INFLUENCE OF ENVIRONMENTAL ENRICHMENT EXPOSURE ON COGNITIVE PERFORMANCE AND NEUROPROTECTION OF DIABETIC, STRESSED RAT DENTATE GYRUS AND AMYGDALA

Narendra Pamidi ¹ and Satheesha Nayak B²

¹Faculty of Medicine, Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Kuala Lumpur, Malaysia,
²Department of Anatomy, Melaka Manipal Medical College, Manipal University, Manipal, Karnataka, India

ABSTRACT: Environmental enrichment (EE) exposure is known to influence structural changes in the neuronal network of brain areas to improve learning and memory functions. The present study was aimed to evaluate the effectiveness of environmental enriched treatment on the cognitive behavior and neurons of diabetic and stressed dentate gyrus (DG), amygdala of rat brain. Rats were made hyperglycemic with streptozotocin (40mg/kg). Diabetic and stressed rats were housed in enriched cages daily for 6 hrs. Blood sugar levels and body weight were measured at regular intervals. Behavioral studies were performed at regular intervals. On day 30, rat brains were processed for cresyl violet staining for quantification of neurons. EE exposure improved the learning and memory abilities and minimized the neuronal damage (increased the number of surviving neurons) in diabetic and stressed rats. In the DG, the cell death rate in diabetic group was 38.43% and in diabetic+stressed group was 59.53%. A significantly decreased cell death rate (P<0.001) was observed in diabetic+ EE exposed (24.46%) and in diabetic+stress+EE exposed rats (40.56%). In the amygdala also, the number of surviving neurons was significantly increased (P<0.001) in diabetic and EE exposed (Cell death rate, lateral nucleus: 27%, basal nuclei: 42%), diabetic+stress+EE groups (lateral nucleus: 42%, basal nuclei: 29%) compared to diabetic (lateral nucleus: 39.5%, basal nuclei: 54%) and diabetic+stressed groups (lateral nucleus: 67%, basal nuclei: 66%). Enriched cage housing significantly improved the cognitive abilities and decreased the amount of neuronal damage caused by diabetes and stress

Key words: Behavior, brain, diabetes, enriched environment, stress

INTRODUCTION
Diabetes mellitus (DM) is an endocrine, metabolic disorder resulting from inadequate insulin release (Type 1 diabetes) or insulin insensitivity coupled with the inadequate compensatory insulin release (Type 2 diabetes). Insulin replacement, drugs, diet and exercise can control diabetes and its long-term complications. The neurological consequences of DM in the central nervous system (CNS) have gained attention most recently (Reagan LP et al., 1999). Studies proved that the streptozotocin (STZ) induced diabetes produced a dramatic decrease in cell proliferation in the DG of hippocampus and altered the hippocampal synaptic plasticity. Depression and anxiety are about 2-folds higher in diabetic patients than in the general population. Findings and evidences in the research revealed an association between DM and reduced learning and memory activities (Jackson-Guilford J et al., 2000).
DG is a substructure of the hippocampal formation of the brain. DG contributes to new memories (Chen Q et al., 2010) and orthogonalizes sensory information cumulated from the entorhinal cortex (EC). Granule cells are the principal neurons of the DG having an elliptical cell body (Mineur YS, et al., 2007) and are closely packed to form the granular cell layer of DG. Dentate granule cell form distinctive unmyelinated axons that project along the glutaminergic mossy fiber pathway to the pyramidal cells of dentate hilus and cornu ammonis 3 (CA3) of hippocampus. Together with the CA3 region of hippocampus, granule cells are involved in the formation of spatial memories pattern separation and rapid pattern completion (Fairhurst GD et al., 2011).

Like many other parts of the limbic system, amygdala has been considered for controlling various emotional and motivated behaviors. Aamygdal has emerged as the key forebrain structure mediating inborn and acquired emotional responses, as well as processing, interpreting, and integrating various aspects of biology and/or emotionally important information (Knapska E et al., 2007). Amygdaloid body is also involved in motor activities (Guarraci FA et al., 1999) and basic drives like eating and sexual behavior, cardiovascular and endocrine mechanisms, memory and other higher cognitive processes. It has been suggested that the amygdala may also mediate anxiety states (Davis M et al., 1999). Lesions affecting bilateral amygdale induce Kluver-Bucy syndrome with marked behavioral changes include visual agnosia, hypersexuality, hyperorality, a tendency to react to every visual stimulus and memory deficits (Hayman LA et al., 1998).

Stress is a well-known risk factor for neuropsychiatric disorders ranging from mood and anxiety, schizophrenia and attention deficit hyperactivity disorder. Personnel and animals differ to a great extent in their vulnerability to stress; even similar stressors can enhance risk for dissimilar neuropsychiatric states in different people (Yehuda R et al., 2007). Several imaging studies revealed abnormalities in blood flow and glucose metabolism in the limbic and prefrontal regions of brain under stress and depression (Drevets WC 2000, Manji HK et al., 2001). Excessive cortisol produced under stress causes damage to the arteries in the brain with a subsequent increased risk of stroke and damage to the brain regions by initiating apoptosis (Yusim A 2000). Stress exposure triggered fear memory, anxiety and decreased hippocampal-dependent memory performance (Chen Q et al., 2010, Ohl F et al., 1999). Studies have shown that chronic stress exposure resulted in decreased cell proliferation in the DG of hippocampus (Mineur YS et al., 2007, Quirk GJ et al., 2003). Stress exposure caused uncontrolled gamma-aminobutyric acid (GABA) signaling in the baso-lateral amygdala (BLA) complex of the amygdala, which contributed to the pathophysiology of affective disorders in the brain (Patel S et al., 2009).

In recent times environmental enrichtment (EE) exposure treatment is getting attention and is widely accepted as an alternative tool from the traditional medicines for managing fearfulness, unwanted behaviors and stress in laboratory animals (Fairhurst GD et al., 2011). EE is the addition of one or more ‘factors’ to a relatively impoverished environment in order to improve the physical/psychological and social welfare of the animals involved (Jiang HL et al., 2014). EE exposure with colorful objects of different shapes, sizes, textures and high contrast patterns are especially known for stimulating brains to the greatest extent possible and EE exposure is considered as a major therapeutic tool in treating stress and neurodegenerative diseases/disorders. EE cages are having large living space for facilitating more social interaction, and novel stimuli that together provide physical and cognitive stimulation (Rutebemberwa E et al., 2013). EE exposure is known to influence the CNS at the functional, anatomical and molecular level, both during the critical period and during adulthood (Baroncelli L et al., 2010). Increased cell proliferation in the DG of adult hippocampus was observed after treatment with EE exposure (Valero J et al., 2011). EE exposure restored the survival and differentiation of neurons in the hippocampus and prevented the depressive symptoms in chronically stressed rats (Veeja J et al., 2009) and even short duration of EE exposure for 10 days also increased the cell proliferation in the DG of diabetic mice (Beauquis J et al., 2010). 7 day period of enrichment increased Bromodeoxyuridine (BrdU) positive cell numbers in the amygdala and drastically reduced cell death (Okuda H et al., 2009).

Therapeutic effects of EE exposure have been documented on focal ischemia (Nygren J et al., 2005), Alzheimer’s (Rodriguez JJ et al., 2011) and Parkinson’s diseases (Steiner B et al., 2006). But the neuroprotective effect of EE on DG and amygdala in conditions of DM and stress is not well documented. Therefore, in this study, we aimed to experiment the therapeutic uses of EE exposure on improving the behavior and the neuroprotective nature of EE on DG and amygdala of DM as well as combined actions of DM and stressed brains.

**MATERIALS AND METHODS**

**Animals and groups**

Male albino rats (Wistar strain) of 5 weeks old were used. Animals were maintained under 12:12 hour dark: light environment, in the institutional animal house. All the animals were given water ad libitum, the standard pallet (Hindustan lever, India) was used for feeding.
Experiment was conducted in compliance with the guidelines and principles of the institutional animal ethics committee and the research protocol was permitted by the institutional animal ethics committee for experimental clearance. Animals were divided into Normal Control (NC), Vehicle Control (VC), Diabetes (DI), Diabetes+Stress (DI+S), Diabetes+ Environmental enrichment (DI+E), Diabetes+Stress+Environmental enrichment (DI+S+E) groups (n=8 in each group). NC group rats were continued in their home cage and VC group rats were fed with citric acid buffer solution (pH 4.5) as a vehicle.

**Experimental model of diabetes with STZ**

STZ solution (10 mg/ml) was prepared by dissolving STZ powder (Sigma, St, Louis Mo., USA), in ice-cold citrate buffer at pH 4.5. STZ solution was maintained in cold condition by keeping it in a tray of ice cubes to prevent decomposition of STZ by external temperature. Diabetes was induced in DI, DI+S, DI+E, DI+S+E groups with a single intra peritoneal injection of STZ at a dose of 40 mg /kg body weight. Rats were fed with 5% glucose solution to overcome drug-induced hypoglycemia after STZ injection.

**Blood glucose and body weight measurement**

To confirm and observe the consistency of diabetic status, the blood glucose levels and body weight of rats were measured on day 0 (before STZ injection), on day 2 after STZ injection, on day 15 and on day 30 (before sacrifice). Rat blood was collected from orbital plexus and glucose levels were estimated with the ‘Accu-Chek Advantage Glucose Monitor’. 60–130 mg/dl is the range of normal blood glucose level of rats. Rats having blood glucose levels above 225 mg/dl after the injection of STZ were considered as diabetic and were used for the experiment.

**Restraint stress protocol (Figure.1)**

![Figure 1. Photograph showing the animal in wire mesh restrainer cage](image)

The restraint stress practice was introduced on day 2 of STZ injection after confirming the diabetic status by measuring blood glucose levels. A wire mesh restrainer cage was prepared with 12 cm length, 5.5 cm height and 5.5 cm width. It has a wooden base and a stainless steel wire mesh restrainer provided with air holes for ventilation to the rat (McLaughlin KJ et al., 2007, Beauquis J et al., 2010). Rats in DI+S and DI+S+E groups were exposed for 6 hrs. of daily restraint stress from day 2 to day 30 of experiment. On completion of the stress session daily, rats were returned to their home cages. Rats were given access to food before the stress and after the stress sessions. After the final stress session (Day 30), rats were weighed and blood glucose levels were assessed to confirm that diabetic parameters had been maintained in both the groups.

**Enriched environment/ Environmental enrichment protocol (Figure.2)**

EE protocol was also introduced on day 2 of STZ injection after confirming the diabetic status. EE was created in a large wood cage (50 cm Length, 50 cm Width, 29 cm Height) that was filled with a variety of objects, including rotating wheels, plastic tubes and toys of different dimensions, which will allow the rats to explore and interact with different objects. The arrangement and types of items in the cage were different on each day in order to induce novel stimulation in rats (Beauquis J et al., 2010, Majno G et al., 1995). Rats in DI+E and DI+S+E groups were housed in EE cages daily for 6 hrs. until the end of the experiment (Day 30). Animals were placed in the enriched cages in groups of 3-4 rats and after EE exposure they were housed in standard cages.
Figure 2. Photograph showing the animals in enriched cages. The number and type of items in the cage were different and increased on each day in order to induce novel stimulation to rats

Two compartment passive avoidance test (Bures J et al., 1983)
The passive avoidance apparatus consists of a square box with a floor grid of 50 x 50 cm and wooden walls of 35 cm height and was illuminated by a 100 watt bulb. A smaller, darker compartment (15X15cm) with an electrified floor was connected to a constant current stimulator. The opening (6x6 cm) between the light and the dark compartments was closed with a plexiglass sliding door.

Test procedure: The experiment was carried out in three stages as follows:

Exploration
The rat was placed in the center of the box facing away from the entrance to the small compartment (dark). The animal was allowed to explore the apparatus (both compartments) for three minutes. Total time spent in the larger and smaller compartments and the number of crossings (from larger to smaller compartments and vice versa; a measure of exploratory behavior) was recorded. The experiment was repeated for each rat with an inter – trial interval of 5 minutes.

Learning (passive avoidance acquisition): After the last exploration trial, rat was placed in the smaller compartment and the entrance to larger compartment was closed. Three strong electric foot shocks (50 Hz, 1.5 mA 2 sec) with five seconds interval were applied. After five minutes of shock treatment, rat was placed in the larger compartment and allowed to explore both the compartments. As soon as the rat enters the compartment a strong foot shock was given and the rat was placed back in the home cage. Next trail was repeated with an interval of five minutes where the rat was again allowed to explore the compartment. The trial was ended by giving a foot shock as soon as it enters the dark compartment. The trial was repeated till the animals reach the “criterion” (stay in the larger compartment by suppressing the natural behavior) for more than three minutes. Number of trails taken by each animal to reach the criterion was recorded. The experiment was terminated if the animal remained for more than three minutes in the larger compartment in three successive trials. A maximum of ten trails was given for each animal.

Retention test
Retention was carried out over 24 hours of acquisition test. Rat was placed in the larger compartment and allowed to explore the compartments for three minutes. Time spent on the larger and smaller compartment and the number of crossings was recorded.

Cresyl violet staining protocol
On day 30 rats were sacrificed by perfusion method. Rats were anesthetized and the heart was exposed to pump 100 ml of heparinized saline in to the left ventricle through a cannula and the right atrium was cut open for the outflow of saline and blood. After saline pumping, transcardial perfusion of 300 ml of 4% paraformaldehyde (Prepared in 0.1M citrate buffer) was performed. Brains were post fixed for 48 hours in the paraformaldehyde solution and were processed through ascending grades of alcohols (50%-24 hrs, 70%-24 hrs, 90%-12 hrs, 100%- 12 hrs). Clearing of alcohol was done with the xylene for about 1-2 hours and paraffin blocks were made in an embedding bath.
Coronal sections of 5µ thickness were cut from the dorsal hippocampus using a rotary microtome (Leica RM 2245, Nussloch, Germany). Twenty five to thirty sections from each animal were mounted serially on air dried gelatinized slides and were immersed in xylene solution for 15 minutes and then in ascending grades of alcohols (absolute alcohol - 1 minute, 90 % alcohol – 2 minutes, 70 % alcohol – 2 minutes, 50 % alcohol – 2 minutes). Sections were dipped in distilled water for 10 minutes and were stained with 0.01% cresyl violet for 25–30 minutes at temperature 60°. Finally the sections were dipped in distilled water for 5 minutes and processed through ascending grades of alcohols (70 %, 90 % - 2 minutes each).

**Neuronal quantification (Figures. 3 & 4)**

Neuronal quantification was done at 400× magnification with a light microscope (Magnus, Olympus Pvt. Ltd. New Delhi, India). Slides from different groups were coded while counting to avoid manual bias. Both the suprapyramidal blade and infrapyramidal blade of DG were chosen for quantification, in the case of amygdala the lateral (LA) and basal nuclei (BL- baso lateral, BM-baso medial) were selected for quantification. A total of 50-60 fields per rat (Ten sections from each rat) were selected for quantification. The numbers of viable neurons having a clear nucleus were included and the neurons stained dark with fragmented nuclei, the shrunken cell body was excluded from quantification. The mean and SEM was derived with the average value of each section per group.

**Data analysis**

Results were analyzed using analysis of variance (ANOVA) with Bonferroni’s multiple comparison test as a post hoc test (Graph Pad Prism 2.01 software, Inc. USA). Values are expressed as mean ± SEM and a probability level of P<0.05 was used as the criterion for statistical significance expression.
RESULTS

Blood glucose levels (mg/dl) (Figure 5.)
On day 0 the blood glucose levels of rats in NC, VC, DI, DI+S, DI+E, DI+S+E were in normal range before inducing diabetes with STZ and there was no significance difference among them (Groups mean: 92.12). Blood glucose levels of NC and VC groups are stable and are in the normal range throughout the experimental period. On day 2 (After 2 days following the STZ injection) rats in DI, DI+S, DI+E, DI+S+E groups had become hyperglycemic (Blood glucose more than 250 mg/dl, $P<0.001$) and the blood glucose levels were significantly increased (Groups mean: 291.64) in comparison to NC (Mean: 94.25). On day 15 the blood glucose levels were elevated significantly ($P<0.001$) in all the experimental groups (DI, DI+S, DI+E, DI+S+E group) compared to NC group (91.17). On day 30 (Before sacrifice) blood glucose levels were significantly higher ($P<0.001$) in DI, DI+S, DI+E, DI+S+E group rats (Groups mean: 470.2) compared to NC rats (Mean: 93.33). Exposure to EE did not show any effect in reducing blood sugar levels in DI+E, DI+S+E rats.

![Blood glucose levels graph](Figure 5. Graph showing the blood glucose levels. NC vs VC: ns, NC vs DI: $***P<0.001$ (Days 2, 15 & 30), NC vs DI+S: $###P<0.001$ (Days 2, 15 & 30), DI vs DI+E: ns, DI+S vs DI+S+E: $bP<0.05$ (Day 15) $bbbP<0.001$(Day 30). One way ANOVA, Bonferroni's multiple comparison tests. Each data represents mean±SEM.)

Body weight (grams) (Figure. 6)
On day 0 and day 2 the body weight of rats in NC, VC, DI, DI+S, DI+E, DI+S+E groups were in a similar range (Groups mean: 92.93). There was no significance decrease in body weight was observed on day 2 (NC mean: 108.22 and DI, DI+S, DI+E, DI+S+E groups mean: 100.78). On day 15, a significant decrease ($P<0.001$) in body weight was observed in experimental groups (DI, DI+S, DI+E, DI+S+E, group mean 90.41) compared to NC group (144.33). On day 30 a highly significant decrease in body weight ($P<0.001$) was observed in DI, DI+S, DI+E, DI+S+E groups (Groups mean: 79.94) compared to NC (Mean: 218). EE exposure did not show any influence in gaining body weight of DI+E and DI+S+E rats.

Passive avoidance test (Figures. 7 & 8)
Diabetic rats housed in EE cages (DI+E, DI+S+E) showed higher memory retention scores compared to rats in DI, DI+S groups. On day 0 there was no significant difference between all the animal groups during exploration and retention phases. On day 15 during retention phase, rats in DI (332±7.27) and DI+S (378.3±15.91) groups spent significantly more time in the dark compartment compared to NC group (252.5±9.26) and DI+E (274.8±9.8), DI+S+E (301.7±5.01) groups spent significantly less time in the small compartment compared to DI and DI+S groups. On day 30 also rats in DI (355.±5.4) and DI+S (392.7±14.4) groups spent significantly more time in the small compartment compared to NC (257.8±7.9). DI+E (265.2±10.2), DI+S+E (288±7.9) groups spent significantly less time in the small compartment compared to DI and DI+S groups.
Figure 6. Graph showing the body weights. NC vs VC: ns, NC vs DI: *** $P<0.001$ (Days 15, & 30), NC vs DI+S: ### $P<0.001$ (Days 15, & 30), DI vs DI+E: ns, DI+S vs DI+S+E: ns. One way ANOVA, Bonferroni’s multiple comparison tests. Each data represents mean±SEM.

Figure 7. Passive avoidance-Exploration. Graph showing the time spent in small/dark compartment in exploration phase. NC vs VC: ns, NC vs DI: *** $P<0.001$ (Day 30), NC vs DI+S: * $P<0.05$ (Day 15) ### $P<0.001$ (Day 30), DI vs DI+E: ns, DI+S vs DI+S+E: bbb $P<0.001$ (Day 30). One way ANOVA, Bonferroni’s multiple comparison tests. Each data represents mean±SEM.

Figure 8. Passive avoidance-Retention. Graph showing the time spent in small/dark compartment in Retention phase. NC vs VC: ns, NC vs DI: *** $P<0.001$ (Days 15 & 30), NC vs DI+S: ### $P<0.001$ (Days 15 & 30), DI vs DI+E: ns, DI+S vs DI+S+E: bbb $P<0.001$ (Days 15 & 30). One way ANOVA, Bonferroni’s multiple comparison tests. Each data represents mean±SEM.
Effect of EE exposure on diabetic and stressed neurons of DG (Table 1 & Figure. 9)

Granule cells of DG exhibited significant and detectable changes under microscopic examination in DI, DI+S, DI+E, DI+S+E groups. Neurons in the NC group were well stained with cresyl violet, the appearance and density of surviving granule cells were normal under 400× light microscopy. The density of surviving cells was significantly decreased in DI and DI+S groups and on gross visual examination the granule cells of DI and DI+S revealed obvious damage and cell death. Granule cells of DI and DI+S groups, stained with cresyl violet are characterized by pyknotic nuclei and remodeling of chromatin material. The DI group exhibited a significant decrease in the mean number of surviving neurons (Cell death rate 38.43%, P<0.001) compared to NC. Granule cells in DI+S group also exhibited a highly significant decrease in the number of surviving neurons (Cell death rate 59.53%, P<0.001) compared to NC rats. Upon quantification rats in DI+E group showed a significant increase in the number of surviving neurons (Cell death rate 24.46%, P<0.001) compared to DI rats. DI+S+E group also showed significantly increased number of surviving neurons (Cell death rate 40.56%, P<0.001) in comparison to the DI+S group.

Table 1. Numerical cell density (Number of surviving neurons in 400µ area)

<table>
<thead>
<tr>
<th>Groups</th>
<th>DG</th>
<th>Lateral Nucleus (Amygdala)</th>
<th>Basal Nuclei (Amygdala)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>97.16±4.48</td>
<td>33.17±0.43</td>
<td>63.5±0.86</td>
</tr>
<tr>
<td>VC</td>
<td>96.30±4.89</td>
<td>34.73±0.49</td>
<td>61.45±1.29</td>
</tr>
<tr>
<td>DI</td>
<td>59.83±5.57***</td>
<td>20.07±0.76***</td>
<td>29.23±1.32***</td>
</tr>
<tr>
<td>DI+S</td>
<td>39.33±4.29***</td>
<td>11.27±0.79***</td>
<td>21.73±1.31***</td>
</tr>
<tr>
<td>DI+E</td>
<td>73.40±8.30aaa</td>
<td>27.03±0.67aaa</td>
<td>49.67±1.25aaa</td>
</tr>
<tr>
<td>DI+S+E</td>
<td>57.76±6.61aaa</td>
<td>19.17±0.82aaa</td>
<td>30.25±1.5aaa</td>
</tr>
<tr>
<td>P value</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>

Numerical cell density- DG & amygdala (Number of surviving neurons in 400µ area) : On day 30 of the experiment, DI had significantly less number of neurons compared to NC group. (ii) DI+E group had significantly more number of neurons compared to DI group. (ii) DI+S+E group had significantly more number of neurons compared to DI+S group. ns: not significant. NC vs VC: ns, NC vs DI: ***P<0.001, NC vs DI+S: **P<0.001, DI vs DI+E: *aa P<0.001, DI+S vs DI+S+E: *bb P<0.001. One way ANOVA, Bonferroni’s multiple comparison tests. Each data represents mean±SEM.

Effect of EE exposure on diabetic and stressed neurons of amygdala (Table. 1, Figures. 10 &11)

Quantification of type 1 neurons (Pyramidal neurons) of amygdala presented significant and measurable degenerative changes DI, DI+S, DI+E, DI+S+E groups. Neurons in both the amygdala nuclei (LA, BL & BM) of NC group were stained sufficiently with cresyl violet dye. They appeared normal and are densely packed with more number of surviving neurons under 400× light microscopy (lateral nucleus: 33.17±0.4365, basal nuclei: 63.5±0.866) Whereas the density and number of surviving neurons were significantly reduced in DI and DI+S groups and on gross visual examination, the neurons of DI and DI+S revealed obvious damage and cell death, characterized by pyknotic nuclei may be due to remodeling of chromatin material. Amygdala of DI group rats exhibited a significant decrease in the mean number of surviving neurons (Cell death - Lateral nucleus: 39.5%, basal nuclei: 54%) compared to NC. Neurons in DI+S group also exhibited a highly significant decrease in the number of surviving neurons (Cell death -lateral nucleus: 67%, basal nuclei: 66%) compared the NC rats.

A significant increase in the mean number of surviving neurons (Cell death-lateral nucleus: 27%, basal nuclei: 42%) was observed in the DI+E group upon quantification compared to DI rats. DI+S+E group also showed significantly increased number of surviving neurons (Cell death- lateral nucleus: 42%, basal nuclei: 29%) in comparison to DI+S group.
Figure 9: Photomicrograph of the dentate gyrus (400× magnification, cresyl violet stain) showing the effect of diabetes (DI) and combined actions of diabetes and stress (DI+S) on the neurons. Decreased number of surviving neurons (Marked with dark arrow, clear rounded cells having distinct nucleus) and cell shrinkage was observed in DI and DI+S groups compared to NC group. Enriched environment exposure resulted in increased number of surviving neurons in DI+E and DI+S+E groups compared to DI and DI+S group respectively. Bar 20 µm.

Figure 10: Photomicrograph of the amygdala (Lateral nucleus) (400× magnification, cresyl violet stain) showing the effect of diabetes (DI) and combined actions of diabetes and stress (DI+S) on the neurons. Decreased number of surviving neurons (Marked with dark arrow, clear rounded cells having distinct nucleus) and cell shrinkage was observed in DI and DI+S groups compared to NC group. Enriched environment exposure resulted in increased number of surviving neurons in DI+E and DI+S+E groups compared to DI and DI+S group respectively. Bar 20 µm.
DISCUSSION

Results from the passive avoidance experiment clearly indicate that the exposure to EE improved learning and memory abilities in the diabetic and stressed rats. Analysis of neurons in the DG of the hippocampus, basal and LA nuclei of amygdala exhibited significant cell death and decreased number of surviving neurons within 30 days of diabetic period compared to NC group. The degenerative changes in the amygdala neurons may lead to alterations in the anxiety responses, emotional and motivated behaviors. These results suggest that the neuronal cell bodies are sensitive to stressful conditions such as diabetes and support the previous findings that proved the STZ induced diabetes produced a dramatic decrease in cell proliferation in the DG of hippocampus and altered the hippocampal synaptic plasticity. The alterations in the neurons of DG may leads to deficits in learning and memory functions (Jackson-Guilford J et al., 2000).

The observed degenerative changes in neurons might be due to oxidative stress. Diabetes as well as hyperglycemia accelerates the formation of oxidative stress in the brain. Free radical causes cellular damage by initiating apoptotic changes such as cell shrinkage, nuclear chromatin condensation, chromatin fragmentation, nuclear membrane lysis and apoptotic body formation (Li PA et al., 2001, Britton M et al., 2003) in neurons and neuroglia. Apoptotic events can happen even at the early stages of diabetes (Dagon Y et al., 2007). In addition to oxidative stress, hyperglycemia may disrupt the blood brain barrier (BBB) by bringing structural changes in arteriovenous shunting, endothelial and perivascular edema, neurotransmitter movement in the micro vessels and alterations in pericytes and astrocytes to contribute for the neurological complications (Mooradian AD et al., 1997, Hawkins BT et al., 2008).
It is well known that hyperglycemia increases lipid peroxidation, which may contribute to long-term tissue damage (Bhor VM et al., 2004). Long term hyperglycemia in the rat brain will down regulate the glucose transporter (GLUT) expression, to prevent additional glucose entry into the brain cells, this may result in cellular injury (Hou WK et al., 2007).

Highly significant nissl damage was seen in diabetic rats exposed to stress. This may be due to excessive cortisol produced under stress causes damage to the arteries in the brain with a subsequent increased risk of stroke and damage to the brain regions by initiating apoptosis (Yusim A et al., 2000, Ohl F et al., 1999). The restraint stress paradigm used in our study is known to increase glucocorticoids (GC) levels in the brain. Excessive GCs secretion due to stress will initiate the neural damage by inhibiting the glucose transporter expression of neurons; to reduce the glucose utilization (McCall AL et al., 1995). GCs bring out changes in synaptic function and plasticity by binding to their associated receptors in hippocampus (Joels M et al., 2000). The structural damage observed in DG of DI+S rat brains may lead to memory deficits and decreased the hippocampal-dependent memory performance (Chen Q et al., 2010, Ohl F et al., 1999). Restraint stress exposure altered the genes of glutamate receptors in amygdala, and hippocampus. Increased amygdala excitatory postsynaptic signaling and altered metaplasticity were observed after repeated restraint stress exposure (Mozhui K et al., 2010). The neuronal damage seen in the DI+S rat amygdala may lead to failure in mediating anxiety states, sense of smell and pheromone-processing and emotional modulation of food intake (Ganong WF 2003, Pape HC & Pare, 2010).

In the present study, a significant improvement in learning and memory in the form of behavior pattern and the recovery/prevention of neuronal damage caused by diabetes and combined effects of diabetes and stress was observed after treatment with EE. EE exposure significantly prevented the amount of neurodegeneration caused by the diabetes and combined effects of diabetes and stress in terms of qualitative and quantitative parameters. EE exposure brings changes in transcriptional, translational and posttranslational actions which are crucial for neuronal structural modification (Huang FL et al., 2006). EE exposure enhances endothelial cell proliferation in CNS and increases capillary branching surface area and this activity may lead to improvement in cerebral blood flow. The disrupted BBB and blood flow due to diabetes as well as the combined effects of diabetes and stress might have been modified by the activation of vascular endothelial growth factor in response to EE exposure. The neuroprotective activity of EE by increasing the number of survived neurons in our study would have been achieved through activation of the transcription factor adenosine 3′,5′-cyclic monophosphate response element binding, which plays a crucial role in promoting survival of neurons in the DG (Walton M et al., 1999) and also EE exposure might have brought positive alterations in the levels of neurotrophic factors like brain derived neurotrophic factor (BDNF) for bringing vigorous changes to prevent the severity of neuronal damage (Ickes BR et al., 2000).

EE had might have brought down corticosterone levels which were higher due to diabetes and stress in pre-enrichment period. Increase in neurotransmitters levels like acetylcholine and increased expression of the gene for the serotonin1A receptor in EE treatment was observed in EE exposure (Rasmussen S et al., 1998). EE treatment might have increased the long term potentiation (LTP) in hippocampus, and amygdala for maintenance of synaptic plasticity (Foster TC et al., 1996, Gagne J et al., 1998) and BDNF for proximal processes growth of neurons (Kuczewski N et al., 2009). Treatment with EE had shown inhibition in the rate of natural apoptosis in the rat hippocampus by 45% (Young D et al., 1999) and EE treatment promoted survival of newly formed granule cells in the DG which was found to be associated with improved cognitive act (Kempermann G et al., 1998). Based on the observations discussed above and findings from our study, EE exposure treatment is an excellent and an alternative tool in treating learning and memory deficiets and neurodegenerative disorders/diseases. It is inexpensive and easy to create an enriched environment without modifying the maintenance of the animal’s facilities and in agreement to the previous findings our results suggest that, EE exposure had a potent neuroprotective activity.

CONCLUSION
From our observations uncontrolled diabetes as well as single housing stress procedure resulted in highly significant behavioral deficits and pathological changes in neurons of DG and amygdala. But upon EE exposure the memory retention was improved and degeneration of neurons was prevented significantly in DI and DI+S groups. In conclusion, EE exposure significantly prevented the neural insults of diabetes and stressed DG and amygdala brain areas; this can ameliorate cognitive impairment and other depressive disorders in the diabetic and stressed brains.

ACKNOWLEDGEMENTS
The authors wish to expresses sincere thanks to Manipal University (MU), Manipal, India for permitting to conduct the work and for providing the laboratory facilities to perform this work.

CONFLICT OF INTEREST
The authors declare no conflict of interest to report.
REFERENCES


