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Brain and behavioral perturbations in rats following Western diet access

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Abstract

Energy dense “Western” diets (WD) are known to cause obesity as well as learning and memory impairments, blood-brain barrier damage, and psychological disturbances. Impaired glucose (GLUT1) and monocarboxylate (MCT1) transport may play a role in diet-induced dementia development. In contrast, ketogenic diets (KD) have been shown to be neuroprotective. We assessed the effect of 10, 40 and 90 days WD, KD and Chow maintenance on spontaneous alternation (SA) and vicarious trial and error (VTE) behaviors in male rats, then analyzed blood glucose, insulin, and ketone levels; and hippocampal GLUT1 and MCT1 mRNA. Compared to Chow and KD, rats fed WD had increased 90 day insulin levels. SA was decreased in WD rats at 10, but not 40 or 90 days. VTE was perturbed in WD-fed rats, particularly at 10 and 90 days, indicating hippocampal deficits. WD rats had lower hippocampal GLUT1 and MCT1 expression compared to Chow and KD, and KD rats had increased 90 day MCT1 expression compared to Chow and WD. These data suggest that WD reduces glucose and monocarboxylate transport at the hippocampus, which may result in learning and memory deficits. Further, KD consumption may be useful for MCT1 transporter recovery, which may benefit cognition

Keywords

Obesity; GLUT1; MCT1; Vicarious Trial and Error; Spontaneous Alternation; Hippocampus

Introduction

In the United States more than one in three adults and one in six children can be classified as obese (Flegal, Carroll, Ogden, & Curtin, 2010; Ogden, Carroll, Kit, & Flegal, 2014).

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Obesity during childhood typically persists into adulthood (Deshmukh-Taskar et al., 2005; Wang, Beydoun, Liang, Caballero, & Kumanyika, 2008), and is associated with increased disease risk as well as neurological and psychological deficits (Agranat-Meged et al., 2005; Cserjési, Molnár, Luminet, & Lénárd, 2007; Kamijo et al., 2014; Yau, Castro, Tagani, Tsui, & Convit, 2012).

Though obesity and the metabolic syndrome can develop via multiple routes, there is evidence that "Western" diets (WD) high in fat and sugar play a substantial role in their etiologies, as well as the often comorbid learning and memory deficits. In adult humans, WD not only induces weight gain, but also increases risk for dementia (de la Monte, 2009; Whitmer, Gunderson, Barrett-Connor, Quesenberry, & Yaffe, 2005). Hippocampal function appears to be particularly vulnerable to dietary insult. For example, adult rats fed WD diet show impairments on radial maze learning (Kanoski, Zhang, Zheng, & Davidson, 2010), Morris water maze performance (Molteni, Barnard, Ying, Roberts, & Gomez-Pinilla, 2002; Pistell et al., 2010; Stranahan et al., 2008; A. Wu, Ying, & Gomez-Pinilla, 2004), spontaneous alternation (Kaczmarczyk et al., 2013), deprivation-discrimination learning (Sample, this issue), reversal learning (Kanoski, Meisel, Mullins, & Davidson, 2007); and feature negative discrimination (Davidson et al., 2013; Davidson et al., 2012; Kanoski et al., 2010) but are not impaired on tasks such as simple discrimination and non-spatial maze learning, which do not depend on the hippocampus (Davidson et al., 2013; Kanoski et al., 2007; Kanoski et al., 2010). WD also reduces hippocampal BDNF (Molteni et al., 2002; A. Wu et al., 2004) and increases hippocampal blood-brain barrier (BBB) permeability (Davidson et al., 2013; Davidson et al., 2012; Kanoski et al., 2010).

These deficits do not appear to be specific to adult animals. In juvenile rats, two months WD exposure is associated with impairments in spatial reference memory on the Morris water maze, delayed spatial reference learning, and an enhancement of lipopolysaccharide (LPS)-induced inflammation in the hippocampus (Boitard et al., 2014; Boitard et al., 2012). Compared to their lean counterparts, obese human children also show impairments in academic performance and deficits in the inhibitory "NoGo" task (Kamijo et al., 2012), while adolescents with the metabolic syndrome have lower overall IQ, impaired attentional abilities, reduced mental flexibility, smaller hippocampal volumes, and damaged white matter tracts (Yau et al., 2012), suggesting that the hippocampus is vulnerable to dietary insults throughout development.

We have noted previously (Davidson et al., 2013; Kanoski et al., 2007) that diet composition plays a significant role in the development of these cognitive deficits. We have also noted that rats fed a very high fat ketogenic diet (KD) did not show hippocampal impairments when circulating ketone bodies were high, despite markedly increased body adiposity levels (Davidson et al., 2013).

KD is known to be an effective treatment for many types of epilepsy (Barañano & Hartman, 2008; Kwon, Jeong, Kim, Choi, & Son, 2008; Lutas & Yellen, 2012), and has been shown to exert numerous other neurological benefits. For instance, peripheral inflammation was significantly reduced in obese men after 12 weeks maintenance on KD (Forsythe et al., 2008), even prior to the onset of weight loss (Sharman et al., 2002; Sharman & Volek, 2004;

Volek et al., 2004). Adults with Alzheimer's disease, probable Alzheimer's disease, mild cognitive impairment, and age-associated memory impairments have demonstrated improvements after consuming AC-1202, a medical food known to increase circulating ketone levels (Costantini, Vogel, Barr, & Henderson, 2007; Henderson et al., 2009).

Ketones act as an alternate energy source for neurons, particularly when there are problems with glucose transport. In DeVivo syndrome, which is characterized by a mutation to the SLC2A-1 gene, GLUT1 transporter levels are reduced or abolished, thereby preventing glucose from crossing the BBB. Individuals with DeVivo syndrome have a cluster of neurological and behavioral symptoms, including seizures, developmental delays, and cognitive deficits that include spatial deficits, suggesting that the hippocampus may be particularly affected by this condition (Akman et al., 2010; De Vivo & Wang, 2008). The majority of these symptoms can be improved or averted via maintenance on KD (Friedman et al., 2006; Ramm-Pettersen, Stabell, Nakken, & Selmer, 2014). This suggests that many of the deficits in De Vivo syndrome are not due to reduced glucose transport *per se*, but a general energy deficit within the brain which can be overcome by switching to a ketone metabolism.

Under normal circumstances, GLUT1 expression increases in response to low, and decreases in response to high, circulating glucose levels, thereby maintaining glucose homeostasis within the brain interstitial fluid (Kumagai, Kang, Boado, & Pardridge, 1995; Pardridge, Triguero, & Farrell, 1990). When glucose levels are low, the primary source of energy for neuronal tissues becomes monocarboxylates, which include lactate, pyruvate, leucine, and ketone bodies such as beta-hydroxybutyrate (BHB), all of which are escorted across the BBB via the MCT1 transporter, which unlike GLUT1 is typically regulated via positive feedback mechanisms in proportion to monocarboxylates (Cortes-Campos et al., 2011; Nehlig, 2004; Vannucci & Simpson, 2003). Therefore, under conditions of starvation (where glucose levels are low, but ketone bodies are relatively high), expression of both GLUT1 and MCT1 are increased. Low levels of both transporters have been associated with cognitive deficits (De Vivo & Wang, 2008; Ding, Yao, Rettberg, Chen, & Brinton, 2013). Low GLUT1 levels have been measured in patients with AD (Bailey, Rivara, Rocher, & Hof, 2004; Mooradian, Chung, & Shah, 1997; Z. Wu et al., 2005), particularly in regions such as the hippocampus and neocortex (Kalaria & Harik, 1989); MCT1 levels are significantly lower in mouse models of AD (Ding, Yao, Rettberg, et al., 2013).

While it is unknown whether this is the result of degeneration at the neurovascular unit or an adaptive response to chronic hyperglycemia, it is clear that AD and related syndromes are associated with progressive reductions in glucose metabolism at the hippocampus and cortex (de la Monte, 2009; Mosconi, 2005; Mosconi et al., 2009), which suggests that the dementia-afflicted neurons may be deprived of energy for extended periods of time. Because GLUT1 and MCT1 transporters can be regulated quickly and play a role in cognitive function, we hypothesized that they may play a role in WD-induced cognitive deficits.

These experiments were conducted with three objectives in mind. First, we assessed the role of short- (10 day), moderate- (40 day) and long-term (90 day) exposure to WD and KD on body weight, markers of the metabolic syndrome, and circulating BHB levels. Our second

goal was to determine the relationship between diet and exposure duration on behavior. We accomplished this by analyzing spontaneous alternation (SA) behavior, a task which is noninvasive and, importantly, requires no food restriction. Because we wished to investigate changes in hippocampal functioning as a result of diet, we assessed vicarious trial and error (VTE) behaviors during the SA task.

VTE is measured by recording the total number of head turns an animal performs at choice points during a maze task. There is evidence (Schmidt, Papale, Redish, & Markus, 2013) that these behaviors are analogous to the "mental time travel" humans engage in during task acquisition (Suddendorf & Corballis, 2007), whereby an individual accesses declarative memories of past events in order to predict future outcomes. During planning and goal-directed learning, the hippocampus is strongly activated (Addis, Moscovitch, & McAndrews, 2007; Addis & Schacter, 2008; Buckner & Carroll, 2007), and animals with hippocampal lesions do not engage in VTE behaviors (Hu & Amsel, 1995; Voss et al., 2011). Therefore, this measure served as an index of hippocampal involvement.

Finally, because we hypothesized that high circulating glucose levels could decrease expression of GLUT1 transporter at the hippocampus, and because we were interested in the effects of WD and KD on the MCT1 transporter, we performed qPCR on hippocampal homogenates for GLUT1 and MCT1 mRNA expression.

Materials and Methods

Animals and Diets

Male Sprague-Dawley rats (n = 93; Harlan Laboratories, Indianapolis, IN) weighing between 275-300g were housed individually in hanging-wire cages in a temperature- and humidity-controlled room, and maintained on a 12h light/dark cycle. Rats were provided with *ad libitum* tap water. All procedures were approved by the Purdue University Animal Care and Use Committee.

Animals were handled for two weeks prior to experimentation, and regular body weight measurements began six days prior to experimental diet administration. During the acclimation period, rats were maintained on standard chow (2018, Harlan Teklad, Indianapolis, IN), after which they were weight-matched into diet treatment groups. Diets included Chow, a high-fat, high-dextrose "Western" diet (WD; Harlan TD.10768), and a low-carbohydrate, high-fat ketogenic diet (KD; Research Diets, New Brunswick, NJ; D06040601); for macronutrient composition, see Table 1. Rats from each diet group were then matched into groups based on exposure duration, and were fed their diets *ad libitum* for 10 (n = 10 per diet), 40 (n = 10 per diet) or 90 (n = 11 per diet) days, except for the two hours immediately prior to glucose testing and sacrifice. For Chow and WD rats, food was delivered in stainless steel hoppers. KD rats received their food in small, enameled cups, which were changed daily to prevent spoilage. Each week, daily food intake was estimated by weighing food containers and spillage on two consecutive days. Data are expressed in kCal.

Rats were weighed 6 hours into the light cycle, twice per week. Body composition was analyzed approximately once every 10 days using an EchoMRI whole-body composition analyzer (EchoMRI-900, Echo Medical Systems, LLC, Houston, TX), and expressed as percent body fat (grams fat mas / grams total body weight × 100).

Behavioral Assessment – Spontaneous Alternation Task

Spontaneous alternation (SA) behavior was analyzed in a Y maze apparatus as an assessment of general cognitive function. During SA trials, vicarious trial and error (VTE) behaviors were quantified as an index of hippocampal involvement in the task. The Y maze consisted of three black, wooden arms radiating 120° from a midpoint. Each arm was 1m long and 20cm wide, and was surrounded by a 20cm high wall on all sides. Lightly soiled bedding was placed inside the maze in order to neutralize the effect of odor cues; bedding was tossed thoroughly between trials.

During testing, rats were placed into the center of the apparatus and allowed to explore the maze for 8 min while an overhead digital camera recorded the rat's behaviors. After 8 min, the rat was returned to its home cage.

The sequence of arm entries was scored by hand according to the following criteria: a rat was deemed to have entered an arm if all four of its paws had crossed the threshold between the center triangle and the rectangular arm space. Animals were considered to have re-entered the same arm if all four paws were withdrawn from and returned to the same arm without simultaneously penetrating another branch. To facilitate scoring, arms were numbered. Number sequences were tabulated, and the number of triplets in which the rat entered exactly three arms were quantified and expressed as a ratio of the total possible number of triplets (the number of arm entries minus two).

During spontaneous alternation performance, VTE, or the total number of lateral head movements at choice points, was quantified and expressed as a ratio of total arm entries. Choice points were defined as occasions prior to the entry of a new arm when the rat's head was within 5 cm of the new arm opening. Lateral head movements were the total number of head turns toward either of the arm choices. Lateral head movements could be accompanied by limb movements, but quantification was paused if the animal turned 180° within the same arm or moved backward such that the head was no longer within 5 cm of the arm opening.

Blood Glucose Quantification

On the day of sacrifice, a tail nick was performed, and blood glucose was analyzed in duplicate via handheld glucometer (Novamax Plus, Nova Diabetes Care, Inc., Billerica, MA). Because brains were to be dissected for fasting-sensitive gene expression analyses immediately following blood collection, rats were exposed to a mild two hour fast in lieu of a more typical overnight food deprivation. If values were disparate by > 20 mg/dL, a third reading was obtained, and the two closest values were averaged.

Sacrifice and Tissue Processing

Rats were anesthetized via isoflurane inhalation and rapidly decapitated with a guillotine. Trunk blood was collected into K3EDTA+ tubes and temporarily stored on ice. Brains were then rapidly rinsed in 1× phosphate buffered saline (PBS), hippocampi were dissected on ice, placed into nuclease-free microcentrifuge tubes, and stored on dry ice for the duration of sacrifice. Blood samples were centrifuged for 15m at 4°C, at 2500 RPM, after which plasma was aspirated and stored in microcentrifuge tubes. Samples were stored at −80°C until further analysis could be performed.

Plasma Analyses

Plasma insulin was assessed in duplicate via radioimmunoassay (Rat Insulin RIA Kit, Millipore, St. Charles, MO) according to the instructions provided by the manufacturer. Sample values were interpolated from a standard curve that was generated using enclosed known insulin quantities. The range of sensitivity for the kit was from 0.081-10 ng/mL.

Plasma beta-hydroxybutyrate (BHB) was measured in duplicate using the beta-hydroxybutyrate LiquiColor® Test (Stanbio Laboratory, Boerne, TX) with a microplate, using instructions provided by the manufacturer. Data were expressed in mg/dL as a function of a known standard value.

RNA Extraction, Quantification, Purification and cDNA Synthesis

Dissected brain tissues were homogenized in TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH), after which RNA was extracted by separating the RNA-containing aqueous phase from the interphase and organic phase (which contain DNA and proteins) using bromochloropropane (Molecular Research Center). RNA was then isolated via alcohol precipitation, and analyzed via spectrophotometry. Prior to cDNA synthesis, RNA was incubated with RNAse-free DNase I and $MnCl_2$ buffer (Fermentas Maxima, Thermo Scientific, Waltham, MA). A First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA) was used to synthesize cDNA in 20 μL PCR reactions according to instructions provided by the manufacturer. A negative control (RT-) was also created by substituting nuclease-free water for the Enzyme Mix.

Quantitative Polymerase Chain Reaction

In order to quantify the hippocampal expression of GLUT1 and MCT1 (relative to GAPDH), SYBR green qPCR was performed using an iCycler iQ Real-Time PCR Detection System with MyIQ software (Bio-Rad Laboratories, Hercules, CA). For each primer set (Table 2), both amplification and melt curve analyses of serially diluted cDNA were conducted to verify the effectiveness of the primer, confirm the absence of primer dimers, and determine the reaction efficiency. Primers were optimized such that the correlation coefficient was 0.98-1.0 and the PCR efficiency was within the range of 98-107%. The RT-control was also compared to a matched cDNA sample for the GAPDH primer. As amplification did not occur in the RT- sample, it was concluded that RNA samples used in cDNA synthesis were void of residual DNA contamination. Amplification of sample cDNA was performed in triplicate. The thermal profile began with a 10 min initiation phase at 95°C, followed by 45 cycles of 95°C (30 s), 62°C (30 s) and 72°C (60 s). Primers are listed

in Table 2**.** Efficiency of primers were 98.2%, 98.5% and 96.7% for GLUT1, MCT1 and GAPDH, respectively. Water controls were run on each plate to assess contamination; there were no cases of water control amplification. Threshold values were standardized across plates for a given primer, such that all plate thresholds were within the exponential region of amplification. Changes in mRNA expression were determined by comparing the CT values of the genes of interest to that of GAPDH using the Pfaffl method (Pfaffl, 2001).

Statistical Analyses

Differences in behavioral and physiological measures between diet groups were analyzed via two-way ANOVA, with Bonferroni posttests where appropriate. Correlations were also performed to analyze the relationship between behavioral tests, with significance set to $p < 0.05$.

Results

Effect of Diet Type on Food Intake, Body Weight, and Adiposity

Caloric intake was measured during the first day of diet presentation, then weekly by weighing hopper on and off weights during a 24h period. Data from each exposure duration group were analyzed separately via two-way, repeated measures ANOVA (Figure 1A), all three of which revealed significant interactions ($F_{2,27} = 7.228$, $p < 0.001$ at 10 days; $F_{8,108} = 10.27$, $p < 0.0001$ at 40 days; $F_{20,300} = 7.813$, $p < 0.0001$ at 90 days) and main effects of both diet ($F_{2,27} = 19.88$, $p < 0.0001$ at 10 days; $F_{2,108} = 22.08$, $p < 0.0001$ at 40 days; $F_{2,300} = 12.14$, $p < 0.0001$ at 90 days) and exposure time ($F_{1,27} = 36.66$, $p < 0.0001$ at 10 days; $F_{4,108} = 51.17$, $p < 0.0001$ at 40 days; $F_{10,300} = 27.53$, $p < 0.0001$ at 90 days). All three cohorts showed the same pattern of WD-induced hyperphagia at day 1 ($p < 0.001$ for all cohorts, compared to both Chow and KD). On day 7, WD rats from the 40 day group consumed more than KD rats ($p < 0.05$); though there was a trend towards increased WD intake compared to both Chow and KD in all cohorts at this time.

Terminal body weight gain was analyzed via two-way ANOVA and is expressed in Figure 1B. At the time of sacrifice, there were no interactions or effects of diet; however, a main effect of exposure duration was observed ($F_{2,84} = 565.7$, $p < 0.0001$).

Terminal body fat was also analyzed via NMR for all exposure duration cohorts via two-way ANOVA (Figure 1C). There were no interactions, but significant main effects of both diet ($F_{2,84} = 43.46$, $p < 0.0001$) and exposure time ($F_{2,84} = 56.01$, $p < 0.0001$) were observed. Compared to Chow, adiposity was significantly higher at sacrifice for WD and KD rats at all times (for WD, $p < 0.001$ at days 10 and 90, $p < 0.05$ at day 40. For KD, $p < 0.01$ at day 10, and $p < 0.001$ at days 40 and 90). These data suggest that while rats fed WD or KD were not markedly heavier than Chow rats, they carried a significantly greater proportion of their body weight in adipose tissue.

Effect of Diet on Behavior

Because previous analyses revealed that WD is capable of inducing cognitive deficits, we analyzed SA and VTE performance in rats after 10, 40, or 90 days access to Chow, WD, or

KD. Data were analyzed via two-way ANOVA with Bonferroni post-hoc tests, and are depicted in Figure 2. Correlations were calculated for each group for % Sequential Arm Entries × Number of Arm Entries and % Sequential Arm Entries × VTE Episodes per Alternation.

Spontaneous Alternation—The total percentage of arm entry triplets without redundant entries were quantified as % Sequential Arm Entries (Figure 2A). A significant interaction between diet treatment and duration was noted ($F_{4,84} = 3.039$, $p = 0.0218$), though no main effects of diet or treatment duration were detected. After 10 days diet access, rats fed WD had significantly fewer sequential arm entries than either Chow ($p < 0.05$) or KD ($p < 0.01$). The total number of arm entries was also analyzed (Figure 2B), and an interaction ($F_{4,84} = 3.788$, $p = 0.007$) and main effect of time ($F_{2,84} = 11.90$, $p = 0.0001$) were found. After 10 days, WD rats had entered significantly more arms than Chow ($p < 0.05$). A significant and robust negative correlation between the total number of arm entries and SA performance was noted for WD rats ($r = -0.6402$; $p = 0.0002$). This correlation was absent in KD and Chow-fed rats.

Vicarious Trial and Error—We then analyzed VTE behaviors by counting the total number of head turns at choice points. Data are expressed as the total number of head turns per alternation (Figure 2C). A significant interaction between diet and duration was observed ($F_{4,84} = 2.687$, $p = 0.0367$), along with a main effect of diet ($F_{2,84} = 14.48$, $p < 0.0001$). After 10 days diet access WD rats engaged in fewer VTE behaviors than Chow ($p < 0.001$) and KD ($p < 0.01$). There were no differences observed at 40 days, but after 90 days diet access, WD rats engaged in fewer VTE behaviors compared to Chow ($p < 0.05$). To be sure that these data were not an artifact of increases in total arm entries, total number of VTE behaviors over the entire 8 minute session were analyzed via two-way ANOVA (Figure 2D). There was no interaction, but main effects of both diet ($F_{2,84} = 27.36$, $p < 0.0001$) and time ($F_{2,84} = 22.95$, $p < 0.0001$) were observed, with rats engaging in progressively fewer VTE behaviors over time, and WD rats engaging in fewer VTE movements than Chow and KD rats. This suggests that rats fed WD were less inclined to engage in these behaviors, which may indicate diminished hippocampal involvement. There were no correlations between VTE Episodes per Alternation and SA performance for any group.

Effect of Diet on Glucose, Insulin, and BHB

Blood was collected for analysis after a two-hour fast on the day of sacrifice (10, 40 or 90 days maintenance on Chow, WD or KD), and levels of glucose, insulin and BHB were measured (Figure 3). Data were analyzed via two-way ANOVA with Bonferroni posttests. For glucose (Figure 3A), no interactions between diet and time were detected; however, main effects of diet ($F_{2,84} = 4.023$, $p = 0.0215$) and duration ($F_{2,84} = 3.925$, $p = 0.0235$) were observed. At all time points, glucose levels were marginally higher in WD rats compared to Chow and KD.

Plasma insulin (Figure 3B) was measured in duplicate using a commercially available radioimmunoassay kit. A significant interaction between diet and exposure time was present

($F_{4,84}$ = 2.516, p = 0.0484), along with a main effect of diet treatment ($F_{2,84}$ = 6.101, p = 0.0035). Post-hoc tests revealed that 90 day insulin levels were significantly higher in WD rats, compared to both Chow and KD rats ($p < 0.01$). There were no differences between other groups or time points.

Plasma BHB (Figure 3C) was quantified in duplicate using a microtiter assay. An interaction approached significance ($F_{4,84}$ = 2.065, p = 0.0926), and robust main effects of diet ($F_{2,84}$ = 50.55, $p < 0.0001$) and time ($F_{2,84}$ = 47.24, $p < 0.0001$) were observed. At all time points, KD rats had significantly higher levels of BHB compared to both Chow and WD rats ($p <$ 0.01 at 10 days, and $p < 0.001$ at 40 and 90 days), indicating that, as expected, maintenance on KD led to increases in circulating ketone bodies. The lack of BHB differences between Chow and WD rats suggests that despite its high fat content, WD was not ketogenic.

Effects of Diet on Hippocampal mRNA Expression

Hippocampal glucose (GLUT1) and monocarboxylate (MCT1) transporter expression was measured via qPCR in rats fed Chow, WD or KD relative to GAPDH, and analyzed via two-way ANOVA with post-hoc Bonferroni contrasts. Two samples were excluded due to poor cDNA quality, one from the 10 day KD group, and one from the 90 day Chow group. Data are presented in Figure 4.

Though no interaction was detected, a main effect of diet on hippocampal GLUT1 (Figure 4A) expression was observed ($F_{2,82}$ = 5.822, p = 0.0043). At all time points, GLUT1 mRNA was lower in WD rats compared to Chow and, to a lesser extent, KD; however, the magnitude of this difference was most pronounced at the 10 day time point.

We then analyzed hippocampal MCT1 mRNA (Figure 4B), and noted a significant interaction between diet and time ($F_{4,82}$ = 4.223, p = 0.0037) and a main effect of diet ($F_{2,82}$ = 10.23, $p < 0.0001$). Bonferroni post-hoc revealed that MCT1 levels were significantly lower in WD rats at 10 days compared to both Chow ($p < 0.001$) and KD ($p < 0.05$) rats. At 90 days, KD rats had MCT1 mRNA expression levels significantly higher than both Chow and WD ($p < 0.01$).

Discussion

These data are in agreement with previous reports that hippocampal-dependent cognitive performance is affected by exposure to WD (Davidson et al., 2013; Davidson et al., 2012; Kanoski et al., 2007; Kanoski et al., 2010) but not KD, despite KD-fed rats' propensity to maintain extremely high levels of body fat (Davidson et al., 2013). We also replicated previous findings suggesting maintenance on KD does not induce excess weight gain, but does lead to increases in overall fat mass (Davidson et al., 2013; Kinzig, Hargrave, Hyun, & Moran, 2007; Kinzig, Honors, & Hargrave, 2010).

We now report significant decreases in hippocampal GLUT1 and MCT1 mRNA in after 10 days WD access, and higher levels of MCT1 mRNA in KD-fed rats after 90 days. The increased 90 day MCT1 levels in KD rats were not surprising given the marked increase in BHB expression at this time. However, BHB levels were also higher in KD rats at 10 and 40

days without an accompanying MCT1 up-regulation. Whether MCT1 up-regulation depends on duration of exposure to ketones, ketone levels, or both, is currently unknown.

The 10 day decrease in GLUT1 and MCT1 mRNA expression exhibited by WD rats (which appear to be associated with hyperphagia and diet change, rather than weight or body composition) cannot be explained by blood glucose or BHB levels, which did not differ significantly from Chow rats. It is possible that an increased fasting duration prior to blood collection would have revealed significant differences in glucose expression. Further, glucose levels in the brain interstitial fluid represent only a small fraction of the levels in circulation, and could therefore differ from blood glucose levels measured. Given the hyperphagia that was present throughout the first week of WD access, but not during the testing phases, it is plausible that transient, intake-induced hyperglycemia precipitated a reduction in transporter levels, which may not have normalized by 10 days. Typically, glucose-induced changes in GLUT1 mRNA peak approximately 24 hours following experimental metabolic manipulations (Ruben J. Boado & Pardridge, 1993; Kumagai et al., 1995), but other insults can lead to more sustained GLUT1 changes (McCall et al., 1996). Whether the present reduction in GLUT1 was sustained across days 7 through 10 despite normalized glucose levels, or was in response to brain interstitial hyperglycemia or high fasting glucose levels is not known.

GLUT1 levels may also be decreased in response to another factor, such as neurotrophins, which are decreased following WD exposure (Cordeira & Rios, 2011; Kanoski et al., 2007; Molteni et al., 2002), and are positively correlated with GLUT1 expression (Rubén J. Boado, 1996). Alternately, since GLUT1 is a critical component of the BBB (Maher, Vannucci, & Simpson, 1994; Maurer, Canis, Kuschinsky, & Duelli, 2004; Roberts et al., 2008), which is compromised after extended WD exposure (Davidson et al., 2013; Kanoski et al., 2010), GLUT1 may also be decreased in response to BBB damage.

The regulation of the MCT1 transporter has not been fully elucidated; however, it is likely that factors beyond BHB are capable of altering its expression patterns. For instance, PPARα has been shown to up-regulate MCT1 in peripheral tissues (König et al., 2008) Another possibility is that other monocarboxylates (e.g., lactate and pyruvate) are capable of regulating neuronal MCT1 expression and were altered following maintenance on WD, though this relationship has not yet been established. However, peripheral administration of pyruvate and lactate have been shown to reduce the hyperphagia associated with WD intake (Nagase, Bray, & York, 1996). While glucose and ketone bodies have generally been considered the primary energy substrates within the brain, there is evidence that lactate is also an effective energy source for neurons (Pellerin et al., 2007; Schurr, West, & Rigor, 1988; Suzuki et al., 2011; Zilberter, Zilberter, & Bregestovski, 2010). Further investigation should be conducted to determine whether WD has the potential to alter pyruvate and/or lactate levels and the effect this has on central MCT1 expression.

Disruptions to astrocytic MCT1 transporter expression in the hippocampus have been shown to induce amnesia, which is restored following lactate, but not glucose, administration (Suzuki et al., 2011). Very low GLUT1 levels limit the rate of glucose transport into the brain interstitial fluid (Ding, Yao, Zhao, et al., 2013; Qutub & Hunt, 2005), which is

associated with cognitive deficits (de la Monte, 2009; De Vivo et al., 1991; De Vivo & Wang, 2008; Ding, Yao, Rettberg, et al., 2013). The point at which GLUT1 and MCT1 levels are low enough to limit the rate at which glucose or monocarboxylates enter the interstitial fluid is not currently known. Future research should analyze the composition of the brain interstitial fluid of WD-fed rats to assess whether this down-regulation of GLUT1 and MCT1 simply normalized glucose concentration levels or induced brain-specific reductions in glucose, ketone bodies, or lactate. If paradoxical reductions in glucose, BHB, or lactate are indeed present in the hippocampus of rats fed WD, energy-deprived neurons could sustain damage that manifests as cognitive deficits.

In addition to the novel transporter data, we have observed several WD-induced behavioral outcomes, including slight reductions in SA after 10 days exposure, significant decreases in VTE episodes per alternation after 10 and 90 days exposure, and reductions in total VTE behaviors after all WD exposure durations. The total number of arm entries was also significantly higher in WD rats after 10 days, and was negatively correlated with % sequential arm entries, suggesting SA performance may be influenced by the animal's activity patterns.

Notably, we observed performance deficits in a non-appetitive task, suggesting that WD does not merely influence cognitive behaviors directly related to ingestion. Furthermore, while there was a significant main effect of diet on body weight over the course of the study, no such difference was present between groups at the time of behavioral testing or sacrifice, suggesting WD can induce behavioral deficits even in normal weight animals. Throughout the study we observed reductions in VTE behaviors in WD-fed rats, which remained significant after accounting for the total number of alternations at both 10 and 90 days. VTE depends on the hippocampus (Addis et al., 2007; Addis & Schacter, 2008; Buckner & Carroll, 2007; van der Meer, Kurth-Nelson, & Redish, 2012), and reduced VTE behaviors may indicate diminished behavioral flexibility. Reductions in VTE following WD maintenance may make food consumption more mindless and less malleable via cognitive inputs (Blass et al., 2006; Cserjési et al., 2007; Delgado-Rico, Río-Valle, González-Jiménez, Campoy, & Verdejo-García, 2012). This may impair an animal's ability to exert inhibitory control over their feeding patterns, thereby increasing inappropriate responding to food cues and impairing adherence to dietary regimens, ultimately exacerbating any innately obesogenic properties of WD and leading to a vicious cycle of obesity and cognitive decline (Benoit, Davis, & Davidson, 2010; Davidson, Kanoski, Schier, Clegg, & Benoit, 2007; Kanoski & Davidson, 2011).

A consideration in interpreting the VTE data within this experiment is that VTE behaviors were observed during the SA task, rather than during a traditional, appetitive spatial learning paradigm. While SA behaviors are quite robustly expressed, the underlying behavioral and neural mechanisms have not yet been elucidated. SA is often touted as a "manifestation of curiosity" (Gerlai, 1998), but it is not known if SA behaviors are due to an animal's "lose-shift" foraging strategy (Estes & Schoeffler, 1955), preference for novelty (Dember & Fowler, 1958), or "obsessive-compulsive"-like behaviors (Albelda & Joel, 2012; Yadin, Friedman, & Bridger, 1991). Further, mazes such as that used in the SA task can be navigated using either place or response strategies, which (respectively) rely on extra-maze

spatial cues and habitual motor responses (such as turning in the same direction at each choice point).

Unlike place strategies, response strategies do not require an intact hippocampus (Packard & McGaugh, 1996), and are associated with reductions in VTE (Schmidt et al., 2013). Though alternation can be reduced following elimination of external cues (Gerlai, 1998), these cues are not required for SA behaviors to occur (Lennartz, 2008). If maintenance on WD induces hippocampal deficits and biases rats toward a response strategy, it would be expected that SA, but not VTE, performance would remain intact.

The neural correlates of SA are predominantly (but not exclusively) limbic structures (Lalonde, 2002). Many have noted SA changes in response to hippocampal manipulations. For example, alternation was reduced in mice with ibotenic lesions to the hippocampus, or with genetically-induced LTP deficits (Gerlai, 1998). SA performance is also enhanced via administration of glucose to the septum (Stefani, Nicholson, & Gold, 1999), a region with numerous connections to the hippocampus. This suggests that this task may depend on adequate nutrient transport to these brain regions, and may be sensitive to reductions in hippocampal GLUT1 or MCT1 expression.

However, several studies (e.g., Asin & Fibiger, 1984; Caston, Vasseur, Delhaye-Bouchaud, & Mariani, 1997; Divac, Wikmark, & Gade, 1975) suggest that SA is not hippocampal-dependent. Since alternation can be influenced by behavioral strategy, learning, and environmental factors (Lalonde, 2002), it is understandable that lesions induce divergent effects.

Whether WD-induced GLUT1 and MCT1 reductions are due to an immediate effect of the diet which is compensated for later, or as a result of the hyperphagia present during the first week of WD access is currently unknown. Whatever the mechanism, changes in transporter levels may be one mechanism by which short-term, diet-induced cognitive impairments develop. In adults, diet-induced changes in hippocampal function could facilitate overeating, whereas in juvenile organisms, perturbations in neuronal glucose homeostasis could lead not only to cognitive deficits (Boitard et al., 2014), but also to atypical hippocampal development (Boitard et al., 2012; Ekdahl, Kokaia, & Lindvall, 2009) which may in turn influence the development of other neurological structures (Lipska, Aultman, Verma, Weinberger, & Moghaddam, 2002) and result in psychiatric illness (Agranat-Meged et al., 2005; Schaefer et al., 2000).

Fortunately, isolated deficits in GLUT1 and MCT1 are likely to be modifiable via dietary changes such as maintenance on a ketogenic diet, which has been demonstrated to up-regulate both GLUT1 and MCT1 (Leino, Gerhart, Duelli, Enerson, & Drewes, 2001) and ameliorate the cognitive deficits associated with GLUT1 deficiency syndrome (Akman et al., 2010; De Vivo et al., 1991; De Vivo & Wang, 2008), Alzheimer's disesase (Van der Auwera, Wera, Van Leuven, & Henderson, 2005) and other conditions. Ketone bodies, or other monocarboxylates such as lactate, may represent safe, effective supplements for diet-induced cognitive deficits. Further investigation may be useful for identifying the

mechanism behind the purported therapeutic properties of these compounds and identify pharmaceutical targets for more focused treatments.

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Highlights

- Short-term western diet access impairs spontaneous alternation performance.
- Vicarious trial and error is disturbed throughout the course of western diet treatment.
- Hippocampal GLUT1 and MCT1 mRNA levels decreased following western diet maintenance.
- Hippocampal MCT1 mRNA levels increased following long-term access to ketogenic diet.

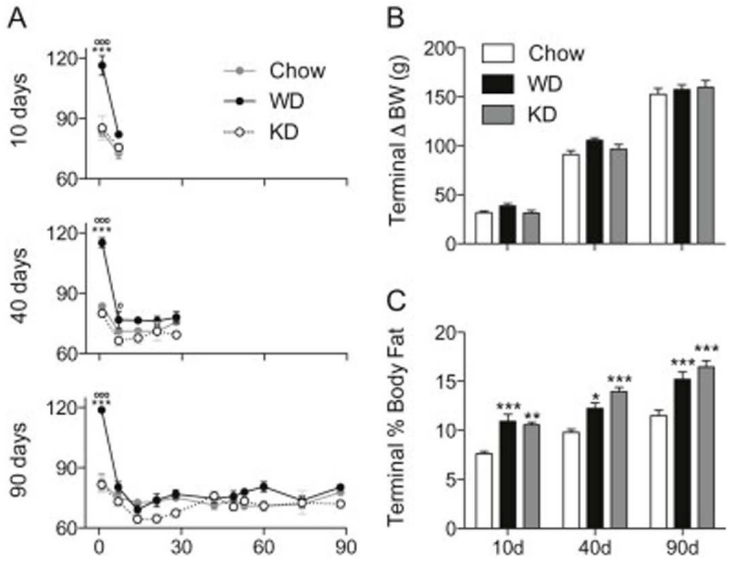

Figure 1.

Caloric intake, body weight, and adiposity data for rats fed Chow, Western diet (WD) and ketogenic diet (KD). A) Amount consumed (kCal) for rats belonging to the 10, 40, and 90 day cohorts throughout the experiment. There was an overall effect of diet on caloric consumption, with WD rats consuming significantly more than Chow ($p < 0.05$) and KD-fed ($p < 0.01$) rats. According to post-hoc tests, WD-associated hyperphagia was present for only one week, with WD rats consuming more than Chow and KD at day 1 ($p < 0.001$, for all cohorts) and compared to KD at day 7 in the 40 day cohort ($p < 0.05$). No differences were detected at any other time points. (B) Change in body weight at sacrifice for Chow, WD, and KD rats. There were no differences in terminal weight gain between Chow, WD, and KD rats at any time point. (C) Body adiposity at sacrifice. Chow-fed rats had lower adiposity than WD ($p < 0.001$ at days 10 and 90; $p < 0.05$ at day 40), and KD ($p < 0.01$ at day 10; $p < 0.001$ at days 40 and 90) rats at the time of sacrifice. (*) indicates significant difference between WD and Chow; (o) indicates significant difference between WD and KD; (^) indicates significant difference between Chow and KD.

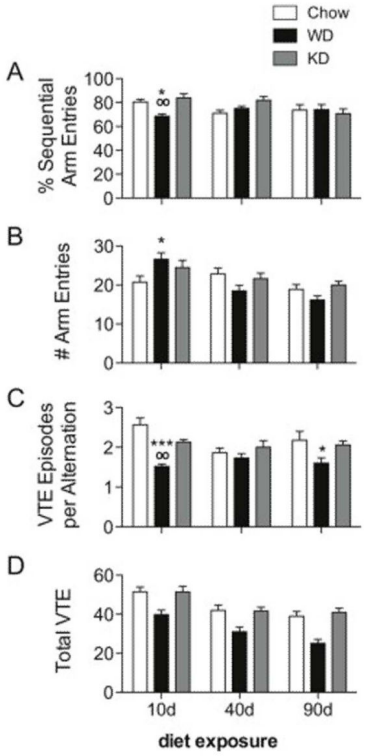

Figure 2.

Spontaneous alternation (SA) and vicarious trial and error (VTE) behaviors for Chow, WD and KD rats. (A) An interaction between diet and exposure duration was detected for SA performance (expressed as percent sequential arm entries; $p = 0.0218$). After 10 days, WD rats had fewer sequential arm entries than Chow ($p < 0.05$) and KD ($p < 0.01$), but no differences were detected at later time points. (B) A similar interaction ($p = 0.007$) was noted for the total number of arms explored. At 10 days, WD rats entered significantly more arms than Chow ($p < 0.05$). (C) VTE was measured by the total number of head turns per alternation. An interaction between diet and time was noted ($p = 0.0367$), with WD rats engaging in fewer VTE behaviors than Chow rats at 10 ($p < 0.001$) and 90 days ($p < 0.05$), and than KD rats after 10 days ($p < 0.01$). (D) The total number of head turns per 8 minute session were also analyzed. There was no interaction, but main effects of diet and time ($p < 0.0001$) were observed, with WD rats engaging in fewer VTE behaviors than Chow and KD rats. (*) indicates significant difference compared to Chow; (o) indicates significant difference compared to KD.

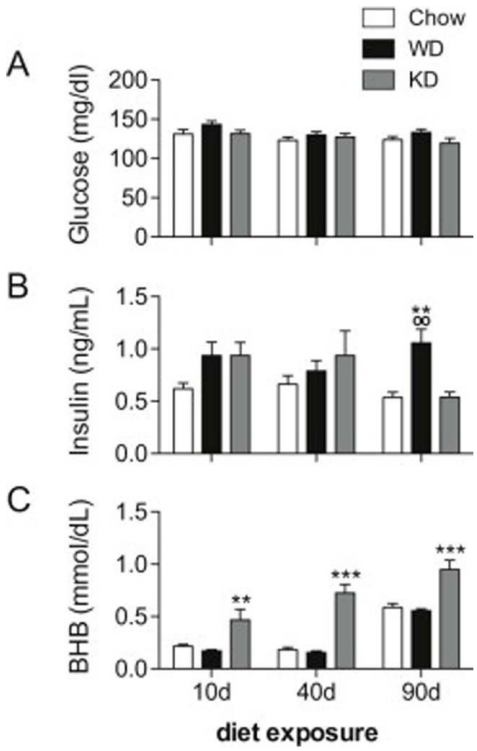

Figure 3.

Blood glucose, plasma insulin, and plasma beta-hydroxybutyrate (BHB) from rats fed Chow, WD, and KD for 10, 40 and 90 days. (A) Main effects of diet ($p = 0.0215$) and time ($p = 0.0235$) on blood glucose were detected. Glucose levels were higher in WD-fed rats, compared to Chow and KD. (B) An interaction between diet and time was observed for plasma insulin ($p = 0.0484$), and post-hoc tests revealed that WD-fed rats had higher insulin levels at 90 days, compared to Chow and KD ($p < 0.01$). (C) A main effect of diet was observed ($p < 0.0001$), and KD rats had higher BHB levels at all time points. A marginally significant interaction was also present ($p = 0.0926$); post-hoc tests revealed that KD rats had elevated BHB than Chow and WD rats at all time points ($p < 0.01$ at 10 days, $p < 0.001$ at 40 and 90 days). (*) indicates significant difference compared to Chow; (o) indicates significant difference compared to KD.

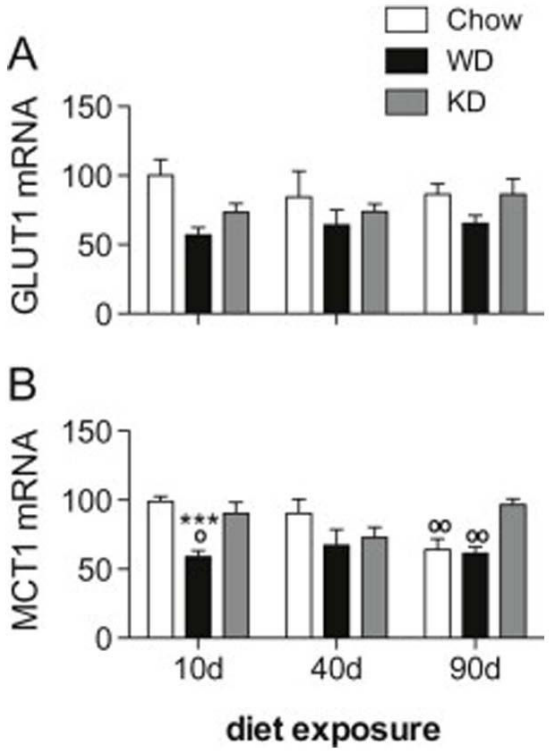

Figure 4.

Hippocampal GLUT1 and MCT1 mRNA expression for rats fed Chow, WD and KD. (A) A main effect of diet on GLUT1 was observed, and expression was lower in WD rats compared to Chow and KD, particularly after 10 days diet access. (B) An interaction between diet and time ($p = 0.0037$), and a main effect of diet ($p < 0.0001$) on MCT1 mRNA expression were observed. Post-hoc tests revealed that MCT1 levels were significantly lower in WD rats compared to Chow ($p < 0.001$) and KD ($p < 0.05$) rats at 10 days, and higher in KD rats, compared to Chow rats at 90 days ($p < 0.01$). (*) indicates significant difference compared to Chow; (o) indicates significant difference compared to KD.

Table 1

Macronutrient composition of diets.

	Chow Harlan 2018	WD Harlan TD.10768	KD Research Diets D06040601
% kCal Fat	18	38	80
% kCal Dextrose	-	20	-
% kCal Other Carb.	58	18	5
% kCal Protein	24	24	15
Caloric Density	**3.4 kCal/g**	**4.5 kCal/g**	**6.1 kCal/g**

Table 2

Primer sequences and reference numbers.

Target	FWD sequence	REV sequence	NCBI Reference
GLUT1	TGG CCC CTA CGT CTT CAT CAT CTT CA	TCG CCC GAG ATC TGT CAG TTT GGA AG	NM_138827.1
MCT1	TGT GGA GCA TGA AGA GAG CAG GTG TG	CCC CAT ATT CTT TGT CAA CCA CTC CC	NM_012716.2
GAPDH	ACA GCA ACA GGG TGG TGG AC	TTT GAG GGT GCA GCG AAC TT	NM_017008