RESEARCH PAPER

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The enriched environment prevents degeneration of cerebellum Purkinje cells layer of rats

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Neurodegeneration is characterized by loss of neurons causing changes that lead individuals to debilitating conditions; the most common of this condition is the Alzheimer's disease. It has been related that enriched environment (EE) induces experience-dependent plasticity mechanisms, improving the performance of the animals in learning and memory tests. This study evaluated the effects of EE on histological parameters of the cerebellum in rats that received intracerebroventricular streptozotocin. In the standard environment, streptozotocin (STZ) promoted a significant increase between the gaps in the Purkinje layer of approximately 20%. On the other hand, in an enriched environment, the control result (EE) was similar to the result under streptozotocin effect (STZEE). In the standard environment (SE) group a 26% significant reduction in Purkinje cell density was observed under STZ presence. By analyzing the results of the density of Purkinje cells under the effect of streptozotocin in a standard environment (STZSE) against the density of the layer of Purkinje cells also under the effect of streptozotocin in an enriched environment (STZEE), a significant reduction of approximately 76% in density was observed of Purkinje cells in standard environment (STZSE), the mean number of Purkinje cells in enriched environments was not reduced, despite of STZ. According to the results, treatment with STZ and exposure to EE did not change the cerebellum general morphology/cytoarchitecture, hence was no significant difference in the layers thickness. These facts demonstrate that the enriched environment appears to protect the Purkinje cells layer of cerebellum from possible degeneration.

Key words: neurodegeneration, Alzheimer's disease, standard environment, enriched environment, cerebellum, Purkinje cell

INTRODUCTION

The human central nervous system (CNS) follows a typical mammalian developmental pattern. Building it requires precise coordination between different cellular and molecular processes with a wide variety of cells (Silbereis et al., 2016). The neurogenic process expands the number of cells during the embryonic phase and remains active after birth only in certain brain areas. This phenomenon, in adults, occurs mainly in the subventricular zone of the lateral ventricles and in the subgranular zone of the hippocampus. Dysregulation of these processes affects the structure and function of the CNS and can lead to neurological or psychiatric disorders (Mavroudis et al., 2010; Torrão et al., 2012; Pino et al., 2017; DeTure and Dickson, 2019).

One of the essential components of the nervous system is the cerebellum, responsible for learning motor

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skills, controlling and executing several functions, including motor coordination, muscle tonus and locomotion (Horne and Butler, 1995; Armstrong and Hawkes, 2000; Hernández-Fonseca et al., 2009; Meneses et al., 2011; Koziol et al., 2014; Schmahmann, 2016; 2019). Purkinje neurons represent the dominant element in cerebellar information processing, thus they are GABAergic cells that form the only output of the cerebellar cortex (Apfel et al., 2002). Studies have shown that degenerative diseases and focal lesions also affect cerebellar functioning (Schmahmann and Sherman, 1998; Baldaçara et al., 2008; Koziol et al., 2014; Schmahmann, 2019; El-Baz et al., 2020; Salomova et al., 2021). Injuries in the cerebellar layers generate changes in its thickness, as well as modify the amount of Purkinje cells (Hirano et al., 1986; Apfel et al., 2002). Mavroudis and colleagues (2010) observed in Alzheimer's disease (AD), two types of deposits in the cerebellar cortex, diffuse plaques in the molecular layer and plaques with compact amyloid nuclei in the Purkinje layer and granular cells. Based on these reports, experimental models were developed (Puzzo et al., 2014; Penney et al., 2019) and one of them uses intracerebroventricular streptozotocin (ICVSTZ) (Pfutzenreuter et al., 2020). This protocol alters cerebellar motor functions, spatial learning and memory (the Morris water maze test). It also causes loss of weight in the brain and cerebellum by the impairment of neurons in the frontal cortex and cerebellar cortex (Hernández-Fonseca et al., 2009; Meneses et al., 2011).

It is known that environmental and chemical stimuli can promote neurogenesis (Ernst and Frisén, 2015) and prevent neurodegenerative processes. Tests conducted on adult rats, when housed in an enriched environment containing toys and gym equipment, provided increased sensory, cognitive and motor stimulation, which resulted in an increase in the number of new cells (Anacker and Hen, 2017). It has been related that environmental enrichment (EE) improved the cognitive function in an AD animal model (APPsw transgenic mouse) (Arendash et al., 2004; Jankowsky et al., 2005). In this path, it was also demonstrated that EE added physical activities have better neuroprotective effects than standard environmental in memory deficits in AD model used (Prado Lima et al., 2018). In some experimental protocols the animals have been previously hemicerebellectomized to analyze whether EE exposure would compensate for the motor and cognitive functions loss. The exposure to the enriched environment seems compensated the effects of the hemicerebellectomy. The motor and cognitive functions were similar to those of the control animals the changes induced by the EE suggested to compensate the cerebellar damage (Foti et al., 2011). In other words, performing activities with exercises and enrich environment seem to delay neurodegeneration processes (Kempermann et al., 1997; Fischer, 2016; Salomova et al., 2021).

This study aimed to evaluate the effects of EE on cerebellar morphology in rats that received ICVSTZ (Chen et al., 2012; Kwan et al., 2012; Torrão et al., 2012; Pfutzenreuter et al., 2020). Cyto- and histomorphometric characteristics of the cerebellar tissue of rats carried in enriched or not enriched environments were studied; in addition to the histological characterization of the cerebellar layers, Purkinje cells were quantified. These analyzes allowed us to evaluate the effects of enriched environments on the prevention of neurodegeneration of the cerebellar tissue of rats (Salomova et al., 2021).

METHODS

Animals

All experiments were conducted using 11-month-old male Wistar rats weighting average 350 g, from the animal facility at the Universidade Positivo. The animals at the age of 21 days were randomly assigned to standard laboratory cages or artificial-enriched cages (toys, running wheels, cognitive tasks). The rats remained under controlled conditions including light/dark cycle of 12 h each (lights from 07 h up to 19 h) and temperature 21°C with water and food provided *ad libitum*. All the experimental procedures were approved by The Ethics Committee on Animal Use in Research of Universidade Positivo (Nº 227).

ICV injection of STZ

After 6-7 months of exposure to the respective environments, the animals were submitted to stereotactic surgery for ICV administration of STZ. The animals received STZ (3 mg/kg) or saline in the same final volume (4 µL). For stereotaxic surgery, the rats were anesthetized with a combination of ketamine (90 mg/kg) and xylazine (12 mg/kg). After a skin incision, two access were bilaterally made in skulls, following coordinates measured from bregma (–0.96 mm on the anteroposterior, ± 1.8 mm on the medial–lateral and –3.6 mm on the dorso-ventral axis, according to the stereotaxic atlas) (Paxinos and Watson, 1987). The STZ was diluted in saline and injected into bilateral intracerebroventricular access. The control group was injected with saline, thus the following experimental groups have been established: control maintained in

a standard environment (SE, n=4); control maintained in enriched environment (EE, n=4); Streptozotocin maintained in standard environment (STZSE, n=4); and streptozotocin maintained in enriched environment (STZEE, n=4).

Tissue collection and processing

Three months after the STZ injection, all the rats were anesthetized with an overdose of sodium pentobarbital (50 mg/kg) and transcardially perfused so that the cerebellum was extracted. Under anesthesia the perfusion was performed as per previously described methods (Lambert et al., 2016). After perfusion-fixation the tissues were dissected and post-fixed in 10% formalin for 48 h. The cerebellar tissues were left in xylol solution to make them transparent. The cerebellum was cut in the transversely plane and embedded in paraffin. Afterwards, the paraffin blocks were cut using a microtome (Leica™) into histological serial sections (4 µm thick) with a 20 µm interval. Hematoxylin and Eosin (H&E) staining was performed after dewaxing sections in xylene and rehydrating them in graded ethanol solutions.

Histological and image analysis studies

The H&E stained slides were scanned at 40× magnification. Stacks of images were captured by a Zeiss Axio Scan.Z1 (ZEISS, Jena, Germany) slide scanner, and images were exported using the ZEN 3.1 blue edition (ZEISS, Jena, Germany) software.

Cerebellar layer thickness measurements

In this study, the measurements of molecular layer, Purkinje cells, and granular layers were made using arbitrary line probe of the ZEN 2.3 (ZEISS, Jena, Germany). The variations of the layers of the cerebellum (µm) were measured at the apex of the cerebellar randomly selected leave within each case, in 3 sections per animal (magnification ×10).

Cell quantification

The Purkinje neurons density quantification was performed in some cerebellum's leaves. This quantification was made in the Purkinje layer using H&E stained sections. As read out, the blinded observer manually focused on the tissue surface and quantified the Purkinje cells using three criteria: darkened cytoplasm, irregular cell shape and nucleus or nucleolus as counting criterion. Cells that presented the score 2 in at least 2 criteria were regarded as degenerating. Purkinje neurons were manually counted using ZEN 2.3 software in 3 sections per animal (magnification 40×).

Quantification of the distance between Purkinje cells

The gaps (distance from one Purkinje cell body up to another Purkinje cell body along the layer) between Purkinje cells were determined using a nearest neighbor analysis, calculated from the plugin distance using ZEN 2.3 software (Choe et al., 2016). For this, a free-hand line was designed between adjacent Purkinje cell bodies along the entirety of the Purkinje layer within a given image. The gaps were determined from twelve images and then averaged together to determine the mean nearest neighbor distance (µm) for each group (magnification 40×).

Statistics

Results are expressed as mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey *post hoc* test. $p<0.05$ is considered significant differences.

RESULTS

In order to investigate the effect of exposure to EE, animals treated with STZ were evaluated for the general morphology of the cerebellum, as well as the cellular organization of the layers by morphometric analysis in different environments. The morphometry, verified by the length of each layer (µm), was performed at the apex of the leaves of the cerebellum as indicated in Fig. 1. According to the results, treatment with SZT and exposure to EE did not change the general morphology of the cerebellum, since there was not significant difference in the thickness of the layers of the samples studied.

Fig. 2 A shows a sample of a leaf of the cerebellum, detailing the markings with the measurements of the gaps between the Purkinje cells. The graph (Fig. 2B) represents the results of the gaps obtained in standard (SE) and enriched (EE) environments. The control value (SE) was 50.16 ± 2.3 µm and under the effect of streptozotocin (STZSE) 60.43 ± 3 µm. In the SE streptozotocin promoted a significant increase between the gaps in the Purkinje layer of approximately 20%. In an enriched

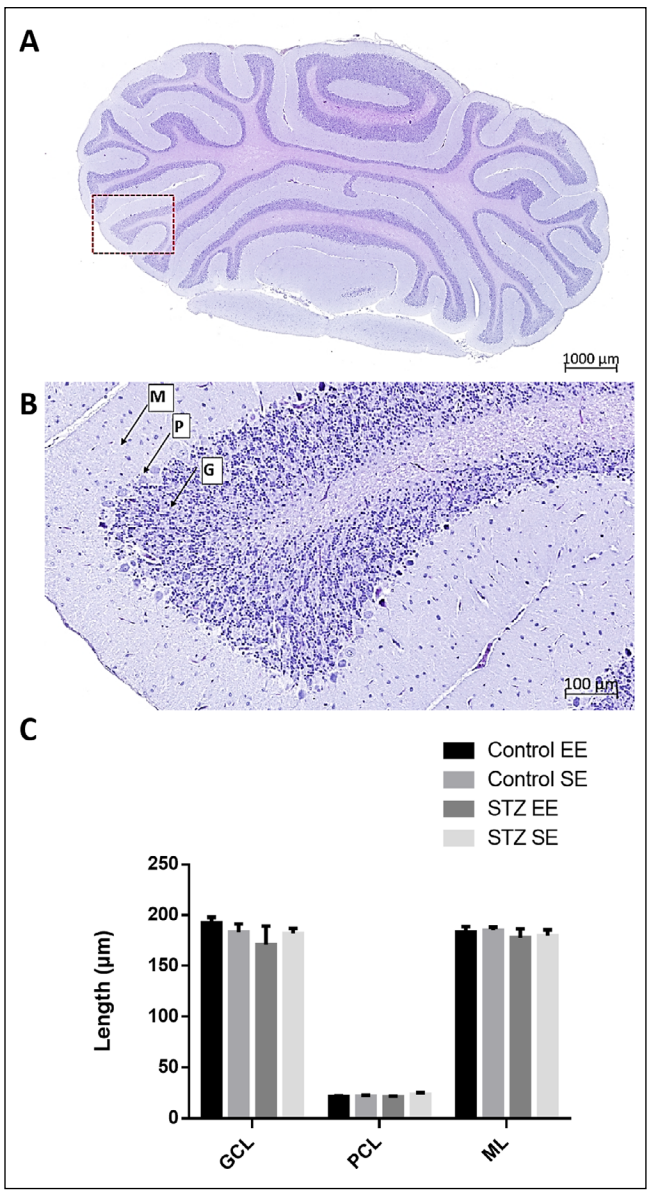

Fig. 1. Representative of the histological evaluations considering the thickness of the cerebellar layers of rats submitted to SE/EE and STZSE/STZEE environments. Cerebellar cross-sections (6 µm) were stained with H&E. (A) Overview of a rat cerebellar slide. In the area demarcated in the rectangle, a sample of the region of the analyzed cerebellar leaves can be observed. (B) Region of the leaves of the cerebellum with its layers: Molecular layer (M), Purkinje layer (P), granular layer (G) The experimental groups were performed, obtained at the apex of the cerebellum leaves. (C) The morphometry (µm) of the cerebellar layers (granular cells layers (GCL)).

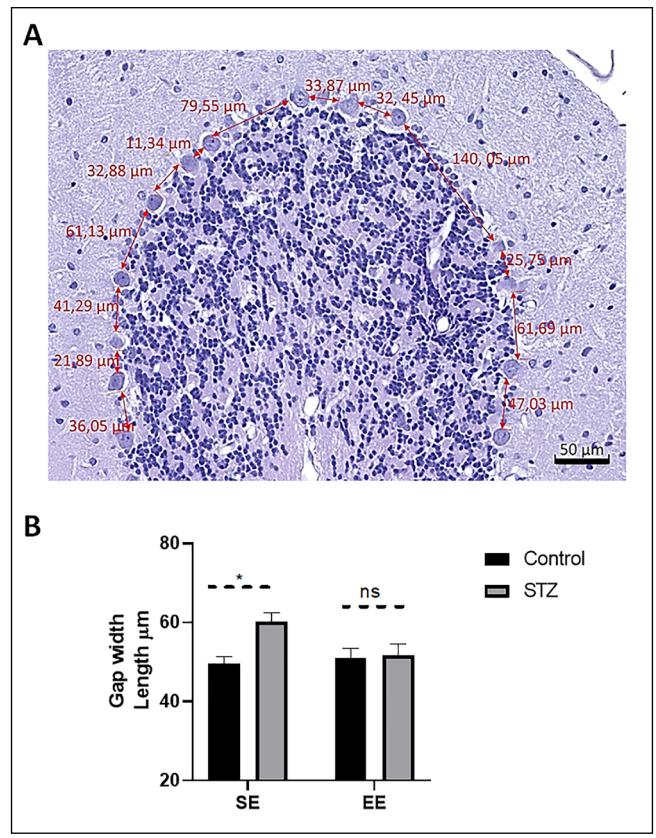

Fig. 2. Assessment of gaps between Purkinje cells from rats subjected to SE/EE and STZSE/STZEE environment. Quantification of gaps between Purkinje cell bodies in H&E stained cerebellar sections. (A) Red arrows show measurements (µm) of gaps between Purkinje cells. (B) Measurements of gaps between Purkinje cells in the different experimental groups.

environment, the control result (EE) was 50.75 ± 3.4 µm, similar to the result under the effect of streptozotocin (STZEE) 51.74 ± 4.2 µm. There was no significant difference between the gaps in the enriched environment. It is possible to infer a potential protective effect of the enriched environment according to these results.

Fig. 3 in standard environment, when comparing results SE and STZSE there was a significant decrease (p<0.05) of approximately 26% in the density of the Purkinje cell layer under the effect of streptozotocin. There was an increase in Purkinje cell density in the enriched environment control EE (Fig. 3C) compared to the standard environment control SE (Fig. 3A) indicating an EE protective effect but the result was not statistically significant.

When comparing the results of the density of Purkinje cells from the control in standard environment (Fig. 3A) SE against the density of the layer of Purkinje cells under the effect of streptozotocin in enriched environment (Fig. 3D) STZEE a significant decrease was also observed (p<0.05) of approximately 30% in SE. In an enriched environment, the results were compared between the layer of Purkinje cells of control (Fig. 3C) EE against the density of the layer of Purkinje cells under the effect of streptozotocin (Fig. 3D) STZEE, a significant gain of approximately 16% was observed (p<0.05) in this layer. When analyzing the re-

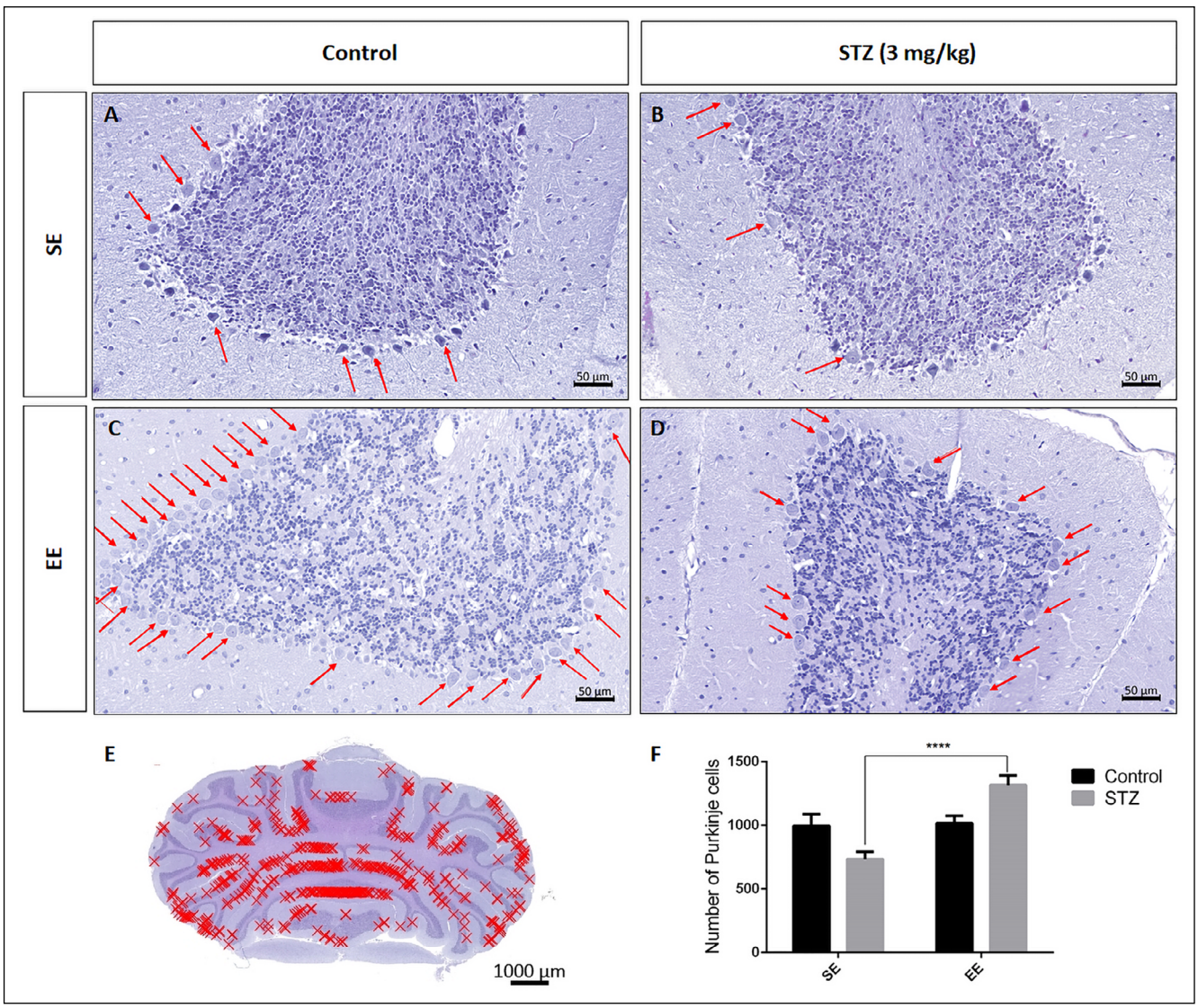

Fig. 3. Evaluation of the density of Purkinje cells from rats submitted to standard environment (SE/EE) and under the effect of streptozotocin (STZSE/STZEE). (A) In standard environment (SE), representative cross-sections of the cerebellum showing the density of the Purkinje cell layer with large pear-shaped cell bodies and nuclei (arrows). (B) In a standard environment and under the influence of streptozotocin (STZEE), representative cross-sections of the cerebellum showing the density of the Purkinje cell layer with large pear-shaped cell bodies and nuclei (arrows). (C) In enriched environment (EE), representative cross-sections of the cerebellum showing the density of the Purkinje cell layer with large pear-shaped cell bodies and nuclei (arrows). (D) In an enriched environment under the influence of streptozotocin (STZEE), representative cross-sections of the cerebellum showing the density of the Purkinje cell layer with large pear-shaped cell bodies and nuclei (arrows). (E) Representation of the total area (µm^2) of Purkinje cell count. (F) Representative graph of cell number density in the Purkinje layer.

sults of the density of Purkinje cells under the effect of streptozotocin in standard environment (Fig. 3B) STZSE against the density of the layer of Purkinje cells also under the effect of streptozotocin in enriched environment (Fig. 3D) STZEE a significant decrease in cell density was observed of approximately 76% in the standard environment (SE) (p<0.05). Fig. 3E shows the total area (µm^2) of Purkinje cell count. For its quantification, three criteria were considered: darkened cytoplasm, irregular cell shape, and nucleus. Cells that scored on at least two criteria were considered to be in the process of degeneration. Fig. 3F is the graph that summarizes the results described above of the effects of standard or enriched environments, under the effect of STZ or not, on the density of cells in the Purkinje layer. In view of these results, it is also possible to infer that the enriched environment (EE) promotes a protective effect on the Purkinje layer.

DISCUSSION

Among the NS structures affected by AD, the cerebellum has long been considered secondary and has received little attention (Guo et al., 2016; Gellersen et al., 2021). Recently, studies have revealed certain AD-specific changes in the cerebellar structure, suggesting that it also undergoes degenerative changes during the course of the pathology (Jacobs et al., 2018; Di Lorenzo et al., 2020; Olivito et al., 2020). Therefore, studying neurodegeneration in the cerebellum in AD can help to elucidate the mechanisms that lead to the pathology. In previous reports, the intracerebral injection (IC) protocol of STZ was used to provoke cerebral metabolic dysfunction, as occurs in human sporadic AD (Jankowsky et al., 2005; Fischer, 2016; Penney et al., 2019). Thus, the use of intracerebral STZ has been a suitable experimental model for the characterization of AD neurotoxicity and studies of neuroprotective interventions (Tong and Dominguez, 2016; Boelter et al., 2020). The effects of the enriched environment (EE) on AD-type deficits in the cerebellum are still scarce in the literature, and with this condition, it was possible to investigate the role. Also, it has been reported that long term exposure to an enriched environment (Cutuli et al., 2011; Cendelin et al., 2016) similar to this study, can prevent reduction on cortical volume after traumatic brain injuries (Maegele et al., 2015; Gelfo and Petrosini, 2022), as well as mitigate nervous system impairments after chronic cerebral hypoperfusion in rats (Song et al., 2020). When comparing short- and long-term effects of the enriched environment on brain functions, including motor and cognitive, Kapgal and colleagues (2016) observed that short-term exposure did not prevent functional deficits on these functions, while long term exposure showed a positive effect. Considering these studies, our attempt to expose these animals for a long period in the enriched environment after the treatment aimed to increase the benefits of the enriched environment, as observed in previous studies.

In this work, cerebellar neurodegeneration was induced to verify the protective effect of the enriched environment mainly on Purkinje cells layer. EE is considered an effective exercise protocol to increase sensory, cognitive, and motor stimuli, in addition to promoting interaction in rodents (Lambert et al., 2016: Di Lorenzo et al., 2020). It is known that the use of objects and their exchange in the environment contribute to the increase of hippocampal plasticity, being able to promote changes in the nervous system, such as in memorization and learning skills (Gardner et al., 1975; Leggio et al., 2005). It has been shown that in rodents EE can attenuate neurodegeneration (Dhanushkodi et al., 2007). Furthermore, a decrease in Purkinje cell density has been observed in AD patients and in animal models of AD (Gellersen et al., 2021). These reports are in line with our results. It was verified that the measurement of gaps between Purkinje cells did not change in the enriched environment even in the samples under STZ. But in the standard environment there was a 20% increase in this distance between the control (SE) and the STZSE treated samples (Fig. 2B). It was also observed in standard environment (SE) samples there was a 26% reduction in the density of these cells under STZ action (STZSE). On the other hand, our data demonstrated that the enriched environment (EE) protected (16%) the Purkinje cell layer from degeneration even under STZ. Corroborating with these findings the protective effect of the enriched environment was even bigger (76%) when comparing the results of Purkinje cells in enriched environment (STZEE) with the data of Purkinje cells in a standard environment (STZSE). Our results are in agreement with the studies by Dhanushkodi et al. (2007) who demonstrated that EE can prevent cell degeneration in the AD model.

CONCLUSIONS

According to our results, it is possible to infer that the degeneration of the layer of Purkinje neurons, induced by streptozotocin, can be minimized by the environment where the animals are, evidencing the beneficial effects of the enriched environment in the prevention of neurodegeneration of this layer in AD models. It is suggested that the promotion of preventive therapies, which are able to improve neural plasticity, may be extremely important in order to delay the progression of cerebellar neurodegeneration triggered by Alzheimer's disease.

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