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Environmental enrichment preserves hippocampal neurons in diabetes and stressed rats

N. Pamidi[1], C.G. Yap[1] and S. Nayak[2]

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

Summary. This study evaluated the effect of Environmental Enrichment (EE) on neuron morphology in the CA1, CA3 and dentate hilus (DH) regions of the hippocampus by quantitating the total dendritic arborizations. EE is a potential intervention for stress and diabetes. It is capable of mitigating diabetes and stress-induced cognitive and memory deficit. Diabetes and stress were induced in male Wistar rats (4-5 weeks). Diabetic and stressed rats were exposed to EE on Day 2 post STZ injection and subsequently once daily for 30 days. All animals were sacrificed on Day 30. The hippocampus was dissected and processed for Golgi staining to quantitate dendritic arborizations at the CA1, CA2 and DH regions. Diabetes (D) and Diabetes+stress (D+S) groups had significantly fewer apical and basal dendritic branching points (ADBP, BDBP) at CA1 ($p<0.01$), CA3 ($p<0.001$) and DH ($p<0.001$) relative to control group (NC). Diabetes and stressed rats exposed to EE: [D+EE and D+S+EE groups] exhibited significantly denser ADBP and BDBP at all regions relative to D ($p<0.001$) and (D+S+EE) ($p<0.001$) groups respectively. EE significantly preserved neuronal arborizations in hippocampus of diabetic and stressed rats, suggesting a potential entity of diabetes and stress management.

Key words: Diabetes, Stress, Hippocampus, Dendritic branching

Introduction

Diabetes mellitus (DM) is a metabolic disorder preceded by the interplay of multiple risk factors. It is an epidemic globally, with an estimated 463 million people living with DM in 2019 and is predicted to rise to 10.2% by 2030 (Saeedi et al., 2019). Commonly, management of diabetes aims to achieve homeostatic glycemic control and prevent the onset of its complications. Diabetes is a systemic disease, therefore people with diabetes have an increased risk of developing co-morbidities commonly involving the cardiovascular system (heart and blood vessels), the kidneys, eyes, and the nerves. The rapidly increasing prevalence of DM globally is a public health concern largely due to the associated end-organ complications. Nevertheless, DM (both type 1 and type 2) is a significant risk factor for cognitive dysfunction. Diabetes is associated with a 60% increased risk of dementia (Zilliox et al., 2016) and Alzheimer's disease (AD) (Vagelatos and Eslick, 2013). Cognitive dysfunction commonly includes memory deficit, a decline in the abilities to formulate reasoning and communicate.

Cognitive processes are formulated and integrated at a region in the brain called the "hippocampus formation". Therefore, the hippocampus' main function in humans and animals is learning and memory (Anand and Dhikav, 2012). The hippocampus' normal physiological functioning depends on the optimal viability of the abundant pyramidal cells within the hippocampus formation (Amin et al., 2013). The pyramidal cells' role is to channel and disseminate neuronal information to and fro within the hippocampus formation and other parts of the brain. Hence, the pyramidal neurons' morphological structure is paramount for effective learning and memory in our daily life.

Neuronal cell damage due to hyperglycemia, has been demonstrated in both human and animal studies (Sonneville et al., 2012), consistent with the broad spectrum of catastrophic effects of a hyperglycemic milieu (Schaffer et al., 2012). In humans, cognitive dysfunction caused by degenerative brain disease is commonly managed using pharmacological interventions. The common therapeutic focus is on ameliorating the symptoms as well as delaying the rate of progressive damage. Cholinesterase inhibitors are commonly used to prevent memory loss and

Corresponding Author: Dr. Christina Gertrude Yap, Jeffrey Cheah School of Medicine and Health Sciences, No 1, Pesiaran Lagoon Selatan, Monash University Malaysia, 46150 Selangor, Kuala Lumpur, Malaysia. e-mail: christina.yap@monash.edu

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antipsychotics to control behavioral changes (Mendiola-Precoma et al., 2016). Currently, it is uncertain how rapidly neuronal injury occurs in diabetes. Hence, it is not practical to treat diabetic patients with pharmacological interventions as prophylactics for cognitive dysfunction.

In diabetes, the main goal in patient management is achieving and maintaining a normal glycemic level. Nevertheless, since the pathophysiology of diabetes leads to more than just hyperglycemia alone (Chilelli et al., 2013; Vasanth et al., 2017; Yu and Lyons, 2017), a holistic intervention approach is paramount to benefit the patient fully. Environmental enrichment (EE) (Coleman and Novak, 2017) is currently a popular concept that has evidently improved learning and memory in animal models (Jin et al., 2017; Yu and Lyons, 2017) and humans (Khan et al., 2016; McDonald et al., 2018). EE's neuroprotective effects to date were explored in randomized control studies, and the outcomes were measures of changes seen in cognitive scores (Khan et al., 2016; McDonald et al., 2018). Given the physiological significance of the morphology of pyramidal neurons in the hippocampus, to-date no study has demonstrated whether EE actually protected the morphology of neurons in diabetic and stress models, which enabled better cognitive functions in neurological disorders seen in reported animals and human studies. Therefore, our study aims to evaluate the effects of EE's on changes of pyramidal neuron morphology in the hippocampus of diabetic and stressed rats.

Materials and methods

Animals and experimental groups

5 week old Male albino rats (Wistar strain) were housed in a dark (12h) / light (12h) cycle at our animal holding facility. We allowed the animals to acclimatize for 7 days prior to the start of our experiments. After the acclimatization period we randomly grouped the rats into 6 experimental groups: Normal Control (NC), Vehicle Control (VC), Diabetes (D), Diabetes+Stress (D+S), Diabetes+Environmental enrichment (D+EE), Diabetes+Stress+Environmental enrichment (D+S+EE) groups (n=8). All the animals were allowed water ad libitum and fed with standard rat pellets (Hindustan lever, India). VC group rats were fed with citric acid buffer solution (pH 4.5) during the acclimatization period (7 days). Animal ethics clearance has been approved by the Institutional animal ethics committee (IAEC/KMC/07/2007–2008).

Experimental induction of diabetes with STZ

Diabetes was experimentally induced in D, (D+S), (D+EE), (D+S+EE) groups using Streptozotocin (STZ) (40 μg/g body weight). STZ induces the destruction of beta cells in the pancreas. Thereafter, diabetic rats were fed with 5% glucose solution to sustain STZ-induced hyperglycemia. 10 mg/ml STZ stock solution was prepared by dissolving STZ powder (Sigma, St, Louis Mo., USA) in ice-cold buffer (pH 4.5) and kept refrigerated until used.

Measurement of blood glucose and body weight

Blood glucose was quantitated to confirm the diabetic status of the experimental animals and ensure consistency of diabetic status throughout the experimental period. Blood samples were collected by aseptic venipuncture at the tail vein on Day 0 (before STZ injection), Day 2, Day 15, and Day 30 (before sacrifice). Random blood glucose levels were estimated using "Accu-Check Advantage Glucose Monitor". Normal reference limits for non-fasting blood glucose level in rats is 86-162 mg/dl (Grant et al., 2012). Blood glucose levels above 225 mg/dl post-STZ injection were classified as diabetes and were retained for this study. All rats were weighed on Day 2, Day 15 and Day 30.

Induction of restraint stress

Induction of restrained stress commenced on Day 2 post-STZ injection on rats in groups (D+S) and (D+S+EE). The stress induction cage was fabricated using stainless steel wire mesh and a wooden base with dimensions: 12 cm (l) x 5.5 cm (h) x 5.5 cm (w). Holes on the wire mesh provided ventilation for the experimental animals (Fig. 1). Rats in (D+S) and (D+S+EE) groups were exposed to 6 hours of restraint stress daily at a consistent time during the day from Day 2 till Day 30 of the experimental period. On completion of the daily stress session, rats from the (D+S) group were returned to their home cage while those from the

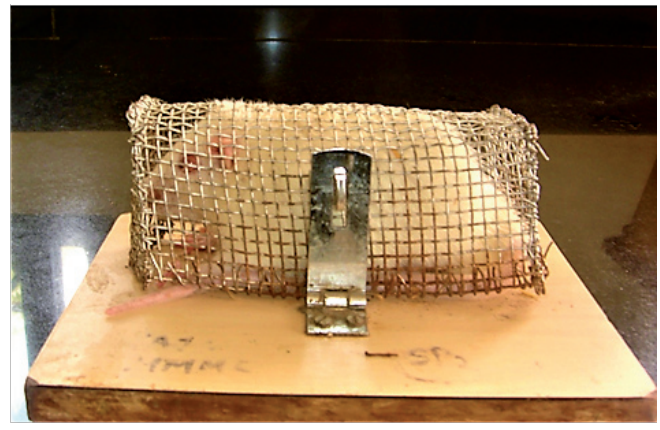

Fig. 1. Photograph of an experimental rat in the wire mesh restrainer cage. On Day 2 till Day 30 post STZ injection, rats in groups D+S and D+S+EE were placed individually in this cage at a consistent time for 6 hours per day to induce stress. D+S group will be returned into their normal housing cage after “restrained stress” sessions, while the D+S+EE group will be transferred into the EE cage for 6 hours before returning into their normal housing cage.

(D+S+EE) group were transferred into the environmental enrichment cages (Fig. 2) for a subsequent 6 hours before returning them into their home cage. Rats were given access to food before and after the experimental stress induction. After the final stress session (Day 30), the rats were weighed, and blood glucose levels were assessed to ensure that hyperglycemia had been maintained in both the groups.

Enriched environment (EE) as treatment for diabetic and stressed rats

EE exposure was introduced to rats in groups (D+EE) and (D+S+EE) on Day 2 post-STZ injection after confirming the diabetic status. An enriched environment (EE) was created in large wooden cages with dimensions: 50 cm Length (l) x 50 cm (w) x 29 cm (h) (Fig. 2). EE cages were fitted with various objects, such as rotating wheels, plastic tubes, and objects of different dimensions and colors which attracted the rats to explore and interact with different objects. The orientations and types of objects in the EE cages varied each day to stimulate the process of learning and memory in rats. Rats in (D+EE) and (D+S+EE) groups were housed in EE cages for 6 hours daily until the end of the experimental period (Day 30). Animals were placed in the enriched cages in groups of 3-4 rats per session. After EE exposure, they were returned into their home cages.

Rapid golgi staining

All rats were sacrificed on Day 30 by anesthetic (ether) overdose. The brains were dissected and processed for histological investigation of the morphology of neurons in the hippocampal region. Reagents were prepared fresh and tissues sections were processed according to Winer and Morant (1983).

Tracing of neuron dendritic branching using camera lucida

Dendritic branching was traced at 400x magnification using a Biolux research microscope with a camera lucida attached. Numbers of dendritic branching were manually counted on 10 well-strained neurons of sections from each rat in all experimental groups.

The Biolux microscope was modified to

Fig. 2. Photograph showing experimental rats in EE simulation cages. The orientation and types of items in each cage will be changed daily to induce cognitive stimulation. Day 2 till Day 30 post STZ injection rats in groups (D+EE) and (D+S+EE) will be placed in these cages (3-4 rats per cage) for 6 hours per day. After 6 hours of exposure to EE all rats were returned to their normal housing cages.

accommodate a wide mirror and a beam-splitting prism. To enhance visualization, a red light was aligned to the pencil tip as a guide to clearly visualize the dendritic branching through the prism in a dark room.

Selection of neurons

Neurons for dendritic quantification were selected according to standardized criteria:
1. Neurons must be confined to CA1, CA3 and dentate hilus regions of the hippocampus.
2. Neurons must be well stained and homogeneously impregnated with silver nitrate throughout all arborizations.
3. Only non-overlapping neurons were selected.
4. Dendritic quantification constitutes the total counts of: a) Dendritic intersections, b) Dendritic branching points.

Dendritic quantification

The counting process of dendritic intersections and branching points was done using the Scholl concentric circle (Fig. 3) as a guide (Khan et al., 2016). Measurements of the concentric circles were calibrated using a stage micrometer at 400x magnification. Circles were drawn on a transparent sheet with a radial distance of 20 µm between two adjacent concentric circles. Traced neurons (as described in subsection 2.7) were placed on the concentric circles with the neuron's cell body in the center of the innermost circle.

The numbers of branching points between the two adjacent concentric circles and the total number of branching points were counted. The dendritic intersection is the point where a dendrite touches or intersects the given concentric circle. Both branching points and intersections of the total dendritic tree were counted from the center of the soma. The apical dendritic intersections, apical branching points, basal dendritic intersections and basal branching points were recorded.

Data analysis

Results were analyzed using the analysis of variance (ANOVA) statistics followed by the Bonferroni's post

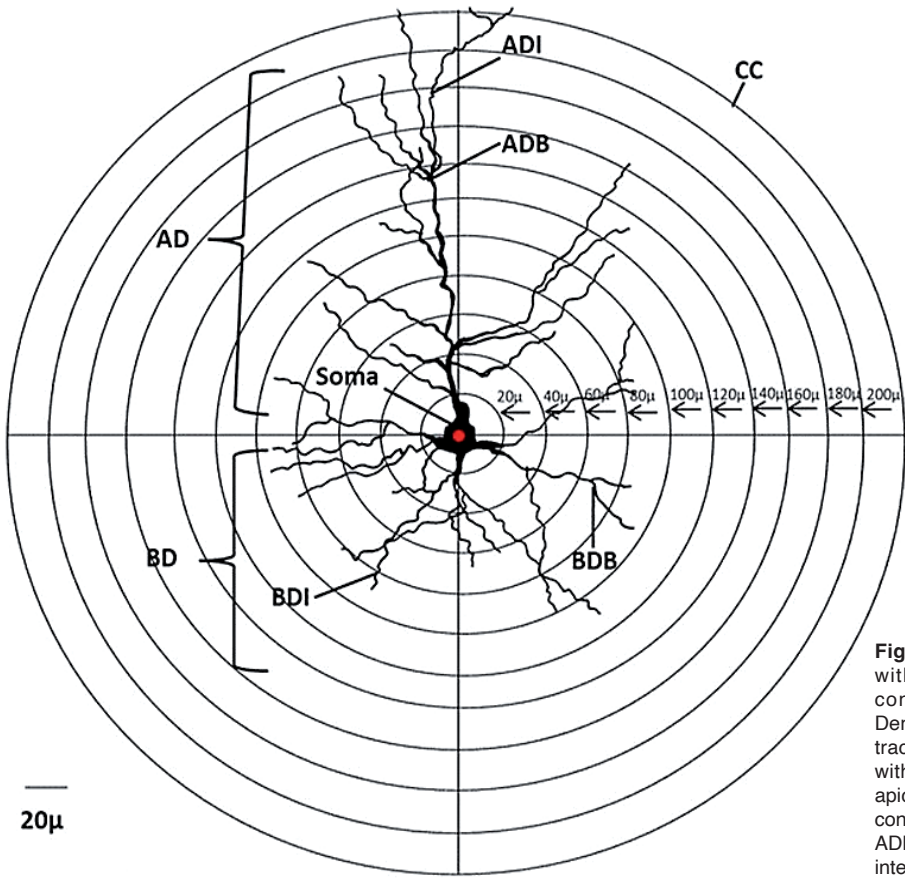

Fig. 3. Scholl concentric rings. Concentric circles with the radial distance between two adjacent concentric circles being equivalent to 20 μm. Dendritic quantification is performed by placing the traced neuron, aligning the center of the cell body with the center of concentric circles as seen. AD, apical branching; BD, basal dendritic branching; CC, concentric circles; ADI, Apical dendritic intersection; ADB, Apical dendritic branching; BDI, Basal dendritic intersection; BDB, Basal dendritic branching.

hoc test for multiple comparison. (Graph Pad Prism 2.01 software, Inc. USA). Values are expressed as mean ± SEM. Total counts of arborizations in test groups [D, (D+S), (D+EE), (D+S+EE)] were compared to the control group (NC). A significant difference is reported when $p<0.05$.

Results

Blood glucose levels (mg/dl)

The baseline blood glucose level at Day 0 was 92.12 mg/dl (Mean of all experimental groups). No significant difference was seen between all groups [NC, VC, (D+S), (D+EE), (D+S+EE)]. Rats in NC and VC groups had consistent blood glucose levels, and all were within normal reference limits throughout the experimental period. Two days post STZ injection (Day 2) rats in D, (D+S), (D+EE), (D+S+EE) groups had developed hyperglycemia (Blood glucose>250 mg/dl, $p<0.001$) and blood glucose levels were significantly increased (Group mean=291.64 mg/dl) relative to NC rats (Group mean=94.25 mg/dl). On Day 15 blood glucose levels were significantly increased ($p<0.001$) in all the experimental groups [D, (D+S), (D+EE), (D+S+EE)] groups (Group mean=374.95 mg/dl) relative to NC (Mean=91.17 mg/dl). On Day 30 (Before sacrificing) blood glucose levels were significantly high ($p<0.001$) in D, (D+S), (D+EE) and (D+S+EE) group (Group mean=470.2 mg/dl) relative to NC rats (Mean=93.33 mg/dl). Exposure to EE did not show any effect in reducing blood sugar levels in rats in (D+EE) and (D+S+EE) groups (Table 1).

Body weight (g)

On Day 0, there were no significant differences in body weight of rats in all experimental groups [NC, VC, D, (D+S), (D+EE), (D+S+EE)] groups; Groups mean=92.93 g on Day 0 ($p>0.05$) and Day 2 ($p>0.05$). Mean body weight for the NC group was 108.22 g, and groups mean for all diabetes groups [D, (D+S), (D+EE), (D+S+EE)] was 100.78 g. On Day 30, a significant decrease in body weight ($p<0.001$) was seen in D, (D+S), (D+EE) and (D+S+EE) groups (Groups mean=79.94 g) compared to NC (Mean=218 g). However, the EE treated groups (D+EE; D+S+EE)) exhibited significantly less (D+EE vs. D: $p<0.001$; D+S+EE vs. D+S: $p<0.001$) weight reduction relative to groups that were not exposed to EE (D and D+S) (Table 2).

Table 1. Blood glucose levels (mg/dl) in experimental groups.

Groups	Day 0 (mg/dl)	Day 2 (mg/dl)	Day 30 (mg/dl)
Normal Control (NC, n=6)	95.17±2.023	99.33±3.18	91.17±1.249
Vehicle Control (VC, n=6)	91.67±1.892	94.5±3.041	95.5±2.553
Diabetes (D, n=6)	96.33±2.789	295.5±19.51***	389.5±41.56***
Diabtes+Stress (D+S, n=6)	99.5±2.156	321.5±26.43###	405.7±28.51###
Diabetes+Environmental Enrichment (D+EE, n=6)	93.33±1.542	276.8±19.67aaa	372.8±31.79aaa
Diabetes+Stress+Environmental Enrichment (D+S+EE, n=6)	96.5±3.519	289.8±11.06bbb	463.8±32.23bbb

Data represents mean ± SEM. Statistical analysis was done using the one-way ANOVA with Bonferroni's multiple comparison tests. Blood glucose levels in D, (D+S), (D+S+EE) groups were significantly higher relative to NC group on Day 2 ($p<0.0001$) and Day 30 ($p<0.0001$). NC vs VC: ns, NC vs D: ***$p<0.0001$; NC vs (D+S): ###$p<0.0001$; D vs (D+EE): aaa$p<0.0001$; (D+S) vs D+S+EE: bbb$p<0.0001$. ns=non-significant; n=number of rats per group.

Table 2. Body weight (g) of experimental groups.

Groups	Day 0 (g)	Day 2 (g)	Day 30 (g)
Normal Control (NC, n=8)	98.33±3.303	117.2±1.014	220.3±6.059
Vehicle Control (VC, n=8)	96.83±2.574	113.2±1.973	218.5±5.169
Diabetes (D, n=8)	97.5±3.433	107.8±3.859	82.83±7.534*
Diabtes+Stress (D+S, n=8)	102.3±2.155	112.2±1.887	58.17±4.679**
Diabetes+Environmental Enrichment (D+EE, n=8)	99.83±1.222	109.8±2.626	86±4.442***
Diabetes+Stress+Environmental Enrichment (D+S+EE, n=8)	93±1.438	108.7±3.93	64.67±5.044****

Changes in body weight with and without exposure to EE. Data presented represents mean ±SEM. One-way ANOVA with Bonferroni's multiple comparison tests demonstrated no significant reduction in body weight in D, (D+S), (D+S+EE) groups relative to NC group respectively on Day 30. NC vs VC: ns; D vs NC: *$p<0.0001$; (D+S) vs NC: **$p<0.0001$; (D+EE) vs D: ***$p<0.0001$; (D+S+EE) vs (D+S): ****$p<0.0001$. EE showed prevention of body weight gain in STZ induced diabetic and stressed rats. ns=non-significant; vs=versus; n=number of experimental animals per group.

Dendritic morphology at hippocampal CA1 region after exposure to EE

Dendritic intersections

Apical dendritic intersections. Apical dendritic intersections were significantly decreased in D group rats at 40 µm ($p<0.001$), 60 µm ($p<0.001$), 80 µm ($p<0.001$), 100 µm ($p<0.001$), 120 µm ($p<0.001$), 140 µm ($p<0.001$) and 200 µm ($p<0.001$) circles relative to the NC group. Rats in (D+S) group exhibited a significant decrease in the apical dendritic intersections at 40 µm ($p<0.01$), 60, 80, 100, 120, 140 µm ($p<0.01$ respectively), 160 µm ($p<0.05$), 180 µm ($p<0.05$) and at 200 µm ($p<0.001$) circles relative to NC. Group (D+EE) rats after being exposed to the enriched environment (EE) had exhibited significantly increased numbers of interactions at 40 µm ($p<0.05$), 60 µm ($p<0.001$), 80 µm ($p<0.001$), 100, 120, 140 µm ($p<0.01$respectively), 160µm ($p<0.01$), and at 200 µm ($p<0.01$) circles in comparison to the D group rats. (D+S+EE) rats also showed a significant increase in the apical dendritic intersections at 40 µm ($p<0.05$), 60 µm, 80 µm, 100 µm, 120 µm ($p<0.01$), 140 µm ($p<0.05$), and at 200 µm circles ($p<0.01$) relative to rats in the (D+S) group.

Basal dendritic intersections. Rats in the D group exhibited a significant decrease in the basal dendritic intersections at the 40 µm, 60 µm, 80 µm, 100 µm circles ($p<0.001$) relative to the NC. (D+S) group rats exhibited significant decrease in the basal dendritic intersections at the 20 µm ($p<0.01$), 60 µm, 80 µm and 100 µm circles ($p<0.001$) relative to the NC group. (D+EE) rats showed a significant increase in the basal dendritic intersections at 20 µm ($p<0.05$), 60 µm, 80 µm ($p<0.01$) and 100 µm ($p<0.001$) circles relative to rats in the D group. (D+S+EE) rats also exhibited a significant increase in the basal dendritic intersections at the 40 µm ($p<0.05$), 60 µm, 80 µm ($p<0.001$) and at 100 µm ($p<0.01$) circles relative to rats in the (D+S) group.

Dendritic branching points

Apical dendritic branching points. Pyramidal neurons in the hippocampus of the D group rats exhibited a significant decrease in the numbers of apical dendritic branching points at the 60-80 µm ($p<0.01$), 80-100 µm, 100-120 µm ($p<0.001$), 120-140 µm ($p<0.05$), 140-160 µm ($p<0.01$) and 180-200 µm ($p<0.001$) zones relative to rats in the NC group. Also, significant reductions in the apical dendritic branching points at the 20-40 µm ($p<0.05$), 60-80 µm, 80-100 µm, 100-120 µm ($p<0.001$), 120-140 µm ($p<0.01$), 140-160 µm ($p<0.001$), 160-180 µm ($p<0.05$), and 180-200 µm ($p<0.001$) zones were observed in (D+S) group rats relative to NC rats. On the contrary, (D+EE) group rats exhibited a significant increase in the apical dendritic branching points at the 60-80 µm ($p<0.05$), 80-100 µm, 100-120 µm, 140-160 µm ($p<0.01$), 160-180 ($p<0.05$) and 180-200 µm ($p<0.001$) zones relative to the D group rats. At 60-80 µm ($p<0.05$), 80-100 µm, 100-120 µm, 140-160 µm ($p<0.01$), 160-180 µm ($p<0.05$) and 180-200 µm ($p<0.001$) zones (D+S+EE) rats had a significant increase in the apical dendritic branching

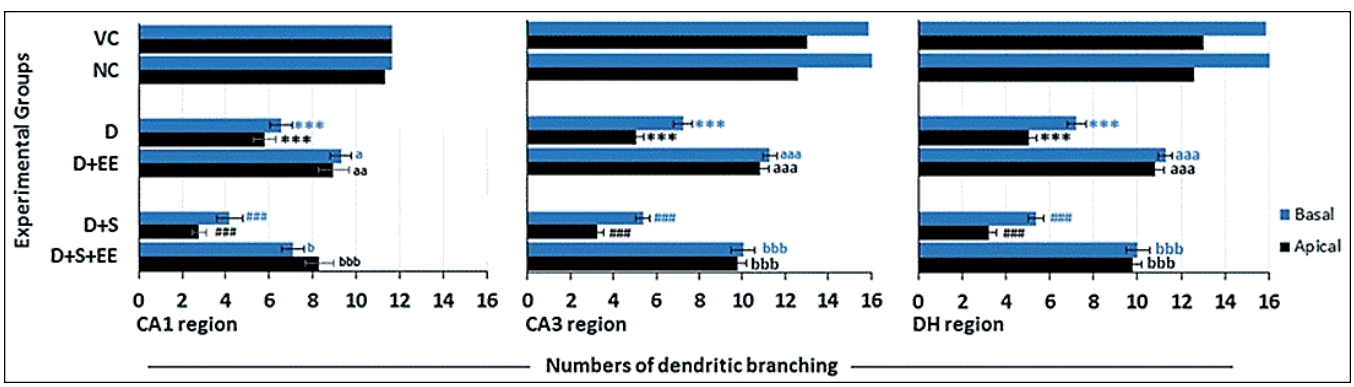

Fig. 4. Total dendritic branching at CA1, CA3 and DH regions of hippocampus in diabetic and stressed diabetic rats. All data were analyzed using one-way ANOVA and Bonferroni's multiple comparison tests. Data are presented as mean±SEM. Each bar represents total numbers of apical (black bars) and basal (blue bars) dendritic branching points on neurons at the CA1, CA3 and DH regions of rat hippocampus. Hippocampus tissues were dissected from all animals on Day 30 of the experimental schedule, and processed for histological examination. Diabetes and non-diabetes stressed rats (D and S groups) had significantly lower numbers of apical and basal dendritic branching points at CA1, CA3 and DH hippocampal regions with reference to the normal control group (NC). Diabetic rats exposed to environmental enrichment (D+EE) had significantly higher numbers of apical and basal dendritic branching points on neurons at the CA1, CA3 and DH regions of hippocampus compared to D group (unexposed to EE). Diabetic + Stress + Environmental Enrichment (D+S+EE) group also had significantly higher numbers of apical and basal dendritic branching points at all three regions of rat of hippocampus compared to Diabetic + Stress (D+S) group. CA1 region statistical analysis: NC vs VC = ns (ns=non-significant); NC vs D = ***$p<0.001$; NC vs (D+S) = ##$p<0.01$; D vs (D+EE) = aap<0.01; (D+S) vs (D+S+EE) = bbbp<0.001. CA3 region statistical analysis: NC vs VC = ns; NC vs D = ***$p<0.001$; NC vs (D+S) = ###$p<0.001$; D vs (D+EE) = aaap<0.001; (D+S) vs (D+S+EE) = bbbp<0.001. DH region statistical analysis: NC vs VC = ns; NC vs D = ***$p<0.00$; NC vs (D+S) = ###$p<0.001$; D vs (D+EE) = aaap<0.001; (D+S) vs (D+S+EE) = bbbp<0.001.

points relative to (D+S) group (Fig. 4).

Basal dendritic branching points. Basal dendritic branching points were significantly reduced in D group rats at 20-40 μm ($p<0.001$), 40-60 μm ($p<0.05$), 60-80 μm and 80-100 μm ($p<0.001$) zones relative to the rats in the NC group. (D+S) group also demonstrated significantly fewer basal dendritic branching points at the 20-40 μm ($p<0.001$), 40-60 μm, 60-80 μm, 80-100 ($p<0.001$) zones relative to NC. On the contrary, significantly increased basal dendritic branching points were observed at the 20-40 μm ($p<0.001$), 60-80 μm, and 80-100 μm ($p<0.01$) zones in the CA1 hippocampus region of the (D+E) group relative to the D group. Similarly, significantly more basal dendritic branching points were observed at 20-40 μm ($p<0.001$), 60-80 μm ($p<0.05$), 80-100 μm ($p<0.01$), zones in group (D+S+EE) rats relative to the (D+S) group.

Total number of dendritic branching points on neurons at the CA1 region

Overall, groups D and (D+S) demonstrated significantly fewer total apical ($p<0.001$, $p<0.01$ respectively) and basal ($p<0.001$) dendritic branching points relative to the NC group. (D+EE) and (D+S+EE) groups demonstrated significantly higher numbers of total apical ($p<0.01$, $p<0.001$respectively) and basal ($p<0.05$) dendritic branching points at the CA1 region of rat hippocampus relative to D and (D+S) groups respectively (Fig. 4).

Dendritic morphology at hippocampal CA3 region after exposure to EE

Dendritic intersections

Apical dendritic intersections. The numbers of apical dendritic intersections were significantly reduced at 40 μm ($p<0.05$), 60 μm ($p<0.01$), 80 μm ($p<0.001$), 100 μm ($p<0.01$), 120 μm ($p<0.01$), 140 μm ($p<0.001$) and 160 μm ($p<0.001$) circles in D group rats relative to NC group. Rats in (D+S) group exhibited a significant reduction in the number of apical dendritic intersections at 40 μm ($p<0.05$), 60 μm ($p<0.01$), 80 μm, 100 μm, 120 μm, 140 μm, 160 μm ($p<0.001$) circles relative to rats in the NC group. Interestingly, (D+EE) group of rats demonstrated significantly more apical dendritic intersections at 40 μm, 60 μm ($p<0.05$), 80 μm ($p<0.01$) 100, 120, 140 ($p<0.05$), and 160 μm ($p<0.001$) circles relative to rats in D group. Similarly, (D+S+EE) group of rats also demonstrated significantly more apical dendritic intersections at 60 μm ($p<0.01$), 80 μm, 100 μm ($p<0.001$), 120 μm ($p<0.05$), 140 μm ($p<0.001$) and 160 μm ($p<0.001$) circles relative to the rats from the (D+S) group.

Basal dendritic intersections. Numbers of basal dendritic intersections in rats from Group D exhibited significantly fewer basal dendritic intersections at 40 μm, 60 μm, 80 μm and 100 μm circles ($p<0.001$) relative to the rats in NC group. Similarly, (D+S) group rats exhibited a significant decrease in the basal dendritic intersections at the 40 μm, 60 μm, 80 μm,100 μm circles ($p<0.001$) relative to the NC rats. After exposure to enriched environment, interestingly, group (D+EE) rats had a significant increase in the basal dendritic intersections at the 40 μm ($p<0.05$), 60 μm ($p<0.001$), 80 μm ($p<0.01$), 100 μm ($p<0.001$) circles relative to group D rats. (D+S+EE) group rats also exhibited significantly increased numbers of basal dendritic intersections at 40 μm, 60 μm, 80 μm ($p<0.01$), 100 μm ($p<0.001$) circles relative to the (D+S) group rats.

Dendritic branching points

Apical dendritic branching points. Significantly reduced numbers of apical dendritic branching points were observed at the 40-60 μm ($p<0.01$), 60-80 μm, 80-100 μm,100-120 μm, 120-140 μm, 140-160 μm ($p<0.001$) circle zones in D group rats. A similar reduction in numbers of apical dendritic branching points were observed in the (D+S) group at 20-40 μm ($p<0.01$), 60-80 μm, 80-100 μm, 100-120 μm, 120-140 μm, 140-160 μm ($p<0.001$) circle zones relative to NC group. Similar to observations in the CA1 hippocampal region, after therapeutic exposure to EE the neurons in the CA2 region of (D+E) group rats also exhibited a significant increase in numbers of apical dendritic branching points at the 40-60 μm ($p<0.05$), 60-80 μm ($p<0.01$), 80-100 μm ($p<0.05$), 100-120 μm ($p<0.01$), 120-140 μm, 140-160 μm ($p<0.001$) circle zones relative to D group rats. The same outcome was observed in the (D+S+EE) group rats. A significant abundance of the apical dendritic branching points was seen at 20-40 μm ($p<0.05$), 40-60 μm, 60-80 μm ($p<0.01$), 80-100 μm ($p<0.001$), 100-120 μm, 140-160 μm ($p<0.01$) circle zones relative to the (D+S) group.

Basal dendritic branching points. Significantly reduced basal dendritic branching points were observed at the 20-40 μm, 40-60 μm, 60-80 μm, 80-100 ($p<0.001$) circle zones relative to NC group. Reduced numbers of dendritic branching points were observed in the (D+S) group rats at the 0-20 μm ($p<0.05$), 20-40 μm, 40-60 μm, 60-80 μm, 80-100 μm ($p<0.001$) circle zones relative to the rats in the NC group. (D+EE) group demonstrated a significant increase in the basal dendritic branching points at 20-40 μm ($p<0.05$), 60-80 μm ($p<0.001$), and 80-100 μm ($p<0.01$) circle zones relative to D group rats. A significant increase in the basal dendritic branching points at 0-20 μm ($p<0.01$), 20-40 μm ($p<0.05$), 60-80 μm ($p<0.001$), 80-100 μm ($p<0.001$) circle zones were seen in D+S+EE group relative to (D+S) group.

Total number of dendritic branching points at CA3 hippocampal region

Overall, diabetes significantly reduced the quantity

of dendritic branching points on neurons at the CA3 hippocampal region. When diabetic rats were subjected to restraint stress, a significant further reduction of dendritic branching points was observed. D, (D+S) group rats demonstrated a significant reduction of total apical ($p<0.001$) and basal ($p<0.001$) dendritic branching points relative to the NC groups. EE treatment significantly demonstrated neuroprotective effects on diabetic and stressed rats. (D+EE), (D+S+EE) groups exhibited a significant increase in total apical ($p<0.001$) and basal ($p<0.001$) dendritic branching points relative to D, (D+S) groups respectively (Fig. 4).

Dendritic morphology at hippocampal dentate hilus (DH) region after exposure to EE

Dendritic intersections

Apical dendritic intersections. At the DH (CA4) region of rat hippocampus, apical dendritic intersections

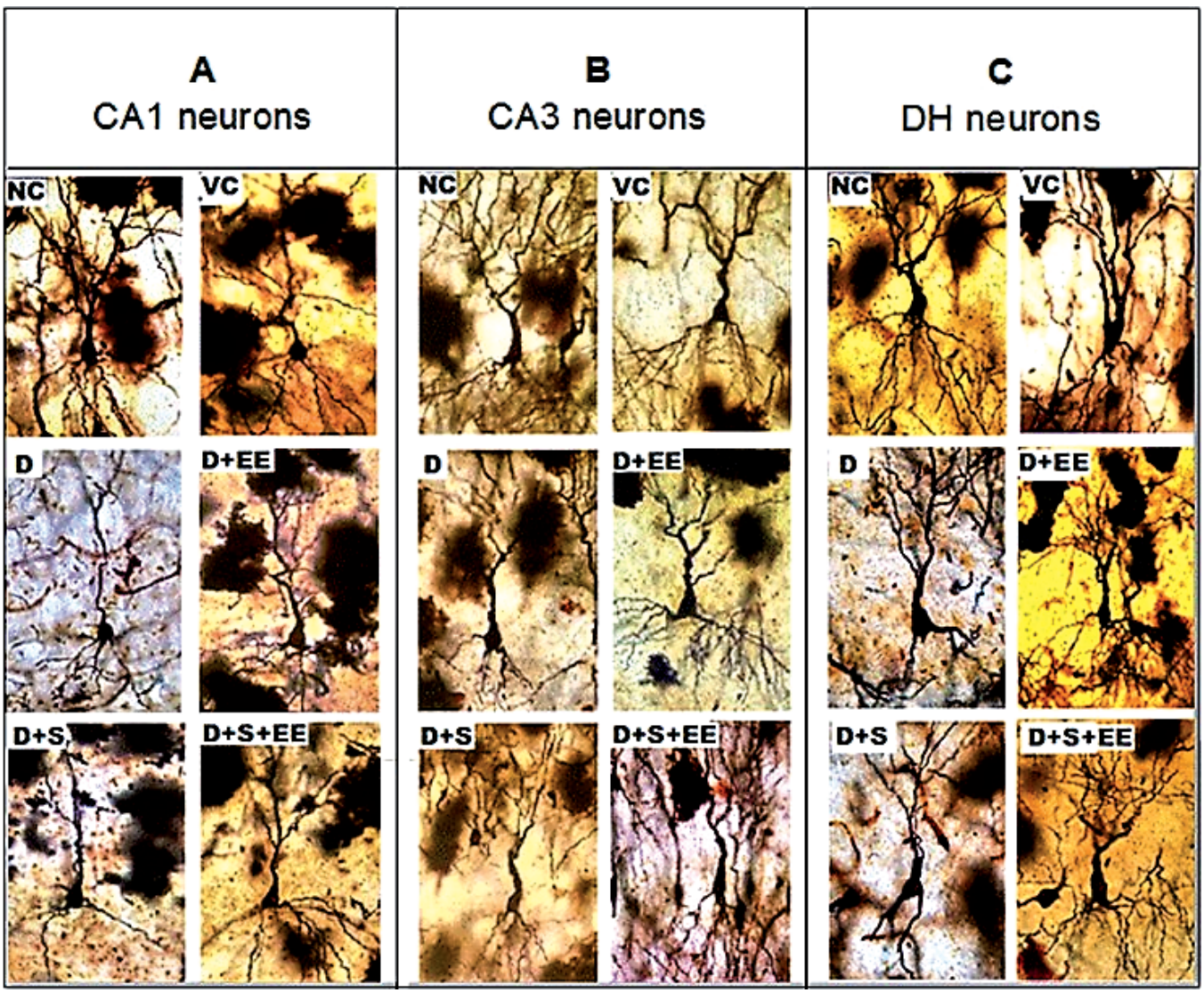

Fig. 5. Morphological evidence of dendritic branching densities on neurons at CA1, CA3 and DH regions of hippocampus from diabetes and stressed rats exposed to environmental enrichment. Photomicrographs of histological sections stained with the rapid Golgi staining protocol. Panels A, B and C exhibit a representative stained neuron at the cornu ammonis 1 and 3 (CA1, CA3) and Dentate Hilus (DH) regions of the hippocampus respectively. Visual analysis, manual quantitation, followed by statistical analysis demonstrated that dendritic arborizations on neurons from CA1, CA3 and DH were significantly decreased in the diabetes (D) and diabetes plus stress (D+S) groups relative to the normal control group (NC), ($p<0.001$). Significantly increased dendritic arborizations were seen in diabetes rats and diabetes plus stress rats which were exposed to environmental enrichment [(D+EE) and (D+S+EE) groups] in comparison to the D and (D+S) groups respectively. CA1 region statistical analysis: NC vs VC = ns (VC=Vehicle control; ns=non-significant); NC vs D = $p<0.001$; NC vs (D+S) = $p<0.01$; D vs (D+EE) = $p<0.01$; (D+S) vs (D+S+EE) = $p<0.001$. CA3 region statistical analysis: NC vs VC = ns; NC vs D = $p<0.001$; NC vs (D+S) = $p<0.001$; D vs (D+EE) = $p<0.001$; (D+S) vs (D+S+EE) = $p<0.001$. DH region statistical analysis: NC vs VC = ns; NC vs D = $p<0.001$; NC vs (D+S) = $p<0.001$; D vs (D+EE) = $p<0.001$; (D+S) vs (D+S+EE) = $p<0.001$. No significant difference in the quantity of dendritic arborizations was seen between the Normal Control (NC) and Vehicle Control (VC) groups. EE = environmental enrichment.

were significantly reduced in D group at 40 µm ($p<0.05$), 60 µm, 80 µm ($p<0.001$), 100 µm ($p<0.05$), 120 µm, 140 µm ($p<0.001$) circles and at 40 µm, 60 µm ($p<0.01$), 80 µm, 100 µm, 120 µm, 140 µm ($p<0.001$) circles in (D+S) group relative to the NC group. (D+EE) rats demonstrated a significant increase in apical dendritic intersections at 60 µm ($p<0.01$), 80 µm ($p<0.001$), 120 µm ($p<0.01$), 140 µm ($p<0.001$) circles relative to D group rats. A significant increase in apical dendritic intersections was also seen in rats from (D+S+EE) group at 80 µm ($p<0.01$), 140 µm ($p<0.01$) circled in the apical dendritic intersections relative to (D+S) group rats.

Basal dendritic intersections. Similar to the CA1 and CA3 hippocampal regions, DH region demonstrated significantly reduced ($p<0.001$) basal dendritic intersections at the 20 µm, 40 µm, 80 µm, 100 µm, 120 µm, 140 µm circles in D group rats relative to the NC group. A significant reduction in the basal dendritic intersections was exhibited in the (D+S) group rats at the 20 µm, 40 µm, 80 µm, 100 µm, 120 µm, 140 µm circles ($p<0.001$) relative to the NC group. On the other hand, after exposure to EE, (D+EE) group rats exhibited a significant increase in quantity of basal dendritic intersections at 20 µm ($p<0.05$), 40 µm ($p<0.001$), 80 µm ($p<0.001$), 100 µm ($p<0.01$), 120 µm, 140 µm ($p<0.001$) circles relative to the D group rats. (D+S+EE) group rats also exhibited a significant increase in the basal dendritic intersections at 20 µm ($p<0.05$), 40 µm ($p<0.01$), 60 µm ($p<0.05$), 80 µm, 100 µm ($p<0.001$) 120 µm, 140 µm ($p<0.01$) circles relative to the (D+S) group.

Dendritic branching points

Apical dendritic branching points. Apical dendritic branching points were significantly reduced in D group rats at 20-40 µm ($p< 0.001$), 40-60 µm ($p<0.01$), 60-80 µm, 100-120 µm, 120-140 µm, 140-160 µm ($p<0.001$) circle zones. A similar increase in apical dendritic branching points was seen (D+S) group and at 20-40 µm, 40-60 µm, 60-80 µm ($p<0.001$), 80-100 µm ($p<0.05$), 100-120 µm, 120-140 µm ($p<0.001$) circle zones relative to NC groups. (D+EE) group rats exhibited significantly increased quantities of apical dendritic branching points at 20-40 µm, 40-60 µm ($p<0.001$), 60-80 µm ($p<0.001$), 100-120 µm, 120-140 µm ($p<0.01$) circle zones relative to D group rats. (D+S+EE) group rats also exhibited significantly higher quantities of the apical dendritic branching points at 40-60 µm ($p<0.05$), 60-80 µm ($p<0.01$) circle

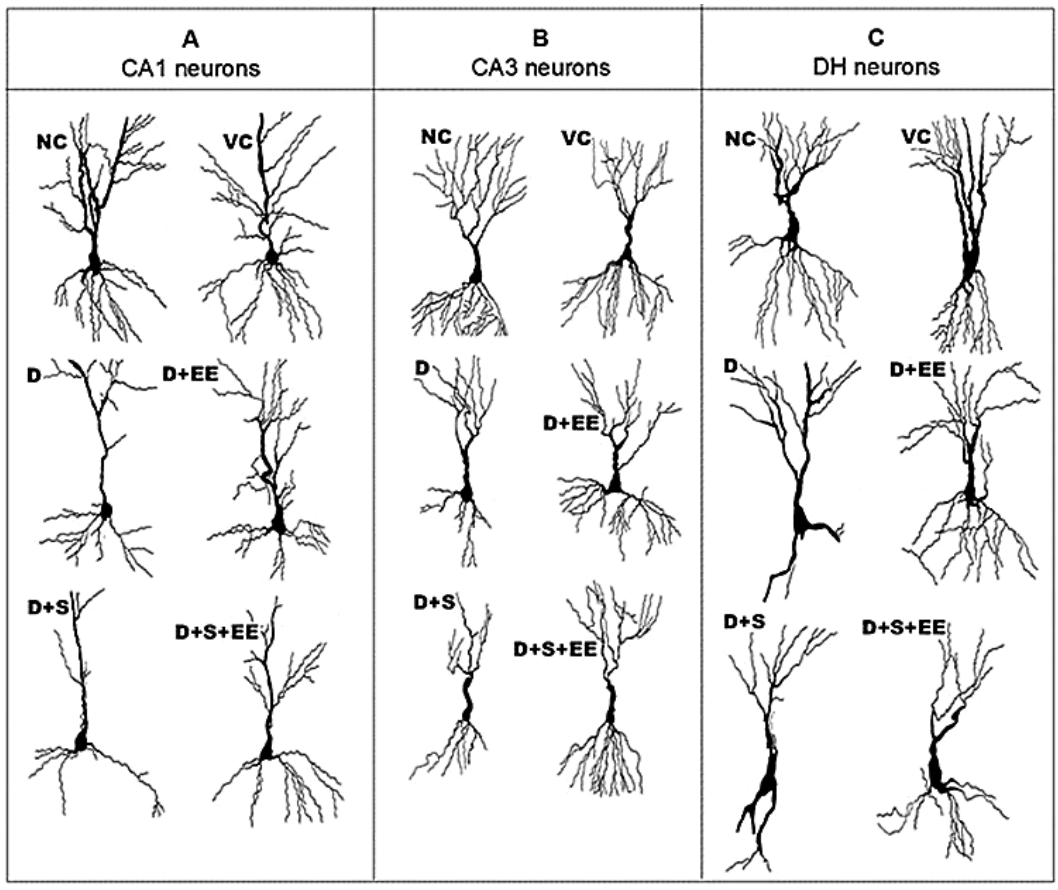

Fig. 6. Camera Lucida aided tracings of neuronal arborizations. NC, Normal control; VC, Vehicle control D=Diabetic; (D+S), Diabetes+Stress; (D+EE), Diabetes Environmental Enrichment; (D+S+EE), Diabetes+Stress+ Environmental Enrichment. Significantly decreased dendritic arborizations were observed in D and (D+S) groups relative to NC. Dendritic arborizations significantly increased in groups exposed to environmental enrichment. D vs. (D+EE), $p<0.001$; (D+S) vs. (D+S+EE), $p<0.001$. No significant difference was seen between NC and VC groups.

zones relative to (D+S) group (Fig. 4).

Basal dendritic branching points. (D) group rats exhibited a significant reduction in quantity of basal dendritic branching points relative to NC group at 20-40 µm ($p<0.01$), 40-60 µm, 60-80 µm, 80-100 µm, 100-120 µm, 120-140 µm ($p<0.001$) circle zones. Stress induced diabetes rats (D+S) exhibited significant reductions in quantities of basal dendritic branching points at 20-40 µm, 40-60 µm, 60-80 µm, 80-100 µm, 100-120 µm, 120-140 µm ($p<0.001$) circle zones relative to (NC) group. Exposure to EE (D+EE) significantly increased quantities of basal dendritic branching points at 20-40 µm ($p<0.05$), 40-60 µm, 60-80 µm, 80-100 µm ($p<0.01$), 100-120 µm, 120-140 µm ($p<0.01$) circle zones relative to D group rats which were not exposed to EE. D+S+EE rats also exhibited a significant increase in basal dendritic branching points at 0-20 µm, 20-40 µm, 60-80 µm, 80-100 µm ($p<0.01$), 100-120 µm, 120-140 µm ($p<0.001$) circular zones relative to (D+S) group which was unexposed to EE.

Total number of dendritic branching points at the DH (CA4) hippocampal region

Overall, the neurons at the DH region of the rat hippocampus exhibited a significant reduction in total apical ($p<0.001$) and basal ($p<0.001$) dendritic branching points in D group and (D+S) group relative to NC. Diabetic and stressed rats [(D+EE) and (D+S+EE) groups] exhibited significantly higher quantities of total apical ($p<0.001$) and basal ($p<0.001$) dendritic branching points (Fig. 4).

Discussion

The hippocampus is the region in the brain responsible for formulation of memory, emotion, spatial navigation and adaptive behaviors (Rubin et al., 2014). CA1, CA2, CA3 and CA4 (Dentate hilus) are sub-division regions within the hippocampus formation where neurons in each region contribute to unique physiological functions. Regions in the hypothalamus are highly interconnected locally within the hypothalamus formation as well as with other parts of the central nervous systems (Bartsch et al., 2011; Dudek et al., 2016; Sadeghi et al., 2016). In diabetes, neurons at the hippocampus undergo apoptosis (Cherubini and Miles, 2015), commonly leading to memory deficit, cognitive impairments and psychomotor decline (Ware, 2008).

The STZ-induced-diabetes model in rats simulates type 1 diabetes and advanced type 2 diabetes where there is total destruction of pancreatic β-cells resulting in little or no insulin secretion, hence hyperglycemia abruptly prevails. In the current study the experimental groups developed hyperglycemia on Day 2 after STZ injection (Table 1). In the hyperglycemic milieu, a vicious cycle of reactive oxygen species (ROS) is generated (Wrighten et al., 2009) via the overactivity of intracellular glucose metabolism pathways and shunting towards the polyol pathway. Glucose molecules are capable of oxidizing intracellular structures and along with ROS, exacerbate injury to the neuronal cells in the hippocampus (Yap et al., 2013) initiating apoptosis (Kaneto et al., 2010).

Stress is a state of feeling overwhelmed and incapable of coping with the lifestyle challenges, from traffic jams, career uncertainties, and financial strain to various health conditions. All societies globally experience various levels of stress in their day-to-day living. Stress is the cornerstone of diabetes in terms of both its onset and exacerbation. Stress triggers the hippocampus to signal the activation of hypothalamo-pituitary-adrenal (HPA) axis. Subsequently, stress hormones (cortisol, epinephrine and norepinephrine) are released from the adrenal glands and play crucial roles in antagonizing insulin functions initiating the development of insulin resistance (Ware, 2008), contributing further to neuronal apoptosis at the hippocampus.

In the current study, restrained stress is employed on experimental animals to simulate real-life stress in humans. Diabetes and stress related neuronal cell damage in selected hippocampal regions was evaluated by quantitating neuronal arborizations. Fig. 5 exhibits histology photomicrographs of representative neurons at the CA1, CA3 and HD regions from all experimental groups. Fig. 6 exhibits the hand traced branching morphology of the hippocampal neurons.

Our results evidently demonstrated that EE preserved the morphology of neurons at the CA1, CA3 and CA4 regions. Diabetic rats exposed to EE [Group (D+EE)] at consistent duration (6 hours) and frequency (every week; for 4 weeks) had significantly higher numbers ($p<0.001$) of arborizations on neurons at all 3 hippocampal regions (CA1, CA3 and DH) relative to the diabetic rats which were not exposed to EE. Similarly, diabetic rats which were challenged with stress and exposed to EE [Group (D+S+EE)] had significantly denser ($p<0.001$) arborizations relative to the group that was not treated with EE [Group (D+S)]. Our findings complement another similar study which demonstrated that EE induced neuronal plasticity and improved spatial learning and memory function (Rajashree et al., 2019).

Conclusion

The findings of this study demonstrate that EE is potentially a convincing preventive measure for protecting against diabetes-induced cognitive and memory deficits caused by neural damage in the brain. EE should be included as part of a management plan for diabetes patients alongside hypoglycemic medications, since our findings revealed that EE did not significantly lower the blood glucose levels in STZ-induced diabetic rats. EE in reality, may be daily relaxing or strolling in a serene park or home garden filled with colorful objects, flora and fauna.

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