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RESEARCH REPORT

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Microglia cells treated with synthetic vasoactive intestinal peptide or transduced with LentiVIP protect neuronal cells against degeneration

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Abstract

A common pathological hallmark of neurodegenerative disorders is neuronal cell death, accompanied by neuroinflammation and oxidative stress. The vasoactive intestinal peptide (VIP) is a pleiotropic peptide that combines neuroprotective and immunomodulatory actions. The gene therapy field shows long-term promise for treating a wide range of neurodegenerative diseases (ND). In this study, we aimed to investigate the in vitro efficacy of transduction of microglia using lentiviral gene therapy vectors encoding VIP (LentiVIP). Additionally, we tested the protective effects of the secretome derived from LentiVIP-infected "immortalized human" microglia HMC3 cells, and cells treated with Synthetic VIP (SynVIP), against toxin-induced neurodegeneration. First, LentiVIP, which stably expresses VIP, was generated and purified. VIP secretion in microglial conditioned media (MG CM) for LentiVIP-infected HMC3 microglia cells was confirmed. Microglia cells were activated with lipopolysaccharide, and groups were formed as follows: 1) Control, 2) SynVIP-treated, or 3) LentiVIP-transduced. These MG CM were applied on an in vitro neurodegenerative model formed by differentiated *(d)*-SH-SY5Y cells. Then, cell survival analysis and apoptotic nuclear staining, besides measurement of oxidative/inflammatory parameters in CM of cells were performed. Activated MG CM reduced survival rates of both control and toxin-

Abbreviations: *(d)*-SH-SY5Y cells, Differentiated SH-SY5Y cells; AAVs, Adeno-associated viruses; ALS, Amyotrophic lateral sclerosis; BBB, Blood brain barrier; CNS, Central nervous system; DAT, Dopamine transporter; DMEM, Dulbecco's Minimum Essential Media; DPP-4, Dipeptidyl peptidase-4; EIA, Enzyme immune assay; ELISA, Enzyme-Linked Immunosorbent Assay; GDNF, Glial cell line-derived neurotrophic factor; HMC3, Human embryonic microglia clone 3; IF, Immunofluorescence; IL, Interleukin; iNOS, Induced nitric oxide synthase; LentiVIP, Lentiviral gene therapy vectors encoding VIP; LPS, Lipopolysaccharide; MAO-B, Monoamine oxidase-B; MG CM, Microglial conditioned media; MMP-9, Matrix metalloproteinase-9; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; ND, Neurodegenerative diseases; NFkB, Nuclear factor kappa B; NO, Nitric oxide; NRTN, Neurturin; PACAP, Pituitary adenylyl cyclase-activating polypeptide; PD, Parkinson's disease; PGP9.5, Protein gene product 9.5; RA, Retinoic acid; ROS, Reactive oxygen species; SNpc, Substansia nigra pars compacta; SynVIP, Synthetic VIP; TAC, Total antioxidant capacity; TGF-β1, Transforming Growth Factor-beta1; TLR-4, Toll-like receptor-4; TNF, Tumor necrosis factor; TOC, Total oxidant capacity; VIP, Vasoactive intestinal peptide; VIPR1, Specific type 1 VIP receptor; VIPR2, Specific type 1 VIP receptor.

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applied *(d)*-SH-SY5Y cells, whereas LentiVIP-infected MG CM and SynVIP-treated ones exhibited better survival rates. These findings were supported by apoptotic nuclear evaluations of *(d)*-SH-SY5Y cells, alongside oxidative/inflammatory parameters in their CM. LentiVIP seems worthy of further studies for the treatment of ND because of the potential of gene therapy to treat diseases effectively with a single injection.

KEYWORDS
gene therapy, LentiVIP, microglia, neurodegeneration, vasoactive intestinal peptide

1 | INTRODUCTION

Over the past decade, remarkable progress has been made in our understanding of the pathophysiology of neurodegenerative diseases (ND). In this context, recent data has contributed to our knowledge of the potential pathways in the process of apoptosis and neuronal loss, and the oxidative/antioxidative mechanisms, which are involved in the pathogenesis of ND, as well (X. Sureda et al., 2011). The crucial role of immune cells, especially of microglia and mast cells, in the pathogenesis of ND has also recently been demonstrated. Namely, overactivation and excessive proliferation of microglia by various stimuli can induce the release of multiple mediators that induce neuroinflammation and oxidative stress, two important processes associated with neurodegeneration (Roqué & Costa, 2017).

Microglia are small, stellate cells located primarily along capillaries of the central nervous system (CNS) that function as phagocytotic cells. Since microglial cells are considered as a part of the mononuclear phagocytotic system, they play a defensive and beneficial role in maintaining an environment that supports the functioning and survival of neurons, partially through their ability to mediate controlled inflammatory reactions (Block et al., 2007). They normally account for about 5% of all glial cells in the adult CNS, but in regions of injury and disease, they proliferate and become actively phagocytotic. In response to injury or numerous pathological stimuli, activated microglia move through a series of morphological and functional changes, and become reactive microglial cells. When activated, they begin to proliferate, retract their ramified extensions, become more amoeboid in shape, and more motile, besides gaining the increased ability to phagocytize debris (Stence et al., 2001). In the activated state, microglia are known to release both protective and cytotoxic factors, through which they can influence neuronal cell viability and neuron functions (Block et al., 2007; Luo & Chen, 2012; Ransohoff & Perry, 2009).

In case the process of microglial response is dysregulated, reactive microglia may release many pro-inflammatory cytokines and induce the production of reactive oxygen species (ROS). In this situation, microglia may act as a major contributor to oxidative and neuroinflammatory damage (Block et al., 2007). These cells can switch phenotypes when exposed to specific growth factors or cytokines, as well. In vitro exposure to lipopolysaccharide (LPS), which is a lipid-linked polymer of bacterial cell wall components found in gram-negative bacteria, has been associated with morphological alterations from ramified (rest or M0 phenotype) to amoeboid (activated or M1) phenotype of microglia which have long been associated with neuroinflammation (Timmerman et al., 2018).

LPS, first identified as a Toll-like receptor 4 (TLR-4) ligand (Beutler, 2000; Yang et al., 2018), enables microglia in the CNS to be activated by expressing TLR-4 and eventually produce neuroinflammatory cytokines that mediate neuronal cell death (Lysakova-Devine et al., 2010). It mainly initiates the production of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and ROS in both glial cells and neurons (Boche et al., 2013; Sica &

Mantovani, 2012). Administration of LPS to animals is thought to cause behaviors very similar to clinically relevant symptoms of ND in humans (Choi et al., 2012). Additionally, LPS is also known to cause withdrawal of neurites and loss of neuron viability particularly in the CNS cells, and stimulation of nitric oxide (NO) production which contributes to neurotoxicity (Hoozemans et al., 2002; Shi et al., 2010).

Vasoactive intestinal peptide (VIP), a 28-amino acid neuropeptide/neurotransmitter is an essential endogenous peptide molecule involved in many physiological processes. It recently appears to be a molecule that has the potential to exert beneficial effects against ND, such as Parkinson's disease (PD) (Delgado & Ganea, 2013). VIP is identified in the central and peripheral nervous systems, and also recognized as a widely distributed neuropeptide in nerve terminal and intra-organ ganglia (Said & Rosenberg, 1976). VIP is known to influence both innate and adaptive immune responses, and acts as an important anti-inflammatory mediator in animal models of inflammatory/autoimmune diseases, suggesting that VIP/VIP receptor system could serve as a target for novel therapeutic strategies in immune disorders. VIP also enhances glycogen metabolism in the cerebral cortex and promotes neuronal survival (Brenneman et al., 1990; Sorg & Magistretti, 1992). Its neuroprotective effect can be direct through receptors or indirect by promoting release of neurotrophic factors from microglial cells.

Owing to the mediatory effect of the specific type 1 VIP receptor (VPAC1), VIP and its structurally related peptide pituitary adenylyl cyclase-activating polypeptide (PACAP) have been shown to inhibit pro-inflammatory cytokines TNF-alpha, IL-1beta, IL-6, and NO production from LPS-activated microglia by inhibiting p65 nuclear translocation and nuclear factor-kappaB (NFkB)-DNA binding (Delgado et al., 2003). It has also been reported that VIP and PACAP inhibit the production of TNF-alpha from activated microglia by a cyclic adenosine monophosphate-dependent pathway (Kim et al., 2000). Moreover, VIP has been shown to reduce the monocyte-induced neutrophil chemotaxis, presumably through the inhibition of IL-8 production (Delgado & Ganea, 2003a). Furthermore, Gonzalez-Rey et al. have reported the inhibitory effect of VIP on cyclooxygenase expression and subsequent production of Prostaglandin E2 by activated macrophages, dendritic cells, and microglia, being mediated through VPAC1 (Gonzalez-Rey & Delgado, 2008). VIP and PACAP have also been shown to inhibit the expression of the microglia-derived CXC (MIP-2 and KC) or CC (such as MIP-1alpha, −1beta) chemokines and NFkB binding mediated through VPAC1. The inhibition of chemokine production by VIP/PACAP leads to a significant reduction in the chemotactic activity generated by activated microglia for peripheral leukocytes, which in turn contributes to the control of inflammation in the CNS (Delgado et al., 2002). These findings indicate that VIP and/or PACAP released by neurons promote neuronal survival via limiting the inflammatory process (Delgado et al., 2002; Ganea & Delgado, 2002).

Moreover, VIP and PACAP have been shown to control the gene expression of interferon-gamma (IFN-γ)-inducible protein-10, CD40, and iNOS, three microglia-derived mediators, through their effect on IFN-gamma-induced Jak/STAT1 pathway, that play an essential role in several pathological conditions, including inflammatory and autoimmune disorders (Delgado, 2003). In addition, Broome et al. have reported that co-treatment of rotenone with PACAP or VIP prevents rotenone-induced increase of NO, CD11b, Matrix metalloproteinase (MMP)-9 and IL-6 in BV2 microglia cells, supporting the protective effects of these peptides against inflammation (Broome et al., 2022). Recent reports also show that VIP suppresses the inflammatory response of microglia in in vivo models of neurodegeneration. PD is characterized by the selective degeneration of dopaminergic neurons in substansia nigra pars compacta (SNpc). Dopaminergic cells are therefore an obvious candidate for cell-based therapies for PD (Skidmore & Barker, 2023). In an MPTP model of PD, VIP treatment significantly decreased MPTP-induced dopaminergic neuronal loss in SNpc and nigrostriatal nerve-fiber loss. VIP has also been reported to prevent MPTP-induced activation of microglia in SNpc and striatum and the expression of the cytotoxic mediators, iNOS, interleukin 1beta, and TNF-alpha (Delgado & Ganea, 2003b).

On the other hand, VIP has also been shown to inhibit the neurodegeneration induced by β-amyloid, a characteristic feature of Alzheimer's disease (AD), by indirectly inhibiting the production of some inflammatory and neurotoxic agents by activated microglia cells via blocking signaling through the p38 MAPK, p42/p44 MAPK, and NFkB cascades (Delgado et al., 2008). In an in vivo model of spared nerve injury, mice deficient in VIP have been investigated in terms of immune responsiveness to the nerve lesion. VIP-deficient mice had a stronger early pro-inflammatory cytokine response and a more augmented microglial reactivity compared with wild-type controls, suggesting a role of VIP in neuropathic states (Gallo et al., 2017).

Finally, VIP mediates biological responses by activating two related receptors, VIPR1 and VIPR2, and the use of native VIP cannot distinguish between these two receptors. Besides VIP's rapid metabolism, there is a challenge in VIP therapy related to receptor specificity, since activation of both receptors may cause secondary toxicities. This led to the development of metabolically stable

and receptor-selective agonists that provide better pharmacokinetic and pharmacodynamic therapeutic end points. Olson et al. have investigated the protective effects of selective agonists against MPTP-induced mice PD model. Treatment with VIPR2 agonist caused increased neuronal sparing, reduced microglial responses, and diminished release of proinflammatory cytokines, such as IL-17A, IL-6, and IFN-γ (Olson et al., 2015).

Despite such beneficial effects of VIP, the duration of VIP action may be limited by its short biological half-life because of dipeptidyl peptidase-4 (DPP-4) mediated degradation. Its short plasma half-life after intravenous administration and the difficulty in administration routes limit its clinical application. This led to the development of long-acting VIP analogues, in combination with appropriate drug delivery systems (Onoue et al., 2007). As a strategy to increase the short half-life of the cognate VIP molecule, VIP analogues, either shorter or nonpeptide are used, however, they show limited advantages, while the application of protease inhibitors could lead to undesired side effects (Klippstein & Pozo, 2015). Moreover, the transportation of peptide and protein therapeutics from blood to brain are generally prevented by blood–brain barrier (BBB). In this context, chimeric peptides are formed by coupling a non-transportable peptide therapeutic to a BBB drug transport vector (Bickel et al., 2001). In ND, the efficacy of pharmacological treatments reduces as the neurodegenerative process progresses. Significant side effects may occur, because high dosage of medication is required, since BBB significantly prevents systemic agents from reaching therapeutic parenchymal levels. Intracerebral drug delivery, specifically gene therapy, is a promising strategy for overcoming these challenges in medical therapy.

Gene therapy may allow correction of the underlying pathogenic mechanism, or may exert neuroprotective/restorative effects, by altering or inducing the expression of specific proteins (Sun & Roy, 2021). Modified viral vectors are used to deliver genes of interest to the brain. Vectors derived from adeno-associated viruses (AAVs) are the most widely used ones in clinical trials for CNS disorders. On the other hand, Lentiviral vectors have a great advantage, since they can carry a larger DNA payload than AAV vectors. A Phase 1/2 open-label clinical trial of gene therapy in PD has been reported. In this trial, researchers achieved to produce a continuous and stable production of dopamine in the motor region of the putamen, by using 'ProSavin', a lentiviral vector that encodes dopamine biosynthetic enzymes (Palfi et al., 2014). Furthermore, two promising gene therapy candidates of neurotrophic/regenerative factors that support the survival of dopaminergic midbrain neurons are glial cell line-derived neurotrophic factor (GDNF) and neurturin (NRTN) (Collier & Sortwell, 1999). In this context, Phase 1 clinical trials are ongoing to determine the safety of bilateral AAV2-GDNF injections into the putamen, whereas Phase 1 clinical trial delivering AAV2-NRTN to the putamen have been reported to be well-tolerated, but not to have succeeded an improvement in motor functions (Marks et al., 2010).

In the light of these data, gene therapy vectors with a capability of VIP expression could serve as a continuous VIP source to obtain neuroprotective/restorative effects. Lentiviral vectors may provide long-term gene expression that is suitable for treating diseases, especially of complex genetic disorders (Tasyurek et al., 2018). In a recent Type 1 Diabetes mellitus model of mice, LentiVIP provided suppression of diabetes-induced inflammation that resulted in the protection of pancreatic beta cells from apoptosis, in addition to restored beta-cell proliferation (Erendor et al., 2020).

The aforementioned data indicates the potential for lentiviral gene therapy vectors encoding VIP (LentiVIP) as a novel gene therapy agent, to treat ND owing to its anti-inflammatory, anti-apoptotic and neuroprotective properties. Collectively, these advantages have led us to test the protective properties of LentiVIP gene delivery against microglial toxicity and toxin-mediated neurodegeneration on an in vitro neurodegenerative model. In this context, we constructed a neurodegenerative model by treating neuron-like human differentiated *(d)-SH-SY5Y* cells with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is frequently used to build 'in vivo' and 'in vitro' PD models (Chen et al., 2018; Goksu Erol et al., 2022). This toxin is metabolized into the toxic cation 1-methyl-4-phenylpyridinium (MPP^+) by the enzyme, monoamine oxidase B (MAO-B) (Frim et al., 1994). Dopaminergic neurons are selectively vulnerable to the effects of MPP^+, because their dopamine reuptake is mediated by dopamine transporter (DAT) that displays high affinity for MPP^+. As SH-SY5Y neuroblastoma cells become differentiated, they acquire the ability to transport dopamine (Willets et al., 1993) and norepinephrine (Murphy et al., 1991), which make them capable of metabolizing MPTP into MPP + via MAO-B (Song et al., 1996, 1997). The versatility of SH-SY5Y cells in recapitulating key aspects of neuronal physiology and their susceptibility to neurotoxic insults make them a valuable tool for elucidating disease mechanisms, evaluating potential therapeutic interventions, and screening drug candidates.

In the light of all this information, this study aimed to evaluate the protective effects of the secretome that is derived from microglia transfected with LentiVIP against toxin-induced neurodegeneration using a cellular model. Synthetic VIP treated microglial secretome was also evaluated in terms of neuroprotection.

2 | MATERIALS AND METHODS

2.1 | Reagents and chemicals

SynVIP (V3628-Sigma) was dissolved in acetic acid (AA, 64–19-7-EMSURE, Merck, Germany) as a 100 mM stock solution, then serial dilution of test solutions was performed. Since the doses higher than 10^{-6} M did not show the desired effect of VIP on cells in our preliminary experiments, and the recommended dose is between the range of 10^{-8}–10^{-6} M, we applied SynVIP in cell culture at 10^{-7} and 10^{-8} M (Delgado & Ganea, 2003a; Festoff et al., 1996; Nicol et al., 2004). In addition, its vehicle, AA was also tested at the doses required for dissolving 10^{-7} and 10^{-8} M VIP.

Lipopolysaccharide (LPS, L2654-Sigma Aldrich, St. Louis, MO) was used to activate microglia cells. An effective concentration of '5 μg/ml' was selected based on previous studies (Kang, 2014; Kocanci et al., 2024; Xu et al., 2022).

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, M0896-Sigma Aldrich), a neurotoxin that triggers oxidative stress and apoptosis in neurons, was applied on differentiated *(d)*-SH-SY5Y cells to induce cell degeneration (Chen et al., 2018; Frim et al., 1994; Murphy et al., 1991; Song et al., 1996, 1997; Willets et al., 1993). 10 mg MPTP was dissolved in 1 ml of double distilled water. The solution was filter-sterilized and stored in dark at −20°C. Concentration titration assay of MPTP, including four different concentrations, as 100, 500, 1000, and 1500 μM, for cell viability was performed, as described in our previous study (Goksu Erol et al., 2022).

2.2 | Production and purification of HIV-based lentivirus encoding VIP and their functionality analysis

Lentivirus vectors were produced by mixing the transfer vectors of pLentiVIP (Transfer plasmid carrying CMV-driven VIP encoding sequence) or pLentiLacZ with three packaging plasmids [gag/pol, rev and VSV-G] as described by Tasyurek et al. (Tasyurek et al., 2018). Concentrated viral samples were then purified by AEX chromatography, as described by Olgun et al. (Olgun et al., 2019). The titer of lentiviral vector stocks was determined by qPCR method, and the functionality analysis of constructed lentiviral vectors (both LentiVIP and LentiLacZ as control vector) was performed by Glucose-Stimulated Insulin Secretion method as described our previous study (Erendor et al.) (Erendor et al., 2020).

2.3 | Confirmation of the in vitro expression of LentiVIP

Before investigating the in vitro therapeutic efficacy of the LentiVIP gene therapy on neurodegenerative model, first the expression of VIP from the newly generated vector LentiVIP was verified in human HepG2 hepatocellular carcinoma cell line - as control that does not expresses VIP. HepG2 cells were transduced with LentiVIP or LentiLacZ. 24 hours later, the supernatant was refreshed to remove polybrene which was regarded as 'zero point'. 24 h and 72 h later than the 'zero point', cell groups were morphologically assessed, then their supernatants were removed for analysis, and immunostaining was performed. To quantitate the amount of VIP found in cell culture supernatants, Enzyme Immune Assay (EIA) for VIP was performed according to the manufacturer's instructions (Peninsula Laboratories, LLC, Bachem; S-1183, San Carlos, CA). Immunocytochemical analysis was also performed using anti-VIP antibody (Abcam, ab8556) to confirm VIP expression in HepG2 (Erendor et al., 2020). Also, the level of VIP secretion in microglial cells transduced with LentiVIP/LentiLacZ or treated with SynVIP/or its vehicle, AA, depending on the dose and time points, was determined with Enzyme Immune Assay.

2.4 | Cell culture

2.4.1 | HepG2 cell line

HepG2 hepatocellular carcinoma cell line was obtained from ATCC (HepG2, HB 8065™). Cells were cultured in High Glucose Dulbecco's Minimum Essential Media (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (04–007-1A-Biological Industries, Beit Haemek, IL), 1% non-essential amino acids (11140–050-Gibco, Waltham, MA), and 1% (v/v) antibiotic-antimycotic (L0010–020-Biowest, Nuaille, FR), and maintained in a humidified atmosphere, 5% CO_2 at 37°C. Cells were plated out as 1.0×10^5 cell/cm^2 in 24-well plates.

2.4.2 | HMC3 and human SH-SY5Y neuroblastoma cell lines

The SV-40 immortalized human microglia cell line HMC3 were purchased from ATCC (CRL-3304, Manassas, VA). Cells were cultured in Eagle's Minimum Essential Media (EMEM) (11,095–080-Thermo Fisher Scientific, Waltham, MA), supplemented with 10% (v/v)

fetal bovine serum (FBS) (04–007-1A-Biological Industries, Beit Haemek, IL), 1% non-essential amino acids (11,140–050-Gibco, Waltham, MA), and 1% (v/v) antibiotic-antimycotic (L0010–020-Biowest, Nuaille, FR), and maintained in a humidified atmosphere, 5% CO_2 at 37°C.

The human neuroblastoma SH-SY5Y cell line (ATCC, Rockville, MD, USA) was obtained from SAP Institute (Ankara, Turkey). These cells have been used as a cell model for studying multiple pathways linked to PD pathology and novel treatment options (Song et al., 1997). The cells were cultured in a 1:1 mixture of DMEM/F12 (Gibco) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (10,500,064-Gibco). In addition, 1% (v/v) penicillin/streptomycin (15,140,122-Sigma Aldrich) and 1% (v/v) L-glutamine (25,030,024-Sigma Aldrich), and 40% MCDB-201 (M6770-Sigma Aldrich) were also added to the culture media. The media were changed every other day.

For neuronal differentiation, cells were seeded in sterile 96 or 6-well plates (1.0×10^5 cell/cm^2) and treated with 10 μM Retinoic acid (RA) (R2625-Sigma) once every other day in DMEM/F12 with 2% FBS for 6 days. Treated with 10 μM RA (R2625-Sigma) once every other day in DMEM/F12 with 2% (v/v) FBS for 6 days. The differentiated *(d)*-SH-SY5Y cells with acquired neuronal properties were further used in our experiments involving MPTP application and microglial CM treatments (Yamchuen et al., 2017).

2.4.3 | Experimental design

HMC3 microglial cells were plated out as 1.0×10^5 cell/cm^2 in 96, 24, or 6-well plates. Microglia cells were treated with LPS (5 μg/ml). Then SynVIP or LentiVIP treatments were performed. SynVIP group cells were treated with 10^{-7} and 10^{-8} M VIP/AA. LentiVIP group cells were transduced with 5, 25 and 125 MOI LentiVIP/25 or 125 MOI LentiLacZ together with polybrene (6 μg/ml). The control group was treated with no virus but polybrene. 24 hours after transduction, the cell media was refreshed with a whole culture medium to remove the polybrene (This timepoint was regarded as 'zero point'). All cell groups were incubated for an additional 72 hours, then cell viability was determined with MTT assay in microglia culture groups (in 96 well plates), whereas MG CMs (in 6 well plates) were collected. CMs were stored at −80°C for later application to *(d)*-SH-SY5Y cells or subjected to analysis for TGF-β1 and NO levels, besides TOC/TAC. Additionally, IF staining with Anti-CD11b antibody was performed on these cellular groups.

(d)-SH-SY5Y cells were treated with microglial CM groups of SynVIP or LentiVIP in the presence or absence of MPTP. 24 hours following the MG CM treatments, groups in 96-well plates were undergone cell viability analyses. All supernatants in the 6 or 24-well plates were collected and stored at −80°C for subsequent analysis of TOC, TAC, NO, and TGF-β1 levels; IF staining and apoptotic nuclear assessment after Hoechst staining were also performed. (The major applications and their timepoints were visualized in graphical abstract.).

2.4.4 | Cell viability assay

Microglia and *(d)*-SH-SY5Y cell groups were cultured in 96-well plates at a density of 1.0×10^4 cell/well. The cell viability rates in 1- Microglial cell groups (*that were treated with LPS and Lenti/SynVIP*), 2- with or without MPTP (*d*)-SH-SY5Y cell groups (*that were treated with various concentrations of MG CM*) were investigated using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium (MTT) assay. MTT, a membrane-permeable dye solution, was added to the wells at a concentration of 1 mg/ml, and the mixture was incubated at 37°C for about 4 h. After the supernatant was removed, the pellets were dissolved in 200 μl/well of DMSO. The blue crystals were solubilized, and the colorimetric intensity was measured at 570 nm and 690 nm on a microplate reader (Thermo Scientific Multiskan Spectrum). The percentage of cell viability was calculated relative to the colorimetric intensity of control cells (Zhang et al., 2020). MTT is used to assess cell viability and proliferation. Besides evaluating the cell viability rates, the MTT assay is a measure of the metabolic activity of the cells analysed; namely, the more metabolic activity in the sample, the higher will be the signal obtained.

2.4.5 | Immunofluorescence (IF) staining of microglia and *(d)*-SH-SY5Y cells

Groups of microglial cells in six-well plates were employed to assess alterations in the expression intensity of the following treatments with LPS/SynVIP/LentiVIP. To observe alterations in the microglial activation caused by LPS/SynVIP/LentiVIP treatments, HMC3 microglial cells were plated in 6 well plates (1.0×10^5 cell/cm^2) and groups were formed.

On the other side, to observe the MPTP-related changes in *(d)*-SH-SY5Y cells, SH-SY5Y cells were plated in 6 well plates (1.0×10^5 cell/cm^2), and differentiated with RA treatment for 6 days, and with or without MPTP groups were formed.

At the end of the treatments, cells were fixed with paraformaldehyde for 20 min, and blocked with phosphate buffered saline (PBS) containing 0.1% Tween-20 (v/v) and 5% bovine serum albumin for 1 h at room temperature. Microglia were immunostained with anti-CD-11b antibody (nb11089474 pAB-Novus, Boston, MA antibody (1:400) (Jurga et al., 2020), whereas *(d)*-SH-SY5Y cells were immunostained with anti-PGP9.5 antibody (1:600) (ab8189-Abcam). Following incubation at 4°C overnight, all cell groups were incubated with secondary antibodies (HMC3: 35552-Goat Anti-Rabbit IgG, DyLight 488, Thermo Scientific) (1:400), (*(d)*-SH-SY5Y: A10037-Alexa Fluor 568 donkey anti-mouse IgG (H + L)- Invitrogen) (1:400)), and then stained with DAPI at room temperature.

Expression of CD-11b, a marker indicative of microglial activation, and PGP9.5, a neuron specific protein, were evaluated as fluorescent staining intensity in the cytoplasm of microglia and *(d)*-SH-SY5Y cells, respectively, using a Leica DMi8 Microscope. Photographs were taken using LasX Software (Fan et al., 2018). The fluorescence intensity of CD-11b, PGP9.5, and DAPI expression was assessed utilizing NIH ImageJ, as outlined in a previous study (Varghese et al., 2014).

2.4.6 | Nuclear DNA staining with Hoechst 33342

SH-SY5Y cells were seeded into 6 well plates at a density of 1.0×10^5 cell/cm^2. After seeding, cells were differentiated with RA treatment for 6 days and grouped as (a) Non-activated MG CM treated (Control), (b) Activated MG CM treated, (c) SynVIP treated activated MG CM treated, and (d) LentiVIP transfected activated MG CM treated groups. The cells were stained with 15 μg/ml of Hoechst 33342 staining (H-3570 for 10 min in the dark, washed with PBS, and immediately visualized to observe morphological changes using Leica DMi8 Fluorescence Microscope (×10 and ×20 objective).

2.4.7 | Measurement of Total oxidant and Total antioxidant capacities

TOC and TAC levels of the samples were measured as previously described (Malik et al., 2018; Yaribeygi et al., 2019). TOC analysis is a colorimetric method based on the oxidation of ferrous iron (Fe^{+2}) to the ferric iron complex (Fe^{+3}). In an acidic medium, ferric iron forms a colored compound. The intensity of this colored compound was then measured at 600 nm by a spectrophotometer. H_2O_2 was used for the calibration and the result was given as μmol H_2O_2 Equiv./L. The expected coefficient of variation of the method is <5%. TAC was determined by the antioxidant-induced color change in 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) measured at 660 nm using a spectrophotometer. Vitamin E was used for the calibration and the result was expressed as μmol H_2O_2 Equiv./L. The expected coefficient of variation of the method is <3%.

2.4.8 | Transforming growth factor-β1 (TGF-β1) enzyme-linked immunosorbent assay

After cell-free culture supernatants of microglia and *(d)*-SH-SY5Y cells were collected, they were assayed for anti-inflammatory cytokine TGF-β1 with Enzyme-Linked Immunosorbent Assay (ELISA) using Human/Mouse TGF-β1 ELISA kit (88–8350-eBioscience).

2.4.9 | Nitrate/nitrite colorimetric assay

After cell-free culture supernatants of *(d)*-SH-SY5Y cells were collected, they were assayed for NO levels with Nitrate/Nitrite Colorimetric Assay Kit (78,001-Cayman, MI) according to the manufacturer's instructions. Nitrate and nitrite in culture supernatants were converted to NO.

3 | STATISTICAL ANALYSIS

All data were presented as the mean ± S.E.M. of four independent experiments in which triplicate samples were performed. Statistical differences between the control group and agent treated groups were determined by unpaired two-tailed Student's t-test, ANOVA (parametric) or Mann–Whitney U (non-parametric). Multiple group comparisons were performed using oneway analysis of variance (ANOVA) followed by posthoc Tukey's HSD test for pairwise comparisons between the groups. Kruskal–Wallis was used to compare non-parametric data. The statistical analysis of data was performed using GraphPad Prism version 9.3.1 for Windows (San Diego, CA, USA).

4 | RESULTS

4.1 | Verification of in vitro expression of LentiVIP vector

VIP levels in culture supernatants of SynVIP treated microglia at 24 h and 72 h after 'zero point' indicated

that high VIP levels persisted in the supernatants until 72 h of culture after 'zero point', with a slightly decreasing pattern. The highest level of VIP was detected in microglial cells treated with 10^{-7} M SynVIP at 24 h after 'zero point' (Figure 1a).

HepG2 cells that do not express VIP were transduced with LentiVIP or LentiLacZ (control vector) to confirm VIP expression from LentiVIP. VIP levels increased in dose and time-dependent manner in LentiVIP infected cell CM, and the amount of VIP in the LentiLacZ-infected cells did not show any significant difference compared with uninfected cells (Figure 1b).

Moreover, VIP levels in culture supernatants of LentiVIP infected microglia displayed higher levels of this

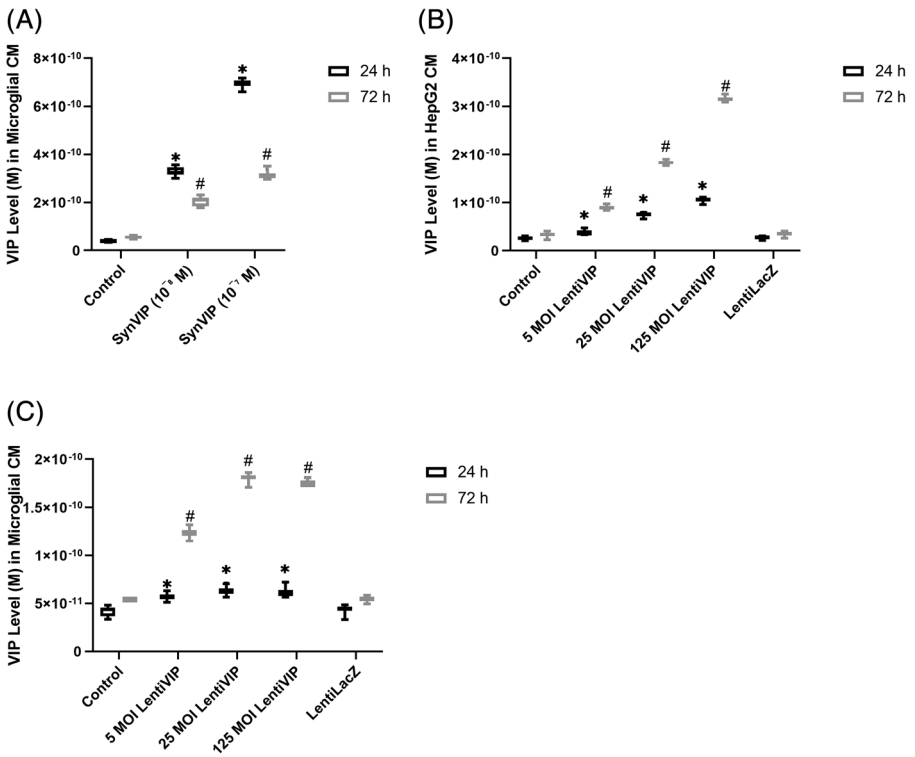

FIGURE 1 (a–c). In vitro expression of the LentiVIP vector. The levels of VIP were measured at 24 and 72 hours after 'zero point' in the CM of microglial cells treated with SynVIP (10^{-8} and 10^{-7} M) (a), HepG2 cells infected with LentiVIP (5, 25, 125 MOI) or LentiLacZ (b), and microglial cells infected with LentiVIP (5, 25, 125 MOI) or LentiLacZ (c). The presented data represent the mean ± S.E.M. from four independent experiments, each consisting of triplicate samples, expressed as a percentage relative to the control (*$p < 0.05$ compared with untreated cells as control, 24 h; #$p < 0.05$ compared with untreated cells as control, 72 h). The LentiLacZ vector was utilized at an MOI of 125 (for zero point, see Graphical abstract).

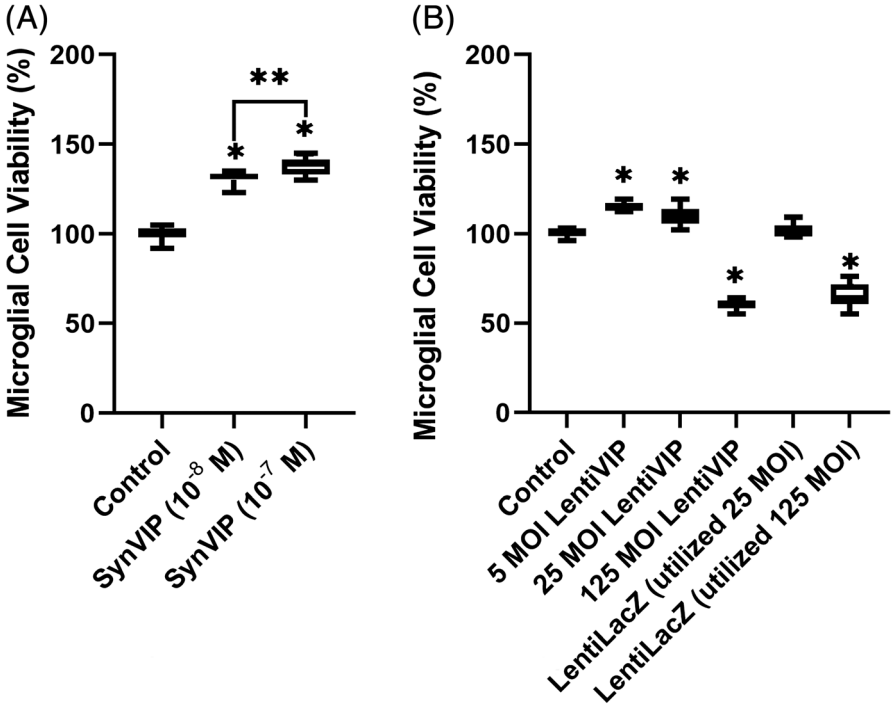

FIGURE 2 (a and b). The effect of SynVIP treatment at concentrations of 10^{-8} and 10^{-7} M (a) and LentiVIP transduction at 5, 25, and 125 MOI (b), on microglial cell viability rates. The presented data represent the mean ± S.E.M. from four independent experiments, each with triplicate samples, expressed as a percentage relative to the control (*$p < 0.05$ compared with untreated cells as the control; **$p < 0.05$ compared among treatment groups). The LentiLacZ vector was utilized at an MOI of 25 and 125 (for detailed timepoints of applications, see Section '2.3.3. Experimental Design' and 'Graphical abstract').

peptide in a time and dose-dependent manner with LentiVIP concentration used for transduction. The amount of VIP in the LentiLacZ infected cells was indistinguishable from uninfected cells. The highest VIP level was detected at the 72 h after 'zero point in the CM of 25 MOI LentiVIP infected microglia (Figure 1c).

4.2 | Effects of SyntheticVIP and LentiVIP on cell viability of microglia

As illustrated in Figure 2a, application of SynVIP on LPS-treated (activated) microglial cells resulted in a statistically significant increase in cell viability rates. Specifically, both doses (10^{-8} and 10^{-7} M) exhibited a substantial cell-proliferative effect, with viability rates reaching 1.31 and 1.38 times that of the control, respectively (for both, $p < 0.05$). Furthermore, the solvent for VIP, AA did not cause any significant effect on cell viability rates ($p > 0.05$) (data not shown in the figure).

As depicted in Figure 2b, LentiVIP-infected microglia exhibited a significant increase in cell viability rates at concentrations of 5 and 25 MOI (for both, $p < 0.05$). However, microglia infected with 125 MOI of LentiVIP and LentiLacZ displayed a notable decrease in viability rates ($p < 0.05$ for both), indicating a cytotoxic effect at this high concentration. Additionally, the viability rate of LentiLacZ-infected microglia cells at 25 MOI was indistinguishable from that of uninfected cells ($p > 0.05$).

Since 25 MOI concentration of LentiVIP and LentiLacZ did not show any cytotoxic effect, we applied 5 and 25 MOI of LentiVIP in subsequent experiments.

4.3 | SynVIP treated/LentiVIP infected microglial CM is less detrimental to *(d)*-SH-SY5Y cells

To determine the concentrations that induce 30–40% of degeneration in (d)-SH-SY5Y cell groups, we *exposed the cells to five different concentrations of MPTP (100, 250, 500, 1000, 2000 μM) for a 24-hour period. It was observed that an increase in the MPTP dose resulted in a proportional reduction in cell viability (ranging from 5% to 75% cell death). Among these various concentrations, 1000 μM was selected as it yielded an approximate survival rate of 65–70% in (d)-SH-SY5Y cells (Data not shown). This concentration was subsequently employed in our experimental applications to establish our* in vitro *neurodegenerative models* (Goksu Erol et al., 2022).

When different concentrations of activated microglial conditioned media (½, 1/4, 1/8) were applied to (d)-SHSY5Y cells, a negative correlation between cell viability rates and the increasing concentrations of CM were observed (5 ± 2, 75 ± 4, $35 \pm 5\%$ of cell viability rates, respectively). '1/4' ratio of microglial conditioned media to (d)-SH-SY5Y cell culture medium was found to be appropriate for subsequent experiments (Data not shown).

As shown in Figure 3a, CM of LPS-activated microglia led to a significant decrease in the viability of without or with MPTP-*(d)*-SH-SY5Y cells ($50 \pm 5\%$ and $75 \pm 6\%$, respectively) ($p < 0.05$). CM from activated microglia treated with both 10^{-8} and 10^{-7} M SynVIP provided significantly increased survival rates of with or without MPTP- *(d)*-SH-SY5Y cells compared with non-treated

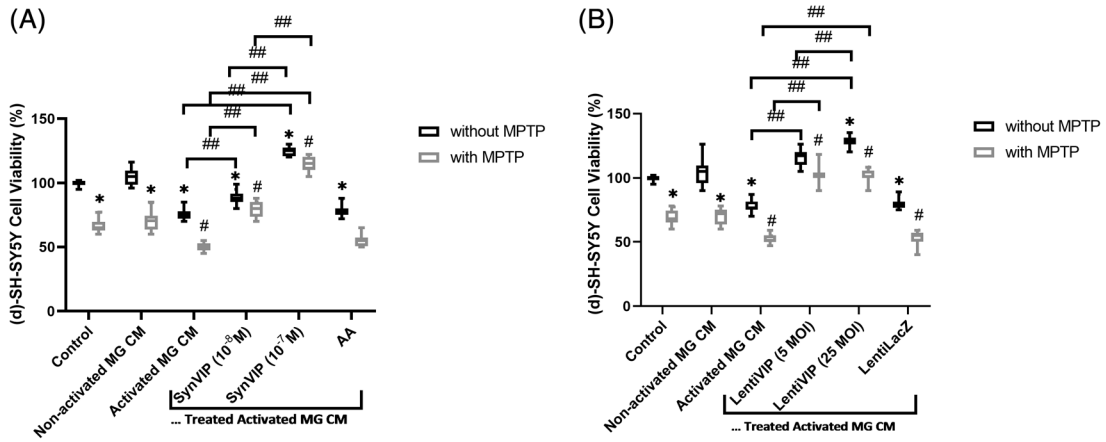

FIGURE 3 (a and b). Effects of various microglial conditioned media (MG CM) groups (a – SynVIP or AA [employed as the vehicle for 10^{-7} M SynVIP] treated, b – LentiVIP/LentiLacZ transduced MG CM) on with or without MPTP-(d)-SH-SY5Y cell viability for 24 h. (Activated MG CM = 5 μg/ml LPS treated MG CM). (The presented data represent the mean ± S.E.M. from four independent experiments, each conducted with triplicate samples, and are expressed as a percentage relative to the control (*$p < 0.05$ compared with untreated cells as control, $^{\#}p < 0.05$ compared with only MPTP treated CM group, $^{\#\#}p < 0.05$ compared among MG CM groups). (For detailed timepoints of applications, see Section '2.3.3. Experimental design' and 'Graphical abstract').

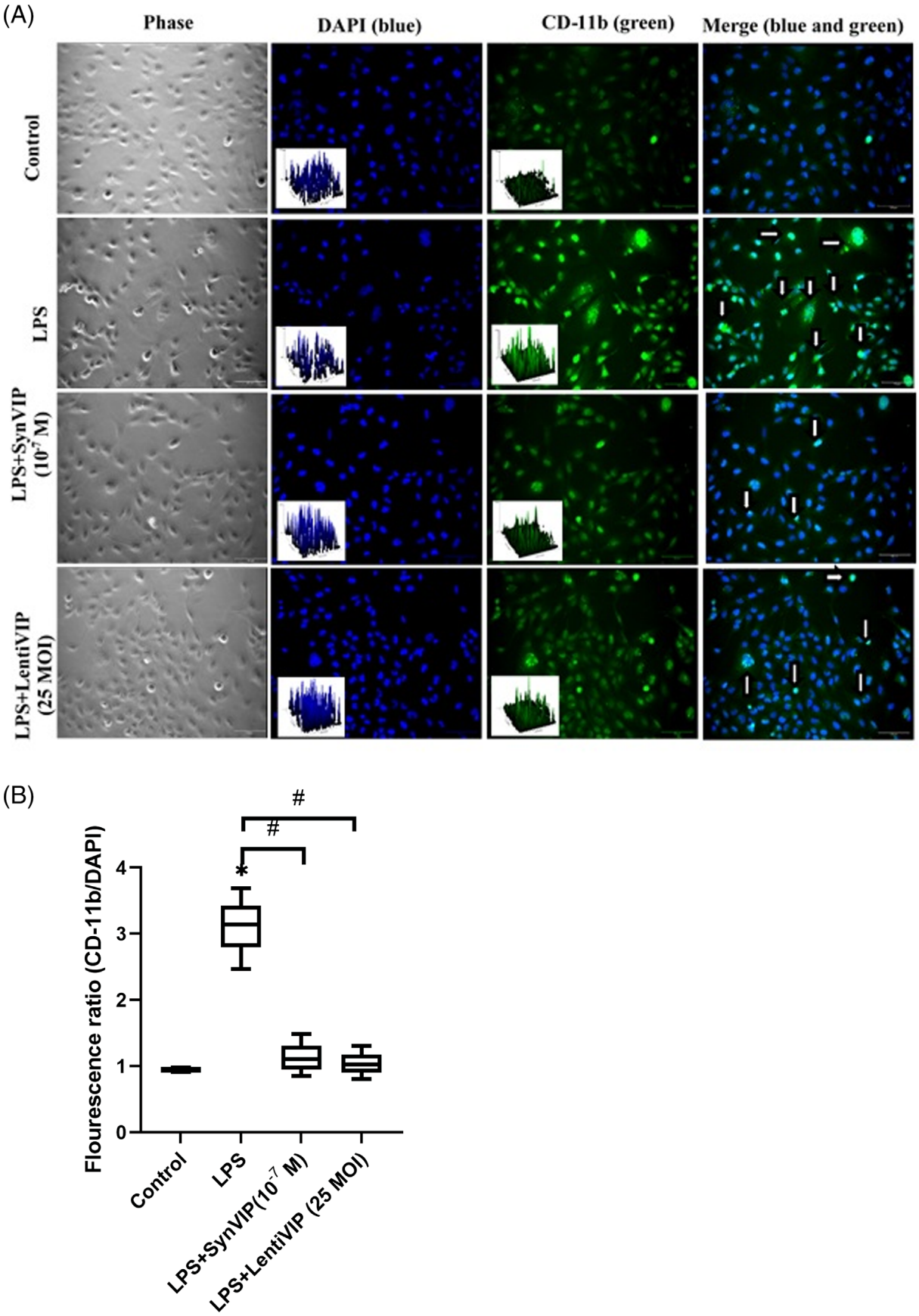

FIGURE 4 Legend on next page.

ones ($p < 0.05$). In contrast, the application of AA, the SynVIP vehicle, did not exert a proliferative effect on *(d)*-SH-SY5Y cell viability compared with the control, indicating that the vehicle alone did not induce any significant changes in cell viability. 10^{-7} M SynVIP group showed significantly better cell viability rates, thus we used 10^{-7} M SynVIP application in subsequent experiments.

As shown in Figure 3b, when CM of activated microglia that were transduced with LentiVIP were applied to *(d)*-SH-SY5Y cells, it was observed that both 5 and 25 MOI LentiVIP-infected microglia CM were associated with a significant increase in viability rates compared with non-infected activated microglia CM ($p < 0.05$). 25 MOI LentiVIP-infected microglial CM caused better viability rates significantly better cell viability rates, thus we used 25 MOI LentiVIP application in subsequent experiments ($p < 0.05$).

In addressing the concern regarding the potential presence of residual VIP in synthetic VIP-treated conditioned medium, we conducted an investigation by assessing the impact of VIP alone on *(d)*-SH-SY5Y cell viability. VIP was applied at concentrations of 3×10^{-10} and 7×10^{-10} M, chosen to be equivalent to the levels of VIP detected in the microglial conditioned medium (MG CM) -24-hour after zero point- following the application of 10^{-8} and 10^{-7} M SynVIP, respectively, as depicted in Figure 1a). Both concentrations of SynVIP showed insignificant changes in viability rates of *(d)*-SH-SY5Y cells.

4.4 | IF images of microglia cells

As depicted in Figure 4, the assessment of fluorescent images of microglia, stained with the microglial activation marker, CD11b, revealed distinct patterns. In the control group, which was not exposed to LPS, a moderate number of microglial cells, a few ameboid microglia cells scattered throughout all areas, and low-intensity CD-11b staining were observed. In the group treated with 5 μg/ml LPS, an increased number of microglial cells, intensified CD-11b staining, and an elevated presence of ameboeid microglia cells were evident. In contrast, the VIP-treated groups (both SynVIP and LentiVIP) exhibited a reduction in CD-11b expression and a decreased number of ameboeid microglia cells, as further illustrated in Figure 4a. As seen in the quantitative analysis of the fluorescence ratio of CD-11b/DAPI (Figure 4b), VIP treatments (both SynVIP and LentiVIP) diminished the LPS-derived elevation of the aforementioned ratio. Collectively, these results indicate that VIP attenuates microglial activation.

4.5 | IF images of *(d)*-SH-SY5Y cells

As seen in Figure 5, PGP9.5 activation was found to be significantly decreased in MPTP-treated *(d)*-SH- SY5Y cells when compared with non-treated control, supported by the results of quantitative analysis of the fluorescence ratio of PGP9.5/DAPI, which revealed the values of for the control group (0.83 ± 0.03), significantly higher than that of MPTP-treated group (0.40 ± 0.30) ($p < 0.05$).

4.6 | Apoptotic nuclear assessment of *(d)*-SH-SY5Y cells

Apoptotic nuclear morphology was examined using Hoechst 33342 staining. Activated MG CM treatment on *(d)*-SH-SY5Y cells expectedly caused a significant increase in apoptotic cell number when compared with non-activated MG CM treated ones. On the other hand, the groups of 'SynVIP/LentiVIP treated activated MG CM showed significantly reduced number of apoptotic cells, when compared with 'activated MG CM' treated group (Figure 6 a-e).

4.7 | SynVIP treated/LentiVIP infected microglia display higher levels of TGF-β1 in their conditioned media

The level of TGF-β1 showed a significant reduction in CM of LPS (5 μg/ml) treated microglial group (239 ± 46 pg/ml) compared with the control group (414 ± 23 pg/ml) ($p < 0.05$). Conversely, a substantial increase in TGF-β1 levels was observed in SynVIP (10^{-7} M) treated group (537 ± 33 pg/ml) compared with control

FIGURE 4 (a and b). Photomicrograph of microglia cells (phase/immunostained with CD11b antibody [a microglial activation marker]/DAPI and their merge), and 3D surface plot illustrating intensity (arbitrary units) (a), and quantitative analysis of the fluorescence ratio of CD-11b/DAPI, (b) of microglia cells that were treated with LPS: 5 μg/ml, SynVIP: 10^{-7} M or Lenti VIP: 25 MOI. Observation was made under a fluorescent microscope at ×20 objective [scale Bar = 100 μm]. (Amoeboid microglia are indicated by arrows). The quantitative analysis of fluorescence ratio of CD-11b/DAPI was conducted on a sample size of n = 5. The intensity range was set from 0 to 255, where 0 corresponds to the darkest shade, and 255 represents the lightest shade.

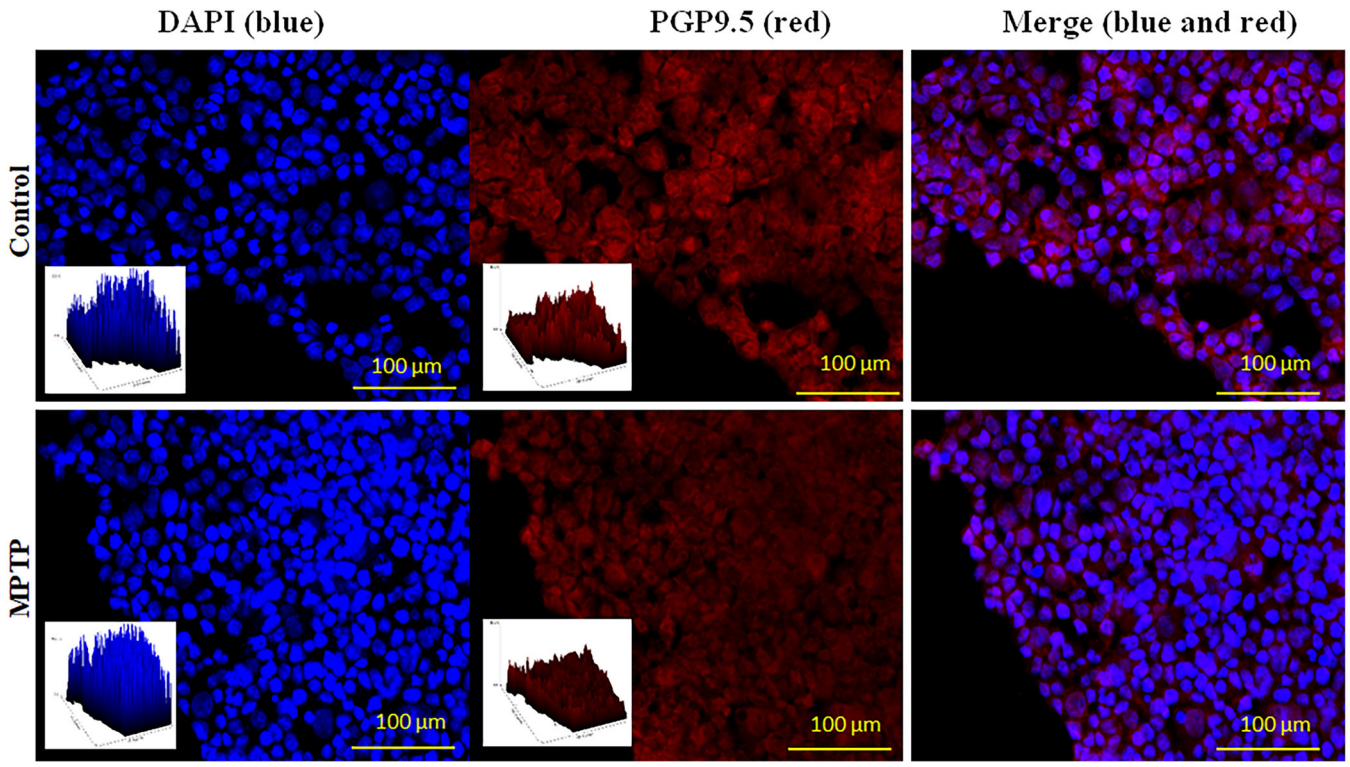

FIGURE 5 Photomicrograph of control and MPTP treated *(d)*-SH-SY5Y cells immunostained with *anti*-PGP9.5 antibody (a neuronal marker), and 3D surface plot illustrating intensity (arbitrary units) of these cells. SH-SY5Y cells that were differentiated within 6 days of RA treatment were treated with 1000 µM MPTP for 24 h. observation was made under a fluorescent microscope at ×20 objective. [scale Bar = 100 µm]. The cytoplasm of control *(d)*-SH-SY5Y cell groups exhibit a pronounced high intensity of PGP9.5 expression, whereas the ones treated with MPTP display a significantly diminished intensity in their cytoplasm, indicative of neurite degeneration. The quantitative analysis of fluorescence ratio of PGP9.5/DAPI, was conducted on a sample size of n = 5. The intensity range was set from 0 to 255, where 0 corresponds to the darkest shade, and 255 represents the lightest shade.

($p < 0.05$). Moreover, TGF-β1 levels were higher in LPS + SynVIP (10^{-7} M) treated group, (495 ± 41 pg/ml) compared with only LPS group (239 ± 46 pg/ml) ($p < 0.05$) (Figure 7a).

Compared with control (385 ± 23 pg/ml), a significant increase in TGF-β1 levels was observed in the 5 and 25 MOI LentiVIP transduced MG CM (546 ± 21 and 563 ± 34 pg/ml, respectively) ($p < 0.05$ for both) (Figure 7b).

4.8 | High TGF-β1 level and total antioxidant capacity and low levels of nitric oxide and total oxidant capacity in CM of *(d)*-SH-SY5Y cells treated with LentiVIP infected microglial CM

The CM from LPS-treated microglia led to a significant decrease in TGF-β1 levels within the CM of *(d)*-SH-SY5Y cells, compared with control ($p < 0.05$). The levels of this anti-inflammatory cytokine were notably higher in *(d)*-SH-SY5Y cell groups treated with '5 and 25 MOI LentiVIP-infected' activated MG CMs (396 ± 21, 413 ± 34 pg/ml, respectively) compared with the group treated with LPS alone (146 ± 21) (significant for both, $p < 0.05$) (Figure 8a).

Activated MG CM caused an increase in NO levels ($p < 0.05$). When compared with the group treated with LPS alone, the presence of 25 MOI LentiVIP-infected microglia CM led to significantly lower NO levels in *(d)*-SH-SY5Y cell CM ($p < 0.05$) (Figure 8b). Furthermore, activated MG CM resulted in increased TOC levels (Figure 8c) and decreased TAC levels compared with the control group (Figure 8d) (significant for both $p < 0.05$). However, these levels were reversed in the CM of *(d)*-SH-SY5Y cells that were exposed to LentiVIP-MG CM.

5 | DISCUSSION

Both neuro-inflammation and oxidative stress play important roles in PD (Graeber et al., 2011; Kraft &

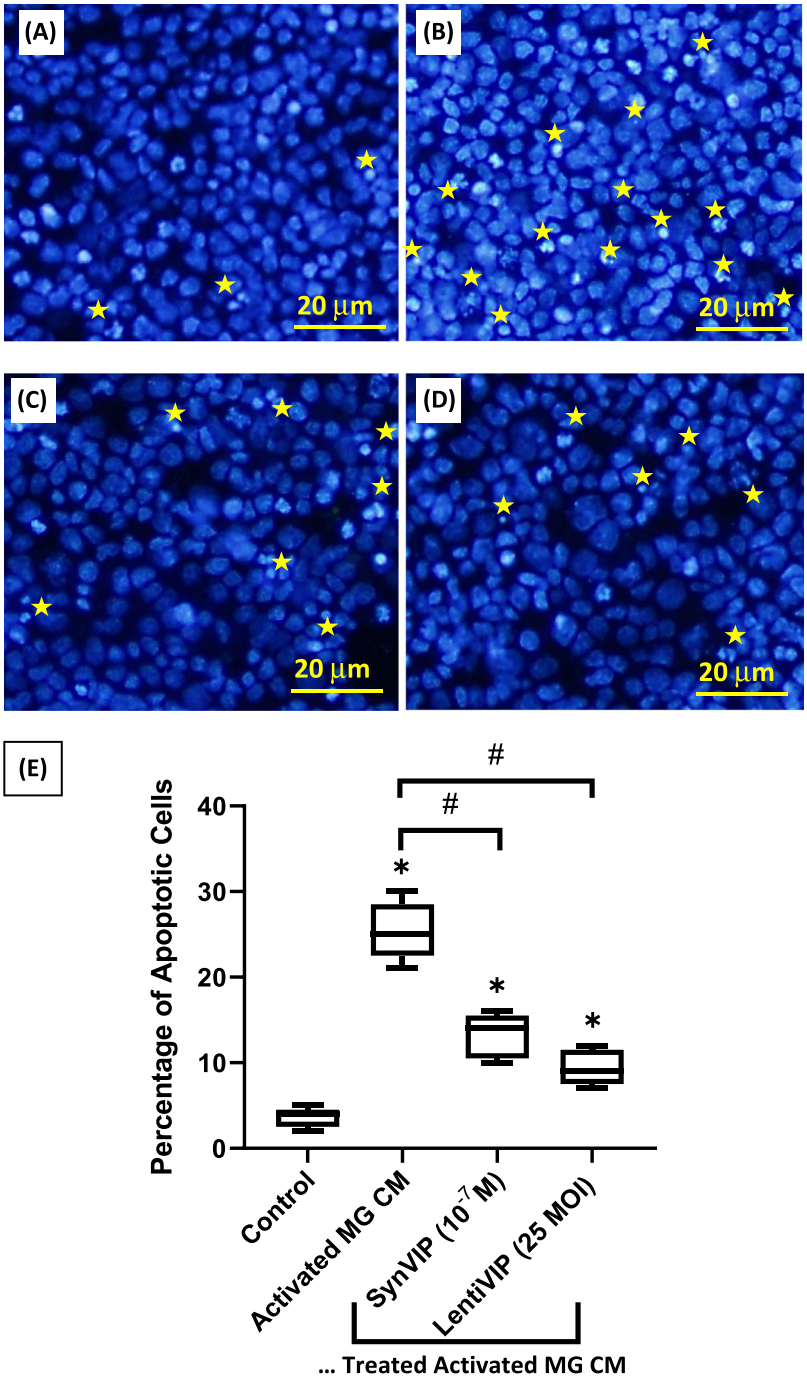

FIGURE 6 (a–e). Representative images for apoptotic nuclear assessment with Hoechst 33342 staining of *(d)*-SH-SY5Y cells after 24 h incubation with non-activated MG CM as control (a), activated MG CM (b), SynVIP (10^{-7} M)-treated activated MG CM (c), LentiVIP (25 MOI)-infected- activated MG CM (d). (*represents apoptotic cells). The number of apoptotic cells were counted and averaged from five areas. The percentage of apoptotic cells is represented as a histogram (e). Data are expressed as mean ± SEM (n = 5). (*$p < 0.05$ as compared with the non-activated MG CM treatment as control; $^{\#}p < 0.05$ as compared with activated MG CM treatment). Observation was made under a fluorescent microscope at ×20 objective [scale bar = 20 µm]. [activated MG CM = conditioned media of microglia activated with 5 µg/ml LPS].

Harry, 2011), which is the second most common cause of ND after AD. PD is primarily characterized by the loss of dopaminergic neurons in the SNpc, yet the precise molecular mechanisms underlying neurodegeneration in PD remain incompletely understood. Nevertheless, it is well-established that various pathophysiological mechanisms, including α-synuclein aggregation, inflammation, oxidative stress, mitochondrial dysfunction, and activation of apoptotic pathways, are implicated as causative factors (Aarsland et al., 2017). In patients with PD, the expression of enzymes responsible for generating harmful oxygen species, such as NADPH oxidase, induced nitric oxide synthase (iNOS), and myeloperoxidase, is elevated in the SNpc (Birben et al., 2012). The generation of reactive species can activate microglial cells, which are resident macrophage-like cells and serve as the initial and primary form of active immune defense in the CNS. Activated microglial cells can release proinflammatory cytokines such as TNF-α, IL-1β, and IL-6, all of which attract lymphocytes into the inflammatory process.

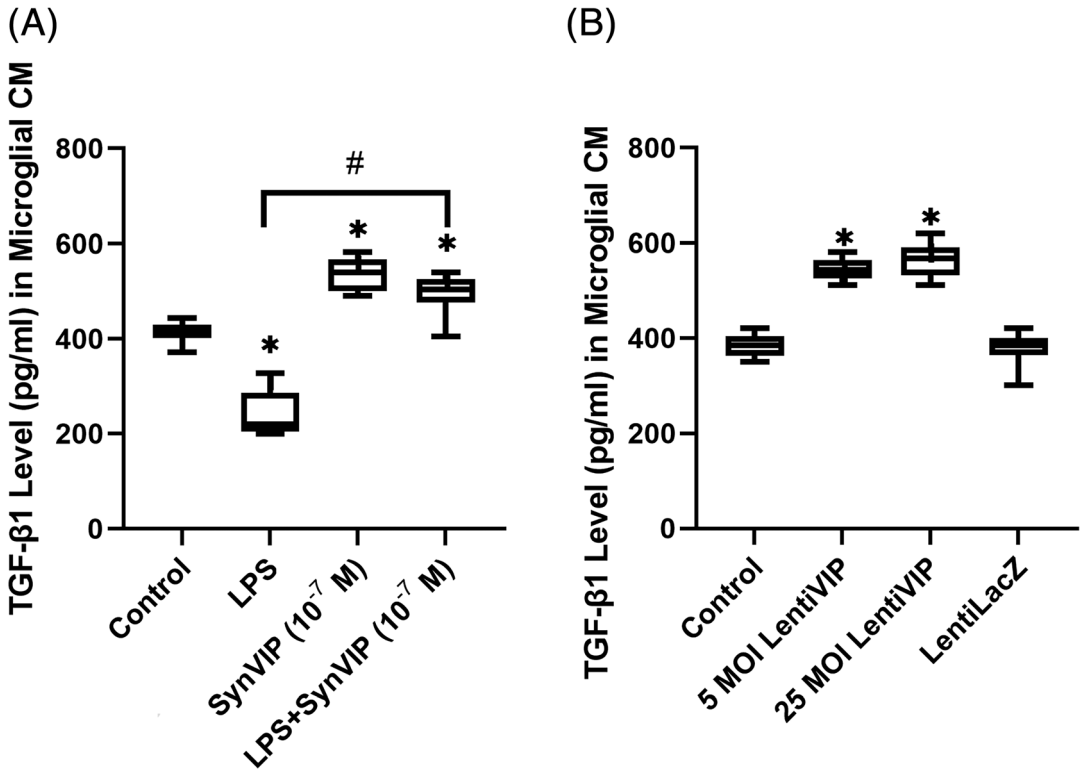

FIGURE 7 (a and b) The TGF-β1 levels in CM of microglia that were compared among non-treated (control), treated with LPS (5 μg/ml) and/or SynVIP groups (a), that were compared among non-treated (control), transduced with LentiVIP (5 and 25 MOI)/LentiLacZ (25 MOI) (b). The levels of TGF-β1 were assessed in comparison to the control. The presented data represent the mean ± S.E.M. from five independent experiments, each consisting of one sample, expressed as a percentage relative to the control (*$p < 0.05$, compared with the untreated cells as control, #$p < 0.05$, as compared among treatment groups). (for detailed timepoints of applications, see Section '2.3.3. Experimental design' and 'Graphical abstract').

Furthermore, these cytokines can indirectly stimulate excessive production of ROS and pro-inflammatory cytokines or directly induce receptor-mediated cytotoxicity (Dushanova, 2012). Clearly, microglial cells play active roles in the pathogenesis of PD, as well as AD, multiple sclerosis and autism (Block et al., 2007; Goldmann & Prinz, 2013; Hong et al., 2016; Qian & Flood, 2008; Ransohoff & Perry, 2009; Takano, 2015; Zhan et al., 2014).

Inhibiting abnormal microglial activation, reversing apoptotic processes, or reducing oxidative stress may represent effective strategies in prevention or treatment of such diseases (Slemmer et al., 2008). In this context, many antioxidant compounds have been investigated to prevent neurodegenerative disorders by scavenging of ROS (Slemmer et al., 2008; Suematsu et al., 2011). Given that the immunomodulatory and antioxidant properties of VIP in both in vivo and in vitro models of ND, that have been previously documented, there is substantial potential for its use in the treatment of such diseases (Abad & Tan, 2018; Delgado & Ganea, 2013). In this regard, VIP induces brain-derived neurotrophic factor and activity-dependent neuroprotective protein, which are growth factors involved in neuroprotection (Rangon et al., 2006).

Recent reports have highlighted the modulatory effects of VIP on CNS function in conditions such as multiple sclerosis, stroke, and AD (Fernandez-Martin et al., 2006; Song et al., 2012; Yang et al., 2015). However, VIP's clinical efficacy is often limited because of several factors, including its vulnerability to endopeptidases. To overcome these limitations and explore the clinical applicability of VIP, structural modifications of VIP were investigated, identifying shorter active VIP fragments that enhanced its neuroprotective activity (Deng & Jin, 2017). Another option to ameliorate deficits of traditional VIP treatment is application of gene therapy which has the potential to treat human diseases with a single injection.

In recent years, several gene therapy modalities for different types of ND have been progressed into clinical development (Martier & Konstantinova, 2020). Within clinical trials involving patients with PD, innovative gene therapy vectors expressing neurotrophic factors, like glial

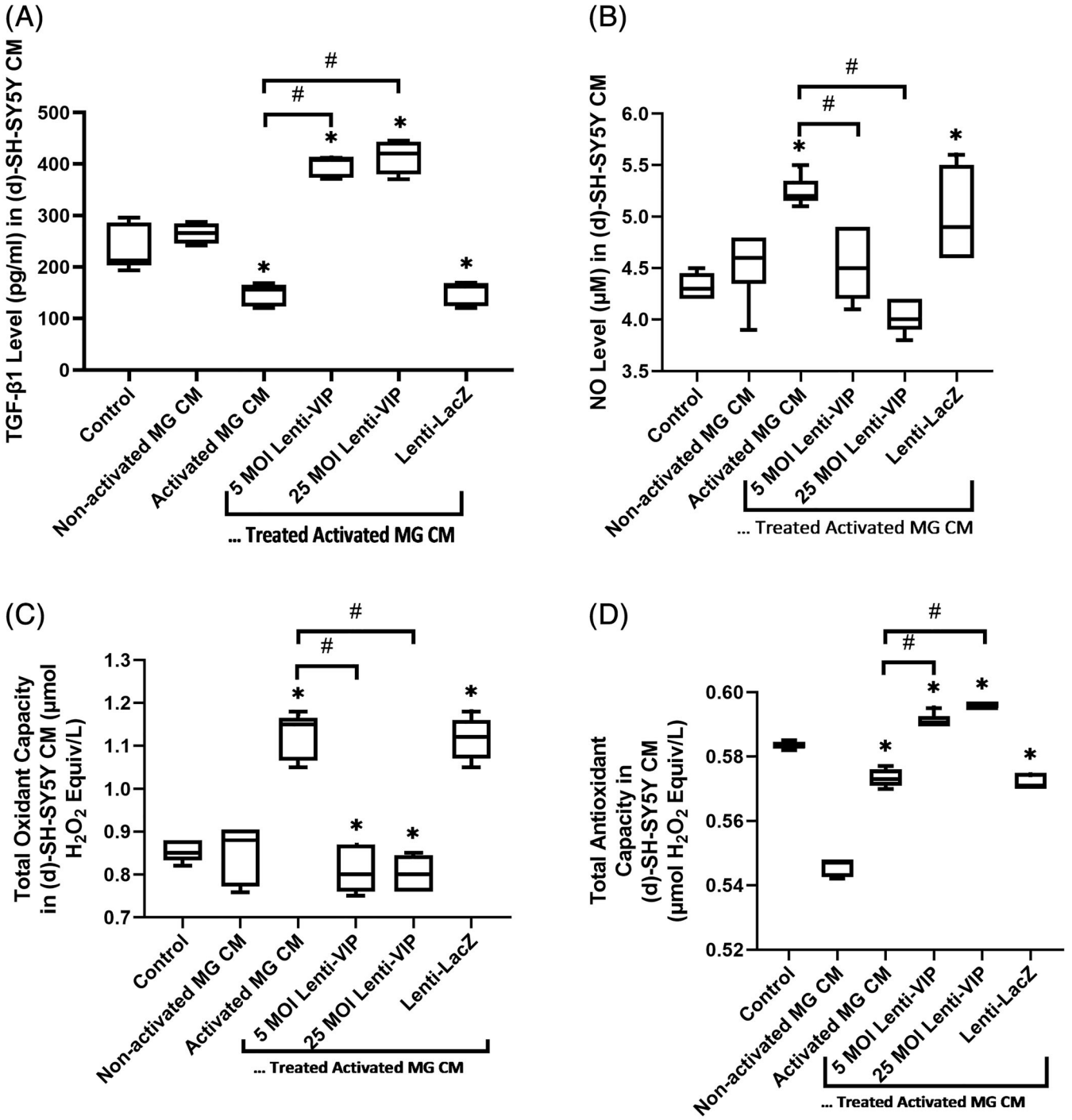

FIGURE 8 (a–d). Changes in TGF-β1 and NO levels, and total oxidant/antioxidant capacity in CM of *(d)*-SH-SY5Y cells. MG CM were collected from non-activated microglial groups or activated microglia (after treatment with 5 μg/ml LPS) alone or in combination with 5 MOI LentiVIP or 25 MOI LentiVIP/LentiLacZ. Subsequently, these conditioned media were applied to *(d)*-SH-SY5Y cells for a duration of 24 hours. Following this exposure, the levels of TGF-β1 (a), NO (b), the Total oxidant (c) and Total antioxidant capacities (d) in *(d)*-SH-SY5Y cell CM were measured. The presented data represent the mean ± S.E.M. from five independent experiments, each consisting of one sample, expressed as a percentage relative to the control ($^{*}p < 0.05$ as compared with the untreated cells as control, $^{\#}p < 0.05$, as compared with the activated MG CM treated group). (for detailed timepoints of applications, see Section '2.3.3. Experimental design' and 'Graphical abstract').

cell-derived neurotrophic factor neurturin (NTN) and glial cell line-derived neurotrophic factor (GDNF) have been explored. The rationale behind delivering these neurotrophic factors was to provide support to the degenerating neuronal population rather than targeting specific causative pathological molecular pathways. Although the

delivery of these neurotrophic factors via AAV was well-tolerated in patients, their efficacies remained unclear. Nevertheless, these studies played a crucial role in demonstrating the feasibility and safety of intraparenchymal gene therapy delivery directly into the human brain (Björklund et al., 2000; Marks et al., 2008; Marks et al., 2010).

Today lentiviral vectors are known to be the most potent of all integrative vector systems (Zufferey et al., 1998). Among the various viral vectors tested, the latest generation of lentiviral vectors stands out as some of the safest and most efficient tools for achieving stable gene transfer. These vectors possess the capacity for sustained, long-term gene expression, making them valuable for addressing complex ND (Robbins & Ghivizzani, 1998). The mentioned advantages justify the use of lentiviral vectors for delivering the VIP transgene in the treatment of ND (Erendor et al., 2020; Tasyurek et al., 2018). Recently, the therapeutic efficacy of lentivirus-mediated VIP gene delivery was explored in mouse models of both Type 1 and Type 2 diabetes mellitus. LentiVIP delivery not only enhanced insulin sensitivity and glucose tolerance in diet-induced obese Type 2 models (Tasyurek et al., 2018), but also improved glucose tolerance, reduced hyperglycemia, and prevented weight loss in streptozotocin-induced Type 1 diabetic models (Erendor et al., 2020).

Additionally, Cobo et al. reported beneficial effects of LentiVIP gene therapy in a mouse model of chronic multiple sclerosis, where LentiVIP-infected mesenchymal stem cell treatment reduced astrocyte activation and decreased neuronal cell death (Cobo et al., 2013). Furthermore, several studies have illustrated the potential of lentivirus vector-based gene therapies for addressing ND, despite the mixed outcomes observed in some clinical studies (Björklund et al., 2000; Martier & Konstantinova, 2020). The utilization of lentivirus as a delivery tool has been demonstrated to be both safe and effective in clinical trials. To date, clinical trials employing these vectors have not raised concerns related to integration-induced mutagenesis. Notably, in a mouse model of severe rheumatoid arthritis, a single injection of LentiVIP was found to reduce autoimmune and inflammatory responses, resulting in highly effective treatment with complete regression of established disease (Delgado et al., 2008). In a separate study conducted by the same research team, a single administration of dendritic cells transduced with LentiVIP during differentiation from bone marrow cells was proven to be therapeutic when administered before the onset of experimental autoimmune encephalomyelitis (Toscano et al., 2010).

In our study, we investigated the protective effects of VIP through lentivirus-mediated VIP gene delivery and SynVIP treatments against the toxicity of microglial secretome and MPTP-induced neurotoxicity (Javitch et al., 1985; Notter et al., 1988; Westlund et al., 1985) using an in vitro cell culture model. Initially, we demonstrated that the transduction of microglial cells with LentiVIP resulted in a robust expression of VIP. Subsequently, we observed that LentiVIP transduction of microglia exerted protective effects against cell viability loss induced by both neurotoxin and the secretome of activated microglia. Furthermore, LentiVIP transduction of microglia led to increased expression of the anti-inflammatory molecule TGF-β1 from microglia. Neuron-like cells treated with CM from LentiVIP -transduced microglia exhibited decreased NO levels and TOC, along with elevated levels of TGF-β1 and TAC, providing evidence of the antioxidative properties associated with LentiVIP transduction.

It is well-established that TGF-β is typically found at low levels in the brain until inflammation occurs (Lu et al., 2005). There is also evidence supporting the antiinflammatory role of TGF-β and its significance in neuroprotection (Brionne et al., 2003; Lu et al., 2005; Nagai et al., 2001). Therefore, the stimulatory effect of VIP on TGF-β1 expression appears to be a crucial indicator of its neuroprotective actions. In line with our findings, Reynolds et al. demonstrated the immunomodulatory and neuroprotective activities of VIP (Reynolds et al., 2010). Additionally, VIP has been shown to induce the development of regulatory T cells (Treg) with concomitant anti-inflammatory and neuroprotective responses in MPTP-intoxicated mice (Delgado & Ganea, 2003c; Ganea & Delgado, 2002; Reynolds et al., 2010).

Furthermore, we observed that LentiVIP exerted not only an anti-apoptotic effect, but at the same time a cell-proliferative effect on neuronal cells. Namely, the decrease in the survival rate of *(d)*-SH-SY5Y cells because of the application of activated MG CM, has been improved by LentiVIP (and also by SynVIP), as verified by MTT results. On the other hand, our apoptotic nuclear analysis showed that LentiVIP infected (and also SynVIP treated) activated MG CM caused lesser apoptotic cell percentage of *(d)*-SH-SY5Y cells when, compared with high apoptotic cell number because of activated MG CM. However, the MTT results we obtained were reflecting a very high cell survival rate in VIP groups above the anti-apoptotic action of VIP, indicating also a cell proliferative effect of this peptide. Similar findings have been reported concerning the cell-proliferative and anti-apoptotic effects of LentiVIP by the study of Erendor et al. in which LentiVIP has been demonstrated to suppress inflammation and apoptosis in pancreatic beta cells, induced by diabetes, and even promoted cell proliferation (Erendor et al., 2020).

It is crucial to underscore the comparable results observed in our study between SynVIP and LentiVIP treatments. Although the outcomes of SynVIP treatment demonstrated similarity to those of LentiVIP, it is noteworthy that LentiVIP gene therapy emerges as a potentially advantageous option. Specifically, when the VIP gene was delivered into microglial cells via Lentivirus, and VIP expression was compared with SynVIP, the VIP expression resulting from low-dose SynVIP treatment closely resembled that achieved through high-dose LentiVIP transduction, as illustrated in Figures 1a and c.

It is important to acknowledge that VIP is a peptide prone to rapid degradation by DPP-4. Therefore, if beneficial effects are observed even at lower expression levels, effective treatment might not necessitate higher concentrations of LentiVIP. Furthermore, the integration of the VIP gene into the microglia genome, characteristic of 3rd generation lentiviral vectors, facilitates permanent gene transfer. In contrast, SynVIP requires regular administration, while LentiVIP, utilizing the CMV promoter, offers sustained low-level but long-term expression.

However, it is essential to clarify that the primary objective of this publication was not a direct comparative analysis between the effects of SynVIP and LentiVIP. Instead, our focus was on evaluating the in vitro efficacy of microglial transduction using lentiviral gene therapy vectors encoding VIP and investigating the protective effects of LentiVIP against activated microglial toxicity. We successfully substantiated these aims in this study. Aforementioned knowledge emphasizes the potential advantages of LentiVIP gene therapy over SynVIP.

Furthermore, our results indicated the antioxidant effects of VIP, as the levels of NO and TOC were significantly lower in the CM of neuronal cells exposed to LentiVIP-infected or SynVIP treated microglial secretome. These findings support a previous study that has reported that VIP effectively blocked microglial activation and the production of neurotoxic factors, including TNF-α, IL-1β, and NO, in a model of PD and brain trauma (Delgado & Ganea, 2003b; Delgado & Ganea, 2003c). The elevated levels of NO and oxidant molecules, along with inflammatory cytokines in MG CM may serve as indicators of the toxicity of the microglial secretome. Indeed, the activation of microglia involves the secretion of multiple cytokines/chemokines and reactive species (Graeber et al., 2011). Activated microglia can generate reactive oxygen and nitrogen species, including NO, primarily through the upregulation of the inducible form of iNOS, also known as NOS2 (Dello Russo et al., 2018). Previous studies have also reported that IL-1β and NO which are derived from microglia could be related to neuroinflammation and neuron injury in the CNS (Black & Waxman, 2012; Ransohoff & Perry, 2009; Sperlágh & Illes, 2007; Yang et al., 2016). VIP has also been found effective in preventing LPS-induced neurodegeneration and microglial activation in in-vivo neuroinflammation models (Delgado & Ganea, 2003b; Delgado & Ganea, 2003c), as well as in suppressing neuronal death associated with ND in both in vitro and in vivo settings (Dejda & Soko, 2005).

In our study, in order to make more accurate evaluations, we should consider those two separate effects of CM of 'VIP-treated microglia' on *(d)*-SH-SY5Y cells: 1-The indirect 'deactivation/detoxifying' effect of VIP on microglia (less inflammatory and cytotoxic factors and more anti-inflammatory mediators in CM). 2-Effects of VIP that is secreted by microglia and found in CM. It is necessary to take into account these both effects when VIP-treated MG-CM is applied on *(d)*-SH-SY5Y cells.

While this study primarily evaluates the indirect 'detoxifying' effect of VIP on microglia, it is relevant to briefly allude to ongoing research from our laboratory. Specifically, in a separate investigation currently undergoing further analysis, we explored the protective potential of direct VIP application against degeneration of *(d)*-SH-SY5Y cells induced by MPTP/activated MG CM. Our preliminary results indicate that, following the application of activated MG CM, treatment with SynVIP at a concentration of 10^{-7} M significantly increased the mean viability rate of (d)-SH-SY5Y cells compared with the group treated with activated MG CM alone. Intriguingly, direct application of VIP did not correct the effects of microglial toxicity to the extent of the results we obtained in this study (the very high rate of improvement in (d)-SH-SY5Y cell viability).

Our overall results show that VIP's effect on the neuron, is both direct and indirect in terms of function and viability. The presence of VIP within the MG CM also provides extra benefit on *(d)*-SH-SY5Y cells. From here, the effect of VIP against neurodegeneration should be evaluated based on the sum of both effects. One of the limitations of this study is that we were not able to test both of these effects in our study. We plan more advanced models in the future involving application of VIP-blocking antibody in the experimental design, so that, VIP's effect on the reduction of activated MG CM toxicity and the direct effects of it will be more clearly distinguished from each other.

Furthermore, our in vitro neurodegenerative model, which involves activated microglia, may replicate and simulate findings observed in ND. For instance, in AD, microglia surrounding plaques undergo a morphological change from ramified to amoeboid and exhibit positive staining for activation markers (Bolmont et al., 2008; Itagaki et al., 1989). Likewise, a substantial number of activated microglia are present in the CNS and spinal

cords of patients with amyotrophic lateral sclerosis (ALS) and in SOD1 mouse models of ALS (Hall et al., 1998; McGeer et al., 1993). Consequently, this model holds promise as a faithful representation of the in vivo environment where neurons interact with activated microglia, as observed in ND. It is well-suited for conducting drug trials.

In summary, our study highlights the promising therapeutic potential of LentiVIP and SynVIP as valuable strategies in mitigating neuronal damage caused by neurotoxins and microglial secretome, offering hope for effective interventions in the context of ND.

Incidentally, it would be useful to mention here the importance of using VIP in other diseases, as well. A potential therapeutic role of a VIP agonist have been reported on asthma, pulmonary hypertension, chronic obstructive pulmonary disease, cystic fibrosis, and sarcoidosis. Simultaneously, limited clinical trials have reported that novel stabilized inhaled VIP agonists with less side effects are promising alternative drug candidates (Mathioudakis et al., 2013). These agonists have been proven to exhibit immunoregulatory effect in sarcoid alveolitis in humans and been proposed as an attractive future therapy to control augmented immune responses in lung disorders (Prasse et al., 2010). Moreover, Aviptadil, a SynVIP has been shown to be effective in the treatment of sepsis- related severe respiratory failure and some other lung injuries. Ttrials are still ongoing to clarify the effectiveness of this drug in the treatment of COVID 19 (Lahiry et al., 2022).

Lastly, BBB is one of the most important challenges in the treatment of ND, including PD. Because of the presence of the BBB, that involves endothelial cells, astrocytes, pericytes, and basal membranes providing enclosure of the capillaries, only small lipophilic molecules can enter the brain. Large hydrophobic charged molecules can enter through facilitated transport, whereas pharmacophore designed drugs and dopamine are restricted for entry. The use of nanotechnology overcomes the ineffectiveness of CNS-related disorder treatments because of the BBB. By this technology, the parenteral route of drug administration enables direct systemic exposure of nanoparticles, and provides the complete bioavailability of the drug, at the same time (Shankar et al., 2021).

Gene therapy has the potential to effectively treat ND, because it has potential to provide direct correction of pathogenic mechanisms, and also to achieve neuroprotection/restoration, or diminish symptoms at the same time (Sudhakar & Richardson, 2019). Since the CNS has a large vascular structure, it would be easy to deliver gene therapy vectors if the BBB was not present. However, efforts continue to overcome the limitations imposed by BBB. An effective method for this is the transport of viral vectors across the BBB, achieved by temporary disruption of the endothelial tight junctions of the brain microvasculature; or by using receptor-mediated transcytosis, which is in phase I clinical trials in humans (Fu & McCarty, 2016). Recently, accurate vector delivery has been achieved by Interventional MRI-guided convection-enhanced delivery (iMRI-CED), which is an advanced neurosurgical technique, and this technique promotes the translation of preclinical therapies, being developed for ND, into clinical therapies (Sudhakar & Richardson, 2019).

6 | CONCLUSION AND LIMITATIONS

Our study sheds light on the promising therapeutic potential of LentiVIP in the context of ND, particularly PD. This is attributed to its anti-inflammatory, antioxidant, and anti-apoptotic capabilities against both MG CM/neurotoxin -induced neurodegeneration. Moreover, the observed reduction in neurotoxicity in our in vitro model suggests that VIP-based gene therapy approaches may be clinically applied in treating ND and show long-term promise for treating a wide range of CNS diseases.

Considering these findings, it is imperative to further investigate the therapeutic efficacy of LentiVIP in ND using advanced in vitro models, such as induced pluripotent stem cell-derived dopaminergic/motor neuron models, as well as in vivo ND models. Since our study only partially predicts the in vivo behavior of neuronal cells, our initial observations should be validated through further investigations involving acutely isolated cells, organoids, or other animal models that allow for a more precise quantification of in vivo behavior. Further exploration through advanced techniques at the molecular level is also essential to unravel the precise molecular mechanisms underlying LentiVIP's effects. Given that this peptide exhibits therapeutic potential for neurodegenerative disorders, the role of LentiVIP in neuroprotection worth further investigation for the development of innovative treatments.

AUTHOR CONTRIBUTIONS

Azize Yasemin Goksu: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; software; supervision; validation; visualization; writing—original draft; writing—review and editing. **Fatma Gonca Kocanci:** Data curation; formal analysis; resources; software; supervision; validation; visualization; writing—

original draft; writing—review and editing. **Ersin Akinci:** Conceptualization; funding acquisition; investigation; methodology; project administration. **Devrim Demir-Dora:** Conceptualization; funding acquisition; methodology; project administration. **Fulya Erendor:** Investigation. **Salih Sanlioglu:** Conceptualization; funding acquisition; methodology; project administration. **Hilmi Uysal:** Conceptualization; funding acquisition; project administration.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

We placed our data on Figshare, a public access repository. https://doi.org/10.6084/m9.figshare.24746250.

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