Hyperglycemia Decreased Medial Amygdala Projections to Medial Preoptic Area in Experimental Model of Diabetes Mellitus

Yousef Mohamadi[1], Seyed Behnam-edin Jameie[2], Mohammad Akbari[1], Masumeh Staji[1], Fatemeh Moradi[2], Tahmineh Mokhtari[2], Maryam Khanehzad[2], and Gholamreza Hassanzadeh[1]

[1] Department of Anatomy, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[2]Department of Anatomy, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

Received: 14 Jan. 2014; Accepted: 25 Jan. 2014

Abstract- In Wistar rats, reproductive behavior is controlled in a neural circuit of ventral forebrain including the medial amygdala (Me), bed nucleus of the stria terminalis (BNST) and medial preoptic area (MPOA) via perception of social odors. Diabetes Mellitus (DM) is a widespread metabolic disease that affects many organs in a variety of levels. DM can cause central neuropathies such as neuronal apoptosis, dendritic atrophy, neurochemical alterations and also causes reproductive dysfunctions. So we hypothesized damage to the nuclei of this circuit can cause reproductive dysfunctions. Therefore in this project we assessed diabetic effect on these nuclei. For this purpose neuron tracing technique and TUNEL assay were used. We injected HRP in the MPOA and counted labeled cells in the Me and BNST to evaluate the reduction of neurons in diabetic animals. Also, coronal sections were analyzed with the TMB histochemistry method. Animals in this study were adult male Wistar rats (230 ± 8g) divided to control and 10-week streptozotocin-induced diabetic groups. After data analysis by SPSS 16 software, a significant reduction of HRP-labeled neurons was shown in both Me and BNST nuclei in the diabetic group. Moreover, apoptotic cells were significantly observed in diabetic animals in contrast to control the group. In conclusion, these alterations of the circuit as a result of diabetes might be one of the reasons for reproductive dysfunctions.

Acta Medica Iranica, 2015;53(1):1-7.

Keywords: Diabetes mellitus; Medial preoptic area; Medial amygdala; Bed nucleus of the stria terminalis; Apoptosis; Streptozotocin; HRP

Introduction

Diabetes mellitus (DM) is a chronic endocrine disease that described as a disorder in carbohydrate metabolism. Two factors are mentioned as leading causes of DM including destruction of pancreatic β-cells (type 1 diabetes) and insulin insensitivity (type 2 diabetes) (1). Over 171 million people in the world are suffering from diabetes, and it is estimated to grow up to rate of 366 million people in 2030 (2). In Iran, 7.7% of adult people had diabetes in 2008 (3).

Peripheral and central neuropathies caused by diabetes are well known (4). For example, DM results in stress oxidative (5), morphological plasticity such as decrease of neuron diameter (6), neuroaxonal dystrophy (7),dendritic atrophy and decrease in spine density in CA1 pyramidal neurons (8), neuronal apoptosis (9), behavioral problems (10,11), finally electrophysiological and neurochemical alterations (12).

Numerous studies reported reproductive dysfunctions as another important complication of diabetes (13-15). Although peripheral organs perform reproductive behavior such as spermatogenesis, ejaculation, fertility and androgenic hormone secretion, but it controls in central nervous system.

In many rodent species, proper reproductive behaviors depend on reception and perception of social odors (16), then these chemosensory signals merge with hormonal signals and process in a neural circuit between three nuclei in forebrain including medial amygdala (Me), bed nucleus of stria terminalis (BNST) and medial preoptic area (MPOA) (17,18). Particularly BNST and Me both receive chemosensory signals (19) and are densely connected to each other and to MPOA (18, 20, 21). In addition, according to studies conducted on male hamsters, damage of Me (22) and MPOA (23)

Corresponding Author: G. Hassanzadeh
Department of Anatomy, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
Tel: +98 21 88953008, Fax: +98 2166419072, e-mail address: hassanzadeh@tums.ac.ir

eliminates copulatory behavior and causes severe deficits in preference for volatile opposite-sex odors (23,24). However, in lesion of BNST in male hamsters, the elimination just was observed in odor preference (25) and deficits in copulatory behavior were temporal and more subtle (26,27). Consequently, it is concluded that MPOA, Me and BNST act as a neural network in the regulation of reproductive behavior. According to these findings and this fact that these nuclei contain glucose-sensing neurons which express glucokinase (that plays an important role in regulation of glucose level through insulin secretion) mRNA and respond directly to availability and changes of blood glucose levels (28,29), in this project we hypothesize diabetes causes reproductive dysfunction through damage to this circuit as a controlling center for reproductive functions.

Materials and Methods

Animals

24 adult male Wistar rats (230 ± 8 g) are used in this study. The animals were holding on 12 hours light/dark cycle. Water and food were available all the time. Animals were assigned into diabetic and control groups randomly. Type 1 diabetes (n=12) was induced by a single intraperitoneal injection of 60 mg/kg b.w. Streptozotocin (Sigma-Aldrich, St. Louis, MO, The USA). A week after the induction, glucose level was assessed using blood-glucose monitoring test strips. Blood glucose level was >300 mg/dl in all STZ-injected animals. Diabetic animals were maintained for 10 weeks before the study.

Neuron is tracing, and TUNEL staining was used to study the effects of diabetes on the Me and BNST.

Stereotaxic injection of HRP

After anesthesia using a single intraperitoneal injection of ketamine and xylazine (80 mg/kg and 8 mg/kg respectively), the animal head was placed into a stereotaxic apparatus using ear bars. The skull was exposed, and lambda and bregma were aligned by regulating the incisor bar. 200 nl HRP (Sigma, type VI) was injected into MPOA (by 1 µl syringe, Hamilton, Reno, NV, USA) according to coordinates in Paxinos and Watson Atlas (2007). Following injection, HRP is transported retrogradely via axonal endings to perikarya of Me and BNST projections to the injection site. Subjects were recovered for 48 hours. After deep anesthesia with ketamine and xylazine perfusion was performed transcardially with 200 ml normal saline followed by 300 mL 4% paraformaldehyde and 200 ml sucrose buffer 10% and soaking in 10% glycerin overnight at 4°C. The brains were removed and cut in coronal sections (40 µm) on a freezing microtome (Cryocut 1800, ELICA). The sections were reacted with tetramethyl benzidine (Sigma. St Louis. Mo, The USA) following the procedure of Mesolam *et al*., (30) to distinguish HRP-labeled neurons. After mounting and counterstaining with 1% neutral red, the slides were studied by light microscope and digital photos were taken. The injection site was evaluated, and optika software counted diffusion of labeled cells in Me and BNST. SPSS 16.0 software was used for data analysis.

TUNEL assay

After deep anesthesia, perfusion was performed with 200 ml of normal saline followed by 10% formalin. Paraffin-embedded brains were cut coronally (7 µm) by microtome. After deparaffinized, the sections were incubated with proteinase K (Sigma, St. Louis, MO, and The U.S.A.) to strip nuclei of tissue of proteins. Endogenous peroxidase activity was quenched with 2% H202 in phosphate- buffered saline (PBS). The slices were incubated in a humidified chamber at 37°C for 1 h with terminal deoxynucleotidyl transferase (TdT) and dUTP-digoxigenin to label 3'-OH DNA ends in apoptotic nuclei. Incubation with the stop/wash buffer at 37°C for 30 min was done to stop the reaction. The slices were colorized with diaminobenzidine /H202 solution after incubation with anti-digoxigenin-peroxidase. Finally, coronal sections were counterstained with hematoxylin and studied by light microscopy.

Results

Blood glucose level and body weight

Animal weight and blood glucose level were recorded during the study in both control and diabetic groups. Body weight normally increased in control animals (from 228 Â±10 to 334 Â ±10) and blood glucose level was 80-110 mg/dl with no change. In contrast, body weight of animals in the diabetic group was decreased (230 Â±7 to 171 Â±4) and blood sugar was >300 mg/dl during the disease (Figure 1).

Neuron tracing

HRP was injected in MPOA (Figure 2) and labeled cells were seen in Me and BNST. The cells were counted in both nuclei ipsilateral to the injection site. Based on Paxinos Atlas, 6 sections per nucleus in each animal were selected to study by light microscope.

Labeled neurons in these sections were counted. In both Me and BNST, number of labeled neurons in first and sixth sections was less in contrast to other sections (19%). In the control group the total mean of labeled neurons of Me and BNST were 33.83± 2/13 and 41.66±1/96 respectively. These numbers in the diabetic group were 26.83± 2/31 and 33.83±1.16 in Me and BNST respectively. These data show a significant reduction (20.69% in Me and 18.79% in BNST) of labeled cells in 10-week diabetic group ($P \leq 0.05$) (Figure 3 and 4).

Apoptosis

Apoptosis (DNA fragmentation) was investigated With the ApopTag kit staining. A neuron with a wrinkled and condensed nucleus (due to the DNA fragmentation) was considered as an apoptotic cell. We counted apoptotic neurons of the Me and BNST in two groups. In control animal's brain sections, a considerable amount of apoptosis did not appear in any nuclei. Cell death was approximately equal in Me and BNST (~0-3 per section) in this group. In case animals, the diabetes had affected on neuron survival, and the number of apoptotic cells had increased significantly ($P \leq 0.05$). Nearly 20 apoptotic neurons per section were seen in the both nuclei. In this group, a number of apoptosis in the Me and BNST was equivalent (Figure 5).

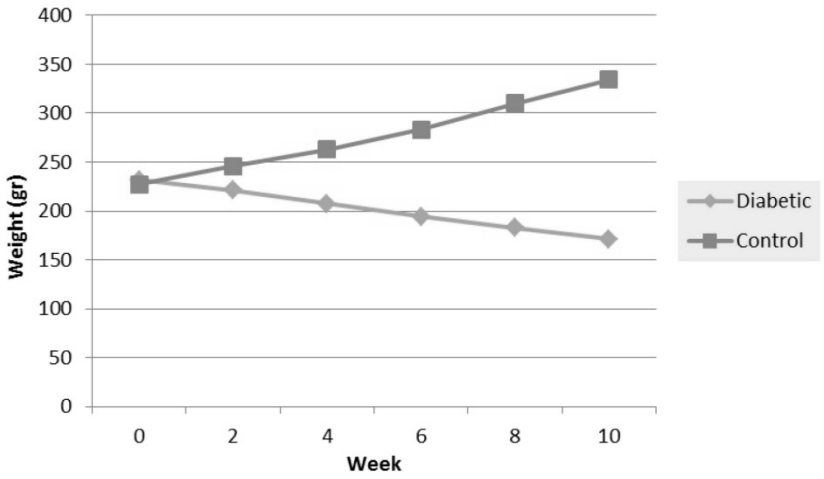

Figure 1. Changes of body weight in diabetic and control groups in duration of the study

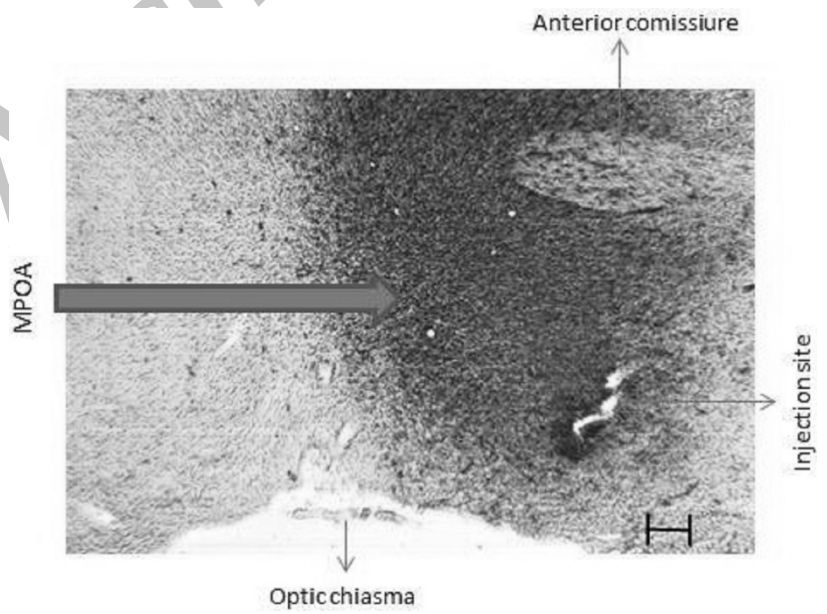

Figure 2. Injection site

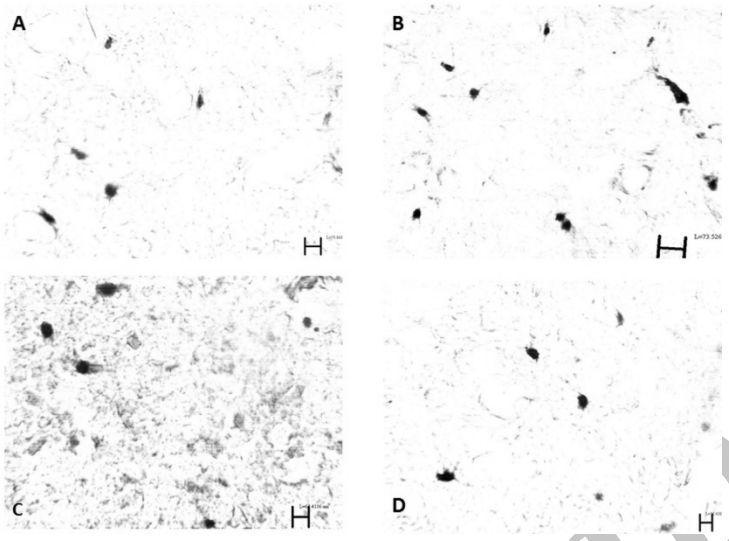

Figure 3. HRP-labeled cells
A: Me and B: BNST in the control group. C: Me and D: BNST in diabetic group (×400)

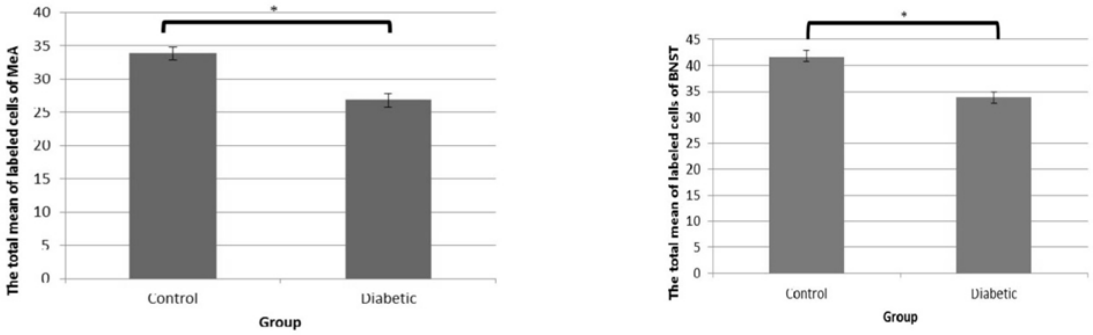

Figure 4. The number of labeled cells of Me (the left chart) and BNST (the right chart) in control and diabetic groups
*Significant differences between control and diabetic groups

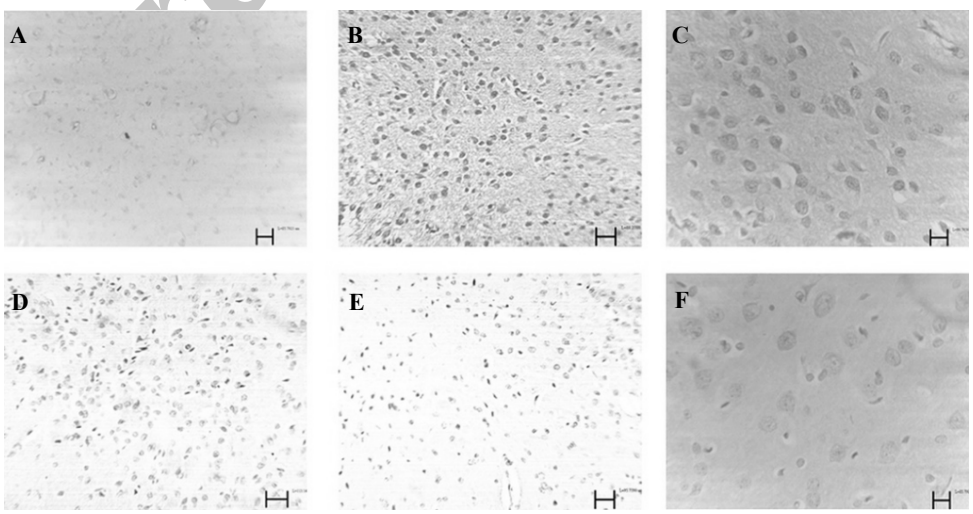

Figure 5. Apoptotic activity in the control group
(**A**: Me ×200, **B**: BNST ×200, **C**: Me ×400) and in the diabetic group (D:Me ×200, E: BNST ×200, F: Me ×400)

Discussion

This study shows that the STZ-induced diabetes decreases the retrogradely axonal transport of HRP in the Me and BNST projections to the MPOA. Other studies showed this reduction as well as cell death in peripheral nerves following DM (31). Also, cell death was confirmed in our study by TUNEL assay. Here the apoptosis was detected in the Me and BNST after 10 weeks diabetes in agreement with Rizk and Li research that observed apoptosis in hippocampus and cortex in 6-week diabetic rats (32,33). However, Li had not observed the apoptosis in the hippocampus after 2 months diabetes in 2002 (9). In both control and diabetic groups labeled, neurons in BNST were more than of those in Me. This fact shows more projections to MPOA from BNST than Me. So we realize a dense interconnection between MPOA and BNST. Although the reduction of retrogradely transport has usually be seen at the late stages of diabetes after four months (34, 35) but we observed this change in 10-week diabetic rats in this study. However, the reduction was be seen in raphe nucleus-projecting neurons to striatum after two months diabetes although it was not significant (36). On the other hand marked decreased retrogradely transport of neurotrophic protein nerve growth factor (NGF) was observed in the sciatic nerve in diabetic rats after 2 months (37). The mechanisms of these reductions are not known, but some reasons can be alterations in access to transportable agent and alterations in agent receptors as the significant decrease of NGF-receptor saturation was observed in Hellweg research (37). These findings suggest that the diabetes can decrease transport of each biological agent by similar mechanisms.

We think that the reduced number of neurons and their apoptosis in our study were due to the sensitivity of them to changes of blood glucose level (28,29). In addition to these changes, some researchers have shown that diabetes affects the Me and BNST at physiological and neurobiological level (38-40). Many studies show these nuclei contribute and control reproductive functions via reception and merging of chemosensory and hormonal signals (17,18). Therefore, deficits in this network can cause reproductive dysfunctions that reported as a complication of diabetes in many studies (13,41). For this reasons, we suspect diabetic effects on these nuclei can be a neural mechanism for reproductive dysfunctions. In conclusion, our findings demonstrate that the diabetes induce apoptosis and affect retrogradely axonal transport in projecting neurons from Me and BNST to medial preoptic area.

References

1. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus. Provisional report of a WHO consultation. Diabet Med 1998;15(7):539-53.
2. Prevalence of diabetes worldwide. WHO. (Accessed in May 2014, 18, at http://www.who.int/diabetes/facts/world_figures/en/).
3. Esteghamati A, Gouya MM, Abbasi M, et al. Prevalence of Diabetes and Impaired Fasting Glucose in the Adult Population of Iran National Survey of Risk Factors for Non-Communicable Diseases of Iran. Diabetes Care 2008;31(1):96-8.
4. Reagan LP. Diabetes as a chronic metabolic stressor: causes, consequences, and clinical complications. Exp Neurol 2012;233(1):68-78.
5. Hosseini A, Sharifzadeh M, Rezayat SM, et al. Benefit of magnesium-25 carrying porphyrin-fullerene nanoparticles in experimental diabetic neuropathy. Int J Nanomedicine 2010;5:517-23.
6. Hosseini A, Abdollahi M, Hassanzadeh G, et al. Protective Effect of Magnesium□25 Carrying Porphyrin□Fullerene Nanoparticles on Degeneration of Dorsal Root Ganglion Neurons and Motor Function in Experimental Diabetic Neuropathy. Basic Clin Pharmacol Toxicol 2011;109(5):381-6.
7. Schmidt RE, Plurad SB, Parvin C, et al. Effect of diabetes and aging on human sympathetic autonomic ganglia. Am J Pathol 1993;143(1):143-53.
8. Martínez-Tellez R, Gómez-Villalobos Mde J. Alteration in dendritic morphology of cortical neurons in rats with diabetes mellitus induced by streptozotocin. Brain Res 2005;1048(1-2):108-15.
9. Li Z-G, Zhang W, Grunberger G, et al. Hippocampal neuronal apoptosis in type 1 diabetes. Brain Res 2002;946(2):221-31.
10. Sharma AN, Elased KM, Garrett TL, et al. Neurobehavioral deficits in db/db diabetic mice. Physiol Behav 2010;101(3):381-8.
11. Meehan WP, Leedom LJ, Nagayama T, et al. Agonistic behavior patterns in mice with streptozotocin-induced diabetes mellitus. Physiol Behav 1986;38(3):301-6.
12. Kamal A, Biessels G-J, Gispen WH, et al. Synaptic transmission changes in the pyramidal cells of the

hippocampus in streptozotocin-induced diabetes mellitus in rats. Brain Res 2006;1073-1074:276-80.
13. Pontes DA, Fernandes GSA, Piffer RC, et al. Ejaculatory dysfunction in streptozotocin-induced diabetic rats: the role of testosterone. Pharmacol Rep 2011;63(1):130-8.
14. Scarano WR, Messias AG, Oliva SU, et al. Sexual behaviour, sperm quantity and quality after short-term streptozotocin-induced hyperglycaemia in rats. Int J Androl 2006;29(4):482–8.
15. Chaiban J, Azar S. Erectile dysfunction in diabetic patients. Leban Medl J 2004;52(4):217.
16. Baum MJ, Kelliher KR. Complementary roles of the main and accessory olfactory systems in mammalian mate recognition. Ann Rev Physiol 2009;71:141-60.
17. Wood RI, Newman SW. Integration of Chemosensory and Hormonal Cues Is Essential for Mating in the Male Syrian Hamster. J Neurosci 1995;15(11):7261-9.
18. Been LE, Petrulis A. Chemosensory and hormone information are relayed directly between the medial amygdala, posterior bed nucleus of the stria terminalis, and medial preoptic area in male Syrian hamsters. Horm Behav 2011;59(4):536-48.
19. Scalia F, Gallousis G, Roca S. Differential projections of the main and accessory olfactory bulb in the frog. J Comp Neurol 1991;305(3):443-61.
20. Wood R, Swann J. The bed nucleus of the stria terminalis in the Syrian hamster: subnuclei and connections of the posterior division. Neuroscience 2005;135(1):155-79.
21. Coolen LM, Wood RI. Bidirectional connections of the medial amygdaloid nucleus in the Syrian hamster brain: simultaneous anterograde and retrograde tract tracing. J Comp Neurol 1998;399(2):189-209.
22. Maras PM, Petrulis A. Chemosensory and steroid responsive regions of the medial amygdala regulate distinct aspects of opposite-sex odor preference in male Syrian hamsters. Eur J Neurosci 2006;24(12):3541-52.
23. Been LE, Petrulis A. The role of the medial preoptic area in appetitive and consummatory reproductive behaviors depends on sexual experience and odor volatility in male Syrian hamsters. Neuroscience 2010;170(4):1120-32.
24. Lehman MN, Winans SS, Powers JB. Medial nucleus of the amygdala mediates chemosensory control of male hamster sexual behavior. Science 1980;210(4469):557-60.
25. Been LE, Petrulis A. Lesions of the posterior bed nucleus of the stria terminalis eliminate opposite-sex odor preference and delay copulation in male Syrian hamsters: role of odor volatility and sexual experience. Eur J Neurosci 2010;32(3):483-93.
26. Claro F, Segovia S, Guilamón A, et al. Lesions in the medial posterior region of the BST impair sexual behavior in sexually experienced and inexperienced male rats. Brain Res Bull 1995;36(1):1-10.
27. Liu Y-C, Salamone JD, Sachs BD. Lesions in medial preoptic area and bed nucleus of stria terminalis: differential effects on copulatory behavior and noncontact erection in male rats. J Neurosci 1997;17(13):5245-53.
28. Lynch RM, Tompkins LS, Brooks HL, et al. Localization of Glucokinase Gene Expression in the Rat Brain. Diabetes 2000;49(5):693-700.
29. Zhou L, Podolsky N, Sang Z, et al. The Medial Amygdalar Nucleus: A Novel Glucose-Sensing Region That Modulates the Counterregulatory Response to Hypoglycemia. Diabetes 2010;59(10):2646-52.
30. Mesulam M-M, Van Hoesen GW, Pandya DN, et al. Limbic and sensory connections of the inferior parietal lobule (area PG) in the rhesus monkey: a study with a new method for horseradish peroxidase histochemistry. Brain Res 1977;136(3):393-414.
31. Abbate SL, Atkinson MB, Breuer AC. Amount and speed of fast axonal transport in diabetes. Diabetes 1991;40(1):111-7.
32. Rizk NN, Rafols J, Dunbar JC. Cerebral ischemia induced apoptosis and necrosis in normal and diabetic rats. Brain Res 2005;1053(1-2):1-9.
33. Li Z-g, Britton M, Sima AA, et al. Diabetes enhances apoptosis induced by cerebral ischemia. Life sciences. 2004;76(3):249-62.
34. Hassanzadeh G, Zendedel A, Akbari M, et al. Effect of Diabetes on Median and Dorsal Raphe Nuclei Efferent Fibers to CA3 Hippocampal Area in Rat. Acta Medica Iranica 2008;48(1):1-8.
35. Lee P-G, Hohman TC, Cai F, et al. Streptozotocin-induced diabetes causes metabolic changes and alterations in neurotrophin content and retrograde transport in the cervical vagus nerve. Exp Neurol 2001;170(1):149-61.
36. Hassanzadeh G, Ghorbani R, Mokhtari T, et al. Decreased Efferents of Mesencephalic Raphe Nuclei to Striatum in Animal Model of Streptozocin-Induced Diabetes. Arch Neurosci 2013;1(2):55-9.
37. Hellweg R, Raivich G, Hartung H-D, et al. Axonal transport of endogenous nerve growth factor (NGF) and NGF receptor in experimental diabetic neuropathy. Exp Neurol 1994;130(1):24-30.
38. Lakhman S, Kaur G. Effect of alloxan-induced diabetes on acetylcholinesterase activity from discrete areas of rat brain. Neurochem Int 1994;24(2):159-63.
39. Lakhman SS, Kaur G. Effect of experimental diabetes on monoamine oxidase activity from discrete areas of rat brain: relationship with diabetes associated reproductive failure. Mol Cell Biochem 1997;177(1-2):15-20.
40. Lakhman SS, Sharma P, Kaur G, et al. Changes in glucose metabolism from discrete regions of rat brain and its

relationship to reproductive failure during experimental diabetes. Mol Cell Biochem 1994;141(2):97-102.

41. Balasubramanian K, Sivashanmugam P, Thameemdheen S, et al. Effect of diabetes mellitus on epididymal enzymes of adult rats. Indian J Expe Biol 1991;29(10):907-9.