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Early Cognitive and Hippocampal Alterations During Diet-Induced Metabolic Dysfunction in Sprague–Dawley Rats

Bayan Alsamarah
Jordan University of Science and Technology
Ibtihal Smadie
Jordan University of Science and Technology
Rasha Khader
Jordan University of Science and Technology
Khawla Nuseir
kqnuseir@just.edu.jo

Jordan University of Science and Technology

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Early Cognitive and Hippocampal Alterations During Diet-Induced Metabolic Dysfunction in Sprague–Dawley Rats

Authors[1]

[1]Department of Clinical Pharmacy, Faculty of Pharmacy, Jordan University of Science & Technology, Irbid, Jordan

Corresponding Author:

Khawla Nuseir, PhD

Department of Clinical Pharmacy

Faculty of Pharmacy

Jordan University of Science and Technology

Irbid, Jordan, 22110

kqnuseir@just.edu.jo

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Abstract

Background: Diet-induced obesity and insulin resistance are associated with an increased risk of cognitive impairment, yet the onset of these changes before overt type 2 diabetes (T2DM) remains poorly understood. This study investigated cognitive and hippocampal alterations associated with diet-induced metabolic dysfunction in a pre-diabetic rat model. **Methods:** Forty-eight male and female Sprague–Dawley rats were fed either a high-fat diet (HFD) with fructose water or a regular diet for 21 weeks. Behavioural assessments were performed after 15 weeks of dietary intervention, before streptozotocin (STZ) administration, to evaluate cognitive function during the pre-diabetic stage. Metabolic parameters were measured throughout the study, and hippocampal and serum biomarkers of neurodegeneration, oxidative stress, and neuroplasticity were analysed at the study endpoint. **Results:** HFD significantly increased body weight, fasting blood glucose, and insulin resistance (all $P < 0.0001$). Behavioural testing revealed impaired long-term memory consolidation without affecting spatial learning. Hippocampal analysis demonstrated increased MMP-9, phosphorylated tau, and β-amyloid levels, indicating early neurodegenerative changes. Unexpectedly, hippocampal BDNF levels were elevated in HFD male rats ($P < 0.05$), suggesting a potential compensatory response during early metabolic dysfunction. **Conclusion:** Diet-induced metabolic dysfunction was associated with early cognitive impairment and hippocampal molecular alterations before the development of overt T2DM. These findings suggest that metabolic disturbances may initiate neurobiological changes preceding clinical diabetes and support the existence of an early window for intervention.

Introduction

Metabolic dysfunction can affect the brain long before the clinical onset of diabetes, suggesting that cognitive vulnerability may emerge during the earliest stages of impaired metabolic regulation(Garcia-Serrano & Duarte, 2020; Soares et al., 2013). Type 2 diabetes mellitus (T2DM), which represents the majority of diabetes cases, is characterized by progressive insulin resistance and pancreatic β-cell dysfunction (Haines, 2023; Hossain et al., 2024). Beyond its well-established metabolic and vascular complications, T2DM has increasingly been associated with cognitive impairment, with mild cognitive impairment reported in substantial proportion of affected individuals (You et al., 2021). Epidemiological studies have further linked diabetes and related metabolic disorders to an increased risk of dementia and Alzheimer's disease (AD) (Jeffry, 2023; Tseng, 2026), suggesting that metabolic abnormalities may contribute to neurodegenerative processes (Mehla et al., 2014; Zhang et al., 2017). Insulin signaling within the brain contributes to neuronal growth, synaptic plasticity, and memory formation (Agrawal et al., 2014; Mehla et al., 2014). Chronic metabolic disturbances can impair central insulin signaling and promote oxidative stress, neuroinflammation, and synaptic dysfunction, processes that are closely associated with cognitive decline (Agrawal et al., 2014; Garcia-Serrano & Duarte, 2020). Importantly, accumulating evidence suggests that these alterations may begin during earlier metabolic dysfunction, before the onset of clinically apparent diabetes (Garcia-Serrano & Duarte, 2020; Soares et al., 2013). Despite growing evidence linking diabetes with cognitive decline, most experimental investigations have focused on established diabetes after persistent hyperglycemia has developed (Palazzo et al., 2024). Consequently, the early effects of metabolic dysfunction on brain function remain insufficiently understood. High-fat diet (HFD)-fed rodents provide a useful model of obesity, insulin resistance, and early glucose dysregulation that precede overt diabetes

(Gannon et al., 2022; Soares et al., 2013). Although cognitive deficits have been reported in these models (Soares et al., 2013), it remains unclear whether these early metabolic disturbances are accompanied by hippocampal changes in neuroplasticity, neurodegenerative signaling, and oxidative stress before hyperglycemia becomes established (Gannon et al., 2022).

The hippocampus is particularly vulnerable to metabolic stress because of its critical role in learning and memory and its dependence on intact insulin signaling and synaptic plasticity (Spinelli et al., 2017, 2019). Therefore, this study examined multiple molecular pathways implicated in metabolic-associated cognitive dysfunction. BDNF (abbreviations are defined in Supplementary Table S1) and NT-3 were assessed as indicators of synaptic plasticity and neurotrophic support (Agrawal et al., 2014; Garcia-Serrano & Duarte, 2020)whereas Aβ and P-tau were evaluated as markers associated with neurodegenerative processes (Dubey et al., 2022; Kim et al., 2009; Mehla et al., 2014). MMP-9 was examined for its role in synaptic remodeling, blood-brain barrier integrity, and neuroinflammation (Dubey et al., 2022; Kim et al., 2009). In addition, AGEs and GPX were measured to assess metabolic and oxidative stress responses (Qaed et al., 2025; Tsiknia et al., 2022). Because sex can influence metabolic responses, cognitive outcomes, and susceptibility to neurobiological stress, potential sex-dependent differences were also investigated (Shin et al., 2020).

Accordingly, this study investigated whether prolonged HFD exposure induces cognitive and hippocampal alterations during diet-induced metabolic dysfunction before the development of overt diabetes. Behavioral assessments were conducted before streptozotocin administration to isolate the effects of diet-induced metabolic changes on cognition. We hypothesized that prolonged metabolic dysregulation would impair memory performance and be associated with early hippocampal molecular alterations before overt hyperglycemia develops.

Materials and Methods

Animals and Treatments

A total of 48 five-week-old Sprague–Dawley (SD) rats (body weight: 65–125 g) were obtained from the animal facility at Jordan University of Science and Technology (JUST). Experimental procedures were initiated after approval from the Animal Care and Use Committee (ACUC) at JUST (approval no. 72/12/4/16) and were conducted in accordance with the National Institute of Health's guide for the Care and Use of Laboratory Animals. The rats were maintained under standard laboratory conditions at a temperature of 24 ± 1 °C with a 12-hour light/dark cycle beginning at 07:00 a.m. Animals had ad libitum access to water and standard laboratory feed prior to dietary manipulation.

Rats were naive to drug treatments and experimentation at the beginning of all studies. All tests were conducted between 08:00 a.m. and 04:00 p.m. Following one-week acclimatization period, rats were randomly assigned according to dietary intervention and sex into four experimental groups (n = 12/group): high-fat diet male group (HFDM) with STZ injection; high-fat diet female group (HFDF) with STZ injection; regular diet male group (RDM) without STZ injection; and regular diet female group (RDF) without STZ injection. The RD groups served as

the controls. The experimental timeline is illustrated in **Figure 1**.

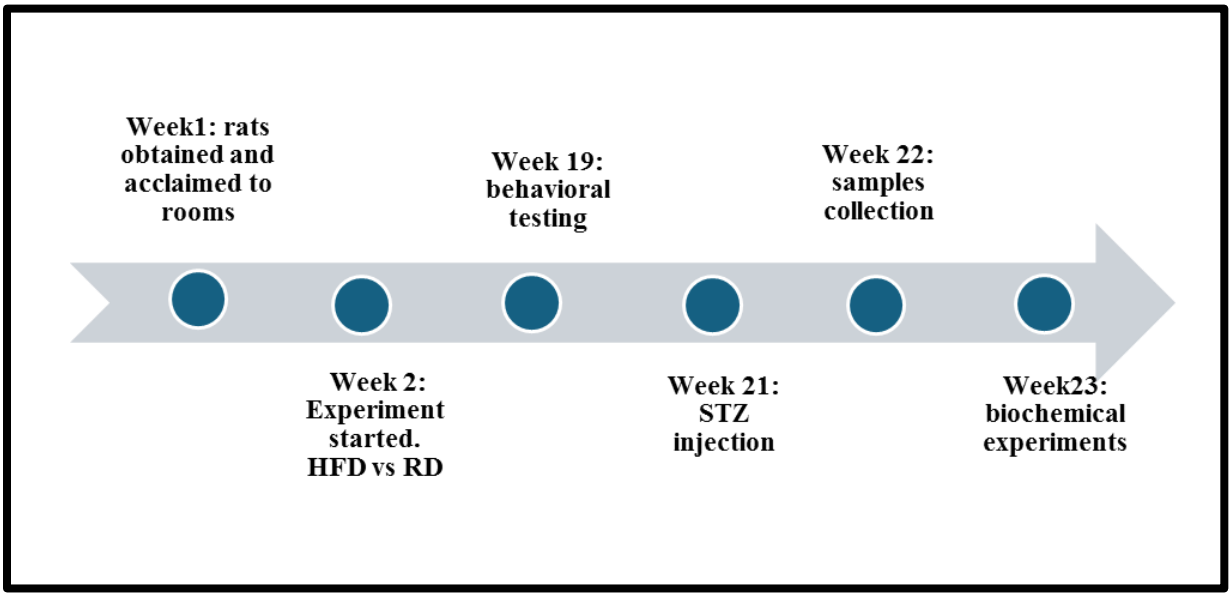


Figure 1. Schematic Representation of The Project Timeline

Behavioral Experiments

Behavioral assessments were conducted after 15 weeks of dietary manipulation and prior to STZ administration to evaluate cognitive changes associated with diet-induced metabolic dysfunction before induction of overt diabetes. STZ was administered following behavioral testing, and hippocampal and serum biomarkers were assessed at the study endpoint. Rats with fasting blood glucose levels ≥ 200 mg/dL four days after STZ administration were considered diabetic. All rats completed behavioral testing. One RDF rat was excluded from biochemical analyses due to detection of an abnormal mass following behavioral testing. Mortality following STZ administration resulted in further exclusion of seven HFDM and three HFDF animals from biochemical analyses. (**Supplementary Table S2**).

Induction of Hyperglycemia Following Dietary Intervention

Diabetes was induced in the rats following the method described by Anoka A. Njan et al (Njan et al., 2020). In short, HFD (31.4% fat, 12% carbohydrate, and 13% protein). Fructose water was provided concurrently at a concentration ranging from 10% to 20%, adjusted based on observed consumption; concentration was increased when intake was low and decreased when consumption was adequate, to ensure consistent fructose exposure across the study period. Following the dietary intervention period, rats were fasted for 6 hours and injected intraperitoneally with STZ (MedChemExpress® CO., Monmouth junction, USA). STZ was dissolved in 0.1M citrate buffer, pH 4.4, and administered at a dose of 40 mg/kg. The STZ-injected rats were given a 10% glucose solution for 3 days to prevent death from STZ-induced hypoglycemic shock. To assess and confirm diabetes, blood samples were obtained from the tail vein and evaluated for fasting blood glucose (FBG) (Akbarzadeh et al., 2007).

Preparation of High Fat Diet (HFD)

During the 21-week dietary intervention, rats had ad libitum access to HFD (62.1% kcal from fat), or RD (10.4% kcal from fat) according to their experimental group assignment. The fat source consisted of sheep rump fat, which was melted and incorporated into standard rat chow according to calculated proportions. The mixture was homogenized for 30 min and converted into pellet. Food was replaced daily to maintain freshness (Kolahdouzi et al., 2019).

Metabolic Parameters

The following parameters were measured during the experimental period.

Rats' Body Weight

Rats' body weights were measured and recorded at baseline and weekly thereafter using an electronic weighing scale.

Fasting Blood Glucose (FBG)

FBG was determined using a GlucoLab® meter (Osang Healthcare Co., Anyang, Republic of Korea). FBG levels were measured at the following time points: 15 weeks (before the behavioral tests), 21 weeks (just after completion of behavioral tests and before STZ injection), two days after STZ injection (to exclude hyperglycemia), and five days after STZ injection.

Fasting Insulin (FI)

FI was determined using a Rat Insulin Enzyme-linked immunosorbent assay (ELISA) kit (Genochem World Co., Valencia, Spain). FI levels were measured at: 15 weeks (before the behavioral tests), 21 weeks (just after completion of behavioral tests and before STZ injection), and five days after STZ injection.

Insulin Resistance Assessment

Insulin resistance was expressed as the homeostasis model assessment-insulin resistance (HOMA-IR): HOMA-IR= fasting Insulin (μU/mL) x [fasting Glucose (mg/dL)/405]

Behavioral Experiments

Open field test (OFT)

Open field experiment was run as previously explained ((Nuseir et al., 2024)). Briefly, rats were placed in the middle of a clear box divided into 16 squares and left to explore freely for 15 minutes. Video record was taken to be assed later for parameters such as time spent in each area and number of crossings of each.

Elevated Plus Maze (EPM)

This test was performed as previously described (Nuseir et al., 2024). In brief, rats were put in the center of two raised arms and allowed to freely choose either the closed or open arm. Video recordings of 5 minutes exploration were later assessed for number and time of entries.

Radial Arm Water Maze (RAWM)

RAWM investigates learning and memory including short- and long-term memory performance in models of aging and neurodegenerative diseases like AD **[8]**. The test was performed using a black circular stainless-steel pool (diameter:167 cm; height: 55 cm; depth: 43 cm). Six V-shaped stainless-steel plates (height:49 cm; length: 55 cm) were used to form a swimming arena with six arms (width: 35 cm). A hidden platform was placed at the end of one arm and served as the goal. The test was conducted in four phases: acclimation, learning, short-term memory evaluation, and long-term memory evaluation. During acclimation, animals underwent six trials. The goal platform was positioned 2 cm above the water surface under normal lighting conditions and without visual cues. On the following day, learning and memory were assessed using a 12-trial protocol, with 5-min rest period after the first six trials. During this phase, the goal platform was submerged 2 cm below the water surface, lighting was dim, and visual cues were provided. Rats were allowed up to 60 s to locate the platform. Animals failing to reach the platform within this period were gently guided to it, and an error was recorded. The number of errors was the number of entries into an arm other than the arm containing the goal. Rats were required to successfully complete at least 80% of learning trials to be considered eligible for memory testing.

Short-term memory was evaluated as a single trial conducted 30 minutes after the learning phase, while long-term memory was assessed in single trials performed 5 h and 24 h after training.

Brain Dissection and Blood Collection

Rats were fasted for 6 hours prior to terminal blood and tissue collection. One week after STZ injection, rats were euthanized by rapid decapitation. The brains were immediately extracted, and the hippocampi were carefully dissected. Brain tissues were placed on filter paper over crushed ice and rinsed with normal saline to remove residual blood. The isolated hippocampus was transferred to pre-labeled 1.5 ml tubes, immediately snap-frozen in liquid nitrogen, and subsequently stored at −80 °C until analysis. Blood samples were collected and centrifuged at 4,500 rpm for 10 min at room temperature to obtain serum which was then stored at −20 °C.

For ELISA analysis, hippocampal samples were homogenized by adding 400 µL of freshly prepared phosphate-buffered saline (PBS) with protease inhibitors to each sample. The tissues were manually homogenized using a spatula and subsequently centrifuged at 15,000 rpm for 20 min at room temperature. The resulting supernatant was aliquoted into three 1.5 ml tubes and stored at −20 °C for further analysis.

Enzyme-Linked Immunosorbent Assay (ELISA)

Commercially available ELISA kits (Genochem World, Valencia, Spain) were used to quantify serum markers of oxidative/metabolic stress (GPX and AGEs) and hippocampal markers associated with neuroplasticity and neurodegenerative pathways (BDNF, NT-3, Aβ, phosphorylated tau, and MMP-9).

The concentrations of these biological markers in serum and hippocampal samples were determined using ELISA according to the manufacturer's instructions. Sample concentrations were calculated from a standard curve using GainData Arigo ELISA data calculator. Hippocampal markers concentrations were normalized to the total hippocampal protein content, which was determined using the standard Bio-Rad® protein assay.

Statistical Analysis

Data distribution was assessed using the Shapiro–Wilk normality test (α = 0.05; **Table S3**). Body weight, glucose levels, metabolic parameters, behavioral outcomes, and biochemical markers were analyzed using two-way ANOVA with diet and sex as factors, followed by Tukey's multiple comparisons test when significant effects were detected. For glucose measurements obtained at multiple time points, repeated-measures analysis was performed where appropriate. When comparisons were limited to two groups, an unpaired Student's t-test was applied. Outlier analysis was performed only for individual biomarker datasets using GraphPad Prism criteria. All analyses were performed using GraphPad Prism 9.5.1 (GraphPad Software, La Jolla, CA, USA), and data are presented as mean ± SEM. Statistical significance was set at $P < 0.05$.

Sensitivity analysis

Outliers were identified using the ROUT method (Q = 1%) in GraphPad Prism.

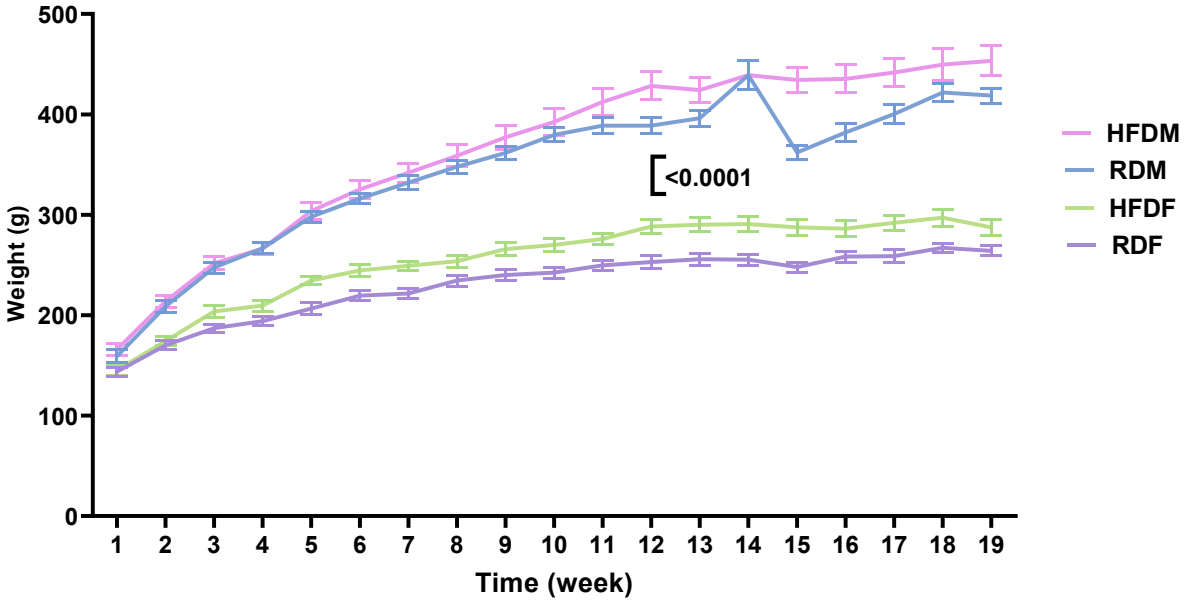
Sensitivity analysis was performed for the RAWM dataset by repeating the statistical analysis after exclusion of identified outliers. The same statistical approach used in the primary analysis was applied to the sensitivity dataset to allow direct comparison. Results were consistent with the primary analysis, with no changes in the direction or statistical interpretation of the observed effects (**Figure S1**), indicating that the findings were not substantially influenced by extreme values.

Results

HFD increased body weight compared with RD

During the first 12 weeks of dietary intervention, body weight did not differ significantly between dietary groups within either sex. From week 12 onward, HFD-fed rats exhibited significantly greater body weight gain compared with sex-matched RD controls ($F_{(18, 834)} = 217.9$, $P < 0.0001$;

241 **Figure 2**).



242

243 **Figure 2. Rats' Body Weight Over Time.**

244 Body weight was recorded weekly throughout the dietary intervention. Two-way ANOVA with
245 Tukey's multiple comparisons test demonstrated a significant effect of diet beginning at week 12.
246 RDM: regular diet male; RDF: regular diet female; HFDM: high-fat, high-fructose diet male;
247 HFDF: high-fat, high-fructose diet female. Data is presented as mean ± SEM of N = 11–12 rats
248 per group.

249 ***Rats in HFD groups had higher fasting blood glucose levels than RD groups.***

250 Fasting blood glucose levels were assessed at three key time points: week 15 of dietary
251 manipulation (just before behavioral testing), week 21 (immediately after behavioral testing),
252 two days after STZ administration (only for HFD group), and one week following STZ
253 administration. Because neither sex nor the diet × sex interaction significantly affected fasting
254 blood glucose levels, male and female groups were combined for diet-based comparisons (Two-
255 way ANOVA followed by Tukey's multiple comparisons test ($F_{(1, 44)} = 0.1298$, $P = 0.7204$)). At

week 15, rats in the HFD group showed a significant elevation in fasting blood glucose compared to the RD group ($t_{(46)} = 2.050$, $P = 0.0461$; **Figure 3A**). By week 21, no significant differences were observed between diet groups ($t_{(45)} = 0.895$, $P = 0.3758$; **Figure 3B**). Following STZ administration, HFD-fed rats developed marked hyperglycemia compared with RD controls ($t_{(35)} = 22.05$, $P < 0.0001$; **Figure 3C**)

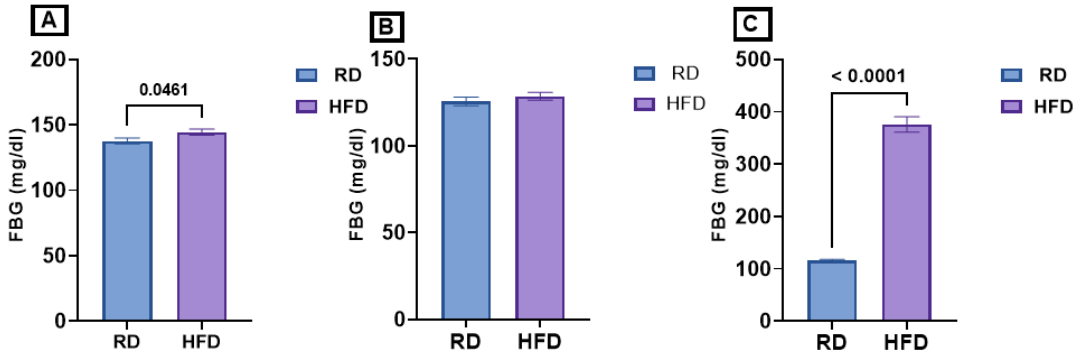


Figure 3. Fasting Blood Glucose Levels.

Fasting blood glucose levels were measured at three time points: (A) week 15 (before behavioral testing), (B) week 21 (after behavioral testing), and (C) one week following STZ injection. Unpaired t-tests revealed significant differences between diet groups at week 15, and one week after STZ injection, but not at week 21. Error bars represent mean ± SEM of $N = 14–24$ rats per group.

HFD combined with STZ administration increased HOMA-IR

Insulin resistance was assessed using the HOMA-IR model at three points corresponding to fasting glucose and insulin measurements (week 15, week 21, and one week after STZ). At weeks 15 and 21, HFD rats showed a non-significant tendency toward higher HOMA-IR values compared with

RD controls. Following STZ administration, HOMA-IR was significantly increased in HFD rats compared with RD controls ($F_{(3, 33)} = 86.61$, $P < 0.0001$; **Figure 4**).

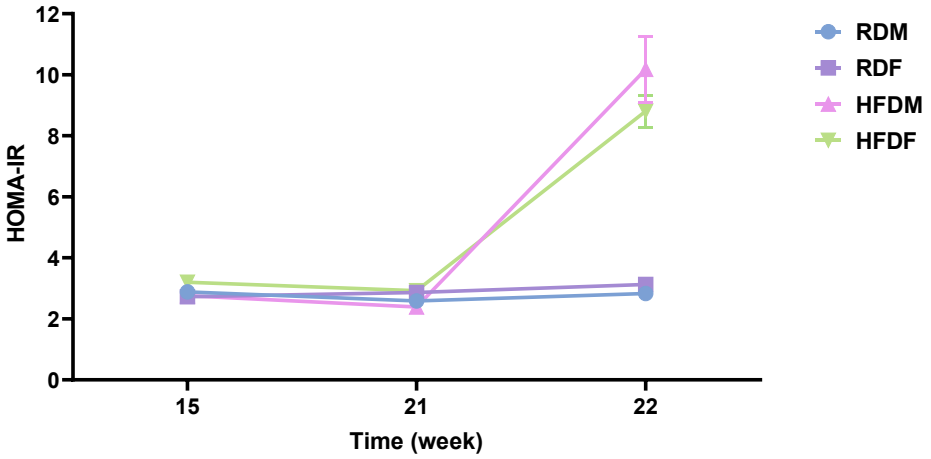


Figure 4: Insulin Resistance Assessed by HOMA-IR.

Insulin resistance values were calculated using the HOMA-IR equation at weeks 15 and 21 and one week after STZ. Two-way ANOVA followed by Tukey's multiple comparisons test revealed a significant increase in insulin resistance was observed in the HFD groups compared to RD groups one-week post-STZ Data are presented as mean ± SEM of $N = 5–12$ rats per group.

Female rats in the HFD group exhibited higher numbers of rearing in the open field test.

Locomotor activity and anxiety-like behaviors were assessed using OFT following 15 weeks of dietary intervention (**Figure 5**). Six behavioral parameters were analyzed: number of rearing episodes, time spent in the center, number of crossed squares, number of grooming episodes, number of defecation pellets, and number of freezing episodes. HFDF rats displayed significantly

increased rearing frequency compared with HFDM rats ($F_{(1, 43)} = 15.41$, $P = 0.0013$; **Figure 5A**). No significant effects of diet or sex were observed for the remaining behavioral parameters.

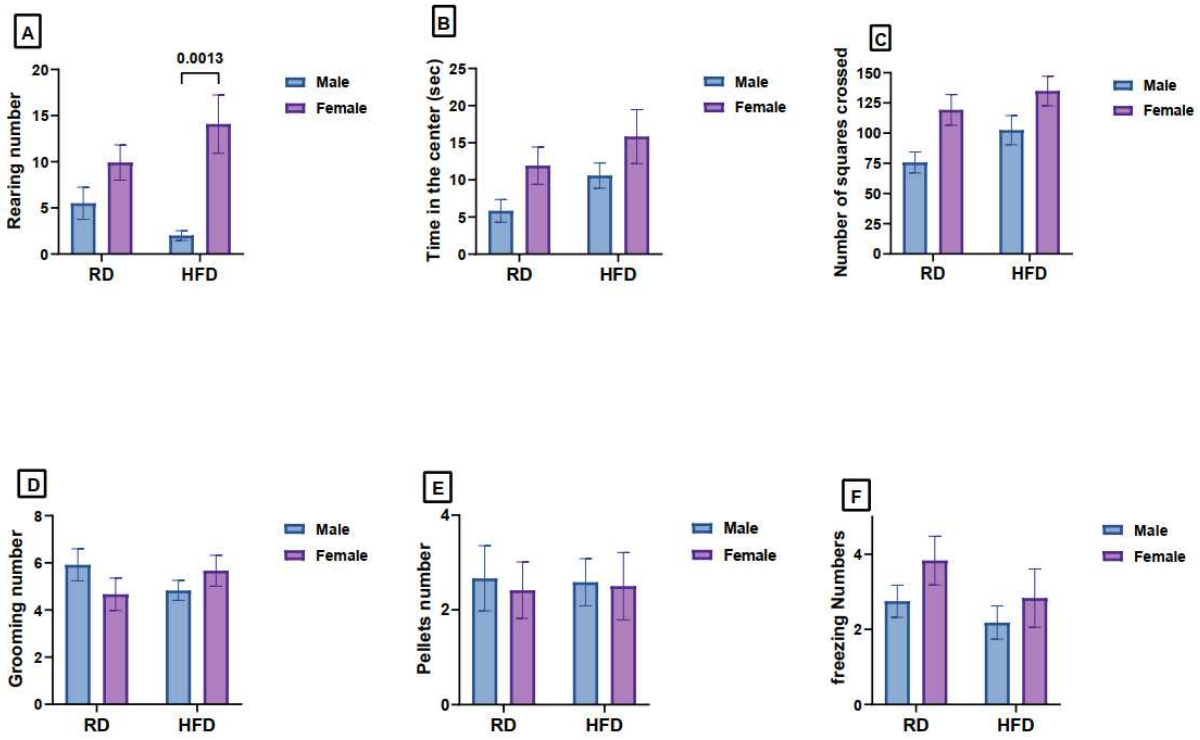


Figure 5: Open Field Test Results

Assessed parameters included: (A) rearing frequency, (B) time spent in the center, (C) number of crossed squares, (D) number of grooming episodes, (E) number of defecation pellets, and (F) number of freezing episodes. Two-way ANOVA followed by Tukey's multiple comparisons test was performed and revealed a significant difference in rearing frequency between HFDM and HFDF groups. Data represented as mean ± SEM of $N = 12$ rats per group.

HFD reduced open-arm exploration in female rats

Anxiety-like behaviors were assessed using the EPM test following 15 weeks of dietary intervention (**Figure 6**). Four behavioral parameters were analyzed: number of open arm entries,

number of closed arm entries, time spent in open arms, and time spent in closed arms. The HFDF group entered the open arms less frequently than the RDF group ($F_{(1, 37)} = 8.494$, $P = 0.0060$; **Figure 6A**). No significant differences were observed in the number of closed arm entries or time spent in open arms between diet or sex groups (**Figure 6B, C**). HFDF rats spent significantly less time in the closed arms compared to RD female rats ($F_{(1, 35)} = 10.06$, $P = 0.0031$; **Figure 6D**).

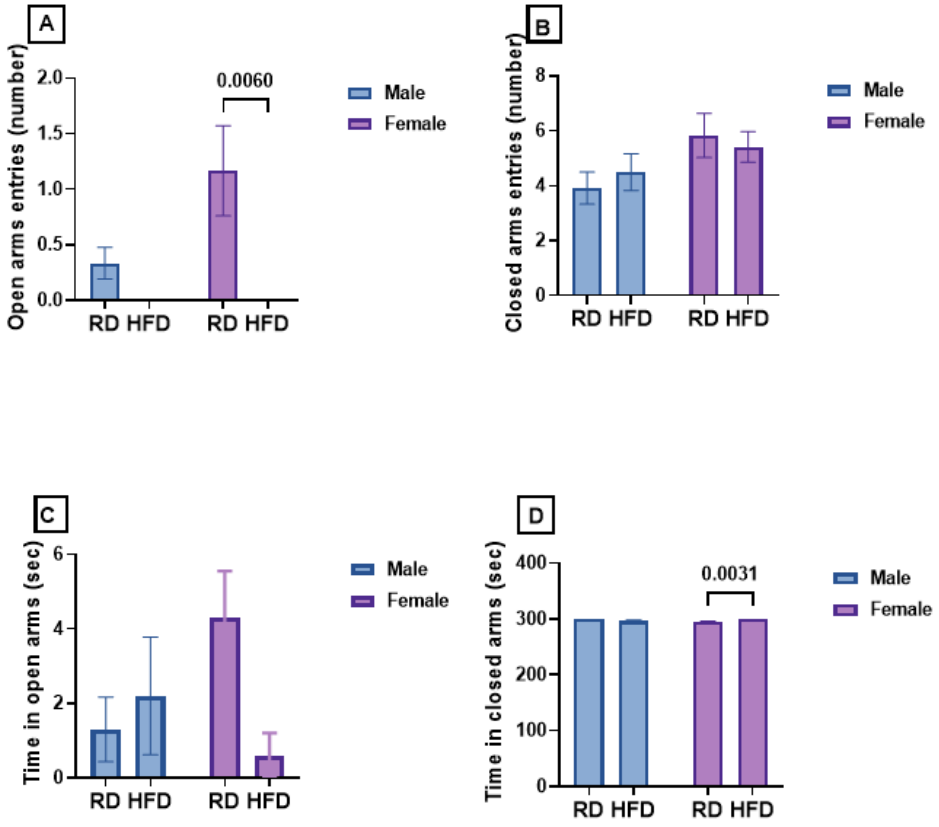


Figure 6: Elevated Plus Maze Results

Assessed parameters included: (A) number of open arm entries, (B) number of closed arm entries, (C) time spent in open arms, and (D) time spent in closed arms. Two-way ANOVA followed by Tukey's multiple comparisons test revealed a significant main effect of diet on open arm entries ($F(1,37) = 8.494$, $P = 0.0060$). Post hoc analysis showed that HFDF rats entered the open arms

significantly less frequently than RDF rats (adjusted P = 0.0407). A significant diet × sex interaction was observed for time spent in the closed arms (F (1,35) = 10.06, P = 0.0031). No significant differences were observed in the number of closed arm entries or time spent in open arms. Error bars represent mean ± SEM of N = 12 rats per group.

HFD impaired long-term spatial memory performance

Short-term memory performance was not affected by diet; however, females exhibited a higher number of errors than males ($F_{(1, 39)}$ = 18.64, P = 0.0001; **Figure 7A**). At 5 h post-training, a significant diet × sex interaction was observed, with HFDF rats showing fewer errors than RDF rats ($F_{(1, 39)}$ = 9.099, P = 0.0045; **Figure 7B**). At 24 h post-training, HFD animals of both sexes demonstrated impaired long-term memory, with increased errors compared with RD controls ($F_{(1, 39)}$ = 12.38, p = 0.0011; **Figure 7C**).

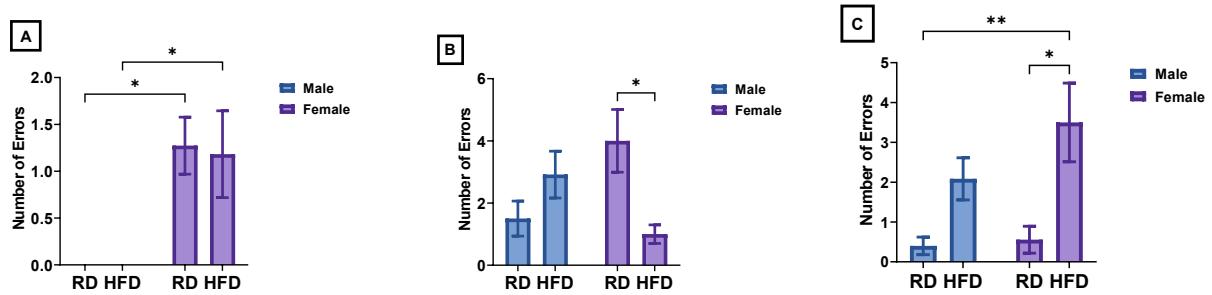


Figure 7. Radial Arm Water Maze Test Results.

Rat memory performance was assessed at (A) 30 min, (B) 5 h, and (C) 24 h post-training. Two-way ANOVA followed by Tukey's multiple comparisons test was performed and revealed the following: (A) only sex (but not diet) has a significant effect. (B) Unlike male rat groups, HFDF rats made significantly lower errors than RDF rats. (C) HFD rats of both sexes committed

significantly more errors than RD rats. Error bars represent mean ± SEM of N = 11–12 rats per group.

Hippocampal Molecular Markers

To assess molecular alterations associated with the observed behavioral outcomes, hippocampal levels of neuroplasticity and neurodegeneration-related markers were measured at the study endpoint. Normalized BDNF levels were significantly higher in the HFDM group compared with both RDM (P = 0.0004) and HFDF (P = 0.0048) (**Figure 8A**). A similar pattern was observed for normalized MMP-9 (**Figure 8B),** with significantly higher levels in HFDM compared with RDM (P = 0.0014) and HFDF (P = 0.0080). P-Tau levels (**Figure 8C)** were likewise significantly elevated in HFDM relative to RDM (P = 0.0055) and HFDF (P = 0.0252), and Beta-amyloid levels (**Figure 8D**) followed the same trend HFDM significantly higher than both RDM (P = 0.0058) and HFDF (P = 0.0273).

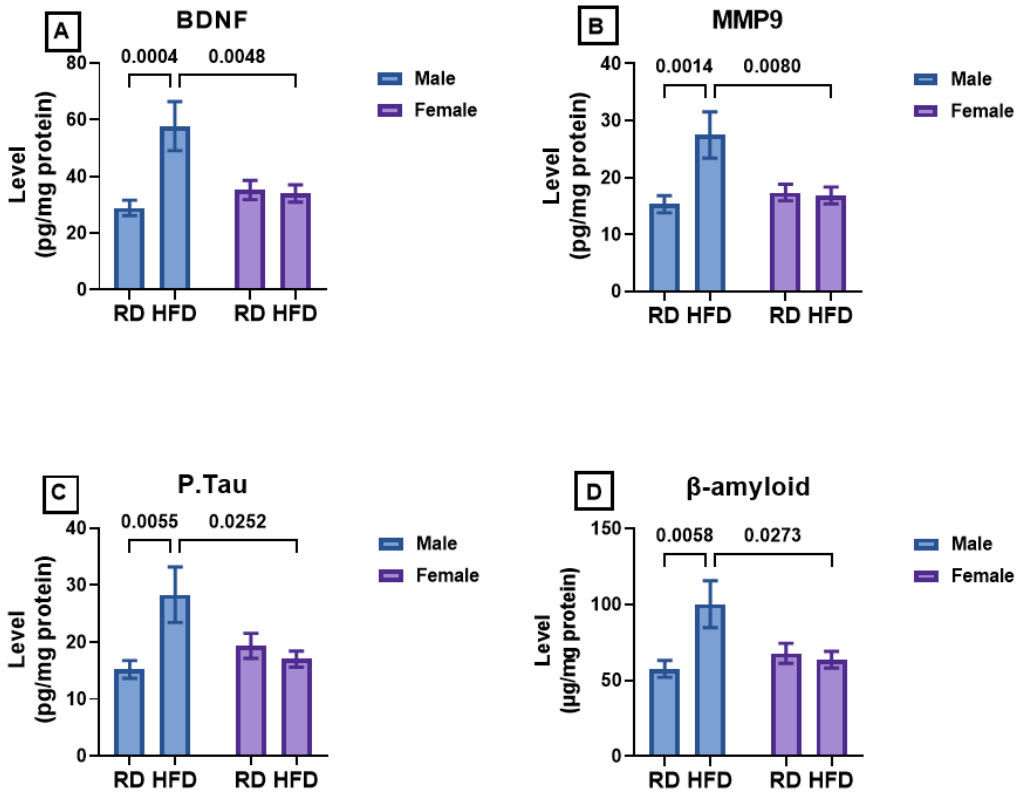


Figure 8. Hippocampal Cognitive Biomarkers.

Following a long-term high-fat, high-fructose diet (HFD) and STZ administration, levels of (**A**) BDNF, (**B**) MMP-9, (**C**) P-Tau, and (**D**) Beta-amyloid were quantified. Two-way ANOVA followed by Tukey's multiple comparisons test was performed and revealed significant differences between HFDM and both RDM and HFDF groups. Concentrations were normalized to total hippocampal protein. Error bars represent mean ± SEM; $N = 5–12$ rats per group.

Serum metabolic and neurotrophic markers

To evaluate whether these hippocampal alterations were reflected systemically, serum levels of neurotrophic and oxidative stress-related markers were also measured at the end of the study. Serum AGEs levels were not significantly affected by diet or sex (**Figure 9A**). However, HFDM rats exhibited increased GPX levels compared with RDF ($P = 0.0458$) and HFDF ($P = 0.0184$)

(**Figure 9B**). Serum levels of the neurotrophic factor NT-3 were significantly elevated in HFDM compared with RDM ($P = 0.0418$; **Figure 9C**).

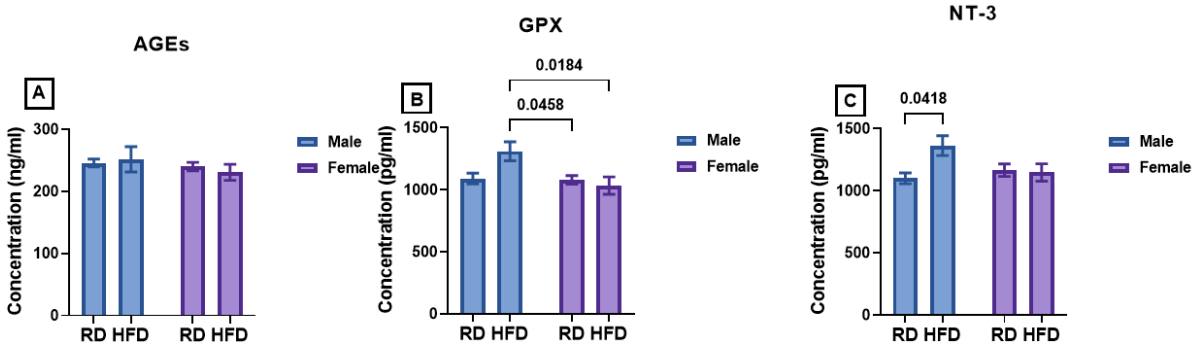


Figure 9. Serum Oxidative Stress and Neurotrophic Markers.

Following a long-term high-fat, high-fructose diet (HFD) and STZ administration, levels of (**A**) AGEs, (**B**) GPX**,** and (**C**) NT-3 were quantified in all groups. Statistical significance was determined by two-way ANOVA (Diet × Sex) followed by Tukey's multiple comparisons test. Error bars represent mean ± SEM; $N = 5–12$ rats per group.

Discussion

Metabolic consequences of HFD exposure before overt diabetes

The present findings indicate that cognitive and neurobiological alterations were associated with diet-induced metabolic dysfunction before the development of overt diabetes. Behavioral assessment was performed before STZ administration, whereas molecular analyses were conducted after STZ-induced metabolic progression. Therefore, these findings represent distinct stages of metabolic alteration rather than a single experimental time point.

Consistent with previous reports, rats fed HFD exhibited significant weight gain beginning at week 12 of dietary intervention, which persisted until the end of the study, even in the absence of STZ

administration (Zhao et al., 2022)[23]. Notably, male rats in the HFD group gained more weight than their female counterparts. Increased body weight is a key feature of diet-induced metabolic dysfunction and is commonly associated with adipose inflammation, insulin resistance, and progression towards T2DM (Barriere et al., 2018; Buettner et al., 2006; Hafizur, 2015; Malicevic et al., 2025; Zaman et al., 2011)[24-28]. These findings confirm the metabolic impact of the HFD model and its relevance to metabolic syndrome development.

Despite increased fasting glucose, fasting insulin and HOMA-IR remained unchanged during this stage, suggesting that early metabolic impairment occurred without marked systemic insulin resistance. This patternmatches prior observations in SD rats (Hafizur, 2015; Marques et al., 2016; Zhao et al., 2022)[23, 25, 29] The reduction in glucose levels after behavioral testing may partly reflect increased physical activity associated with repeated swimming exposure during RAWM testing (Caponi et al., 2013; Ghiasi et al., 2015)[30, 31]; however, physical activity was not directly measured in this study and this interpretation remains speculative. One week following STZ administration, both FBG and HOMA-IR increased markedly in the HFD groups, confirming progression toward a diabetic metabolic phenotype. Notably, the effects were more pronounced in males, paralleling their greater weight gain and suggesting sex-specific differences in metabolic vulnerability. These findings are consistent with previous studies demonstrating that low-dose STZ induces progressive pancreatic stress while early endocrine function may be partially preserved. (Barriere et al., 2018; Hu et al., 2013; Malicevic et al., 2025; Zhao et al., 2022)[23, 27, 28, 32].

Behavioral alterations associated with diet-induced metabolic dysfunction

In the OFT, locomotor activity was unaffected by diet or sex, suggesting that differences in cognitive performance were unlikely to be explained by altered general activity. The observed

differences in rearing behaviour indicate a possible sex-dependent behavioural response to dietary challenge. In the EPM, HFD females exhibited reduced open-arm entries and increased closed-arm preference compared with RD females, whereas males showed no significant differences (Attrill et al., 2025)[33]. The pattern of behavioral effects differed between tests, suggesting task-dependent and sex-specific expression of anxiety-related behavior (Shukitt-Hale et al., 2004)[34]. Spatial learning and memory were assessed using the RAWM, a hippocampus-dependent task (Ghafarimoghadam et al., 2022)[35]. All groups showed progressive reductions in errors during acquisition, indicating intact learning across conditions (Alqudah et al., 2022)[36]. In the short-term memory test, females showed more errors than males regardless of diet (Srivastava et al., 2023)[37].

At the 5-hour time point, a sex-dependent interaction was observed: RDF rats committed significantly more errors than HFDF rats, whereas male groups were unaffected by diet. This unexpected finding does not indicate impairment in HFD females; rather, it may reflect baseline variability or other factors influencing intermediate memory performance and should therefore be interpreted cautiously. By 24 hours, memory impairment was evident in both HFD groups, consistent with impaired long-term memory retention following prolonged HFD exposure (Alqudah et al., 2022; Attrill et al., 2025; Barriere et al., 2018; Shukitt-Hale et al., 2004)[28, 33, 34, 36].

Hippocampal molecular responses to metabolic stress

Hippocampal biomarker analysis revealed a male-predominant elevation across BDNF, MMP-9, P-Tau, and Aβ. Notably, increased hippocampal Aβ levels, a marker associated with AD-related pathology (Malicevic et al., 2025; Ohta et al., 2014)[27, 38], were observed in HFD males, which

is consistent with memory impairment observed in the RAWM. Elevated hippocampal MMP-9 levels in HFD males further indicate activation of pathways associated with synaptic remodelling and neuroinflammatory processes (Li, 2025; Tsiknia et al., 2022; Wang et al., 2014; Whelan et al., 2019)[18, 39-41].

In parallel, hippocampal phosphorylated tau levels were significantly elevated in HFD male rats. Tau hyperphosphorylation disrupts microtubule stability and synaptic integrity and is a key contributor to neurodegeneration in AD (Liang et al., 2023; Peng et al., 2013)[42, 43]. Together, these molecular changes provide support for the observed cognitive deficits.

Contrary to expectations, BDNF and NT-3 were not reduced in the HFD groups; both were maintained or elevated, with males showing a notable increase. This aligns with evidence that short-term metabolic stress does not necessarily suppress neurotrophic signaling (Omidi et al., 2020; Shin et al., 2020)[19, 44]. The absence of reduced neurotrophic markers may reflect compensatory responses to metabolic stress, temporal differences in biomarker expression, or other adaptive mechanisms(Marmigère et al., 2003; Sugiyama et al., 2020) [45, 46]. Alternatively, the timing of tissue collection relative to metabolic exposure may have influenced these results (de Paula et al., 2021)[47].

Serum AGEs, an important mediator associated with diabetic complications, were not significantly elevated in this study. This finding fits with evidence indicating that AGEs accumulation requires prolonged hyperglycemia and high-protein intake (Qaed et al., 2025; Xue et al., 2025)[17, 48], conditions not yet fully established at the one-week post-STZ endpoint examined here.

In contrast, serum GPX, an antioxidant enzyme, was significantly elevated in HFDM relative to RDF and HFDF, suggesting a sex-specific oxidative stress response to diet-induced metabolic

challenge in males. Considered alongside the unchanged AGEs levels, this may reflect an early antioxidant compensatory response that precedes measurable glycation-related injury.

Sex-dependent responses to metabolic dysfunction

Across these outcomes, a sex-dependent pattern was observed. Females showed distinct behavioural responses, including altered anxiety-related measures and sex-related differences in short-term memory performance, whereas males showed greater hippocampal molecular alterations. This divergence aligns with evidence of sex-specific susceptibility to metabolic stress and indicates that diet-induced metabolic dysregulation is associated with distinct behavioral and neurobiological profiles in males and females.

Implications for early brain vulnerability during metabolic progression

Collectively, these findings support a conceptual model in which HFD-induced metabolic stress is associated with early behavioral and hippocampal molecular alterations, while overt peripheral metabolic dysfunction becomes evident only after STZ administration. This temporal separation suggests that the brain may undergo functional and molecular changes during early metabolic disturbance, before conventional markers reach diagnostic thresholds for diabetes. These findings may provide insight into early cognitive vulnerability associated with insulin-resistant and metabolic dysfunction, where neurobiological alterations may precede clinically apparent metabolic disease.

Limitations and future directions

Several limitations should be considered when interpreting these findings. First, behavioural testing and molecular analyses were conducted at different stages of metabolic progression; therefore, cognitive outcomes reflect diet-induced metabolic dysfunction before STZ administration, whereas molecular findings represent the subsequent STZ-induced metabolic state. Second, the relatively short post-STZ period may not fully capture long-term neurobiological changes associated with chronic diabetes. Additionally, the absence of inflammatory cytokine measurements limits assessment of neuroinflammatory mechanisms underlying the observed alterations. The use of SD rats, which may exhibit relative metabolic resistance, and the mortality following STZ administration reduced sample sizes and may have limited sex-based comparisons. Future studies incorporating longitudinal metabolic and behavioural assessments, inflammatory profiling, and optimized STZ protocols will help clarify the mechanisms linking early metabolic dysfunction to cognitive decline.

Conclusion

This study demonstrates that prolonged HDF exposure is associated with early cognitive and hippocampal molecular alterations during diet-induced metabolic dysfunction before overt diabetes. Memory impairment was primarily evident during long-term retention and was accompanied by increased hippocampal levels of amyloid-β, phosphorylated tau, and MMP-9, whereas neurotrophic markers were maintained or elevated. Sex-dependent responses were observed, with females showing behavioural alterations and males exhibiting more pronounced hippocampal molecular changes. These findings highlight the potential vulnerability of the brain to metabolic stress before the onset of established diabetes.

Declarations

Ethical statement

All animal experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize suffering and stress, such as using a minimum number of rats per experiment. All experimental procedures were approved by the Animal Care and Use Committee (ACUC) of the Jordan University of Science and Technology (Grant Number: 794/2024).

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Author Contributions

Conceptualization: BS, KN, RK; Methodology: BS, KN, RK; Investigation: BS, IS, KN; Formal analysis: KN, RK; Validation: BS, KN, RK; Supervision: KN, RK; Resources: KN, RK; Writing - Original Draft: BS, IS; Writing - Review & Editing: KN, RK, IS.

Availability of data and material

Raw data are available upon reasonable request

Financial and Competing Interests Disclosure Statement

The authors report no conflicts of interest.

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