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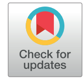
Original Article

Neuronal Protective Effect of Nosustrophine in Cell Culture Models



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Abstract

Background and objectives: Alzheimer's disease (AD) is characterized by the progressive degeneration of neurons and pathological activation of glial cells. The present study aimed to investigate the potential protective effects of Nosustrophine, a nootropic supplement derived from young porcine (*Sus scrofa domesticus*) brains on the progression of neurodegeneration.

Methods: Different concentrations of the lyophilized Nosustrophine extract were added into the SH-SY5Y neuroblastoma cell line, hepatocarcinoma heppG2 cell line and rat neuronal and glial cells with or without different treatments. The viability of cells and the response of neurons, astrocytes and microglia to oxidative stress were measured and compared.

Results: The cell viability of SH-SY5Y cells treated with low concentrations of Nosustrophine was notably improved, when compared to control cells. In the HepG2 hepatocarcinoma cell line, Nosustrophine had a moderate, concentration-dependent impact on cell viability, with the most significant effects observed at concentrations greater than 1 mg/mL. However, Nosustrophine did not confer any toxic effects on human cell lines, sustain neuronal survivability rates, or significantly enhance the astroglial cell survival in mouse primary neuronal and glial cells. The protective effect of Nosustrophine on microglia was inversely correlated to the drug concentration in the culture medium. It was found that Nosustrophine was protective against Aβ1-42-induced neurodegeneration in mouse organotypic hippocampal slice cultures.

Conclusions: Nosustrophine has potent neuroprotective properties, enhances neural plasticity, and may be a potential therapeutic option for degenerative diseases.

Introduction

The gradual death of neurons in diverse parts of the nervous system is a feature of neurodegenerative diseases.[1] This loss of nerve cells leads to distinct neurological and cognitive symptoms that are specific to each condition. A prominent pathological feature in Alzheimer's disease (AD) is the loss of neurons and synapses in the cerebral cortex and subcortical regions.[2] This causes the atrophy of some affected areas due to pathological abnormalities, including the accumulation of β-amyloid protein and cellular material in thick, insoluble deposits around and outside of neurons. The accumulation of Aβ and tau proteins in the brain has led to the definition of AD as a condition that produces proteotoxic proteins.[3] Previous studies have attempted to identify new therapeutic approaches. However, effective medications that can slow or halt the progression of neurodegenerative diseases, such as AD, are yet to be discovered.[4–8]

In the majority of cases, the supplementation of culture media with serum or other substances is required. However, there are a number of drawbacks to using serum, including high costs, unknown composition, and greater risk of contamination with accidental chemicals. For these reasons, the porcine (*Sus scrofa domesticus*) brain is a reliable source of a number of growth factors.[9] Indeed, the growth factors released from neural tissues, such as those in the brain and retina, are powerful mitogens for mesoderm-

Keywords: Alzheimeŕs disease; Cellular screening; Nosustrophine; Neurodegeneration; Neuroprotection.

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid beta; BDNF, brain-derived neurotrophic factor; CA1-2, hippocampal subfields; GDNF, glial cell line-derived neurotrophic factor; HepG2, hepatocarcinoma cell line; LDH, lactate dehydrogenase; MHC, major histocompatibility complex; NST, Nosustrophine; OGD, oxygen and glucose deprivation; OHSCs, organotypic hippocampal slice cultures; PBE, porcine brain extract; SH-SY5Y, neuroblastoma cell line.

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derived cells, particularly for vascular endothelial cells and some ectoderm-derived cells.[10] These chemicals are known as pituitary and brain fibroblast growth factors, due to the ability to stimulate fibroblast proliferation.[11] A large number of sympathetic,[12] sensory,[13] and parasympathetic[14] neurons respond to neurotrophic chemicals by maturing, growing, and/or needing maintenance. The importance of substrate-binding neurite-promoting factors (NPFs), which are required under particular culture conditions for neurotrophic factors to affect peripheral neurons, has been highlighted.[14–18]

The number of astrocytes in the mammalian brain is consistently steady throughout maturity,[19,20] and this is most likely caused by the coexistence of specific mitogens and mitogen inhibitors.[21] Specific astroblast mitogen inhibitors exist in rat brain,[22,23] and one such inhibitor, neurostatin, was recently discovered in rat and bovine brain extracts.[24] Neurostatin shares epitopes with human blood types and the carbohydrate moiety of the epidermal growth factor (EGF) receptor.[22,23] The elimination of an experimental rat brain tumor *in vivo* was aided by neurostatin.[22,25,26] Furthermore, the artificial oligosaccharide counterparts of neurostatin can prevent astrocyte, glioma, and neuroblastoma cell division in culture. In addition, the brain-derived trophic peptides used to treat endogenous neurotrophic factor deficiencies can decrease the degeneration of neurological diseases.[27,28] In order to protect neurons from oxidative stress-induced degeneration, these peptides promote cell survival and possible cell growth, in addition to other beneficial effects, *in vitro* and *in vivo*.[29,30] Neurotrophic drugs may also improve neuronal metabolism and cell performance, which can restore synaptic plasticity through the growth of new axons, enhance cognitive function by increasing neural connectivity, and enhance long-term memory.[31] There is presently no effective treatment to stop the progressive degeneration of affected brain areas, and standard care typically concentrates on palliative medications to postpone dementia. Therefore, pharmacogenomic methods would directly contribute to improve pharmaceutical treatment responses for people with AD or other similar disorders.[32] Nosustrophine is a novel pleiotropic epigenetic bioproduct, which is a nootropic supplement produced from young porcine (*Sus scrofa domesticus*) brain through non-denaturing biotechnological methods.[33] The intended function of this formulation is to stimulate endogenous neuropeptide synthesis and release by activating neuro-enzymatic processes.

The present study aims to examine the neuroprotective effects of Nosustrophine against cellular degeneration, which lead to the development of neuropathologies. In order to determine the response of neurons, astrocytes, and microglia to Nosustrophine at different concentrations, cell culture models were used in the presence or absence of oxidative stress. The present findings revealed that Nosustrophine can reduce the activation of microglia, and has a neuroprotective effect on neurons and astrocytes in culture.

Materials and methods

Biochemical characterization of Nosustrophine

Nosustrophine is a biological extract and an epigenetic bioproduct[33] synthesized from the brain of *Sus scrofa domesticus* using non-denaturing biotechnological methods (Patent ID: P202230047/ES2547.5).

Compound analysis: The nutrient profile and analysis of the catecholamines, serotonin, L-dopa and neurotrophic factors of the powdered extract have been examined, and previously published.[33]

Experimental design

Treatment preparation: A stock solution (20 mg/mL) of lyophilized Nosustrophine extract (young porcine brain extract [PBE]) was sonicated in sterile filtered 0.9% NaCl, and centrifuged at 3,000 g for three minutes. Then, the supernatant was collected and used for all cell culture experiments.

For the analytic assays, 4.5×10^5 cells were grown for 24 hours in 6-well plates at 37°C. Then, these cells were exposed to 10 µg/mL and 50 µg/mL of Nosustrophine for 0, 3 and 24 hours.

Cell line culture assays

Cell lines: Human neuroblastoma SH-SY5Y and hepatocarcinoma HepG2 cell lines were maintained in Roswell Park Memorial Institute (RPMI, Gibco) or Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with 1% penicillin/streptomycin (Gibco) and 10% heat-inactivated fetal bovine serum (Gibco). Then, the cells were incubated at 37°C in a humidified incubator with 5% CO_2. These cells were kindly provided by Dr. Ana Aranda (Instituto de Investigaciones Biomédicas, Madrid). The SH-SY5Y neuroblastoma cell line is a commonly used experimental model for studying the molecular mechanisms underlying AD, due to its ability to differentiate into neurons, low cost, and ease of handling.

Cell viability assay: Cell viability was determined by Presto-Blue Cell Viability assay (Thermo Fisher). Cells (1×10^4) were incubated with different concentrations of Nosustrophine (0.05–10.00 mg/mL) for 72 hours in 96-well plates. Then, the Presto Blue reagent (10 µL) was added to each well, and incubated for three hours. Afterwards, the absorbance was recorded at 570 nm, with the absorbance at 630 nm used as the reference. Eight replicates were performed for each condition, and the experiment was repeated twice.

Primary cultures of cortical neurons

Obtaining the cells: All experimental procedures were performed in accordance with the European Community Law (86/609/EEC), European Union Directive 2016/63/EU, and the Spanish Royal Decree (R.D. 1201/2005). Study procedures were reviewed and approved by the Ethics Committee of the International Center of Neuroscience and Genomic Medicine.

Before starting the dissociation process, the culture plates were treated with poly-L-lysine to enhance the cell adhesion to the surface. These cells were obtained from 17-day gestation Wistar rat fetuses. These rats were decapitated, and the fetuses were extracted and washed with washing buffer (150 mM of NaCl, 8 mM of $Na_2HPO_4 \cdot 2H_2O$, 2.7 mM of KCl, 1.45 mM of KH_2PO_4 and 2.6 mM of $NaHCO_3$, pH = 7.2). Then, the cerebral cortices were obtained by transferring the fetuses to a plate that contained commercial dissection medium (L-15). Next, the meninges were removed from the cortex before homogenization. Then, the cleaned cortex was transferred to a plate that contained the incubation medium, which consisted of 80% (v/v) Minimum Essential Medium (MEM), 10% (v/v) horse serum, 10% (v/v) fetal serum, 1.98 mM of glutamine, 3.3 mM of glucose, and 16 mg/L of gentamicin sulfate. Afterwards, the tissue was homogenized after mechanical disruption.

Sub-culturing: After homogenization, the number of obtained cells was counted. In order to count these cells, the Trypan Blue exclusion method was used in a Neubauer chamber. When the number of cells obtained was known, these were suspended in "incubation medium" up to the required density, which was 2×10^5 cells/cm^2 for the present study. Then, these were seeded in 12-unit multi-well Petri dishes (∅ = 2.2 cm).

Cell culture maintenance: During the first four days, the cells were maintained in the incubation medium. On the fourth day, this was changed to a growth medium (90% [v/v] MEM, 10% [v/v] horse serum, 1.98 mM of glutamine, 3.3 mM of glucose, and 16 mg/L of gentamicin sulfate), which included a cytostatic agent, cytosine arabinoside, in order to prevent the growth of different proliferating cells, such as glia. At one week after these were plated, the medium was changed to a new growth medium that excluded cytosine. With this treatment, a homogenous neuronal culture was obtained that contained an approximately 5% glial cell population.

Primary cultures of glial cells

Obtaining the glial cells: In order to obtain a culture with a mainly glial cell population, the procedure performed was similar to that for obtaining neurons with some modifications. The seeding density was 5×10^4 cells/cm^2, and no cytosine arabinoside was added on the fourth day after seeding. In this manner, the proliferation and growth of the glia was allowed. The percentage of each cell population was, as follows: 15 ± 3% neurons, 75 ± 8% astrocytes, and 10 ± 2% microglia. In addition, the experiments were conducted at two weeks after seeding.

Oxygen and glucose deprivation (OGD)

The experiments were performed at nine or 10 days after sub-culturing. In order to simulate the ischemia *in vitro*, OGD was performed in a chamber (Forma Scientific) at 37°C, with the total absence of glucose, and an anaerobic nitrogen atmosphere (95% N_2/5% CO_2). Before commencing any treatment, the cells were washed twice with the "ischemia buffer" (130 mM of NaCl, 5.4 mM of KCl, 1.8 mM of $CaCl_2$, 0.8 mM of $MgCl_2$, 1 mM of NaH_2PO_4 H_2O, and 26 mM of $NaHCO_3$; pH = 7.2) used during OGD, in order to remove the growth medium. During the OGD, the pressure (0.5 psi) and temperature of the system were kept constant. The control group was maintained in an aerobic atmosphere with the glucose buffer (the same as the ischemia buffer, but contains 33 mM of glucose) for the same duration. At the end of the OGD period, the cells were washed twice with reperfusion medium (the same as the growth medium, but gentamicin was replaced with 0.15 ng/mL of penicillin), and fixed in this medium for a 24-hour reperfusion period. The OGD duration in the glutamate release experiment was 150 minutes. The time range of 140–160 minutes was chosen, because glutamate was observed to be released at this time, and there was no lactate dehydrogenase (LDH) release. Thus, experiments in which the LDH values of the control and OGD groups significantly differed were not considered. This ruled out the possibility of unwanted glutamate release due to cell rupture and its subsequent consequences (excitotoxicity), which would interfere with the study of the release mechanisms and the effect of Nosustrophine in this process. For the experiments, the OGD duration was 150 minutes. After the experiments, different cell viability parameters, such as LDH, were measured. Then, the medium was removed after 150 minutes, and the cells were washed with reperfusion buffer and kept in an incubator at 37°C. This allowed the LDH to be measured at 0, 3 and 24 hours after OGD. These experiments were performed to verify the occurrence of cell death after OGD, and determine whether Nosustrophine is capable of reducing this. Different concentrations of Nosustrophine (10, 50 and 100 µg/mL) were added at the start of the OGD period.

Preparation and treatment of mouse organotypic hippocampal slice cultures (OHSCs)

The OHSCs were prepared from postnatal day 4–6 mice, following an established protocol.[34] After decapitation, the brains were removed, and the hippocampi were dissected and transversely cut in 350 µm sections using a McIlwain tissue chopper. In the intact state, the hippocampal sections were selected and placed onto porous polyethylene (PTFE) membrane inserts (PICM0RG50, Merck Millipore), with three sections per insert. Then, the inserts were transferred to 6-well plates, with each well containing 1.2 mL of culture medium. Next, the culture medium comprised of MEM supplemented with 2 mM of GlutaMAX™ (Thermo Fisher Scientific), and this was adjusted to pH 7.3. In addition, the medium was supplemented with 20% heat-inactivated horse serum, 0.00125% ascorbic acid, 1 µg/mL of insulin, 1 mM of $CaCl_2$, 2 mM of $MgSO_4$, 13 mM of D-glucose, and 1 mM of GlutaMAX™. Then, the OHSCs were cultured at 37°C in a humidified CO_2-enriched atmosphere, and the medium was changed twice each week for the subsequent 2–3 weeks.

In order to prepare the Aβ1-42 peptide solution, 1 mg of human amyloid β-peptide (1-42) (Tocris, Bio-Techne, Wiesbaden, Germany) was dissolved in 1 mL of sterilized distilled water, and stored at −20°C. Then, the peptides were aggregated by incubation at 37°C for 72 hours.[35,36] In order to induce the Aβ1-42-induced neurotoxicity, the slices in the serum-free medium were exposed to a final concentration of Aβ1-42 (25 µM) on day 22 of the *in vitro* culture. The culture medium (300 µL) that contained the Aβ1-42 peptides were applied on top of the slices, and 700 µL was added underneath the slices. The control slices were only treated with serum-free medium. For slices that were exposed to both Aβ1-42 and Nosustrophine, the OHSCs were initially pretreated with 50 µg/mL or 100 µg/mL of Nosustrophine in serum-free media for 72 hours at 37°C. Then, these slices were exposed to 25 µM of Aβ1-42 in the presence of Nosustrophine (50 µg/mL or 100 µg/mL) for 48 hours. Finally, the slices were washed with serum-free media and harvested.

Immunofluorescence

A total of nine cultured hippocampal explants were fixed in 4% paraformaldehyde, and blocked with 5% bovine serum albumin (BSA; Sigma, Japan) in phosphate buffered saline (PBS), which contained 0.1% Triton X-100, for 1.5 hours. Then, these were permeabilized with 0.3% Triton-X 100 in 0.1 M of PBS for 30 minutes, and blocked again in 5% BSA in 0.1 M of PBS, which contained 0.1% Triton X-100, for 1.5 hours. Afterwards, the slices were incubated overnight with the primary antibody against the neuron-specific protein NeuN (1:1,000; MAB-377, Millipore), and detected using the Alexa Fluor-488-tagged secondary antibody (Thermo Fisher Scientific). The specificity of the fluorescent immunostaining for each antibody was confirmed by omission of the primary antibody. Then, the slices were counterstained with DAPI (Vector Laboratories). Several images of the three hippocampal explants from each treatment group were captured using the Leica DM6 B upright microscope and LAS X software. The mean density among the triplicates of immunofluorescence cell markers relative to the background in each explant image was quantified using the area/pixel analysis software (Pixcavator 4).

Determination of lactate dehydrogenase activity

LDH is a cytosolic enzyme released into the extracellular space as a consequence of cell lysis. The demand for ATP, when compared to aerobic ATP supply, causes the accumulation of ADP, AMP and pyruvate. This glycolytic flux leads to the production of pyruvate, which exceeds the metabolic capacity of pyruvate dehydrogenase and other shuttle enzymes that metabolize pyruvate. This mecha-

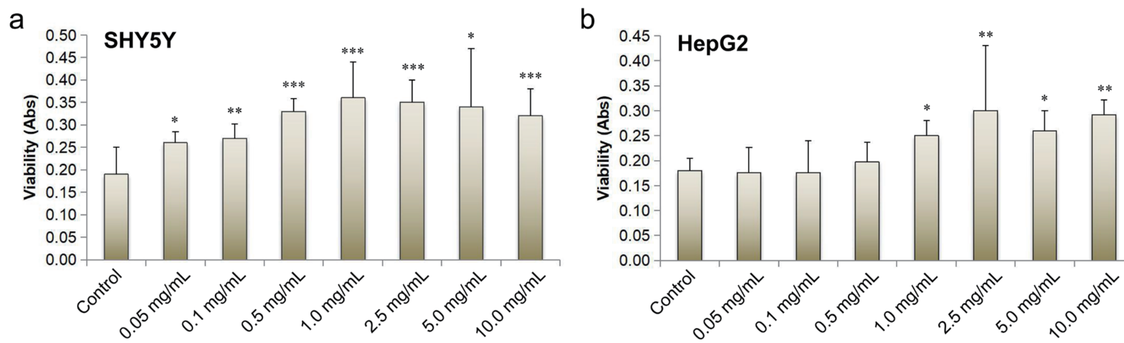

Fig. 1. Nosustrophine increases cell viability. (a) Viability assay in the neuroblastoma SH-SY5Y cell line; (b) Viability assay in hepatocarcinoma HepG2 cell line. Cells were treated with 0.05–10.00 mg/mL of Nosustrophine, and the cell viability was measured after 72 hours of incubation; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. HepG2, hepatocarcinoma cell line; SH-SY5Y, neuroblastoma cell line.

nism directs the flow of pyruvate and NAD+ via LDH, producing lactate and NADH in the process. In order to measure this, after OGD, a volume of medium was removed and mixed with the same amount of phosphate/NADH/pyruvate buffer (a final concentration of 350 µM of NADH, and 900 µM of sodium pyruvate; pH 7.4). Then, the kinetics of the different cell groups with specific ATP concentrations (0, 1 and 3 mM) were measured for 150 seconds in room temperature using a spectrophotometer at 340 nm. This wavelength was used to measure the increase in fluorescence, which is an indirect method to record the disappearance of NADH upon oxidation, due to the presence of LDH in the medium. Specifically, the reaction was, as follows:

LDH was expressed as a percentage of the total LDH. In order to calculate this value, the cells were lysed with Triton X-100, and measured at 340 nm. Then, the value, together with the values previously obtained at 0, 3 and 24 hours, were recorded as the total value of LDH. Thus, the released LDH value was determined, as follows:

$$\text{LDH (\%)} = 100 \times \frac{\text{LDH (mean)}}{\text{LDH (mean)} + \text{LDH (cells)}}.$$

Statistical analysis

The data was tested for the normality and equality of variances using the Shapiro-Wilk test and Levene's test. The statistical significance was determined using one-way ANOVA with post-hoc Bonferroni correction: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. All values were expressed as the mean ± standard error of the mean (SEM) of the number of experiments indicated in each case. A p-value of < 0.05 was considered statistically significant (Newman-Keuls test).

Results

Effect of Nosustrophine on the viability of human cell lines

The impact of Nosustrophine on the viability of human cell lines was investigated by evaluating the cell viability rates of two distinct cell lines: hepatocarcinoma (HepG2) and neuroblastoma (SH-SY5Y) cells. In order to assess the cytotoxicity of Nosustrophine, the different increasing concentrations of the drug that was added to the SH-SY5Y cell culture medium were tested (Fig. 1a). The control group presented with the expected low viability rates, since Nosustrophine was not added to the medium. However, as the concentration of Nosustrophine increased in the medium, there was a corresponding significant effect on cell viability. Even the low concentration of Nosustrophine (0.05 mg/mL) resulted in a considerable difference in viability rate, when compared to the control group. The most substantial effect was detected at a concentration of 5 mg/mL of Nosustrophine. The present primary findings demonstrate that Nosustrophine has a significant effect on the viability of neuroblastoma SH-SY5Y cells at concentrations greater than 1 mg/mL (Fig. 1a).

Given its integral role in drug metabolism, the liver represents a suitable organ for assessing drug-induced cytotoxicity. The HepG2 cell line, which is derived from human liver carcinoma, was used as the experimental model system for analyzing the possible adverse effects of Nosustrophine. In order to evaluate the potential impact of this drug on cell viability, a standardized experimental protocol was used, and increasing concentrations of Nosustrophine were applied to the culture medium for HepG2 cells (Fig. 1b). The control group under OGD, in which no Nosustrophine was introduced, had low rates of viability, akin to those detected in the SH-SY5Y cell line (Fig. 1a). Conversely, a moderate, concentration-dependent impact on the viability of HepG2 cells upon the administration of Nosustrophine was observed (Fig. 1b). Furthermore, a modest increase in cell viability rates was observed with Nosustrophine concentrations of greater than 0.5 mg/mL, and the most significant effects were observed with Nosustrophine concentrations that exceeded 1 mg/mL (Fig. 1b). These results collectively suggest that Nosustrophine, even at high doses, may not confer any toxic effects on human cell lines, including both the SH-SY5Y and HepG2 cell lines (Fig. 1a, b).

Effect of Nosustrophine on rat neuronal and glial cell viability

As a model of oxidative stress, primary rat neurons were subjected to OGD (150 minutes). This induced neuronal death, as shown by the LDH viability marker in the control group. However, the administration of Nosustrophine to the culture medium sustained the neuronal survivability rate at all three studied time points. Compared to control cells, the amount of LDH released to the medium after OGD decreased at 0, 3 and 24 hours (Fig. 2). Neurons treated with Nosustrophine (10, 50 and 100 µg/mL) had similar levels of cell viability at zero and three hours after OGD. Nevertheless, at 24 hours after OGD, all Nosustrophine-treated groups presented with a small increase in viability levels (Fig. 2). The LDH marker indicated a significant loss in astroglial cell viability in the OGD-exposed control group. However, the astroglia that were treated with various concentrations of Nosustrophine (10, 50 and 100 µg/

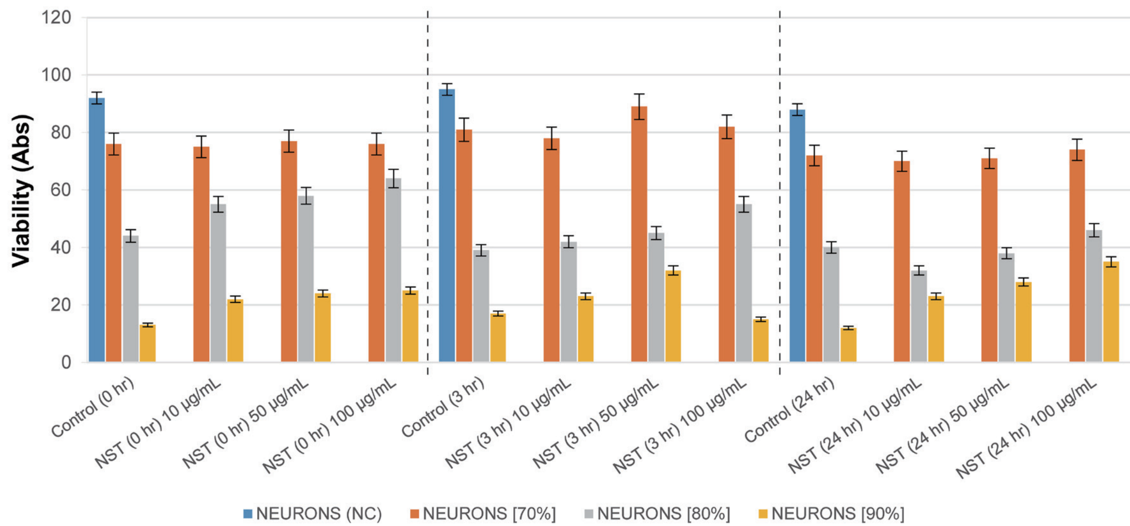

Fig. 2. Nosustrophine modified the survival of the primary culture of rat neurons after oxygen and glucose deprivation. The 150-minute OGD process induced neuronal death in the model used through a cell viability marker (LDH). The incubation with Nosustrophin (10, 50 and 100 µg/mL) significantly modified the LDH values at all different confluence cell rates. LDH, lactate dehydrogenase; NST, Nosustrophine; OGD, oxygen and glucose deprivation.

mL) presented with significant improvements in viability levels, when compared to the control group (Fig. 3). This effect was positively associated with the concentration of Nosustrophine across all three time points. These data suggest that Nosustrophine significantly enhances astroglial cell survival.

In cultured microglial cells, the identical OGD methodology was used on two separate sets of microglia, depending on the type of major histocompatibility complex (MHC) molecule expressed. Both kinds of microglial cells had a similar rate of viability reduction at all three time points, with varying concentrations of Nosustrophine (10, 50 and 100 µg/mL). The protective effect of Nosustrophine on microglia was inversely correlated to the drug concentration in the culture medium (Fig. 4). OHSCs offer a sensitive model for investigating pathogenic responses. The treatment with both Nosustrophine (50 µg/mL) and Aβ1-42 (25 µM) resulted in pyknotic changes, and the loss of pyramidal neurons (Fig. 5b), mainly in the CA1-CA2 and dentate gyrus regions, when compared to the control slices (Fig. 5a). However, higher concentrations of Nosustrophine (100 µg/mL) protected against the Aβ1-42-induced neurodegeneration, preserved the neuronal structure, and led to fewer pathogenic manifestations (Fig. 5c), when compared to 50 µg/mL of Nosustrophine.

Discussion

Neurotrophic factors have potential as treatments for neurodegenerative diseases, but its clinical application remains limited by challenges related to its transport to the brain and suboptimal pharmacokinetic profiles. In order to address this, safe delivery methods and the investigation of the duration of its effects are necessary. The encapsulation of natural neuroprotective extracts is a

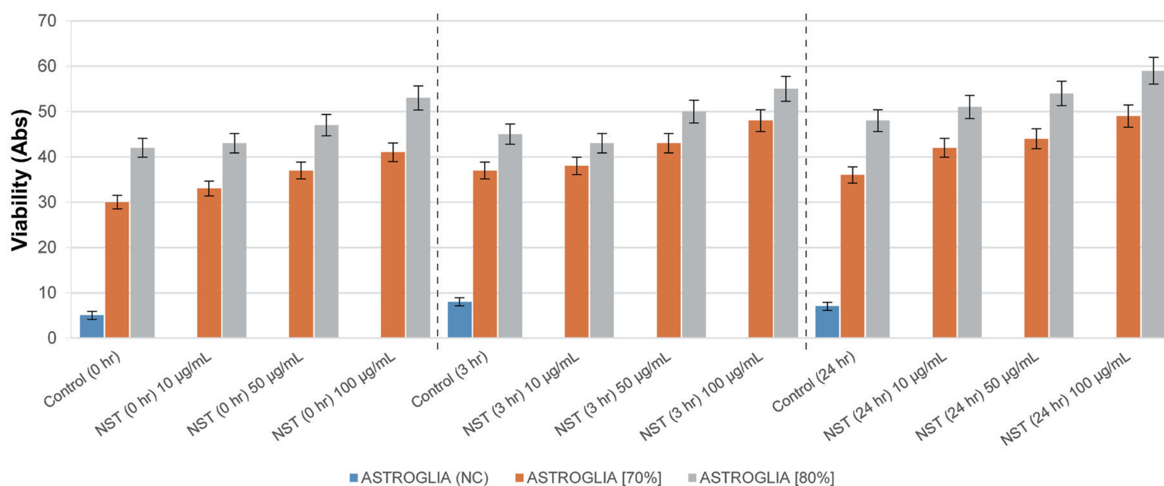

Fig. 3. Nosustrophine improved the survival of astroglial cells after oxygen and glucose deprivation. The results for the glutamate release were significantly increased by the presence of Nosustrophin in cultures of microglia exposed to OGD. The incubation with nosustrophin (10, 50, and 100 µg/mL) significantly modified the LDH values at all different confluence cell rates. LDH, lactate dehydrogenase; NST, Nosustrophine; OGD, oxygen and glucose deprivation.

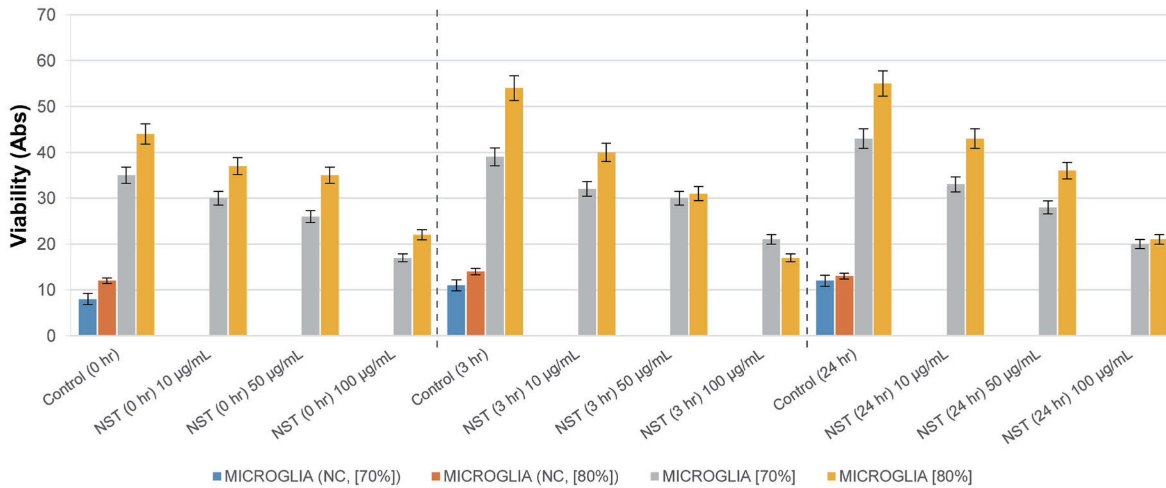

Fig. 4. Nosustrophine improved the survival of microglia cells after oxygen and glucose deprivation. The results for the glutamate release, although not significant, were modified by the presence of Nosustrophin in mixed-neuron-glia cultures exposed to OGD at all different confluence cell rates. NST, Nosustrophine; OGD, oxygen and glucose deprivation.

promising approach for delivering neurotrophic factors to the brain during neurodegenerative disease phases. Combined with stem cell transplantation, this approach can enhance the neuroprotection, and promote tissue repair. The present study demonstrated the potential of Nosustrophine as a biological compound for harnessing the advantageous traits of PBE. Earlier studies have demonstrated the neuroprotective effects of PBE in hypoxia-induced diseased animal models, and the enhanced proliferation and differentiation of primary cells from the ovary, uterus and heart of rats, when PBE was added to the culture media.[9,37] However, the neuroprotective effects of PBE on mouse and human cell lines remain undocumented. The present study revealed that Nosustrophine, which is a pure PBE molecule, can promote high levels of cell viability and survival rates, in both animal and human cultured cells. The present findings suggest that Nosustrophine has neuroprotective effects, which increase glial density and promote neuronal survival. This effect may be beneficial for reducing common neuropathologies, since the cellular densities of the tested cell lines were higher than those of the control group. Furthermore, the present findings align with the findings of earlier reports that demonstrated the neuroprotective and neurotrophic properties of cerebrolysin, which is a commercially available porcine-derived brain extract.[38,39]

Growth factors regulate cell growth and proliferation *in vivo* and in culture. Growth factors are required for the proliferation of non-transformed cells in culture, and numerous factors are usually required. Since these deplete faster than other components of the culture media, these factors are rate-limiting for cell proliferation. Neoplastically altered cells may lack or require less growth factors, which may provide a growth advantage, and this is a distinguishing feature of cancer cells. At increasing concentrations, Nosustrophine exhibited a considerable impact on the viability of hepatocarcinoma HepG2 and neuroblastoma SH-5YS cells, possibly indicating the interaction with these protein factors. Furthermore, in the present study, the HepG2 cell line data indicated that

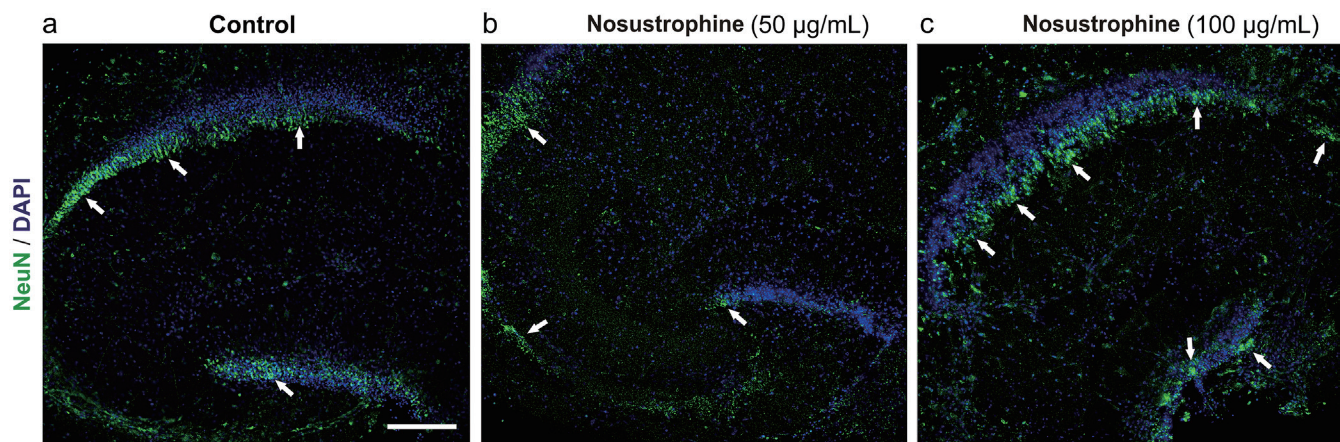

Fig. 5. Nosustrophine is protective against Aβ1-42-induced neurodegeneration in organotypic hippocampal slice cultures. Transverse slices of hippocampi obtained from neonatal mice pups were maintained on Millipore inserts for 22 days. The slices were exposed to (a) the vehicle (control), (b) Nosustrophine (50 µg/mL) with Aβ1-42 (25 µM), and (c) Nosustrophine (100 µg/mL)+Aβ1-42 (25 µM). Then, these were fixed and immunostained for NeuN (green). Afterwards, the nuclei were counterstained with DAPI (blue). The images were processed for maximal intensity projection. The white arrows point to the neurons. Scale bar: 80 µm. DAPI, neuronal nuclei; NeuN, marker of postmitotic neurons.

the Nosustrophine treatment had no adverse effects on the liver. However, research into the molecular mechanisms related to the proteins in Nosustrophine remains at its early phases.

The organotypic hippocampal slice culture is a suitable model system for studying the mechanisms of neurodegeneration, since this preserves several key features of the hippocampal circuitry *in vitro*, including synapse maturation and intrinsic signaling pathways.[40] The investigators previously reported that in 8-9-month-old transgenic AD (APP/BIN1/COPS5) mice, Nosustrophine substantially reduced the Aβ1-42 immunoreactivity levels, when compared to saline-treated mice. The present study confirmed that finding, and revealed that the treatment with 100 µg/mL of Nosusrophine was protective by reducing cellular damage and neuronal loss after 48 hours of co-exposure to Aβ1-42. However, the treatment on hippocampal slices with 50 µg/mL of Nosustrophine did not prevent the Aβ1-42-induced neuronal death after 48 hours of exposure. Since Nosustrophine improves neurological injury outcomes through mechanisms other than reducing oxidative damage, gaining an understanding of the molecular basis of its protective effect can help to identify effective therapeutic targets against neurodegeneration.

Neurotrophins are an important group of chemicals that play a vital role in neuronal survival in vertebrates. These molecules are synthesized as large precursor forms, and undergo proteolytic processing to produce mature, and biologically functional ligands.[41,42] Brain-derived neurotrophic factor (BDNF) regulates synaptic plasticity, neuronal survival, and differentiation, and this has been considered a promising molecular target for the treatment of neurological disorders.[43] Furthermore, BDNF plays a significant role in neuronal growth and cell survival, and is essential for chemical processes that underlie synaptic plasticity. Thus, BDNF may provide new therapeutic options for neurodegenerative and neuropsychiatric disorders. In situations, such as epilepsy and persistent pain sensitization, the pathological levels of BDNF-dependent synaptic plasticity may be a contributing factor. Neurotrophic factors, including BDNF, are significant pharmacological targets of AD.[44,45] Low levels of BDNF are associated with synaptic loss and neurite atrophy in the brain of AD patients, while high levels of BDNF slow the AD progression and cognitive decline.[46,47] Furthermore, the injection of BDNF into the hippocampus reverses the learning deficits in the A1-42-induced AD rat model.[48] An *in vivo* experiment conducted using conditional knockout mice that targeted glial TrkB by crossing TrkBflox/flox mice with GFAP-Cre[49] revealed that the stimulation of the BDNF-TrkB signaling pathway in glial cells produces neuroprotective effects.[49–51] In addition, CNTF and bFGF levels are upregulated in BDNF-treated cultured Müller glia, conferring neuroprotective effects.[52] These findings suggest that glial BDNF-TrkB signaling induces an independent neuroprotective effect by upregulating several neurotrophic factors that promote prosurvival signaling in neurons and glia. Furthermore, earlier studies have revealed that several neurotrophic substances derived from PBE exhibit a positive effect on cultured brain cells. The neurotrophin nerve growth factor (NGF) enhances the development, differentiation, and survival of cholinergic neurons in the basal forebrain, making it an ideal cholinergic therapeutic agent.[53] Exogenously-applied NGF improves cognitive function in old, impaired, or cholinergic-depleted rats, and rescues cholinergic neurons in the basal forebrain.[53,54] These studies provide conclusive evidence of the neuroprotective effect of BDNF on brain cell culture models, which support the use of Nosustrophine as a reliable adjuvant medication for treating AD-related dementia. Free-radicals play a crucial role in the pathophysiology of brain damage following cerebral ischemia, and antioxidants reduce this impairment by boosting scavenger enzyme activity.[55] The positive effects of Nosustrophine were consistent with these findings, suggesting that its neuroprotective impact may be due to the increase in scavenger enzyme activity, which reduce oxidative stress or free-radicals in the various cell cultures examined. The pleiotropic effects of Nosustrophine may be attributed to the more precise targeting of its active components toward specific cellular target domains.

The present study aims to investigate the effects of Nosustrophine on the viability of astroglial and microglial cells, and the expression of MHC molecules, with the objective of assessing the neuroprotective potential of Nosustrophine. Inflammation is linked to microglia-mediated tissue damage, which underscores the importance of understanding the role of neurotrophic factors in maintaining tissue integrity and healing. The present data indicates that the Nosustrophine administration at various concentrations had a positive impact on the survival and proliferation of astroglial and microglial cells. This suggests that Nosustrophine may contain neurotrophic factors that regulate the apoptosis of enteric glial cells.[56] The disruption of this system may contribute to more severe inflammation. In addition, the availability of neurotrophic factors is essential for the survival and function of dopaminergic neurons, which are associated with neurodegenerative disorders, such as Parkinson's disease. Although the link between GDNF and degenerative diseases has not been conclusively proven, GDNF has the potential to improve the function of surviving dopaminergic neurons, and correct behavioral abnormalities that resulted from nigrostriatal degeneration.[57,58] Therefore, GDNF has emerged as a promising treatment option for Parkinson's disease.

Age is a significant risk factor for neurodegenerative disorders, and the neurotrophic factor expression decreases with age.[59] The present preliminary findings suggest that active porcine brain proteins and neurotrophic factors are neuroprotective against cognitive dysfunction in AD through the regulation of various intracellular processes. These findings support the potential use of neurotrophins as therapeutic agents for AD. However, more extensive research is required to evaluate the effectiveness of Nosustrophine across different stages of the disease. The present study was conducted on a limited number of cell lines, and further investigations are needed to establish the clinical efficacy of Nosustrophine. Nonetheless, the effectiveness of PBE in regulating scavenger enzymes and neurotrophic factors supports the potential of Nosustrophine as a neuroprotective agent. Two aspects should be given particular consideration for future research: First, PBEs are multi-component and multi-target drugs that can modulate neurotrophins in brain pathologies. However, the effective composition of Nosustrophine and its therapeutic effects require further investigations to optimize its clinical outcomes and prescription designs. Second, more comprehensive studies are required to evaluate the therapeutic effects of PBE. Scientific research in these areas would advance the development of PBE treatment for brain disorders, and provide a fair assessment of the clinical outcomes.

Future directions

The understanding of the molecular underpinnings of this nootropic substance may be enhanced by clarifying the mechanism of Nosustrophine neuroprotection in the human neuroblastoma SH-

SY5Y cell line. It may be possible to develop new therapies for the treatment of Parkinson's disease and other dopaminergic neurodegenerative processes by evaluating the molecular mechanisms underlying the neuroprotective effects of Nosustrophine, and other related neurotrophic factors derived from young porcine (*Sus scrofa domesticus*) brains. In order to preserve the high degree of compatibility and stability of the cell model, more effectively simulate the *in vivo* environment, and comprehend the mechanism of the disease state, it is crucial to choose the appropriate research object and preparation techniques. Future studies should concentrate in developing more precise *in vitro* models that might be utilized to create brand-new medications that target the affected areas in the central nervous system, as these would offer huge benefits to people.

Conclusions

The present study investigated the effect of Nosustrophine, which is an epigenetic bioproduct derived from the *Sus scrofa domesticus* brain using non-denaturing biotechnological processes, on the progression of neurodegeneration in the human neuroblastoma SH-SY5Y cell line. The present *in vitro* data indicated that Nosustrophine has therapeutic properties that prevent selective dopaminergic neuronal loss in the central nervous system, and reduce secondary degenerative effects caused by chronic neuroinflammation. Furthermore, Nosustrophine exhibited neuroprotective and anti-inflammatory effects, when this was administered before or after toxic neuroinduction. The present cell culture data highlights the potential of Nosustrophine as a preventive strategy against neuropathological damage. Nevertheless, further preclinical studies are required to validate these findings.

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Conflict of interest

Prof. Ramón Cacabelos has been an Editor-in-Chief of the *Journal of Exploratory Research in Pharmacology* since July 2015. The authors declare no other conflict of interests.

Author contributions

RC, IC, VL and LC conceived the experiments; IC, OMI, VN and VL conducted the experiments; RC, IC, OMI, LC and VL analyzed the results. All authors reviewed the manuscript, and accepted the final manuscript for publication.

Ethical statement

All experimental procedures were performed in accordance with the European Community Law (86/609/EEC), European Union Directive 2016/63/EU, and the Spanish Royal Decree (R.D. 1201/2005). Study procedures were reviewed and approved by the Ethics Committee of the International Center of Neuroscience and Genomic Medicine.

Data sharing statement

No additional data are available.

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