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Research article

Human neural progenitor cells ameliorate NMDA-induced hippocampal degeneration and related functional deficits

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Abstract: It has been established that the CA3 region of the hippocampus is involved in consolidating short-term memory to long-term memory and aids in spatial navigation retention. Seizures and many neurologic diseases induce damage to that region of the hippocampus, resulting in deficits in memory consolidation and spatial navigation. Drug treatments have been proven to have limited effectiveness, but cell replacement therapy has demonstrated to be more promising. Celavie Biosciences have developed a multipotent, nontumorigenic human neural progenitor cell (hNPC) line shown to have the ability to migrate *in situ*, reducing structural and functional deficits in neurodegenerative animal models. Here, we examined whether transplanted hNPCs would reestablish the memories of Han-Wistar rats subjected to hippocampal excitotoxic lesioning. The rats were lesioned in the CA3c regions at 50 days bilaterally with the neurotoxin NMDA (1 µl containing 7.5 mg/ml; −3.5 mm AP; ±2.0 L and −2.5 V). At 54 days of age, live hNPCs (500000 cells in 5 µl cell suspension media), frozen-killed hNPCs (500000 cells/5 µl), HEK293Tcells (500000 cells/5 µl) or vehicle (cell suspension media; 5 µl) were bilaterally implanted directly into the NMDA damaged area. The rats were tested two weeks later with three different memory tests: novel and place-object assays and the water-maze task. Results showed that rats receiving live hNPC implantation performed significantly better in the water maze task than control groups; yet, novel and place-object test results showed no significant differences among treatments. Histology confirmed the survival of implanted hNPCs after 28 days post-implantation as well as showing neuroprotective effects. This study showed that Celavie's hNPCs were able to survive and improve some but not all hippocampal functionality, emphasizing the promise for cell replacement therapeutics for neurodegenerative disorders.

Keywords: CA3c region; replacement cell therapy; water maze; hippocampus; novel object test

Abbreviations: DAB: Diaminobenzidine; hNPC: Human neural progenitor cell; IGF-1: Insulin-like growth factor-1; PBS: Phosphate buffer saline; PFA: Paraformaldehyde; SZD: Subventricular zone-derived

1. Introduction

The mammalian hippocampus plays a crucial role in cognitive function including memory formation, spatial navigation [1] and depressive behaviors [2]. Historically, hippocampal function has been analyzed using chemical lesioning studies; for example, subepileptic injections of excitotoxins like kainic acid or NMDA have been used to study the roles of specific regions on theformation of different types of memory. Using this approach, the CA3 region has been shown to be one of the areas of the hippocampus involved in the consolidation of short-term memory into long-term memory, and spatial navigation [3,4]. While lesioning of the CA3 with a low dose of excitotoxins support its link with memory formation, lesioning with high doses can lead to seizures and complete CA3 neurodegeneration [5,6]. Current drug treatment and physical therapy have only limited efficacy in reducing memory impairments in patients with hippocampal disorders like Alzheimer's, epilepsy and stroke.

While current drug treatments (for example, imipramine and rivastigmine) and physical therapies are not curative, cell replacement strategies in mammalian models have been shown to be successful as potential restorative therapy for many neurological disorders [7,8], including hippocampus-based disorders like epilepsy [9]. In a recent study, scientists were able to repair unilateral hippocampal lesions in rats (made by administration of an excitotoxin) by grafting neuronal progenitor cells (acquired from neonatal pups) into specific locations of hippocampal damage [7]. These researchers found that these rat neuronal progenitor cells were able to differentiate into multiple neuron types and migrate to specific hippocampal areas. Rats that received the cell grafts showed a decrease in memory deficits, suggesting effectiveness of the transplants. In a related study, human neural stem cells over-expressing choline acetyltransferase were utilized to restore cognitive function in an animal model of Alzheimer disease [10]. In this particular study, researchers unilaterally damaged the CA3 region of rat hippocampi, then four weeks later, human stem cells were implanted into the region of damage. They found that these stem cells differentiated into astrocytes and hippocampal neurons, leading to improvements in cognitive function. In yet another related study, researchers were able to prevent hippocampal degeneration and improve cognitive function by implanting subventricular zone-derived (SZD) neural stem cells overexpressing IGF-1 in an animal model of temporal lobe epilepsy [11]. In this study [11], a neurotoxin was unilaterally injected into the hippocampi of mice, causing neurodegeneration and seizures. Four days following the hippocampal injury, mice received a unilateral implantation of SZD stem cells into the damaged area. Mice that received stem cell implants showed improved cognitive function when compared with control mice that received vehicle. In summary, utilizing cell replacement therapies seem to be an encouraging avenue to develop new curative treatments patients with hippocampal injuries.

Celavie Biosciences LLC (Oxnard, CA) has developed a human multipotent, nontumorigenic, hypoimmunogenic neural progenitor cell (hNPCs) line with a normal karyotype. These hNPCs have

demonstrated a unique ability to migrate to the site of brain lesions following microenvironmental cues and differentiate according to the severity and nature of the injury [12]. Ultimately, these neural progenitor cells have shown the ability to reduce structural and functional deficits in some animal models of neurodegenerative diseases. For example, our lab has shown that implantation of Celavie's hNPCs (for background refer to references [13,14]) into the cerebellum of the ataxic *spastic* Han-Wistar rat exhibiting progressive Purkinje cell degeneration resulted in significantly improved motor activity scores compared with control animals that received dead hNPC implantation [13,14]. In a subsequent long-term study [15], these neural progenitor cells were shown to extend longevity and significantly diminish ataxic symptoms in the *spastic* Han-Wistar rat model of cerebellar ataxia. Taken together, we have verified that Celavie's hNPCs have the ability to reduce and even eliminate structural damage and concomitant symptoms. Consequently, will these same human neural progenitor cells that were effective in ataxia also work to reverse the memory dysfunction inherent with animals with bilateral hippocampal damage?

To answer the preceding question, we investigated whether implantation of hNPCs in NMDA-lesioned CA3c regions of the rat hippocampus could alleviate and reverse the neurotoxin-caused memory deficits. The efficacy of Celavie's human neural progenitor cells to repair the damaged CA3c neurons was tested through a series of cognitive tests. The previously reported abilities of hNPCs to ameliorate a wide range of symptoms from consequential neuronal damage would suggest that these human-derived cells could be used to develop an effective restorative treatment for people with hippocampal neurodegenerative disorders.

2. Materials and methods

2.1. Animals

A total of 50 normal, male Han-Wistar rats were obtained from the California State University Northridge (CSUN) breeding colony. The animals were housed in standard rat cages, provided with LabDiet 5001 rodent chow (Purina), and water *ad libitum*. The module room temperature was maintained at 23 ± 1 ℃ with a 12/12-hour light/dark cycle. Male littermates were randomly divided into a live stem cell treatment group (n = 21), a dead stem cell control group (n = 17), a HEK293T cell group (n = 7), and a cell suspension media (vehicle) control group (n = 5). Animals were assessed for their overall health by taking their weight weekly. This study was in compliance with prior approval of CSUN's IACUC committee, protocol number 1011-009c.

2.2. Human neural progenitor cells (NPCs)

The human neural progenitor cells utilized in our study were acquired from fetal brain tissue (six-weeks) via sterile aspiration with informed consent from the donor in accordance with NIH guidelines. The hNPCs were grown in culture medium consisting of ADCF MEM/EBSS basal medium. Dead hNPCs were used as a negative control for possible paracrine effects of cell inoculations. These dead cells controls were obtained from live hNPC populations and killed by placing them into a −20 ℃ freezer until use.

2.3. Immunosuppression

At 45 days of age, all animals were implanted with an Alzet Osmotic Pump (Model# 2ML4; Durect Corp., Cupertino, CA) containing an immunosuppressive agent, cyclosporine (15 mg/kg/day for 28 days). Animals were anesthetized with 2.5% isoflurane, and a small subcutaneous pocket was prepared in the midscapular area. The pump was inserted, and the pocket was closed using wound clips. At the end of the experiment, all pumps were removed and found to be completely devoid of cyclosporine. In our studies with cyclosporine, we have found that one dose (via pumps) is effective for much longer than 28 days [15].

2.4. Neurotoxin damage and neuronal progenitor cell therapy

At 50 days of age (five days after cyclosporine treatment began), all animals were given bilaterally injections of the neurotoxin NMDA (1 µl; 7.5 mg/ml) in the CA3c region of the hippocampus (coordinates from Bregma: −3.5 anteroposterior, ±2.0 mediolateral, and −2.5 dorsoventral) using a stereotaxic table (David Kopf Instruments, Tujunga, CA, USA). At 54 days of age, treatments began with rats injected with one of the following four treatments: 5 µl of cell suspension media that contained 500000 live hNPCs, 500000 freeze-killed hNPCs (in 5 µl of cell suspension media), 500000 live HEK293T cells (in 5µl of cell suspension media) or a 5 µl injection of cell suspension media (vehicle) only. All injections took place in the same CA3c region of the hippocampus that received NMDA injections four days earlier (preliminary studies showed that NMDA was no longer neurotoxic after 4 days).

2.5. Memory testing

At 68 days of age (14 days post-implantation), animals from all four groups were habituated to an empty field arena (100 cm by 100 cm with 30.5 cm walls) for three, 10 minute daily sessions, 24 hours apart. Eight days after the final habituation, all animals were subjected to three different types of memory tests (in the following order): novel-object recognition trial, place-object trial, and water maze trial. The novel-object recognition trials and place-object recognition trials were conducted on alternating days during the first six days of memory testing for a total of three trials apiece. The water maze trial was conducted during the last two days of the animal testing. All memory tests trials were recorded using a video camera. The recorded videos were later analyzedfor memory trial quantification.

In the *novel-object recognition trial*, the animal was placed into the field arena with two identical objects (yellow, conically-shaped plastic toys; 10.5 cm high with a 10 cm diameter base) and allowed to explore for five minutes. The animal was then removed from the arena for a five minute rest period. After, the animal was placed back into the arena with one of the original objects and one novel object (green, cylindrically-shaped plastic toy; 8 cm high with a 10 cm diameter base) for a three minute trial. Videos of the trials were analyzed to measure the time (in seconds) spent at the original object versus novel object. An Object Preference Ratio was generated by determining the time the animal's nose placed within 2 cm of the novel object divided by total time spent (again by the animal's nose within 2 cm) at both objects (see Figure 1).

In the *place-object trial*, the animal was placed into the arena with two identical objects and allowed to explore for five minutes. Then, the animal was removed from the arena, and after a five

minute rest period, it was placed back into the arena for a three minute trial with one object moved to a new location. Videos of the trials were analyzed to measure the time (in seconds) spent at the original object versus moved object. Another Object Preference Ratio was generating by taking the time the animal's nose moved the within 2 cm of the moved object divided by total time spent (again determined by nose proximity) at both objects (see Figure 1).

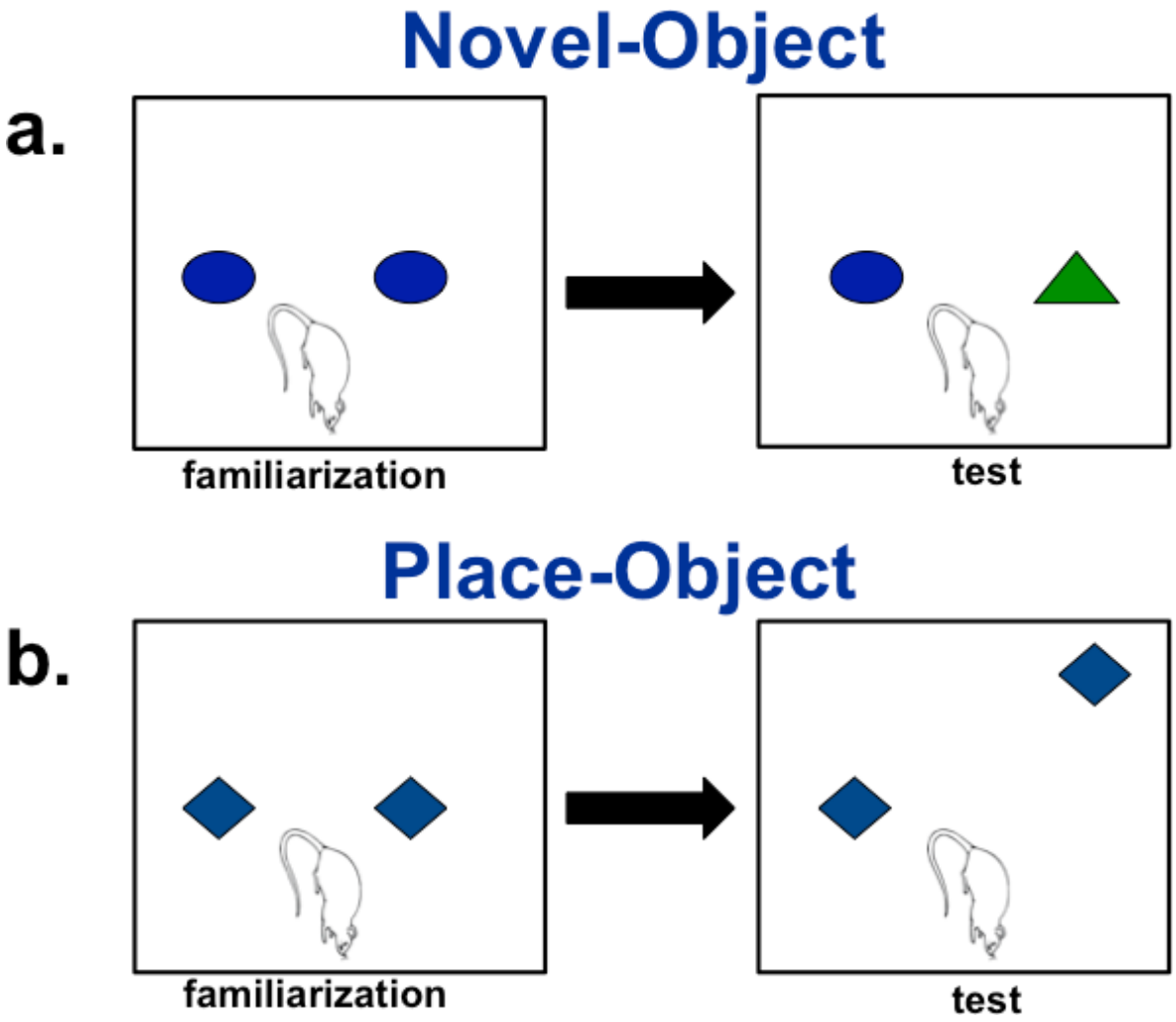

Figure 1. Memory tests were used to assess cognitive and spatial improvements of implanted hNPCs on the NMDA-lesioned rats. On the day of testing, the rats were placed into the field arena with two identical objects and allowed to explore for five minutes. The animal was then removed from the arena for a five minute retention period. Then, the rats were tested using the following two assays: (a) Novel-Object Trial. The Novel-Object test assayed the animal's familiarization of objects in an arena. After habituation, the rat was placed back into the arena for three minutes with one of the familiar objects replaced by a novel object at the previous location. (b) Place-Object Trial. The Place-Object tests comprised of the animal's familiarization of objects in a specific location. After the habituation period, the rat was placed back into the arena for a three minute trial with one object moved to a new location within the arena. In both cases, an Object Preference Ratio was generated by determining the time the animal's nose moved within 2 cm of the novel or moved object divided by total time spent at both objects.

The *water maze trial* was conducted over two days, a training day and a trial day. The test was conducted in a 1.5 m diameter, 45 cm deep circular pool filled with water to a depth of 26.5 cm and made opaque with powdered milk. A small, circular platform, 25 cm high and 12 cm in diameter was placed into the tank at a fixed location in the center of one quadrant. For the training sessions, a red dart was affixed to the platform, making the platform location visible above the waterline. Each animal received three training sessions which began with the animal positioned on the platform for a 10 second retention period. The animal was then released back into the water in one of the three empty quadrants not containing the platform and was given 90 seconds to find the platform. If the platform was not found within 90 seconds, the experimenter guided the animal to swim to the platform. This training was repeated in the other two quadrants not containing the platform. On the next day ("trial" day), the red dart was removed from the platform. Each animal was given four swim trials in which the animal was released in the water at either one of two alternate locations (*right entry* or *left entry*) equidistant from the platform. The animal was allowed to swim for up to 90 seconds to locate the platform, if it failed to locate the platform, escape was assisted. A rest period of 30 minutes was given between each trial. Trial videos were analyzed to measure latency swim time (in seconds) starting from initial entry location into the water maze to when the rat found the hidden platform.

2.6. Perfusion

Following final memory testing, all animals were sacrificed. Animals were injected with chloral hydrate (400 mg/kg; IP) and transcardially perfused with 0.9% saline solution followed by 4% paraformaldehyde (PFA) in 0.1M phosphate buffer saline (PBS). Brains were extracted, post-fixed in PFA for two days, and then placed in 20% sucrose in 4% PFA/0.1 M PBS for 24 hours prior to sectioning. Using a cryostat, the hippocampus was sliced on the coronal plane (35 μm sections). The sections were placed on glass slides and examined histologically for the presence of human cells at the same stereologic level for all animals. Slides were visualized to identify the original needle track and were stained within one week of slicing.

2.7. Histology

A double-staining technique was utilized to determine presence and location of surviving transplanted cells. First, an anti-human nuclear stain was used to detect the presence of human stem cells inside the foreign rat tissue. Microscope slides containing the hippocampal tissue were rinsed three times for 5 minutes in PBS containing 0.1% Triton X-100 (Sigma Chemical, St. Louis, MO, USA). The slides were then incubated with endogenous blocker solution (10% methanol, 10% hydrogen peroxide, and 80% PBS containing 0.1% Triton X-100) for 1 hour at room temperature. The slides were again rinsed three times for 5 minutes in PBS containing 0.1% Triton X-100 to remove excess endogenous blocker. The slides were then incubated for 1 hour in a serum blocker containing 4% normal horse serum (VECTASTAIN Elite ABC Kit Mouse IgG, PK-6102; Vector Laboratories, Burlingame, CA USA) and 96% PBS containing 0.1% Triton X-100. The slides were then incubated in anti-human nuclear (Catalog #MAB1281; Millipore, Temecula, CA, USA) primary antibody solution containing 1% normal horse serum, 1% anti-human nuclear antibody and 98% PBS containing 0.1% Triton X-100 for a minimum of 15 hours. The slides were then rinsed three times in PBS containing 0.1% Triton X-100. At room temperature, the slides were incubated with the secondary

antibody solution containing 1% normal horse serum, 0.5% biotinylated antibody (VECTASTAIN Elite ABC Kit Mouse IgG, PK-6102) and 98% PBS containing 0.1% Triton X-100 for 1 hour. Following incubation, the slides were rinsed three times for 5 minutes in PBS containing 0.1% Triton X-100. ABC Reagent (VECTASTAIN Elite ABC Kit Mouse IgG, PK-6102) was prepared 30 minutes prior to its use by adding 75 ml of reagent A with 75 ml of reagent B to 3750 ml of PBS containing 0.1% Triton X-100. The slides were incubated in the ABC Reagent for 1 hour at room temperature and rinsed three times for 5 minutes in PBS containing 0.1% Triton X-100. The slides were then placed in a diaminobenzidine (DAB; Sigma Chemical) solution containing 0.01 g DAB, 20 ml PBS containing 0.1% Triton X-100 and 25 ml of 30% hydrogen peroxide for 5 minutes.

After immunostaining was completed, the slides were then counterstained with cresyl violet to visualize the nuclei of the cells within the hippocampus. Microscope slides were first dehydrated using a series of ethanol dilutions (100%, 95%, and 70%) for 2 minutes each. This step was followed by a 2 minute hydration rinse in water. The slides were then immersed in the cresyl violet stain (Sigma Chemical) for 30 seconds, followed by a 2 minute water rinse. Next, the slides were destained in acetic formalin for 5 minutes, rinsed again in water for 2 minutes and were dehydrated using three, 2 minute ethanol rinses at 95%, 100% and 100% dilution. Finally, ethanol was removed using xylene, and slides were mounted with coverslips using Permount (Themo-Fisher Scientific;Waltham, MA).

2.8. Statistical analysis

Statistical analysis of weight gain was analyzed with repeated measures ANOVA using Systat. For analysis on the memory tests, a one-way ANOVA was used to determine significance. If differences were found, then we used a t-test to determine significance among means. Significance levels were set at $p < 0.05$ for all analyses. All values presented were means ± SEM.

3. Results

3.1. Animal health

To determine if animal health was affected by the combination of cyclosporine immunosuppression, hippocampal lesioning using NMDA, and subsequent cell or vehicle (cell suspension media) implantation, all animals were weighed on a weekly basis. There were no significant differences in weight gain (Repeated Measures ANOVA; $F = 0.435$, $p = 0.729$; Figure 2) among the treatment groups: cell suspension media ($n = 5$), HEK293T cells ($n = 7$), live hNPC ($n = 21$), and dead hNPC ($n = 17$).

3.2. Novel-object recognition and place-object trials

Novel Object-Recognition and Place-Object behavioral trials were used to test object recognition and positioning memory in rats injected with cell suspension media, HEK293T cells, live hNPC, or dead hNPC into their NMDA-damaged hippocampi. The data indicate that live hNPCs implantation did not affect Novel Object-Recognition (ANOVA; $F = 0.855$, $p = 0.471$; Figure 3A) or Place-Object (ANOVA; $F = 0.730$, $p = 0.539$; Figure 3B) memories in NMDA lesioned rats. In fact, there were no statistical differences among all treatments in these Novel Object-Recognition or Place-Object assays.

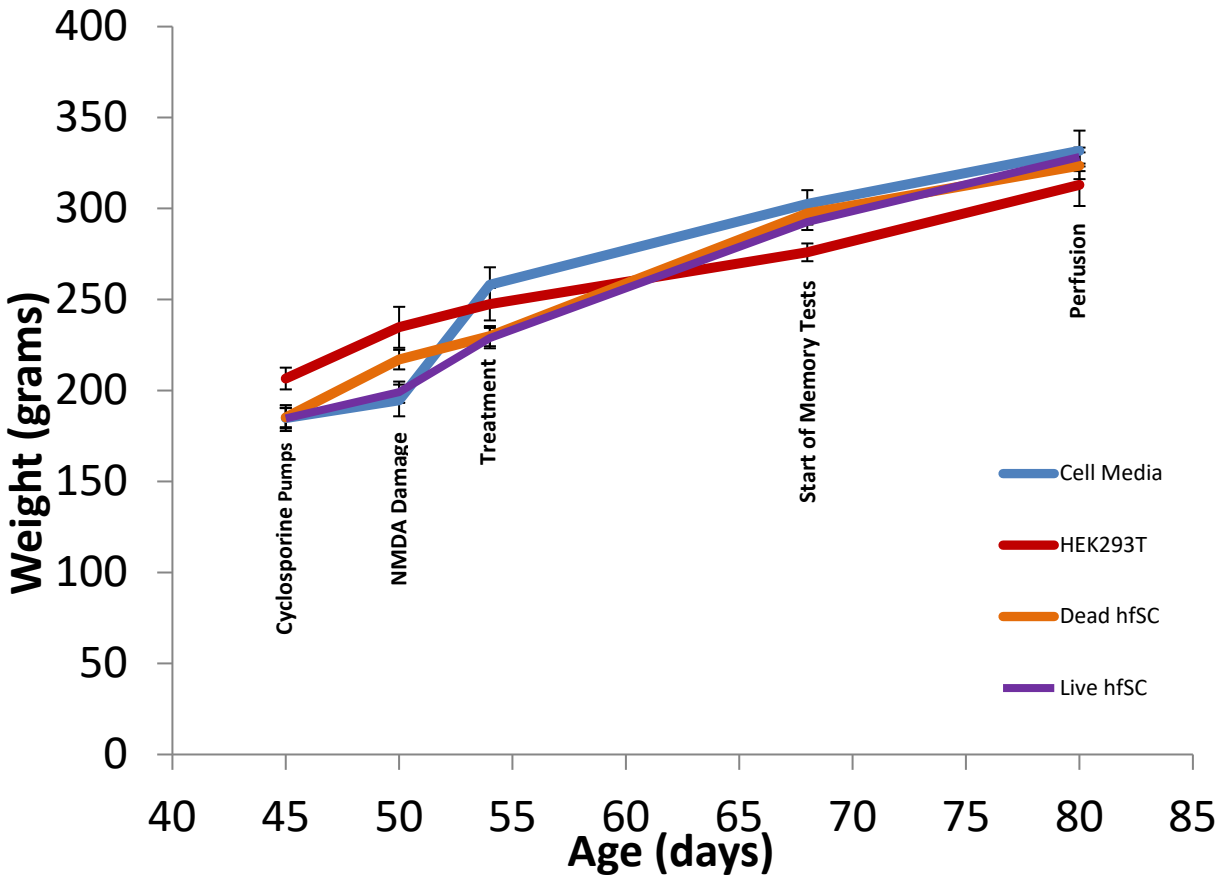

Figure 2. Mean weight gain for rats whose hippocampi were lesioned with NMDA and subsequently injected with cell suspension media (n = 5), HEK293T cells (n = 7), live hNPC (n = 21), or dead hNPC (n = 17). All treatment groups displayed statistically similar weight gains over the course of the experiment, demonstrating that hNPC implantation did not adversely affect rat health as measured by weight gain (Repeated Measures ANOVA; F = 0.435; p = 0.729). All values are means ± SEM.

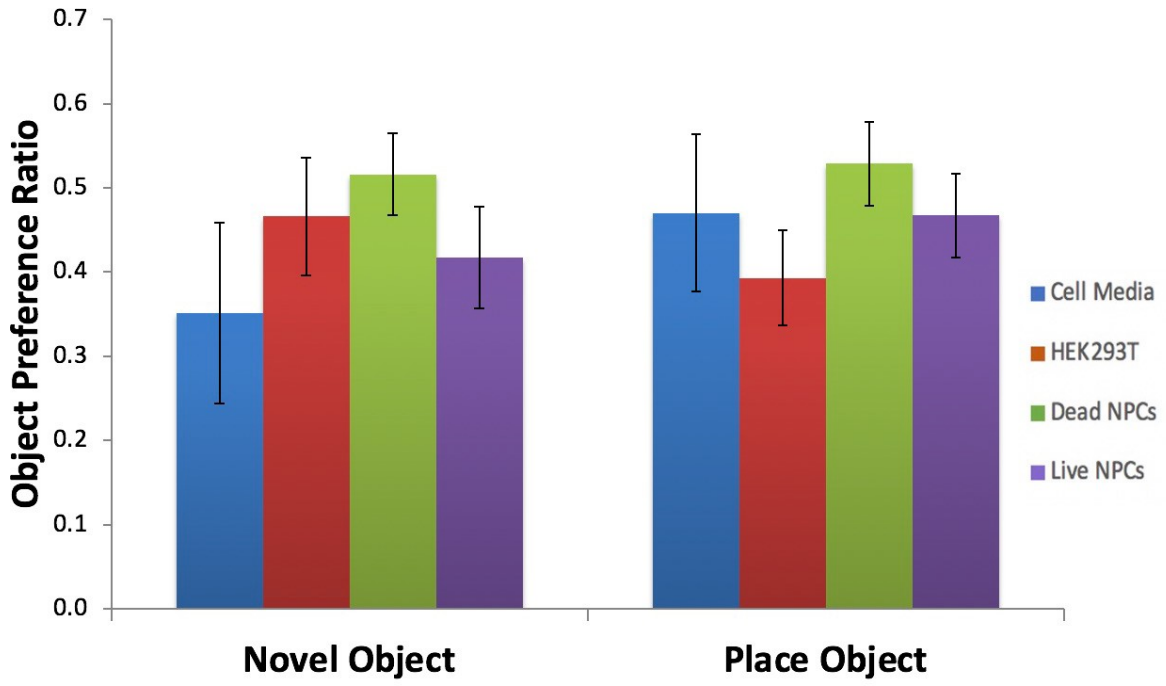

Figure 3. Novel Object-Recognition (A) and Place-Object (B) assays were used to test object recognition and place memory in rats injected with cell suspension media (n = 5), HEK293T cells (n = 7), live hNPC (n = 21), or dead hNPC (n = 17) into their NMDA-damaged hippocampi. The data revealed that stereotactic injection of live hNPCs did not affect object recognition or spatial memory in NMDA-lesioned rats compared to the other treatment groups (ANOVA, Novel-Object: $F = 0.855$, $p = 0.471$; Place-Object: $F = 0.730$, $p = 0.539$). All values are means ± SEM.

3.3. Water maze

Water Maze trials were used to test spatial memory in rats injected with cell media, HEK293T cells, live hNPC, or dead hNPC in NMDA-damaged hippocampi. In contrast to the Novel Object-Recognition and Place-Object behavioral trials, statistical differences were observed regardless of entry position (Left Entry Point, ANOVA; $F = 14.465$, $p < 0.001$; Right Entry Point: ANOVA; $F = 9.638$, $p < 0.001$, Figure 4) in the water maze trials. Statistically, lesioned rats that were transplanted with live hNPCs performed significantly better ($p < 0.001$) than those rats implanted with the various controls, including cell suspension media (vehicle), HEK293T cell, or dead hNPC treatment groups.

3.4. Histological analyses

The combination of immunostaining using human nuclear antibodies (detected with DAB) and cresyl violet counterstain was used to analyze location and survival of human NPCs in hippocampal sections. All rats received 1 µl of 1 mg/ml NMDA injected bilaterally into the CA3c region at day 50 of age, followed by treatment at day 55 of age. At 80 days of age, all animals were perfused. Rat brains were sliced and processed within one week of perfusion, and the histological results are displayed in Figure 5. The HEK293T control group showed abnormal, tumor-like growth in the hippocampus

region (Figure 5a; arrow shows the tumor); this type of tumorigenic growth has been observed previously with HEK293T brain implantation [16]. No tumors were observed in any other treatment, including live hNPC transplants. The dead hNPC control group showed clearly within the damaged CA3c region as a visible clump of darkly-stained, disfigured cells (Figure 5b; as shown by arrow). Finally, the large nucleated, human NPCs were clearly visible (darkened DAB immunostaining illuminated by cresyl violet counterstain) using a both low magnification (4×) and especially under higher magnification (40×) (Figures 5c—as shown by an arrow, and Figure 5d; highlighted by an arrow). Note, the much larger stained nuclei in Figure 5d compared to the surviving rat hippocampal cells within the CA3c region. Immunostaining (darkened by DAB reactivity) revealed that hNPCs survived *in vivo* for at least 28 days post-implantation as the higher magnification panel highlights many viable hNPCs in the damaged CA3c region (Figure 5d).

In addition, please examine the rat hippocampal cells within the dentate gyrus in Figure 5. Note that the CA3c region is devoid of cells in both sets of negative control panels (Figures 5a and b). Now, compare the paucity of cells in these panels with the CA3c regions depicted in Figures 5c and d. Note, the healthy CA3c cells were found only in those slices with the live NPC treatment. This observation is in agreement with our past experiments showing that these human NPC cells provide significant neuroprotection to neighboring rat cells.

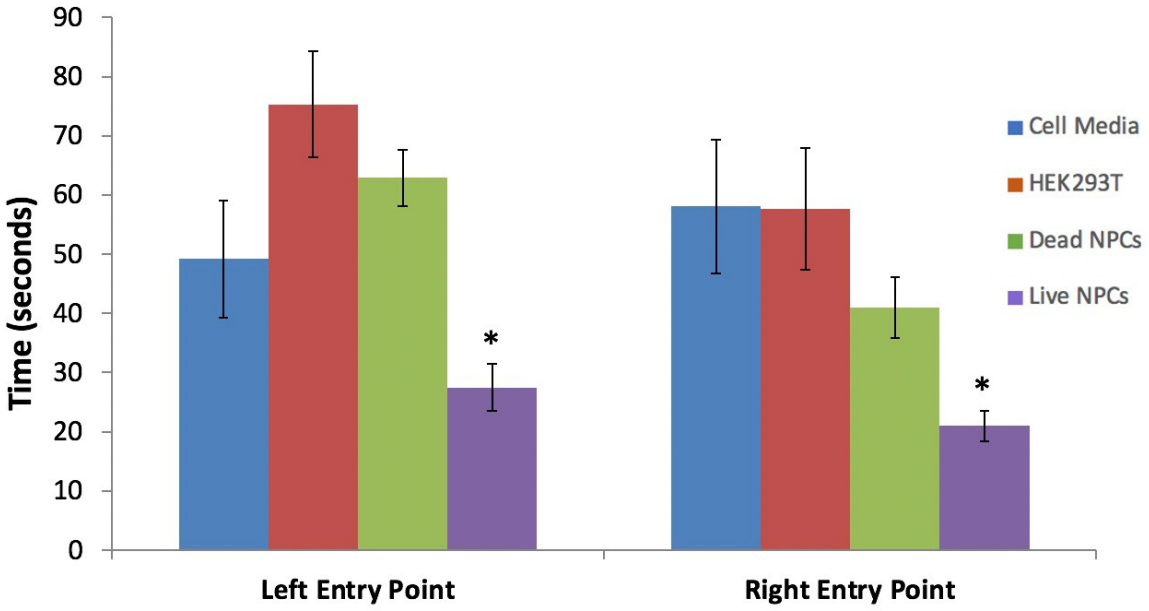

Figure 4. Water Maze behavioral trials were used to test spatial memory in rats injected with cell suspension media (n = 5), HEK293T cells (n = 7), live hNPC (n = 21), or dead hNPC (n = 17) in NMDA-damaged hippocampi. Rats with live hNPC implantation performed significantly better (Left Entry Point, ANOVA; $F = 14.465$, $p < 0.001$; Right Entry Point: ANOVA; $F = 9.638$, $p < 0.001$) than those implanted with cell media, HEK293T cells or dead hNPC in NMDA lesioned rats. Asterisks represent statistical differences. All values are means ± SEM.

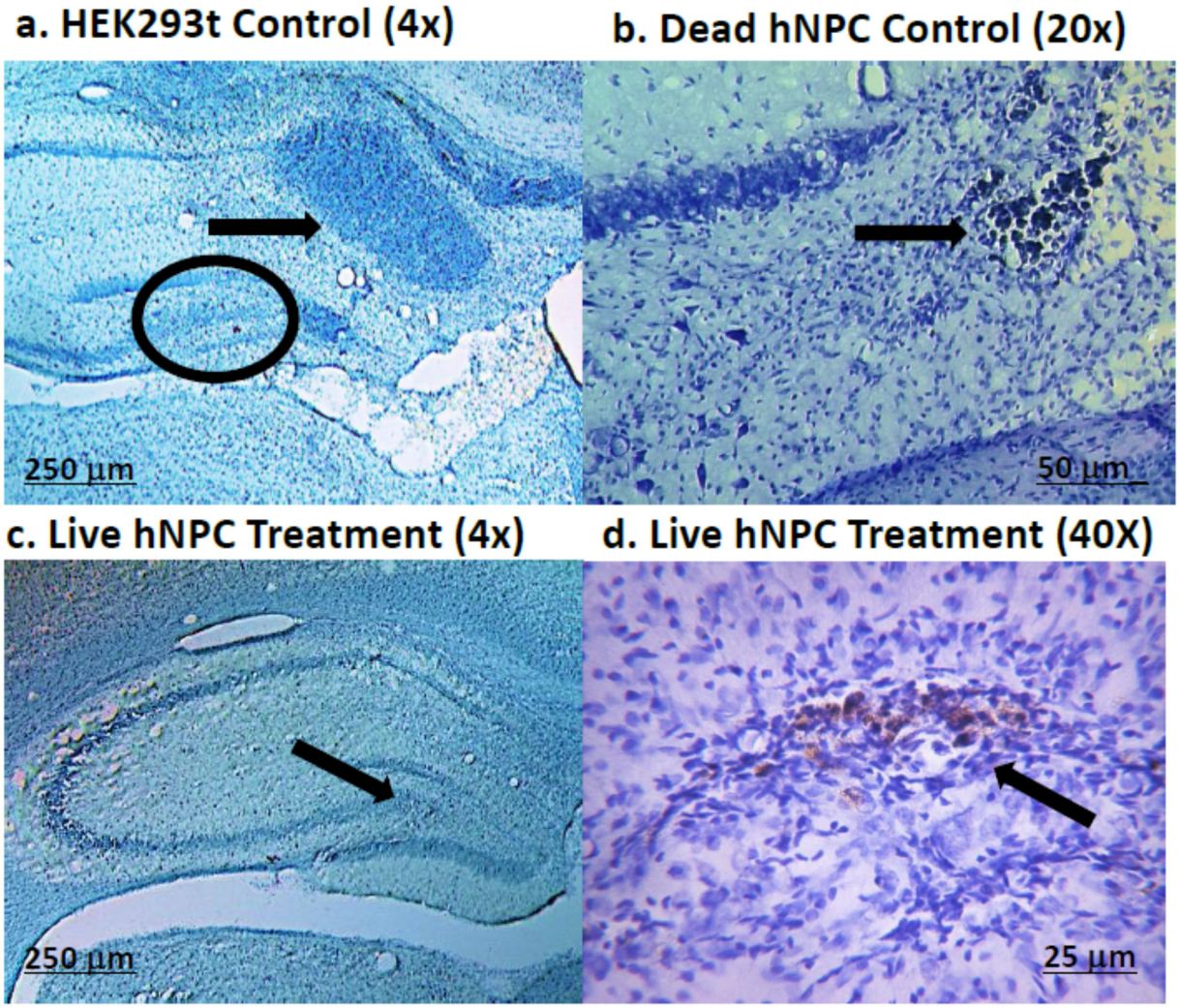

Figure 5. Photomicrographs of double stained (human nuclear immunostaining counterstained with cresyl violet) hippocampi of rats that received 1 µl of 1 mg/ml NMDA injected into the CA3c region at day 50 of age, followed by injection of HEK293T (Figure 5a), dead hNPC (Figure 5b), or live hNPC (Figures 5c and 5d) implantation at day 54 of age. Figure 5a shows the typical tumor growth (arrow) caused by HEK293T cell implantation. Also, note the paucity of surviving CA3c cells (within the circle) within the dentate gyrus. Figure 5b displays the damaged CA3c region and a DAB-enhanced clump of dead hNPCs (arrow). Figure 5c reveals many small, labeled hNPCs growing within the CA3c region (arrow); while Figure 5d is a higher magnification image showing location of surviving, double-labeled CA3c cells (arrow). Also, note the survival of rat CA3c neurons scattered around the human cells. The brown color is indicative of anti-human nuclear antibody immunostaining (DAB labeling).

4. Discussion

The ability of human neuronal progenitor cells (hNPCs) to ameliorate the behavioral effects of bilateral NMDA lesioned CA3c region of the hippocampus was examined. To summarize, our results

showed that rats that received live hNPC implantation performed significantly better in the water maze task than control groups (Figure 4), demonstrating the potential ability of hNPCs to improve spatial memory following hippocampal injury. Surprisingly, both the novel and place object assays showed no significant differences among the treatment groups (Figure 3). Histological results confirmed the survival of implanted hNPCs up to 28 days post-implantation (Figure 5c) with hNPC presence observed within the CA3c region (Figure 5d). The implantation of a different human cell, HEK293T, did not improve performance in any memory trials, reiterating the observation that only specific human replacement neural progenitor cells must be used to ameliorate damage in the rat CNS.

We chose to lesion the CA3c region because of its extensive interconnections known to be involved in working spatial memory as well as memory consolidation [17]. In studies using focal injection of a presynaptic zinc chelator diethyldithiocarbamate to inactivate temporarily the CA3 region during the consolidation phase of the water maze task, researchers found that mice showed significant deficits in working spatial memory performance when compared with untreated control animals [18,19]. We also confirmed the role of the CA3c in memory consolidation since our controls (media, HEK or dead hNPC treatments) did not alter the adverse effects of neurotoxin-induced CA3c region damage especially in the water maze memory test. Only those rats provided with implanted hNPCs were able to augment functional memories in the water maze task.

Additionally, we were able to observe that hNPC transplantation had no apparent impact on short-term memory formation which has limited storage and rapidly decays over time. Shorter term memory was tested using the novel object and place object trials since these animals were given a short, five-minute familiarization period with two identical objects before they were moved to their cages for only a five-minute retention period. In contrast, in the water maze test the animals were given three training sessions on one day, and longer-term memory trials were conducted on the following day (24 hours), allowing the animals significant time to consolidate spatial navigation information.

In previous studies, implanted human stem cells have been shown to not only differentiate into neuronal cells and migrate to areas of CNS damage but also alter behavior [20–22]. Celavie's human NPCs have also demonstrated that ability to migrate to the site of brain lesions and differentiate according to the nature of the lesion or neurodegeneration [12–15]. In this current study, our immunohistochemistry results confirmed that hNPCs survived within the lesioned CA3c region, reversing some of the functional impairments caused by NMDA-induced lesioning (Figure 4). While, this study did not determine if these cells had matured into either neurons or glia, the improvements in longer-term spatial memory demonstrate the potential ability of hNPCs to ameliorate hippocampal damage. In addition, the transplanted hNPCs demonstrated their unique ability to provide neuroprotection to the neighboring rat CA3c cells within the dentate gyrus (Figure 5). This observation of hNPC's neuroprotection of host cells in the rat hippocampus corroborates the similar findings in the rat cerebellum [14,15].

Replacement therapies and its effects on memory have been studied where the whole hippocampus or a specific portion were either bilaterally or unilaterally lesioned. Researchers [7,10,11] performed unilateral lesion studies where they grafted replacement cells into lesioned hippocampal areas. These research groups found that their replacement cells were effective as all animals showed improvements in cognitive function, restoring hippocampal function. However, the technique of unilateral hippocampal lesioning with implanted replacement cells may not account solely for functional improvements since the ability of the surviving contralateral hippocampus may compensate for damage in the lesioned ipsilateral side. In a study comparing unilateral and bilateral hippocampal

lesions, researchers found that unilaterally lesioned rats performed similar to sham rats in spatial memory tasks whereas bilaterally lesioned rats performed significant worse than sham controls [23]. In a similar study [24], unilateral hippocampal lesioned rats were able to recover working memory and spatial memory in the radial arm maze task, performing similar to sham control rats following 21 days of repeated training. In our study, the CA3 region was bilaterally damaged, thereby preventing any contralateral hippocampal compensation, emphasizing the positive effects of hNPC implantation.

In addition, the hippocampus has the characteristic ability to compensate for neuronal loss by synaptic reorganization. Hippocampal neurons have the ability to rewire with surviving neurons following focal lesioning. Specifically, loss of CA3c neurons has been shown to trigger granule cell axons (mossy fibers) to seek new synaptic connections with surviving CA3 pyramidal neurons to maintain hippocampal circuitry and functionality [25,26]. In our experiment, we do not believe that the process of synaptic reorganization influenced the observed significant differences between the live hNPC treatment group and the other control groups in the water maze trials. Although previous studies have shown that synaptic reorganization takes place following traumatic brain injury, researchers have found this process to be insufficient in compensating for significant memory loss [27,28]. In a study examining cognitive function immediately following traumatic brain injury, researchers found that it took up to 50 days for lesioned animals to perform similar to sham controls in water maze tests [29]. Recall, we tested rats at a much earlier post-lesioning age (28 days). In addition, synaptic reorganization in the hippocampus may not be beneficial for a hippocampal-lesioned rat. It has been determined that CA3 synaptic reorganization may lead to increased seizure activity, due to the formation of hyper-excitable neuronal synapses [30]. No seizures were observed in our current study either directly after NMDA injections or after cell implantations. Thus, only those rats that received live cell implants showed significant memory enhancement, implying that the hippocampus had not utilized another compensating mechanism (like synaptic reorganization) to restore function.

Finally, one other mechanism of compensation can occur in the lesioned hippocampus: neurogenesis. Following traumatic brain injury, endogenous hippocampal stem cells found in the dentate gyrus have been shown to migrate, differentiate and form synaptic reconnections [31]. Human neurogenesis occurs throughout the hippocampi in healthy, non-cognitively impaired aging humans [32]. Yet, the process of hippocampal neurogenesis takes approximately 60 days for stem cells to fully mature into functional neurons in rats [27]. Because of the time factor, neurogenesis was unlikely a major influence in our study since cognitive tests were conducted 2–3 weeks following lesioning and subsequent hNPC implantation which would not allow time for hippocampal neurogenesis and comprehensive neurodevelopment [27]. Despite the ability of the hippocampus to compensate for neural damage via neurogenesis or synaptic reorganization, this compensation often falls short of full recovery of memory in damaged hippocampi [28].

Since this was a pre-clinical study designed to examine behavioral effectiveness of cell transplantation, we did not evaluate how hNPCs induced memory consolidation in the NMDA-induced lesioned hippocampus. However, based upon previous work with this cell line in ataxic rats, we can speculate that cell-specific paracrine factors may have initiated the restorative effects observed in these experiments. Although we did not quantify neurotrophic levels in the lesioned rat hippocampus, a previous study [15] confirmed that cell-released permeable factors likely elicited the observed neuroprotective effects. Based on an unpublished proteomic study (performed by National Research Center of Canada), Celavie's hNPCs were found to secrete the following paracrine factors: 1. Neuromodulin (GAP-43)—which promotes neuronal growth, neuronal plasticity, and neuronal

protection during neural development and regeneration after traumatic brain injury [33–35]; 2. Hyaluronan and proteoglycan link protein 1—shown to trigger the formation of perineuronal nets [36–38]; 3. 14-3-3 epsilon protein—found to be responsible for regulating signaling and neural development [39,40]; 4. Ceruloplasmin—a serum ferroxidase that has been shown to be linked to the pathogenesis found in AD, PD and cerebellar ataxia [41,42]; 5. Insulin Growth Factor II (IGF II)—discovered to promote neuroprotection and neuroplasticity recovery [43]. These factors may play important roles in the functional recovery we observed in our experiments with hNPCs. Further *in situ* biochemical analysis would help confirm that these localized changes were induced by the same neurotrophic factors associated with Celavie's hNPC line.

5. Conclusions

The implantation of hNPCs improved spatial memory significantly in rats who had their CA3c regions lesioned by NMDA. This result is of immense clinically importance since there are currently very limited treatments to repair fully hippocampal damage. It is also important to note that the hNPC implantation did not have an adverse effect on animal health. This study has demonstrated that Celavie's human-derived neuronal progenitor cells were able to survive *in vivo* and improve hippocampal functionality, thereby confirming the potential promise for replacement cell treatment of brain damage in neurodegenerative diseases.

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Conflict of interest

The authors declared no impending conflicts of interest with regard to this study, authorship, or publication of this article.

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