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MEMORY AND HIPPOCAMPAL ARCHITECTURE FOLLOWING SHORT-TERM MIDAZOLAM IN WESTERN DIET-TREATED RATS

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Abstract

The impact of short-term benzodiazepine exposure on cognition in middle-aged or older patients is a highly debated topic among anesthesiologists, critical care physicians and public media.

“Western diet” (WD) consumption is linked to impaired cognition as well. The combination of benzodiazepines with substantial exposure to WD might set the stage for increased hippocampal vulnerability for benzodiazepines leading to exaggerated cognitive impairment in the postoperative period. In this study, Fischer 344 rats were fed either WD or standard rodent diet from 5 to 10.5 months of age. Rats were exposed to midazolam or placebo two days prior to an MRI scan using Diffusional Kurtosis Imaging (DKI) to assess brain microstructural integrity, followed by behavioral testing using a water radial arm maze. Hippocampal tissue was collected to assess alterations in protein biochemistry in brain regions associated with learning and memory. Our results showed that rats exposed to the combination of midazolam and WD had significantly delayed time of learning and exhibited spatial memory impairment. Further, we observed an overall increase of kurtosis metrics in the hippocampus and increased expression of the mitochondrial protein VDAC2 in midazolam-treated rats. Our data suggest that both the short-acting benzodiazepine midazolam and WD contribute to negatively affect the brain in middle-aged rats. This study is the first application of DKI on the effects of midazolam and WD exposure, and the findings demonstrate that diffusion metrics are sensitive indicators of changes in the complexity of neurite architecture.

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Keywords

Midazolam; diffusional kurtosis imaging; Western diet; spatial memory

Introduction

Benzodiazepines (BZD) have been used for decades by anesthesiologists for anxiolysis in patients of all ages undergoing surgical procedures, moderate sedation in non-surgical procedures [1] and in critically ill patients in the Intensive Care Unit (ICU). Midazolam (MDZ) is preferred for sedation over other BZDs because of its short elimination half-life, combined with its water solubility and its suitability for continuous infusion [2,3]. However, short-term BZD exposure, particularly MDZ, and its impact on brain microarchitecture and cognition in the elderly is still a controversial issue. Mechanisms of action, confounding factors and synergisms such as the influence of premorbid status and/or surgical stress leading to postoperative or post-anesthetic cognitive impairment are not well understood [4–6].

Industrialized countries experienced a shift in dietary composition over the past three decades, from a diet rich in complex carbohydrates and fibers to what is known as a “Western diet” (WD), a high-energy diet rich in saturated fats and highly refined simple sugars [7]. It is estimated that 68 % of adults in the United States are overweight or obese [8]. Obesity is related to metabolic syndrome, which includes visceral adiposity, hypertension, dyslipidemia, glucose intolerance and insulin resistance [9]. WD exposure in animal models leads to impaired cognitive function, disrupting hippocampal-dependent learning and memory processes involved in spatial memory and executive memory function [10,11]. Recent epidemiological studies suggested that WD may contribute to age-related cognitive impairment in humans [12,13], and increases the risk for Alzheimer’s disease [14]. Mechanisms may include dietary-induced reductions in brain-derived neurotrophic factor (BDNF), and increased oxidative stress and neuroinflammation, which could result in impaired blood brain barrier integrity [12,15,16].

Diffusion magnetic resonance imaging (dMRI) is a powerful method for probing brain microstructure abnormalities, changes of brain microstructure in normal aging and its disruption in several neurological diseases [17,18] both in humans and animals. Diffusional kurtosis imaging (DKI) is a specific dMRI technique that extends diffusion tensor imaging (DTI) by quantifying non-Gaussian behavior of water diffusion, contributing additional information beyond that provided by DTI [19–21]. DKI also provides the metrics of diffusional non-Gaussianity, such as mean (MK), axial ($K_{//}$) and radial ($K_{\perp}$) kurtoses. These additional metrics can further help in our understanding of normal and pathologic brain tissue cytoarchitecture. DKI is already yielding promising preliminary results in studies of normal aging and brain diseases [22–30]. Our work on animal models, as well as studies from other groups, have shown that DKI metrics are sensitive to changes in brain microstructural complexity that may be associated with brain development [31], aging [32], Down syndrome [33], amyloid-beta (A β) deposition [34], and myelin abnormalities [35].

One of the two benzodiazepine receptor types (translocator protein, TSPO) is located in the outer mitochondrial membrane and interacts with a voltage-dependent anion channel (VDAC) [36]. We were therefore interested in whether alterations in VDAC protein expression might be an underlying mechanism by which midazolam affects cognition and hippocampal circuitry in the brain.

The goal of this study was to investigate effects of MDZ exposure in middle-aged rats exposed to regular diet and in a WD “pre-conditioned” brain by assessing behavior, DKI measures, and brain biochemistry. Results may elucidate mechanisms of BDZ-WD interaction in the brain leading to altered therapeutic interventions in individuals with metabolic syndrome.

Materials and Methods

Animal model and Experimental Design

Twenty-two 5 month old male Fischer 344 rats (Harlan Inc., Indianapolis, IN) weighing 357 ± 15 g were housed in pairs and were given one week to acclimate to the vivarium and were then randomly allocated to one of these groups: a) conventional rodent chow controls (NC, n=8); b) conventional rodent chow injected with midazolam (NC-MDZ, n=6), and c) Western diet (WD) injected with midazolam (WD-MDZ, n=8).

The Western diet (D12079B, Research Diets Inc., Brunswick, NJ) provided (in kcal) 17% protein, 41% fat and 43% carbohydrates. The control diet consisted of standard rodent chow and provided (in kcal) 14.7% protein, 9.4% fat and 75.9 % carbohydrates (Table 1). Rats were fed the standard or the Western diet for a total of 5.5 months. Weights were obtained for the whole body, liver and intra-abdominal fat.

Preservative-free midazolam (MDZ) (Hospira, Inc., Lake Forest, IL) with a concentration of 1 mg/mL was provided by Medical University of South Carolina research pharmacy. Rats from the NC-MDZ and WD-MDZ groups received either placebo or midazolam (1 mg/kg, ip) every 12 hours during the two days prior to MRI imaging to mimic a clinical model of anxiolysis as practiced in the pre- and postoperative or critical care setting. Animals were under close observation for behavioral changes and respiratory distress following injections. All experimental animal procedures were approved by the Medical University of South Carolina, Charleston Institutional Animal Care and Use Committee, and conformed to National Institute of Health guidelines.

Behavioral Testing: Water Radial Arm Maze (WRAM)

All rats underwent behavioral testing in the 8-arm radial maze following MRI imaging. The 8-arm radial maze was used to assess working and reference memory as previously described [10]. The maze was constructed of galvanized steel, painted black, placed in a room with extra-maze cues and filled with water at room temperature. On the first day of testing, all rats were subjected to a simple visible platform task during which the time they spent to reach the platform was recorded in order to estimate their swimming ability. Then, for the rest of the testing period, escape platforms were placed 1 cm below the water surface close to the end of the arms. Four of eight arms were baited with platforms; the four baited

arms were assigned randomly and kept consistent over the 12 days of testing for each rat. Each session consisted of four trials (3 minutes maximum each). Each time the subject found a platform it was removed, allowing for a win-shift paradigm [37]. Three types of errors were quantified: Working Memory Correct (WMC), Reference Memory (RM), and Working Memory Incorrect (WMI) [37]. WMC errors were first and repeat entries into an arm that previously contained a platform, RM errors were first entries into any arm that never contained a platform, and WMI errors were repeat entries into an arm that never contained a platform, i.e. repeat entries into a reference memory arm [38]. The total number of entered arms (NOE) was also recorded for each trial. Data were blocked into initial, or acquisition (days 2–6) and latter, or asymptotic (days 7–12) phases.

Diffusion Magnetic Resonance Imaging

All rats were anesthetized with isoflurane/O₂ (4–5% for induction/1–3% for maintenance) for MRI scans. The *in vivo* MRI experiments were performed on a 7T/30 Bruker BioSpec (Billerica, MA) animal scanner. A two shot spin-echo planar imaging (EPI) diffusion sequence with 64 diffusional directions and 4 b-values (0, 650, 1300, 2000 s/mm²) was used for Diffusional kurtosis imaging (DKI) acquisition. Other imaging parameters were: TR/TE = 4750/32.5 ms, FOV = 30 mm × 30 mm, matrix = 128 × 128, in plane resolution = 0.23 × 0.23 × 1 mm³, δ/γ = 5/18 ms, and number of excitations (NEX) = 2. Nineteen axial slices with no gap were collected with a slice thickness of 1 mm. DKI post-processing was performed using DKE software [39] (<http://nitrc.org/projects/dke>). Parametric maps were obtained by fitting dMRI signal measurements to the DKI signal model for each voxel using a linearly constrained weighted linear least squares fitting algorithm. Parametric maps of the conventional diffusion tensor (DT) metrics of mean (MD), axial ($D_{||}$) and radial ($D_{\perp}$) diffusivities, as well as the additional DK metrics of MK, $K_{||}$, and $K_{\perp}$ were subsequently computed. All of these metrics were estimated from the diffusion and diffusional kurtosis tensors [19,21]. Regions of interest (ROI) at the level of the hippocampus was manually drawn on the b=0 image, using ImageJ (<http://rsb.info.nih.gov/>). Anatomical guidelines for outlining these regions were determined by comparing anatomical structures in the MRI slices with a standard rat atlas [40]. The average regional value for each diffusion MRI metric was obtained from the voxels within each ROI. To minimize the effect of cerebrospinal fluid (CSF) contamination, all voxels with MD > 1.5 $\mu\text{m}^2/\text{ms}$ were excluded from the ROIs prior to parameter quantification.

Protein Quantification

Rats were euthanized with an overdose of inhalative isoflurane and the brain rapidly removed. Frozen hippocampal tissues were homogenized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and protease and phosphatase cocktail inhibitor, pH 7.5) Total amounts of 15 μg protein per sample were separated on 10% sodium 4–15% Mini-Protean TGX Precast gels, and transferred to nitrocellulose membranes (BioRad Laboratories). After blocking, membranes were incubated overnight at 4°C with primary antibodies to VDAC1 and VDAC2 (anti-VDAC1 1:7,500, Abcam and anti-VDAC2 1:7,500 Abcam). The membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare) 1 hour at room temperature. Blots were developed with

electrochemiluminescence (ECL Prime, GE Healthcare) and imaged on Kodak Molecular imaging system including analysis with ImageJ Gel Analysis software. All samples were run in one blot for each marker and β -actin was used as a loading standard. Protein levels were normalized to β -actin levels in the respective blots.

Statistical Analysis

All data are expressed as mean $\pm$ standard error of the mean (SEM). For the diffusion MRI metrics, one-way analysis of variance (ANOVA) corrected for multiple comparisons using the Sidak method was performed to compare the means of the groups. A one-way ANOVA followed by Sidak's multiple comparison post hoc test was used to compare body weight gain, intra-abdominal fat and liver weights, as well as hippocampal VDAC1 and VDAC2 expression levels among the groups. For all behavior errors analyses, two-way repeated measures ANOVA were utilized with a primary focus on the treatment effect for learning vs. asymptotic phases during Trial 4. Post hoc analyses used the Sidak's multiple comparison test. Pearson correlation was used to examine the association between diffusion MRI metrics, VDAC1 and VDAC2 protein levels and behavioral measures. All reported p-values were considered statistically significant at $p \leq 0.05$.

Results

Body, liver and intra-abdominal fat weights

Rats were administered the control or Western diet for 5.5 months. The weight gain after the diet treatment period was significantly affected by the diet [$F_{2,19} = 21.33$, $p < 0.0001$]. Post hoc analysis showed that the rats fed the Western diet gained a significantly greater amount of weight compared to NC rats ($p = 0.003$) and NC+MDZ rats ($p < 0.0001$). As a consequence of the diet, WD-MDZ rats also had significantly higher intra-abdominal fat and liver weights compared to NC and NC-MDZ rats (Fig. 1).

Diffusion MRI Assessment

Representative parametric maps of all diffusion metrics of a single slice at the level of hippocampus are shown in Fig. 2. Estimates of the hippocampus diffusion metrics (mean $\pm$ SEM) are presented in Table 2. DT metrics showed no group differences, but DK metrics showed significant differences for MK and $K_{//}$ between NC and NC-MDZ ($p = 0.013$ and $p = 0.012$, respectively) and the WD-MDZ groups ($p = 0.009$ and $p = 0.015$, respectively).

Behavioral Testing

All rats were subjected to a 12-day water radial arm maze following MRI scanning [10,38]. On day 1 of testing, the time spent by the rats to reach a visible platform was recorded, and showed that there was no significant difference between the three groups ($F_{2,17} = 1.81$, $p = 0.194$). Testing days were divided into two blocks: the learning phase (days 2–6) and the asymptotic phase (days 7–12) (Fig. 3). Each day had four trials with increasing working memory load (Trials 1–4), such that rats had to remember both the four reference memory arms (no platforms) and three of the four working memory arms (with previous platforms) in Trial 4. Evaluation of the number of entered arms (NOE) during Trial 4, which represents the highest working memory load, throughout the 12 days of testing, revealed a significant

main effect of treatment [$F_{2,19} = 16.22$, $p < 0.0001$], with both NC-MDZ and WD-MDZ rats entering significantly more arms compared to NC rats ($p = 0.002$ and $p = 0.03$, respectively) during the learning phase, as well as during the asymptotic phase ($p = 0.002$ and $p = 0.001$, respectively, Fig. 3A).

While no significant group effects were observed for the WMI or the RM measures of the WRAM outcome, significant overall effects of the treatment [$F_{2,19} = 4.901$, $p = 0.019$] was observed for WMC errors performed on Trial 4 (Fig. 3B). Sidak's multiple comparisons test revealed that both NC-MDZ and WD-MDZ groups had significantly more WMC errors compared to NC rats during the asymptotic phase ($p = 0.013$ and $p = 0.037$, respectively). There were, on the other hand, significant correlations between kurtosis measures, VDAC expression levels, and RM and WMI errors as described below.

A significant overall effect of the treatment [$F_{2,19} = 5.394$, $p = 0.014$] was observed for the time needed to complete the task during Trial 4 (Fig. 3C). Sidak's multiple comparisons test revealed that WD-MDZ rats spent significantly more time to find the platform, compared to NC rats during the learning phase ($p = 0.003$; Fig. 3C).

Protein quantification

Protein expression of VDAC1 and VDAC2 was examined in frozen hippocampal formation by Western blot analysis, to determine whether alterations in these mitochondrial proteins were responsible for triggering apoptosis and neuroinflammatory cascades leading to altered behavioral performance caused by the MDZ and/or WD exposure. Expression of mitochondria-related protein expression (VDAC1 and VDAC2) for both NC-MDZ and WD-MDZ groups showed a trend for increased expression when compared with the NC group. Statistical analysis (ANOVA) revealed a statistically significant increase in VDAC2 protein levels in the hippocampus in the NC-MDZ group compared to the NC control group ($p = 0.038$; Fig. 4).

Pearson correlations

Pearson correlations were conducted in order to determine if any relationships existed between the dMRI, behavioral, and biochemical measures. For the NC group, VDAC1 had a statistically significant positive relationship with $D_{\perp}$ ($r = 0.99$; $p = 0.002$). VDAC2 had a statistically significant negative relationship with NOE (asymptotic phase; $r = -0.76$; $p = 0.047$) and a statistically significant positive relationship with WMC (learning phase; $r = 0.77$; $p = 0.044$).

For the NC-MDZ group, VDAC1 exhibited several statistically significant correlations with the MRI metrics ($K_{//}$, $r = -0.93$; $p = 0.02$; MD, $r = -0.89$; $p = 0.044$; $D_{//}$, $r = -0.92$; $p = 0.026$; $D_{\perp}$, $r = -0.88$, $p = 0.05$), suggesting that VDAC1 expression is affected by water diffusion in the hippocampus. Time was also significantly correlated with $D_{\perp}$ ($r = 0.83$; $p = 0.04$). VDAC1 had also statistically significant relationship with WMC (learning phase; $r = -0.93$; $p = 0.02$).

For the WD-MDZ group, no correlation was observed between DKI and VDAC1 or VDAC2 and behavior measures, but MD ($r = 0.71$; $p = 0.049$) and $D_{\perp}$ ($r = 0.71$; $p = 0.048$) were

significantly correlated with WMC (learning phase), and $D_{//}$ was significantly correlated ($r = 0.76$; $p = 0.029$) with NOE (learning phase) such that dMRI parameters were related to poor memory performance. This correlation points to the suggestion that diffusion kurtosis can, indeed, be used for predicting memory decline at least in animal models of cognitive impairment, and may therefore represent a validation of the hypothesis posed in this study. VDAC1 was negatively correlated with Time (asymptotic phase, $r = -0.79$; $p = 0.036$) and VDAC2 was positively correlated with WMI (asymptotic phase; $r = -0.84$; $p = 0.035$). These correlative measures indicated that expression of the hippocampal mitochondrial proteins VDAC1 and 2 may be of importance for memory performance in the water radial arm maze, and also for overall hippocampal cytoarchitecture as indicated by the VDAC correlations with DKI measures.

Discussion

We investigated the effects of short-term midazolam treatment and Western diet exposure on cognitive function and microstructural changes in the hippocampus in a non-surgical animal model using middle-aged male rats. The first main finding of this study was that both midazolam and Western diet exposure contributed to negatively affect the brain in middle-aged rats, indicated both by the learning parameters in the behavioral task and by MRI measures. The second main finding was that there were readily detectable changes in the cerebral microenvironment following pharmacological treatment, using *in vivo* diffusional kurtosis imaging, even after short-term MDZ administration. Furthermore, although a correlation cannot definitely pose a cause and effect, the significant correlations between DKI parameters and memory function indicate that this imaging method can be used to detect cognitive impairment, at least in animal models.

The overall effects of benzodiazepines on memory and cognition are well documented [41–43], and benzodiazepines have been associated with an increased risk of Alzheimer's disease [44]. However, the effects of midazolam on the brain microenvironment are still not fully understood, and very few imaging studies have been performed [45]. This study is the first application of DKI on the effects of midazolam and Western diet exposure. The findings demonstrate that these diffusion metrics are sensitive indicators of changes in the complexity of neurite architecture.

We found statistically significant increases in mean and axial kurtoses in the hippocampal tissue between control rats compared with midazolam exposed rats, regardless of their diet. The interpretation of changes in diffusion MRI metrics is complex. DKI enables the degree of diffusional non-Gaussianity to be quantified *in vivo*. Since diffusional non-Gaussianity is believed to arise from diffusion barriers, such as cell membranes and organelles, and water compartments (e.g., extracellular and intracellular), DK metrics can be regarded as indices of the complexity and heterogeneity of the tissue's microenvironment and can be altered by water exchange or by diffusion barriers [19]. The overall increase in kurtosis metrics may be related to the increase in mitochondrial membrane permeability, mitochondrial swelling and axonal and synapse degeneration due to the exposure to midazolam [6] and Western diets [46,47]. The increase in DK metrics in the hippocampus was accompanied by negative behavior changes and changes in mitochondrial proteins.

Rats exposed to midazolam showed significantly altered memory function in the water radial arm maze in the learning phase (days 2–6), indicative of delayed learning of the task. NC-MDZ and WD-MDZ exposed groups performed significantly worse during the learning phase as well as during the asymptomatic phase (days 7–12) compared to the NC group. The WD-MDZ exposed rats did not show significant improvement in performance across the two phases of the task. The lack of learning in this group was not related to the ability to swim, since WD-MDZ rats spent more time in the water compared to the other groups, instead it indicates that these rats needed the entire test period to complete the task, demonstrating that MDZ exposure gave rise to reduced memory function [10].

Western blot analysis of VDAC1 and VDAC2 expression revealed increased levels for both proteins in both of the midazolam treated groups, with statistical significance reached for the VDAC2 levels in the NC-MDZ group. VDAC1 and VDAC2 are found in the outer mitochondrial wall and are also part of the mitochondrial permeability transition pore (mtPTP), known as intracellular peripheral benzodiazepine binding receptor (PBR) [48]. Reduced VDAC1 was identified to be protective against Alzheimer's disease [49]. Studies have shown that enhanced expression and/or activation of the mitochondrial Translocator Protein (TSPO) leads to cell death [50], and activation of TSPO affects the function of VDAC-mediated transport of proteins in mitochondria [51]. Recent data also suggest that mitochondrial function is intricately involved in inflammatory processes, both in the brain and in the periphery [53]. Pearson correlations suggested that the two mitochondrial proteins might play a role for the behavioral and dMRI alterations observed, but further in-depth studies are needed to determine the impact of elevated VDAC1 or VDAC2 levels on the behavioral and imaging outcomes.

Midazolam and Western diet exposure have been associated with neurotoxicity, both in humans [54,55] and in animal studies [56]. For instance, in humans, it has been demonstrated that the administration of midazolam to “recovered” stroke and transient ischemic attack (TIA) patients causes transient reemergence of previous cerebral functional deficits [54,55]. In addition, midazolam has negative effects on brain maturation in rodents. Mice exposed to midazolam in the first 30 days of life exhibited thinner cerebral cortex with signs of apoptotic neurodegeneration [56]. Similarly, our group [10] and others [57,59] have demonstrated that high-fat diets are associated with cognitive impairment, with a specific emphasis on learning and memory functions that are dependent on the integrity of the hippocampus. However, this is the first study to demonstrate the negative effects on cognition and brain cytoarchitecture caused by MDZ and Western diet exposure. Future longitudinal studies will address other potential targets for intervention and also determine if these brain changes are permanent or reversible. Future studies will also be designed to further explore the potential of WD effects on liver function and the relationship between these observed changes in liver weight and brain alterations observed herein.

In conclusion, exposure to the short-acting benzodiazepine MDZ and Western diet seem to exhibit detrimental effects on the brain in middle-aged rats. We demonstrated alterations in behavior, VDAC expression and in DKI metrics in the hippocampus of rats exposed to midazolam and/or a combination of midazolam and Western diet. Our findings are timely and suggest a negative effect of anesthetic agents with diet in an animal model of middle-

aged rats. Translational studies need to confirm the effects of diet, aging and anesthetic drugs on cognition in humans. Diffusional kurtosis imaging is a powerful, clinically available *in vivo* technique to visualize microstructural changes leading to cognitive impairment triggered by an unhealthy diet and anesthetic drug exposure.

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Figure 1. Body weight gain, abdominal fat and liver weight for the three groups
 (A) Change in body weight, (B) abdominal fat and (C) liver weight after a 5.5 month exposure to the Western or control diets. Data are represented as mean $\pm$ SEM. Statistical differences were tested with one-way ANOVA followed by Sidak's post hoc test. Significant differences are signified in the bargraph: $p < 0.01$ *; $p < 0.001$ **; $p < 0.0001$ ***.

Figure 2. DKI maps

Example of diffusion MRI parametric maps: mean (MD), axial ($D_{\parallel}$) and radial ($D_{\perp}$) diffusivities; mean (MK), axial ($K_{\parallel}$) and radial ($K_{\perp}$) kurtoses. Notice the different diffusion contrast present on each parametric map.

Figure 3. Water Radial Arm Maze (WRAM) performance
 (A) Number of entered arms (NOE), (B) Working Memory Correct (WMC) errors and (C) time to complete the task during Trial 4 by the three groups during the learning phase (D2–D6) and the asymptotic phase (D7–D12). Data are represented as mean ± SEM. Statistical differences were tested with 2-way ANOVA followed by Sidak's post hoc test. Statistically significant differences were observed with: $p < 0.05$ *, $p < 0.01$ **.

Figure 4. VDAC protein levels in the hippocampus

Mean relative changes in VDAC1 and VDAC2 ($\pm$ SEM) obtained by Western blot analysis of the hippocampus region from NC (Control), NC-MDZ (rodent chow with MDZ) and WD-MDZ (Western diet with MDZ) rats. Statistical differences were tested with a one-way ANOVA followed by Sidak's post hoc test. The MDZ treatment increased VDAC2 levels significantly in the normal diet treated group ($p < 0.05^*$, and marginally in the WD-MDZ treated group).

Table 1

Diet composition.

Ingredient (g)	Control	Western
Casein	14	19.5
DL-Methionine	-	0.25
L-Cystine	0.18	-
Corn Starch	49.57	5
Maltodextrin 10	12.5	10
Sucrose	10	34.1
Cellulose	5	5
Corn oil	-	1
Milk fat	-	20
Soybean Oil	4	-
Cholesterol	-	0.15
t-butylhydroquinone	0.0008	-
Mineral Mix	3.5	3.5
Vitamin mix	1	1
Calcium carbonate	-	0.4
Ethoxyquin	-	0.004
Choline Bitartrate	0.25	-
% Calories from protein	14.7	17
% Calories from fat	9.4	41
% Calories from carbohydrate	75.9	43

Table 2Diffusion metrics estimates (mean $\pm$ SEM).

	MD ($\mu\text{m}^2/\text{ms}$)	$D_{//}$ ($\mu\text{m}^2/\text{ms}$)	$D_{\perp}$ ($\mu\text{m}^2/\text{ms}$)	MK	$K_{//}$	$K_{\perp}$
NC	0.808 $\pm$ 0.004	0.888 $\pm$ 0.005	0.766 $\pm$ 0.003	0.583 $\pm$ 0.013	0.624 $\pm$ 0.020	0.567 $\pm$ 0.015
NC-MDZ	0.817 $\pm$ 0.004	0.907 $\pm$ 0.006	0.772 $\pm$ 0.004	0.647 $\pm$ 0.015	0.722 $\pm$ 0.023	0.603 $\pm$ 0.017
WD-MDZ	0.805 $\pm$ 0.004	0.889 $\pm$ 0.005	0.764 $\pm$ 0.003	0.645 $\pm$ 0.013	0.711 $\pm$ 0.020	0.617 $\pm$ 0.015
<i>p values</i>						
NC vs. NC-MDZ	0.322	0.070	0.652	0.013	0.012	0.337
NC vs. WD-MDZ	0.953	0.998	0.935	0.009	0.015	0.079
WD-MDZ vs. NC-MDZ	0.151	0.097	0.349	1.000	0.981	0.901

Conventional rodent chow controls (NC); conventional rodent chew injected with midazolam (NC-MDZ) and Western diet injected with midazolam (WD-MDZ). Mean diffusivity (MD); axial diffusivity ($D_{//}$); radial diffusivity ($D_{\perp}$); mean kurtosis (MK); axial kurtosis ($K_{//}$); radial kurtosis ($K_{\perp}$).