Effect of Diabetes Mellitus on Rat Cognitive Functions and Related Hippocampal Synaptic Plasticity Markers

SHAIMAA N. AMIN, M.Sc.*; SANDRA M. YOUNAN, M.D.*; MIRA F. YOUSSEF, M.D.**; LAILA A. RASHED, M.D.*** and IBRAHIM MOHAMADY, M.D.*

The Departments of Physiology*, Histology**, Biochemistry***, Kasr El-Eini, Faculty of Medicine, Cairo University

Abstract

Cognitive dysfunction is a common complication of diabetes mellitus however, less addressed and recognized. This study aimed to investigate the effect of type 1 and 2 diabetes on cognitive functions and related markers of hippocampal synaptic plasticity and the possible impact of blocking NMDA receptors by memantine. Seven rat groups were included in this study: Non-diabetic, non-diabetic-memantine treated, type-1 diabetic: Untreated, treated with insulin alone and treated with insulin and memantine and type 2 diabetic groups: Untreated and memantine treated. Cognitive functions were assessed by Morris Water Maze and passive avoidance test and immunohistochemistry was used for detection of hippocampus pre and post-synaptic markers: Synaptophysin and postsynaptic density protein-95 (PSD-95) respectively. Both type 1 and 2 untreated diabetic groups showed significantly impaired cognitive performance with concomitant decrease in synaptophysin and PSD-95 compared to the non-diabetic group. Treating type 1 diabetic group with insulin alone significantly improved cognitive performance and PSD-95 compared to untreated type 1 group. Blocking NMDA receptors by memantine (30mg/kg/day) for 3 weeks significantly increased cognitive performance and synaptophysin in type 1 insulin-memantine group compared to type 1-insulin group and significantly increased synaptophysin and PSD-95 in type 2-memantine group compared to the untreated type 2 diabetic group. In conclusion, cognitive functions are impaired in both types of diabetes mellitus and can be improved by blockage of NMDA receptors which may spark future therapeutic role of these receptors in diabetes-associated cognitive dysfunction.

Key Words: Diabetes – Cognitive functions – Synaptophysin – Postsynaptic density protein-95 – Immunohistochemistry.

Introduction

MANY organ systems are adversely affected by diabetes mellitus, including the brain. Patients with either type 1 or type 2 diabetes are more prone to show cognitive dysfunction including impaired memory and learning as well as Alzheimer’s disease compared to age-matched nondiabetic subjects [1]. Cellular mechanisms that explain how diabetes negatively influences brain functioning are still not well understood and the most appropriate methods to diagnose and treat cognitive dysfunction in diabetes have not yet been defined.

The hippocampus is a key brain area for many forms of learning and memory and is particularly sensitive to changes in glucose homeostasis. Analyses of behavioral performance and hippocampal synaptic plasticity in experimental models of diabetes have yielded inconsistent findings. While some studies suggested that water maze performance and hippocampal long-term potentiation (LTP) as measures of synaptic plasticity are reduced, others reported that these measures are unaffected [2,3].

Changes in synaptic strength can occur within minutes of stimulation. For these changes to represent memory, they must persist for days and months. Synaptophysin is a major neuronal presynaptic vesicle protein present in the hippocampus reported to be reduced in Alzheimer associated cognitive dysfunction [4].

N-Methyl-D-Aspartate receptors (NMDARs) are ionotropic glutamate receptors found in CNS and it is thought that the flow of Ca$^{2+}$ through these receptors can cause both LTP and long-term depression (LTD) vital for memory and learning [5]. However, overstimulation of these receptors causes neurodegeneration and excitotoxicity [6]. Trafficking of intact, functional NMDARs is regulated by the postsynaptic density protein-95 (PSD-95), a multivalent synaptic scaffolding protein at excitatory synapses [7].

Since the impairment of synaptic plasticity in streptozotocin-rats was linked to an inappropriate
level of NMDA receptor stimulation required for the induction phase of long term-potentiation [8], this study aimed to investigate the effect of type 1 and type 2 diabetes on cognitive functions and hippocampal synaptic plasticity markers and the impact of partial NMDARs blocking on these parameters.

**Material and Methods**

**Experimental design and animals:**

42 male albino rats 5-6 months old weighing 200-250g constituted the animal model in this study. Rats were housed each in a cage at a constant temperature (22-24°C) and light controlled room on an alternating 12: 12h light-dark cycle and had free access to food and water. Rats were fed a standard commercial pellet diet except groups for type 2 diabetes which were fed high fat diet (HFD).

Rats were divided into the following groups (n=6/group):

**Control groups:**
- Non-diabetic
- Non-diabetic memantine treated.

**Type 1 diabetes mellitus (DM) groups:**
- Untreated type 1 DM.
- Type 1-DM-insulin-memantine.
- Type 1 DM-insulin.

**Type 2 DM groups:**
- Untreated type 2 DM.
- Type 2 DM-memantine.

**Induction of diabetes mellitus:**

Type 1 diabetes was induced in rats fed on standard diet (6.5% Kcal fat) by single intraperitoneal (i.p.) injection of 65mg/kg streptozotocin (STZ) (Biomedicals, LLC, France) dissolved in cold citrate buffer (pH 4.5) [9]. Type 2 diabetes was induced by feeding the rats HFD (58% Kcal fat) for a period of 2 weeks followed by i.p. injection with a single low dose of streptozotocin 45mg/kg. Both the low dose of STZ and the high fat diet are essential elements to induce type 2 diabetes with insulin resistance [10]. Rats were continued on their respective diets till the end of the study. Non-diabetic group received (i.p) injection of 1ml citrate buffer.

The diagnosis of diabetes mellitus was confirmed by measuring blood glucose one week after STZ injection.

**Insulin treatment:**

Groups treated with insulin received (1U/100gm) of insulin once/day S.C in the evening before the rat activity phase [11].

**Memantine treatment:**

Memantine was used in the form of commercial tablets: Memantine hydrochloride (Ebixa, Lundbeck, A/S, Denmark 10mg/tablet). Tablets were crushed, dissolved in water and the calculated dose (30mg/kg/day) was given orally by gavage feeding for 3 weeks [12]. Memantine treatment was started 4 weeks after induction of diabetes in corresponding groups i.e. after diagnosis of cognitive dysfunction.

**Learning and memory tests:**

These tests were performed in the Physiology Department Kasr El-Eini Faculty of Medicine, 4 weeks after induction of diabetes to allow time for development of the diabetic-associated behavioural changes [13] and then repeated at the end of the study to assess the effect of the different treatment protocols.

**A- Passive avoidance test:**

Passive avoidance test is generally regarded as a measure of long-term memory and was performed according to method described previously by Almonte et al. [14]. An illuminated compartment (floor side; 13.5x10cm, height 8.5cm, 20W) connected to a dark compartment (floor side; 15.5x10cm, height 8.5cm) through a guillotine door was used. On habituation day, the rat was placed in the illuminated compartment and allowed to explore freely for 30s, then the door was raised and once the rat entered the dark compartment with four paws, the door was closed and the latency to enter was recorded (from the time the door was lifted). On the following day, one learning trial was given by repeating steps of the habituation trial and 3 seconds after the door was closed, an unavoidable scrambled electric foot shock (0.5mA for 2s) was delivered through the grid floor of the dark compartment and the rat was removed 30s latter to its home cage. Retention of the passive avoidance response (task) was tested 24h later by placing the animal on the lighted compartment, and measuring the latency in re-entering the dark compartment, increased escape latency to dark compartment is a good index of long-term memory.

**B- The Morris water maze test:**

The Spatial learning and memory of rats was tested according to the method of Morris [15]. Morris water-maze with submerged platform and a video tracking system (ANY-mazeTM Video...
Tracking System; version 4.73) was used. The Morris water maze consisted of a circular tank, (diameter: 120cm, height: 30cm) filled to a depth of 24cm. The water temperature was 26 °C and a 10-cm clear circular platform was submerged 1 cm below the water level in the northwest quadrant of the maze.

- **Cue discrimination:**
  A Visible platform test was performed to exclude drug or experimental manipulation-induced changes in visual acuity. The video tracker system was not used and only a stop watch was used in this test. Rats were first habituated to the pool by allowing 30 seconds free swim and four trials to climb into a platform from four different directions. The platform in this test was extended 1 cm above the water level. The rats were given three blocks of five trials of cue training for a total of 15 trials with approximately 10 minutes intervals. No intentional cues other than the platform were available to the rat during this phase [16].

- **Spatial discrimination:**
  For spatial discrimination, the escape platform was hidden approximately 1.5cm beneath the water level and placed in an area of the pool different from the location used for “cue discrimination” training and water rendered opaque by adding powdered milk. The platform remained in the same location relative to the distal cues in the room. Training consisted of six blocks of three trials. Intertrial intervals were approximately 10 minutes. On each trial, the rat was placed in the pool from one of four equally spaced start locations (north, south, east and west) and was given 60 seconds to escape during each trial; if they did not escape within the allotted time, they were gently guided to the platform where they remained for 10 seconds. Start locations were randomized across each rat and trial. The water was stirred between trials to eliminate the use of odor trails as cues. Between each trial and following performance of the swim task, rats were towel dried and returned to their home cages. Latency to escape (seconds to reach the platform), distance traveled (centimeters traversed in maze) and proximity (% of time spent within 40cm of the platform) were measured.

- **Probe trial:**
  After the fifth block of training, rats received a probe trial where the platform was removed from the pool and the animal was allowed to swim for 60 seconds. Rats were subsequently given the “reminder” sixth block of training following this immediate probe trial, which was not included in the assessed learning. The following day, the platform was again removed for a free-swim probe trial to measure 24-hour retention of the task. Proximity was measured during immediate and late probe trials as % time spent within 40cm to where the platform was previously placed.

**Biochemical analysis:**
At the end of experimental period, fasting serum glucose was measured using oxidase-peroxidase method and fasting serum insulin was analyzed using enzyme-linked immunosorbent assay (ELISA) (DRG diagnostics, Germany) according to the manufacturer’s instructions. To estimate insulin resistance, the homeostasis model assessment for insulin resistance (HOMA-IR: Insulin resistance index) was calculated as the product of fasting insulin (in microunits/ml) and fasting glucose (in mmol/l) divided by 22.5, which has been used previously in rodents [17].

**Histological staining:**
Brain sectioning and staining: The rats were anesthetized with ether followed by harvesting of brain tissues, placement in 10% paraformaldehyde for 2 hours [18]. The brains were removed and placed in a new formaldehyde solution for 24 hrs before being dehydrated using ethanol (70% for 24hs, 90% for 1h and 100% for 1h) then cleaned in xylene and embedded in paraffin. Coronal sections were cut with a microtome at 5 µ thicknesses, mounted on glass slides and stained with routine hematoxylin & eosin techniques.

**Immunohistochemical techniques:**
Serial sections cut at 5 µ thickness were also mounted on positive charged glass slides for Immunohistochemical staining using secondary “Ultravision detection system” (ThermoScientific-USA, catalog no TP-015-HD). Sections were deparaffinised and hydrated in graded descending concentrations of alcohol, then incubated with hydrogen peroxide blocking solution for 15 minutes. Incubation was done in humid chambers at room temperature, and slides were continuously kept wet starting from this step onwards. Slides were washed twice in phosphate buffer; incubated with pepsin digestive enzyme and washed 4 times in buffer. Ultra Violet block was applied and incubated for 5mins. Primary antibodies were then applied on the serial sections and incubated as follows: Synaptophysin (ThermoScientific-USA) 1: 60 for 30 minutes and PSD-95 (Lifespan Biosciences, Inc-USA) 5 µg/ml for 30 minutes.

Sections were then washed and biotinylated goat antipolyvalent antibody (secondary antibody)
was applied for 10mins, washed, then followed by streptavidin peroxidase for 10mins and washed after. To develop colour reaction, one drop of DAB Plus chromogen was added to 2ml of DAB Plus substrate, mixed and applied on tissues for 5-15mins. Sections were then counterstained with Mayer’s hematoxylin. Coverslip was applied using mounting media. Positive reaction appeared as brown color [19].

Quantitative morphometric study:

Morphometric study was done at Histology Department at Kasr El-Eini Faculty of Medicine-Cairo University. Ten high power fields were measured in each of the serial sections in the different studied groups. Area % was measured for PSD-95; and optical density was measured as regard synaptophysin expression.

The data were obtained by using Leica QWin 500 image analyzer computer system (England). The image analyzer consists of Olympus microscope, a colored video camera, colored monitor and a hard disc of Leica IBM personal computer connected to the microscope and controlled by Leica QWin 500 software. Data were statistically described in terms of, mean and standard deviation (Mean±SD) for area %, and as sum of grey and mean grey measures for optical density. Photography was performed by Panasonic camera connected to Olympus microscope connected to the computer.

Statistics:

The results were analyzed using SPSS computer software package, version 16 (Chicago, IL, USA). Data were presented as mean ± S.D. Differences among the parameters of the three groups were compared by one-way ANOVA followed by post-hoc test. Results were considered statistically significant at $p<0.05$.

Results

Metabolic parameters in the different studied groups:

As revealed from Table (1), administration of memantine to non-diabetic rats significantly increased serum glucose of the non-diabetic-memantine group compared to the non-diabetic group. No statistical difference was observed in serum insulin and HOMA-IR between both groups.

As expected, induction of type 1 diabetes mellitus significantly increased serum glucose level and HOMA-IR and significantly decreased serum insulin in the untreated type 1 DM group ($p<0.05$) compared to the non-diabetic one.

Table (1): Metabolic parameters in the different groups.

<table>
<thead>
<tr>
<th>Measured parameters</th>
<th>Non-diabetic</th>
<th>Non-diabetic-memantine</th>
<th>Untreated type 1 diabetic</th>
<th>Type 1 diabetic-insulin</th>
<th>Untreated type 2 diabetic</th>
<th>Type 2 diabetic-memantine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum glucose (mg/dl)</td>
<td>76.6±6.2</td>
<td>113.3±1.36*</td>
<td>172.3±23*</td>
<td>185.3±9.6</td>
<td>131.1±5.87</td>
<td>@</td>
</tr>
<tr>
<td>Serum Insulin (µU/ml)</td>
<td>4.1±0.76</td>
<td>4.2±0.5</td>
<td>3.2±0.55*</td>
<td>5.3±0.53*</td>
<td>6.9 ±0.62</td>
<td>@</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.77±0.16</td>
<td>1.17±0.15</td>
<td>1.38±0.37*</td>
<td>2.4±0.27*</td>
<td>2.26 ±0.27</td>
<td>4.27±0.48*</td>
</tr>
</tbody>
</table>

*: Significant compared to non-diabetic group. @: Significant compared to type-1 DM-insulin group. +: Significant compared to non-diabetic-memantine group. $: Significant compared to untreated type 2 DM group.

Table (2): Comparison of hippocampal expression of synaptic plasticity markers by immunohistochemistry among the studied groups.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>Non diabetic Control</th>
<th>Non diabetic Memantine</th>
<th>Untreated Type 1 diabetic</th>
<th>Type 1 diabetic</th>
<th>Type 1 Diabetic-insulin-memantine</th>
<th>Untreated Type 2 diabetic</th>
<th>Type 2 Diabetic-memantine</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synaptophysin (Optical density)</td>
<td>Non diabetic Memantine</td>
<td>.681±</td>
<td>.473±</td>
<td>.498±</td>
<td>.504±</td>
<td>.571±</td>
<td>.539±</td>
<td>.657±</td>
<td>.000</td>
</tr>
<tr>
<td></td>
<td>Untreated Type 1 diabetic</td>
<td>.03±*</td>
<td>.05*</td>
<td>.04*</td>
<td>.05*</td>
<td>.059@#+*</td>
<td>.06+*</td>
<td>.08&amp;$@#++</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Type 1 diabetic</td>
<td>.98±*</td>
<td>.85±</td>
<td>.84±*</td>
<td>.85*</td>
<td>.859@#++</td>
<td>.86*</td>
<td>.08&amp;$@#++</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Type 1 Diabetic-insulin-memantine</td>
<td>3.51±*</td>
<td>3.52±*</td>
<td>3.53±*</td>
<td>3.54±*</td>
<td>3.55±*</td>
<td>3.56±</td>
<td>.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Untreated Type 2 diabetic</td>
<td>3.14±*</td>
<td>3.15±*</td>
<td>3.25±*</td>
<td>3.35±*</td>
<td>3.45±*</td>
<td>3.46*</td>
<td>.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Type 2 Diabetic-memantine</td>
<td>3.15±*</td>
<td>3.16±*</td>
<td>3.25±*</td>
<td>3.35±*</td>
<td>3.45±*</td>
<td>3.46*</td>
<td>.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$p$ value</td>
<td>3.56$@#++</td>
<td>3.57$@#++</td>
<td>3.58$@#++</td>
<td>3.59$@#++</td>
<td>3.60$@#++</td>
<td>3.61$@#++</td>
<td>.01</td>
<td></td>
</tr>
<tr>
<td>PSD-95 (area % of the field)</td>
<td>Non diabetic Memantine</td>
<td>35.24±</td>
<td>34.26±</td>
<td>34.27±</td>
<td>34.28±</td>
<td>34.29±</td>
<td>34.30±</td>
<td>34.31±</td>
<td>.000</td>
</tr>
<tr>
<td></td>
<td>Untreated Type 1 diabetic</td>
<td>5.25*</td>
<td>5.74*</td>
<td>5.26*</td>
<td>5.27*</td>
<td>5.28*</td>
<td>5.29*</td>
<td>.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Type 1 diabetic</td>
<td>3.74*</td>
<td>3.75*</td>
<td>3.76*</td>
<td>3.77*</td>
<td>3.78*</td>
<td>3.79*</td>
<td>.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Untreated Type 2 diabetic</td>
<td>6.13±*</td>
<td>6.14±*</td>
<td>6.15±*</td>
<td>6.16±*</td>
<td>6.17±*</td>
<td>6.18*</td>
<td>.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Type 2 Diabetic-memantine</td>
<td>6.14±*</td>
<td>6.15±*</td>
<td>6.16±*</td>
<td>6.17±*</td>
<td>6.18±*</td>
<td>6.19*</td>
<td>.01</td>
<td></td>
</tr>
</tbody>
</table>

* : Significant compared to control injected with buffer. $: Significant compared to type-1 insulin + memantine. +: Significant compared to non-diabetic-memantine treated. &: Significant compared to untreated type-2.
Type 1 insulin-treated group showed a significantly increased serum insulin and HOMA-IR ($p<0.05$) with no significant change in serum glucose compared to the untreated type 1 diabetic group (Table 1). Type 1 DM group treated with both insulin and memantine showed a significantly decreased serum glucose and a significant increase in the serum insulin level compared to type 1 DM group treated with insulin alone ($p<0.05$).

Induction of type 2 diabetes significantly increased serum glucose and insulin levels as well as HOMA-IR in untreated type 2 DM group compared to the non-diabetic group. Type 2 DM group treated with memantine showed a significant decrease in serum insulin and HOMA-IR with no significant change in serum glucose compared to untreated type 2 DM group although these parameters were still significantly increased compared to non-diabetic-memantine group (Table 1, $p<0.05$). These results indicate that memantine may improve insulin resistance in diabetes mellitus.

**Effect of diabetes and memantine on cognitive functions:**

Results indicate that both types of diabetes induced deficiency in learning and spatial memory in rats during the passive avoidance and Morris water maze tests. Untreated type 1 and 2 diabetic groups compared to the non-diabetic group exhibited a significant decrease in the escape latency to dark compartment in passive avoidance test (Fig. 1A,B, $p<0.05$), a significant increase in the escape latency and the travelled distance to hidden platform (Fig. 2A,B and Fig. 3A,B, $p<0.05$) and a significant decrease in proximity as measured by the % time spent within 40cm of the platform (Fig. 4A,B, $p<0.05$) in all Morris maze training blocks. Also a significant decrease was observed in the proximity of both the untreated diabetic groups during immediate and 24h probe trials compared to the non-diabetic group (Fig. 5A,B, $p<0.05$).

Type 1 diabetic group treated with insulin showed partial improvement of the cognitive functions compared to the untreated type 1 diabetic group as revealed by a significant increase in the escape latency to dark compartment of the passive avoidance test (Fig. 1A), a significant decrease in the escape latency (Fig. 2A) and distance travelled (Fig. 3A) to hidden platform in all blocks of training and a significant increase in proximity only during the 1st and 5th blocks of training and immediate probe trial during MWM test (Figs. 4A,5A, $p<0.05$). However all tests were still impaired compared to the non-diabetic group ($p<0.05$).

Treating type 1 diabetic group with both insulin and memantine had better impact on learning and spatial memory compared to insulin treatment alone. A significant increase was observed in the escape latency to dark compartment of the passive avoidance test (Fig. 1A) compared to the insulin-treated type 1 group ($p<0.05$). Also a significant decrease in escape latency to hidden platform (Fig. 2A) was revealed in the 4th and 5th training blocks and in the distance travelled to hidden platform in all training blocks of the MWM test (Fig. 3A) compared to the type 1 diabetic-insulin group ($p<0.05$) indicating a beneficial effect of memantine over the insulin treatment alone. Although all cognitive tests were still significantly impaired compared to non-diabetic group.

Proximity in type 1 insulin-memantine group was significantly increased in all blocks of training (Fig. 4A) and in immediate and 24h probe trials (Fig. 5A) compared to that of the type 1 diabetic-insulin group ($p<0.05$).

Treating type 2 diabetic group with memantine, significantly increased escape latency to dark compartment of the passive avoidance test (Fig. 1B) compared to the non-diabetic-memantine and untreated type 2 diabetic groups. Also it significantly decreased the escape latency to hidden platform (Fig. 2B, $p<0.05$) compared to untreated type 2 diabetic group in all MWM training blocks although still significantly increased compared to non-diabetic-memantine in the 1st 4 blocks and only significantly decreased in the 5th training block.

Distance travelled to hidden platform in the MWM was significantly decreased in type 2-memantine group compared to untreated type 2 group in all training blocks and to non-diabetic-memantine group in the first three blocks (Fig. 3B, $p<0.05$) with statistical insignificant difference in the last two blocks, which indicate a positive effect of memantine on learning and spatial memory.

Proximity was significantly increased in type 2-memantine group in the last 4 training blocks and in immediate and 24h probe trials compared to untreated type 2 DM and in the last three training blocks and the immediate probe trial compared to the non-diabetic-memantine group (Figs. 4B, 5B, $p<0.05$) which suggest that memantine may consolidate the long-term memory in type 2 diabetes.

Administration of memantine to non-diabetic group had less beneficial effects with significant decrease in the escape latency to dark compartment
in the passive avoidance test (Fig. 1, $p<0.05$) and a significant increase in the escape latency and distance travelled to hidden platform during all training blocks of the MWM test compared to the non-diabetic group (Figs. 2, 3, $p<0.05$). Proximity was also significantly reduced in all training blocks and in the immediate and 24h probe trials compared to the non-diabetic group (Figs. 4, 5, $p<0.05$), which suggest that memantine has no role in learning and memory consolidation in the non-diabetic state.

Fig. (1): Escape latency recorded during passive avoidance test. Latency to enter the dark compartment in type 1 diabetic (A) and type 2 diabetic (B) subgroups. *: Significant compared to non-diabetic group, +: Significant compared to non-diabetic-memantine group, #: Significant compared to untreated type 1 diabetic group, @: Significant compared to type 1-insulin group, $: Significant compared to untreated type 2 diabetic group at $p<0.05$.

Fig. (2): Latency to reach the hidden platform during training blocks of Morris water maze in the different groups. A: Escape latency to hidden platform in type 1 diabetic subgroups. B: Escape latency to hidden platform in type 2 subgroups. *: Significant compared to non-diabetic group, +: Significant compared to non-diabetic-memantine group, #: Significant compared to untreated type 1 diabetic group, @: Significant compared to type 1-insulin group, $: Significant compared to untreated type 2 diabetic group at $p<0.05$. Data are presented as mean ± S.D.
Fig. (3): Distance travelled to reach the hidden platform during training blocks of Morris water maze in the different groups. A: Distance travelled to hidden platform by type 1 diabetic subgroups. B: Distance travelled to hidden platform by type 2 subgroups. *: Significant compared to non-diabetic group, +: Significant compared to non-diabetic-memantine group, #: Significant compared to untreated type 1 diabetic group, @: Significant compared to type 1-insulin group, $: Significant compared to untreated type 2 diabetic group at \( p < 0.05 \). Data are presented as mean \( \pm \) S.D.

Fig. (4): Proximity during training blocks of Morris water maze in the different groups. Proximity (% time spent within 40 cm of the platform) in type 1 (A) and in type 2 (B) diabetic subgroups. *: Significant compared to non-diabetic group, +: Significant compared to non-diabetic-memantine group, #: Significant compared to untreated type 1 diabetic group, @: Significant compared to type 1-insulin group, $: Significant compared to untreated type 2 diabetic group at \( p < 0.05 \). Data are presented as mean \( \pm \) S.D.
Effect of Diabetes Mellitus on Rat Cognitive Functions & Related Hippocampal

Fig. (5): Proximity during immediate and 24 h probe trials in Morris water maze of the different groups. Proximity (% time spent within 40 cm to where the platform was previously present) in type 1 (A) and in type 2 (B) diabetic subgroups during immediate (open bars) and 24 h (filled bars) probe trials. *: Significant compared to non–diabetic group, +: Significant compared to non-diabetic-memantine group, #: Significant compared to untreated type 1 diabetic group, @: Significant compared to type 1-insulin group, $: Significant compared to untreated type 2 diabetic group at $p<0.05$. Data are presented as mean ± S.D.

Fig. (6): Histological examination of non-diabetic groups:

Non diabetic control: a) normal pyramidal cell layer, and b) normal granular cell layer.

Non diabetic-Memantine: a) disorganized pyramidal layer with apoptosis, and b) retracted processes in granular layer (arrowheads) (H & E x200).
Fig. (7): Histological examination of type 1 diabetic groups. Untreated type 1DM: a) shrunken pyramidal cells, clumped neuronal processes, wide capillary (c); b) shrunken dark granular cell region, preserved mol layer, many astrocytes (*). DM type 1 treated with insulin alone: a) preservation of pyramidal cell layer; b) granular cells with dark nuclei, disorganized arrangement and many vacuolations, with enlarged cells of molecular layer. Type 1 DM treated with insulin and memantine: a) preserved pyramidal layer in areas, others are shrunken, & widened capillaries; b) granular layer and molecular layers preserved. (H&E x200).

Fig. (8): Histological examination of type 2 diabetic groups. DM type 2 untreated: a) shrunken pyramidal cells, areas of cell loss (arrow); b) cluster of dead cells within granular layer, lost cell junctions, vacuolation, astrocytes are in excess (*). Type 2 DM-memantine: a) shrunken pyramidal cells with dark nuclei; b) granular cells with disrupted connections (arrow), preserved molecular layer with many astrocytes (*) (H&E x200).
Effect of Diabetes Mellitus on Rat Cognitive Functions & Related Hippocampal

A: Synaptophysin immunostaining:

Fig. (9): Synaptophysin and PSD-95 immunostaining.

a) non diabetic control, b) non diabetic memantine, c) untreated type 1 DM, d) type 1 DM + insulin, e) type 1 DM + insulin-memantine, f) untreated type 2 DM, g) type 2 DM + memantine.

A: Synaptophysin immunostained sections show: homogenous moderate staining of neuronal processes in a, as opposed to patchy lamellar staining in b (moderate & clumped proximal to cell bodies of CA regions, & more mild in center of molecular layer). Both c & f show clumping of staining (non homogenous distribution), while d regained homogenous staining. Also e & g both showed homogenous staining pattern similar to a. Note that cell bodies were always left unstained. (Synaptophysin immunostaining x200).

B: PSD-95 immunostained sections show: a) homogenous intense reaction in cell bodies, moderate reaction in axonal processes of fimbrial part above CA regions, and moderate to mild reaction in molecular regions; b) irregular patchy staining of cell bodies; c) & f) both show decreased staining in cell bodies as well as in cell processes; d) & e) both regained homogenous staining of cell bodies & moderate staining of axonal processes similar to a; g) also regained homogenous reaction of cell bodies as well as moderate reaction in processes. (PSD 95 immunostaining x200).
Hippocampal synaptophysin by optical density of the field (A) and % area in the hippocampal field of postsynaptic density-95 (PSD-95) (B) assessed by immunohistochemistry in non-diabetic groups, type 1 and type 2 subgroups. *: Significant compared to non-diabetic group, +: Significant compared to non-diabetic-memantine group, #: Significant compared to untreated type 1 diabetic group, @: Significant compared to type 1-insulin group, $: Significant compared to untreated type 2 diabetic group at p<0.05. Data are presented as mean ± S.D.

Results of histological examination of hippocampal tissue sections:

Hematoxylin and Eosin stained sections of hippocampus revealed the differences in layer structure between the different studied groups. Non-diabetic control sections showed compact arrangement of pyramidal cell layers of cornu ammonis: CA1 & CA3. Cells had pale nuclei and deep basophilic cytoplasm. Molecular layer contained neuronal processes, neurons and astrocytes (Fig. 6a,b).

Non diabetic-memantine group showed marked decrease of pyramidal cell density, with marked retraction of neuronal processes in many areas (Fig. 6a,b).

Untreated type 1 DM group showed many shrunken pyramidal cells with dark nuclei, clumping of neuronal processes and wide capillaries, as well as shrunken dark granular cell layer and preserved molecular layer with many astrocytes (Fig. 7a,b).

Type 1 DM treated with insulin alone showed proper preservation of pyramidal cell layer, whereas granular cells still showed darkened nuclei and disorganized layers. Molecular layer had many enlarged cells (Fig. 7a,b).

Meanwhile, type 1 DM treated with both insulin and memantine showed that layer of pyramidal cells was well, but others were shrunken. Granular and molecular layers were also well preserved. Many widened capillaries could be seen (Fig. 7a,b).

Type 2 DM group that is left untreated showed many shrunken pyramidal cells with areas of cell loss. Granular cells had many clustered dead cells, and many other dark ones with loss of cell junctions and with vacuolations. Astrocytes among molecular layer were present in excess (Fig. 8a,b).

Type 2 DM that was treated with memantine showed markedly shrunken pyramidal cells with dark nuclei, as well as granular cells with disrupted connections. Molecular layer was mostly preserved but with more astrocytes in between (Fig. 8a,b).

Synaptic plasticity markers in the hippocampus detected by immunohistochemistry:

Immunohistochemical staining for synaptophysin in non diabetic control showed homogenous moderate staining of neuronal processes and wide capillaries, as well as shrunken dark granular cell layer and preserved molecular layer with many astrocytes (Fig. 9A,a&b).

Untreated type 1 and type 2 DM both lost homogenous pattern of staining, where reaction was generally less than that non diabetic control group; reaction also showed clumping within processes of molecular region (Fig. 9A,c&f). Treatment of type 1 DM with insulin alone resulted in marked regaining of pattern to be similar to non diabetic control group. Treatment with memantine (with insulin in type 1 DM or alone in type 2 DM) also
had very significant effect on regaining of normal organization and of normal intensity of staining in the studied groups (Fig. 9A,e&g).

Immunohistochemical staining for PSD 95 non-diabetic group showed characteristic pattern that included homogenous intense reaction in cell bodies, moderate reaction in axonal processes of fimbrial part above CA1 region, and moderate to mild reaction in synapses of molecular regions. Non diabetic group receiving memantine lost organized pattern, where reaction showed irregular distribution in cell bodies of neurons (Fig. 9B,a&b). Both untreated diabetic groups showed decreased irregular staining in cell bodies as well as decreased staining in neurons when compared to non diabetic control (Fig. 9B,c&f). Treatment with insulin alone or insulin and memantine of type 1 diabetic groups both led to regaining of normal organization and intensity of staining reaction. Also treatment of type 2 with memantine alone led to significant improvement and regaining of normal pattern (Fig. 9B,e&g).

Quantitative study by optical density for synaptophysin expression and area % for PSD-95, revealed a significant decrease in the hippocampal synaptophysin (Fig. 10A) and the postsynaptic density protein-95 (Table 2 \( p < 0.5 \)) (Fig. 10B) in untreated type 1 and 2 diabetic groups compared to the non-diabetic group (\( p < 0.05 \)).

Treating type 1 diabetic group with insulin alone significantly increased hippocampal PSD-95 (Fig. 10B, \( p < 0.05 \)) compared to the untreated type 1 without significantly changing the synaptophysin (Fig. 10A, Table 2 \( p > 0.05 \)).

Type 1 diabetic group treated with both insulin and memantine showed a significant increase in hippocampal synaptophysin (Fig. 10A), compared to the type 1 DM-insulin group with no statistical significant difference in PSD-95 (Fig. 10B, Table 2 \( p > 0.05 \)).

Synaptophysin was still significantly decreased in type 1 DM-insulin and type 1 DM-insulin-memantine compared to control group while PSD-95 was insignificantly changed (Table 2).

Treating type 2 diabetic group with memantine significantly increased synaptophysin and PSD-95 compared to the untreated type 2 diabetic group (Fig. 10A&B, \( p < 0.05 \)) and significantly increased synaptophysin compared to non-diabetic-memantine, however, the PSD-95 was still significantly decreased.

Administration of memantine to non-diabetic group significantly decreased synapophysin and insignificantly changed PSD-95 compared to the non-diabetic group (Table 2 \( p > 0.05 \)).

**Discussion**

Diabetes is associated with several adverse effects on the brain, some of which may result primarily from direct consequences of chronic hyperglycemia. In this work, both type 1 and 2 untreated diabetic groups showed significantly impaired learning and spatial memory in rats during the passive avoidance and Morris water maze tests and many shrunken pyramidal cells compared to the non-diabetic group in agreement with previous studies [1,20].

Insulin treatment of type 1 diabetic group after development of cognitive dysfunction (reversal experiment) partially improved cognitive functions and preserved pyramidal cells involved in spatial memory compared to the untreated type 1 group; however, all tested cognitive functions were still impaired compared to the non-diabetic group and the granular layer was still disorganized. These results strongly support previous one suggesting that intervention with insulin failed to reverse water maze learning and partially affected long-term potentiation needed for memory retention unlike insulin treatment commenced at the onset of diabetes [21].

Cognitive dysfunction and impaired synaptic plasticity in both types of diabetes has been linked to hyperglycemia, insulin deficiency and/or insulin resistance and altered insulin signaling [22,23] as well as to hypophyseal-pituitary axis hyperactivity and elevated glucocorticoid levels [24]. Insulin diminishes hypothalamic-pituitary-adrenal-axis activity [25] and modulates neurotransmitter levels [26] thus promotes physiologic processes critical for memory.

Serum glucose in type 1 DM-insulin group was elevated comparable to that of the untreated DM group similar to that reported previously as reactive hyperglycemia to administrated insulin [27]. Hyperglycemia increases NMDA receptor-mediated calcium entry into the neurons and may induce neuronal excitotoxicity through activation cascade ending by the release of ROS [28]. Memantine an uncompetitive NMDA receptor antagonist significantly improved serum insulin and insulin resistance in both types of diabetes and serum glucose in type 1 diabetic group compared to the insulin-treated type 1 group. Also memantine significantly
improved microscopic pattern of pyramidal, granular and molecular hippocampal cell layers and all tested cognitive functions in type 1 DM group (with the exception of the escape latency to hidden platform in the first three training blocks) compared to type 1 group treated with insulin alone and in type 2 DM group compared to the untreated type 2 diabetic group which indicate a positive effect of blocking NMDA receptors on memory and synaptic plasticity in diabetes.

Similar dose of memantine has been shown to improve cognitive function and to slow cognitive and functional decline in Alzheimer disease transgenic rat models [12]. The importance of maintaining normal synaptic NMDA signaling which has the special property of allowing calcium to enter the postsynaptic spine only when presynaptic activation by glutamate and postsynaptic depolarization occur at the same time was demonstrated by Papadia et al. [29]. Memantine cannot act or accumulate in NMDA channel when the channel is open for several milliseconds as occurring during normal synaptic activity but only inhibit the prolonged influx of Ca$^{2+}$ ions and blocks abnormal glutamate excitatory signals [30].

In this study, both types of diabetes decreased hippocampal presynaptic synaptophysin and postsynaptic density protein (PSD-95) consistent with previous studies showing that both types of diabetes affect pre and post-synaptic activities in the hippocampus [31,32]. Meanwhile, our results disagree with a study performed by Grillo et al. [33] reporting increased hippocampal synaptophysin and postsynaptic density-95 (PSD-95) expression in streptozotocin-induced diabetes on both a short-term (1 week) and long-term (5 weeks) course of diabetes. The decrease in synaptophysin expression in frontal cortex and hippocampus lead to decrease in neurotransmitters release and may be one of the factors leading to diabetic dementia [34].

Kurihara et al. [35] found a relationship between active renin-angiotensin system and angiotensin-I receptor (AT 1R) signaling and down regulation of synaptophysin in diabetes-induced retinal dysfunction and neuronal damage. Since previous reports showed synaptic expression of AT1R in the hippocampus [36] similar mechanism could be suggested.

Hippocampal cells bear a great number of insulin and glutamatergic receptors [24] at the synapses so it seemed to be an anatomy of choice to examine effect of both insulin and memantine on synaptic markers. The present study demonstrates that while treatment with memantine could reverse diabetic-induced decrease in hippocampal synaptophysin and PSD-95, insulin treatment could only increase PSD-95 without significantly changing synaptophysin.

These results agree with those of Lee et al. [37] which showed that insulin can increase protein synthesis of PSD-95 through its receptors on neurons and glial cells in a phosphatidylinositol-3 kinase (PI3) dependent manner, however disagree with those of Francis et al. [18] suggesting that insulin could increase hippocampus synaptophysin in type 1 diabetes.

In consistent with our results, memantine increased synaptophysin in the cortex of transgenic mice with memory impairments [38] and improved soluble oligomeric amyloid-$\beta$ (A$\beta$) [39] which are known to downregulate PSD-95 and synaptophysin during early stages of Alzheimer's disease [40].

However the positive effects of memantine were absent in the non-diabetic group. Memantine significantly increased serum glucose and significantly impaired all tested cognitive performances compared to the non-diabetic group. Also there was disruption of normal distribution with decreased synaptophysin and normal PSD 95 indicating that the beneficial effects of memantine on memory consolidation are only evident in the disease state.

In conclusion, both types of diabetes are associated with cognitive dysfunction and decreased pre- and post-synaptic synaptic markers. Insulin in type 1 DM and memantine in both diabetic types could improve cognitive functions and PSD-95. Memantine had an advantage to be able to improve synaptophysin in both types of diabetes which may spark future therapeutic role of the NMDARs antagonists in diabetes-associated cognitive dysfunction.

References

4. PROCTOR D.T., COULSON E.J. and DODD P.R.: Reduction in post-synaptic scaffolding PSD-95 and SAP-102 protein levels in the Alzheimer inferior temporal


