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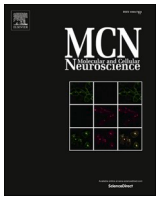

Stable apelin-13 analogues promote cell proliferation, differentiation and protect inflammation induced cell death

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ABSTRACT

Emerging evidence indicates that apelin, an adipokine, plays a critical role in numerous biological functions and may hold potential for therapeutic applications; however, its efficacy is constrained by rapid plasma degradation. Thus, the search for novel apelin analogues with reduced susceptibility to plasma degradation is ongoing. We have previously shown novel modified apelin-13 analogues, providing exciting opportunities for potential therapeutic development against Alzheimer's disease. In this study we explored novel insights into the neuroprotective effects of stable fatty acid modified (Lys8GluPAL) apelin-13-amide and amidated apelin-13 amide in mitigating cellular damage in SH-SY5Y neuroblastoma cells exposed to palmitic acid (PA) and lipopolysaccharide-induced (LPS) stress. Both apelin-13 analogues were found to modulate ER stress response and reduce oxidative stress by suppressing PA- and LPS-induced ROS production (36 % and 42 % reductions in GSH/GSG ($p < 0.005$). The peptides attenuated apoptosis by reducing caspase 3/7 activity and restoring bcl2 expression ($p < 0.05$) in cells treated with PA and LPS. They also downregulated pro-apoptotic genes, protected neurites from stress-induced damage, and promoted neurite outgrowth. The observed protective effects could be due to activation of the AMPK pathway, a critical regulator of cellular energy homeostasis and survival. These findings provide insight into novel, enzymatically stable apelin-13 analogues and highlight their potential to be developed as therapeutic agents against neuroinflammation and neurodegenerative disease, including Alzheimer's disease.

1. Background

Neurodegenerative conditions like Alzheimer's disease (AD) are associated with neuroinflammation, synaptotoxicity, changes in dendritic spines shape and progressive neuronal death, leading to cognitive impairment (Tzioras et al., 2023). Globally, 50 million people suffers from AD, and with increased prevalence of AD with age, it is predicted to reach 152 million by 2050 (Hou et al., 2019; Guerchet et al., 2020) which will have a great socioeconomic and healthcare burden (Wong, 2020). There is lack of understanding regarding AD pathophysiology, but growing evidence suggests a central role of neuro-inflammation (Heneka et al., 2015; Leng and Edison, 2021). Interestingly, neuroinflammation is also linked to metabolic syndromes such as obesity and diabetes (O'Brien et al., 2017; van Dyken and Lacoste, 2018). Especially, the Long chain saturated fatty acids (LCSFA) are implicated with central obesity, insulin receptor resistance and development of systemic and neuronal inflammation (Sergi and Williams, 2020). LCSFA induced lipotoxicity, plays a prominent role in multiple abnormalities including development of oxidative stress and microglial activation, leading to neuro-inflammation and impaired protein clearance in AD (Vesga-Jiménez et al., 2022; Cleland and Bruce, 2024). Lipopolysaccharides (LPS), an endotoxin, can exacerbate pro-inflammatory pathways, amplify neuro-inflammatory response and promote apoptosis (Mohammad and Thiemermann, 2021). Currently available pharmacological intervention for AD primarily focuses on symptom alleviation and have no positive curative effects on either halting or slowing the progression of AD. There remains an urgent need for predictive therapeutic interventions capable of minimising the pathophysiological

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impact of AD. However, before such interventions can be applied clinically, *in vitro* studies are essential to validate their efficacy and safety. These studies are crucial in developing strategies that could effectively target inflammation and neurodegeneration-driven cellular loss, potentially slowing or halting AD progression.

Apelin is a circulating adipokine, produced and secreted by white adipose tissue (WAT) (Boucher et al., 2005). APLN gene, located on chromosome 11 (11q12) (O'Dowd et al., 1993) produces preproapelin, a 77-amino-acid precursor, which is subsequently cleaved into several bioactive smaller peptides, including apelin-12, −13, −16, −17, −19 and 36 (Kawamata et al., 2001). Apelin receptor, also called APJ receptor, (of which Apelin is a cognate ligand), belongs to family of G-protein-coupled receptors (GPCRs) and is ubiquitously expressed in various tissues (Castan-Laurell et al., 2011). APJ/Apelin signalling has been identified to play central role in metabolic signalling including cardiovascular regulation and glucose homeostasis (Wysocka et al., 2018; Hu et al., 2021; Li et al., 2022). Recent studies have indicated apelin's role in cell proliferation in neuroblastoma cell lines (Jiang et al., 2018; Chen et al., 2020) and reduction in neuro-inflammation by affecting leukocyte recruitment by modulating cell adhesion molecules (Park et al., 2024).

Native apelin peptides have a short half-life (O'Harte et al., 2017) making it unsuitable for pharmacological use. We have previously shown that structural modification of native Apelin-13 at N- and C terminals could prolong clearance and improve bioactivity (Parthsarathy et al., 2018; O'Harte et al., 2018). Various studies have utilised both Palmitic acid (a LCSFA) and LPS for induction of inflammation as they are potent in upregulating pro-inflammatory cytokines, ROS (Reactive oxygen species) generation, and ultimately cell suicide in microglial (Zhao et al., 2019a; Zhang et al., 2018; Lu et al., 2021; Chmielarz et al., 2023), neurons (Zhao et al., 2019b; Sergi et al., 2020; Zhang et al., 2021a) and microglial-neuronal co-culture (Beaulieu et al., 2021) and various other tissues (Yamada et al., 2006; Alnahdi et al., 2019). In this study we have evaluated the effects of stable apelin 13-amide and (Lys8GluPAL) apelin-13-amide in mitigating PA and LPS induced cell growth arrest, cell death, cell survival and cellular health.

2. Materials and methods

2.1. Peptides

All apelin analogous used in the study were purchased from Synpeptide®; with a certification of >95 % purity. Structural identity and purity of peptides were confirmed in house as described in an earlier study (Parthsarathy and Hölscher, 2013). To confer resistance against cleavage by angiotensin converting enzyme 2 (ACE2), native peptides were modified by amidation of the C-terminus and/or addition of a gamma-glutamyl spacer with palmitate adjunct (GluPAL) to the side chain Lys^8 which promotes plasma protein binding and reduces renal clearance (Parthsarathy and Hölscher, 2013; Parthsarathy et al., 2016). Similar substitutions were used previously in our work with other small peptides and been tested previously for stability and potency.

2.2. Cell culture and differentiation

Human neuroblastoma cells line (SH-SY5Y) was obtained from University of Aston, UK and stored in liquid nitrogen. Upon thawing, cells were cultivated as monolayers in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 supplemented with L-glutamine, heat inactivated FBS (10 % *v*/v), antibiotics penicillin (100 U/ml) and streptomycin (0.1 mg/ml) (Gibco, Strathclyde, UK). Cells were incubated in humidified environment of 95 % air/5 % CO2 at 37 °C. The culture medium was renewed every 3 days and sub-cultured or seeded at 90 % confluency or when required, differentiated with 10 mM Retinoic acid for 3 days in dark at 37 °C as described previously (Karacaoğlu, 2021).

2.3. Cell viability and toxicity assessments

Cell viability was evaluated using the CyQUANT ™ MTT Cell Viability Assay Kit (Thermofisher, UK) and CellTiter-Glo® Luminescent Cell Viability assay kit (Promega, UK). Assays were conducted following manufacturer's protocols. Briefly, 96 well plate (MTT assay- clear, Luminescent assay-opaque) were seeded with SH-SY5Y cells at a density of 1×10^4 cells/well, incubated for 24 h and then treated. Labelling reagents were added and incubated for set period. For MTT, reaction was stopped by adding DMSO, and absorbance measured at 570 nm.. Lactate dehydrogenase (LDH), a marker for cell toxicity was analysed using CyQUANT™ LDH Cytotoxicity Assay Kit (Thermofisher Scientific, UK) following manufacturer's protocol. Briefly, 1×10^4 cells/well were seeded into 96-well plates. Following treatments, 50 ml of cell culture supernatant was incubated with reagent substrate, reaction stopped, and absorbance measured at 450 nm. For AMPK inhibition, SH-SY5Y cells were pretreated with 5 μM Compound C (6-[4-(2-Piperidin-1-ylethoxy) phenyl]-3-pyridin-4-ylpyrazolo [1,5-a]pyrimidine) a cell-permeable AMPK inhibitor, for 30 min before treatments with peptides for 6 h. Cell viability (CellTiter-Glo® Luminescent Cell Viability assay kit) and toxicity (CytoTox 96® Non-Radioactive Cytotoxicity assay kit) was used as per vendor's instructions.

2.4. Neurite outgrowth assay

SHSY-5Y cells were seeded at density of 1×10^5/well in 24-well plate and differentiated with Retinoic acid (10 μM). Following 3 days of incubation at 37 °C cells were treated with peptides for 24 h and fixed with freshly prepared paraformaldehyde. Following washing steps with PBS (Phosphate buffered saline), fixed cells were stained with Coomassie brilliant blue and imaged at 20× magnification. Neurite growth (vertical axis) was quantified as the percentage of cells bearing axodendritic processes longer than two times cell diameters in length. The double the body of cell neurite outgrowths were counted and analysed by Image J (NIH).

2.5. Reactive oxygen species measurement (ROS), GSH/GSSG (Glutathione/Glutathione disulfide) and Caspase assays

Levels of ROS production in treated cells were analysed with ROS-Glo™ H_2O_2 Assay kit (Promega, UK), Total and oxidized glutathione was measured with GSH/GSSG-Glo™ Assay (Promega, UK) and Caspase-3 and caspase-7 activities in adherent cells were measured with Caspase-Glo® 3/7 assay (Promega, UK) as per manufacturer's protocol.

2.6. Mitochondrial membrane potential ($\Delta\Psi_m$) measurement

Mitochondrial membrane potential was measured by JC-1 dye (Thermofisher, UK). Briefly, 5×10^4 cells/well were seeded on Poly-D lysin coated coverslips and incubated. Following treatments, cells were washed and incubated with JC-1 dye for 30 min and then fixed (4 % paraformaldehyde), washed, mounted with fluoroshield and images captured using Olympus XI83 Inverted fluorescence microscope at 20× magnification for green (monomers) (excitation 470 nm, emission 525 nm) and red (aggregates) (excitation 560 nm, emission 595 nm) fluorescence and images analysed using Image J (NIH).

2.7. Western blot

Standardised procedures were followed for western blot analysis. Cells (2×10^6 cells/well) were grown, and following serum starvation, treated with stressors, with or without apelin analogues. Cells were lysed in ice-cold RIPA (Radioimmunoprecipitation assay) buffer and BCA (Bicinchoninic acid assay) was used to assess protein concentration. Proteins were separated on polyacrylamide gels and transferred to PVDA membrane, followed by blocking and subsequent incubation with

primary antibodies Bax (89477), bcl2 (51071) and GAPDH (5174S) (dilutions were 1:1000 for 89,477; 1:1000 for 51,071; and 1:3000 for 5174S, Cell Signalling, UK). All the primary antibodies were raised in mouse except for GAPDH which was raised in rabbit. Following wash steps, blots were incubated with HRP-linked secondary antibodies against corresponding species (1:1000, Cell signalling, UK). Upon exposer with BCL, band intensity was quantified using LI-COR software. GAPDH served as loading control and utilised to normalise the relative peak intensity of the markers.

2.8. Statistical analysis

The statistical package PRISM (v.10.0, GraphPad Software Inc., San Diego, CA, USA) was used to analyze the data. One-way ANOVA followed by Tukey's post-hoc test was used to compare the difference between groups. All data were presented as mean ± SEM for given numbers of replications (n) as indicated in individual figs. *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Apelin-13 analogues restore palmitic acid and lipopolysaccharide -induced cell toxicity and cell growth arrests

We evaluated the efficacy of stable apelin analogues in protecting the human neuroblastoma cells *in-vitro* from the acute and chronic stress and inflammation caused by Palmitic acid (PA) and lipopolysaccharides (LPS). PA, a saturated fatty acid is a lipotoxic agent and LPS is inducer of neuroinflammation. SH-SY5Y cells were treated with apelin-13 amide or (Lys8GluPAL) apelin-13 amide, with or without the presence of the stressors PA (0.6 and 1.0 mM) and LPS (30 and 50 μg). A one-way ANOVA with *post hoc* analysis further revealed a decline in metabolically active, viable cells in a dose and time dependent manner in both PA (Fig. 1A-C, 0.6 mM- 22–32 % reduction in metabolic active cells; $p <$ 0.01-$p < 0.0001$; 1.0 mM-30-34 % reduction; $p < 0.01$-$p < 0.0001$; $F_{8,126} = 2.049–3.693$; $R^2 = 0.3483–0.987$) and LPS treatments (Fig. 1D-F, 30 μg- 28-31 % reduction, $p < 0.01$-$p < 0.001$; 50 μg- 40-57 % reduction; <0.0001; $F_{8,126} = 0.6862–2.939$; $R^2 = 0.4346–0.68$) compared to control conditions. On the contrary, cellular release of LDH gradually increased in the supernatants of cells treated with PA (Fig. 2A-C, 0.6 mM-4- 31 % $p < 0.0001$; and 1.0 mM-17-35 %, $p < 0.001$-$p < 0.0001$; $F_{8,129} = 0.897–2764$; $R^2 = 0.5073–0.5984$) and LPS (Fig. 2D-E, 30 μg-19-44 % $p < 0.001$-$p < 0.0001$; and 50 μg −20-51 %, $p < 0.001$-$p < 0.0001$; $F_{8,126} = 1.54–4.074$; $R^2 = 0.3851–0.5295$).

Importantly, co-treatment with Apelin-13 analogues counteracts the reduction in cell viability of SH-SY5Y microcultures caused by PA (Fig. 1A; 0.6 mM- 21 %, $p < 0.0001$; Fig. 1B-C, 1.0 mM- 11–23 %, $p <$ 0.05-$p < 0.0001$) and LPS (Fig. 1D-E, 30 μg - 13-33 %, $p < 0.01$-$p <$ 0.001; Fig. 1 *E*-F, 50 μg - 11-36 %, $p < 0.05$-$p < 0.0001$). Similarly, Apelin-13 co-treatment normalises abnormal LDH activity in both PA (Fig. 2A-C, 0.6 mM- 4-17 %, $p < 0.05$; Fig. 2A-C, 1.0 mM- 6-26 %, $p <$ 0.05-$p < 0.0001$) and LPS (Fig. 2D-E, 30 μg-19-29 %, $p < 0.001$-$p <$ 0.0001; Fig. 2D-E, 50 μg - 10-23 %, $p < 0.05$-$p < 0.0001$).

To confirm the aforementioned results, we evaluated cell viability *via* ATP synthesis. Significant cell growth arrests and suppression of ATP production was observed in SH-SY5Y cells treated with PA (Fig. 3A, 0.6 mM-28 % reduction, $p < 0.01$; and 1.0 mM-38 %, $p < 0.0001$; $F_{8,18} =$ 0.5306; $R^2 = 0.8983$) or LPS (Fig. 3B, 30 μg-30 %, $p < 0.0001$; 50 μg 43 %, $p < 0.0001$; $F_{8,18} = 0.8139$; $R^2 = 0.9484$). Interestingly, Apelin-13 amide induces an increase in metabolically active cells when stressed with higher concentration of PA (Fig. 3A, 26 % increase, $p < 0.01$) and with both concentration of LPS (Fig. 3B, 21–32 % increase, $p < 0.001$-$p <$ 0.0001), while (Lys8GluPAL) apelin-13-amide was less potent with increase only observed in higher concentration of both stressors (Fig. 3A-B, 26–36 % increase, $p < 0.01$).

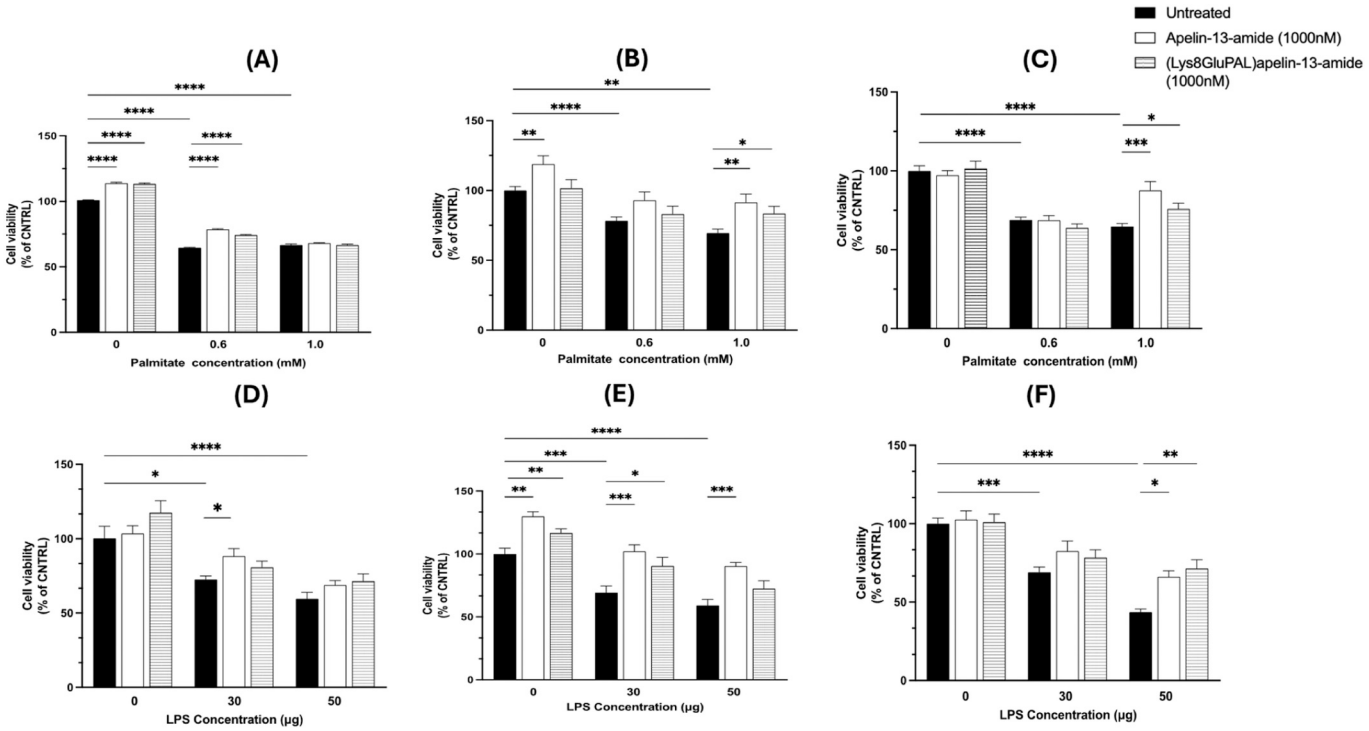

Fig. 1. Apelin-13 analogues restore impaired cell viability upon persistent PA and LPS stress. Following 24 h cell adherence and 8 h serum starvation, cells were exposed to PA (0.6 and 1.0 mM) or LPS (30, 50 μg) in the presence or absence of peptides apelin-13 amide and (Lys8GluPAL) apelin-13-amide for (A, D) 2 h, (B, E) 4 h, (C, F) 24 h and assayed for reduction of MTT by mitochondrial dehydrogenase and presented as percentage of control. Values represents mean ± SEM from three independent experiments. Data was analysed by one-way ANOVA, where $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

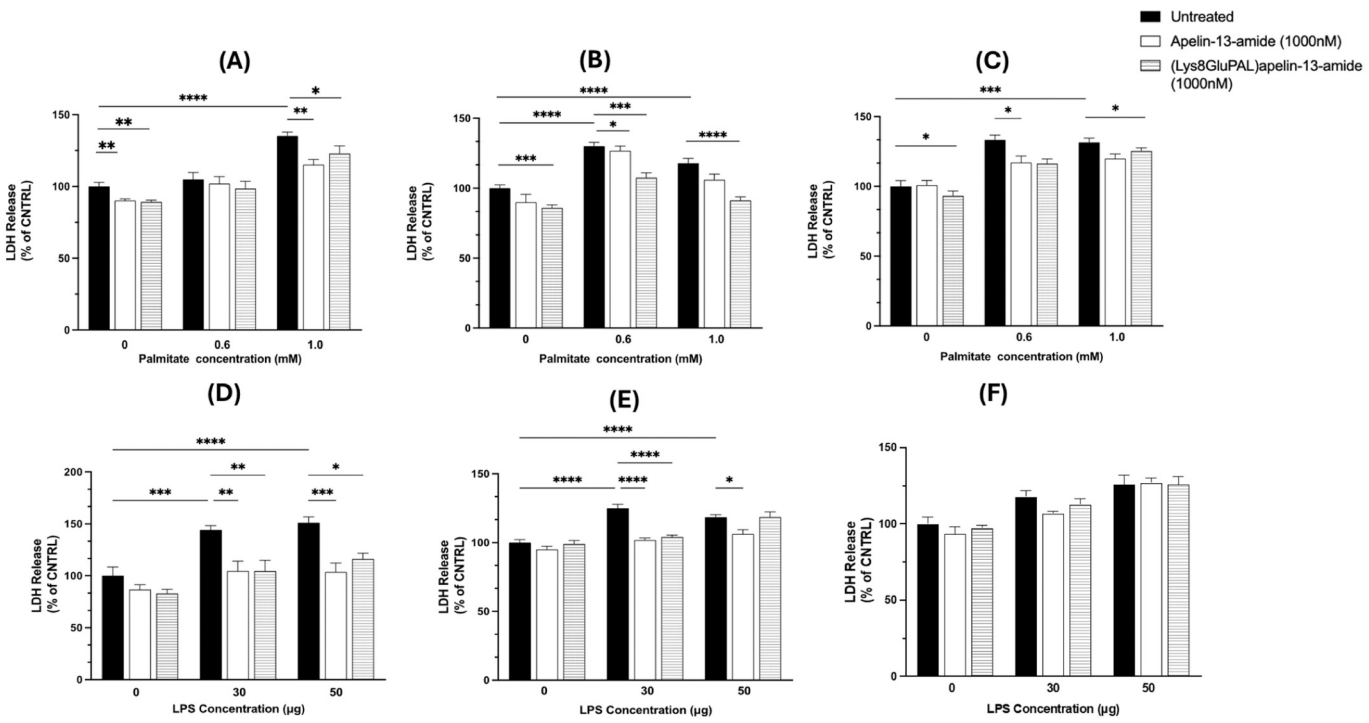

Fig. 2. Apelin-13 analogues restore abnormal cytotoxicity upon persistent PA and LPS stress. Following 24 h cell adherence and 8 h serum starvation, cells were exposed to PA or LPS in the presence or absence of peptides apelin-13 amide and (Lys8GluPAL) apelin-13-amide for (A, D) 2 h, (B, E) 4 h, (C, F) 24 h and assayed for Lactate dehydrogenase release presented as percentage of control. Values represents mean ± SEM from three independent experiments. Data was analysed by one-way ANOVA, where *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

3.2. Protective effects of apelin is mediated by AMPK

To determine the involvement of AMPK in apelin's role in promoting survival and death in SH-SY5Y cells, we pre-treated the cells with specific inhibitor of AMPK, Compound C. The results showed that Compound C blocked the protective effects of apelin in cell viability assay (Fig. 3C) and cell toxicity assay (Fig. 3D).

3.3. Apelin-13 analogues improve cellular health, inhibits ROS production and reduces apoptosis induced by stressors

To assess the role of apelin-13 in improving cellular health, a ratio of reduced GSH to oxidized GSH (GSSG) was measured. Reduced levels of GSH/GSSG ratio, suggesting reduction in antioxidant homeostasis are seen in pathological conditions, like neurodegeneration. Both Apelin-13 amide and (Lys8GluPAL) apelin-13-amide increased the GSH/GSSH ratio (Fig. 4A-B, 34–36 % increase, $p < 0.01$) compared to untreated controls. Significant reduction in GSH/GSSG ratio induced with PA (Fig. 4A, 36 % reduction, $p < 0.01$; $F_{5,12} = 0.3558$; $R^2 = 0.9307$) and LPS (Fig. 4B, 42 %, $p < 0.001$; $F_{5,12} = 0.7668$; $R^2 = 0.8516$) was reversed by apelin-13 analogues (Fig. 4A, 31–37 % increase, $p < 0.05$-$p < 0.01$; Fig. 4B, 43–56 % increase, $p < 0.05$-$p < 0.01$). Both Apelin-13 amide and (Lys8GluPAL) apelin-13-amide had potent effect in reducing LPS induced production of reactive oxygen species {(such as hydroperoxide, superoxide, hydroxy radical and singlet oxygen) and oxygen free radicals)}, markers for oxidative stress, in SH-SY5Y cells (Fig. 4D, 30 µg- 28-37 % reduction, $p < 0.001$; $F_{8,18} = 0.657$; $R^2 = 0.914$) but failed to have a positive response against lipotoxic stress (Fig. 4C; $F_{8,18} = 0.3289$; $R^2 = 0.638$). Interestingly, both analogues showed protective effect against cell apoptosis, evaluated by caspase-3/7 activity, on its own (Fig. 4E-H, 23–30 % reduction, $p < 0.05$-$p < 0.01$), against lipotoxic (Fig. E-F, 36–95 % reduction, $p < 0.0001$; $F_{5,12} = 0.6077$–1.006; $R^2 = 0.9351$–0.9993) and inflammatory (Fig. G-H, 17–60 % reduction, $p < 0.0001$; $F_{5,12} = 0.3253$–1.303; $R^2 = 0.7673$–0.9915) stress.

3.4. Apelin-13 analogues restore mitochondrial membrane potential in SH-SY5Y cells

Mitochondrial depolarization and loss of membrane potential ($\Delta\Psi_m$) is often associated with apoptosis (Ly et al., 2003). To evaluate the effect of our peptides, immunostaining of cells with J aggregate forming cationic dye (JC-1) was utilised. At low potential the dye presents as green (monomeric) and at higher potential, as red (aggregates) fluorescence. A decrease in red/green intensity ratio indicates mitochondrial depolarization. Increased mitochondrial membrane potential was observed when treated with Apelin-13 amide (Fig. 5C-D, 1.83 fold increase, $p < 0.01$) but not with (Lys8GluPAL) apelin-13-amide. Interestingly, only Apelin-13 amide reversed the detrimental effect of lipotoxic (Fig. 5C, 2.3 fold increase, $p < 0.01$; $F_{5,26} = 1.422$; $R^2 = 0.8049$) and inflammatory (Fig. 5D, 6 fold increase, $p < 0.05$; $F_{8,36} = 1.350$; $R^2 = 0.7988$) stress exposure on membrane potential, with (Lys8GluPAL) apelin-13-amide only effective against LPS (Fig. 5D, 4.8 fold increase, $p < 0.05$) stress.

3.5. Apelin-13 modulates expression of key apoptotic proteins

Both PA and LPS induced a significant increase in BAX expression (Fig. 6A, C, 20 %, 35 % respectively, $p < 0.01$; $F_{5,12} = 0.3775$; $R^2 = 0.7052$) but not of bcl2 (Fig. 6B, D, 12–10 % decline, $p > 0.05$; $F_{5,12} = 1.581$; $R^2 = 0.6613$). Apelin-13 amide and (Lys8GluPAL) apelin-13-amide, mitigated the increase in BAX in both PA exposed cells (Fig. 6A, 17 % and 16 % reduction respectively, $p < 0.05$) and LPS exposed cells (Fig. 6C, 20 % and 35 % reduction respectively, $p < 0.01$; $F_{5,12} = 0.3378$; $R^2 = 0.8167$). Only (Lys8GluPAL) apelin-13 amide was effective in restoring reduction in bcl2 expression in PA (Fig. 6B, 80 %, $p < 0.01$) and LPS (Fig. 6D, 26 %, $p < 0.05$; $F_{5,12} = 0.6888$; $R^2 = 0.7848$) treated cells, however, apelin-13-amide was effective only against LPS (Fig. 6B, 27 %, $p < 0.01$). Increase in the ratio of BAX/bcl2 confirmed pro-apoptotic properties of both PA (Fig. 6E, 32 % increase, $p > 0.05$;

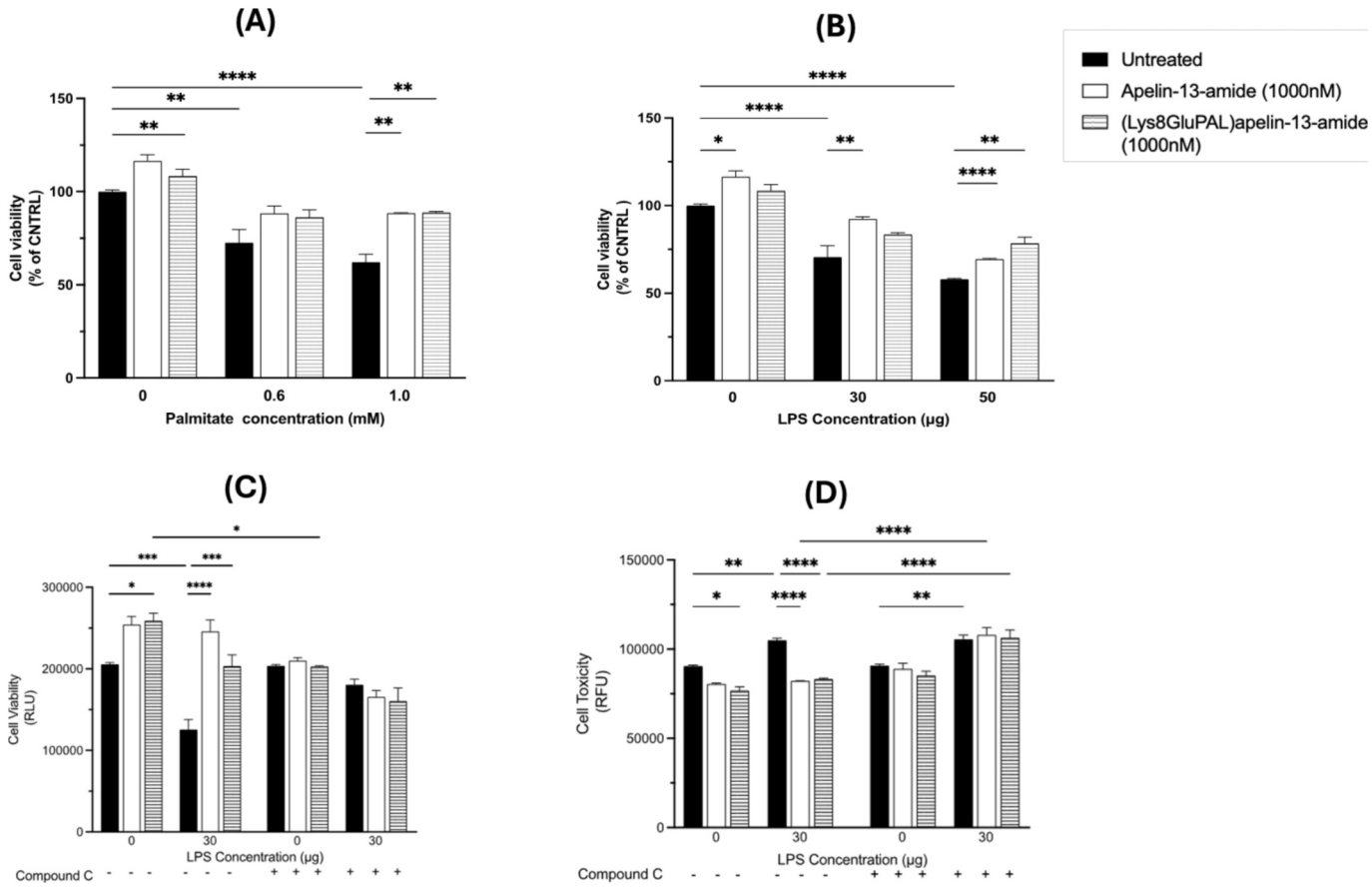

Fig. 3. Apelin-13 analogues reversed cell growth arrests and suppression of ATP production upon persistent PA and LPS stress. Cells were exposed for 6 h to PA (A) or LPS (B) in the presence or absence of apelin-13 amide and (Lys8GluPAL) apelin-13-amide. For AMPK pathway involvement, cells were pre-treated with 5 μM Compound C and then treated with peptides in the presence or absence of LPS (C—D). Cell viability, measured by ATP production was assessed using CellTiter-Glo® viability assay kit and presented either as percentage of control (A-B) or relative Luminescent units (C). CellTox™ Green cytotoxicity assay were used to measure the toxicity and presented as relative fluorescent units (D). Cell Values represents mean ± SEM from three independent experiments. Data was analysed by one-way ANOVA, where $*p < 0.05$, $**p < 0.01$, $***p < 0.001$and $****p < 0.0001$.

$F_{5,12} = 0.4247$; $R^2 = 0.7333$) and LPS (Fig. 6F, 51 % increase, $p < 0.01$; $F_{5,12} = 0.4496$; $R^2 = 0.8691$), both our peptides promoted cell survival by mitigating apoptosis in cells treated with lipotoxic (Fig. 6E, 33–52 % reduction, $p < 0.05$-$p < 0.01$) and inflammatory agents (Fig. 6F, 48–55 % reduction, $p < 0.01$-$p < 0.001$).

3.6. *Apelin-13 analogues counteract reduction in neurite growth caused by PA and LPS*

We investigated the effect of our peptides on neurite outgrowth in SH-SY5Y cells. Cells bearing axodendritic process longer than two times cell diameter in length were quantified using Image J software. Significant reduction in neurite growth was observed in cells treated with PA (Fig. 7 A, 1.9 fold reduction, $p < 0.001$; $F_{5,178} = 4.481$; $R^2 = 0.3184$) and LPS (Fig. 7B, 1.85 fold reduction, $p < 0.001$; $F_{8,90} = 1.578$; $R^2 = 0.7491$) both of which were counteracted with the treatment of Apelin-13 amide (Fig. 7A-B, PA-1.7 fold, LPS-1.8fold increase, $p < 0.0001$ and $p < 0.05$ respectively) and (Lys8GluPAL) apelin-13-amide (Fig. 7A-B, PA-1.4 fold increase, LPS-1.5fold-1.9 fold increase, $p < 0.05$-$p < 0.0001$).

4. Discussion

This study has investigated the effects of stable apelin 13 -amide and (Lys8GluPAL) apelin-13-amide in mitigating PA and LPS induced cell growth arrest, cell death, cell survival *via* rejuvenating cellular activity. Inflammation induced neuronal injury, as discussed in the introduction, is a significant contributor to the pathogenesis of multiple neurodegenerative disorders, including AD (Zhang et al., 2023; Adamu et al., 2024). In addition to the pathological hallmarks of amyloid- β (A β) and tau, brains of AD are marked by microglia-mediated inflammation, metabolic dysregulation, including glucose-hypometabolism, mitochondrial dysfunction, lipid imbalances and oxidative stress (Leng and Edison, 2021; Misrani et al., 2021; Jurcău et al., 2022; Zhou et al., 2023). Chronic inflammation in the brain is largely driven by persistent activation of microglia cells. Reactive microglial cells release pro-inflammatory cytokines, initiating a cascade, exacerbating protein misfolding and neuronal death. Additionally, increased oxidative stress disrupts mitochondrial activity, escalating neuronal dysfunction and apoptosis (Norat et al., 2020). LCSFA is an effective inducer of ROS in various tissues, including cardiomyocytes (Joseph et al., 2016), hepatocytes (Yu et al., 2021), adipocytes (Shin, 2022), pancreatic β cells (Sato et al., 2014) and neuronal cells (Urso and Zhou, 2022). Herein, we demonstrate PA affect cell viability in SH-SY5Y cell as shown previously by others in various *in-vitro* cell lines (Hsiao et al., 2014; González-Giraldo et al., 2018). LPS, part of gram negative bacteria's outer membrane, activates Toll-like receptor (TLR) 4 (Michot et al., 2023), which then recruits downstream adaptors, like TIR-domain-containing adaptor-inducing interferon- β (TRIF), TRF-related adaptor molecule (TRAM), and myeloid differentiation primary response protein 88 (My88) (Fitzgerald et al., 2003; Ruckdeschel et al., 2004), all are essential in activating transcription factors related to induction of pro-inflammatory genes (Ruckdeschel et al., 2004; Zughaier et al., 2005).

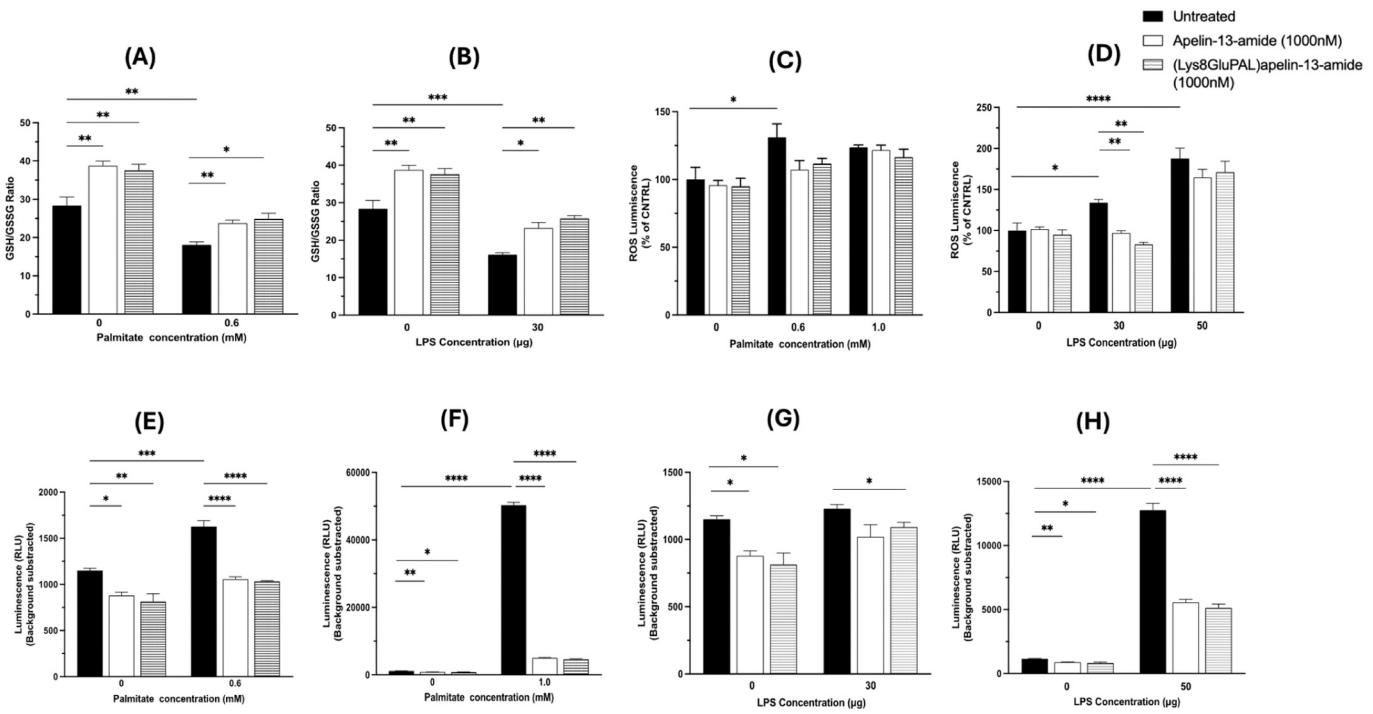

Fig. 4. Apelin-13 analogues scavenge ROS production and reduces cell apoptosis in PA and LPS induced stress. SH-SY5Y cells treated with PA and LPS in the presence or absence of apelin-13 amide and (Lys8GluPAL) apelin-13-amide. ROS production was assessed by measuring (A-B) total and oxidized glutathione (GSH/GSSG) generation, (C—D) H_2O_2 generation and assessing (*E*-H) caspase activity using Caspase-Glo® 3/7 assay. Data presented as presented as ration (GSH/GSSG) percentage of control (ROS) and relative luminescence unit (Caspase activity). Values represents mean ± SEM from three independent experiments. Data was analysed by one-way ANOVA, where *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

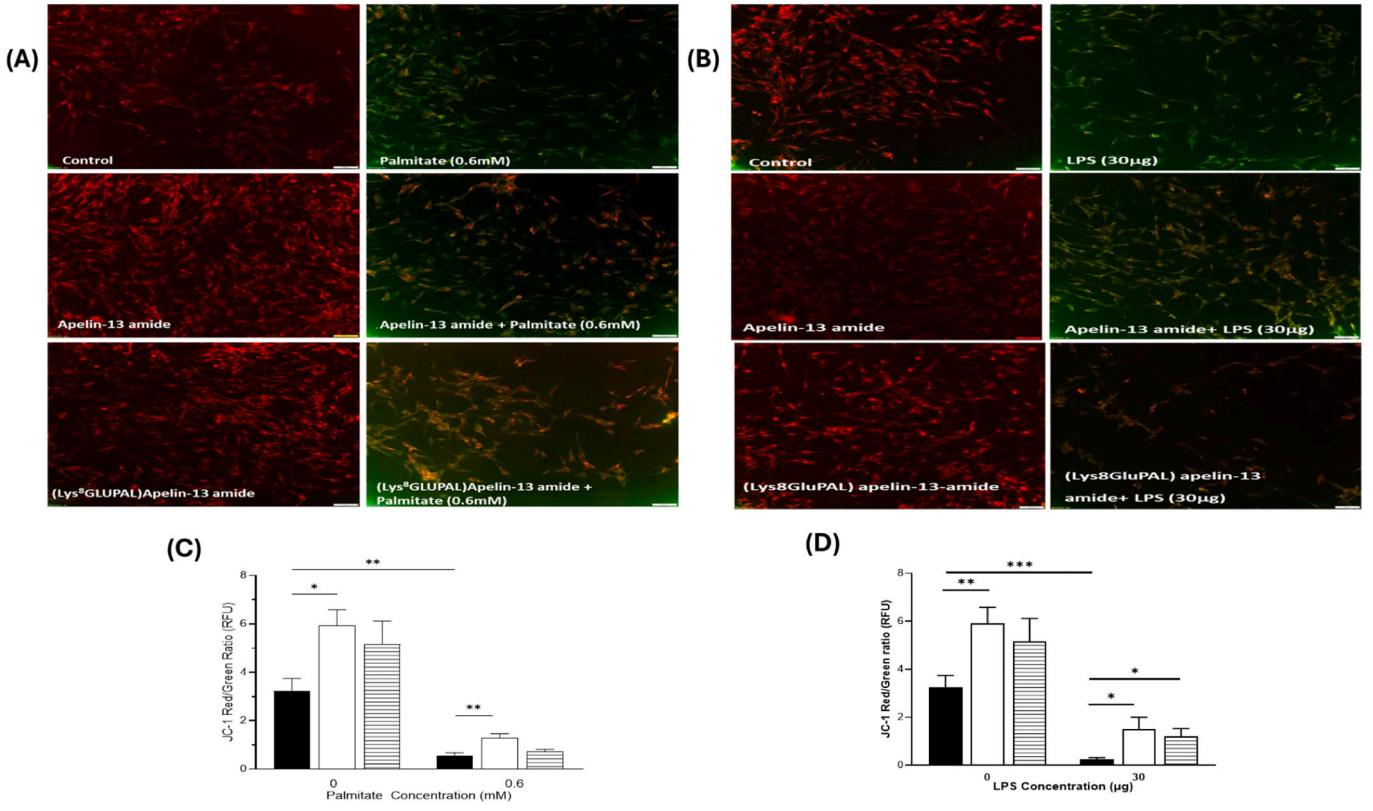

Fig. 5. Apelin improves mitochondrial membrane potential (ΔΨm) in PA and LPS stressed SH-SY5Y neuroblastoma cells. (A) Representative images of JC-1-stained SH-SY5Y cells (B) JC-1 Red/Green fluorescence ratio of cells treated with Apelin-13-amide, (Lys^{8}GluPAL) apelin-13-amide and palmitate. Values represents mean ± SEM for $n = 3$ where *p < 0.05, **$p < 0.01$.

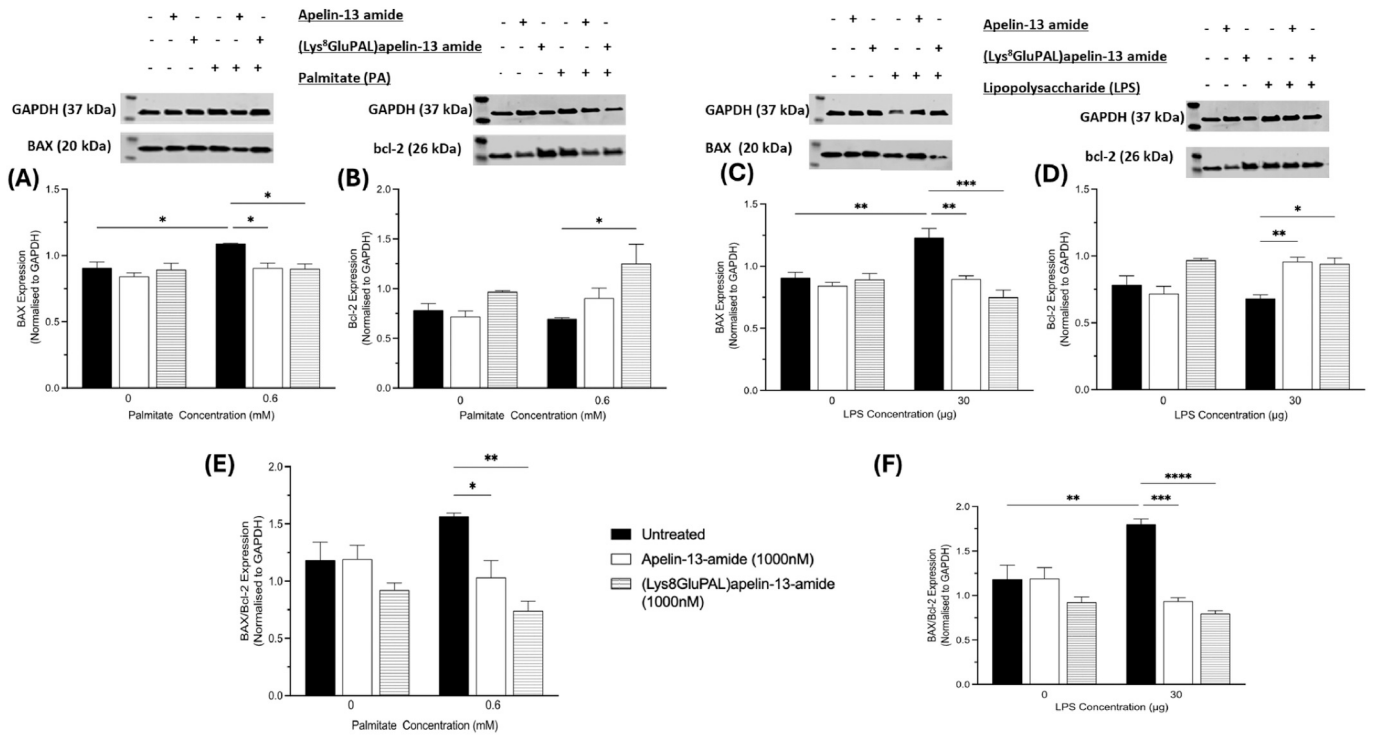

Fig. 6. Apelin-13 analogues protect cell from apoptosis by modulating expression of pro and anti-apoptotic protein expression. Following treatments with stressor in the presence or absence of apelin-13 amide and (Lys8GluPAL) apelin-13-amide, cells were harvested, and probed for BAX (A, C) and bcl2 (B, D) expression. Ratio of BAX/bcl2 was calculated as shown (E, F). Values represents mean ± SEM from three independent experiments. Data was analysed by one-way ANOVA, where *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

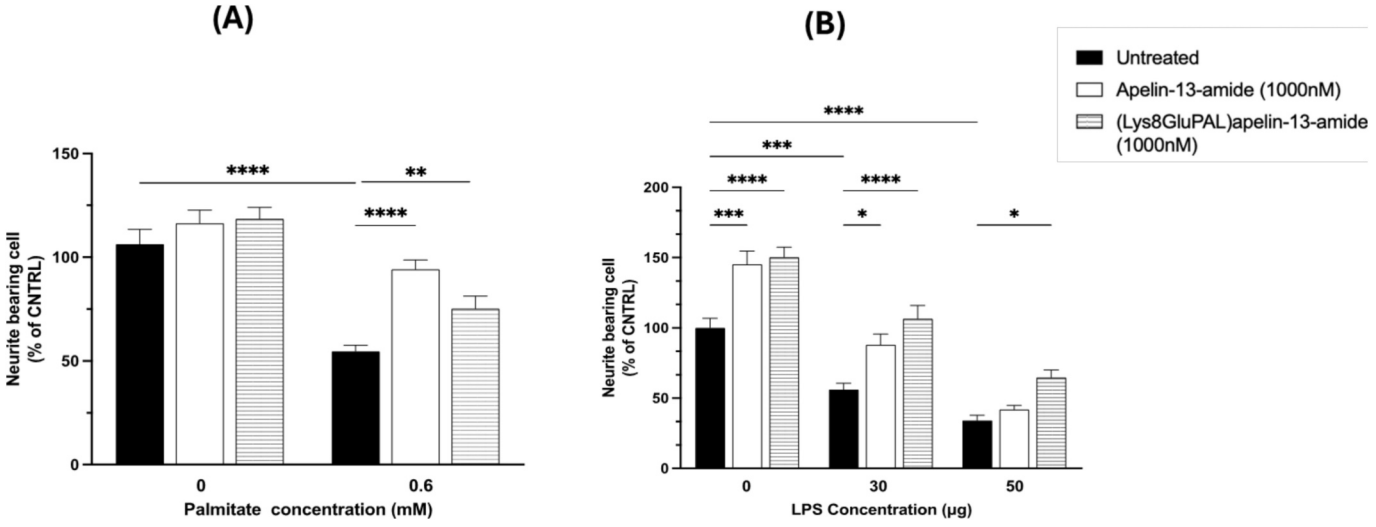

Fig. 7. Apelin protects and promotes neurite growth in response to PA and LPS stress. Neurite growth (vertical axis) was quantified as the percentage of cells bearing axodendritic processes longer than two times cell diameters in length for cells treated with stressors (A) PA and (B) LPS. Negative control consists of SH-SY5Y cells in complete medium. Values represents mean ± SEM from three independent experiments. Data was analysed by one-way ANOVA, where *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

Moreover, *in-vitro* and *in-vivo* models utilizing LPS-induced neuroinflammation has been shown to promote Aβ deposition (Sheng et al., 2003) and presynaptic disruption (Sheppard et al., 2019). We additionally show that treatment with PA and LPS triggers dose and time dependent reduction in cell viability, reduced mitochondrial membrane potential, leading to ROS generation, increased expression of pro-apoptotic genes and release of lactate dehydrogenase, a marker of cell death.

Hence, restoring mitochondrial membrane potential, alleviating endoplasmic and oxidative stress, and reversing pro-apoptotic gene expressions may reduce neuroinflammation, protect cells from apoptosis and can provide therapeutic benefits for neurodegenerative disorders (Nakka et al., 2016; Liu et al., 2024). A previous study conducted by our group has shown that structural modification of native apelin-13 improved its stability and bioactivity (O'Harte et al., 2017; O'Harte et al., 2018). We have also extensively studied the benefits of these novel compounds in both *in-vitro* and *in-vivo* models of type1 and type 2 diabetes (O'Harte et al., 2017; O'Harte et al., 2018; O'Harte et al., 2020).

Additionally, apelin analogues have been previously shown to improve cell survival in various *in-vitro* and *in-vivo* studies including oxygen glucose deprivation cell models (Zhang et al., 2021a; Kamińska et al., 2024). Apelin analogues have prevented aberrant apoptosis of skeletal muscles cells, hippocampal neurons and SH-SY5Y neuroblastoma cells exposed to excitotoxic abuse, neurotoxins, hypoxia (Jiang et al., 2018; Chen et al., 2020; O'Donnell et al., 2007; Wu et al., 2018; Dong et al., 2020; Saral et al., 2021). The present study indicates acute and chronic exposure to palmitate induces ROS-dependent lipotoxicity, which are in agreement to previously conducted studies reporting detrimental effects of PA on viability of cells (Thombare et al., 2017). In this study, we have consistently reported that stable and novel apelin-13-amide and (Lys8GluPAL) apelin-13 amide showed restorative/preventive effect against PA and LPS induced cellular dysfunction and promotes cell proliferation in dose and time dependent manner.

Our data also consistently show that both PA and LPS results in elevated ROS generation, along with diminished GSH/GSSG activity and a loss of mitochondrial membrane potential. Our study corroborates previous findings showing PA and LPS can directly enhance ROS generation, decreases neuronal concentration of mitochondrial fusion protein, mitofusin 2 (MFN2), leading to Mitochondrial-ER stress (Alnahdi et al., 2019; Schneeberger et al., 2013; Xu et al., 2015; Yi et al., 2016; Zhang et al., 2021b; Sánchez-Alegría and Arias, 2023). Interaction with mitochondrial carrier proteins by PA and LPS could lead in reduction of mitochondrial membrane potential, leading to opening of permeability transition pore (PTP), resulting in inner mitochondrial membrane permeabilization (IMM), which releases mitochondrial DNA (mtDNA) in the cytoplasm and exposes cardiolipin to outer membrane of mitochondria, thus activating NLRP3 (nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3) inflammasome and secretion of cytokines IL-1β and IL-18 (González-Giraldo et al., 2018; Yu and Lee, 2016; Esteves et al., 2023; Han et al., 2023). As predicted, apelin analogues were able to restrain PA and LPS induced mitochondrial dysfunction by regulating mitochondrial membrane potential.

For cells to resist oxidative damage, physiological antioxidants like GSH play an important role. GSH is a vital scavenger of ROS, converting to GSSG to neutralize ROS, thus GSH/GSSG ratio is commonly used to assess levels of oxidative stress (Zitka et al., 2012). Cellular antioxidant deficiencies, justified by decrease in reduced-to-oxidized GSH (GSH/GSSG) ratio, could arise from impaired production or increased utilization as seen in the presence of stressors like PA and LPS (Kurutas, 2016). Strikingly, we found that apelin had a potent antioxidant effect, significantly restoring GSH/GSSG ratio and leading to notable reduction in ROS levels, highlighting its promising role in mitigating cellular oxidative stress.

Both PA and LPS increased the expression of anti-apoptotic gene, Bcl-2 and reduced the pro-apoptotic Bcl-2 associated X protein (Bax) expression, both involved in regulation of apoptosis, belonging to B-cell lymphoma (Bcl-2) family. We also observed increased concentration of caspase 3/7, proteases which are critical in apoptotic cascades in SH-SY5Y cells. However, treatment with stable apelin significantly attenuated Bcl-2, cleaved caspase 3/7 and increased Bax expression, suggesting apelin's positive role in alleviating PA and LPS induced neuronal apoptosis by regulating Bax, Bcl-2 and caspase. We have shown both PA and LPS impaired neurite extension, most likely by induction of oxidative stress and disruption of cytoskeletal dynamics (Jo et al., 2021). Interestingly, apelin analogues were able to prevent and stimulate neurite length in differentiated SH-SY5Y cells. Promotion of synaptic and neurite outgrowth will maintain cell volume, improve memory and learning in AD patients (Udomruk et al., 2020). However, studies conducted by Boato and colleague (Boato et al., 2011) showed Interleukin-1 beta (IL-1β), with potent pro-inflammatory characteristic, increased neurite growth in cortical slices. It would be interesting to look at the synergistic action of apelin with IL-1β.

Likewise, AMPK pathway is indicated to play an essential role in cell survival and apoptosis and is a key signalling molecule in antioxidant properties (Han et al., 2016). PA and LPS induced production of oxidative stress have shown to impair phosphorylation of Akt and activation of AMPK, both reduce cell survival and increase apoptosis by supressing mTORC1 (mammalian target of rapamycin complex 1) activity (Lee et al., 2014; Calvo-Ochoa et al., 2017). Our data suggests that blocking of AMPK by pharmacological inhibitor Compound C, attenuated the protective actions of our peptide, suggesting an important role of AMPK in apelin-mediated protection against oxidative stress induced cells death. Apelin modulates activation of AMPK which reduces ER stress-mediated oxidative stress and neuroinflammation (Xu et al., 2019). In presence of apelin, AMPK, has shown to increase and reduce the concentrations of NLRP3 (NOD-like receptor protein 3) and TXNIP (Thioredoxin-interacting protein), thus relieving ROS and inflammation induced nerve injury in subarachnoid haemorrhage (Xu et al., 2019). Previous studies have shown that apelin influence PI3K/Akt (Phosphoinositide 3-kinases/Protein kinase B) and MAPK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinases) pathways, responsible for cell survival and growth (Chen et al., 2020). Stimulation of PI3K/Akt leads to mTORC1 activation, a key regulator for cell growth and protein synthesis (Ramasubbu and Devi, 2023), while activation of ERK has shown to promote neurite growth, resulting in long term potentiation and memory (Jo et al., 2021).

Our findings suggests the dose- and time dependent reduction in cell viability, driven by loss of mitochondrial membrane potential, ROS generation, upregulation of pro-apoptotic genes and caspase activity, indicating increase cell death. Concurrent treatment with our stable apelin analogues effectively counteracts these deleterious effects in SH-SY5Y cells. Our peptide not only protected neurites against stress but also stimulated neurite outgrowth. These finding underscore therapeutic potential of our novel, stable apelin analogues in mitigating inflammation induced cellular damage and promoting survival, making it a promising candidate for further research in finding potential therapeutic agent against AD.

Abbreviations

AD	Alzheimer's disease
LCSFA	Long chain saturated fatty acid
PA	Palmitic acid
LPS	Lipopolysaccharides
WAT	White adipose tissue
GPCRs	G-protein-coupled receptors
ROS	Reactive oxygen species
ACE2	Angiotensin converting enzyme 2
PBS	Phosphate buffered saline
GSH/GSSG	Glutathione/Glutathione disulfide
RIPA	Radioimmunoprecipitation assay
BCA	Bicinchoninic acid assay
AMPK	Adenosine monophosphate-activated protein kinase
TLR	Toll-like receptor
TRIF	TIR-domain-containing adaptor-inducing interferon- β
TRAM	TRF-related adaptor molecule
My88	Myeloid differentiation primary response protein 88
MFN2	Mitofusin 2
PTP	Permeability transition pore
IMM	Inner mitochondrial membrane permeabilization
mtDNA	Mitochondrial DNA
mTORC1	Mammalian target of rapamycin complex 1
NLRP3	NOD-like receptor protein 3
TXNIP	Thioredoxin-interacting protein
Akt	Protein kinase B (PKB)
PI3K	Phosphoinositide 3-kinases
MAPK	mitogen-activated protein kinase
ERK	extracellular signal-regulated kinases

CRediT authorship contribution statement

Priya Sharma: Investigation, Formal analysis, Data curation. **Mary Erazo Bastidas:** Investigation. **Usman Ali:** Writing – review & editing, Visualization. **Shivadas Sivasubramaniam:** Writing – review & editing, Supervision, Resources, Project administration. **Vadivel Parthsarathy:** Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mcn.2025.104036.

Data availability

Data will be made available on request.

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