Final Report

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By Prapimpun Wongchitrat, Ph.D

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Abstract

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Project Period : 2 years

Abstract:

Modern societies have promoted the intake of high-calorie diets and sedentary lifestyles, causing the abnormal function of biological clock and accelerated body weight gain which increase the risk to develop metabolic syndrome. Chronic hyperglycemia is a primary factor in the pathophysiology of diabetes. The present study aimed to investigate the effect of high-fat diet (HFD)-fed and streptozotocin (STZ)-induced hyperglycemia condition and to examine the effect of melatonin on the adult hippocampal neurogenesis. In HFD-fed and STZ-treated rats, the reduction of neurogenesis in the hippocampus was observed as shown by the reduction of nestin, doublecortin (DCX) and β-III tubulin immunoreactivities. Likewise, the reduction of ionotropic glutamate receptor (NR2A) and synaptic proteins, synaptophysin, PSD-95 expression were detected whereas, the level of GFAP was increased in the hippocampus of HFD-fed and STZ-treated rats. Melatonin administration significantly increased the level of neurogenesis markers, glutamate receptor and synaptic proteins whereas significantly decreased the GFAP proteins expression in the HFD-fed and STZ-treated rats compared to that observed in the vehicle-injected HFD-fed and STZ-treated rats. Moreover, melatonin improved the decreases of melatonin receptors (MT1 and MT2) and insulin receptor-β, including p-IR-β and p-ERK, its downstream signaling, that occurred in the hippocampus of HFD-fed and STZ-treated rats. Therefore, the results suggest that melatonin ameliorates the decrease of neurogenesis and synaptic formation, and prevents the astrocytic activity which may also be involved in the protection of insulin signaling impairment via melatonin receptor and protect the damage of insulin receptors under the hyperglycemia condition.

Keywords : hippocampus, clock gene, neurogenesis, hyperglycemia

2. Executive summary

The modern society lifestyle, including shift work, nocturnal activity, and late-night dietary which commonly present in our daily life causing accelerated body weight gain in both children and adults. Currently, both industrialized nations and developing countries are experiencing an increase in the rate of metabolic syndrome, which is comprised of several metabolic abnormalities, including central obesity, dyslipidemia, fatty liver, hyperglycemia and hypertension. This syndrome has become a major public health worldwide. More than 2.1 billion people around the world suffers from the diseases associated with metabolic syndrome such as, cardiovascular disease, various types of cancer, diabetes and also promote the age-related disease as neurodegeneration that reduce both human lifespan and quality of life.

Chronic hyperglycemia is a primary factor in the pathophysiology of Type 2 diabetes mellitus (T2DM). T2DM is becoming increasingly which associated with obesity and aging in our modern society. Hyperglycemia contribute the development of insulin resistance and beta cell dysfunction, both of which, in turn, exacerbate hyperglycemia which is linked to the development of many chronic diseases. Several lines of evidence suggest that brain is a target organ in type 2 diabetes (Awad et al., 2004; Greenwood and Winocur, 2005; Messier, 2005; Winocur and Greenwood, 2005). Very high blood glucose concentration cause structural changes in the brain and is associated with cognitive impairment and dementia. Numerous studies report changes in cognitive functioning and brain structure in patients with diabetes relative to controls (Desrocher and Rovet, 2004; Greenwood and Winocur, 2005). Several lines of evidence mostly implicate a role of insulin and glucose metabolism on risk of developing neurodegenerative condition, in particular the loss of cognitive function those related to Alzheimer's disease (AD). However, the exact mechanisms underpinning the association between high blood glucose level and cognitive impairment are unclear. In addition, there are evidences suggest that molecular clock component play an important role on the learning and memory function of the brain. The alteration or disruption the circadian rhythm such as poor quality of sleep and also aging are effect neuronal structure and function in regions that support cognition. Also, epigenetic mechanisms, specifically the role of clock genes in the modification of chromatin to influence gene regulation, also represent an exciting new overlay to regulation of the clock to the age-related disease in metabolic syndrome. Therefore, more research to elucidate the mechanism of cognitive impairment associated with type 2 diabetes may reveal us the potential approaches to understand and gain more knowledge which can help us for the further treatment and prevention of the disease.

Within the hippocampus, changes in the strength of synapses and neurogenesis are critical in certain types of learning and memory. Regulation of synaptic connectivity extends beyond changes in the number and strength of synapses to the de novo addition of new neurons in adulthood (Leuner et al., 2006). Data from studies of animal models suggest that impairments of both hippocampal synaptic plasticity associated with impairment of long-term potentiation (LTP) and adult neurogenesis in diabetes are believed to be a cellular mechanism of cognitive deficits (Kamal et al., 1999). Diabetes and high-fat fed rodents also show lower rates of adult neurogenesis (Zhang et al., 2008), whereas exercise, dietary restriction, and melatonin can enhance synaptic plasticity and adult neurogenesis in hippocampus (Lee et al., 2002; van Praag et al., 1999). Therefore, promoting neurogenesis is the one key target for treatment brain impairment. Previous report have been shown that melatonin (Mel), an indoleamine hormone secreted by the pineal gland, which is a powerful free radical scavenger agent, a lipophilic antioxidant, and neuroprotective agent (Reiter, 1996) can promote proliferation and differentiation of neural stem cell obtained from subventricular and subgranular zone of hippocampus (Sotthibundhu et al., 2010; Tocharus et al., 2014; Yoo et al., 2012a). Moreover, the change in melatonin level was observed in a model of metabolic diseases (Peschke et al., 2007). Melatonin treatment ameliorates metabolic changes associated with obesity in rats fed a high fat diet (HFD) (Agil et al., 2011; Rios-Lugo et al., 2010). In addition, the effect of melatonin on the improvement of the insulin signaling pathway was found in the hypothalamus, skeletal muscle, liver of old obese rat (Zanuto et al., 2013). These results reveal the role of melatonin related to the metabolic regulation.

The present study show that in HFD-fed+STZ-treated rats, the reduction of neurogenesis in the hippocampus was observed as shown by the decreasing of proteins expression related to neurogenesis such as nestin, DCX and β-III tubulin. These results suggest the loss of these marker proteins expression in hyperglycemia animals may represents the loss of function or number of neural stem cells including mature neurons in hippocampus. We also showed a significant downregulation of synaptic proteins expression, synaptophysin and PSD-95. Synaptophysin, an abundant presynaptic protein and PSD-95, a scaffolding protein enriched in post-synaptic densities, in the hippocampus of HFD+STZ-induced hyperglycemia rats. The alteration of pre- and postsynaptic protein expression indicated the abnormality of synaptic function in hippocampal neurons under hyperglycemia condition. Additionally, we found that HFD-fed+STZ-treated rats showed an obvious increase in GFAP level in the hippocampus. Changes in glial activity caused the neuronal cell death or caused the disturbance of the brain functions. Hyperglycemia condition also enhance the proliferation and activation of microglial

that induced the increasing of cell death signaling molecules such as caspase-3 level in the rat brain.

Interestingly, we found that the melatonin administration significantly increased the level of neuronal markers during neurogenesis and synaptic proteins expression while decreased the astrocytic protein in the hippocampal of the HFD+STZ-treated rats compared with the vehicle-injected HFD+STZ-treated rats. The administration of melatonin prevents an increase in oxidative stress and nitric oxide levels in blood plasma and several tissues during diabetes and also modulates neuroinflammation and oxidative stress via NF-kappa B and Nrf2 cascade. Then, melatonin ameliorates the decrease of neurogenesis and synaptic formation of hyperglycemia rat hippocampus by attenuating the oxidative stress induced hippocampal cell damage. The results also demonstrated that in hippocampus of diabetic-like rats, melatonin treatment also improved the reduction of IR-β, the phosphorylation levels of IR-β and of their downstream proteins related with proliferative pathway such as p-ERK which might be associated with neurogenesis. In this study, we found that rats treated by the HFD and low dose of STZ which showed markedly increased of their blood glucose level, was exhibited the low level of melatonin receptors both MT1 and MT2 in the hippocampus. The melatonin treatment could enhance the expression of hippocampal MT1 and MT2 protein levels in HFD-fed and STZ-treated rats. Our data suggest that dysregulation of the insulin signaling pathway during diabetes may provide a convergent mechanism of impaired neurogenesis and synaptic proteins dysfunction in hippocampus, while melatonin can reverse these negative effects. Moreover, melatonin also rescues the reduction of the melatonin receptors in the hippocampus of HFD-fed and STZ induced hyperglycemia rats in order to improve or reduce the pathogenesis of diabetes.

In addition, we observed that high blood glucose condition in our animal's model affected the level of clock gene and *Sirt1* expression in the rat hippocampus. The treatment of melatonin could attenuate the level of *Period2* and also *Sirt1* expression. Therefore the molecular clock genes may one of the potential cause or response to development of hippocampus function impairment in diabetic and melatonin treatment may help to modulate the severe of the disease. However, further study to understand the mechanism related to the function of these clock component on the diabetes and memory formation still are necessary.

In summary, our experiments showed that HFD-fed+STZ treatment resulted in a reduction in proliferation of new neurons, and an increase in the expression of an astrocyte marker. HFD-fed+STZ treatment led to the reduction of crucially functional synaptic proteins and melatonin receptors. Also the alteration in the insulin receptor and its downstream signaling protein related proliferative pathway was observed in the hippocampus of HFD-fed+STZ treatment animals.

Moreover, HFD-fed+STZ treatment also altered the level of clock genes and sirtuin gene. Furthermore, these HFD-fed+STZ treatment-induced alterations in hippocampal rats were attenuated by melatonin administration. These results reveal us the potential approaches to understand and gain more knowledge which can help us for the further treatment and prevention of the disease.

3. Objective

The present study aimed to investigate the effects of melatonin on the alteration of physiological parameters, neurogenesis, astrogliosis, synaptic function, and investigate the related mechanism for these changes in the hippocampus of HFD-fed and streptozotocin (STZ) induced hyperglycemia rat.

4. Research methodology

4.1. Animal and experimental design

6 week old male Wistar rats weighing 200 g were purchased from the National Laboratory Animal Center of Mahidol University, Thailand. The experimental protocol was performed in accordance with experimental protocols approved by the Laboratory Animal Care and Use Committee of Mahidol University (COA. NO. MB-ACUC 2013/003). Rats were housed under a 12h light:12h dark cycle with lights on from 6 am (Zeitgeber time (ZT) 0) at constant temperature. Animals were allowed to accommodate to laboratory conditions for at least 1 week which food and water were available ad libitum. After accommodation, rats will be divided into normal diet control (NFD) and high fat diet groups (HFD). The normal diet control group was fed with standard laboratory chow containing 4-6% energy from fat through the experiment for 7 weeks. The high fat diet group was fed by high energy diet (57% energy from fat, Table 1) for 5 weeks and then was injected intraperitoneally with streptozotocin (STZ) (40 mg/kg body weight, Sigma, USA) (Wu et al., 2011) dissolved in normal saline and used within 15 minutes. The normal diet control group was injected with a vehicle. The body weight was monitored weekly during dietary manipulation. The blood and urine glucose level was monitored by a test strip before and after the vehicle or STZ injection. The rats with the fasting glucose level of $\geq$300 mg/dl were considered diabetic and selected for studies. For the melatonin treatment, the high fat diet group was divided into 2 subgroups. One subgroup (HFD+STZ+Mel) was injected subcutaneously with melatonin (10 mg/kg body weight) dissolved in 0.05 % absolute ethanol once daily at ZT5 for 4 weeks stared at 2 weeks before and after STZ-treatment. While for another high fat diet subgroup (HFD+STZ) and the normal diet control were treated in the same procedure with vehicle at the

same time. Six rats per each group were used. Animals were sacrificed and brains were immediately removed, and the specific tissues areas related to cognitive function, hippocampus, were dissected under a stereo dissecting microscope at 4 ^{o}C. The tissues will be stored at -80 ^{o}C until use.

Table 1. Detail of high fat diet

Nutrient name	g/kg	Energy (kcal/kg diet)	Energy ratio
Protein	330.00	1,320	26.54
Carbohydrate	198.70	795	15.51
Fat	340.00	3,060	57.95
Total	1000.00	5,175	100.00

4.2. Study the profile of mRNA level of expression of clock gene and related clock controlled gene by Real-time PCR

Total RNAs from the brain tissue was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. The RNA quality and concentration was determined using nanodrop UV spectrophotometer. Then RNAs were reverse transcribed to cDNA by using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA, USA) under the condition that was showed in Table 2. Specific primers and TaqMan probes use to analyze the mRNA expression of hippocampal clock gene (*Per1, Per2, Bmal1* and *Rev-erbα*) and *Sirt1* was ordered from Applied Biosystems (assay detail showed in Table 3). The rat beta-actin (*Actb*), an endogenous control gene, was used to normalize the differences in sample RNA content. Real-time PCR will be performed according to the protocol described previously (Wongchitrat et al., 2013). All studied gene reactions were performed duplicate in separate well of each samples. PCR reactions were run on the CFX96 real-time PCR (Bio-rad, USA). The relative mRNA expression will be achieved with Bio-rad CFX manager software (Bio-rad, USA) by performing the comparative Ct method.

Table 2. Detail of condition for cDNA synthesis

	Step 1	Step 2	Step 3	Step 4
Temperature	25 ºC	37 ºC	85 ºC	4 ºC
Time	10 min	120 min	5 sec	∞

Table 3. Detail of used TaqMan assay

Interrogated Sequence			Translated Protein	Exon Boundary	Ampli-con Length
Gene name	Assay no.	Ref. Sequence			
Per1	Rn01496753_g1	NM_001034125.1	NP_001029297.1	12 - 13	142
Per2	Rn01427704_m1	NM_031678.1	NP_113866.1	22 - 23	100
Bmal1	Mm01269616_m1	NM_007489.3	NP_031515.1	9 - 10	100
Rev-erbα	Rn01460659_g1	NM_145775.2	NP_665718.2	2 - 3	123
Sirt1	Rn01428096_m1	rCT60587.0	rCP41658.0	5 - 6	65
Actb	PN 4352931E	NM_031144.2	NP_112406.1	4 - 5	91

4.3. Study the profile of protein level of expression of clock gene and related clock controlled gene by western blot analysis

Hippocampus was homogenized in RIPA lysis buffer containing 150 mM NaCl, 50 mM Tris-base, 1 mM phenyl methanesulfonyl fluoride (PMSF), 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1% protease inhibitor and 1% phosphatase inhibitor, and homogenized by sonicator for 10 s twice. The homogenization mixtures were centrifuged at 12,000 x g for 15 min at 4 ^{o}C, and the supernatant was collected and used for western blots analysis. Protein concentration was determined by Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin (BSA) as a protein standard.

The protein samples were mixed with 2X loading buffer (125 mM Tris–HCl, pH 8.5, 2% SDS, 20% glycerol, 2.5% mercaptoethanol and 2.5% bromophenol blue) and boiled at 95 oC for 5 min. The equal amount of protein sample were loaded onto 12-15% polyacrylamide gel to perform SDS–PAGE electrophoresis and transferred onto PVDF membranes (Amersham Biosciences, Piscataway, NJ, USA). The transfer efficiency was observed by Ponceau-S red solution. Then, the membranes were washed out by distilled water (DW) until the red color of Ponceau-S red disappeared. The blots were blocked with blocking buffer (5% non-fat milk in tris-buffered saline (TBS) containing 0.1% Tween-20, TBST) for 1 h at room temperature and then incubated overnight at 4 °C with the following antibodies; goat polyclonal antibody against doublecortin (DCX) (1:1,000), mouse monoclonal antibody against nestin (1:1,000), mouse monoclonal antibody against β-III Tubulin (1:1,000), rabbit polyclonal against anti-GFAP (1:1,000), mouse monoclonal antibody against synaptophysin (1:10,000), mouse monoclonal antibody against PSD-95 (1:10,000), rabbit anti NR2A (1:1000), mouse anti NR2B (1:1000),

rabbit polyclonal anti-insulin receptor (IR) α-subunit (1:1,000), mouse monoclonal antibody against IR-β (1:1,000), rabbit monoclonal anti-p-IRβ (1:1,000), mouse monoclonal antibody against ERK1/2 (1:1,000), mouse monoclonal antibody against p-ERK1/2 (1:1,000), rabbit polyclonal antibody against MT1 (1:500), rabbit polyclonal antibody against MT2 (1:500), rabbit monoclonal antibody against BMAL1 (1:2,000), rabbit polyclonal antibody against PER1 (1:2,000), rabbit polyclonal antibody against PER2 (1:1,000), Goat polyclonal antibody against REV-ERBα (1:1,000), mouse polyclonal antibody against SIRT1 (1:1,000), and mouse monoclonal antibody against actin (1:10,000). On the next day, the membranes were washed three times with TBST for 5 min before incubated with the following secondary antibodies; rabbit anti-goat IgG HRP linked for DCX (1:20,000), REV-ERBα (1:60,000) anti-rabbit IgG HRP-linked for GFAP (1:5,000 in 5%NFM), insulin receptor (α-subunit) (1:5,000), p-IRβ (1:5,000 in 3% BSA), MT1 (1:1,000), MT2 (1:1,000 in 3% BSA), BMAL1 (1:10,000), PER1 (1:20,000), PER2 (1:20,000) and anti-mouse IgG HRP-linked for nestin (1:2,000), β-III-tubulin (1:5,000), synaptophysin (1:10,000), PSD95 (1:10,000), insulin receptor (β-subunit) (1:5,000 in 5% NFM), ERK1/2 (1:5,000), p-ERK1/2 (1:2,000), SIRT1 (1:5,000) and actin (1:20,000), for 1.30 hr at room temperature. Finally membranes were washed thrice and visualized enhanced chemiluminescence using ECL Prime™ and exposed on X-ray film (Kodak, Rochester, NY, USA). The immunoblot bands were quantified by measuring the density of each band with the Scion image software (National Institutes of Health, Bethesda, MD, USA).

4.4. Statistical analysis

STATISTICA10 statistical software (StatSoft, Tulsa, OK, USA) was used for data analysis. The level of expression of studied genes are presented as means ± SEM from at least four to six animals per each study groups. The data were first analyzed by one-way analysis of variance (ANOVA) and, subsequently, by Least Significant Difference (LSD) post hoc test, if necessary. $P < 0.05$ was considered as statistically significant.

5. Results

5.1. Body weight and blood glucose level

Rat body weight and glucose level at the end of experiment were shown in figure1 and 2, respectively. There was no significant difference in both parameters between studied groups at the start of the experiment and the urine glucose test with strips were negative. The weight of rats was gained with age increase without any significant difference between groups after 5 weeks of dietary feeding. However, the body weight and glucose level in HFD-treated groups

were significantly changed 3 days after STZ injection. At the end of experiment, the body weight in HFD+STZ-treated group (365.74 ± 6.74 g) was significantly decreased compared with the NFD-treated group (394.87 ± 5.18 g) ($P < 0.05$, Fig.1). The glucose levels in HFD+STZ-treated rats (378.6 ± 20.79 mg/dl) were significantly higher than those in control rats (99.8 ± 1.46 mg/dl) ($P < 0.05$, Fig.2). HFD+STZ-treated rats exhibited 3-fold increase of fasting blood glucose level as compare to NFD-treated group and urine test with strips were also positive. In addition, those HFD+STZ-treated rats displayed the symptoms of polyphagia, polyuria and polydipsia as compared to NFD-treated rats by observation. The feeding of HFD and injection of STZ also induce hepatic pathology as compared to control group (Fig.3, 4). The results showed that high fat diet together with single low dose of STZ induced the change of blood glucose in animals.

After 4 weeks of melatonin administration, the slight decrease in fasting blood glucose levels observed in HFD+STZ+Mel-treated rats compare to HFD+STZ treated group (Fig.2). However, the change was not statistically significant. The body weight did not alter by melatonin treatment in HFD+STZ-treated rats.

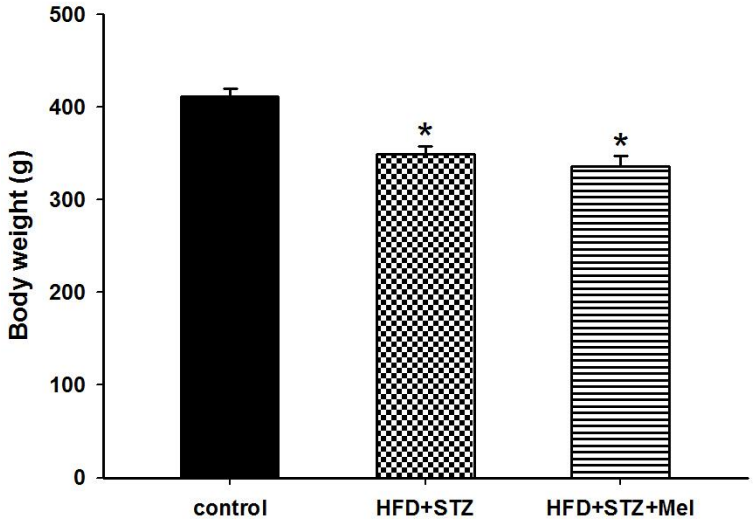

Fig.1 Body weight of animals after HFD fed + STZ injection and melatonin treatment. Parameters were determined at the end of the experiments. Data represented as mean ± SEM. * $P < 0.05$ denotes significant difference compared with the NFD-fed rats (control).

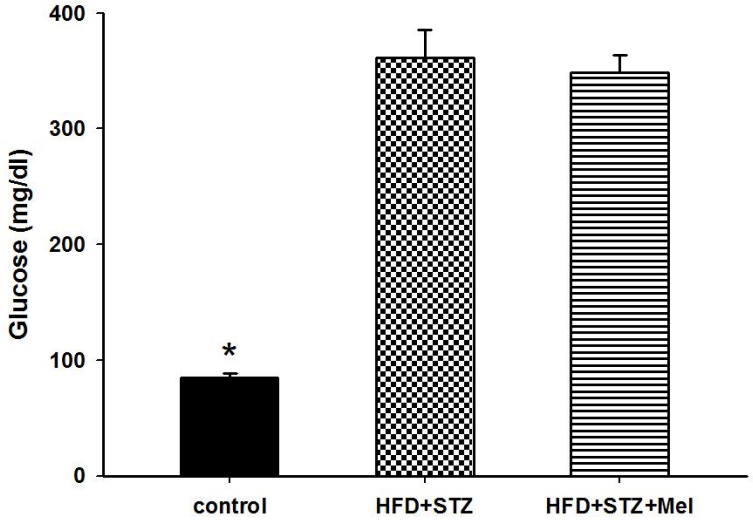

Fig.2 Fasting blood glucose level of animal 3 days after HFD fed + STZ injection and 4 week of melatonin treatment. Parameters were determined at the end of the experiments. Data represented as mean ± SEM. * P < 0.05 denotes significant difference compared with the NFD-fed rats (control).

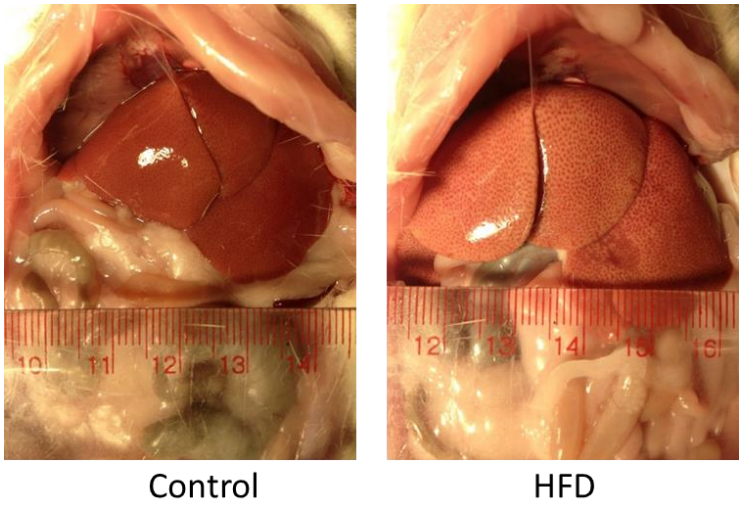

Fig.3 High fat diet (HFD) and low dose STZ treatment induced hepatic pathology in liver after 5 weeks of feeding in HFD rats as compared to normal fat diet fed rats (control)

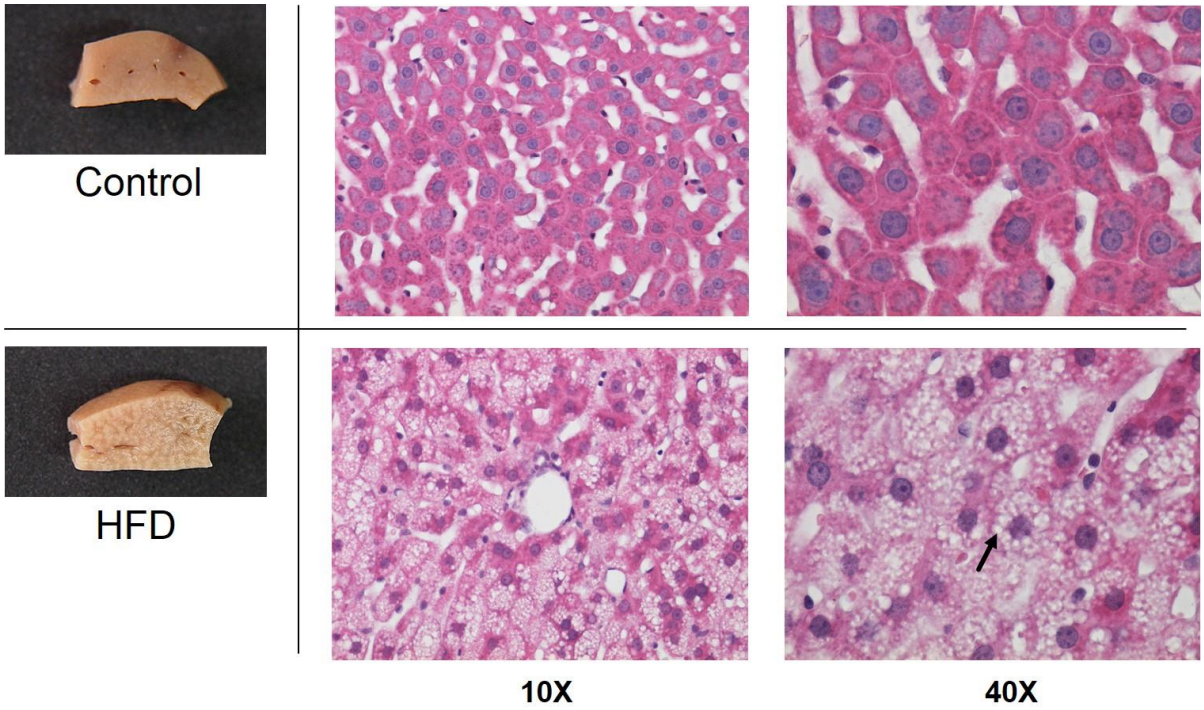

Fig.4 Liver autopsy tissue and liver histology by hematoxylin-eosin stain (H&E) staining of normal fat diet fed (upper panel) and high fat diet fed animals (lower panel). Liver steatosis, which is accumulation of triglycerides in hepatic cells, visualized as clear vesicles (arrow) in the liver of HFD group.

5.2. Hippocampal neurogenesis and astrocytic activity

In order to determine whether HFD+STZ-induced hyperglycemia influenced neurogenesis, the markers of neural stem cells; nestin, migrating immature neuron; DCX, and mature neuron; β-III Tubulin were examined (Fig. 5). HFD-fed+STZ-induced hyperglycemia showed significant reduced of nestin level to 49.49 ± 2.11% ($P < 0.05$, Fig. 5A) and also revealed decrease of DCX level to 71.05 ± 6.00% ($P < 0.05$, Fig. 5B) as well as β-III Tubulin level to 60.72 ± 7.90% ($P < 0.05$, Fig. 5C) in HFD+STZ-treated group when compared with NFD-treated group. This indicated the decrease of hippocampal neurogenesis was occurred during the HFD-fed+STZ treatments. In HFD+STZ+Mel-treated group, the levels of nestin, DCX and β-III Tubulin expression were increased to 94.11 ± 1.54% ($P < 0.05$), 96.58 ± 6.19% ($P < 0.05$), and 91.96 ± 3.50% ($P < 0.05$), respectively when compared with HFD+STZ-treated group. This suggested that melatonin treatment restored the HFD-fed+STZ-induced decrease of neurogenesis in hippocampus.

To determine the effect of HFD+STZ-induced hyperglycemia on the astrocytic activity referring to inflammation in the brain, quantitative GFAP immnoreactivity was evaluated. Western blot analysis showed significant increase in GFAP to 147.6 ± 4.54% ($P < 0.05$; Fig. 5D) in HFD+STZ-treated group when compared with NFD-treated group. Melatonin supplement could significantly decrease level of GFAP to 112.5 ± 4.66% ($P < 0.05$; Fig. 5D) when compared with HFD+STZ-treated rats.

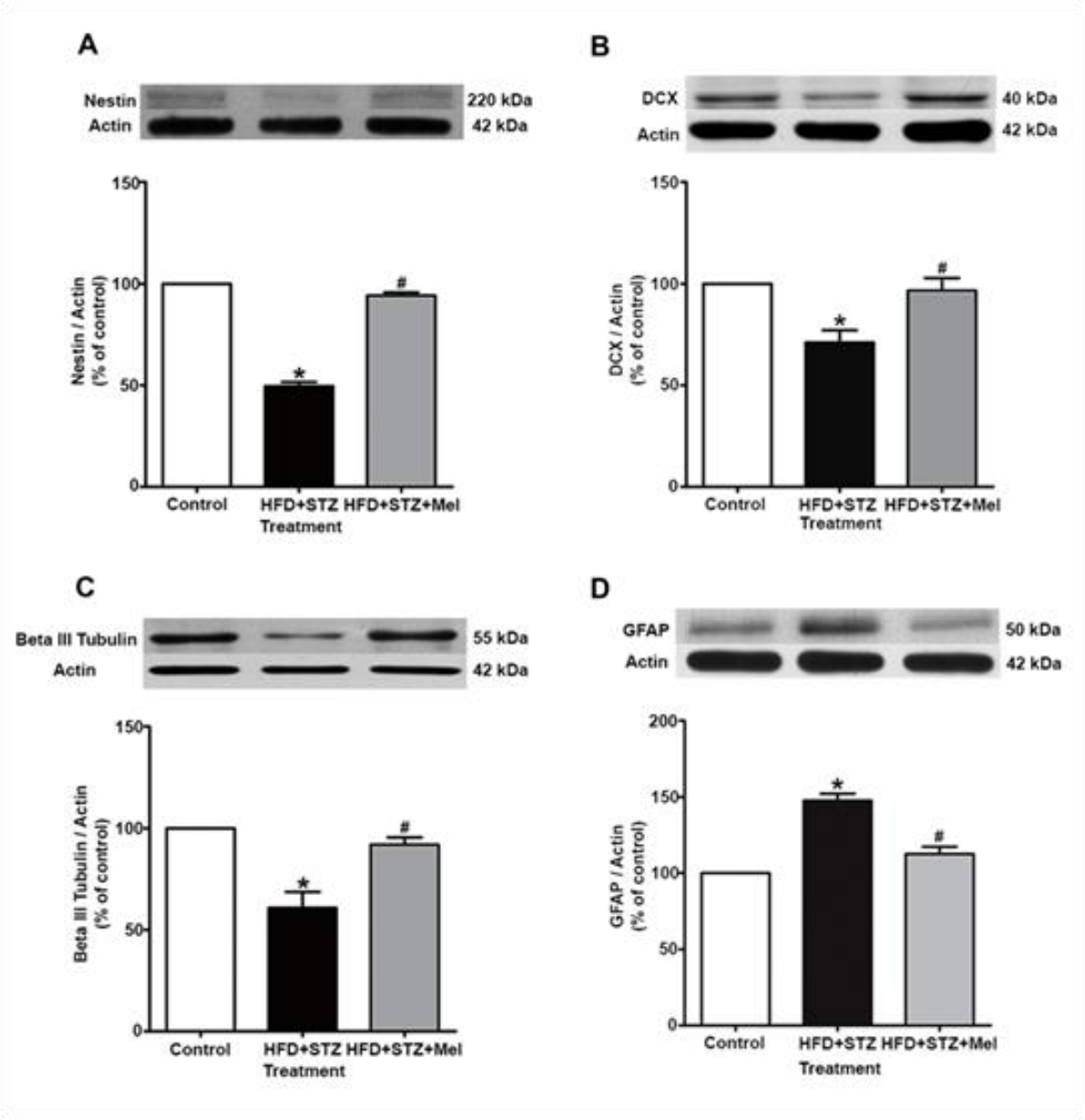

Fig.5 Effect of melatonin on hippocampal neurogenesis and astrogliosis in HFD-fed and STZ-induced rats. 10mg/kg melatonin treatment attenuated a reduction of A) nestin: neural stem cell B) DCX: immature neurons and C) β-III Tubulin: mature neurons whereas decreased D) GFAP in the HFD+STZ-treated rats. The western immunoreactive bands are shown above each graph and the band densities were normalized with actin. Their ratios were calculated as percentage of the respective value of NFD-fed control group. Data represented as mean ± SEM. * denotes significant difference compared with the NFD-fed rats (control) and # denotes significant difference compared with HFD+STZ-induced rats at $P < 0.05$

5.3. Hippocampal NMDA receptor subunits and structural synaptic proteins

Several lines of evidence have documented that the deterioration of memory performance in diabetes is primarily related to synaptic dysfunction, degeneration and also plasticity, which are regulated at pre-synaptic site by modulating the release of neurotransmitter molecules and post-synaptic site by modulating the amount, types, or properties of neurotransmitter receptors and their interactions with post synaptic scaffold protein. Thus, we investigated if HFD+STZ-treated rats display alterations of pre- and post-synaptic proteins in the rat hippocampus, including synaptophysin, synaptic protein integrating the vesicular release machinery as a presynaptic marker and PSD-95 as postsynaptic markers. Western blot analysis showed significantly decreased of the synaptophysin level to 61.73 ± 7.38% ($P < 0.05$; Fig.6A) and PSD-95 level to 47.4± 2.67% ($P < 0.05$; Fig. 6B) in HFD+STZ-treated group when compared with the NFD-fed group, which indicated the occurrence of pre- and post-synaptic disturbance in hippocampus of hyperglycemia induced by HFD+STZ treatment. In HFD+STZ+Mel-treated group, synaptophysin level was increased to 97.95 ± 3.03% ($P < 0.05$; Fig.6A) similar to PSD-95 level was increased to 69.61 ± 2.85% ($P < 0.05$; Fig.6B). This showed that melatonin modulated the effect of HFD+STZ-induced decrease of synaptic modulation in hippocampus.

To further ascertain that the reduction of PSD-95 induced by HFD+STZ-treatment associated with the expression of ionotropic glutamate receptors, N-methyl-D-aspartate (NMDA) receptors, we investigated the alterations of NR2A and NR2B which are the subtype of GluN2 and the key factor regulating this receptor mobility, protein-protein interaction and also post-synaptic membrane trafficking. Immunoreactivity for the NR2A subunit of NMDA receptor complex was dramatically reduced to 43.99 ± 3.64% ($P < 0.05$; Fig.6C) in HFD+STZ-treated group when compared with the NFD-fed group, whereas immunoreactivity for the NR2B subunit was unaffected (Fig.6D). Interestingly, melatonin treatment significantly inhibited the reduction of NR2A level and reversed to 86.45 ± 5.94% ($P < 0.05$; Fig.6C) when compared with HFD+STZ-treated rats. This indicated that melatonin treatment restored the HFD-fed+STZ-induced decrease of synaptogenesis in hippocampus.

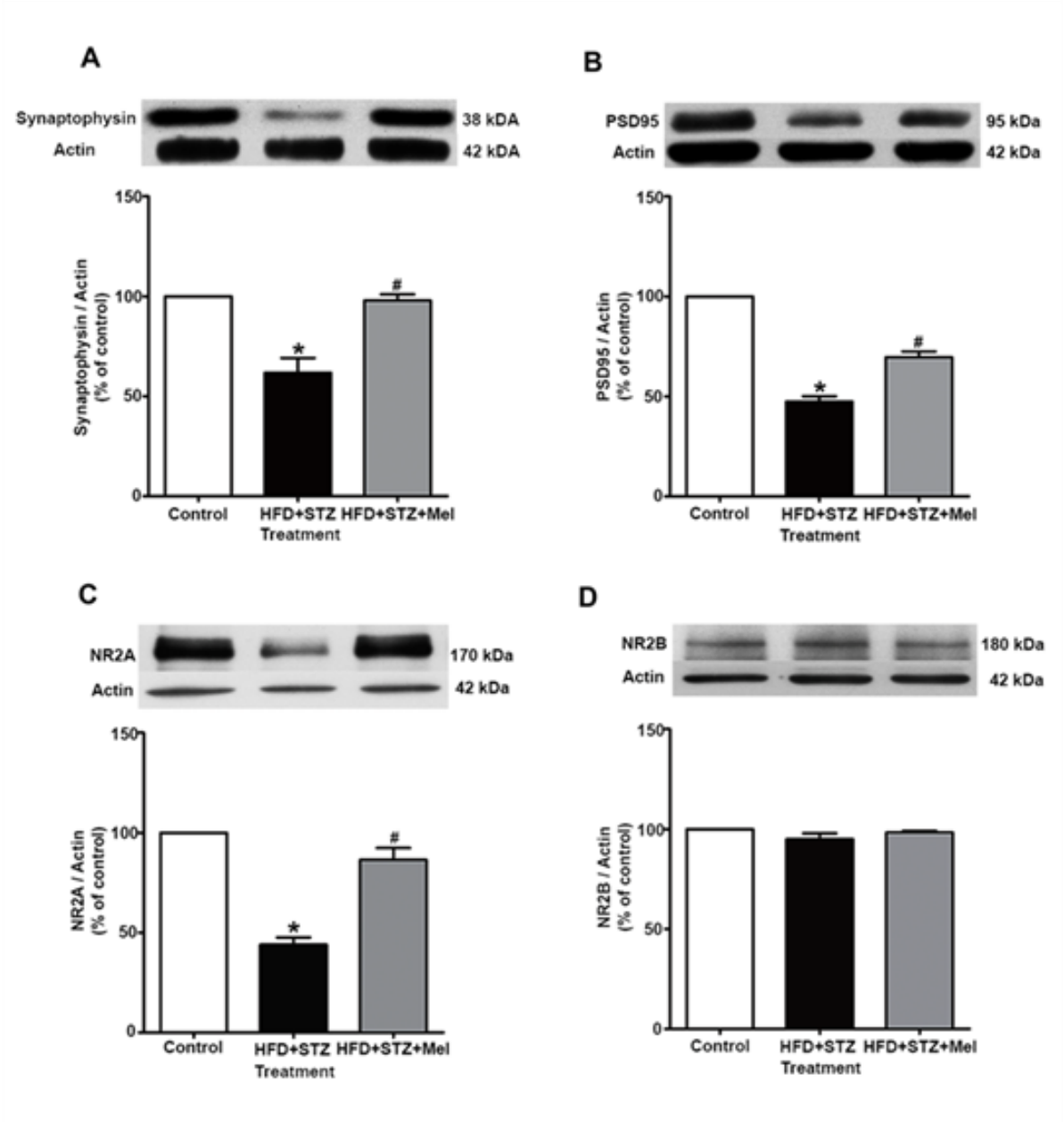

Fig.6 Effect of melatonin on hippocampal synaptic proteins and NMDA receptors in HFD-fed and STZ-induced rats. 10mg/kg melatonin treatment attenuated a reduction of A) synaptophysin: presynaptic marker protein B) PSD-95: postsynaptic marker protein C) NMDA receptor, NR2A subunit but no effect on D) NR2B subunit in the HFD+STZ-treated rats. The western immunoreactive bands are shown above each graph and the band densities were normalized with actin. Their ratios were calculated as percentage of the respective value of NFD-fed control group. Data represented as mean ± SEM. * denotes significant difference compared with the NFD-fed rats and # denotes significant difference compared with HFD+STZ-induced rats at $P < 0.05$

5.4. IR/Erk signaling in hippocampus

Brain insulin signaling plays important roles in the regulation of neuronal proliferation, survival and synaptogenesis as well as in learning and memory. Defective insulin signaling is contributed to decrease in cognitive ability and also development of dementia. Therefore, we investigated if HFD+STZ-treated rats display alterations of proteins related IR/ERK pathway in the rat hippocampus such as IR-α, IR-β, p-IR-β, ERK and p-ERK. In HFD+STZ-induced hyperglycemia rats showed significantly reduced of IR-β level to 49.58 ± 6.04% ($P < 0.05$; Fig. 7B) and also revealed significantly decreased of p-IR-β level to 47.28 ± 1.41% ($P < 0.05$, Fig. 7C) as well as p-ERK level to 41.53± 4.78% ($P < 0.05$; Fig. 7D) when compared with NFD-treated group. After melatonin administration, IR-β, p-IR-β and p-ERK levels in HFD+STZ+Mel-treated rats were increased to 73.55 ± 8.45% ($P < 0.05$; Fig. 7B), 84.98 ± 4.65% ($P < 0.05$; Fig. 7C) and 83.73 ± 5.09% ($P < 0.05$; Fig. 7D), respectively. On the other hand, IR-α level (Fig. 7A) was not showed any significant different between groups. Therefore, HFD together with STZ treatment altered the insulin signaling pathway and melatonin was able to attenuate the decreased of IR-β, p-IR-β, and p-ERK level in hippocampus.

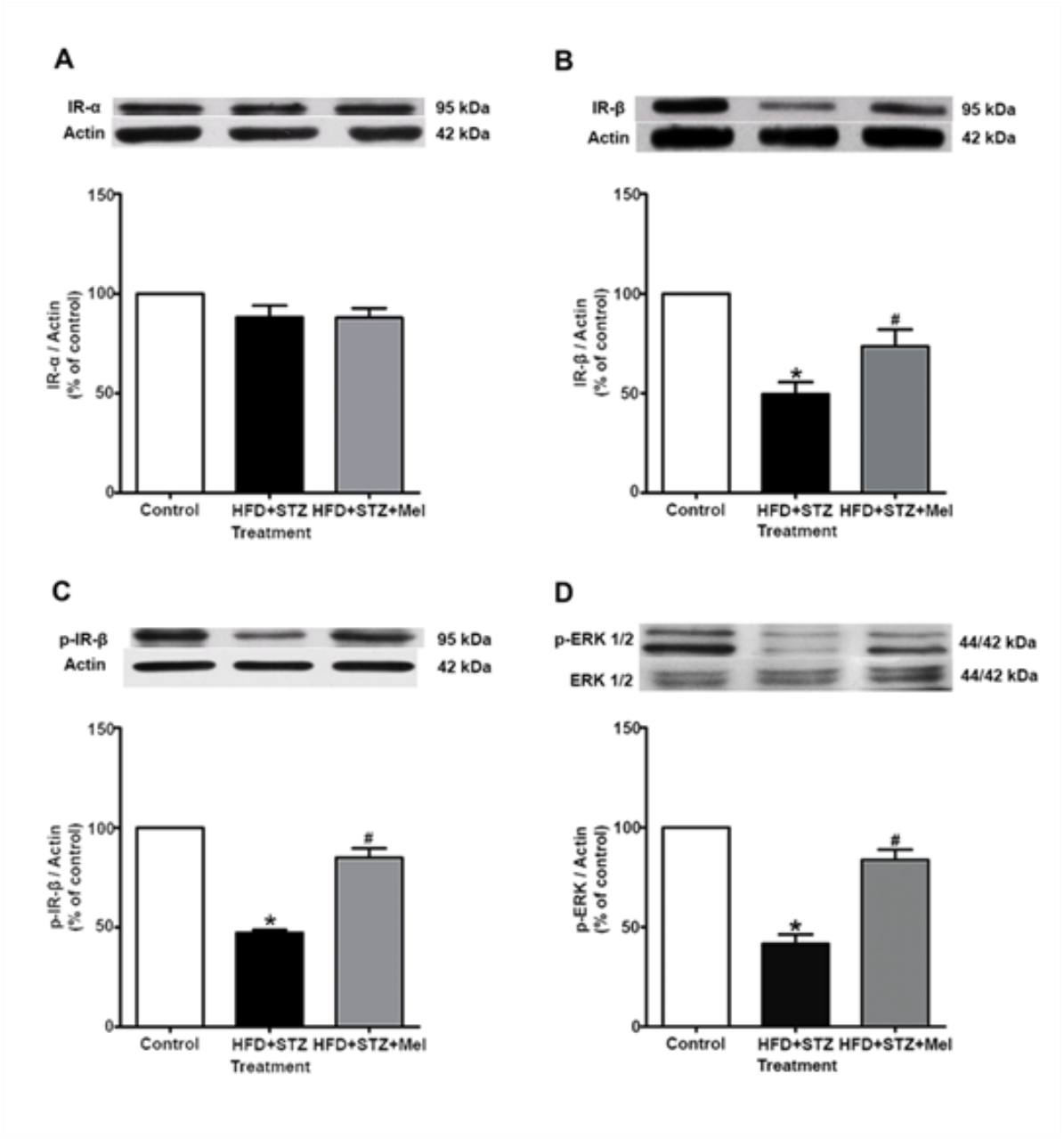

Fig.7 Effect of melatonin on hippocampal insulin signaling proteins in HFD-fed and STZ-induced rats. 10mg/kg melatonin treatment attenuated the alteration of A) IR-α, B) IR-β, C) p-IR-β, D) p-ERK protein levels in the HFD+STZ-treated rats. The western immunoreactive bands are shown above each graph and the band densities were normalized with actin. Their ratios were calculated as percentage of the respective value of NFD-fed control group. Data represented as mean ± SEM. * denotes significant difference compared with the NFD-fed rats and # denotes significant difference compared with HFD+STZ-induced rats at $P < 0.05$

5.5. Hippocampal melatonin receptor

Melatonin receptors, MT1 and MT2 levels were examined due to the action pathway of melatonin to the hippocampus. HFD+STZ-treatment significant decreased the MT1 level to 58.47 ± 1.83% ($P < 0.05$; Fig.8A) as well as MT2 level to 57.46 ± 1.75% ($P < 0.05$, Fig. 8B) in HFD+STZ-treated group when compared with NFD-treated group. In HFD+STZ+Mel-treated group, MT1 level was increased to 89.28 ± 3.63% ($P < 0.05$; Fig 8A) and MT2 level to 90.32 ± 2.89% ($P < 0.05$; Fig. 8B). Then, administration of melatonin reversed the reduction of metatonin receptors expression by HFD-fed and STZ-induced hyperglycemia.

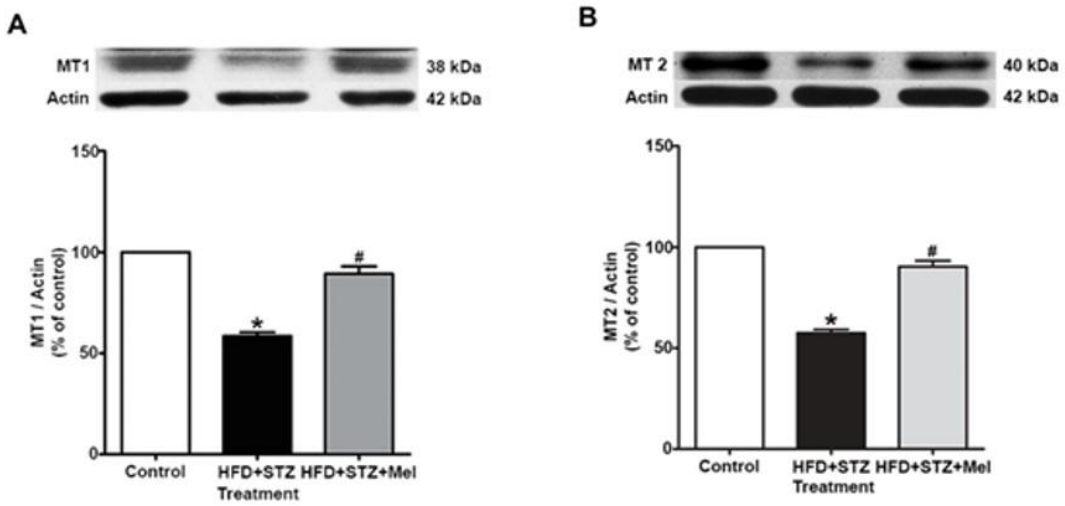

Fig.8 Effect of melatonin on hippocampal melatonin receptors in HFD-fed and STZ-induced rats. 10mg/kg melatonin treatment attenuated a reduction of A) MT1 B) MT2 protein in the HFD+STZ-treated rats. The western immunoreactive bands are shown above each graph and the band densities were normalized with actin. Their ratios were calculated as percentage of the respective value of NFD-fed control group. Data represented as mean ± SEM. * denotes significant difference compared with the NFD-fed rats and # denotes significant difference compared with HFD+STZ-induced rats at $P < 0.05$

5.6. Clock gene mRNA expression in rat hippocampus

First of all we study the daily rhythm of expression of studied clock genes. The results showed that all four genes; *Per1, Per2, Bmal1* and *Rev-erb alpha* expressed with significant the daily variation which *Per1, Per2* and *Rev-erb alpha* display a diurnal pattern whereas *Bmal1* display a nocturnal pattern ($P < 0.05$, Fig. 9).

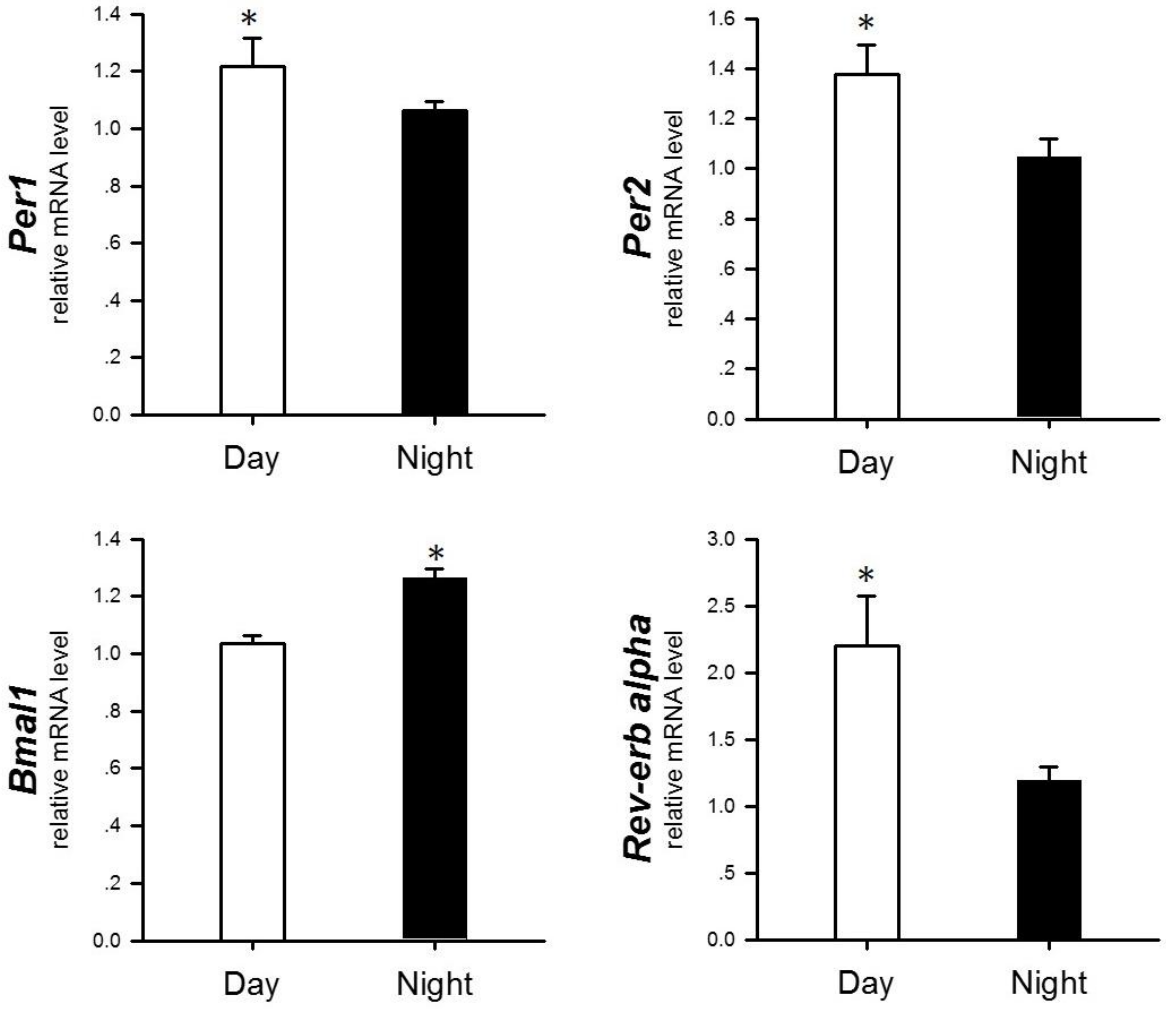

Fig.9 Daily variation of clock genes mRNA expression in the rat hippocampus under LD12:12. Values represent as relative abundance (mean ± SEM from 3 - 6 animals for each group) after normalization to actin. *$P < 0.05$ showed a statistically significant difference between day and night timepoint.

5.7. Effect of melatonin on clock gene expression in the high fat diet fed and streptozotocin-induced hyperglycemia rats.

Next, we examined mRNA and protein level of expression of the core clock components in positive loop; *Bmal1* (Fig. 10), negative loop; *Per1* (Fig. 11) and *Per2* (Fig. 12) and auxiliary loop; *Rev-erb alpha* (Fig. 13) to see whether HFD-fed and STZ induced high blood glucose affected the level of genes expression. The results showed that all studied clock genes were expressed in the rat hippocampus of all groups. HFD-fed and STZ injection had different effect on clock genes mRNA levels. The comparison of mRNA level showed a statistically significant difference between NFD control and HFD+STZ groups for *Per2, Bmal1* and *Rev-erbα* but not for *Per1*. HFD+STZ treatment obviously increased *Per2* and *Bmal1* mRNA levels while slightly decreased the level of *Rev-erbα* mRNA expression. Melatonin injection for 4 weeks had significant ability to attenuate the effect of HFD-fed+STZ treatment on the mRNA level of *Per2* ($P < 0.05$). However, melatonin had no specific effect on other clock gene.

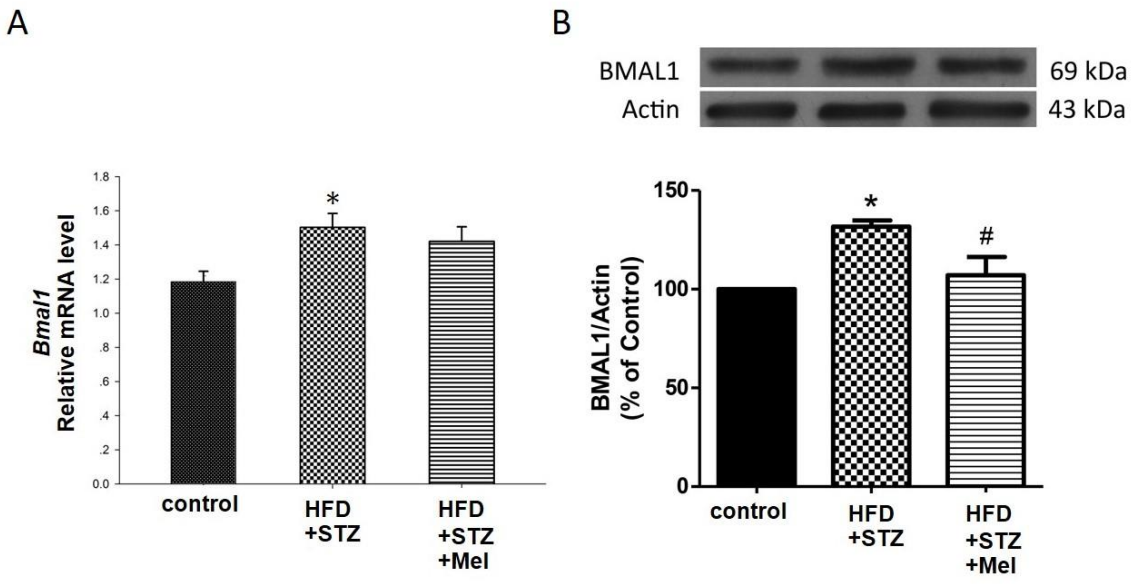

Fig.10 Levels of *Bmal1* (A) mRNA were analyzed by real-time PCR and (B) protein were analyzed by western blot in the hippocampus of high fat diet fed (HFD) and streptozotocin (STZ)-induced hyperglycemia rats undergoing melatonin (Mel) treatment for 4 weeks. Values represent as relative abundance (mean ± SEM from 3 - 6 animals for each group) after normalization to actin. * $P < 0.05$ compared to rat injected with saline as the controls at the same time points. # $P < 0.05$ compared with the HFD+STZ group at the same time points.

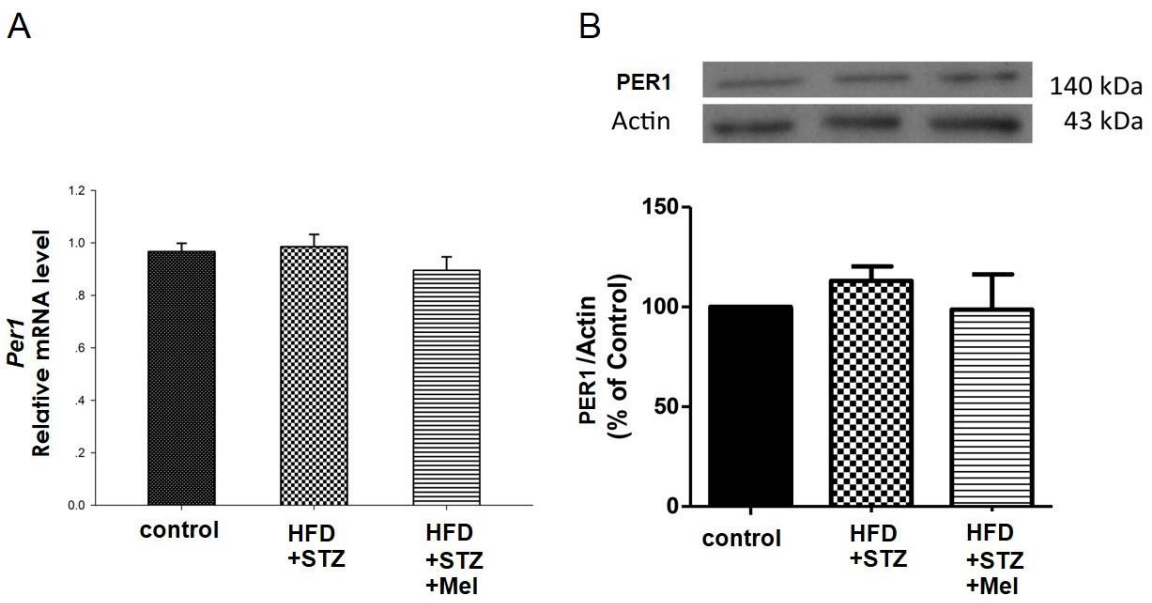

Fig.11 Levels of *Per1* (A) mRNA were analyzed by real-time PCR and (B) protein were analyzed by western blot in the hippocampus of high fat diet fed (HFD) and streptozotocin (STZ)-induced hyperglycemia rats undergoing melatonin (Mel) treatment for 4 weeks. Values represent as relative abundance (mean ± SEM from 3 - 6 animals for each group) after normalization to actin. * $P < 0.05$ compared to rat injected with saline as the controls at the same time points. # $P < 0.05$ compared with the HFD+STZ group at the same time points.

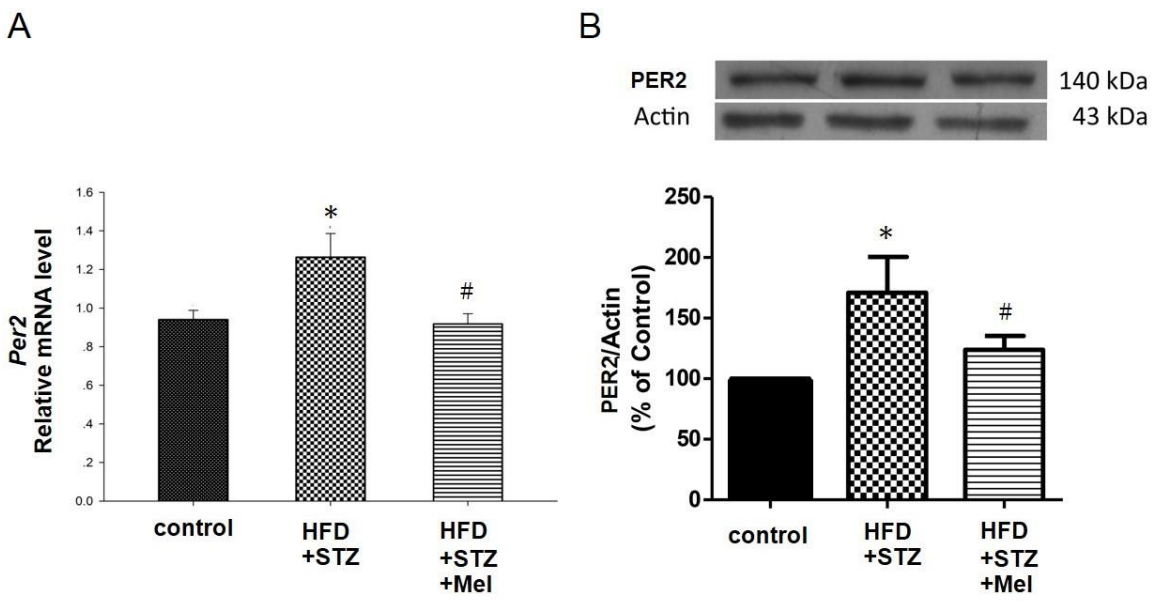

Fig.12 Levels of *Per2* (A) mRNA were analyzed by real-time PCR and (B) protein were analyzed by western blot in the hippocampus of high fat diet fed (HFD) and streptozotocin (STZ)-induced hyperglycemia rats undergoing melatonin (Mel) treatment for 4 weeks. Values represent as relative abundance (mean ± SEM from 3 - 6 animals for each group) after normalization to actin. * $P < 0.05$ compared to rat injected with saline as the controls at the same time points. # $P < 0.05$ compared with the HFD+STZ group at the same time points.

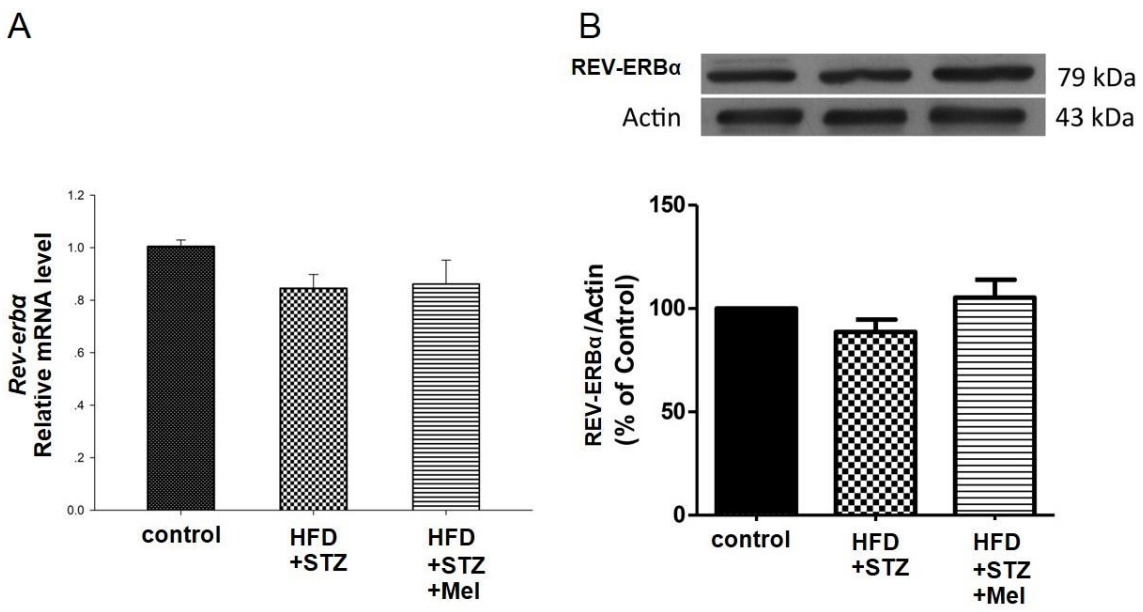

Fig.13 Levels of *Rev-erbα* (A) mRNA were analyzed by real-time PCR and (B) protein were analyzed by western blot in the hippocampus of high fat diet fed (HFD) and streptozotocin (STZ)-induced hyperglycemia rats undergoing melatonin (Mel) treatment for 4 weeks. Values represent as relative abundance (mean ± SEM from 3 - 6 animals for each group) after normalization to actin.

5.8. Effect of melatonin on Sirtuin1 gene expression in the high fat diet fed and streptozotocin-induced hyperglycemia rats.

In order to study the regulation of epigenetic mechanism on the circadian clock component under high blood glucose condition of diabetic in the brain. We examined the alteration of *Sirt1* mRNA level in the hippocampus of the hyperglycemic rats (Fig. 14). The results showed that high blood glucose significantly elevated *Sirt1* expression in HFD+STZ treated rats as compare to normal control rats ($P < 0.05$). The melatonin treatment attenuated the level of *Sirt1* expression to the normal level of expression ($P < 0.05$).

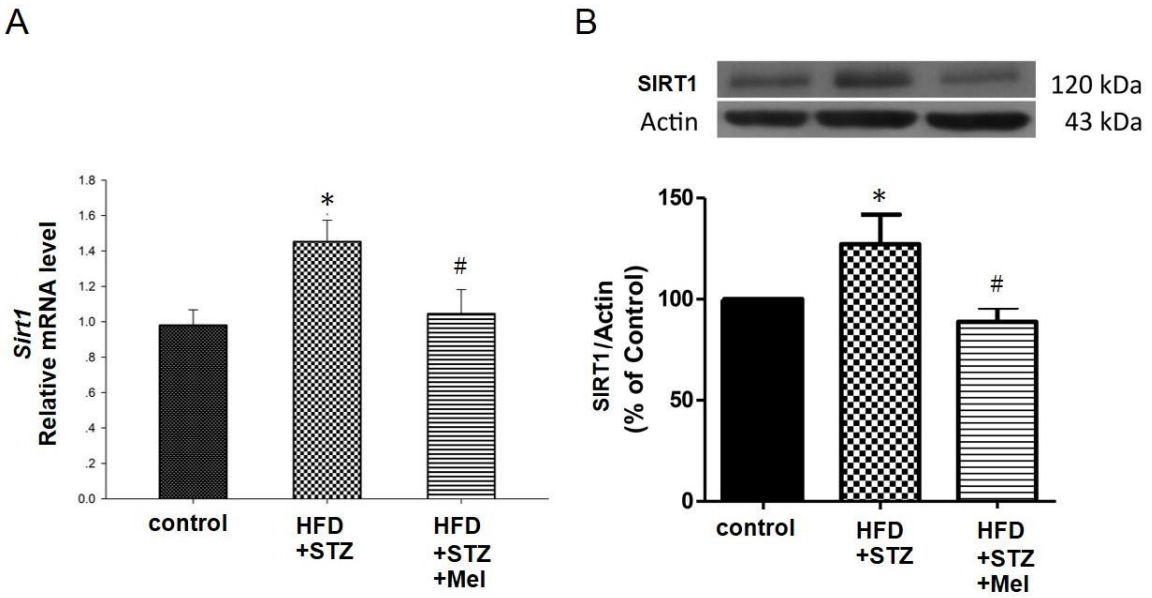

Fig.14 Levels of *Sirt1* (A) mRNA were analyzed by real-time PCR and (B) protein were analyzed by western blot in the hippocampus of high fat diet fed (HFD) and streptozotocin (STZ)-induced hyperglycemia rats undergoing melatonin (Mel) treatment for 4 weeks. Values represent as relative abundance (mean ± SEM from 3 - 6 animals for each group) after normalization to actin. * $P < 0.05$ compared to rat injected with saline as the controls at the same time points. # $P < 0.05$ compared with the HFD+STZ group at the same time points.

6. Conclusion and Discussion

We found that the HFD-fed together with, a low dose, STZ-injected rats were shown diabetic-like appearance which is comparable to previous study (Hu et al., 2013; Miao et al., 2015). The presence of over peritoneal fat in HFD rats due to excess fat consumption could constitute a source of oxidation leading to hyperinsulinemia, a typical feature of insulin resistance (Belfiore and Iannello, 1998). Further, in induced diabetic models, most of the animals requires relatively high dose of STZ (>50 mg/kg) (Lenzen, 2008). However, high dose of STZ usually give dramatic increase mortality rate (Ventura-Sobrevilla et al., 2011). On the contrary, too low dose of STZ (25 mg/kg) did not successful produce hyperglycemia (Srinivasan et al 2005). For this model, we used the combination of HFD-fed and low dose of STZ (40 mg/kg) which gave an effective result in hyperglycemia in harmony with prior study (Hu et al., 2013). High concentration of glucose increase the production of oxidative stress under chronic inflammation can overcome antioxidant defenses leading to intense oxidative stress (Esposito et al., 2002). Both in vivo and in vitro studies demonstrated that high glucose levels affect the production of pineal melatonin (Amaral et al., 2014; Peschke et al., 2008; Srinivasan et al., 2005). The alteration of melatonin synthesis influences the important role played by melatonin as a powerful antioxidant and in the control of sleep and biological rhythms and energy homeostasis including glucose metabolism and insulin secretion. Hippocampal neurogenesis and synaptic plasticity seems to be sensitive to many pathogenic and treatment factors that are assorted with the comorbidity between diabetes and impaired memory (Ho et al., 2013). In this study, we showed that melatonin attenuated the reduction in proliferation of hippocampal neurons and synaptic plasticity and help to decreased astrocytic activity that induced by HFD-fed+STZ treatment.

Hippocampal neurons in adult mammals are vulnerable to an abnormal glycemia. A chronic high blood glucose levels in an experimental model of diabetes showed the reduction of cognitive dysfunction and hippocampal changes (Alvarez et al., 2009; Kerti et al., 2013). Rat with severe hyperglycemia showed the low weight of brain tissue and severity of brain damage and apoptosis especially hippocampus (Tayman et al., 2014). In HFD-fed+STZ-treated rats, the reduction of neurogenesis in the hippocampus was observed as shown by the decreasing of proteins expression related to neurogenesis. Nestin is an intermediate filament protein marker of neural stem cell (Gilyarov, 2008; Park et al., 2010). The reduction of nestin expression in hippocampus in the HFD-fed+STZ-treated rats agree with the previous reports in cardiac (El-Helou et al., 2009) and vascular smooth muscle (Tardif et al., 2014) of diabetic rodent models. The level of DCX, a microtubule-associated protein marker of migrating or immature neuron, decreased in HFD-fed+STZ-treated rats which is similar as the results found in the dentate gyrus

of mice that treated with HFD (Hamilton et al., 2011; Yoo et al., 2012b) We also examined the level of β-III tubulin protein level as a marker of mature neuron and the results showed that there was a reduction of β-III tubulin expression in HFD-fed+STZ-treated animals. These results suggest the loss of these marker proteins expression in hyperglycemia animals may represents the loss of function or number of neural stem cells including mature neurons in hippocampus. Many reports showed in animal models of diabetes that were established by STZ and/or HFD significantly decreased Ki67 (Balu and Lucki, 2009; Hwang et al., 2008; Revsin et al., 2009; Stranahan et al., 2008) BDNF protein level (Yoo et al., 2011; Yoo et al., 2014) and Brdu positive cell in hippocampus (Beauquis et al., 2006; Lindqvist et al., 2006; Saravia et al., 2004). Moreover, the study in the neural stem cells (NSCs) also showed that exposure to the high glucose altered the epigenetic mechanisms in NSCs (Shyamasundar et al., 2013) and altered expression of genes that involved in cell-cycle progression and cell-fate specification during neurulation phase of brain formation in embryo (Fu et al., 2006). Together, these indicate that hyperglycemia can contribute to the impairment of adult hippocampal neurogenesis by inducing many protein dysregulations that involve in cell proliferation, progenitor survival and neuronal differentiation in the hippocampus.

We also showed a significant downregulation of synaptic proteins expression, synaptophysin and PSD-95. Synaptophysin, an abundant presynaptic protein and PSD-95, a scaffolding protein enriched in post-synaptic densities, in the hippocampus of HFD+STZ-induced hyperglycemia rats corresponded to previous studies in STZ-induced diabetes (Duarte et al., 2009; Hou et al., 2012; Wang et al., 2014) and HFD-fed animals (Arnold et al., 2014; Najem et al., 2014). It has been shown that dysregulation of synaptic formation induce memory impairment by interference the process of neurotransmission in hippocampus (Trudeau et al., 2004). As synaptophysin is a constituent of neurotransmitter-containing presynaptic vesicle membranes, the augmentation of this synaptic protein may reflect an increase in neurotransmission leading to improved memory (Edelmann et al., 1995). Apart from PSD-95 immunoreactivity, these HFD-fed+STZ-treated animals also showed a decrease in this synaptic protein. This present results agree with Amold and colleagues who showed that HFD produced brain insulin resistance related to a reduction of hippocampal PSD-95 immunostaining in mice (Arnold et al., 2014). PSD-95 is a cytoskeletal elements adhering to the postsynaptic membrane and serving as scaffold for surface neurotransmitter receptors to promote synaptic efficiency (Delint-Ramírez et al., 2008). Therefore, the decrease in PSD-95 indicates that the strength of synapse may aggravate by distorted clustering of N-methyl-D-aspartate receptor (NMDAR), an ionotropic glutamate receptor. It has been extensively affirmed that NMDAR signaling in the hippocampus

is considerable for encoding the connections between particular events and specific spatial locations within a cognitive map (Simoes et al., 2007). A study using a streptozotocin treated showed that tyrosine dependent phosphorylation of the NR2A/B subunits of the NMDA receptor and CAMKII were reduced and its association to the NMDA receptor complex were impaired in hyperglycemic rats compared with age-matched controls (Gardoni et al., 2002). In this study, we founded that HFD-fed+STZ treatment decreased the expression of NR2A consistent with Delibas et al who found that STZ-diabetes resulted in increased lipid peroxidation and exhibited the reduction of NR2A concentrations (Delibas et al., 2004). NMDARs are good substrates for CAMKII and Src protein kinase (Tezuka et al., 1999).

In neurogenesis aspect, there has evidence showed that in the adult mouse dentate gyrus, the stimulation of the NMDA receptor leads to suppress the expression of both nestin and proliferating cell nuclear antigen (PCNA). Li et al. reported that NR2B-containing NMDARs play a positive role in regulating NSPC proliferation by using a selective NR2B antagonist to prevent the NMDA-induced increase in cell proliferation (Li et al., 2011). Nevertheless, a study by Hu et al. showed that NR2B-containing NMDA receptors negatively regulate neurogenesis and spatial memory (Hu et al., 2008). Moreover, a study in NR2A knockout mice (GluN2A$^{-/-}$) by using golgi staining revealed the deficits in the dendritic growth in maturing dentate granule neurons (Kannangara et al., 2014). In addition, a study of Watanabe et al. concluded that the NR2A receptor subunit promotes cell proliferation by accelerating cell cycling (Watanabe et al., 2008). Therefore, the alterations of pre/postsynaptic and NR2A protein expression indicated the abnormality of synaptic structures which may influence the efficiency of glutamate transmission and cell proliferation in hippocampal neurons under hyperglycemia condition.

Additionally, we found that HFD-fed+STZ-treated rats showed an obvious increase in GFAP level in the hippocampus. This result was corresponded with the previous report in STZ-induced diabetes animals (Amin et al., 2013; Baydas et al., 2003; Coleman et al., 2004). The study in diabetes rats showed the association of the increasing in GFAP expression was related to the phenotypic changes and increased astrocytic number (Nagayach et al., 2014a). Hyperglycemia condition also enhance the proliferation and activation of microglial that induced the increasing of cell death signaling molecules such as caspase-3 level in the rat brain (Nagayach et al., 2014b). Changes in glial activity caused the neuronal cell death or caused the disturbance of the brain functions such a cognitive function and motor function (Amin et al., 2013; Nagayach et al., 2014a; Nagayach et al., 2014b). Therefore, these results suggest that HFD and STZ induced hyperglycemia causes decreased neurogenesis and synapse function while increased astrocyte activity in the hippocampus.

Melatonin has been found to promote learning and memory function. Our previous study found that melatonin could prevent the dexamethasone- induced reduction in neurogenesis and impairment of spatial memory in adult mice (Ruksee et al., 2014) and also modulated the expression of Ki67, DCX, NR2A/B, and CaMKII in adult rat hippocampal progenitor cells induced by methamphetamine (Ekthuwapranee et al., 2015). In the present study, we found that the melatonin administration significantly increased the level of neuronal markers during neurogenesis and also synaptic proteins expression while decreased the astrocytic protein in the hippocampal of the HFD+STZ-treated rats compared with the vehicle-injected HFD+STZ-treated rats. The administration of melatonin prevents an increase in oxidative stress and nitric oxide levels in blood plasma and several tissues during diabetes (Korkmaz et al., 2012; Reiter et al., 2009; Sudnikovich et al., 2007) and also modulates neuroinflammation and oxidative stress via NF-kappa B and Nrf2 cascade (Negi et al., 2011; Permpoonputtana and Govitrapong, 2013). It has been reported that melatonin induced an increase in the number of BrdU/DCX-cell populations in hippocampus. This implied that melatonin induced survival of newly generated neurons by affecting intermediate DCX-expressing stages of adult neurogenesis (Ramirez-Rodriguez et al., 2009). Furthermore, cell viability, differentiation of neural stem cells and BDNF were increased by melatonin (Kong et al., 2008). Our previous studies (Sotthibundhu et al., 2010; Tocharus et al., 2014) exhibited that melatonin directly promoted neural stem cell proliferation via melatonin receptor and improve hippocampal synaptophysin, PSD-95, and NMDA receptor such as NR2A/B under both normal and abnormal conditions (Dilek et al., 2010; Juan et al., 2014; Kaewsuk et al., 2009; Sutcu et al., 2006; Zhou et al., 2011). Therefore, we propose that down-regulation of neurogenesis and synaptic formation, and up-regulation of astrocytic activity implied to occurring inflammation in rat hippocampus would be the effect of hyperglycemia. Melatonin could be beneficial for a therapeutic approach in diabetic patients by attenuating the oxidative stress induced hippocampal cell damage and promoting proliferation.

Recently, the brain insulin/insulin receptors system is obtaining major attention in regulation of brain functions. Insulin receptors are distributed in the brain regions such as hippocampus, hypothalamus and olfactory bulb. The presence of IR in the hippocampus suggests its functional involvement in cognition (Kim and Feldman, 2012). The disturbances of the insulin signaling pathway have revealed new perspective on the link between cognitive impairment associated AD and HFD/STZ-induced diabetes (Agrawal et al., 2011; Blazquez et al., 2014; Havrankova et al., 1979; Schioth et al., 2012). The present results demonstrated that in hippocampus of hyperglycemia rats, melatonin treatment also improved the reduction of IR-β, the phosphorylation levels of IR-β and of their downstream protein activation related with

proliferative pathway such as phosphorylated extracellular signal-regulated kinase-1/2 (p-ERK1/2), which might be associated with neurogenesis (De Fea and Roth, 1997; McNeill et al., 2008; Murray and Holmes, 2011; Pearson et al., 2001; Skolnik et al., 1993). Activation of the insulin receptor-Shc (Src homology collagen peptide)-ERK pathway activates gene expressions by upregulation CREB (cAMP response element-binding protein-cellular transcription factor) phosphorylation that are required for cell and synapse growth, and for cell repair and maintenance. Our results are consistent with previous study in obese rats, which demonstrated that melatonin improve insulin sensitivity in hypothalamus, liver, skeletal muscle, and epididymal adipose tissue (Zanuto et al., 2013). The number of signaling molecules have been implicated in learning and memory functions in different species, such as ERK activity, the important one of downstream insulin signaling protein, is involved in learning and memory (Selcher et al., 1999; Zhao et al., 2004). Moreover, Hwang et al. discovered that ERK2 activation induced mitogenic signaling that promoted the translocation of p21 from the nucleus to the cytoplasm, causing cell cycle progression and proliferation (Hwang et al., 2009). It has been suggested that ERK mediates changes factor for neurogenesis and in the long-term storage of information in the brain, a process that requires participation of gene regulation and expression (Samuels et al., 2008; Shioda et al., 2009; Yan et al., 2007).

Several evidence suggested a physiopathological relationship between the lacked or reduced melatonin level and their receptors exhibited in the diabetes which could be improved by melatonin supplementation (She et al., 2014). Melatonin transmits their actions mediate the specific high-affinity G protein-coupled receptors known as melatonin receptor 1 (MT1) and melatonin receptor 2 (MT2) (Reppert et al., 1994), which found to express in many area of the brain include hippocampus (Mazzucchelli et al., 1996). Melatonin administration increased insulin sensitivity and glucose tolerance in animals fed with either high fat or high sucrose diet (Kitagawa et al., 2012). Recent reports in diabetes model showed that melatonin acts through MT1 and MT2 receptors to activate hypothalamic Akt/PKB, protein kinase B and suppress hepatic gluconeogenesis in rats (Faria et al., 2013) and inhibit insulin release of pancreatic β cells (Karamitri et al., 2013). In addition, use of luzindole, a melatonin antagonist can block some of the metabolic actions of melatonin (Srinivasan et al., 2013). In this study, we found that rats treated by the HFD and low dose of STZ which showed the markedly increased of their blood glucose level, was exhibited the low level of melatonin receptors both MT1 and MT2 in the hippocampus. The study in the melatonin receptor knockout mice showed the alteration of melatonin level that affect the change in several parameters such as body weight, blood glucose level, insulin level which induced the diabetes condition (Bazwinsky-Wutschke et al., 2014;

Contreras-Alcantara et al., 2010). In addition, the genetic studies in patients also demonstrate the association of melatonin receptor genes polymorphism in the diabetes especially the variation in MT2 (Nagorny and Lyssenko, 2012; Zhang et al., 2014).

More interestingly, not only the signal transduction pathway of melatonin, but also a potential cross-talk between melatonin and insulin has been reported. Recently, Gabriel et al. show that melatonin induces a rapid MT1/MT2 membrane receptor-dependent tyrosine phosphorylation and activation of the insulin receptor β-subunit tyrosine kinase (IR) and downstream AKT serine phosphorylation and ERK phosphorylation, respectively, in the rat hypothalamic suprachiasmatic region (Anhe et al., 2004) similar to our study. Then, the loss of melatonin receptor signaling might affect the insulin secretion or function and increased the risk to develop diabetes associated with abnormal brain. In this study, we first report that melatonin treatment could enhance the expression of hippocampal MT1 and MT2 protein levels in HFD-fed and STZ-treated rats. Our data imply that dysreguration of the insulin signaling pathway during diabetes may provide a convergent mechanism of hippocampal impaired neurogenesis and synaptic proteins, and melatonin can reverse these negative effects. Moreover, melatonin also rescues the reduction of the melatonin receptors in the hippocampus of HFD-fed and STZ induced hyperglycemia rats to improve the transmission of the action of melatonin to reduce the pathogenesis of diabetes.

Additionally, high concentration of glucose in blood circulation alters different gene expressions in many organs including the brain. It can cause the change in brain function such as cognitive function of the hippocampus. Several reports have suggested the association of the impairment of the molecular clock function to the development of cognitive impairment in diabetic. However, the precise underlying mechanisms remain unknown. In the present study we investigated the effects of melatonin on clock gene expression in hippocampus of HFD-fed and STZ induced hyperglycemia rats. The results showed that hyperglycemia condition affected both the mRNA and protein level of clock genes; *Per2, Bmal1* and *Rev-erb alpha* expression in the hippocampus of hyperglycemia rats compared to that observed in the vehicle-injected group. Melatonin treatment could attenuate the level of *Per2* expression in the hippocampus of HFD-fed and STZ induced hyperglycemia rats. Therefore, hyperglycemia disrupts the molecular clock genes expression that may lead to the circadian physiological changes of hippocampal functions and melatonin treatment could help to modulate the abnormality, therefore the mechanism still need to elucidate by more research in the future.

References

Agil, A., et al., 2011. Beneficial effects of melatonin on obesity and lipid profile in young Zucker diabetic fatty rats. J Pineal Res. 50, 207-12.

Agrawal, R., et al., 2011. Insulin receptor signaling in rat hippocampus: a study in STZ (ICV) induced memory deficit model. Eur Neuropsychopharmacol. 21, 261-73.

Alvarez, E.O., et al., 2009. Cognitive dysfunction and hippocampal changes in experimental type 1 diabetes. Behav Brain Res. 198, 224-30.

Amaral, F.G., et al., 2014. Melatonin synthesis impairment as a new deleterious outcome of diabetes-derived hyperglycemia. J Pineal Res. 57, 67-79.

Amin, S.N., et al., 2013. A histological and functional study on hippocampal formation of normal and diabetic rats. F1000Res. 2, 151.

Anhe, G.F., et al., 2004. In vivo activation of insulin receptor tyrosine kinase by melatonin in the rat hypothalamus. J Neurochem. 90, 559-66.

Arnold, S.E., et al., 2014. High fat diet produces brain insulin resistance, synaptodendritic abnormalities and altered behavior in mice. Neurobiology of disease. 67, 79-87.

Awad, N., Gagnon, M., Messier, C., 2004. The relationship between impaired glucose tolerance, type 2 diabetes, and cognitive function. J Clin Exp Neuropsychol. 26, 1044-80.

Balu, D.T., Lucki, I., 2009. Adult hippocampal neurogenesis: regulation, functional implications, and contribution to disease pathology. Neurosci Biobehav Rev. 33, 232-52.

Baydas, G., et al., 2003. Inhibitory effects of melatonin on neural lipid peroxidation induced by intracerebroventricularly administered homocysteine. J Pineal Res. 34, 36-9.

Bazwinsky-Wutschke, I., et al., 2014. Influence of melatonin receptor signalling on parameters involved in blood glucose regulation. J Pineal Res. 56, 82-96.

Beauquis, J., et al., 2006. Reduced hippocampal neurogenesis and number of hilar neurones in streptozotocin-induced diabetic mice: reversion by antidepressant treatment. Eur J Neurosci. 23, 1539-46.

Belfiore, F., Iannello, S., 1998. Insulin resistance in obesity: metabolic mechanisms and measurement methods. Molecular genetics and metabolism. 65, 121-128.

Blazquez, E., et al., 2014. Insulin in the brain: its pathophysiological implications for States related with central insulin resistance, type 2 diabetes and Alzheimer's disease. Front Endocrinol (Lausanne). 5, 161.

Coleman, E., et al., 2004. Effects of diabetes mellitus on astrocyte GFAP and glutamate transporters in the CNS. Glia. 48, 166-78.

Contreras-Alcantara, S., Baba, K., Tosini, G., 2010. Removal of melatonin receptor type 1 induces insulin resistance in the mouse. Obesity (Silver Spring). 18, 1861-3.

De Fea, K., Roth, R.A., 1997. Modulation of insulin receptor substrate-1 tyrosine phosphorylation and function by mitogen-activated protein kinase. J Biol Chem. 272, 31400-6.

Delibas, N., et al., 2004. NMDA receptor subunits 2A and 2B decrease and lipid peroxidation increase in the hippocampus of streptozotocin-diabetic rats: effects of insulin and gliclazide treatments. Int J Neurosci. 114, 391-401.

Delint-Ramírez, I., Salcedo-Tello, P., Bermudez-Rattoni, F., 2008. Spatial memory formation induces recruitment of NMDA receptor and PSD-95 to synaptic lipid rafts. Journal of neurochemistry. 106, 1658-1668.

Desrocher, M., Rovet, J., 2004. Neurocognitive correlates of type 1 diabetes mellitus in childhood. Child Neuropsychol. 10, 36-52.

Dilek, M., et al., 2010. Melatonin modulates hippocampus NMDA receptors, blood and brain oxidative stress levels in ovariectomized rats. J Membr Biol. 233, 135-42.

Duarte, J.M., et al., 2009. Caffeine consumption attenuates neurochemical modifications in the hippocampus of streptozotocin-induced diabetic rats. J Neurochem. 111, 368-79.

Edelmann, L., et al., 1995. Synaptobrevin binding to synaptophysin: a potential mechanism for controlling the exocytotic fusion machine. The EMBO journal. 14, 224.

Ekthuwapranee, K., Sotthibundhu, A., Govitrapong, P., 2015. Melatonin attenuates methamphetamine-induced inhibition of proliferation of adult rat hippocampal progenitor cells in vitro. J Pineal Res. 58, 418-28.

El-Helou, V., et al., 2009. The cardiac neural stem cell phenotype is compromised in streptozotocin-induced diabetic cardiomyopathy. J Cell Physiol. 220, 440-9.

Esposito, K., et al., 2002. Inflammatory cytokine concentrations are acutely increased by hyperglycemia in humans role of oxidative stress. Circulation. 106, 2067-2072.

Faria, J.A., et al., 2013. Melatonin acts through MT1/MT2 receptors to activate hypothalamic Akt and suppress hepatic gluconeogenesis in rats. Am J Physiol Endocrinol Metab. 305, E230-42.

Fu, J., et al., 2006. High glucose alters the expression of genes involved in proliferation and cell-fate specification of embryonic neural stem cells. Diabetologia. 49, 1027-38.

Gardoni, F., et al., 2002. Effects of streptozotocin-diabetes on the hippocampal NMDA receptor complex in rats. Journal of neurochemistry. 80, 438-447.

Gilyarov, A.V., 2008. Nestin in central nervous system cells. Neurosci Behav Physiol. 38, 165-9.

Greenwood, C.E., Winocur, G., 2005. High-fat diets, insulin resistance and declining cognitive function. Neurobiol Aging. 26 Suppl 1, 42-5.

Hamilton, A., et al., 2011. Novel GLP-1 mimetics developed to treat type 2 diabetes promote progenitor cell proliferation in the brain. J Neurosci Res. 89, 481-9.

Havrankova, J., Roth, J., Brownstein, M.J., 1979. Concentrations of insulin and insulin receptors in the brain are independent of peripheral insulin levels. Studies of obese and streptozotocin-treated rodents. J Clin Invest. 64, 636-42.

Ho, N., Sommers, M.S., Lucki, I., 2013. Effects of diabetes on hippocampal neurogenesis: links to cognition and depression. Neurosci Biobehav Rev. 37, 1346-62.

Hou, Y., et al., 2012. Changes in hippocampal synapses and learning-memory abilities in a streptozotocin-treated rat model and intervention by using fasudil hydrochloride. Neuroscience. 200, 120-9.

Hu, M., et al., 2008. Negative regulation of neurogenesis and spatial memory by NR2B-containing NMDA receptors. J Neurochem. 106, 1900-13.

Hu, S.-H., et al., 2013. Pioglitazone ameliorates intracerebral insulin resistance and tau-protein hyperphosphorylation in rats with type 2 diabetes. Experimental and Clinical Endocrinology & Diabetes. 121, 220-224.

Hwang, C.Y., Lee, C., Kwon, K.S., 2009. Extracellular signal-regulated kinase 2-dependent phosphorylation induces cytoplasmic localization and degradation of p21Cip1. Mol Cell Biol. 29, 3379-89.

Hwang, I.K., et al., 2008. Strain-specific differences in cell proliferation and differentiation in the dentate gyrus of C57BL/6N and C3H/HeN mice fed a high fat diet. Brain Res. 1241, 1-6.

Juan, W.S., et al., 2014. Melatonin improves neuroplasticity by upregulating the growth-associated protein-43 (GAP-43) and NMDAR postsynaptic density-95 (PSD-95) proteins in cultured neurons exposed to glutamate excitotoxicity and in rats subjected to transient focal cerebral ischemia even during a long-term recovery period. J Pineal Res. 56, 213-23.

Kaewsuk, S., et al., 2009. Melatonin attenuates methamphetamine-induced reduction of tyrosine hydroxylase, synaptophysin and growth-associated protein-43 levels in the neonatal rat brain. Neurochem Int. 55, 397-405.

Kamal, A., et al., 1999. Hippocampal synaptic plasticity in streptozotocin-diabetic rats: impairment of long-term potentiation and facilitation of long-term depression. Neuroscience. 90, 737-45.

Kannangara, T.S., et al., 2014. Deletion of the NMDA receptor GluN2A subunit significantly decreases dendritic growth in maturing dentate granule neurons. PLoS One. 9, e103155.

Karamitri, A., et al., 2013. Minireview: Toward the establishment of a link between melatonin and glucose homeostasis: association of melatonin MT2 receptor variants with type 2 diabetes. Mol Endocrinol. 27, 1217-33.

Kerti, L., et al., 2013. Higher glucose levels associated with lower memory and reduced hippocampal microstructure. Neurology. 81, 1746-52.

Kim, B., Feldman, E.L., 2012. Insulin resistance in the nervous system. Trends Endocrinol Metab. 23, 133-41.

Kitagawa, A., Ohta, Y., Ohashi, K., 2012. Melatonin improves metabolic syndrome induced by high fructose intake in rats. J Pineal Res. 52, 403-13.

Kong, X., et al., 2008. Melatonin regulates the viability and differentiation of rat midbrain neural stem cells. Cell Mol Neurobiol. 28, 569-79.

Korkmaz, G.G., et al., 2012. Melatonin ameliorates oxidative damage in hyperglycemia-induced liver injury. Clin Invest Med. 35, E370-7.

Lee, J., Duan, W., Mattson, M.P., 2002. Evidence that brain-derived neurotrophic factor is required for basal neurogenesis and mediates, in part, the enhancement of neurogenesis by dietary restriction in the hippocampus of adult mice. J Neurochem. 82, 1367-75.

Lenzen, S., 2008. The mechanisms of alloxan- and streptozotocin-induced diabetes. Diabetologia. 51, 216-26.

Leuner, B., Gould, E., Shors, T.J., 2006. Is there a link between adult neurogenesis and learning? Hippocampus. 16, 216-24.

Li, M., et al., 2011. NR2B-containing NMDA receptors promote neural progenitor cell proliferation through CaMKIV/CREB pathway. Biochem Biophys Res Commun. 411, 667-72.

Lindqvist, A., et al., 2006. High-fat diet impairs hippocampal neurogenesis in male rats. Eur J Neurol. 13, 1385-8.

Mazzucchelli, C., et al., 1996. The melatonin receptor in the human brain: cloning experiments and distribution studies. Brain Res Mol Brain Res. 39, 117-26.

McNeill, H., Craig, G.M., Bateman, J.M., 2008. Regulation of neurogenesis and epidermal growth factor receptor signaling by the insulin receptor/target of rapamycin pathway in Drosophila. Genetics. 179, 843-53.

Messier, C., 2005. Impact of impaired glucose tolerance and type 2 diabetes on cognitive aging. Neurobiol Aging. 26 Suppl 1, 26-30.

Miao, Y., et al., 2015. Activation of Hippocampal CREB by Rolipram Partially Recovers Balance Between TNF-alpha and IL-10 Levels and Improves Cognitive Deficits in Diabetic Rats. Cell Mol Neurobiol.

Murray, P.S., Holmes, P.V., 2011. An overview of brain-derived neurotrophic factor and implications for excitotoxic vulnerability in the hippocampus. Int J Pept. 2011, 654085.

Nagayach, A., Patro, N., Patro, I., 2014a. Astrocytic and microglial response in experimentally induced diabetic rat brain. Metab Brain Dis. 29, 747-61.

Nagayach, A., Patro, N., Patro, I., 2014b. Experimentally induced diabetes causes glial activation, glutamate toxicity and cellular damage leading to changes in motor function. Front Cell Neurosci. 8, 355.

Nagorny, C., Lyssenko, V., 2012. Tired of diabetes genetics? Circadian rhythms and diabetes: the MTNR1B story? Curr Diab Rep. 12, 667-72.

Najem, D., et al., 2014. Insulin resistance, neuroinflammation, and Alzheimer's disease. Rev Neurosci.

Negi, G., Kumar, A., Sharma, S.S., 2011. Melatonin modulates neuroinflammation and oxidative stress in experimental diabetic neuropathy: effects on NF-kappaB and Nrf2 cascades. J Pineal Res. 50, 124-31.

Park, D., et al., 2010. Nestin is required for the proper self-renewal of neural stem cells. Stem Cells. 28, 2162-71.

Pearson, G., et al., 2001. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev. 22, 153-83.

Permpoonputtana, K., Govitrapong, P., 2013. The anti-inflammatory effect of melatonin on methamphetamine-induced proinflammatory mediators in human neuroblastoma dopamine SH-SY5Y cell lines. Neurotox Res. 23, 189-99.

Peschke, E., et al., 2007. Melatonin and type 2 diabetes - a possible link? J Pineal Res. 42, 350-8.

Peschke, E., et al., 2008. Increased melatonin synthesis in pineal glands of rats in streptozotocin induced type 1 diabetes. J Pineal Res. 45, 439-48.

Ramirez-Rodriguez, G., et al., 2009. Melatonin modulates cell survival of new neurons in the hippocampus of adult mice. Neuropsychopharmacology. 34, 2180-91.

Reiter, R.J., 1996. The indoleamine melatonin as a free radical scavenger, electron donor, and antioxidant. In vitro and in vivo studies. Adv Exp Med Biol. 398, 307-13.

Reiter, R.J., et al., 2009. Reducing oxidative/nitrosative stress: a newly-discovered genre for melatonin. Crit Rev Biochem Mol Biol. 44, 175-200.

Reppert, S.M., Weaver, D.R., Ebisawa, T., 1994. Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. Neuron. 13, 1177-85.

Revsin, Y., et al., 2009. Glucocorticoid receptor blockade normalizes hippocampal alterations and cognitive impairment in streptozotocin-induced type 1 diabetes mice. Neuropsychopharmacology. 34, 747-58.

Rios-Lugo, M.J., et al., 2010. Melatonin effect on plasma adiponectin, leptin, insulin, glucose, triglycerides and cholesterol in normal and high fat-fed rats. J Pineal Res. 49, 342-8.

Ruksee, N., et al., 2014. Melatonin pretreatment prevented the effect of dexamethasone negative alterations on behavior and hippocampal neurogenesis in the mouse brain. J Steroid Biochem Mol Biol. 143C, 72-80.

Samuels, I.S., et al., 2008. Deletion of ERK2 mitogen-activated protein kinase identifies its key roles in cortical neurogenesis and cognitive function. J Neurosci. 28, 6983-95.

Saravia, F., et al., 2004. Oestradiol restores cell proliferation in dentate gyrus and subventricular zone of streptozotocin-diabetic mice. J Neuroendocrinol. 16, 704-10.

Schioth, H.B., et al., 2012. Brain insulin signaling and Alzheimer's disease: current evidence and future directions. Mol Neurobiol. 46, 4-10.

Selcher, J.C., et al., 1999. A necessity for MAP kinase activation in mammalian spatial learning. Learn Mem. 6, 478-90.

She, M., Laudon, M., Yin, W., 2014. Melatonin receptors in diabetes: a potential new therapeutical target? Eur J Pharmacol. 744, 220-3.

Shioda, N., Han, F., Fukunaga, K., 2009. Role of Akt and ERK signaling in the neurogenesis following brain ischemia. Int Rev Neurobiol. 85, 375-87.

Shyamasundar, S., et al., 2013. Analysis of epigenetic factors in mouse embryonic neural stem cells exposed to hyperglycemia. PLoS One. 8, e65945.

Simoes, P.F., et al., 2007. Methamphetamine induces alterations on hippocampal NMDA and AMPA receptor subunit levels and impairs spatial working memory. Neuroscience. 150, 433-41.

Skolnik, E.Y., et al., 1993. The function of GRB2 in linking the insulin receptor to Ras signaling pathways. Science. 260, 1953-5.

Sotthibundhu, A., Phansuwan-Pujito, P., Govitrapong, P., 2010. Melatonin increases proliferation of cultured neural stem cells obtained from adult mouse subventricular zone. J Pineal Res. 49, 291-300.

Srinivasan, K., et al., 2005. Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: a model for type 2 diabetes and pharmacological screening. Pharmacol Res. 52, 313-20.

Srinivasan, V., et al., 2013. Metabolic syndrome, its pathophysiology and the role of melatonin. Recent Pat Endocr Metab Immune Drug Discov. 7, 11-25.

Stranahan, A.M., et al., 2008. Diabetes impairs hippocampal function through glucocorticoid-mediated effects on new and mature neurons. Nat Neurosci. 11, 309-17.

Sudnikovich, E.J., et al., 2007. Melatonin attenuates metabolic disorders due to streptozotocin-induced diabetes in rats. Eur J Pharmacol. 569, 180-7.

Sutcu, R., et al., 2006. Melatonin increases NMDA receptor subunits 2A and 2B concentrations in rat hippocampus. Mol Cell Biochem. 283, 101-5.

Tardif, K., et al., 2014. Nestin downregulation in rat vascular smooth muscle cells represents an early marker of vascular disease in experimental type I diabetes. Cardiovasc Diabetol. 13, 119.

Tayman, C., et al., 2014. Effects of hyperglycemia on the developing brain in newborns. Pediatr Neurol. 51, 239-45.

Tezuka, T., et al., 1999. PSD-95 promotes Fyn-mediated tyrosine phosphorylation of the N-methyl-D-aspartate receptor subunit NR2A. Proc Natl Acad Sci U S A. 96, 435-40.

Tocharus, C., et al., 2014. Melatonin enhances adult rat hippocampal progenitor cell proliferation via ERK signaling pathway through melatonin receptor. Neuroscience. 275, 314-21.

Trudeau, F., Gagnon, S., Massicotte, G., 2004. Hippocampal synaptic plasticity and glutamate receptor regulation: influences of diabetes mellitus. European journal of pharmacology. 490, 177-186.

van Praag, H., et al., 1999. Running enhances neurogenesis, learning, and long-term potentiation in mice. Proc Natl Acad Sci U S A. 96, 13427-31.

Ventura-Sobrevilla, J., et al., 2011. Effect of varying dose and administration of streptozotocin on blood sugar in male CD1 mice. In: Proceedings of the Western Pharmacology Society. Vol. 54, ed.^eds., pp. 5-9.

Wang, J.Q., et al., 2014. Brain aging and AD-like pathology in streptozotocin-induced diabetic rats. J Diabetes Res. 2014, 796840.

Watanabe, K., et al., 2008. The NMDA receptor NR2A subunit regulates proliferation of MKN45 human gastric cancer cells. Biochem Biophys Res Commun. 367, 487-90.

Winocur, G., Greenwood, C.E., 2005. Studies of the effects of high fat diets on cognitive function in a rat model. Neurobiol Aging. 26 Suppl 1, 46-9.

Wongchitrat, P., et al., 2013. Effect of amphetamine on the clock gene expression in rat striatum. Neurosci Lett. 542, 126-30.

Yan, X.B., et al., 2007. Lithium regulates hippocampal neurogenesis by ERK pathway and facilitates recovery of spatial learning and memory in rats after transient global cerebral ischemia. Neuropharmacology. 53, 487-95.

Yoo, D.Y., et al., 2011. Reduced cell proliferation and neuroblast differentiation in the dentate gyrus of high fat diet-fed mice are ameliorated by metformin and glimepiride treatment. Neurochem Res. 36, 2401-8.

Yoo, D.Y., et al., 2012a. Melatonin improves D-galactose-induced aging effects on behavior, neurogenesis, and lipid peroxidation in the mouse dentate gyrus via increasing pCREB expression. J Pineal Res. 52, 21-8.

Yoo, D.Y., et al., 2012b. Effects of pyridoxine on a high-fat diet-induced reduction of cell proliferation and neuroblast differentiation depend on cyclic adenosine monophosphate response element binding protein in the mouse dentate gyrus. J Neurosci Res. 90, 1615-25.

Yoo, D.Y., et al., 2014. Cell proliferation and neuroblast differentiation in the dentate gyrus of high-fat diet-fed mice are increased after rosiglitazone treatment. J Vet Sci. 15, 27-33.

Zanuto, R., et al., 2013. Melatonin improves insulin sensitivity independently of weight loss in old obese rats. J Pineal Res. 55, 156-65.

Zhang, W.J., et al., 2008. Impairment of hippocampal neurogenesis in streptozotocin-treated diabetic rats. Acta Neurol Scand. 117, 205-10.

Zhang, Y., et al., 2014. Relationship between melatonin receptor 1B and insulin receptor substrate 1 polymorphisms with gestational diabetes mellitus: a systematic review and meta-analysis. Sci Rep. 4, 6113.

Zhao, W.Q., et al., 2004. Insulin and the insulin receptor in experimental models of learning and memory. Eur J Pharmacol. 490, 71-81.

Zhou, L., Zeng, Q.R., Zhou, J., 2011. [Effect of melatonin on glial fibrillary acidic protein, nuclear factor-kB and synaptophysin expressions in aging mice]. Nan Fang Yi Ke Da Xue Xue Bao. 31, 2043-7.

7. Appendix

7.1 Invited researcher

Visiting Award:	International Society for Neurochemistry (ISN)
Title of Project:	The characterization of the clock of the mammalian retina.
Host laboratory:	CNRS UPR 3212 Institut des Neurosciences Cellulaires et Intégratives (INCI), Département Neurobiologie des Rythmes, Centre de Neurochimie, Université de Strasbourg
Address:	5 rue Blaise Pascal 67084 Strasbourg France
Duration:	May 2015-June 2015
Reference person:	Dr. Marie-Paule Felder-Schmittbuhl Chargée de recherche CNRS Email: felder@neurochem.u-strasbg.fr
Document:	A letter from the principal investigator of host laboratory (page 40)

Marie-Paule FELDER-SCHMITTBUHL
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Strasbourg, june 27th 2015

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To whom it may concern,

Dr Prapimpun WONGCHITRAT has spent 2 months in 2015 (May and June) in our laboratory at the Institute for Cellular and Integrative Neurosciences in Strasbourg as an invited researcher. For this visit, she was also awarded a "visiting grant" from the International Society for Neurochemistry. During her stay she took part in our project to characterize circadian clock mechanisms in the distinct cell types of the retina. She more particularly analysed retina cones by using the *Nrl-/-* mice, a mutant in which the retina rods are converted into cones. She used laser capture microdissection to isolate photoreceptor layers from their retinas at distinct time points of the 24h cycle and extracted mRNA to finally analyse kinetics of clock gene expression by qPCR. We have been fruitfully collaborating with Dr Wongchitrat for several years: she is an excellent scientist with very good technical and conceptual skills, always eager to learn new techniques, whenever necessary for her project. She is also a very kind person, with whom it has been agreeable to interact, whatever the context. The topic she has been developing here this year is rather close to the projects she is conducting at Mahidol University in Thailand and I can tell that our interaction is mutually beneficial. I strongly hope that we will be able to maintain this collaboration in the future.

M. Paule Felder

Marie-Paule Felder-Schmittbuhl, HDR

7.2 Guess speaker

Topic: High fat diet and brain aging

Host: งานประชุมวิชาการประจำปีของผู้ได้รับทุน สกว สาขาประสาทวิทยาศาสตร์ เรื่อง “Melatonin and other factors as the potentially useful agents in attenuating brain aging and neurodegeneration and enhancing neurogenesis” ณ ห้องประชุม A107 สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล ศาลายา วันที่ 29 เมษายน 2558

Reference person: Prof. Dr. Piyarat Govitrapong
Head of Research center of Neurosciences, Mahidol University
Email: piyarat.gov@mahidol.ac.th

7.3 International conference

1) The 12nd Biennial meeting of the Asian Pacific Society for Neurochemistry (APSN) Kaoshiun, Taiwan, 23-26 August 2014
   Presentation type: Poster (2 posters)

2) The 18th Thai Neuroscience Society (TNS) Conference 2014 & 2nd Joint CU-NIPS Symposium “Frontier in Neuroscience Research” Bangkok, Thailand, 21-23 December 2014
   Presentation type: Poster (1 related poster and other 3 posters)

7.4 National conference

1) งานประชุมวิชาการประจำปีของผู้ได้รับทุน สกว สาขาประสาทวิทยาศาสตร์ เรื่อง “Melatonin and other factors as the potentially useful agents in attenuating brain aging and neurodegeneration and enhancing neurogenesis” ณ ห้องประชุม A107 สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล ศาลายา วันที่ 29 เมษายน 2558
   Presentation type: Oral (15 min)
   Title: Effect of melatonin on clock gene expression in hippocampus of high fat diet and streptozotocin-induced high blood glucose rat

8. Output

8.1 International Journal Publication

1) Melatonin regulates aging and neurodegeneration through energy metabolism, epigenetics, autophagy and circadian rhythm pathways. Jenwitheesuk A, Nopparat C, Mukda S, Wongchitrat P, Govitrapong P. Int J Mol Sci. 2014;15(9):16848-84. (attachment 1)
2) Effects of melatonin on hippocampal neurogenesis alteration in high-fat diet-fed and streptozotocin-treated rats. (manuscript to be submitted on July 2015) (attachment 2)

8.2 Others

8.2.1 Proceeding

1) Effects of high-fat diet and low-dose streptozotocin injection-induced changes in rat hippocampal synaptic proteins Lansubsakul N, Wongchitrat P, Mukda S, Govitrapong P. The 18th Thai Neuroscience Society (TNS) Conference 2014 & 2nd Joint CU-NIPS Symposium “Frontier in Neuroscience Research” Bangkok, Thailand, 21-23 December 2014 (attachment 3)

8.2.2 Abstract (International conference)

1) The circadian rhythm of sirtuin mRNA expression in the rat brain. Wongchitrat P, Govitrapong P, Prachayasittikul V.
   Journal of Neurochemistry (2014), 130 Suppl. 1, p50 (page 45)

2) Effect of melatonin on the expression of clock genes in the hippocampus of high-fat diet fed and streptozotocin induced diabetic rats. Mukda S, Wongchitrat P, Suwanjang W, Lansabsakul N, Govitrapong P.
   Journal of Neurochemistry 2014, 130 (Suppl. 1), p73-74 (page 46-47)

The circadian rhythm of sirtuin mRNA expression in the rat brain

P. Wongchitrat[1], P. Govitrapong[2], V. Prachayasittikul[3]

[1]Mahidol University, Faculty of Medical Technology, Center for Innovation Development and Technology Transfer, Thailand

[2]Mahidol University, Institute of Molecular Biosciences, Research Center for Neuroscience, Thailand

[3]Mahidol University, Faculty of Medical Technology, Department of Clinical Microbiology and Applied Technology, Thailand

Sirtuins belong to the third class of deacetylase enzymes, which are dependent on NAD+ for their activity that is associated with variety of mechanism in mammals. Sirtuins expressed in several tissues and organs involved in systemic metabolism have been clearly reported. However, the studies of sirtuins in the brain, where is the central of nervous system are remain unknown. The aim of this study was to examine the pattern of Sirtuin mRNA expression in the rat hippocampus and striatum which are related to many of neurodegenerative diseases. Rat brains were dissected and daily profile of *Sirt1* and *Sirt2* mRNA levels were analyzed using semi-quantitative RT-PCR. Results showed that *Sirt1* and *Sirt2* were expressed in a different pattern of each specific brain areas. *Sirt1* mRNA level displayed a circadian rhythm of expression only in the striatum but not in hippocampus. The highest level of *Sirt1* expression was occurred during night time. The diurnal rhythm of *Sirt2* mRNA expression was found only in hippocampus which peaked at ZT3. These results indicate that the rhythm of Sirtuin may play an important role in circadian rhythms of physiological processes in the specific brain area.

Acknowledgement: TRF (MRG5680016) & Mahidol University

Effect of melatonin on the expression of clock genes in the hippocampus of high-fat diet fed and streptozotocininduced diabetic rats

S. Mukda[1], **P. Wongchitrat**[2], W. Suwanjang[2], N. Lansabsakul[1], P. Govitrapong[1]

[1]Mahidol University, Institute of Molecular Biosciences, Research Center for Neuroscience, Salaya Campus, Nakornpathom, Thailand

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The circadian clock is an endogenous system that acts as an internal time-keeping device, generates approximately 24-hour oscillations in physiology and behavior known as circadian rhythms. An important function of the circadian clock is to synchronize different metabolic processes in an organism including energy metabolism, sleep-wake cycles, hormone secretion, and cardiac function. Recent evidence have demonstrated that the dysfunction of the circadian clock is associated with the development of several pathological conditions including diabetes. Moreover, there is evidence that a functional clock exists in many parts in the brain including the hippocampus. Therefore, this study aims to investigate the effect of streptozotocin (STZ)-induced diabetes on the clock genes expression in the rat hippocampus, as well as to investigate whether the exogenous melatonin, a major entraining signal for the circadian systems, can restore the expression of clock genes. Our results show that exogenous melatonin provides a protective effect against an alteration of clock genes in the hippocampus of STZ-induced diabetic rats. These data demonstrate the important of circadian clock impairment associated with diabetes which may provide new insights and treatment targets for this disease.

Acknowledgement: TRF (MRG5680016) & Mahidol University

P01-14

Learning induces sonic hedgehog signaling in the amygdala which promotes neurogenesis and long-term memory formation

H. C. Hung

National Cheng Kung University, Institute of Basic Medical Science, Tainan, Taiwan

It is known that neurogenesis occurs throughout the life mostly in the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricle. Here we investigated whether neurogenesis occurred in the amygdala and its function in fear memory formation. Mice were injected intraperitoneally with 5-bromo-2′-deoxyuridine (BrdU) 2 h before receiving 15 tone–footshock pairings. The number of $BrdU^+/DCX^+$ and $BrdU^+/NeuN^+$ cells was significantly higher in the conditioned mice suggesting that association of tone with footshock induced neurogenesis. To determine the relationship between neurogenesis and memory formation, mice were given cell proliferation inhibitor methylazoxymethanol acetate (MAM). MAM markedly reduced neurogenesis and impaired fear memory formation. Similarly, intra-amygdala infusion of cytosine arabinoside (Ara-C) which interferes with DNA synthesis decreased freezing responses. Sonic hedgehog (Shh), its receptor patched1 (Ptc1) and transcription factor Gli1 protein levels increased at 1 day and returned to baseline at 7 days after fear conditioning. Immunohistochemistry confirmed that Shh^+ cells increased after conditioning. Chronic infusion of cyclopamine (Shh antagonist) through an osmotic pump into amygdala reduced the number $BrdU^+/DCX^+$ cells and decreased freezing responses. Silencing *Shh* gene expression with small hairpin interfering RNA (shRNA) by means of a lentivirus expression system or with a retrovirus vector encoding *Shh* shRNA (Retro-*Shh*-shRNA) which allowed us to knockdown Shh specifically in the mitotic neurons reduced the number of $BrdU^+/NeuN^+$ cells and decreased freezing responses. Taken together, these results suggest that fear learning induces Shh signaling activation in the amygdala which promotes neurogenesis and long-term memory formation.

P01-15

TRIP6 regulates the maintenance of postnatal mouse neural stem cells

M. Y. Li[1], Y. J. Lai[1], C. Y. Yang[1], K. H. Huang[1], J. C. Tsai[1], T. W. Wang[1,2]

[1]*National Taiwan Normal University, Department of Life Science, Taipei, Taiwan*
[2]*National Yang-Ming University, Brain Research Center, Taipei, Taiwan*

Postnatal neurogenesis persists throughout life in the subventricular zone (SVZ)-olfactory bulb pathway in mammals. Extrinsic or intrinsic factors have been revealed to regulate properties of neural stem cells (NSCs). Thyroid hormone receptor interacting protein 6 (TRIP6) belongs to zyxin family of LIM proteins, which interact with various proteins to mediate cellular functions. However, the role of TRIP6 in NSCs is still unknown. By performing double immunofluorescence staining, we found that TRIP6 was expressed by Sox2-positive NSCs in postnatal mouse SVZ. To study the function of TRIP6 in NSCs, we performed overexpression experiments with neurospheres derived from postnatal day 7 SVZ. We found that TRIP6 increased the sphere size and proliferation of NSCs. To test whether TRIP6 regulates multi-potency in NSCs, we overexpressed and knocked down TRIP6 in NSCs cultured in differentiation condition and found that TRIP6 inhibited NSC differentiation. To further investigate the mechanism of TRIP6 in NSCs, we performed luciferase assay and found that TRIP6 activated Notch signalling, a pathway required for NSC self-renewal. In conclusion, our data suggest that TRIP6 regulates postnatal NSC maintenance. Taken together, our results suggest that TRIP6 regulates NSC maintenance in the postnatal mammalian SVZ.

P01-16

The circadian rhythm of sirtuin mRNA expression in the rat brain

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Sirtuins belong to the third class of deacetylase enzymes, which are dependent on NAD+ for their activityt that is associated with variety of mechanism in mammals. Sirtuins expressed in several tissues and organs involved in systemic metabolism have been clearly reported. However, the studies of sirtuins in the brain, where is the central of nervous system are remain unknown. The aim of this study was to examine the pattern of Sirtuin mRNA expression in the rat hippocampus and striatum which are related to many of neurodegenerative diseases. Rat brains were disected and daily profile of *Sirt1* and *Sirt2* mRNA levels were analyzed using semi-quantitative RT-PCR. Results showed that *Sirt1* and *Sirt2* were expressed in a different pattern of each specific brain areas. *Sirt1*mRNA level displayed a circadian rhythm of expression only in the striatum but not in hippocampus. The highest level of *Sirt1* expression was occurred during night time. The diurnal rhythm of *Sirt2* mRNA expression was found only in hippocampus which peaked at ZT3. These results indicate that the rhythm of Sirtuin may play an important role in circadian rhythms of physiological processes in the specific brain area. [Acknowledgement: TRF(MRG5680016) & Mahidol University]

P01-17

In utero analysis of *miR-3099* role in the development and function of the mouse brain

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MicroRNAs (miRNAs) are small non-coding RNAs about 18–24 nucleotides long that are emerging as key regulator of post-

have been reported that many herbal extracts and natural products possessing antioxidant activity could accelerate recovery after nerve injury by enhancing antioxidant enzymes and reducing tissue damage induced by free radicals. The efficacy of therapeutic strategies for diabetic neuropathy is still not in satisfaction level. Recently, it has been reported that substances delivery via transdermal patch can decrease first pass effect giving rise to the decreased therapeutic dose and decreased side effects. Therefore, this study aimed to develop and to evaluate the effect of the health product on functional recovery of the diabetic neuropathy. In this study, Quercetin and Tomato extract were developed as health products as transdermal patch with electrospinning technique and determined enhancing effect on functional recovery of diabetic neuropathy and determined the possible mechanism of transdermal patch. Male Wistar rats were induced diabetes mellitus and crushing the right sciatic nerve. Quercetin-loaded and Tomato-extract-loaded transdermal patches at concentrations of 5, 10 and 15% with the sized of 1x1 cm once daily at the area which the nerve crush injury was performed for 21 days. The evaluation of nerve function was performed using foot withdrawal reflex, De Medinacelli method every 3 days throughout 21-day study period. The results revealed that 5% Quercetin-loaded and 10% Tomato extract-loaded transdermal patches could enhance the functional recovery of lesion nerve. The underlying mechanism of Quercetin-loaded transdermal patch might occur partly via the decreased oxidative stress whereas Tomato extract-loaded transdermal patch might occur via other mechanisms. Therefore, the current data demonstrates the potential as novel health products against diabetic neuropathy.

P03-21

Biochemical, histological and proteomic characterization of contusion and pericontusion during traumatic brain injury

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Traumatic brain injury (TBI) involves pathologically distinct regions of injury called Contusion (injured tissue) and pericontusion (surrounding contused tissue). These regions could be implicated in secondary injury events following injury. We sought to characterize the injured tissue to elucidate the secondary injury pathways. Contusion ($n = 20$) and pericontusion ($n = 16$) tissues obtained from autopsy and from TBI patients undergoing craniotomy. Age matched pathologically normal frontal cortex tissues which were farthest from the site of injury served as controls. Assays for oxidative stress and antioxidant function; immunohistochemistry for GFAP, phosphorylated Neurofilament, ubiquitin and tau; and LC-MS based proteomics followed by bioinformatic analysis was carried out. Study demonstrated extensive oxidative damage evidenced by altered redox markers consistent decrease of antioxidant enzymes such as SOD and GSH metabolic enzymes and ATP depletion in the contused tissues. Total GSH was significantly decreased both in the synaptosomal and mitochondrial fractions both in contusion and pericontusion. Oxidative stress markers such as lipid peroxides, and protein carbonyls were elevated in the contusion. Histological evaluation showed significant demyelination and increased dystrophic neurons in pericontusion. Astrogliosis was prominent in contusion compared to pericontusion. Microglial activation as seen by Iba1 + cells, oedema and axotomy was more in pericontusion compared to contusion. Functional annotation of proteomics data showed down-regulation of synaptic proteins and up-regulation of inflammatory proteins in contusion. Pericontusion showed down-regulation of structural/cytoskeletal proteins and up-regulation of negative cell regulation proteins. We believe that the current data gives insight into the pathology related to secondary damage and subsequently helps in understand the dynamics of injury.

P03-22

Cathepsin C and Cystatin F gene interaction during demyelination

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Cystatin F, a papain-like lysosomal cysteine proteinase inhibitor, and its main substrate, Cathepsin C, have been demonstrated to be crucial factors in demyelinating diseases. It is found that the expression of Cathepsin C and Cystatin F are profoundly elevated and matched with ongoing demyelination/remyelination. However, their accurate functional role in demyelinating diseases is still unclear. To clarify their function in the pathological process of demyelination, we used a spontaneous chronic demyelination mouse model, named heterozygous PLP transgenic 4e ($PLP^{4e/-}$) mice. Meanwhile, Flexible Accelerated –STOP-Tetracycline Operator Knockin (FAST) system is applied to up or down regulate Cathepsin C or Cystatin F gene expression. In situ hybridization revealed that in $PLP^{4e/-}$ mice conditional knock down of Cystatin F gene in microglia lead to the down regulation of Cathepsin C mRNA levels. On the contrary, Cathepsin C gene expression is enhanced by up regulating Cystatin F. It means that Cathepsin C gene and Cystatin F gene interact with each other through some unknown mechanism during demyelination. It has important significance to clarify this mechanism for understanding their function in this disorder. Further study is needed to estimate the possible pathway and substrate of Cystatin F that influences Cathepsin C gene expression.

P03-23

Effect of melatonin on the expression of clock genes in the hippocampus of high-fat diet fed and streptozotocin-induced diabetic rats

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The circadian clock is an endogenous system that acts as an internal time-keeping device, generates approximately 24-hour oscillations

in physiology and behavior known as circadian rhythms. An important function of the circadian clock is to synchronize different metabolic processes in an organism including energy metabolism, sleep-wake cycles, hormone secretion, and cardiac function. Recent evidence have demonstrated that the dysfunction of the circadian clock is associated with the development of several pathological conditions including diabetes. Moreover, there is evidence that a functional clock exists in many parts in the brain including the hippocampus. Therefore, this study aims to investigate the effect of streptozotocin (STZ)-induced diabetes on the clock genes expression in the rat hippocampus, as well as to investigate whether the exogenous melatonin, a major entraining signal for the circadian systems, can restore the expression of clock genes. Our results show that exogenous melatonin provides a protective effect against an alteration of clock genes in the hippocampus of STZ-induced diabetic rats. These data demonstrate the important of circadian clock impairment associated with diabetes which may provide new insights and treatment targets for this disease.

P03-24

Aging and antioxidants modulate amyloid beta metabolism, memory function and survival in rats: implications in the pathogenesis of Alzheimer's disease

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In aging brain varied forms of behavioural and biochemical deficits take place which show many commonalties with Alzheimer's disease (AD) such as increased amyloid beta peptide deposition, mitochondrial dysfunctions, pro-inflammatory reactions and oxidative stress. The aged brain is a useful tool to investigate altered metabolism of amyloid beta peptide that may have implications in the pathogenesis of AD. In the present study, we have shown a significant increase in the amyloid precursor protein (APP) level in the brain cortex of aged rats (22–24 months) without a corresponding increase in the level of APP mRNA. Moreover, the activity of β secretase is elevated (65%) and that of neprilysin diminished (48%) in brain cortex of aged rats compared to that in young rats (4–6 months). All these changes lead to a markedly increased accumulation of Aβ42 in brain cortical tissue of aged rats. Long-term dietary supplementation of rats with α-tocopherol, *N*-acetyl-cysteine and α-lipoic acid has been carried out from 18 months onwards daily till the sacrifice of the animals by 22–24 months. The antioxidant supplementation attenuates all the age-related alterations in amyloid beta metabolism. Further, a significant impairment of spatial learning and memory and survival has been observed in aged rats concomitant with altered brain metabolism of amyloid beta peptide, and the same dietary antioxidant supplementation of aged rats strikingly prevented the former phenomenon. The results indicate the therapeutic potential of this antioxidant combination in ameliorating the amyloid beta load in AD brain.

P03-25

Effect of d-amphetamine on dopaminergic neurons of substantia nigra and expression of tyrosine hydroxylase in the striatum, nucleus accumbens and prefrontal cortex of d-amphetamine treated Wistar rat

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Dopaminergic neurons of the midbrain are the main source of dopamine in the mammalian central nervous system. Dopamine is a chemical messenger active in mesolimbic and mesocortical reward pathways. Dopamine is manufactured in nerve cell bodies in the ventral tegmental area (VTA) and is released in the nucleus accumbens and the prefrontal cortex. To compare dopaminergic neurons of substantia nigra and level of tyrosine hydroxylase (TH) in the striatum, pre- frontal cortex and nucleus accumbens of D-Amphetamine treated wistar mice. 15 wistar rat were injected subcutaneously with amphetamine 10 mg/Kg body weight till 7 days while the controls groups (15 wistar rat) were injected with normal saline in the same dose. On day 7 both the groups were deeply anesthetized, perfused with a fixative of 4% paraformaldehyde in 0.1 sodium phosphate buffer (PBS), PH 7.4. Tissue sectioning was done followed by immunohistochemical (IHC) staining. One way-ANOVA test and *post hoc* Tests was applied. Decreased level of tyrosine hydroxylase was present in striatum, nucleus accumbens and pre-frontal cortex. The percentage of tyrosine hydroxylase in all these 3 areas were highly significant ($p < 0.001$). D-AMPH affects the neuronal cell morphology and decreases expression level of TH. Degeneration of dopaminergic neurons and damaged synaptic connection in substantia nigra, was observed leading to a reduction of stratial dopamine levels. Animal models of Parkinson's disease, Schizophrenia, Alzheimer's disease, introverted personalities, Attention deficit hyperactivity disorder (ADHD) can be designed for the further clinical experiments.

P03-26

Role of caspase-3 in development of neuronal plasticity and memory in rats subjected to prenatal hypoxia

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Caspases (in particular, caspase-3) play an important role in cellular processes underlying brain development, normal cell functioning and apoptosis. Increased caspase activity in pre- and postsynaptic terminals leads to proteolysis of synapse-associated proteins, including cytoskeleton and receptors, and disruption of synaptic activity. As such, inhibition of caspases is considered as a tool for prevention and compensation of various synaptic pathologies. The brain of rats subjected to prenatal hypoxia (E14, O_2 7%, 3 h) is characterised by an increased number of caspase-3-positive neurones and higher activity of this enzyme in the neocortex and hippocampus in the period of intensive synaptogenesis (P20–30) compared to controls. Subsequently, in later life (P30–0) these animals have a reduced number of synaptopodin-positive dendritic

8.2.3 Poster (International conference)

Poster 1: The circadian rhythm of Sirtruins mRNA expression in the rat brain (page 49)

Poster 2: Effect of melatonin on the expression of clock genes in the hippocampus of high-fat diet fed and streptozotocin-induced diabetic rat. (page 50)

Poster 3: The effect of high-fat diet and low-dose streptozotocin injection-induced changes in rat hippocampal synaptic proteins. (page 51)

The circadian rhythm of Sirtuins mRNA expression in the rat brain

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Summary

Sirtuins belongs to the third class of deacetylase enzymes, which are dependent on NAD+ for their activity. Sirtuin activity is associated with a variety of mechanisms in mammal such as gene repression, metabolic control, apoptosis and cell survival, DNA repair, neuroprotection and healthy aging. Sirtuins expressed in several tissues and organs involved in systemic metabolism have been clearly reported. However, the studies of Sirtuins in the brain, where is the central of nervous system are remain unknown.

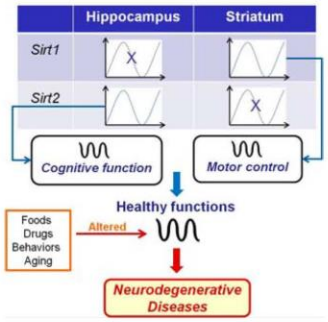
The aim of this study was to examine the normal pattern of Sirtuin (*Sirt*) mRNA expression in the rat hippocampus and striatum which are the brain areas related to many neurodegenerative diseases.

Experimental designs and results

Wistar rats were sacrificed at four different time points along 24 hours. Brains were rapidly removed and hippocampus and striatum were dissected and stored in -80 °C until processing. *Sirt1* and *Sirt2* mRNA levels were analyzed using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) to obtain a daily profile.

Results showed that *Sirt1* and *Sirt2* were expressed in different patterns of each specific brain area. In this study, *Sirt1* mRNA level displayed the variation of its expression during the day or circadian rhythm only in the striatum but not in hippocampus. The highest level of *Sirt1* expression was occurred during night time (ZT21). The diurnal rhythm of *Sirt2* mRNA expression was found only in hippocampus which its level was peaked at ZT3.

Conclusion



The rhythm of Sirtuins expression may play an important role on circadian rhythm of physiological processes in the specific brain areas. The disruption by internal and external cues can cause of the alteration of circadian rhythms which may leads to the development of neurodegenerative diseases.

Results

Hippocampus

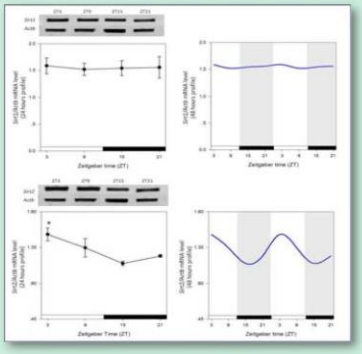


Figure 1. Daily profile of Sirtuin mRNA expression in the rat hippocampus. White and black bars represent light and dark phases, respectively. Values are means ± SEM, with n = 4 for each time point. *$p < 0.05$ was considered as statistically significant.

Striatum

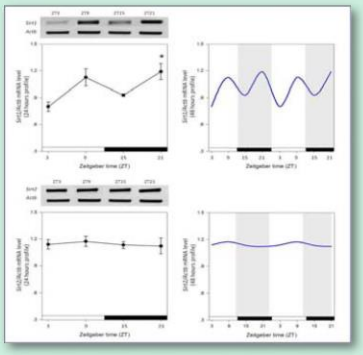


Figure 2. Daily profile of Sirtuin mRNA expression in the rat striatum. White and black bars represent light and dark phases, respectively. Values are means ± SEM, with n = 4 for each time point. *$p < 0.05$ was considered as statistically significant.

ACKNOWLEDGEMENTS

This study was supported by a research fellowship from the Thailand Research Fund No. MRG5680016 to PW and No.DPG 5780001 to PG and a Mahidol University Research Grant. PW was supported by International Society for Neurochemistry (ISN-CAEN) for the travel award.

Effect of melatonin on the expression of clock genes in the hippocampus of high-fat diet fed and streptozotocin-induced diabetic rats

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Introduction

The circadian clock is an endogenous system that acts as an internal time-keeping device, generates approximately 24-hour oscillations in physiology and behavior known as circadian rhythms (Figure 1). An important function of the circadian clock is to synchronize different metabolic processes in an organism including energy metabolism, sleep-wake cycles, hormone secretion, and cardiac function, all of which exhibit daily oscillations.

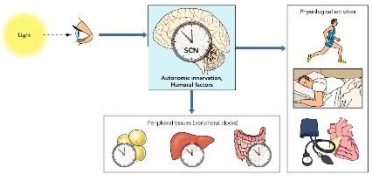


Figure 1: The master clock in mammals located in the suprachiasmatic nucleus (SCN) in the hypothalamus (Froy O., 2011).

The molecular machinery that regulates circadian rhythms consists of a set of genes, known as "clock" genes. The products of "clock" gene regulate the oscillation in gene expression, in protein modifications and hormone secretion, which is primary formed by the transcription-translation feedback loop (Figure 2).

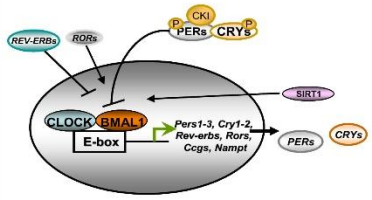


Figure 2: Feedback loop in the core circadian oscillator and the interaction (Modified from Vieira E., 2014).

Recent evidence suggest that the disruption of circadian rhythms on the molecular clock is pivotal in several clinical and pathological conditions including sleep disorders, cancer, depression, inflammation, and the metabolic syndrome.

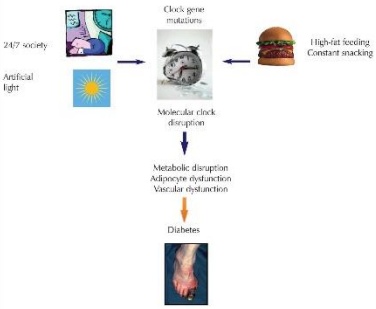
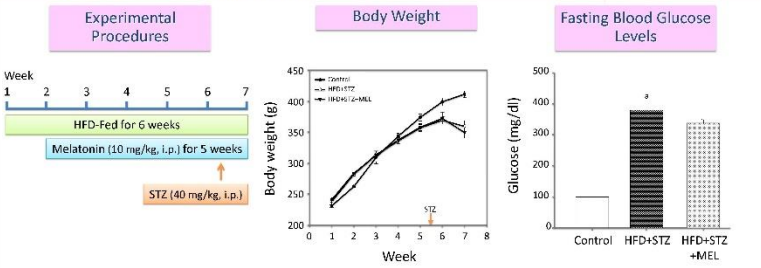


Figure 3: A role for disruption of the molecular clock in diabetes (Modified from Prasai et al 2008).

1. Effects of melatonin on the body weight and fasting blood glucose levels in high-fat diet-fed and low-dose streptozotocin-treated rats

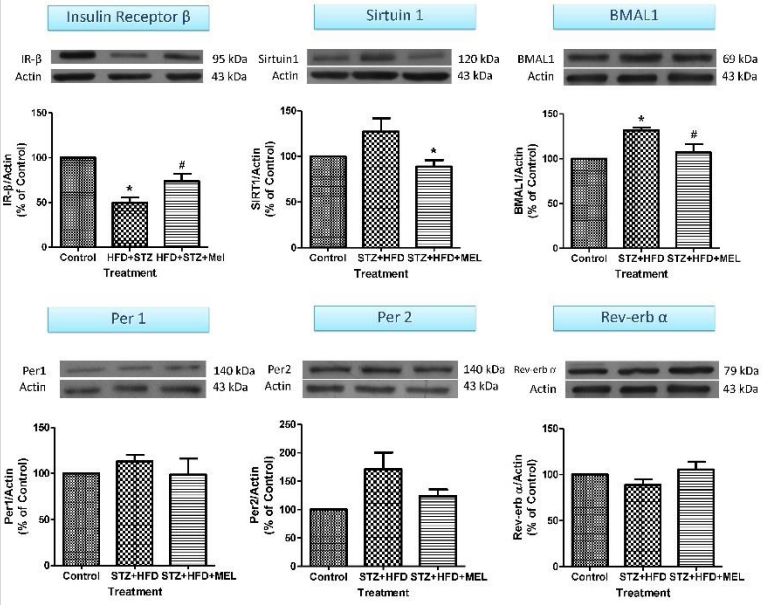


Rats were subcutaneously injected with either 10 mg/kg melatonin or normal saline once daily for 6 weeks, after 1 week feeding with HFD.

No significant differences in body weight between HFD+STZ rats and melatonin-treated HFD+STZ rats.

Melatonin-treated HFD+STZ rats trend to have lower levels of fasting blood glucose levels than the HFD+STZ rats.

2. Effects of melatonin on insulin receptor and circadian genes levels in hippocampus of HFD-fed and streptozotocin-treated rats



Conclusion

This study aims to investigate the effect of streptozotocin (STZ)-induced diabetes on the clock genes expression in the rat hippocampus, as well as to investigate whether the exogenous melatonin, a major entraining signal for the circadian systems, can restore the expression of clock genes.

Our results show that exogenous melatonin provides a protective effect against an alteration of clock genes in the hippocampus of STZ-induced diabetic rats. These data demonstrate the important of circadian clock impairment associated with diabetes which may provide new insights and treatment targets for this disease.

References

1. Froy O. 2011. Circadian rhythms, aging, and life span in mammals. *Physiology* **26**:225-235.
2. Vieira E. 2014. The impact of metformin on circadian clock genes. *J Diabetes Metab Disord Control* 1(3):00016.
3. Prasai et al. 2008. Molecular clocks, typ2 diabetes and cardiovascular disease. *Diabetes Vasc Dis Res* **5**:89-95.

Acknowledgements

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The effect of high-fat diet and low-dose streptozotocin injection-induced changes in rat hippocampal synaptic proteins

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ABSTRACT

Recent evidence has shown an association between diabetes mellitus (DM) and impairments in learning and memory. However, the mechanisms by which cognitive abilities are impaired in diabetes have not been clearly established. The hippocampus plays an important role in spatial learning and memory. Several studies in rodents demonstrate that learning tasks enhance synaptic formation in hippocampus. The aim of this study was therefore to determine the effect of diabetes on synaptic proteins expression in hippocampus of an animal model of high-fat diet (HFD) and streptozotocin (STZ)-induced diabetes. We measured the immunoreactivities of presynaptic protein synaptophysin and postsynaptic density protein-95 (PSD-95) as the indicator of synaptic efficiency. HFD and STZ-induced diabetes produced a dramatic decrease in both synaptophysin and PSD-95 in the rat hippocampus as compared to controls. Therefore, the alteration in synaptic proteins expression in a rat model of diabetes may play a potential role in the cognitive impairment in DM.

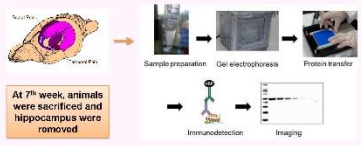
INTRODUCTION

As a result of HFD and sedentary lifestyles, the number of people suffering from DM has increased each year. Previous reports demonstrate that DM is one of the causes of the memory impairment. Rats rendered diabetes by treatment with a high dose STZ, the pancreatic β-cell toxin to induce hyperglycemia, show impaired performance in tests of spatial learning and memory ability similar to HFD-fed rats. Learning and memory disorders suggest the occurrence of the dysregulation of the hippocampal synapse formation process detected by pre- and post-synaptic protein markers, synaptophysin and PSD-95. The synaptotoxicity is an early feature of other conditions related to memory impairment. Thus, the synaptotoxicity has been proposed to be an initial feature resulting from different insidious brain insults include the diabetes. The objective of this present study was to investigate the effect of diabetes on synaptic protein expression in hippocampus of an animal model of HFD and STZ - induced diabetes as a method developed by Srinivasan's group (Srinivasan et al 2005).

METHODS

Experimental Procedures

Western blot analysis

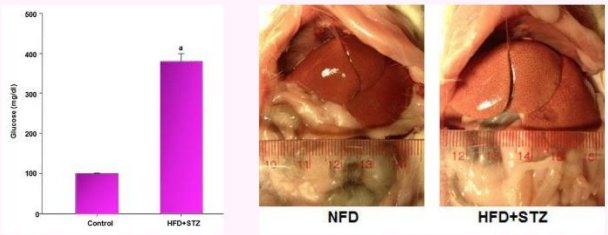


Primary antibodies:
- mouse anti-synaptophysin (1:20,000)
- mouse anti-PSD-95 (1:20,000)
- mouse anti-actin (1:10,000)

Secondary antibodies: anti-mouse IgG HRP-linked

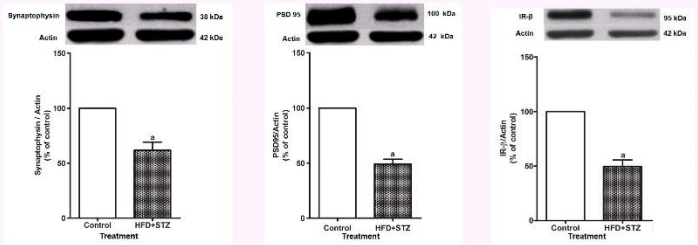
RESULTS

Effect of HFD and low-dose STZ on the blood glucose level and liver necropsy of male Wistar rats



The feeding of HFD and injection of STZ induce the significant increase in fasting blood glucose and hepatic pathology as compared to control group.

Effect of HFD and low-dose STZ-induced diabetes on synaptophysin, PSD-95 and insulin receptor in the rat hippocampus



HFD and injection of STZ induce the significant decrease in both synaptic proteins and insulin receptor as compared to control group.

DISCUSSIONS

Overall, the present study demonstrates that treatment with HFD and low-dose STZ can induce diabetic rats and also shows the obvious decrease in immunoreactivities of synaptophysin as well as PSD-95 in hippocampus. Our finding from this experiment opens a window for concomitant evaluation of synaptic efficiency and diabetic condition. Synaptophysin level was decreased by HFD and STZ treatment in the hippocampal rats indicates that the process of neurotransmitter releasing from glutamatergic presynaptic neuron may be interfered. The decrease in PSD-95 indicates that the strength of synapse may aggravate by distorted clustering of glutamatergic NMDA receptors (Delint-Ramírez et al., 2008). However, relationships among synaptophysin, PSD-95 levels, diabetes and memory are not straightforward and need to further elucidate.

REFERENCES

- Delint-Ramírez I, Salcedo-Tello P, Bermudez-Rattoni F. 2008. Spatial memory formation induces recruitment of NMDA receptor and PSD-95 to synaptic lipid rafts. Journal of neurochemistry 106: 1658-68
- Srinivasan K, Viswanad B, Asrat L, Kaul C, Ramarao P. 2005a. Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: a model for type 2 diabetes and pharmacological screening. Pharmacological Research 52: 313-20

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