kalpouzos, G., Chetelat, G., Baron, J. C., Landeau, B., Mevel, K., Godeau, C., et al. (2009). Voxel-based mapping of brain gray matter volume and glucose metabolism profiles in normal aging. *Neurobiol. Aging* 30, 112–124. doi: 10.1016/j.neurobiolaging.2007.05.019

Kashiwaya, Y., Takeshima, T., Mori, N., Nakashima, K., Clarke, K., and Veech, R. L. (2000). D-beta-hydroxybutyrate protects neurons in models of Alzheimer's and Parkinson's disease. *Proc. Natl. Acad. Sci. U.S.A.* 97, 5440–5444. doi: 10.1073/pnas.97.10.5440

Krikorian, R., Shidler, M. D., Dangelo, K., Couch, S. C., Benoit, S. C., and Clegg, D. J. (2012). Dietary ketosis enhances memory in mild cognitive impairment. *Neurobiol. Aging* 33, 425.e19–425.e27. doi: 10.1016/j.neurobiolaging.2010.10.006

Kuge, Y., Yajima, K., Kawashima, H., Yamazaki, H., Hashimoto, N., and Miyake, Y. (1995). Brain uptake and metabolism of [1-11C]octanoate in rats: pharmacokinetic basis for its application as a radiopharmaceutical for studying brain fatty acid metabolism. *Ann. Nucl. Med.* 9, 137–142. doi: 10.1007/BF03165040

Lapointe, E., Deacon, C., Royer-Perron, L., Cunnane, S. C., Castellano, C. A., and Bocti, C. (2016). Temporal lobe atrophy may be underrecognized in older patients with new-onset epilepsy. *Can. J. Neurol. Sci.* (in press).

London, E. D., Margolin, R. A., Duara, R., Holloway, H. W., Robertson-Tchabo, E. A., Cutler, N. R., et al. (1986). Effects of fasting on ketone body concentrations in healthy men of different ages. *J. Gerontol.* 41, 599–604. doi: 10.1093/geronj/41.5.599

Lying-Tunell, U., Lindblad, B. S., Malmlund, H. O., and Persson, B. (1981). Cerebral blood flow and metabolic rate of oxygen, glucose, lactate, pyruvate, ketone bodies and amino acids. *Acta Neurol. Scand.* 63, 337–350. doi: 10.1111/j.1600-0404.1981.tb00788.x

Maalouf, M., Rho, J. M., and Mattson, M. P. (2009). The neuroprotective properties of calorie restriction, the ketogenic diet, and ketone bodies. *Brain Res. Rev.* 59, 293–315. doi: 10.1016/j.brainresrev.2008.09.002

Mamelak, M. (2012). Sporadic Alzheimer's disease: the starving brain. *J. Alzheimers Dis.* 31, 459–474. doi: 10.3233/JAD-2012-120370

Matsuzaki, T., Sasaki, K., Tanizaki, Y., Hata, J., Fujimi, K., Matsui, Y., et al. (2010). Insulin resistance is associated with the pathology of Alzheimer disease: the Hisayama study. *Neurology* 75, 764–770. doi: 10.1212/WNL.0b013e3181eee25f

Meier-Ruge, W., Iwangoff, P., and Bertoni-Freddari, C. (1994). What is primary and what secondary for amyloid deposition in Alzheimer's disease. *Ann. N. Y. Acad. Sci.* 719, 230–237. doi: 10.1111/j.1749-6632.1994.tb56831.x

Meier-Ruge, W. A., and Bertoni-Freddari, C. (1997). Pathogenesis of decreased glucose turnover and oxidative phosphorylation in ischemic and trauma-induced dementia of the Alzheimer type. *Ann. N. Y. Acad. Sci.* 826, 229–241. doi: 10.1111/j.1749-6632.1997.tb48474.x

Mitchell, G. A., Kassovska-Bratinova, S., Boukaftane, Y., Robert, M. F., Wang, S. P., Ashmarina, L., et al. (1995). Medical aspects of ketone body metabolism. *Clin. Invest. Med.* 18, 193–216.

Mochel, F., DeLonlay, P., Touati, G., Brunengraber, H., Kinman, R. P., Rabier, D., et al. (2005). Pyruvate carboxylase deficiency: clinical and biochemical response to anaplerotic diet therapy. *Mol. Genet. Metab.* 84, 305–312. doi: 10.1016/j.ymgme.2004.09.007

Mochel, F., Duteil, S., Marelli, C., Jauffret, C., Barles, A., Holm, J., et al. (2010). Dietary anaplerotic therapy improves peripheral tissue energy metabolism in patients with Huntington's disease. *Eur. J. Hum. Genet.* 18, 1057–1060. doi: 10.1038/ejhg.2010.72

Moeller, J. R., Ishikawa, T., Dhawan, V., Spetsieris, P., Mandel, F., Alexander, G. E., et al. (1996). The metabolic topography of normal aging. *J. Cereb. Blood Flow Metab.* 16, 385–398. doi: 10.1097/00004647-199605000-00005

Mosconi, L., Sorbi, S., de Leon, M. J., Li, Y., Nacmias, B., Myoung, P. S., et al. (2006). Hypometabolism exceeds atrophy in presymptomatic early-onset familial Alzheimer's disease. *J. Nucl. Med.* 47, 1778–1786.

Newport, M. T., VanItallie, T. B., Kashiwaya, Y., King, M. T., and Veech, R. L. (2015). A new way to produce hyperketonemia: use of ketone ester

in a case of Alzheimer's disease. *Alzheimers Dement.* 11, 99–103. doi: 10.1016/j.jalz.2014.01.006

Nugent, S., Castellano, C. A., Bocti, C., Dionne, I., Fulop, T., and Cunnane, S. C. (2016). Relationship of metabolic and endocrine parameters to brain glucose metabolism in older adults: do cognitively-normal older adults have a particular metabolic phenotype? *Biogerontology* 17, 241–255. doi: 10.1007/s10522-015-9595-7

Nugent, S., Castellano, C. A., Goffaux, P., Whittingstall, K., Lepage, M., Paquet, N., et al. (2014a). Glucose hypometabolism is highly localized but lower cortical thickness and brain atrophy are widespread in cognitively normal older adults. *Am. J. Physiol. Endocrinol. Metab.* 306, E1315–E1321. doi: 10.1152/ajpendo.00067.2014

Nugent, S., Tremblay, S., Chen, K. W., Ayutyanont, N., Roontiva, A., Castellano, C. A., et al. (2014b). Brain glucose and acetoacetate metabolism: a comparison of young and older adults. *Neurobiol. Aging* 35, 1386–1395. doi: 10.1016/j.neurobiolaging.2013.11.027

Ogawa, M., Fukuyama, H., Ouchi, Y., Yamauchi, H., and Kimura, J. (1996). Altered energy metabolism in Alzheimer's disease. *J. Neurol. Sci.* 139, 78–82. doi: 10.1016/0022-510X(96)00033-0

Owen, O. E., Kalhan, S. C., and Hanson, R. W. (2002). The key role of anaplerosis and cataplerosis for citric acid cycle function. *J. Biol. Chem.* 277, 30409–30412. doi: 10.1074/jbc.R200006200

Owen, O. E., Morgan, A. P., Kemp, H. G., Sullivan, J. M., Herrera, M. G., and Cahill, G. F. Jr. (1967). Brain metabolism during fasting. *J. Clin. Invest.* 46, 1589–1595. doi: 10.1172/JCI105650

Owen, O. E., and Reichard, G. A. Jr. (1971). Human forearm metabolism during progressive starvation. *J. Clin. Invest.* 50, 1536–1545. doi: 10.1172/JCI106639

Owen, O. E., Reichard, G. A. Jr., Markus, H., Boden, G., Mozzoli, M. A., and Shuman, C. R. (1973). Rapid intravenous sodium acetoacetate infusion in man. Metabolic and kinetic responses. *J. Clin. Invest.* 52, 2606–2616. doi: 10.1172/JCI107453

Page, K. A., Williamson, A., Yu, N., McNay, E. C., Dzuira, J., McCrimmon, R. J., et al. (2009). Medium-chain fatty acids improve cognitive function in intensively treated type 1 diabetic patients and support in vitro synaptic transmission during acute hypoglycemia. *Diabetes* 58, 1237–1244. doi: 10.2337/db08-1557

Pailla, K., El-Mir, M. Y., Cynober, L., and Blonde-Cynober, F. (2001). Cytokine-mediated inhibition of ketogenesis is unrelated to nitric oxide or protein synthesis. *Clin. Nutr.* 20, 313–317. doi: 10.1054/clnu.2001.0421

Pan, J. W., Telang, F. W., Lee, J. H., de Graaf, R. A., Rothman, D. L., Stein, D. T., et al. (2001). Measurement of beta-hydroxybutyrate in acute hyperketonemia in human brain. *J. Neurochem.* 79, 539–544. doi: 10.1046/j.1471-4159.2001.00575.x

Pascual, J. M., Liu, P., Mao, D., Kelly, D. I., Hernandez, A., Sheng, M., et al. (2014). Triheptanoin for glucose transporter type I deficiency (G1D): modulation of human ictogenesis, cerebral metabolic rate, and cognitive indices by a food supplement. *JAMA Neurol.* 71, 1255–1265. doi: 10.1001/jamaneurol.2014.1584

Perry, E. K., Perry, R. H., Tomlinson, B. E., Blessed, G., and Gibson, P. H. (1980). Coenzyme A-acetylating enzymes in Alzheimer's disease: possible cholinergic 'compartment' of pyruvate dehydrogenase. *Neurosci. Lett.* 18, 105–110. doi: 10.1016/0304-3940(80)90220-7

Petit-Taboue, M. C., Landeau, B., Desson, J. F., Desgranges, B., and Baron, J. C. (1998). Effects of healthy aging on the regional cerebral metabolic rate of glucose assessed with statistical parametric mapping. *Neuroimage* 7, 176–184. doi: 10.1006/nimg.1997.0318

Pifferi, F., Tremblay, S., Croteau, E., Fortier, M., Tremblay-Mercier, J., Lecomte, R., et al. (2011). Mild experimental ketosis increases brain uptake of (11)C-acetoacetate and (18)F-fluorodeoxyglucose: a dual-tracer PET imaging study in rats. *Nutr. Neurosci.* 14, 51–58. doi: 10.1179/1476830510Y.0000000001

Pi-Sunyer, F. X., Hashim, S. A., and Van Itallie, T. B. (1969). Insulin and ketone responses to ingestion of medium and long-chain triglycerides in man. *Diabetes Metab. Res. Rev.* 18, 96–100.

Plecko, B., Stoeckler-Ipsiroglu, S., Schober, E., Harrer, G., Mlynarik, V., Gruber, S., et al. (2002). Oral beta-hydroxybutyrate supplementation in two patients with hyperinsulinemic hypoglycemia: monitoring of beta-hydroxybutyrate levels in blood and cerebrospinal fluid, and in the brain by in vivo magnetic resonance spectroscopy. *Pediatr. Res.* 52, 301–306. doi: 10.1203/00006450-200208000-00025

Rahman, M., Muhammad, S., Khan, M. A., Chen, H., Ridder, D. A., Muller-Fielitz, H., et al. (2014). The beta-hydroxybutyrate receptor HCA2 activates a neuroprotective subset of macrophages. *Nat. Commun.* 5:3944. doi: 10.1038/ncomms4944

Rebello, C. J., Keller, J. N., Liu, A. G., Johnson, W. D., and Greenway, F. L. (2015). Pilot feasibility and safety study examining the effect of medium chain triglyceride supplementation in subjects with mild cognitive impairment: a randomized controlled trial. *BBA Clin.* 3, 123–125. doi: 10.1016/j.bbacli.2015.01.001

Rees, D. A., Udiawar, M., Berlot, R., Jones, D. K., and O'Sullivan, M. J. (2016). White matter microstructure and cognitive function in young women with polycystic ovary syndrome. *J. Clin. Endocrinol. Metab.* 101, 314–323. doi: 10.1210/jc.2015-2318

Reger, M. A., Henderson, S. T., Hale, C., Cholerton, B., Baker, L. D., Watson, G. S., et al. (2004). Effects of beta-hydroxybutyrate on cognition in memory-impaired adults. *Neurobiol. Aging* 25, 311–314. doi: 10.1016/S0197-4580(03)00087-3

Reichard, G. A. Jr., Owen, O. E., Haff, A. C., Paul, P., and Bortz, W. M. (1974). Ketone-body production and oxidation in fasting obese humans. *J. Clin. Invest.* 53, 508–515. doi: 10.1172/JCI107584

Reiman, E. M., Chen, K., Alexander, G. E., Caselli, R. J., Bandy, D., Osborne, D., et al. (2004). Functional brain abnormalities in young adults at genetic risk for late-onset Alzheimer's dementia. *Proc. Natl. Acad. Sci. U.S.A.* 101, 284–289. doi: 10.1073/pnas.2635903100

Robinson, A. M., and Williamson, D. H. (1980). Physiological roles of ketone bodies as substrates and signals in mammalian tissues. *Physiol. Rev.* 60, 143–187.

Roe, C. R., and Mochel, F. (2006). Anaplerotic diet therapy in inherited metabolic disease: therapeutic potential. *J. Inherit. Metab. Dis.* 29, 332–340. doi: 10.1007/s10545-006-0290-3

Ronnemaa, E., Zethelius, B., Sundelof, J., Sundstrom, J., Degerman-Gunnarsson, M., Berne, C., et al. (2008). Impaired insulin secretion increases the risk of Alzheimer disease. *Neurology* 71, 1065–1071. doi: 10.1212/01.wnl.0000310646.32212.3a

Roy, M., Beauvieux, M. C., Naulin, J., El Hamrani, D., Gallis, J. L., Cunnane, S. C., et al. (2015). Rapid adaptation of rat brain and liver metabolism to a ketogenic diet: an integrated study using (1)H- and (13)C-NMR spectroscopy. *J. Cereb. Blood Flow Metab.* 35, 1154–1162. doi: 10.1038/jcbfm.2015.29

Roy, M., Nugent, S., Tremblay-Mercier, J., Tremblay, S., Courchesne-Loyer, A., Beaudoin, J. F., et al. (2012). The ketogenic diet increases brain glucose and ketone uptake in aged rats: a dual tracer PET and volumetric MRI study. *Brain Res.* 1488, 14–23. doi: 10.1016/j.brainres.2012.10.008

Sarda, P., Lepage, G., Roy, C. C., and Chessex, P. (1987). Storage of medium-chain triglycerides in adipose tissue of orally fed infants. *Am. J. Clin. Nutr.* 45, 399–405.

Schioth, H. B., Craft, S., Brooks, S. J., Frey, W. H. II, and Benedict, C. (2012). Brain insulin signaling and Alzheimer's disease: current evidence and future directions. *Mol. Neurobiol.* 46, 4–10. doi: 10.1007/s12035-011-8229-6

Scholl, M., Almkvist, O., Bogdanovic, N., Wall, A., Langstrom, B., Viitanen, M., et al. (2011). Time course of glucose metabolism in relation to cognitive performance and postmortem neuropathology in Met146Val PSEN1 mutation carriers. *J. Alzheimers Dis.* 24, 495–506. doi: 10.3233/JAD-2011-101563

Schrijvers, E. M., Witteman, J. C., Sijbrands, E. J., Hofman, A., Koudstaal, P. J., and Breteler, M. M. (2010). Insulin metabolism and the risk of Alzheimer disease: the rotterdam study. *Neurology* 75, 1982–1987. doi: 10.1212/WNL.0b013e3181ffe4f6

Seaton, T. B., Welle, S. L., Warenko, M. K., and Campbell, R. G. (1986). Thermic effect of medium-chain and long-chain triglycerides in man. *Am. J. Clin. Nutr.* 44, 630–634.

Settergren, G., Lindblad, B. S., and Persson, B. (1976). Cerebral blood flow and exchange of oxygen, glucose, ketone bodies, lactate, pyruvate and amino acids in infants. *Acta Paediatr. Scand.* 65, 343–353. doi: 10.1111/j.1651-2227.1976.tb04896.x

Simpson, I. A., Carruthers, A., and Vannucci, S. J. (2007). Supply and demand in cerebral energy metabolism: the role of nutrient transporters. *J. Cereb. Blood Flow Metab.* 27, 1766–1791. doi: 10.1038/sj.jcbfm.9600521

Sorbi, S., Bird, E. D., and Blass, J. P. (1983). Decreased pyruvate dehydrogenase complex activity in Huntington and Alzheimer brain. *Ann. Neurol.* 13, 72–78. doi: 10.1002/ana.410130116

St-Onge, M. P., and Jones, P. J. (2002). Physiological effects of medium-chain triglycerides: potential agents in the prevention of obesity. *J. Nutr.* 132, 329–332.

Swerdlow, R. H., Burns, J. M., and Khan, S. M. (2014). The Alzheimer's disease mitochondrial cascade hypothesis: progress and perspectives. *Biochim. Biophys. Acta* 1842, 1219–1231. doi: 10.1016/j.bbadis.2013.09.010

Swerdlow, R. H., and Khan, S. M. (2004). A "mitochondrial cascade hypothesis" for sporadic Alzheimer's disease. *Med. Hypotheses* 63, 8–20. doi: 10.1016/j.mehy.2003.12.045

Traul, K. A., Driedger, A., Ingle, D. L., and Nakhasi, D. (2000). Review of the toxicologic properties of medium-chain triglycerides. *Food Chem. Toxicol.* 38, 79–98. doi: 10.1016/S0278-6915(99)00106-4

Tremblay, S., Ouellet, R., Rodrigue, S., Langlois, R., Benard, F., and Cunnane, S. C. (2007). Automated synthesis of 11C-acetoacetic acid, a key alternate brain fuel to glucose. *Appl. Radiat. Isot.* 65, 934–940. doi: 10.1016/j.apradiso.2007.03.015

Tremblay-Mercier, J., Tessier, D., Plourde, M., Fortier, M., Lorrain, D., and Cunnane, S. C. (2010). Bezafibrate mildly stimulates ketogenesis and fatty acid metabolism in hypertriglyceridemic subjects. *J. Pharmacol. Exp. Ther.* 334, 341–346. doi: 10.1124/jpet.110.166504

Veech, R. L., Chance, B., Kashiwaya, Y., Lardy, H. A., and Cahill, G. F. Jr. (2001). Ketone bodies, potential therapeutic uses. *IUBMB Life* 51, 241–247. doi: 10.1080/152165401753311780

Velliquette, R. A., O'Connor, T., and Vassar, R. (2005). Energy inhibition elevates beta-secretase levels and activity and is potentially amyloidogenic in APP transgenic mice: possible early events in Alzheimer's disease pathogenesis. *J. Neurosci.* 25, 10874–10883. doi: 10.1523/JNEUROSCI.2350-05.2005

Veneman, T., Mitrakou, A., Mokan, M., Cryer, P., and Gerich, J. (1994). Effect of hyperketonemia and hyperlacticacidemia on symptoms, cognitive dysfunction, and counterregulatory hormone responses during hypoglycemia in normal humans. *Diabetes Metab. Res. Rev.* 43, 1311–1317.

Viggiano, A., Pilla, R., Arnold, P., Monda, M., D'Agostino, D., and Coppola, G. (2015). Anticonvulsant properties of an oral ketone ester in a pentylenetetrazole-model of seizure. *Brain Res.* 1618, 50–54. doi: 10.1016/j.brainres.2015.05.023

Vossel, K. A., Beagle, A. J., Rabinovici, G. D., Shu, H., Lee, S. E., Naasan, G., et al. (2013). Seizures and epileptiform activity in the early stages of Alzheimer disease. *JAMA Neurol.* 70, 1158–1166. doi: 10.1001/jamaneurol.2013.136

Wilkins, H. M., Koppel, S., Carl, S. M., Ramanujan, S., Weidling, I., Michaelis, M. L., et al. (2016). Oxaloacetate enhances neuronal cell bioenergetic fluxes and infrastructure. *J. Neurochem.* 137, 76–87. doi: 10.1111/jnc.13545

Yao, J., Irwin, R. W., Zhao, L., Nilsen, J., Hamilton, R. T., and Brinton, R. D. (2009). Mitochondrial bioenergetic deficit precedes Alzheimer's pathology in female mouse model of Alzheimer's disease. *Proc. Natl. Acad. Sci. U.S.A.* 106, 14670–14675. doi: 10.1073/pnas.0903563106

Yin, J. X., Maalouf, M., Han, P., Zhao, M., Gao, M., Dharshaun, T., et al. (2016). Ketones block amyloid entry and improve cognition in an Alzheimer's model. *Neurobiol. Aging* 39, 25–37. doi: 10.1016/j.neurobiolaging.2015.11.018

Zilberter, M., Ivanov, A., Ziyatdinova, S., Mukhtarov, M., Malkov, A., Alpar, A., et al. (2013). Dietary energy substrates reverse early neuronal hyperactivity in a mouse model of Alzheimer's disease. *J. Neurochem.* 125, 157–171. doi: 10.1111/jnc.12127

Zuendorf, G., Kerrouche, N., Herholz, K., and Baron, J. C. (2003). Efficient principal component analysis for multivariate 3D voxel-based mapping of brain functional imaging data sets as applied to FDG-PET and normal aging. *Hum. Brain Mapp.* 18, 13–21. doi: 10.1002/hbm.10069

Conflict of Interest Statement: Some MCT used in our more recent studies was provided by Abitec Corporation. SC has participated in *ad hoc* consulting for Keto Products and Bulletproof. All the other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer DR and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read, for greatest visibility

COLLABORATIVE PEER-REVIEW
Designed to be rigorous – yet also collaborative, fair and constructive

FAST PUBLICATION
Average 85 days from submission to publication (across all journals)

COPYRIGHT TO AUTHORS
No limit to article distribution and re-use

TRANSPARENT
Editors and reviewers acknowledged by name on published articles

SUPPORT
By our Swiss-based editorial team

IMPACT METRICS
Advanced metrics track your article's impact

GLOBAL SPREAD
5'100'000+ monthly article views and downloads

LOOP RESEARCH NETWORK
Our network increases readership for your article

Frontiers
EPFL Innovation Park, Building I • 1015 Lausanne • Switzerland
Tel +41 21 510 17 00 • Fax +41 21 510 17 01 • info@frontiersin.org
www.frontiersin.org

Find us on