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ORIGINAL ARTICLE

Time-course study of high fat diet induced alterations in spatial memory, hippocampal JNK, P38, ERK and Akt activity

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Abstract

Consumption of high fat diet (HFD) is a health concern in modern societies, which participate in wide range of diseases. One underlying mechanism in the HFD mediated pathologies is disruption of insulin signaling activity. It is believed that HFD activates several stress signaling molecules such as MAPKs signaling pathway and these molecules participate in harmful effects in different cell populations including hippocampal cells. However, the activity of MAPKs signaling molecules are time dependent, even causing some opposing effects. Given that, MAPKs activity fluctuate with time of stress, there is less cleared how different lengths of HFD consumption can affect hippocampal MAPK. To test how duration of HFD consumption affect hippocampal MAPKs and insulin signaling activity and animal's cognitive function, rats were fed with HFD for different lengths (up to 6 months) and after each point spatial memory performances of animals was tested, then the peripheral indices of insulin resistance and hippocampal MAPKs and insulin signaling activity was evaluated. Results showed that while different time courses of HFD, up to 6 months, did not bring about significant spatial memory impairment, meanwhile the peripheral insulin sensitivity as well as hippocampal insulin and MAPKs signaling showed significant fluctuations during the different time courses of high fat diet regime. These results showed that neuronal responses to HFD is not constant and differ in a time-dependent manner, it seems that in acute phase molecular responses aimed to compensate the HFD stress but in chronic states these responses failed and devastating effects of stress began.

Keywords High-fat diet · Insulin resistance · MAPKs · Akt · Spatial memory

Introduction

Hypercaloric diets such as high fat diet (HFD) consumption is increasing worldwide. The HFD, also known as the Western diet, is one of the most important factors in disruption of the homeostasis and development of many pathological disorder. One of the most prevalent disorders linked to HFD is Diabetes Mellitus (DM) so that, it is well documented that HFD contribute to the observed increase in insulin resistance seen in DM (Freeman et al. 2014). In addition, HFD is a well-accepted method for developing experimental models of insulin resistance in many organs. Despite initial thoughts considering brain as an insulin insensitive organ, it is now a consensus that insulin is present in the brain and disruption of its signaling is an underpinning factor in the pathology of neurodegenerative disorders such as Alzheimer's disease (AD). On the other hand, like peripheral organs, mounting evidences have shown that HFD has detrimental effects on brain functions such as learning and memory (Ghasemi et al. 2013). Accordingly, it has been shown that HFD intake decrease hoppocampal synaptic plasticity (Biessels and Reagan 2015), cell proliferation, neurogenesis (Park et al. 2010; Lindqvist et al. 2006) and LTP (Karimi et al. 2013; Stranahan et al. 2008), all of which contribute to learning and memory impairment. It also has been shown that, like other organs, development of central insulin resistance is one of the mechanisms by which HFD may lead to cognitive

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impairment (Freeman et al. 2014), but the its mechanisms has not been clearly specified, so far. One of the intracellular signaling pathways that play an important role in the pathology of neurodegenerative disorders, participates in insulin resistance development (Hemmati et al. 2014) and also is involved in the cellular responses to the metabolic stresses like HFD (Wu et al. 2006) is Mitogen-Activated Protein Kinase (MAPK) pathway. MAPKs are a group of signaling molecules playing roles in cell response to various extracellular stimuli and are divided into 3 main subgroups; extracellular signal-regulated kinases (ERKs), Jun N-terminal kinases (JNKs) and P38 MAPK (Crowe and Shemirani 2000). These kinases play complex and time-dependent roles in neuronal death or survival (Stanciu et al. 2000; Saurin et al. 2000). As an example, while short-term ERK activity can promote cellular survival but the continuous activity of this kinase can contribute to cellular death (Li et al. 2002). Considering that, the activities of MAPKs are time dependent, but less evidence is available showing how different duration of HFD intake would affect MAPKs signaling pathway as well as insulin signaling transduction. Therefore, the present study aimed to assess the time course effects of HFD consumption on the activity of MAPKs members as well as insulin signaling pathways in the hippocampus of male adult rats as well as animal's cognitive performance in the MWM test. We were also seeking to see if any correlation exist between these two signaling pathways in different HFD consumption durations.

Materials and methods

Materials

Western blot antibodies including Phospho-Akt(ser473) (4060), Total Akt (9272), phospho-P38 (9211), phospho-JNK (4671), phospho-ERK (4377), total-P38 (8690), total -JNK (9252), total -ERK (4695) and secondary HRP-conjugated (7074) were purchased from Cell Signaling Technology Company. Amersham ECL select (RPN2235) reagent kit was purchased from GE healthcare and PVDF membrane (IPVH00010) was purchased from Millipore. Rat Insulin ELISA kit was purchased from Mercodia Company (10–1113-01) and Rat Glucose Assay Kit was purchased from Padginteb Company (Iran). Other reagents were obtained from usual commercial sources.

Animals and diet

Adult male wistar rats weighing 160–180 g obtained from the animal house of the Neuroscience Research Center, Shahid Beheshti University of Medical Science were used in this study. The animals were housed in Plexiglas cages with woodchip bedding in groups of 2–3 per cage at room temperature (25 ± 2 °C) under standard 12 h light-dark cycle (light from 7 am to 7 pm). Food and water were available ad libitum. The animal care was according to the NIH Guide for the care and use of laboratory animals and all protocols and efforts were made to minimize the number of animals used and their suffering. The ethics committee of Shahid Beheshti University of Medical Sciences approved the experimental protocols (Code: IR.SBMU.MSP.REC.1396.87).

One week after arrival, rats were randomly assigned to one control diet (CD) group and seven HFD groups (n = 7–8 in every group). Based on the duration of HFD consumption, rats were divided into different HFD groups (1, 2, 3, 6, 10, 20 and 26 weeks). The CD group received a standard laboratory chow within 26 weeks (highest duration in our study). Energy content calculated for normal diet was 3.8 kcal/g of which 4.75% of energy (%E) was from fat. The energy content of the HFD was 5.35 kcal/g and 58.3% of energy (%E) was from cow butter fat. The fatty acid profile of the diets has been measured according to Institute of Standards and Industrial Research of Iran (ISIRI) No; 4090–4091 (Table 1).

Behavioral test

Morris water maze apparatus

Spatial learning and memory was assessed in Morris Water Maze (MWM). It was a black circular tank (150 cm diameter, 50 cm height) filled with water (20 ± 1 °C) at a depth of 25 cm. A fiberglass platform 10 cm diameter, 23 cm height, was placed 2 cm under beneath of water surface in order to be invisible in black swimming pool. The maze was divided into four virtual quadrants (named geographically NW, SW, NE, SE) and start locations were set at the intersection of each quadrants. In addition, fixed immovable visual cues were provided in room (i.e. camera on ceiling, a door, a column, bookshelves and paper shapes stuck to walls) and these cues remained constant during the length of the experiment. A CCD camera was located above the center of the maze to record rats motions and send to an automated tracking system (Noldus, EthoVision XT 11), then intended parameters (i.e. latency to reach the platform, time spent in target quadrant, and the swimming speed) were analyzed by software.

Procedure

The whole protocol was done in 4 days after the finishing HFD use in each group. In the first three constructive days the fixed position invisible platform was located in the center of SW quadrant. Rats underwent four trials per day with different release points. Before starting the first trial at first day, rats were placed on submerged platform for 20s. Then during each trial the rat had 90s to found the hidden platform. Rats that couldn't find the platform within 90s, were guided to it by

Table 1 shows fatty acid composition and fat-derived energy of the normal and HFD

Type of fatty acid	Common name	Normal diet (%)	HF diet (%)
C4:0	Butyric acid	0.05	1.1
C6:0	Caproic acid	0.0	0.85
C8:0	Caprylic acid	0.03	0.6
C10:0	Capric acid	0.02	2.24
C12:0	Lauric acid	0.3	3.3
C14:0	Myristic acid	0.27	12.4
C14:1	Myristoleic acid	0.0	0.52
C16:0	Palmitic acid	14.4	38.6
C16:1	Sapienic acid	0.0	0.7
C18:0	Stearic acid	3.25	8.26
C18:1 *n-9*	Oleic acid	32.34	27.12
C18:2 *n-6*	Linoleic acid	44.96	2.73
C18:3 *n-6*	α-Linolenic acid (ALA)	3.9	0.1
C20:0	Arachidic acid	0.11	0.18
Others		0.37	1.3
Total saturated fatty acids		18.43	67.53
Total unsaturated fatty acids		81.57	32.47
Percentage of fat-derived energy (%E)		4.75%	58.3%

experimenter. Whenever the rats reached the platform by themselves or by experimenter guidance, they were allowed to stay on the platform for 20s to have rest until the onset of next trial. The fourth day was retrieval testing (probe trial) day. In this test the platform was removed and rats have were released in a fixed releasing point and were allowed to swim for 60s and parameters like cumulative time spent in target quadrant and frequency of crossing over platform site and swimming speed was extracted by Ethovision XT 11 software.

Blood sampling

Blood sampling was performed 1 day after last day of behavioral test in fasting status (16 h). This procedure was done between 8:00 a.m. and 10:00 A.M under CO2 inhalation anesthesia, by cutting the tail. Blood was collected into a microtubes containing 5 μl/ml heparin (5000 IU/ml) and immediately centrifuged at 3000 RPM for 5 min at 4 °C and plasma was extracted and kept at −80 °C until biochemical analysis.

Tissue preparation

After blood sampling animals were allowed to access to their diets for 2 h, after that they were weighted and anesthetized by CO2 inhalation and then decapitated and the hippocampi were quickly isolated on ice and transferred to liquid nitrogen and stored in −80 °C until molecular studies.

HOMA-IR index

The HOMA-IR index, as an indicator of insulin resistance, was calculated using the following formula: HOMA-IR = (Ci × Cg)/22.5, where Ci is fasting insulin level (μU/ml) and Cg is fasting glucose level (mmol/L).

Western blot analysis

Western blotting was used to determine the relative level of protein phosphorylation, as an indicator for their activity. Hippocampi of rats were homogenized in cold RIPA lysis buffer containing protease and phosphatase inhibitor. Samples were centrifuged (14,000 g for 30 min at 4 °C) and supernatants were collected. Next, protein concentration of samples was determined by Lowry method. Finally, equal amount of proteins were boiled in 2× sample buffer for 5 min and 40 μg of protein from each sample were fractionated in 12% SDS-PAGE. Proteins were then transferred to PVDF membranes and after blocking in 5% Bovine Serum Albumin (BSA), the membranes were incubated with primary antibodies (total and phosphorylated forms of MAPKs, Akt and Beta-actin) overnight in 4 °C. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody for 1 h at room temperature. Immunoreactive bands were visualized by ECL select kit. Next, we used an images analysis software (Image J) to quantify bands. After that, the density of each band was normalized to mean control. Finally, the normalized

data from each phosphorylated band was divided to its total antibodies in the same blot and these normalized ratios ($n = 3$ in each group) were compared to control group by unpaired t-test.

Results

Different length of HFD usage did not affect spatial memory acquisition

The effects of HFD or CD on water maze spatial learning and memory is represented in Fig. 1. As it is evident in Fig. 1a, all animals became increasingly adept at finding the hidden platform and displaying spatial memory acquisition. Statistical analysis of the escape latency to hidden platform by repeated measure ANOVA showed no significant difference between the groups [$P = 0.576$, $F_{(7, 53)} = 0.819$].

To compare how rats behave in different days of training, the results of each day were analyzed individually by one way ANOVA. This analysis also failed to reveal any significant differences between groups in the different days. Figure 1b shows bar graphs of mean escape latency to the hidden platform during days 1–3 of training [Day 1: $P = 0.535$, $F_{(7, 53)} = 0.804$; Day 2: $P = 0.398$, $F_{(7, 53)} = 1.066$; Day 3: $P = 0.816$, $F_{(7, 53)} = 0.519$].

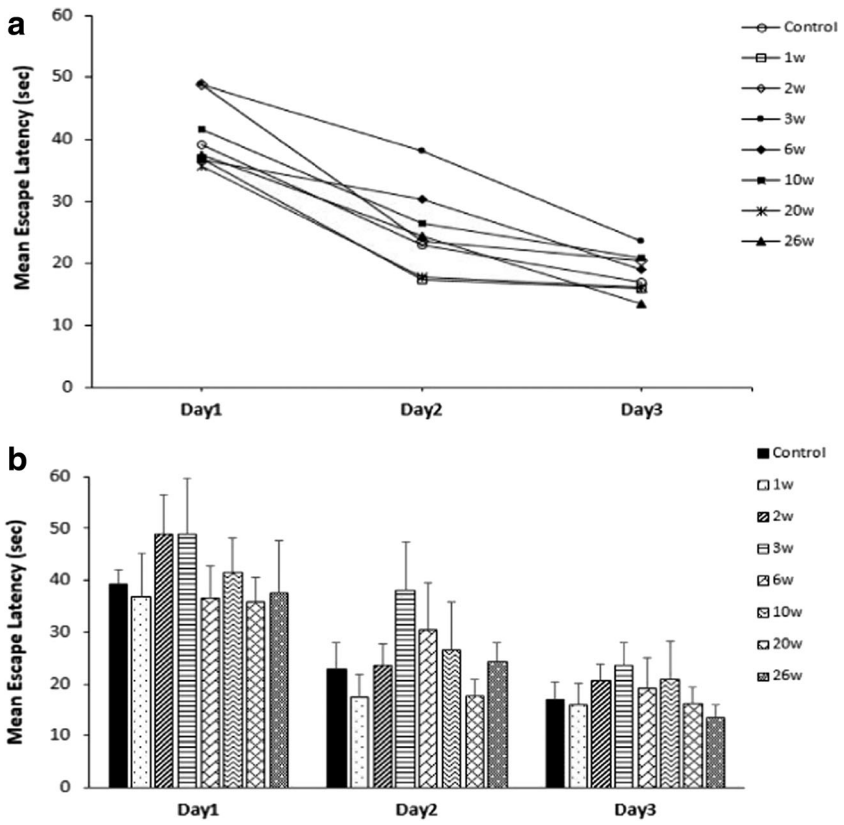

Fig. 1 The effects of HFD or CD on water maze spatial learning and memory. **a** The learning patterns of the animals fed with normal food or different time courses of HFD. **b** The escape latency to the hidden platform during days 1–3 of training. Statistical analysis did not show any significant differences. Data are represented as mean ± SEM

Time courses of HFD neither affect memory retrieval, nor change animal's motivation and sensorimotor function

In order to see if HFD consumptions affect memory retrieval, we conducted a probe test on 4th day and analyzed the mean times spent by animals in the target quadrant by One-way ANOVA. Statistical analysis of the results in CD group or different time courses of HFD groups showed that there was no significant difference between the groups ($P = 0.3217$, $F_{(7, 53)} = 1.195$). The Fig. 2a illustrated results of probe test.

To investigate if the different time courses of HFD consumption change sensorimotor function and/or animal's motivation, we extracted the mean swimming speed of the animals during probe test. As it shown Fig. 2b, one-way ANOVA analysis of the mean swimming speed showed no significant differences between groups ($P = 0.0631$, $F_{(7, 53)} = 2.070$). Furthermore, Fig. 2c shows the effect of HFD or CD on the mean escape latency to the visible platform during visible test. As it is evident, one way ANOVA analysis of mean escape latency to the visible platform did not show significant differences between groups ($P = 0.1568$, $F_{(7, 53)} = 1.596$).

The effect of diet on fasting plasma glucose and insulin concentrations and HOMA-IR index

In order to see how different time courses of HFD affect fasting plasma glucose and insulin level as well as HOMA-

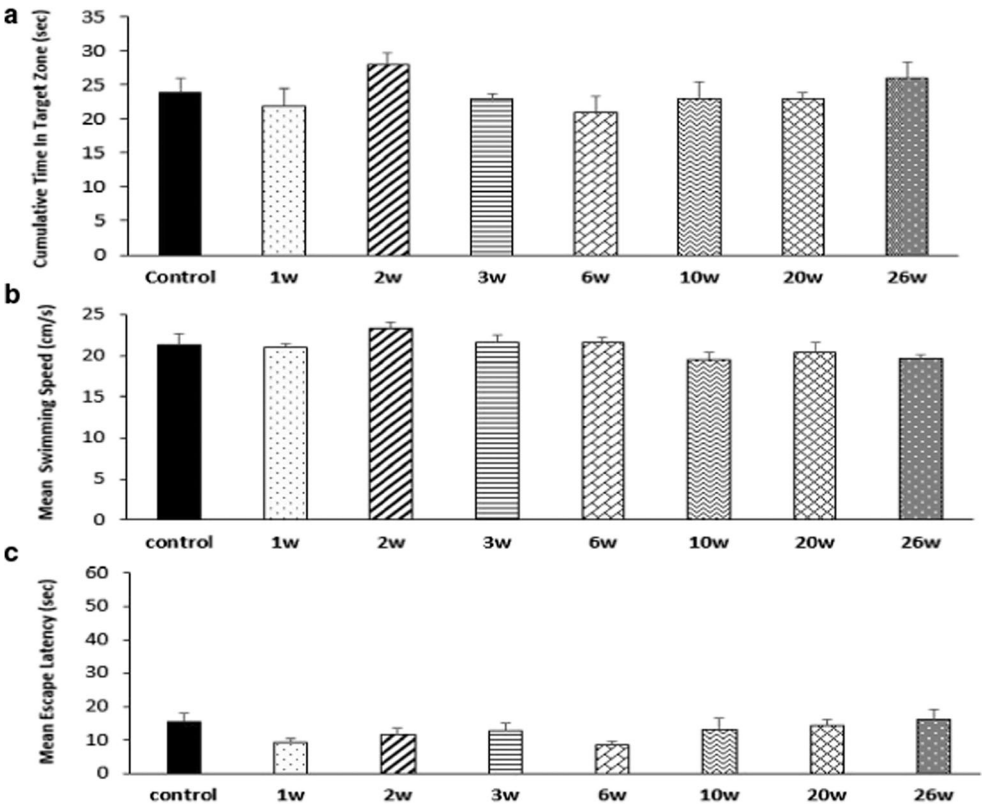

Fig. 2 **a** The effect of CD or different time courses of HFD on cumulative time in target zone. There was no significant difference between the groups in the time spent in the target zone. **b** The effect of CD or different time courses of HFD on mean swimming speed. Swimming speed did not show significant difference between groups. **c** The effect CD or different time courses of HFD on escape latency to the visible platform during day 4 of training. One way ANOVA did not show any significant differences between groups. Data are represented as mean ± SEM

IR index (as an index for peripheral insulin resistance), plasma level of fasting glucose and insulin was extracted and HOMA-IR index was calculated. Thereafter, results from each group was statistically compared to control group by unpaired t-test. Results showed that there is a significant difference between different HFD groups and control group in fasting glucose and insulin concentration (Table 2). Consuming HFD for 1, 3, 6, and 20 weeks causes significant difference in fasting plasma glucose levels compared to the control group. In addition, fluctuating changes were seen in fasting insulin concentration, and these changes was significant in first, 6th, 10th, 20th and 26th weeks when compared to the control group. Moreover, a significant difference in HOMA-IR indexes was evident in different time courses of HFD, when compared with control group (Table 2). Accordingly, consuming HFD leads to significant increases in HOMA-IR level in the first, 6th, 10th, 20th and 26th weeks of HFD.

Western blot results

Western blot experiments was done to assess the effect of different time courses of HFD on hippocampal MAPKs and Akt activity. To analyze the results of western blots, unpaired t-test was used to compare the different weeks of HFD with the control group. Accordingly, to evaluate the effects of 1 week feeding with HFD on activities of MAPKs family and Akt activity (as an indicator for insulin signaling), hippocampal proteins were analyzed by western blotting. Results showing the amounts of P38, JNK and ERK as well as Akt phosphorylation after 1 week of HFD are shown in Fig. 3. It should be noted that MAPKs and Akt are activated through phosphorylation in specific sites (Avruch 2007; Alessi et al. 1996), thus from now on we use the word activity when we talk about phosphorylation of Akt and MAPKs. As it is evident, unpaired *t*-test analysis of normalized ratios of phospho P38 and ERK did not show significant difference

Table 2 Effect of CD or different time courses of HFD on fasting plasma parameters and HOMA-IR index

Group	*Glucose(mMol/l)*	*Insulin(*μU/ml*)*	*HOMA-IR*
control	5.66 ± 0.28	14.10 ± 1.92	3.24 ± 0.56
1w	7.72 ± 0.31**	26.47 ± 4.62**	9.13 ± 2.10**
2w	5.73 ± 0.30	17.47 ± 3.93	4.39 ± 0.97
3w	4.62 ± 0.14**	22.15 ± 4.62	4.54 ± 0.94
6w	7.75 ± 0.44**	54.96 ± 6.75***	19.86 ± 2.31***
10w	6.4 ± 0.48	25.71 ± 4.57***	9.94 ± 2.03***
20w	6.64 ± 0.27*	47.46 ± 4.61***	14.21 ± 1.74***
26w	5.95 ± 0.50	32.24 ± 4.22***	8.75 ± 1.58***

Values are expressed as mean ± SEM ($n = 8$ for CD group; $n = 7$–8 for HFD groups). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. respective control group

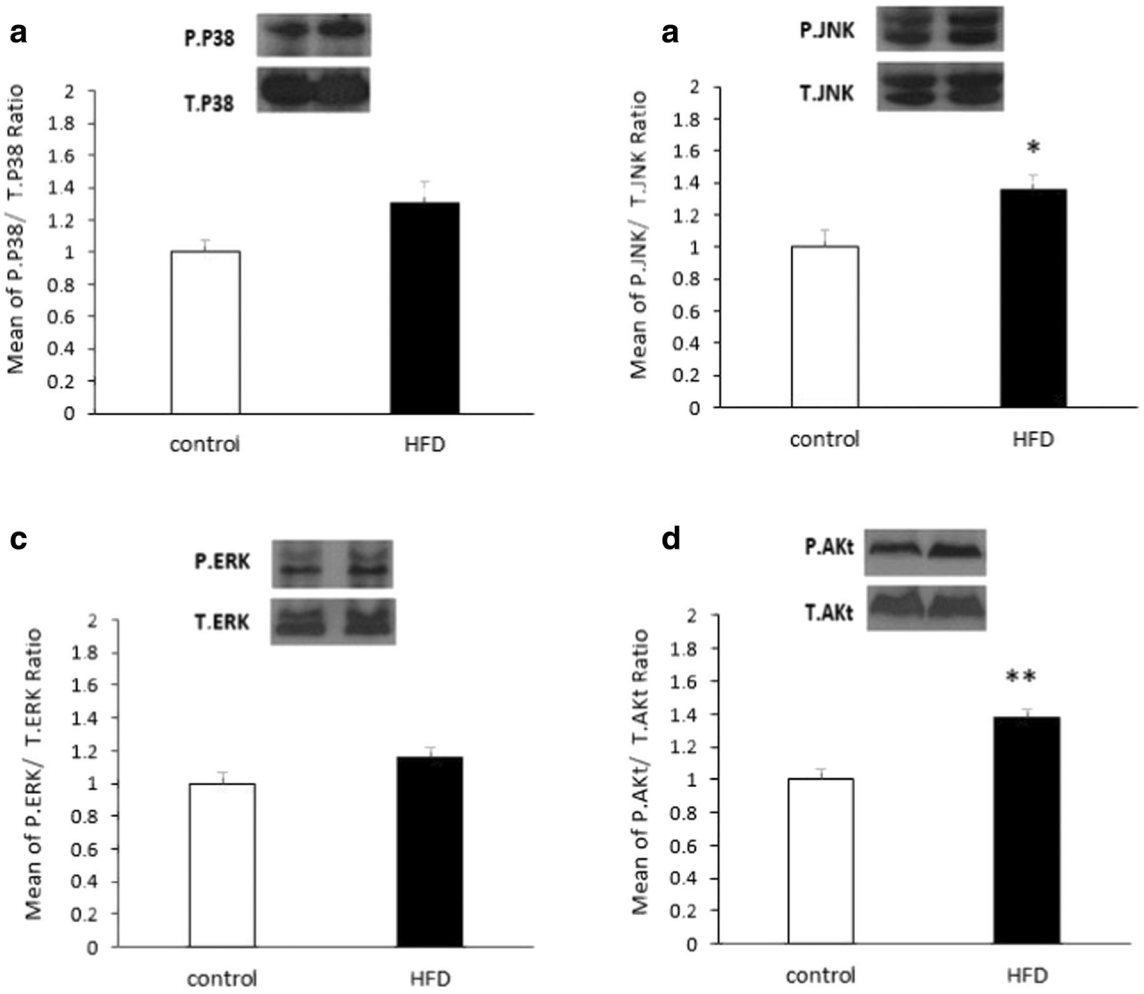

Fig. 3 Western blot analysis showing the effects of 1 week of HFD consumption on phosphorylated P38, JNK. ERK, Akt protein in the hippocampi of rats. Western immunoblots probed with antibodies against phosphorylated (P38, JNK, ERK, Akt) and total (p38, JNK, ERK, Akt) and beta actin. $*P < 0.05$ and $**P < 0.01$, represent the difference between control and the HFD groups in JNK and Akt activity respectively

with control group, however, this short period of HFD caused a significant increase in hippocampal JNK and Akt activity.

Figure 4. shows the effects of 2 weeks of HFD on the activities of MAPKs family and insulin signaling pathway. Unpaired *t*-test analysis showed that activities of P38, JNK and Akt have increased significantly 2 weeks after feeding with HFD in comparison to control group. In this week, activity of ERK did not show significant changes.

Results of western blot evaluating the P38, JNK and ERK as well as Akt activity after 3 weeks of HFD are shown in Fig. 5. As this figure depicts, statistical analysis showed that increasing the duration of HFD regime to 3 weeks would bring back the activity of P38 and JNK to non-significant level. Accordingly, unpaired *t*-test showed no significant difference between P38, JNK and ERK activity in HFD and control diet groups. In addition, the activity of Akt, which was elevated in first 2 weeks of HFD, was reduced to its control level 3 weeks after of HFD feeding.

Figure 6 shows the effects of 6 weeks of HFD on the activities of MAPKs family and insulin signaling pathway. Statistical analysis showed that consumption of HFD for 6 weeks reactivates JNK, as shown by significant increase in JNK phosphorylation. Unpaired *t*-test showed no significant difference in other molecules including P38, ERK and Akt.

To evaluate how extending HFD feeding affects activity of Akt and MAPKs family, hippocampal proteins were analyzed by western blotting. Results showing the amounts of P38, JNK, ERK and Akt phosphorylation after 10 weeks of HFD feeding are shown in Fig. 7. As this figure depicts, unpaired *t*-test analysis of normalized ratios of phospho P38 and JNK show significant difference with control group, however, this period of HFD did not bring about significant difference in hippocampal ERK and Akt activity.

Figure 8 demonstrates the effects of 20 weeks of HFD on the activities of Akt and MAPKs family. As it is evident, statistical analysis showed that in this time point Akt and

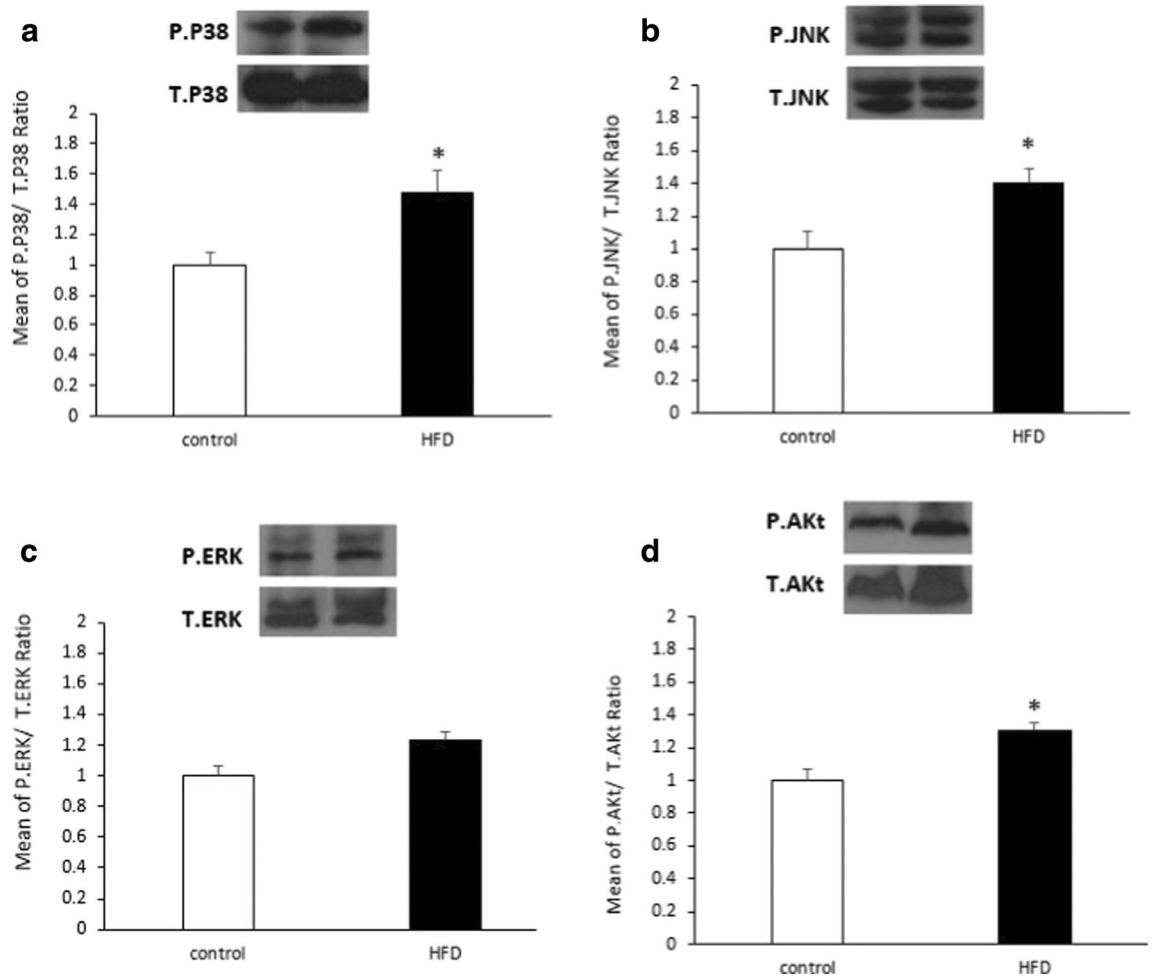

Fig. 4 Western blot analysis showing the effects of 2 weeks of HFD consumption on phosphorylated P38, JNK. ERK, Akt protein in the hippocampi of rats. Western immunoblots probed with antibodies against phosphorylated (P38, JNK, ERK, Akt) and total (p38, JNK, ERK, Akt) and beta actin. $*P < 0.05$ and $**P < 0.01$, represent the difference between control and the HFD groups

ERK activity showed a significant increase in comparison to control group, however, P38 and JNK activty did not show significant changes.

To evaluate how 26 weeks of HFD feeding affects the activities of MAPKs family and insulin signaling pathway, hippocampal proteins were analyzed by western blotting. Results showing the amounts of P38, JNK and ERK, as well as Akt phosphorylation after 26 weeks of HFD are shown in Fig. 9. As it is evident, unpaired *t*-test analysis of normalized ratios of phospho JNK and ERK did not show significant difference with control group, however, in this time point (6 months or 26 weeks) a significant increment in hippocampal P38 and Akt activity could be detected.

Discussion

The relationship between the consumption of HFD and metabolic disorders such as obesity and diabetes has been recognized for a long time (Buettner et al. 2007). Recently, several studies have been published about the effects of HFD on brain function, including behavior and cognition (Oh et al. 2013). Our study aimed to investigate the time course effects of HFD consumption on the CNS both at molecular and behavioral level and compare these effects with peripheral indices of insulin resistance. Accordingly, after different length of HFD feeding, we assessed spatial memory and activity of MAPKs as well as insulin-signaling pathways in the hippocampus of male rats. Simultaneously, we examined animal's blood samples for insulin, glucose concentration and HOMA-IR index to find out if our HFD is efficient enough to bring about systemic metabolic changes and if these changes are correlated with molecular alterations in the hippocampus.

The results showed that none of different time courses of a HFD usage from 1 weeks to 6 months; with the cow butter increased body weight in a linear way (data not presented), however, did not have a significant effect on the spatial memory of the animals. On the other hand, this diet caused significant fluctuating changes in both systemic insulin sensitivity and the insulin and MAPKs signaling pathway in the

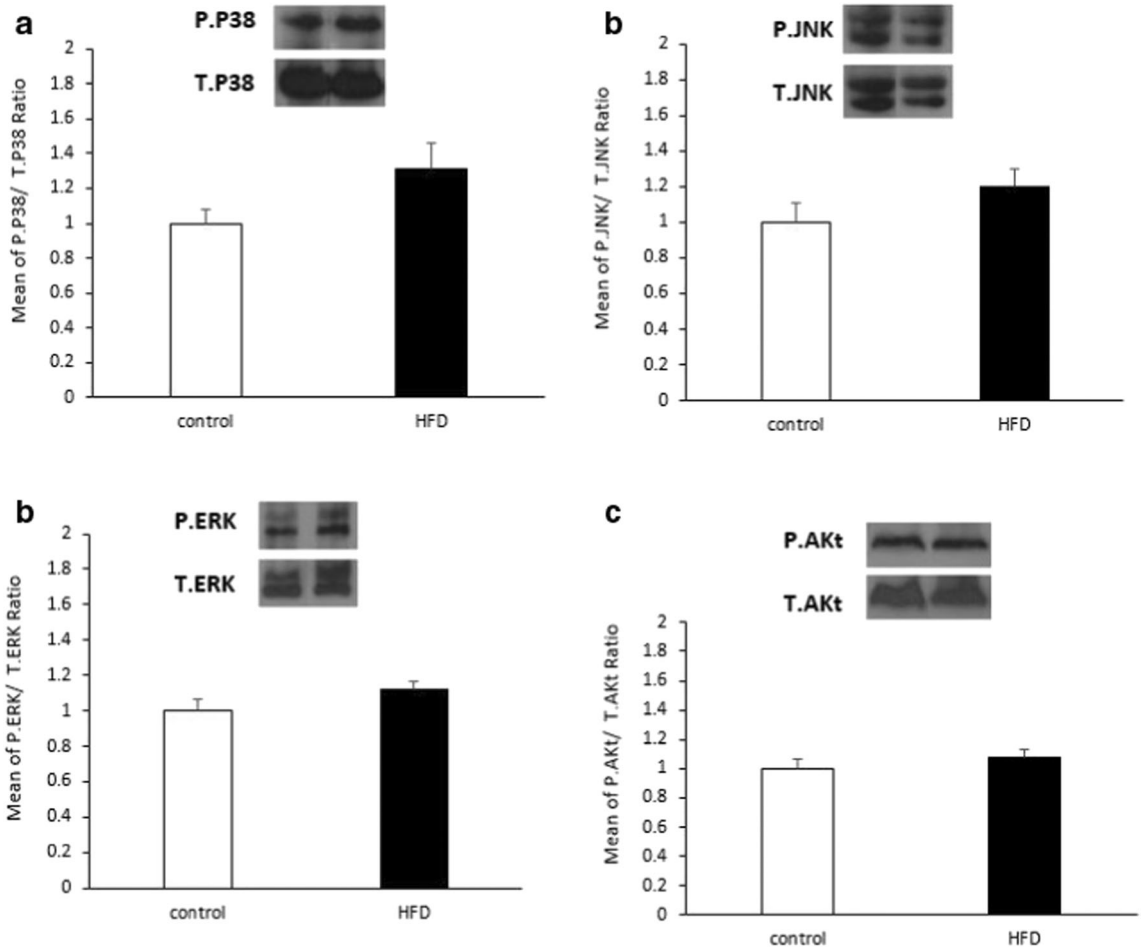

Fig. 5 Western blot analysis showing the effects of 3 weeks of HFD consumption on phosphorylated P38, JNK, ERK and Akt protein in the hippocampi of rats. Western immunoblots probed with antibodies against phosphorylated (P38, JNK, ERK, Akt) and total (p38, JNK, ERK, Akt). $^{**}P < 0.01$, represent the difference between control and the HFD groups

hippocampus. In agreement with our behavioral results, several studies fail to show significant memory impairment after feeding with HFD (Lavin et al. 2011; Nguyen et al. 2017). However, evidences are also available showing that HFD feeding is associated with disruption of learning and memory (Freeman et al. 2014; Greenwood and Winocur 1996). These discrepancies might be derived from different type and percentage of fat, age of the animal at the onset of HFD consumption, the presence of other high caloric substances along with HFD and basic metabolic abnormalities in animals (Boitard et al. 2012; Sodhi and Singh 2013; Woodie and Blythe 2017; Zare et al. 2012).

In the present study, butter base fat was used, however, in many of HFD studies that reported memory impairments, lard base fats have been used, so differences in the dietary fat may be a point (Greenwood and Winocur 1996; Zare et al. 2012; Pratchayasakul et al. 2011; Arnold et al. 2014). Despite the fact that percentage of saturated fatty acids in the cow butter is higher than lard fat (Rohman et al. 2012), but, apparently, it could not negatively affect memory and learning. Moreover, fatty acid profiles are different in these two types of fats and this could be one of the reasons for causing such different effects.

The type of memory test is another important issue that can explain our results. Accordingly, in a study by Knight et al. (2014), 10 months of HFD impaired animal memory in the Y maze test but not in the MWM test (Knight et al. 2014).

Age is another important factor in determining the effects of HFD. In accordance, it seems that memory impairment mainly occurs in animals whom are young (1 month or less) at the onset of a HFD (Greenwood and Winocur 1990; Wang et al. 2016; Hwang et al. 2010), and not in a middle-ages (Boitard et al. 2012; Boitard et al. 2014). The above issue suggests that in the middle-ages, some other factors are needed to strengthen the cognitive effects of HFD. These strengthening factors include prolonged periods of HFD consumption, more severe HFDs, presence of other high-calorie substances along with fat or the presence of metabolic disorders (Stranahan et al. 2008; Arnold et al. 2014; Boitard et al. 2014). In support to this theory, several proofs have been

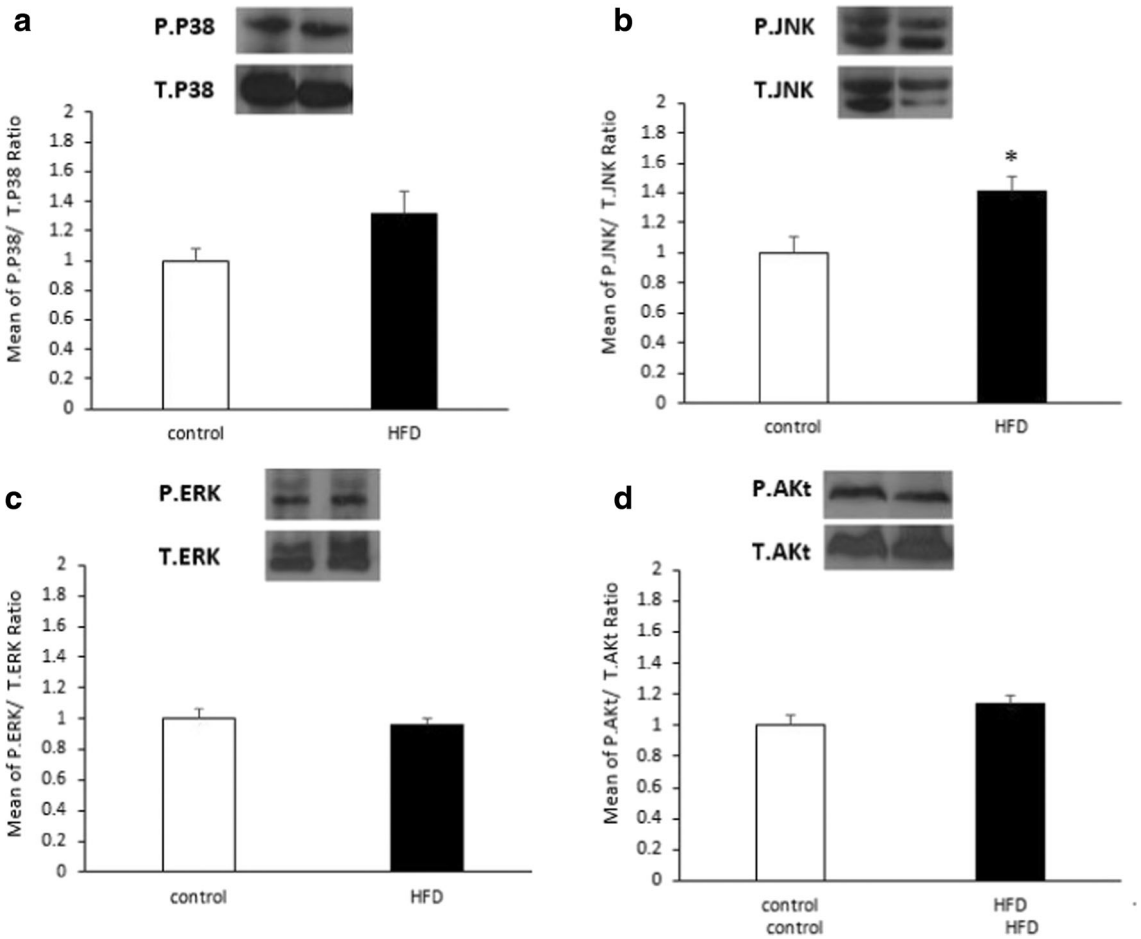

Fig. 6 Western blot analysis showing the effects of 6 weeks of HFD consumption on phosphorylated P38, JNK. ERK and Akt protein in the hippocampi of rats. Western immunoblots probed with antibodies against phosphorylated (P38, JNK, ERK, Akt) and total (p38, JNK, ERK, Akt). *$P < 0.05$ represent the difference between control and the HFD groups

published showing that each mentioned factors could play a determining role in the cognitive effect of HFD. A study by Zare et al. (2012) on diabetic and normal animals showed that the use of HFD causes memory impairment in the passive avoidance memory test only in the diabetic group but not normal group (Zare et al. 2012). Another study conducted on genetic models of Alzheimer's disease, also revealed that HFDs feeding in Alzheimer's transgenic animals can act as a trigger for cognitive impairment by increasing oxidative responses and inflammation in the hippocampal arteries. This suggests that a metabolic or genetic disorder can cause and aggravate memory impairment following a HFD, and healthy animals are more resistant to the adverse effects of this type of diet.As mentioned, combination of other high-calorie substances, such as simple carbohydrates or cholesterol, along with HFD also increases the harmful effects of this diet (Sodhi and Singh 2013; Woodie and Blythe 2017; Jurdak and Kanarek 2009; Kumar et al. 2014). Another affecting factor is the percentage of dietary fat. Despite the fact that using higher percentages of fat might have more adverse effects (Komaki et al. 2015), however, human studies have shown the highest amount of total fat intake does not exceed 50% of total energy (Harika et al. 2013; Austin et al. 2011; Vadiveloo et al. 2014). Therefore, in the present study, we attempted to select and investigate about a percentage of fat that is consistent with its consumption in the society.

In the next step, we investigated the time course effects of HFD on insulin signaling sensitivity in both peripheral tissues and hippocampus. The results showed that consuming HFD for different periods bring about significant time-dependent and fluctuating changes in plasma glucose and insulin concentration. These changes caused a significant increase in the HOMA-IR index (as an indicator for peripheral insulin resistance) in animals receiving HFD for short and long periods (1 week and longer than 6 weeks). Consistent with our study, other studies also suggested that consuming HFD for only a week increases plasma glucose and insulin levels and induces systemic insulin resistance (Wang et al. 2001; Samuel et al. 2004; Winzell and Ahrén 2004; Lee et al. 2011). In line with our results, Krishna et al. (2016) have also monitored insulin resistance after 5, 20 and 33 weeks of a HFD consumption, and their results showed that a significant increase is evident

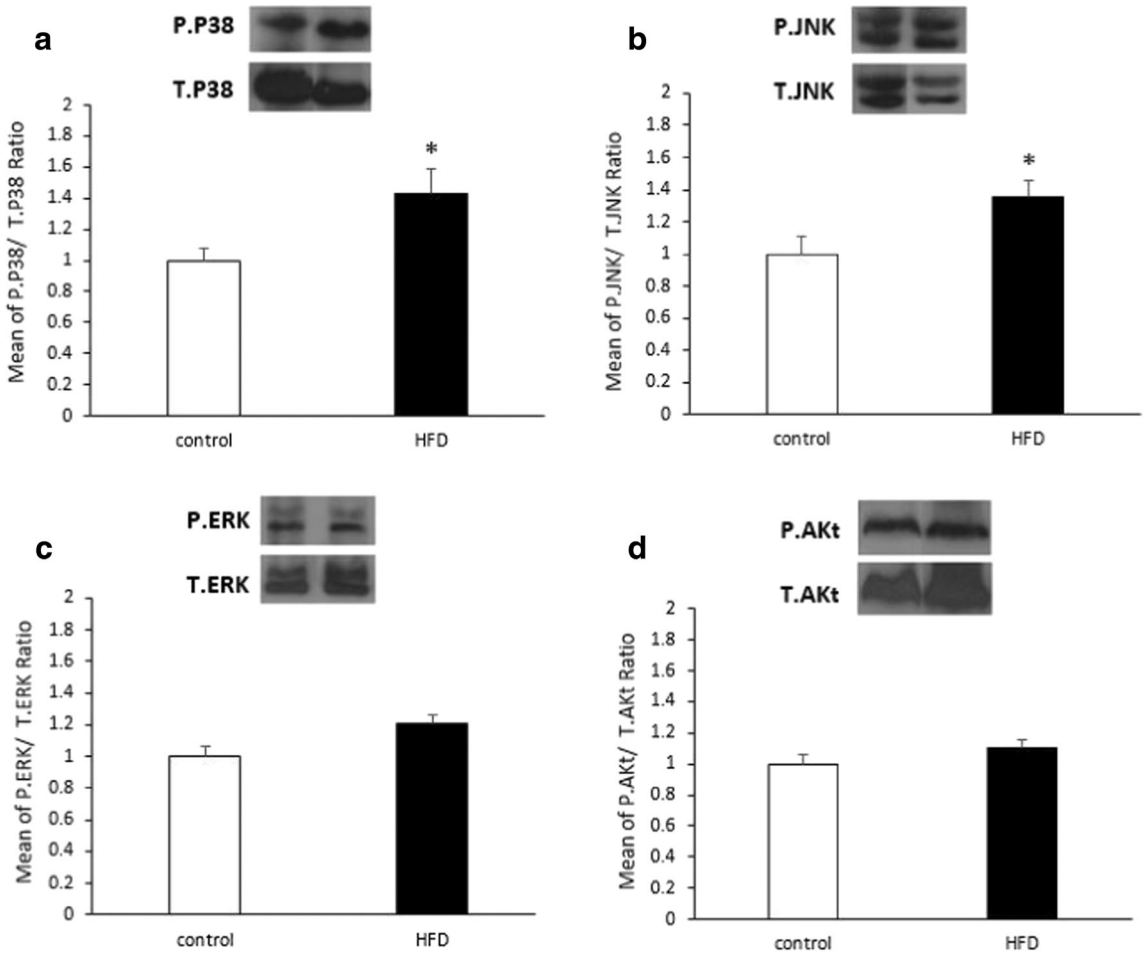

Fig. 7 Western blot analysis showing the effects of 10 weeks of HFD consumption on phosphorylated P38, JNK, ERK and Akt protein in the hippocampi of rats. Western immunoblots probed with antibodies against phosphorylated (P38, JNK, ERK, Akt) and total (p38, JNK, ERK, Akt). $^{*}P < 0.05$ and $^{**}P < 0.01$, represent the difference between control and the HFD groups

only in the 5th and 20th but not 33th weeks (Krishna et al. 2016). In addition, in another study, Winzell et al. fed animals with HFD for 1 year and examined their plasma insulin and glucose changes every 2 weeks, results also revealed fluctuating changes in insulin and glucose levels. However, the overall trend in this study indicates a sharp increase in Insulin values and mild decrease in glucose levels over time (Winzell and Ahrén 2004). Furthermore, Lee et al. (2011) have demonstrated that HFD intake for one and 10 weeks increases the level of plasma glucose levels compared to control, but glucose in the first week, was more than the tenth week (Lee et al. 2011). Other groups have also reported similar periodic and time-dependent changes in insulin and plasma glucose levels after feeding with HFD (Karbaschi et al. 2016; Bonen et al. 2015).

Insulin signaling pathway is conveyed through a complex interconnected system. In the presence of insulin, the receptor causes IRS phosphorylation, which activates other pathways inside the cell (Gual et al. 2005). PI3K/PKB (Akt) pathway is the main pathway for insulin signaling, which is activated by insulin signaling and Akt phosphorylation, could be considered an indicator for insulin signaling activity (Gual et al. 2005; Tanti and Jager 2009; Taniguchi et al. 2006). Since HFD is known as a cellular stress that disrupt the insulin signaling in the brain (Arnold et al. 2014; de la Monte 2012), in current, work we also assessed the Akt activity in the hippocampus of animals receiving HFD for different lengths.

Interestingly, our results showed that the activity of Akt (shown by its partial phosphorylation on ser473 residue) is increased both in short duration (first, second weeks) and long duration of HFD (20th and 26th weeks), but not in intermediary periods after taking HFD. In agreement with our result, other studies on mouse and rat have also reported that 12 weeks of HFD did not alter the level of Akt activity in the brain (Pipatpiboon et al. 2012; Liu et al. 2015). In contrast to ours, some limited reports are also available showing that the short-term intake of high-fat/high fructose diets for 7 days and prolonged periods up to 16 weeks resulted in Akt inhibition in the brain (Calvo-Ochoa et al. 2014; Mi et al. 2017).

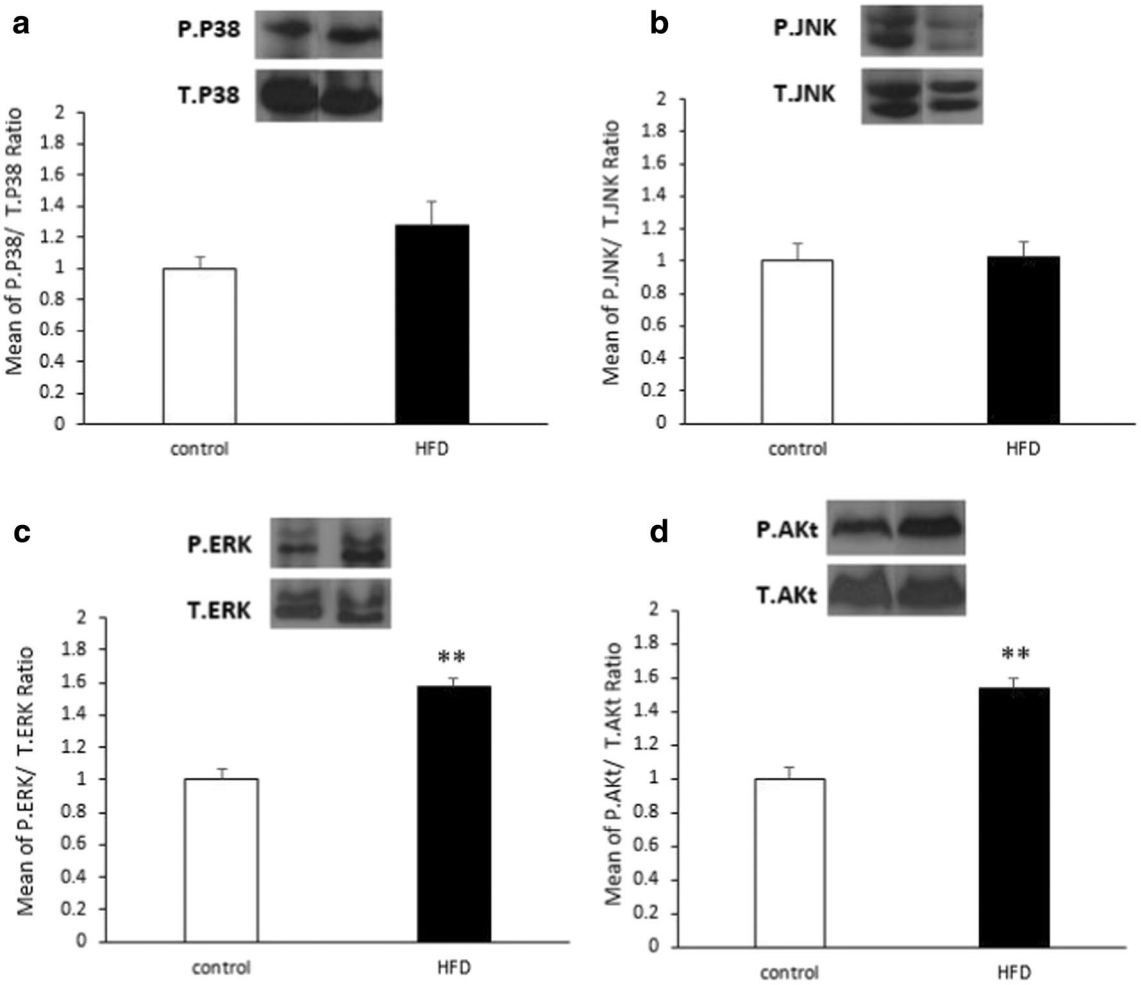

Fig. 8 Western blot analysis showing the effects of 20 weeks of HFD consumption on phosphorylated P38, JNK, ERK and Akt protein in the hippocampi of rats. Western immunoblots probed with antibodies against phosphorylated (P38, JNK, ERK, Akt) and total (p38, JNK, ERK, Akt). $^{**}P < 0.01$, represent the difference between control and the HFD groups

This difference may be due to the more complex diet used in this study, suggesting that high amounts of carbohydrates along with HFDs may exacerbate the adverse effects of HFD on insulin signaling in the brain.

In line with our study Arnold et.al have evaluated the activity of Akt in frontal brain tissue in moderate fat diet (MFD, 45% energy from fat) and HFD (60% energy from fat) groups (Arnold et al. 2014). In this study, after 8 weeks of using a MFD, no change was observed in the activity of Akt in the frontal cortex. While the consumption of HFD for a limited period of 17 days has increased Akt's activity. It is noteworthy to note that when the tissue samples of HFD group were incubated with 10 nM insulin, the Akt did not increase in response to insulin stimulation, while in the control group showed a significant increase in Akt after insulin stimulation. Therefore, it seems that despite the increment seen in Akt activity in the HFD group but HFD has negatively affected insulin signaling, as shown by the weakness in the response to insulin (Arnold et al. 2014). This conclusion could explain our result as well, since according to our results the activity of Akt was increased in short and long periods of HFD usage. Interestingly, in the same periods, indicators of cell stress were also activated, as we will discuss later. This implied that insulin signaling activation in response to HFD my serve to play a compensatory role against the stress, which try to maintain the cellular homeostasis in stressful conditions. It is obvious that this compensatory mechanism may be overwhelmed by increasing the time and or intensity of the stimuli. Furthermore, several lines of evidence have shown that impairment in spatial memory is associated with decreased level of hippocampal Akt activity (Moosavi et al. 2014; Negintaji et al. 2015), therefore, the elevated or unchanged Akt activity obtained in our study is consistent with our behavioural results that did not show significant memory weakening.

In mammalian systems, different signaling molecules are working in a precise harmony to generate and regulate cellular

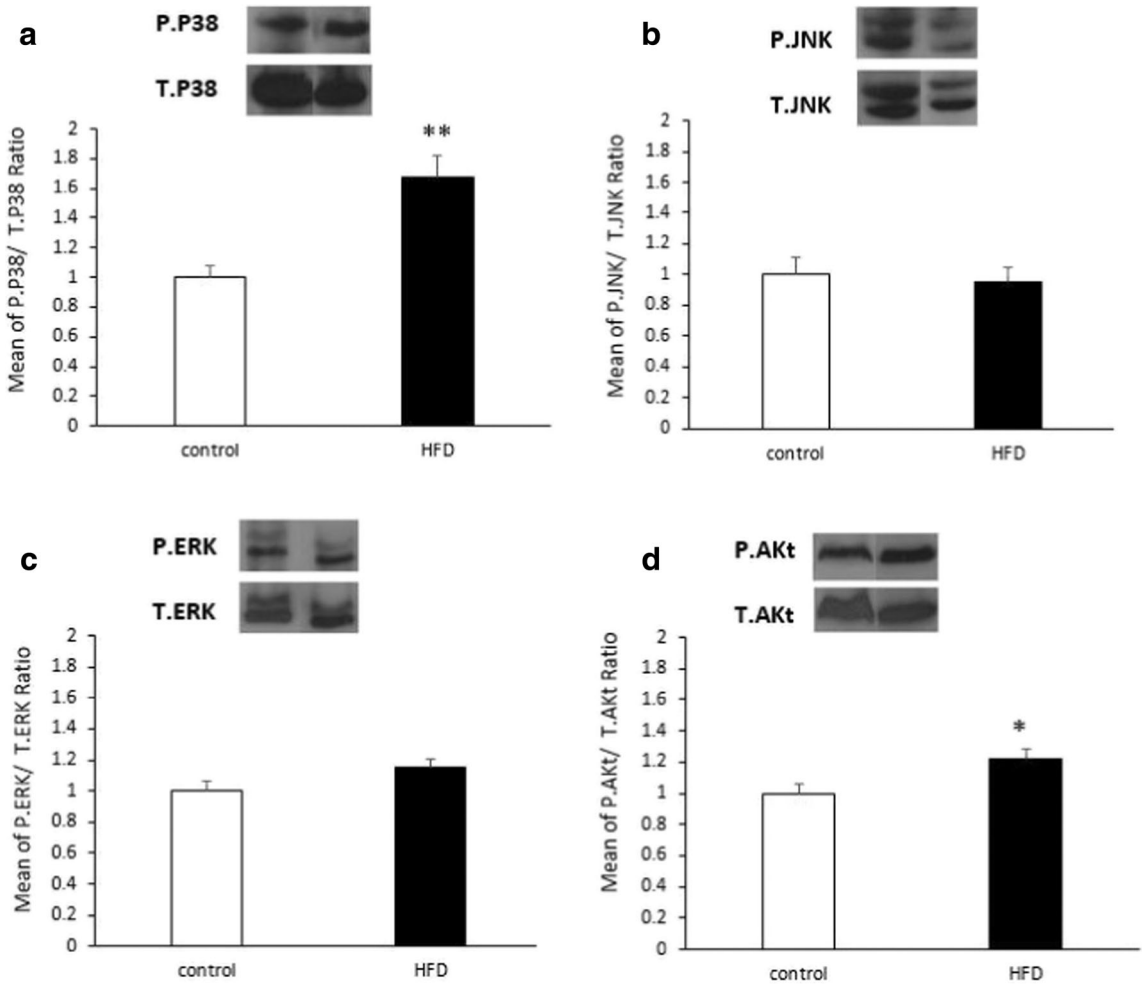

Fig. 9 Western blot analysis showing the effects of 26 weeks of HFD consumption on phosphorylated P38,JNK, ERK, Akt protein in the hippocampi of rats. Western immunoblots probed with antibodies against phosphorylated (P38, JNK, ERK, Akt) and total (p38, JNK, ERK, Akt). *$P < 0.05$ and **$P < 0.01$, represent the difference between control and the HFD groups

responses including proliferation, differentiation, growth, and apoptosis. MAPKs are essential intracellular signaling molecules that serve as important contributors to cellular responses, including responses to metabolic stresses such as HFD intake and contribute to development of insulin resistance (Hemmati et al. 2014; Wu et al. 2006; Peroval et al. 2013). The present study revealed that consuming HFD also affected hippocampal MAPK signaling pathway in a fluctuating manner. Accordingly, in current study, feeding with HFD for 1, 2, 6 and 10 week significantly increased JNK activity. Free fatty acids are well known as JNK activators and studies have shown that after a HFD, JNK activity increases in liver, muscle and adipose tissue (Liu et al. 2015; Hirosumi et al. 2002). Interestingly, our study also showed that HFD intake increased P38 activity at 2nd, 10th and 26th week. In agreement, other studies have reported an increase in the activity of P38 in the liver and brain tissues of animals after 12 and 16 weeks of fat intake (Liu et al. 2015; Mi et al. 2017). This pattern of activity is somehow similar to the JNK (rapid activation at 2th week and being activated in 10 weeks), however unlike JNK we observed that P38 is reactivated after 26 weeks.

Despite the increase seen in JNK and P38 activity in short-term consumption of HFD, but Akt activity is also elevated in these weeks. Therefore, it seems that the acute elevation seen in these stress kinases may be responsible for initiation of an acute compensatory mechanism that was discussed before. However, activation of JNK and P38 in longer durations (6, 10 or 26 weeks) accompanied with blunted Akt phosphorylation. The reactivation of P38 after 26 weeks and partial decrease of Akt activity compared to 20 weeks, suggests that increasing the time of HFD intake (≥6 months) might bring about more profound defects even at cognitive level.

Moreover, we show that ERK protein increased in the twentieth week and did not change significantly in the other time points. Unlike our results, some evidence reported that after 12 or 16 weeks of using HFD decrease ERK activity in the entire brain tissue (Liu et al. 2015; Mi et al. 2017). The temporary increase in ERK's activity seen in the 20th week

apparently has a protective effect, as simultaneously there a sharp increase in the activity of Akt is evident in this time point. The MAPKs members have dual protective or stress-inducing roles in various systems of the body (Sun et al. 2015; Xia et al. 1995). One of the most important factors that can play a role in determining whether MAPKs cause cell survival or cell death is the activity duration of these kinases (Stanciu et al. 2000; Saurin et al. 2000). For instance, while many studies considered ERK as a major neuroprotective factor which is activated in response to insulin and has a positive effect on cell growth (Gual et al. 2005; Uehling and Harris 2015), there are also many studies showing that ERK acts as inducer of neuronal death. Furthermore, it has been shown that ERK participates in insulin signaling malfunction and ERK inhibition can prevent TNFα-induced insulin resistance (Subramaniam and Unsicker 2010; Ghasemi et al. 2014; Engelman et al. 2000). It is believed that the duration of activity is determining factor in these opposing effects. Accordingly, it has been demonstrated that short-term activity of ERK can promote cellular protection, but persistent activity of these kinases can contribute to cell death (Li et al. 2002). The same discrepancy was observed with JNK and P38, and in contrast with most negative and damaging effects of the activation of this stress kinases (Xia et al. 1995), studies have also reported protective effects on JNK or P38 in neuronal and non-neuronal cells (Price et al. 2003; Beguin et al. 2007). Consistently, Svensson et al. (2011) reported that JNK and P38 exhibit anti-apoptotic and pro-survival effects during LPS induced activation of microglia cells (Svensson et al. 2011). Time dependent activity is also reported for P38 and JNK. In this regard, it has been shown that sustained activation of P38 and JNK induces apoptosis in cisplatin-sensitive human ovarian carcinoma cell line, while in resistant subclone showed transient stimulation of P38 and JNK (Mansouri et al. 2003).

In conclusion, this study revealed that however different periods of HFD (1, 2, 3, 6, 10, 20 and 26 weeks) did not impair special memory, but molecular changes have started, from the first week. The HFD in different time courses causes fluctuating changes in the variables and causes systemic insulin resistance and insulin signaling impairments in the hippocampus. Insulin signaling impairment in the hippocampus was shown with reduction of Akt phosphorylation, which appears to be related to the activity of JNK and P38. In addition, ERK activity is likely to have a protective effect and has a role in increasing pAkt in 20th weeks.

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Compliance with ethical standards

Disclosure of interest The authors report no conflict of interest.

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