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CRISPR-Cas9 mediated HMGCL KO in 3xTg AD mice reduces the cognitive deficit improvement seen in an intermittent metabolic switching regimen

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CRISPR-Cas9 mediated HMGCL KO in 3xTg AD mice reduces the cognitive deficit improvement seen in an intermittent metabolic switching regimen

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Abstract

Individuals in modern Western societies are experiencing increasing sedentary lifestyles, overindulgence of high fat, high-sugar diets, and extremely sterilized conditions, putting immense pressure on researchers and clinicians alike to come up with viable treatments for conditions implicated with an aging society. Emerging research have published the benefits of IMS and metabolic switching in a variety of neuroprotective, cellular stress resistance, and neuroplasticity pathways in animal models and clinical results from randomized trials of IMS regimens with susceptible human populations are soon to be published. The application of genome editing and next-generation sequencing (NGS) strategies to clinical and neurodegenerative research continues to elucidate the relationship between a patient's specific genetic background and modern environmental stressors towards disease pathology. This study attempts to utilize novel CRISPR/Cas9 strategies to introduce targeted gene edits and explores the role of reduced ketone-body synthesis/metabolism with 3-hydroxymethyl-3-methylglutaryl-CoA lyase HMGCL KO, in the therapeutic and neuroprotective potential of intermittent metabolic switching in 3xTg mice, genetically predisposed for Alzheimer pathology. IMS-mediated attenuation of hippocampal spatial memory deficits was confirmed in 5-month-old 3xTg mice using Morris Water Maze and Aβ1-40, Aβ1-42, total tau and p-tau levels were calculated accordingly. Mice receiving time-restricted feeding (TRF) and caloric restriction (CR) regardless of KO performed better in the hippocampal-dependent spatial memory test and ELISA analysis of CSF revealed reduced p-tau levels of 3xTg WT TRF + CR mice relative to WT control or the two experimental groups. Overall, genetic modifications of key metabolic enzymes highlight the variable therapeutic results of the glucose to ketone metabolic switch on cognitive deficits depending on an organism's genetic background.

Emergence of Alzheimer Disease

The NIH has stated that the percentage of the world population over 65 years old is expected to double to 1.6 billion people by the year 2050, putting immense pressure on researchers and society alike to provide reproducible solutions to the issues faced by an aging population and their families (Hebert et al., 2013). Accordingly, the rising prevalence of Alzheimer disease (AD) and other dementia-like disorders has placed genetic data analysis and transgenic animal studies at the forefront of pharmacogenetics and clinical research. Progress in understanding the complex etiology of early and late on-set forms of AD have largely stemmed from the application of molecular and cellular manipulations against genetic backgrounds implicated in disease progression and have shed light on the complex interaction of genetic and environmental factors in the pathogenesis of AD. This proposal attempts to address three main hypotheses: Can CRISPR/Cas9 transfected embryonic stem cells and surrogate transplantation lead to viable chimeric HMGCL KO strain in 3xTg AD mice? How does IMS affect cognitive abilities in 6-month-old 3xTg/HMGCL-/- compared to 3xTg/WT littermates? What effect does IMS and gene KO have on levels of Aβ protein and p-tau concentrations? Is cell death observed in hippocampal slices?

Alzheimer Disease Pathology

Traditionally, Alzheimer disease pathology has been well characterized by the amyloid cascade hypothesis (Hardy & Higgins, 1992). This study concludes that the extracellular aggregation of amyloid beta (Aβ) protein from aberrant cleavage of the amyloid precursor protein (APP) leads to neurotoxic amyloid plaques, ultimately contributing to the formation of intracellular neurofibrillary tangles (NFTs), hyper-phosphorylated microtubule associated protein tau pathology, and subsequent cell death (Hardy & Higgins, 1992, Grundke-Iqbal et al., 1986). Since the early identification of mutations associated with APP, researchers have refined the diagnoses of familial early onset forms of AD to also include mutations in presenilin-1 or presenilin-2, which are the catalytic subunits of gamma-secretase, implicated in the pathway that generates the insoluble Aβ1-42 protein aggregates (Lanoiselée et al., 2017, Murphy & LeVine, 2010). Recently, Haass & Selkoe have demonstrated that small soluble oligomer intermediates formed in the aggregation process lead to synaptic dysfunction and that these insoluble Aβ1-42 plaques can act as "reservoirs of bioactive oligomers" in the etiology of AD (2007). Post-mortem analysis of AD patient brain tissue has confirmed both morphological hallmarks of the disease, the extracellular AB protein aggregates, and intracellular hyper-phosphorylated tau protein leading to cytotoxic insult and ultimately cell death, cognitive decline, and disease progression (Selkoe, 1991, LaFerna & Green, 2012, Thal & Brak, 2005). Mutations in this tau protein, responsible for microtubule assembly stability and microtubule binding, result in the protein's hyper-phosphorylation and propensity to form paired helical filaments causing subsequent axonal damage and neurofibrillary tangle formation (Matsue et al., 1994, Grundke-Iqbal et al., 1986). The mutations in the aforementioned genetic loci by themselves are not sufficient to cause cognitive decline associated with AD as studies have shown patients with elevated levels experience normal

physiologies. Therefore, disease progression is dependent on several other known risk factors including the apolipoprotein E allele (APOE-e4) in late-onset AD, inflammatory signatures, diet and exercise, and other environmental factors that affect neuron durability and synaptic coherence (Stritmatter et al., 1993, Wyss-Coray, 2006, Scarmeas et al., 2009, Butterfield et al., 2002, LaFerla & Green, 2012). Attention is given specifically to the complex gene-environment interaction of patients with familial forms of AD by inducing genetic knockouts to pre-existing model organisms through novel CRISPR/Cas9 systems and assessing relevant behavioral and morphological changes associated with the given modification. For this study, the triple transgenic mice (3xTg) contain three mutations associated with familial Alzheimer disease (APP Swedish, MAPT P301L, and PSEN1 M146V) and display progressive AB deposition as early as three to four months of age, extracellular deposition in the frontal cortex noticeable by six months becoming more extensive, and aggregates of altered hyper-phosphorylated tau are detected in the hippocampus (Oddo et al., 2003, Billings et al., 2005). Corresponding behavioral deficits in hippocampal spatial memory deficits and long-term potentiation have been determined in 3xTg mice and have continued to elucidate the age-dependent molecular and physiological manifestations of AD and other disease states (Billings et al., 2005, Clark et al., 2015, Sterniczuk et al., 2010, Orta-Salazar et al., 2013).

Gene Editing by CRISPR/Cas9 Systems

Though the exact nature of the complex interaction of amyloid and tau pathologies in Alzheimer disease progression is still unresolved, researchers have benefitted greatly from the use of transgenic mice model organisms to imitate classical disease conditions and inducible genetic manipulations at sites relevant to human AD pathology through novel CRISPR-Cas9 systems. Recent literature has cited the development of sequence specific DNA nuclease technologies from microbial CRISPR (clustered regularly interspaced short palindromic repeat) systems and programmable Cas9 nucleases that have enhanced the efficiency and accuracy of targeted modifications to genomic sequences of living cells and model organisms (Ran et al., 2013, Jinek et al., 2012, Carlson et al., 2012). Studies have shown that targeted mutagenesis using Cas9 nucleases induce double stranded breaks in an organism's DNA, complementary to the set of guide RNAs encoding for a given gene of interest, and exploit endogenous cellular repair mechanisms such as nonhomologous end-joining or homology-directed repair pathways to produce site-specific knockout at specific genetic loci with minimal toxicity (Katigbak et al., 2018, Cong et al., 2013, Ran et al., 2013, Thurtle-Schmidt & Lo, 2017). Though various strategies exist to increase the specificity of genome editing strategies such as zinc-finger nucleases (ZFN) and transcription activator-like effector nucleases (TALENs) in embryonic cell lines, CRISPR-Cas9 emerges as a reliable mechanism of eukaryotic homologous recombination and genome-scale KO of mammalian cell lines (Shalem et al., 2014, San Filippo et al., 2008, Bozas et al., 2009, Chu et al., 2015). As evident in any field, the cooperation of multiple organizations and individuals with different skill sets is necessary for robust outcomes. Recently, the development of techniques and appropriate culture conditions to introduce genetic manipulations to study human genetic diseases in animal models have expanded the arsenal of researchers and neuroscientists to exploit (Sukoyan

et al., 2002). The formation of in vivo germ-line competent chimeras containing the genetic knockout of 3-hydroxy-3-methylglutaryl-CoA lyase (HMGCL) against a triple transgenic background predisposed to AD pathology and implantation into surrogate mothers was able to produce chimeric offspring (Yamamoto et al., 2013) and relevant behavioral test were conducted.

Intermittent Metabolic Switching

Under normal physiological conditions, an organism will absorb the nutrients from a given food source and will store excess energy in the form of glycogen primarily in the liver and skeletal muscles to maintain healthy blood-glucose levels and supply the tissue with energy in the absence of oxygen (Berg et al., 2002). As an organism begins to burn off the stored energy with activity and liver glucose reserves are used up, the brain and body will recognize low circulating glucose levels and switch the primary energy source of the cells to ketone bodies produced from fatty acids released by adipose tissue (Mattson et al., 2018, Courchesne-Loyer et al., 2017, Camandola & Mattson, 2017). The "G-K" switch from glucose to ketone body use as the major energy source of the cells and subsequent switch back to glucose after food consumption "K-G" has been shown to greatly improve the phenotypic presentations of various inflammatory pathways, resistance to neuronal injury and oxidative stress, shown to improve lifespans and age-dependent cognitive deficits seen in both human and rodent models (Anson et al., 2003, Hatori et al., 2012, Ingram et al., 1987, Means et al., 1993). As such, the role of metabolic disturbances in disease progression and lifestyle intervention strategies have seen increasing salience. A recent review article by Mattson et al. suggests that switching between time-periods of negative energy balance (short fasts/exercise) and positive energy balance (eating and resting) can optimize brain health, improve mood, and create the conditions for cellular stress resistance and cell growth/neuroplasticity pathways in ameliorating neurodegenerative diseases (Longo & Mattson, 2014). Intermittent metabolic switching can be defined as scenarios in which an individual's eating and exercise patterns result in periodic changes in glucose or ketone use as the primary energy source and has been implicated in providing a range of therapeutic effects including increasing spine density and LTP capabilities, reduce seizure induced hippocampal neurodegeneration, and even enhance basal

neurogenesis and neuronal survival (Eckles-Smith et al., 2000, Bruce-Keller et al., 1999, Lee et al., 2002). The physiological effects of time-restricted feeding (TRF) and alternate-day fasting (ADF) methods of experimentally inducing IMS in rodent models have been well characterized in reducing aging-related behavioral deficits seen in 3xTg mice models of AD (Halagappa et al., 2007, Higami et al., 2006, Huber et al., 2017). Similarly, fasting and exercise have been shown to upregulate both BDNF and CREB pathways implicated in cellular repair pathways, long-term memory, insulin-like growth factor (IGF1) signaling and play key roles in mitochondrial biogenesis and LTP (Hirano et al., 2013, Estrada et al., 2009, Llorens-Martin et al., 2009, Hepple 2009, Marosi et al., 2014). Though the benefits of IMS and caloric restriction/exercise on overall brain health and disease pathology have been briefly mentioned role of 3-hydroxy-3-methylglutaryl-CoA lyase knockout in AD progression has not been previously examined. Essentially, training regimens of IMS and periods of fasting/exercise followed by periods of eating/resting can improve the metabolic efficiency and stress tolerance of mitochondria through SIRT3 pathways, regulate protein synthesis through mechanistic target of rapamycin (mTOR) and autophagy pathways, and stimulate the release of peripheral signals that mediate neuroplasticity and behavior (Mattson et al., 2018, Cheng et al., 2016, Palacios et al., 2009, Tang et al., 2002, Alirezaei et al., 2010, Johnson et al., 2013, Kim et al., 2015).

3-Hydroxy-3-Methylglutaryl-CoA Lyase (HMGCL)

The HMGCL gene encodes for a protein that belongs to the HMG-CoA lyase family necessary for ketogenesis and the use of ketone bodies as an alternative energy source by an organisms' cells during periods of low blood glucose or fasting. This protein is a mitochondrial enzyme that catalyzes the final step of leucine degradation in ketone body metabolism and is found on chromosome 1 of the human genome and chromosome four of the mice genome (The Knockout Mouse Project). Genetic knockouts at this target loci may produce challenges to cell viability because of the critical nature of ketone body synthesis and energy expenditure demands of mitochondrion during periods of fasting, and cell death signatures may be experienced prematurely by already susceptible hippocampal neurons of the 3xTg mice. Of note, the few HMGCL mutations that have been investigated in human patients have led to severe metabolic acidosis, acute metabolic degeneration, and the relevant isoforms of HMGCL transcripts and nature of mutations lead to potentially lethal outcomes (Grunert et al., 2017, Fu et al., 2010, Mattson et al., 2018). As such CRISPR-Cas9 mediate genetic knockout of HMGCL in mice models may result in embryonic lethality and gene expression alterations using RNA guided CRISPR-Cas9 systems may help potentiate the complex role of ketone body synthesis and use during periods of fasting (Wang et al., 1998). Regardless of the efficacy of the specific genetic knockout, the use of genome editing strategies to produce high fidelity mutations at specific loci is significant in order to elucidate the interaction of specific genotypes with disease and behavioral phenotypes.

CRISPR/Cas9-mediated 3xTg/HMGCL-/- Chimera Mouse Line from ES Cell

All CRISPR transfection protocol of embryonic cell lines and chimeric mouse generation produced by Synthego (powered.synthego.com, 2018). Single stem cells are extracted from 3xTg mice background and electroporation/nucleofection of cell lines with Cas9 and specifically designed sgRNAs to the HMGCL gene on chromosome four (Catrionia 2013, Fig 1). Multiple sgRNA transcripts that bind to multiple regions of the HMGCL gene adjacent to a NGG PAM sequence included in viral transfection to induce DSB at more specific target sites: 5' CCTGACTCCAAACATGAAAA GG 3', 5' TCCTGACTCCAAACATGAAA GG 3', 5' GACCACTCTGACGTCTTGAA GG 3'. Cell are selectively cultured for those expressed mutant germ line DNA and ES cell aggregates are analyzed with qPCR using sequence specific oligonucleotides to HMGCL labeled with fluorescent EGFP reporter only after hybridization with its complementary strand (TagMan probes). The real-time PCR analysis of whole brain tissue of 3xTg/WT ad libitum and 3xTg/HMGCL-/- ad libitum will be conducted and critical threshold scores are determined. Colocalization of NeuN and HMGCL-EGFP antibody staining was noted in 3xTg/HMGCL-/- ad libitum relative to NonTg/WT ad libitum. Mouse monoclonal antibodies received (Chemicon, Catalog number: MAB377) and IHC-Tek antibody diluent (Cat#IW-1000) used to reduce unspecific staining. Antigen retrieval used IHC-Tek™ Epitope Retrieval Steamer Set (Cat# IW-1102) at 95/100 ºC/room temperature for 20 minutes each. LSAB method for detection was utilized and nuclei staining was observed.

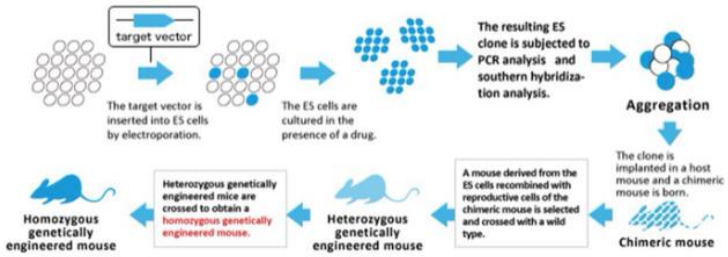

Figure 1. Generalized workflow of gene KO at one-cell embryonic stage to produce non-mosaic gene knockout in all cells followed by surrogate implantation.

Animals

3xTg mice were ordered from The Jackson Lab; available through the JAX MMRRC Stock# 034830. All mice are housed 5 per cage and kept on 12h light/dark cycles with accessible water and minimal animal handling. All euthanization techniques and mice testing/handling meet standards approved by the Institutional Animal Care and Use Committee (IACUC). Intermittent metabolic switching will be experimentally induced in specific cohorts of 3xTg mice using 24-hour periods of restricted feeding with a 40% reduction in caloric intake to determine if the previously established therapeutic effect of IMS is attenuated in mice with differing genetic backgrounds HMGCL KO. Time restricted feeding protocols followed previously established methods and food was deprived every other 24 hours (Halagappa et al., 2007, Anson et al., 2003). At three months of age, 5 groups of five mice each were assigned to the following dietary regimen: non-Tg/WT ad libitum, 3xTg/WT ad libitum, 3xTg/WT TRF + CR, 3xTg/HMGCL-/- ad libitum, and 3xTg/HMGCL-/- TRF + CR. Mice on the CR diet were provided an amount that is 60% of the amount consumed by the 3xTg/WT ad libitum group. At about 6 months of age, the mice were subjected to a hippocampal-dependent spatial memory test to better characterize functional differences in gene knockout and therapeutic efficacy of IMS. CSF fluids were drawn at 6 months of age and animals were euthanized for whole brain and hippocampal dissection and frozen for Aβ1-42, p-tau level analysis and Fluoro-Jade B immunostaining.

Morris Water Maze

Previous studies have shown that both intermittent fasting regimens and caloric restriction contribute to improved cognitive ability and ultimately reduce risk of Aβ deposition and tau pathology seen in mouse models of Alzheimer pathology (Halagappa et al., 2007, Mattson et al., 2015, Patel et al., 2005). The behavioral testing followed previously established protocol. A circular tank (diameter 1.0 m, height 0.40 m, painted white) was filled with water (22 ± 1°C) and water made opaque using non-fat milk. Water will be changed after all mice of a given cohort are tested to prevent detritus buildup. Spatial cues are not provided along the ridge of the tub and mice given one test run to determine escape latency (s). If mice are unable to find the platform hidden 1 cm below the surface of the water before the maximum 2-minute mark, mice were manually guide to the platform and allowed to acclimate for 1 minute. On day 1 of acquisition trials, mice from a given cohort are tested one after another for four cycles as to allow mice to relax between trials and prevent over-handling. Escape latency or the time it takes each mouse to reach the submerged platform was recorded using a stopwatch. A 24-hour delay was administered after day 1 of behavioral testing and subsequent preservation or loss of spatial memory is examined.

ELISA Immunosorbent Assay of Aβ1-42 and p-Tau Levels

Enzyme linked immunosorbent assay (ELISA) kits acquired from ThermoFisher.com for mouse Aβ1-42 (Catalog number: KMB3441) and phosphorylated tau (p-Tau199, Catalog Number: KMB7041) level measurements from cerebrospinal fluid (CSF) of 6-month old mice. In an ELISA, an antigen is immobilized to a solid plate and is complexed with an antibody for a given enzyme. Elevated insoluble Aβ1-42 levels and phosphorylated tau are well characterized biomarkers of AD pathology and subsequent shifts in relative concentrations to the NonTg/WT control were measured (Huber et al., 2017, thermofisher.com). Efficient collection of CSF from cisterna magna of 6-month old mice followed protocol previously outlined using special stereotaxic guides to hold the syringe in place to prevent CSF contamination by blood and other fluids (Zarghami et al., 2013, Rosen et al., 2013). This study could have benefited greatly from repeated measures of minimally-invasive CSF extractions throughout IMS training initiation at 3-months to see how aging and cytotoxic biomarkers contribute to cognitive decline.

Hippocampal Dissection

Hippocampal cell tissue extraction follows standard protocol from previously published studies (Mathis et al., 2011). The ACSF components 124 NaCl, 2KCl, 1.25 KH2PO4, 2MgSo4, 26 NaHCO3, 10 dextrose (in mM)) were combined with distilled H20 were oxygenated vigorously in a 2L Erlenmeyer flask. Oxygenated Ca2+-free ACSF poured into separate Erlenmeyer flask and transferred to ultrafreezer (-80 °C) for 30 minutes to be used during dissection. To produce the media used for brain slice storage after dissection, 2 mM of CaCl2 was added to remaining 1.25 L volume of ACSF and oxygenation resumed with 95%O2//5%CO2. Extracted brain tissue submerged in Ca2+-free ACSF covered with parafilm and prepared for brain slicing from Vibratome sectioning (Leica VT1000 S). Cerebellum and rostral tips of frontal lobe carefully dissected out and brain tissue transferred to the sectioning stage of Vibratome filled with ice-cold Ca2+-free ACSF. Slices collected with wide-mouth paint brush and transferred to small petri dish containing ice-cold Ca2+-free ACSF. Brain slices transferred to holding chamber to bathe in oxygenated Ca2+-containing ACSF and chamber temperature increase +1°C every five minutes to 32°C.

Cellular Degeneration Identification using Fluoro-Jade B

Fluoro-Jade B has shown to exhibit greater target specificity of neurons undergoing degeneration with minimal background staining than earlier Fluoro-Jade staining methods (Schmued & Hopkins, 2000). Fluoro-Jade B positive neuron measurements in this study follow standard procedures from previously cited literature (Laflamme et al., 2016). Tissue sections mounted on Superfrost plus slide (Thermo Fisher Scientific, catalog number: 12-550-17) and fixed for 20 min in paraformaldehyde 4%, and 2 minutes in KPBS (Electron Microscopy Science, catalog number: 19210) Dehydrated with 50%-70%-100% Ethanol for 2 minutes each and rehydrated with 70%-50% Ethanol and KPBS. Incubated in potassium permanganate 0.06% for 5 min at room temperature, rinsed in water, and incubated in Fluoro-Jade B solution on orbital shaker (Merck Millipore Corporation, catalog number: AG310). Slides kept covered from light and dried under vacuum at room temperature after three rinses with water. Slide cleared with Xylene three times at 2 minutes each and slip covered with DPX before drying under hood (Sigma-Aldrich, catalog number:534056, Electron Microscopy Science, catalog number: 13512). Immunofluorescent of Fluoro-Jade positive hippocampal cells counted using EVOS FL Auto Imaging System (SKU# AMAFD1000).

Confirmation of Gene Knockout

Relative fluorescence of qPCR antibodies for anti-HMGCL loci in 3xTg/HMGCL-/- and NonTg/WT hippocampal cell homogenates reveal reduced critical threshold value from NonTg/WT samples. Significant quantities of HMGCL loci binding in NonTg/WT is apparent nearly twice as quickly than in 3xTg/HMGCL-/- mice samples (Fig 2). Colocalization of NeuN and HMGCL/EGFP staining reveal HMGCL preservation in physiologically relevant neurons of the hippocampus but the lack of EGFP-staining suggests genetic knockout compared to the wild-type (Fig 3).

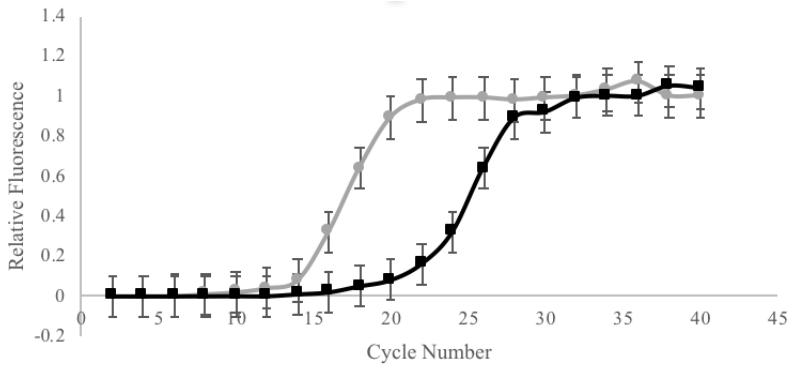

Figure 2. Diminished critical threshold score from quantitative PCR of 3xTg/WT mice compared to 3xTg/HMGCL-/- mice (n=1).

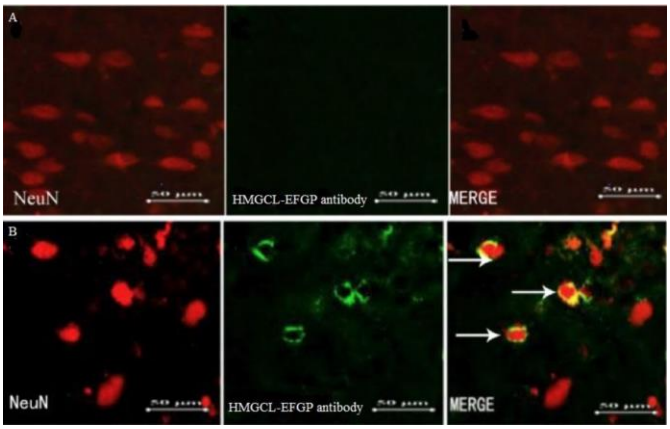

Figure 3. Characterization of NeuN and HMGCL-EGFP in a) 3xTg/HMGCL-/- and b) NonTg/WT

Increased Hippocampal-Dependent Spatial Memory Deficits seen in 3xTg HMGCL-/- mice

Mean escape latency measurements trends suggest similar cognitive phenotypes of NonTg/WT and 3xTg/WT mice on the TRF + CR regimen (Fig. 4). The phenotypic presentation of 3xTg/WT ad libitum mice in spatial memory behavioral tests have been well characterized in recent Alzheimer disease mice models, demonstrate delayed learning speed and altered retention of spatial memory, and serves as the control to compare behavioral data (Huber et al., 2017, Sterniczuk et al., 2010, Gimenez-Llort et al., 2013, Clinton et al, 2007). Latency reduction of 3xTg and memory preservation is evident between 3xTg/WT ad libitum and 3xTg/WT TRF+CR cohorts as evidenced by consistent latency times following the 24-hour break in the IMS induced mice. Interestingly, the escape latencies of both HMGCL KO mice regardless of training regimen experience marked differences in mean latency scores and the IMS training regimen was not sufficient to improve cognitive deficit attenuation seen in 3xTg/WT TRF+CR compared to 3xTg/WT ad libitum trials. The 3xTg/HMGCL-/- mice exhibit significantly longer escape latency times with the mice swimming close to the maximum allotted swim time with each trial, and significant latency reductions were not observed between trials such as in the 3xTg/WT TRF+CR mice (Fig 4.) All groups see a similar upward trend toward the end of their acquisition trials likely due to over-handling.

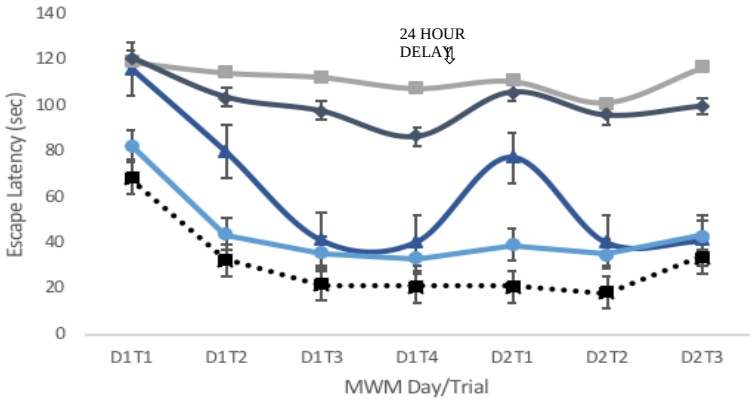

Figure 4. Summary of mean escape latency of hippocampal-dependent MWM cognitive tests (n=5). NonTg/WT = dashed square; 3xTg/WT TRF + CR = blue circle; 3xTg/WT ad libitum = blue triangle; 3xTg/HMGCL-/- TRF + CR = grey diamond; 3xTg/HMGCL-/- ad libitum = grey square

Elevated Aβ1-42 and p-Tau199 from CSF

ELISA Immunosorbent Assay analysis of insoluble Aβ1-42 from the five cohorts reveal significantly elevated concentrations in 3xTg/WT ad libitum compared to NonTg/WT and 3xTg/WT TRF + CR (Fig 5). Interestingly, though 3xTg/WT TRF+CR show slightly elevated concentrations of Aβ1-42 protein, statistical significance is not determined. Mice in the 3xTg/HMGCL-/- TRF + CR cohort show ~35% increase in Aβ concentration compared to 3xTg/WT mice experiencing the same IMS regimen whereas 3xTg/HMGCL -/- ad libitum experience a 250% increase in Aβ accumulation. Analysis of phosphorylation of tau protein at Ser199 of the wild-type and chimeric mice reveal similar concentrations between NonTg/WT ad libitum and 3xTg/WT TRF+CR. A nearly 50% increase in p-tau concentration is noticed in 3xTg/WT ad libitum mice relative to controls for transgene and IMS regimen. Both 3xTg/HMGCL-/- exhibit nearly 200% increases in phosphorylated tau concentrations and correlate with the worst phenotypic presentations in Morris Water Maze.

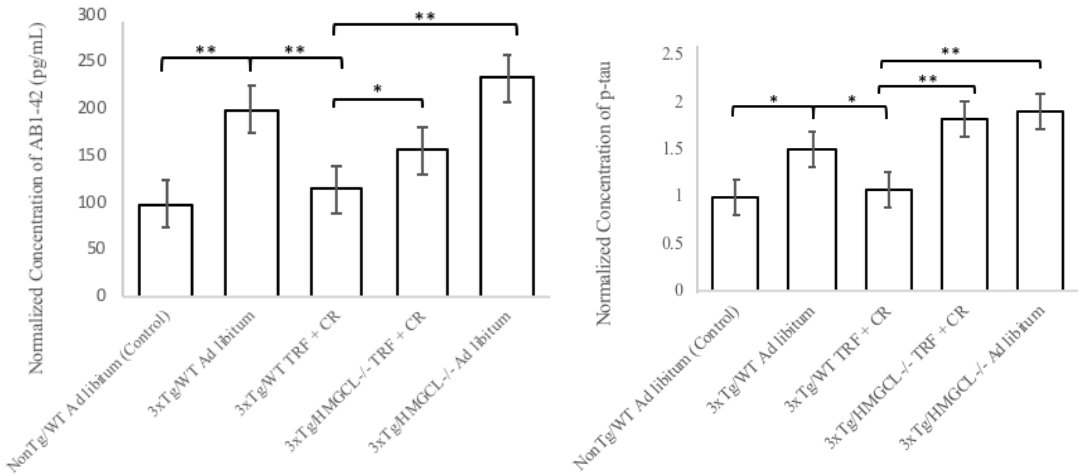

Figure 5. Relative Aβ1-42 and p-tau levels normalized to NonTg/WT mice and significant associations between HMGCL KO and experimental conditions are identified (n=5).

Fluoro-Jade B Positive Staining of Hippocampal Slices

Hippocampal neurons exhibiting positive Fluoro-Jade B staining were elevated to noticeable levels in 3xTg KO mice and IMS training regimen was insufficient to ameliorate cell degeneration processes (Fig 6.) The 3xTg/WT ad libitum mice experience one order of magnitude larger in cell death markers relative to 3xTg/WT TRF+CR and NonTg/WT cohorts. However, insignificant differences were determined between 3xTg/WT ad libitum and 3xTg/HMGCL-/- TRF+CR mice. The elevated counts of Fluoro-Jade B positive hippocampal neurons in 3xTg/HMGCL-/- ad libitum mice were expected but a significant difference existed with the 3xTg/HMGCL-/- TRF+CR.

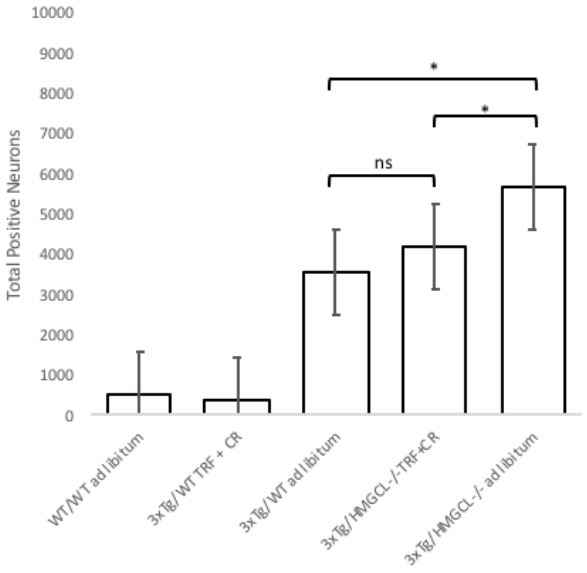

Figure 6. Fluoro-Jade B fluorescent neurons in the hippocampus seen noticeably in HMGCL KO mice and IMS regimen could not recover memory deficits or attenuate Aβ and p-tau levels (n=5).

Discussion

An increasingly sedentary and overindulgent lifestyle of modern Western societies have shown to contribute to complex pathologies of various metabolic and neurodegenerative conditions. The therapeutic potential of IMS and everyday lifestyle management shifts have been attributed to a variety of neuroprotective, synaptic plasticity, and attenuating cognitive decline in both human and AD animal models. Current research continues to elucidate the role of diet and exercise in decelerating disease progression and providing neuroprotective pathways against pathogenesis. This paper has addressed the three specific hypotheses originally posited by successful genetic and visual confirmation of targeted knockout of chimeric mouse strains predisposed for AD pathology, demonstration of the hippocampal-dependent spatial memory deficit mitigation of intermittent metabolic switching regimens to 3xTg mice, and analysis of the classical hallmarks of Aβ1-42, p-tau and cell degeneration biomarkers. As expected, NonTg/WT and 3xTg/WT TRF+CR mice exhibited similar cognitive abilities in MWM and relative concentrations of the enzymes and proteins associated with AD pathology leading to cellular death (Fig 4). IMS has been shown to enhance cell-stress resistance and neuroplasticity, the relatively low levels of Fluoro-Jade B staining of 3xTg/WT TRF+CR a make sense as more neurons are resilient to the degrading nature of Aβ oligomers and phosphorylated tau. Initially, it was expected that mice receiving TRF+CR training regimen would experience overall improvements in behavioral abilities and phenotypic presentations but the 3xTg/HMGCL-/- TRF+CR performed relatively poorly in all aspects. Interestingly, in both HMGCL KO cohorts, marked elevations in Aβ1-42 and p-tau proteins were recognized. The necessary role of HMGCL protein in proper mitochondrial function during the fasting period and subsequent gene editing of this loci may contribute to the increase in cell death markers seen in HMGCL KO cohorts relative

to 3xTg/WT mice. Mice following the IMS diet in the 3xTg/HMGCL-/- group showed decreased relative Aβ1-42, pTau, and Fluoro-Jade B positive staining relative to ad libitum groups. The ability of TRF+CR to prepare neuronal populations for stressful environments and subsequent neuronal degeneration from aberrant amyloid and tau pathologies is evidenced by the decreased Fluoro-Jade staining seen in 3xTg/HMGCL-/- TRF + CR compared to 3xTg/HMGCL-/- ad libitum. The creation of chimeric mouse strains encoding for HMGCL enzyme knockout against a genetic background predisposed for AD progression highlights the complex interaction of the gene and environment on phenotypic presentations of a disease and a patient's response to a given therapeutic. The application of high fidelity genome editing and growing understanding of embryonic cell lines and genomic analysis continue to improve the arsenal of researchers to reliably translate genomic data into personalized treatments. Manipulating CRISPR/Cas9 systems for targeted gene expression modifications instead of permanent genetic knockout is emerging at the forefront of gene therapies and mouse models of disease pathologies. With a growing understanding of functional genomics and endogenous gene expression modifications, researchers may refine the CRISPR/Cas9 systems to non-invasively, non-permanently alter protein expression and functional/phenotypic presentations can be measured. Differing training regimens such as longer food restriction periods or exercise-coupled IMS may contribute to different degrees of cognitive attenuation and the exact nature of IMS regimens could be further developed. Ultimately, an individual's genetic makeup may determine the propensity for aberrant signaling and cellular dysfunction but environmental cues and lifestyle characteristics play a huge role in the pathologies of neurodegenerative diseases. Currently, randomized, double blind clinical trials prescribing individualized training regimens for older patients at risk for developing dementia-like disorders is underway and clinical applications of daily lifestyle management may be seen as early as 2018.

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