



Neuroprotective actions of leptin facilitated through balancing mitochondrial morphology and improving mitochondrial function

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Abstract

Mitochondrial dysfunction has a recognised role in the progression of Alzheimer's disease (AD) pathophysiology. Cerebral perfusion becomes increasingly inefficient throughout ageing, leading to unbalanced mitochondrial dynamics. This effect is exaggerated by amyloid β ($A\beta$) and phosphorylated tau, two hallmark proteins of AD pathology. A neuroprotective role for the adipose-derived hormone, leptin, has been demonstrated in neuronal cells. However, its effects with relation to mitochondrial function in AD remain largely unknown. To address this question, we have used both a glucose-serum-deprived (CGSD) model of ischaemic stroke in SH-SY5Y cells and a $A\beta_{1-42}$ -treatment model of AD in differentiated hippocampal cells. Using a combination of 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) and MitoRed staining techniques, we show that leptin prevents depolarisation of the mitochondrial membrane and excessive mitochondrial fragmentation induced by both CGSD and $A\beta_{1-42}$. Thereafter, we used ELISAs and a number of activity assays to reveal the biochemical underpinnings of these processes. Specifically, leptin was seen to inhibit up-regulation of the mitochondrial fission protein Fis1 and down-regulation of the mitochondrial fusion protein, Mfn2. Furthermore, leptin was seen to up-regulate the expression and activity of the antioxidant enzyme, monoamine oxidase B. Herein we provide the first demonstration that leptin is sufficient to protect against aberrant mitochondrial dynamics and resulting loss of function induced by both CGSD and $A\beta_{1-42}$. We conclude that the established neuroprotective actions of leptin may be facilitated through regulation of mitochondrial dynamics.

KEYWORDS

hippocampal, leptin, mitochondrial fission, mitochondrial fusion, mitochondrion, monoamine oxidase

Abbreviations: AD, Alzheimer's disease; ANOVA, analysis of variance; APP, amyloid precursor protein; $A\beta$, amyloid beta; BACE1, β -secretase; BBB, blood-brain barrier; CGSD, combined glucose and serum deprivation; dbcAMP, N₆,2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt; DMEM, Dulbecco's modified eagle medium; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; LDH, lactate dehydrogenase; MAO, monoamine oxidase; MnSOD, manganese superoxide dismutase; NBF, neutral buffered formalin; NRF-1, nuclear respiratory factor-1; Ob-Rb, Ob (leptin) receptor - b; PBS, phosphate buffered saline; PGC-1 α , peroxisome proliferator-activated receptor γ coactivator 1; PS1, presenilin 1; ROS, reactive oxygen species; SR-2, serum replacement-2; TBS-T, Tris buffered saline with 0.1% Triton X-100; Ψ_m , mitochondrial membrane potential.

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1 | INTRODUCTION

The high energy demands of neuronal cells mean that they are particularly reliant on mitochondrial dynamics and function (Safiulina & Kaasik, 2013). Fission and fusion dynamics promote the distribution of mitochondria along axons so that mitochondria can deliver energy efficiently to synapses-distant locations with high energy requirements (Li, Okamoto, Hayashi, & Sheng, 2004; Westermann, 2012). Aberrant mitochondrial fission and fusion dynamics lead to altered mitochondrial morphology and distribution, which damages the energy supply by impeding mitochondrial function. Abnormal mitochondrial morphology caused by aberrant dynamics represents an essential common pathway which regulates, or intensifies, mitochondrial dysfunction in neurodegenerative disease (Su et al., 2010). Extensive evidence supports the contribution of abnormal mitochondrial function to cellular energy depletion (Divya, Amandine, & Ken, 2013; Flint, 1995), oxidative stress and neuronal loss (Lin & Beal, 2006), which are key features of neurodegenerative diseases (Burté, Carelli, Chinnery, & Yu-Wai-Man, 2015; Johri & Beal, 2012; Lin & Beal, 2006).

Compelling evidence from both in vivo and in vitro studies shows that amyloid-precursor protein (APP) and/or A β causes mitochondrial dysfunction and morphological changes (Reddy & Beal, 2008). APP and/or A β block the import of functional proteins into mitochondria by localizing to mitochondrial membranes (Crouch et al., 2005; Sirk et al., 2007), disrupting the energy metabolism chain and increasing reactive oxygen species (ROS) production by interacting with mitochondrial enzymes (Casley, Canevari, Land, Clark, & Sharpe, 2002; Cha et al., 2012), eventually causing mitochondrial dysfunction and neuronal apoptosis (Reddy & Beal, 2008). In post-mortem AD brains the mRNA for complex I genes is down-regulated, while the genes for complex III and IV constituents are up-regulated revealing molecular defects in oxidative phosphorylation and thus mitochondrial function (Manczak, Park, Jung, & Reddy, 2004); and similar results are observed in APP mutant mice (Tg2576) (Reddy et al., 2004). In vitro studies demonstrate that A β peptides (1-42, 25-35) damage mitochondrial membrane potential ($\Delta\Psi_m$), increase ROS and cause abnormal mitochondrial fragmentation in several cell lines and also in primary neuronal cultures (Barsoum et al., 2006; Cha et al., 2012). A recent study on Alzheimer's disease brains revealed a complex dysregulation of the activities of specific mitochondrial enzymes, monoamine oxidases A and B (MAO A and B) and further confirmed that mRNA, protein and activity of the MAOs varied independently (Quartey et al., 2018). These observations suggest a central role for mitochondria in neurodegeneration that warrants investigation.

The adipose-derived hormone, leptin, is known for its central role in the regulation of energy metabolism. Emerging lines of evidence indicate direct and indirect links between leptin and mitochondria function; however, the vast majority of the empirical studies have been carried out in non-neuronal lineages. It has been found that leptin regulates mitochondrial respiration in prostate cancer cells (Calgani et al., 2016). Leptin treatment ameliorates the

inhibition of mitochondrial respiration in leptin-deficient *ob/ob* mice (Finocchietto et al., 2011). Cultured human adipose tissue fragments inhibit mitochondrial respiration in HCT116 colon cancer cells, an effect purported to be regulated by leptin (Yehuda-Shnaidman et al., 2013). Leptin is also involved in the regulation of mitochondrial enzymes, such as manganese superoxide dismutase in hippocampal neurons (Guo, Jiang, Xu, Duan, & Mattson, 2008) and proxisome proliferator-activated receptor γ coactivator 1 (PGC1- α) in breast cancer cells (Blanquer-Rosselló, Santandreu, Oliver, Roca, & Valle, 2015). All of these mitochondrial enzymes have been linked to neurodegenerative changes in a number of disorders. In addition, other mitochondrial enzymes, such as monoamine oxidases, have been intricately linked to both Parkinson's and Alzheimer's Diseases (Naoi, Riederer, & Maruyama, 2016); and are proposed therapeutic targets in such conditions (Matthew et al., 2019). However, the potential role of leptin in the regulation of the monoamine oxidases remains unknown.

Leptin also has a number of other beneficial effects on neuronal function and survival (Doherty, Oldreive, & Harvey, 2008; McGregor & Harvey, 2019). Neuroprotection by leptin has been described in models of major neurodegenerative diseases such as AD (Doherty, Beccano-Kelly, Yan, Gunn-Moore, & Harvey, 2013; Perez-Gonzalez et al., 2011; Weng et al., 2007), although the underlying molecular mechanism remains to be fully understood. Despite the maladaptive changes to mitochondrial dynamics and function are thought to play in the onset and progression of AD, the potentially important link between leptin and mitochondria in AD has yet to be explored.

Stroke is associated with AD in elderly individuals (Honig et al., 2003). Stroke significantly and increases the risk of AD development independent of genetic factors (Zhou et al., 2015), and disrupted cerebral perfusion has been suggested to contribute to AD neuropathological changes (Austin et al., 2011). Serum starvation-induced neuronal apoptosis is regarded as an established AD-related insult in vitro (Kariya, Takahashi, Hirano, & Ueno, 2003). Serum deprivation leads to the increased secretion of β -secretase that is an initiator in the formation of toxic A β peptides (Stavropoulou, Mavrofrydi, Saftig, & Efthimiopoulos, 2017). Chronic cerebral hypoperfusion exaggerates tau phosphorylation in tau transgenic mice, however, this is attenuated by the expression of the signalling competent form of the leptin receptor (Ob-Rb), and activation of its downstream signalling pathways (AKT/pAKT) (Shimada et al., 2019). Ob-Rb is up-regulated in brains from individuals with AD and cerebrovascular diseases, further indicating an endogenous neuroprotective role of leptin in the crosstalk between stroke and AD (Shimada et al., 2019; Terao et al., 2008). Exploring the effects of leptin in both stroke and AD models will boost our understanding of the neuroprotective role of leptin and its relationship with mitochondrial function. In particular, exploring the protective effects of leptin in a combined glucose and serum deprivation (CGSD) model will further highlight the neuroprotective effects of leptin in the early stages of AD. Therefore, in this study, we examined the effects of leptin on mitochondrial

dynamics, morphology and function in CGSD SH-SY5Y human neuroblastoma cells, an established model of ischaemic stroke (Lorenz et al., 2009), and in $A\beta_{1-42}$ -treated differentiated HT-22 hippocampal neuronal cells as an AD model.

2 | METHODS

2.1 | Materials

Unless otherwise stated all materials and reagents were purchased from Sigma, UK.

2.2 | Cell culture

Ethical approval to work with the cell lines in these experiments was obtained from the University of St Andrews School of Psychology & Neuroscience Ethics committee, approval number PS10538_Doherty. The cell lines used in this study are not listed as a commonly misidentified cell line by the International Cell Line Authentication Committee and the line was last ensured for purity in 2017. The human neuroblastoma cell line SH-SY5Y (#94030304, 2017; RRID:CVCL_0019) and mouse hippocampal neuronal cell line HT-22 (Merck, #SCC129; 2017; RRID:CVCL_0321) were cultured in 10% (v/v) heat-inactivated calf serum (C8056; Fisher Scientific) in Dulbecco's modified Eagle's medium (21885108; Fisher Scientific) supplemented with 4500 mg/L glucose and 10 mg/ml penicillin/streptomycin in a 37°C incubator which provides a humidified 5% CO₂ atmosphere. Cells were plated at densities of 2×10^4 and 5×10^4 on 96-, and 24-well Nunclon coated plates, with or without 13 mm borosilicate glass coverslips (CC7672-7548, CC7672-7458; VWR). Also, they were plated at densities of 3×10^5 and 6×10^5 on 35 and 60 mm Nunclon-coated culture dishes (153066158015; VWR), respectively. SH-SY5Y cells were grown to 70% confluence before treatment. Undifferentiated SH-SY5Y cells were then maintained in 10% medium as a positive control, or subjected to combined serum and glucose deprivation (CGSD; 0% calf serum and 1000 mg/L glucose). CSGD cultures were supplemented with leptin (L4146, 0.1–10 nM) for between 24 and 72 hr depending on the experimental paradigm. All cell lines were used between passage 6 and 20.

2.3 | Differentiation of HT-22 cells

HT-22 cells were grown to 70% confluence before differentiation. Cells were differentiated using 100 μ M N⁶,2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt (dbcAMP) (A9501) in Dulbecco's modified Eagle's medium supplemented with 2% (v/v) serum replacement -2 (S9388), 4500 mg/L glucose and 10 mg/ml penicillin/streptomycin in a 37°C incubator. After 24 hr differentiation, HT-22 cells were then maintained in 2% serum replacement-2

medium as a control, or treated with 10 μ M $A\beta_{1-42}$ (A9810) with or without leptin (0.1–10 nM) for 24 hr.

2.4 | Protein preparation and ELISA assay

Protein was extracted into 500 μ l Tris buffered saline (T6664) supplemented with 1% Triton X-100 (X-100) and 5 μ l protease inhibitor cocktail (S8820). A micro-Bradford assay was performed to measure approximate protein concentration to allow for equal protein loading. ELISA was performed to detect changes in protein expression relative to a loading control. The antibodies for ELISA were as follows: anti-Mfn1 (1:10,000; Santa Cruz, Insight Biotechnologies; #sc-166644, RRID:AB_2142616), anti-Mfn2 (1:5,000; Santa Cruz; #sc-515647, RRID:AB_2811176), anti-Drp1 (1:1,000; Santa Cruz; #sc-101270, RRID:AB_2093545), anti-Fis1 (1:2000; Santa Cruz; #sc-98900, RRID:AB_2246809), anti-VDAC1 (1:5,000; Santa Cruz; #sc-390996, RRID:AB_2750920), anti-MAOA (1:10,000; Santa Cruz; #sc-18396, RRID:AB_2137263), anti-MAOB (1:1,000; Santa Cruz; #c-515354, RRID:AB_2819030), anti-NRF1 (1:5,000; Santa Cruz; #sc-23624, RRID:AB_10613096), anti-PGC1 α (1:5,000; Santa Cruz; #sc-517380, RRID:AB_2755043), anti- α -tubulin (1:2000; Sigma-Aldrich; #T5168, RRID:AB_477579), HRP-conjugated anti-mouse (1:10,000; Sigma-Aldrich; #A9044, RRID:AB_258431), HRP-conjugated anti-goat (1:5,000; Sigma-Aldrich; #A8919, RRID:AB_258425), HRP-conjugated anti-rabbit (1:10,000; Santa Cruz; #sc-2357, RRID:AB_628497). In ELISAs, anti-VDAC1 was used as mitochondrial loading control antibody and anti- α -tubulin was used as cytoplasmic loading control antibody to identify any loading difference across samples. After normalising for any loading differences, the mean absorbance of each sample was further normalised to the control for each experiment.

2.5 | MitoRed staining and Immunocytochemistry

9-[2-(4'-Methylcoumarin-7'-oxycarbonyl) phenyl]-3,6-bis (diethyl-amino) xanthylium chloride (MitoRed; #53271) is a cell membrane permeable rhodamine-based dye that is used to stain mitochondria. Cells were treated with pre-warmed cell culture medium containing 1 μ M MitoRed and returned to the incubator for 45 min. Following fixation in neutral buffered formalin (NBF) (from formaldehyde solution 15,512) for 15 min, cells were washed three times in phosphate buffered saline (PBS BR0014G). 10% horse serum was added and incubated for 10 min to block non-specific antibody binding. Cells were probed with mouse anti-Mfn2 (1:2000; Santa Cruz #sc-515467, RRID:AB_2811176) or rabbit anti-Fis1 (1:500; Santa Cruz #sc-98900; RRID:AB_2246809z) overnight at 4°C followed by staining with FITC-conjugated anti-mouse IgG (1:200, FI-2020, RRID:AB_2336185) or FITC-conjugated anti-rabbit IgG (1:200, FI-1000, RRID:AB_2336197) respectively for 2 hr at room temperature. After washing, the stained coverslips were mounted in fluorescent mountant (1% w/v N-propyl-gallate (O2370) dissolved in 80% v/v glycerol (G5516)+20%v/v PBS) onto microscope slides, sealed with

nail polish and imaged at an excitation wavelength of 495 nm on a Zeiss Axio MR2 microscope (RRID:SCR_016980). Images were captured using the embedded Zen software (RRID:SCR_013672).

2.6 | Mitochondrial morphology analysis

Mitochondrial morphology was quantified using ImageJ software (RRID:SCR_002285). Briefly, a polygon box was drawn around each individual cell. The image background of individual cells was pre-processed. After thresholding images, mitochondrial individual particles were analysed for counts, area and perimeter. From these values, we calculated indices of mitochondrial fragmentation and interconnectivity. The index of mitochondrial fragmentation was calculated from the ratio of the count of individual mitochondria to the total mitochondrial area within the cell. These measure have been described previously (Connolly et al., 2018; Dagda et al., 2009; Senyilmaz et al., 2015). The mean area was divided by the mean perimeter of all analysed particles within individual cells to obtain an index of mitochondrial interconnectivity. As quantitative rather than subjective image analyses were carried out throughout this paper, no blinding was performed.

2.7 | JC-1 assay

To detect variation of the mitochondrial membrane potential ($\Delta\Psi_m$), a cytofluorimetric, lipophilic cationic dye, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, #MAK159) was used. Cells were plated on 13 mm borosilicate glass coverslips (O111530, Pyramid Innovation) and treated as appropriate for experiments. Twenty-five microlitre of 200 \times JC-1 stock solution (1 mg/ml) together with 1 ml of 5 \times JC-1 staining buffer was diluted in 4 ml of ultrapure water. The diluted staining solution was mixed with cell culture medium at a 1:1 ratio before being added into each well. After being incubated for 45 min, the coverslips were mounted in cell culture medium for live imaging at \times 40 and \times 63 magnification. As the red fluorescence fades more quickly than the green, we imaged the red channel (λ_{ex} = 540 nm) first and then the green quickly afterwards (λ_{ex} = 490 nm) and overlaid the images. The intensity ratio of red to green fluorescence was calculated to monitor the $\Delta\Psi_m$.

2.8 | MAO activity assay

MAO activity was determined as previously (Zhou, Diwu, Panchuk-Voloshina, & Haugland, 1997). Briefly, SH-SY5Y cells were washed once in PBS and harvested after 24 hr treatment. Isolation of mitochondria was carried out using a mitochondrial isolation kit (#MITOISO2) in accordance with manufacturer's instructions. Enriched mitochondrial pellets were suspended in a final volume of 100 μ l 50 mM potassium phosphate (P5655) pH 7.5, mixed with

50 μ l tyramine substrate (4 mM) and 50 μ l Amplex Red (90101, 80 μ M) with Horseradish Peroxidase (5 Units/ml). The time-dependent increase in resorufin fluorescence was measured at 30°C using excitation at 535 nm and emission at 595 nm in a fluorescence microplate reader (Molecular Devices FilterMax F5 multi-modal microplate reader).

2.9 | Determination of cell viability

Lactate dehydrogenase assay (LDH assay) and crystal violet assay were performed as described previously (Malekizadeh et al., 2017). The absorbance was determined with a Biohit BP100 plate reader. Readings were collated by conditions and normalized to control to eliminate density differences during plating among individual experiments.

2.10 | Statistical analysis

These experiments were exploratory with no pre-determined endpoint and no data was excluded from the analyses. All statistical analysis was carried out using SPSS 23 (IBM corp.). GraphPad PRISM 5 (Graph Pad Inc.) was used to generate graphs. No samples size calculations were performed. In all experiments, data are expressed as mean \pm SEM. After testing for normality using a D'Agostino-Pearson omnibus normality test, statistical analyses were performed using either one-way analysis of variance (ANOVA) with Dunnett's/Dunn's post hoc test for comparisons between multiple groups or an independent-samples *t*-test for comparisons between two groups. Data that was not normally distributed was analysed by a Mann-Whitney *U* test. Outliers were defined as data points more than two standard deviations from the mean. Data points were excluded from the calculations for Figures 1d, 2e,f, 3e,f, 5c and 6c. *p* < .05 was considered significant.

3 | RESULTS

3.1 | Leptin prevent depolarization of the mitochondrial membrane

$\Delta\Psi_m$ is an important indicator of mitochondrial function and thus cellular health; and we evaluated this using JC-1 dye. In Figure 1, representative images of $\Delta\Psi_m$ variations in both CGSD SH-SY5Y cells (Figure 1a-c) and $A\beta_{1-42}$ -treated differentiated hippocampal HT-22 neurons (Figure 1e-g), with or without leptin treatment for 24 hr are shown. In healthy cells, JC-1 accumulates in the mitochondria as a function of $\Delta\Psi_m$ and forms red fluorescent aggregates in the mitochondrial matrix. JC-1 remaining in the cytosol, indicative of membrane damage, is observed as green fluorescent monomers. In each group, the intensity ratios of JC-1 aggregates (red) to monomers (green) of each group were measured using image analysis

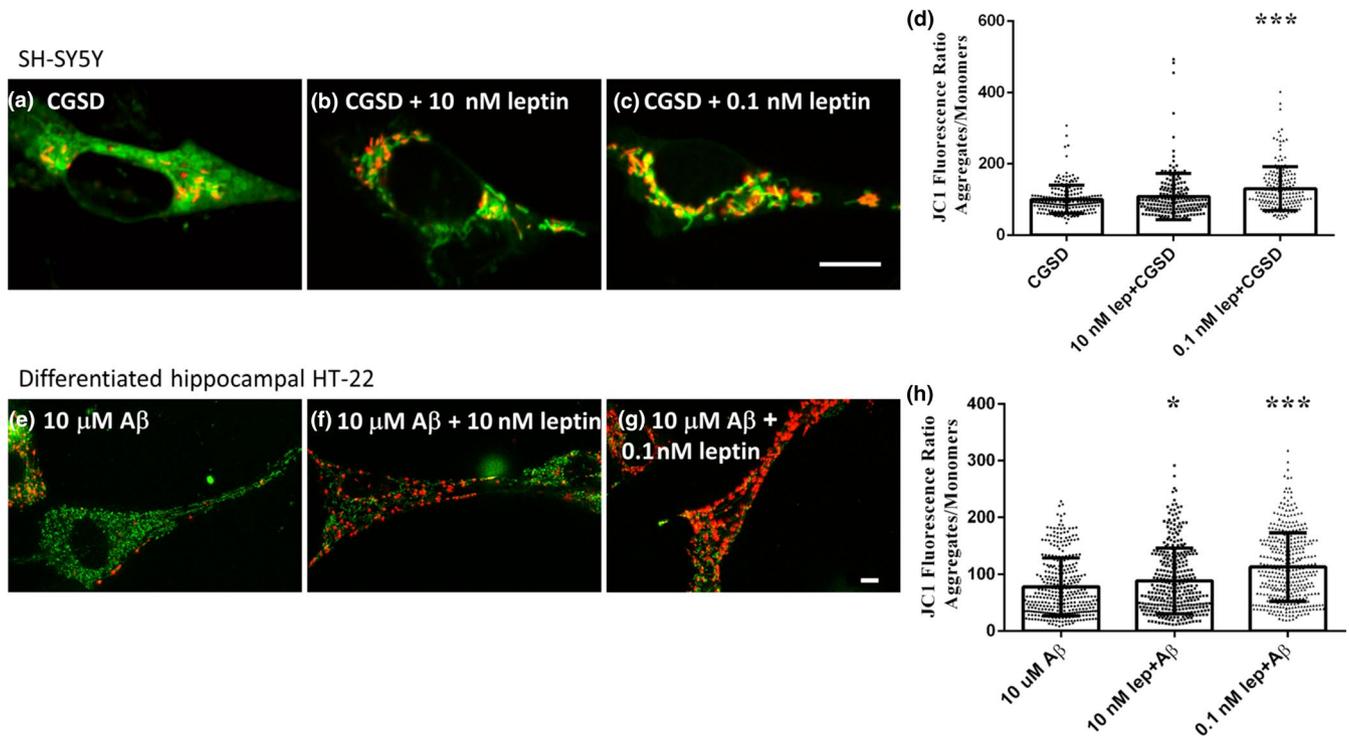


FIGURE 1 Leptin improves both combined glucose and serum deprivation (CGSD)-mediated early $\Delta\Psi_m$ damage and $A\beta_{1-42}$ -mediated $\Delta\Psi_m$ depolarization. $\Delta\Psi_m$ was monitored with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) staining in both CGSD SH-SY5Y cells (a–d) and $A\beta_{1-42}$ -treated HT-22 cells (e–h). Representative fluorescent photomicrographs are shown (a–c; e–g). Scale bars represent 10 μm . Mitochondrial accumulation of JC-1 is demonstrated by the red fluorescence and cytoplasmic JC-1 monomers are observed as green fluorescence. Bar graphs quantifying average J-aggregates/J-monomers ratio of CGSD SH-SY5Y cells with/without 10 or 0.1 nM leptin treatment (d) and $A\beta_{1-42}$ -treated differentiated hippocampal cells with/without 10 or 0.1 nM leptin treatment (h) are also shown. Data are presented as means \pm SEM with individual data points overlaid onto the graphs to demonstrate the distribution of the data. Statistical significance is denoted by * and ***, representing $p < .05$ and $p < .001$ respectively

(Figure 1d,h). In keeping with the previous literature using such analyses, each cell is regarded as an independent event (Connolly et al., 2018). In CGSD SH-SY5Y cells, leptin treatment resulted in more red fluorescent aggregates (Figure 1b,c) compared to CGSD in the absence of leptin (Figure 1a). Application of leptin (0.1 nM) significantly increased $\Delta\Psi_m$ in comparison with CGSD cells that had not been treated with leptin ($p < .001$, Kruskal–Wallis with Dunn's post hoc test; $n > 180$ cells per condition from three independent experiments, Figure 1d).

Previous studies indicated that $A\beta_{1-42}$ reduces $\Delta\Psi_m$ in undifferentiated HT-22 cells that are still in the cell cycle (Cha et al., 2012). Our study expanded on this finding to show that $A\beta_{1-42}$ reduces $\Delta\Psi_m$ in post-mitotic differentiated HT-22 hippocampal cells (Figure 1e). In accordance with our data for the CGSD cells above, leptin treatment promoted the accumulation of JC-1 into mitochondria compared to $A\beta_{1-42}$ -treated cells that had not been co-treated with leptin (Figure 1e–g), indicating increased $\Delta\Psi_m$. Quantitative image analysis demonstrated that both 10 and 0.1 nM leptin significantly ameliorated reductions to $\Delta\Psi_m$ induced by $A\beta_{1-42}$ ($p < .05$, $p < .001$ one-way ANOVA with Dunn's post hoc test; $n > 60$ cells per condition from three independent experiments, Figure 1h). Taken together these data show that leptin ameliorates reductions to $\Delta\Psi_m$ in neuronal models of stroke and AD.

3.2 | Leptin prevents increased mitochondrial fragmentation in an in vitro ischemia model

Given the above beneficial effects of leptin on $\Delta\Psi_m$, we next determined whether leptin modulates mitochondrial morphology, using MitoRed staining. Representative images of CGSD SHSY5Y cells after 72 hr treatment, with different mitochondrial morphologies are shown (Figure 2a–d). In untreated control cells, mitochondrial morphology is predominantly short and tubular (Figure 2a). CGSD for 72 hr resulted in an increase in smaller and rounded mitochondria in comparison to control, (Figure 2b). Leptin co-treatment at 10 or 0.1 nM for 72 hr prevented the CGSD-induced fragmentation, comparably increasing the interconnectivity of the mitochondrial network (Figure 2c,d).

According to the scoring system reported in previous studies (Connolly et al., 2018; Dagda et al., 2009; Senyilmaz et al., 2015), indices of mitochondrial count, size, fragmentation and interconnectivity were calculated. By normalizing the count to the total mitochondrial area, the index of fragmentation was obtained (count/area). The average mitochondrial size was divided by the average mitochondrial perimeter, yielding the index of interconnectivity (mean area/mean perimeter). The index of interconnectivity is sensitive for normal to highly interconnected mitochondria, while the index of

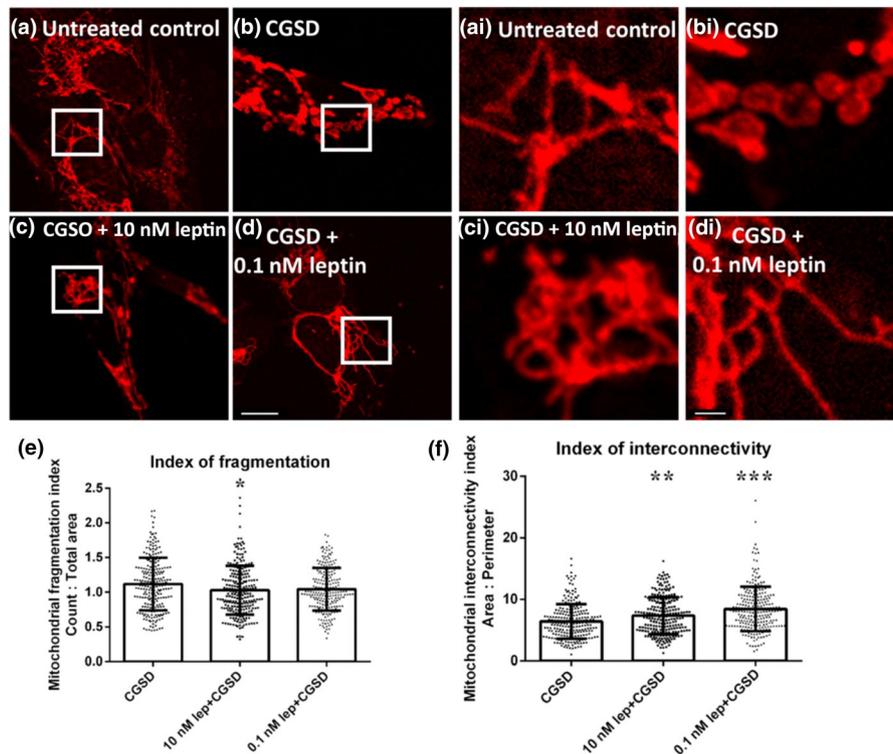


FIGURE 2 Leptin treatment protects against mitochondrial fragmentation induced by combined glucose and serum deprivation (CGSD). Representative images of mitochondrial morphology in 72 hr CGSD SH-SY5Y cells with/without 10 or 0.1 nM leptin treatment are shown (a–d), with expanded views shown (ai–di). In untreated SH-SY5Y cells, the predominant mitochondrial morphology is short and tubular (a, ai). 72 hr CGSD results in the mitochondrial network becoming fragmented and staining appears more punctate. There is also notable mitochondrial swelling (b, bi). Leptin treatment inhibits CGSD mitochondrial fragmentation and promotes mitochondrial fusion. Thus, mitochondria are tubular in leptin-treated cells (c–d, ci–di). Bar graphs demonstrate mitochondrial morphology, quantified by two independent variables: index of mitochondrial fragmentation (e) and interconnectivity (f), with individual data points overlaid onto the graph to demonstrate the variability of the data. Data are presented as means \pm SEM. $N \geq 200$ cells. *, **, and *** represent statistical significance at $p < .05$, $p < .01$ and $p < .001$ respectively. Scale bars represent 10 μ m (a–d) and 2 μ m (ai–di)

fragmentation is sensitive for fragmented to normal short tubular mitochondria. Taken together, these indices provide a thorough investigation of mitochondrial morphology.

From this quantification of mitochondrial morphology, it was demonstrated that serum and glucose withdrawal for 72 hr increased the index of fragmentation, which accords with the above subjective observations. Treatment with leptin at 10 and 0.1 nM prevented CSGD-induced mitochondrial fission by significantly decreasing the fragmentation index ($p < .05$, Kruskal–Wallis with Dunn's post hoc test, Figure 2e) and highly significantly increasing the mitochondrial index of interconnectivity ($p < .01$ for 10 nM leptin treatment; $p < .001$ for 0.1 nM leptin treatment, one-way ANOVA with Dunn's post hoc test, Figure 2f). Taken together, leptin is sufficient to prevent increased mitochondrial fragmentation induced by CGSD.

3.3 | Leptin prevents increased mitochondrial fragmentation in an in vitro AD model

Figure 3 shows the representative mitochondrial morphology of differentiated hippocampal HT-22 neuronal cells in different conditions (Figure 3a–d). In untreated control HT-22 hippocampal neuronal

cells, the majority of mitochondria are short and tubular (Figure 3a). After treatment with 10 μ M $A\beta_{1-42}$ for 24 hr, mitochondria become fragmented, small and punctate (Figure 3b). Following 24 hr of co-treatment with leptin, this effect was largely absent (Figure 3c,d). Indeed, the majority of mitochondria in leptin-treated neuronal cells retained their short tubular morphology.

Leptin treatment prevented the increased index of fragmentation induced by $A\beta_{1-42}$, but significant differences were only observed with 0.1 nM leptin and not 10 nM leptin ($p < .01$, one-way ANOVA with Dunn's post hoc test, $n \geq 60$, Figure 3e). Mitochondria in HT-22 are much smaller than that in SH-SY5Y and a complex mitochondrial network is seldom seen in these hippocampal cells. Therefore, instead of calculating index of interconnectivity, an index of elongation was calculated by adopting the inverse of circularity. Leptin treatment significantly increased the index of elongation in $A\beta_{1-42}$ -treated cells significantly ($p < .05$, one-way ANOVA with Dunn's post hoc test, $n \geq 60$, Figure 3f). Taken together, leptin is sufficient to prevent mitochondrial fragmentation induced by $A\beta_{1-42}$ and promote mitochondrial elongation. This, in conjunction with the data on the $\Delta\Psi_m$, strengthens the evidence that leptin protects mitochondrial function in models of ischaemic stroke and AD in vitro by regulating mitochondrial dynamics.

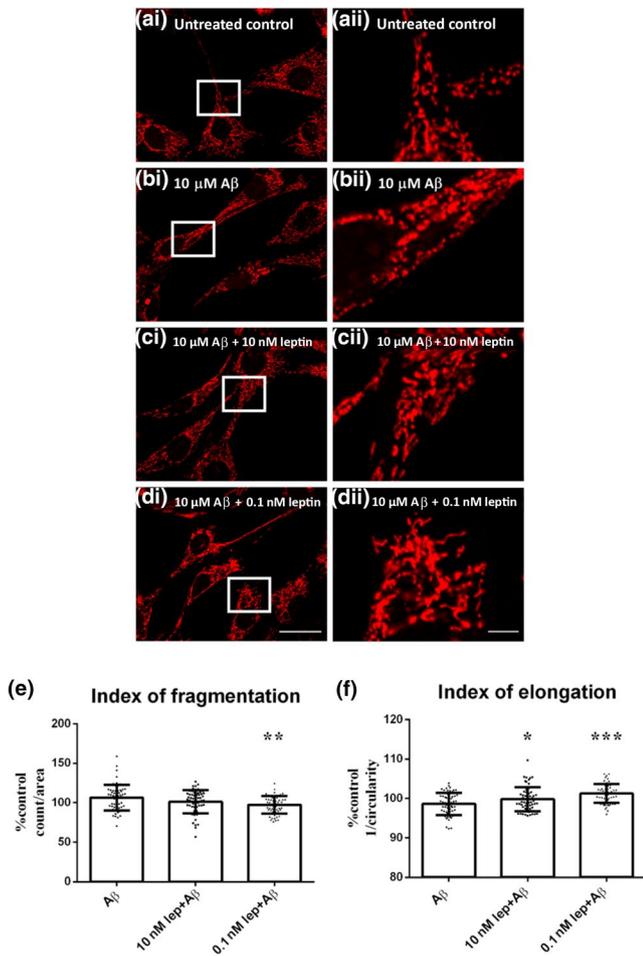


FIGURE 3 Leptin treatment protects against abnormal mitochondrial fragmentation induced by $A\beta_{1-42}$. Representative images of mitochondrial morphology in 24 hr $A\beta_{1-42}$ -treated HT-22 cells with/without 10 or 0.1 nM leptin treatment (ai–di) and digitally expanded views are shown (aii–dii). The expanded areas are denoted by the white quadrangles on ai–di. In untreated differentiated HT-22 cells, the predominant mitochondrial morphology is short and tubular (a). Mitochondria in 24 hr $A\beta_{1-42}$ -treated cells become fragmented and become punctate (b). Leptin inhibits $A\beta_{1-42}$ -triggered mitochondrial fragmentation and promotes mitochondrial fusion. Mitochondria are predominantly observed as short and tubular in leptin and $A\beta_{1-42}$ co-treated cells (c–d). Scale bar represents 30 μm with the scale bar on the expanded views representing 5 μm . The bar graphs show mitochondrial morphology quantified by two independent variables: indices of mitochondrial fragmentation (e) and elongation (f). Data are presented as means \pm SEM, with individual data points overlaid onto the graphs to demonstrate the distribution of the data. $N \geq 200$ cells across the three separate experimental repeats. *, ** and *** represent statistical significance at $p < .05$, $p < .01$ and $p < .001$ respectively

3.4 | Leptin balances mitochondrial dynamics by regulating the expression of mitochondrial fission and fusion proteins

To explore the molecular mechanism by which leptin regulates the dynamics of neuronal mitochondria in vitro, changes of mitochondrial fission and fusion protein expression were studied using ELISA.

Proteins involved in mitochondrial fission (Fis1 and Drp1) and fusion (Mfn1 and Mfn2) were examined. In CGSD SH-SY5Y cells leptin at 10 and 0.1 nM significantly decreased the expression of Fis1 compared to CGSD ($p < .001$, one-way ANOVA with Dunn's post hoc test; $n \geq 4$, Figure 4b). In these experiments, because of the variation in the degree of cell death induced by CGSD, data are compared to CGSD rather than to untreated control. In addition, 0.1 nM leptin treatment significantly increased the expression of Mfn2 in comparison to CGSD ($p < .05$, one-way ANOVA with Dunn's post hoc test, $n \geq 4$, Figure 3d). No significant differences were observed in Drp1 ($p = .5724$, one-way ANOVA with Dunn's post hoc test, $n \geq 4$, Figure 4a) or Mfn1 ($p = .8214$, one-way ANOVA with Dunn's post hoc test, $n \geq 4$, Figure 5). These results reveal that leptin prevents CGSD-induced changes to mitochondrial morphology by reducing Fis1 expression and increasing Mfn2 expression, which is associated with decreased fragmentation and enhanced interconnectivity of the mitochondrial network respectively.

Changes in expression levels of mitochondrial fission and fusion proteins in $A\beta_{1-42}$ -treated HT-22 hippocampal cells are also shown (Figure 4e–h). The expression of Drp1 and Fis1 was increased in $A\beta_{1-42}$ -treated HT-22 neuronal cells compared to untreated control. This effect was abolished by co-administration of 0.1 nM leptin ($p < .05$, one-way ANOVA with Dunn's post hoc test, $n \geq 4$, Figure 4e–f). However, the effect of 10 nM leptin treatment was not significant after post hoc analysis. In addition, the increased level of Mfn1 in $A\beta_{1-42}$ -treated cells was abolished by leptin treatment and was significant at 0.1 nM ($p < .05$, one-way ANOVA with Dunn's post hoc test, $n \geq 4$, Figure 4g). Levels of Mfn2 were increased in all conditions and leptin did not ameliorate these changes in $A\beta_{1-42}$ -treated cells ($p = .7252$, one-way ANOVA with Dunn's post hoc test, $N \geq 4$, Figure 4h). These results suggest that leptin prevents $A\beta_{1-42}$ -induced changes to mitochondrial morphology by reducing the expression of Drp1 and Fis1, and increasing the expression of Mfn1, thereby preventing fragmentation of the mitochondrial network.

To confirm our findings from the ELISA assays, immunocytochemistry was adopted to visualize the expression of Fis1 following CGSD with or without exogenous leptin administration. As shown in Figure 5, the fluorescent labelling and therefore expression of Fis1 following 72 hr CGSD (upper panels) was markedly decreased in 0.1 nM leptin co-treated cells (Figure 5a, lower panels). Cells were co-stained with MitoRed to allow visualisation of the mitochondrial network (Figure 5a). Merged images demonstrating co-localisation of the Fis1 immunoreactivity and the mitochondria are shown. To aid visualisation expanded views of individual cells are also shown (Figure 5b). Quantitative analysis of the immunocytochemistry images was performed. 0.1 nM leptin significantly decreased Fis-1 expressing in CGSD neural cells as compared to those that had not had leptin treatment ($p < .001$, t -test, $n > 200$ cell per condition from at least three separate plate downs, Figure 5c). Control stainings lacking the primary antibody did not exhibit the robust green fluorescence (Figure S1) confirming the specificity of the antibody.

Similarly, immunocytochemical analysis of Mfn2 expression was undertaken in these cells (Figure 6). Mfn2 immunoreactivity

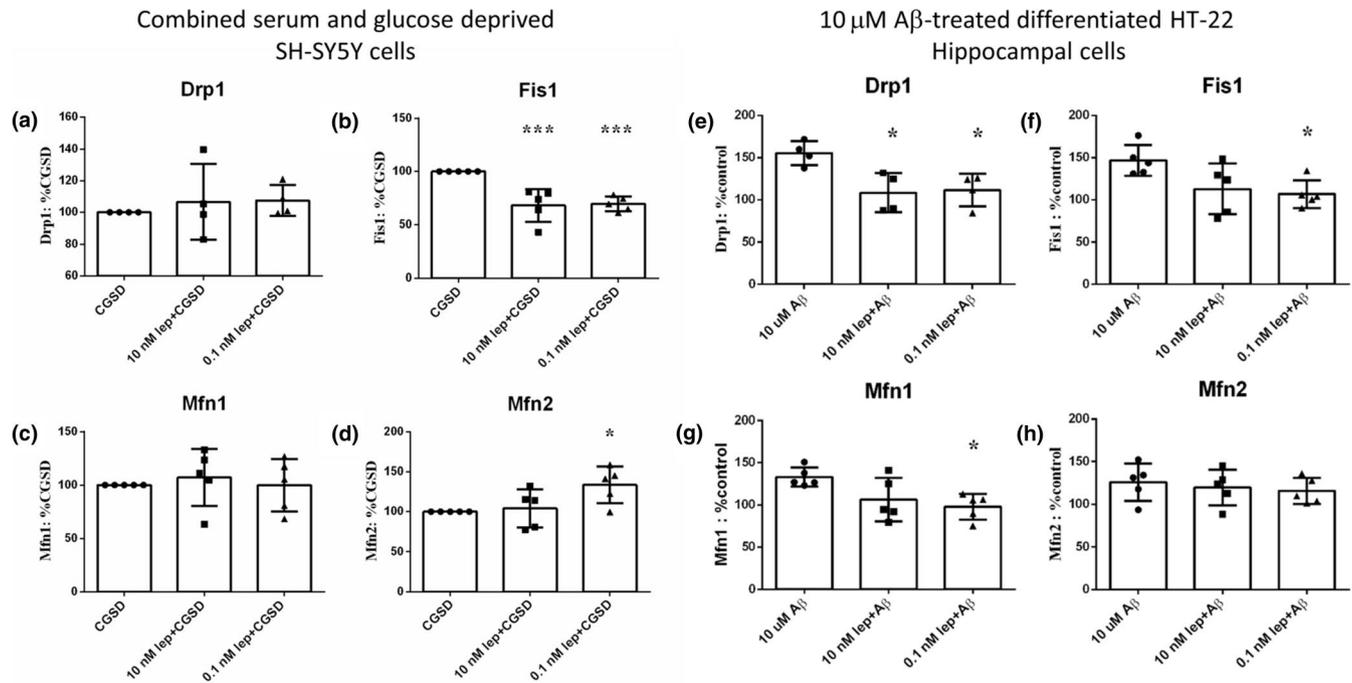


FIGURE 4 Leptin protects against dysregulation of fission and fusion protein expression induced by combined glucose and serum deprivation (CGSD) or $A\beta_{1-42}$. Bar graphs (a–d) show the data from ELISAs to determine the effects of leptin administration on the expression levels of mitochondrial fission proteins (Drp1 (a) and Fis1 (b)) and fusion proteins (Mfn1 (c) and Mfn2 (d)) relative to the CGSD condition in SH-SY5Y cells. Corresponding bar graphs for hippocampal cells (e–h) are also presented showing the effects of leptin administration on the expression levels of mitochondrial fission proteins (Drp1 (e) and Fis1 (f)) and fusion proteins (Mfn1 (g) and Mfn2 (h)) relative to the cells treated with $A\beta$ alone. Data are presented as means \pm SEM, with $n \geq 4$ experimental repeats for each protein. The distribution of the data derived from the individual experimental repeats is demonstrated by the individual data points on each bar. Each experimental data point represents the mean of three technical repeats for each sample. Statistical significance is denoted by *, ** and ***, representing $p < .05$, $p < .01$ and $p < .001$ respectively

after 72 hr CGSD was increased in 0.1 nM leptin treated cells (Figure 6a, lower panels). This is in keeping with the ELISA data (Figure 4). Cells were co-stained with MitoRed to allow visualisation of the mitochondrial network (Figure 6a). Merged images demonstrating co-localisation of the Mfn2 immunoreactivity and the mitochondria are also shown. To aid visualisation expanded views of individual cells are also shown (Figure 6b). Quantitative analysis of the immunocytochemistry images was performed. Percentage areas of Mfn2 staining within the mitochondrial network were calculated. 0.1 nM leptin significantly increased Mfn2 expression in CGSD neural cells ($p < .001$, t test, $n > 200$ cells per condition from at least three separate plate downs, Figure 6c). Control stainings lacking the primary antibody did not exhibit robust green fluorescence (Figure S2) confirming the specificity of the Mfn2 antibody.

3.5 | Leptin regulates enzymes involved in ROS production

Our findings this far demonstrate that leptin inhibits mitochondrial fragmentation in both a CGSD ischaemic stroke model and in an in vitro model of AD by modulating the expression of proteins involved in regulating mitochondrial dynamics. To expand upon these

finding we next examined the effects of leptin on proteins involved in inducing mitochondrial biogenesis and oxidative stress-related enzymes (Figure 7). PGC1- α , a central inducer of mitochondrial biogenesis, induces the expression of nuclear respiration factor-1 (NRF-1). PGC1- α is also a powerful regulator of ROS removal. In CGSD human neural cells, we discovered that leptin (10 or 0.1 nM) decreased the expression of NRF1 ($p < .01$, one-way ANOVA, $n \geq 4$, Figure 7b). No significant differences in PGC1- α in leptin-treated cells were observed ($p = .1196$, one-way ANOVA, $n \geq 4$, Figure 7a). Monoamine oxidases (MAO-A/B) are enzymes that are responsible for the metabolism of monoamine neuro-transmitters, are involved in production of ROS. In CGSD cells, 0.1 nM leptin decreased the levels of MAOB ($p < .01$, one-way ANOVA, $n \geq 4$, Figure 7c) significantly in comparison to CGSD, however, we found no significant differences of MAO-A expression with leptin administration ($p = .8199$, one-way ANOVA, $n \geq 4$, Figure 7d). To determine whether this decrease in MAOB expression resulted in a decrease in the activity of the monoamine oxidase enzymes, we conducted an MAO activity assay as reported previously (Zhou et al., 1997). We uncovered that 0.1 nM leptin significantly decreases the activity of MAO compared to CGSD alone ($p < .05$, one-way ANOVA, $n = 7$, Figure 7e).

In $A\beta_{1-42}$ -treated HT-22 cells, neither the expression of PGC1- α ($p = .3791$, one-way ANOVA, $n \geq 4$, Figure 7f) nor NRF1

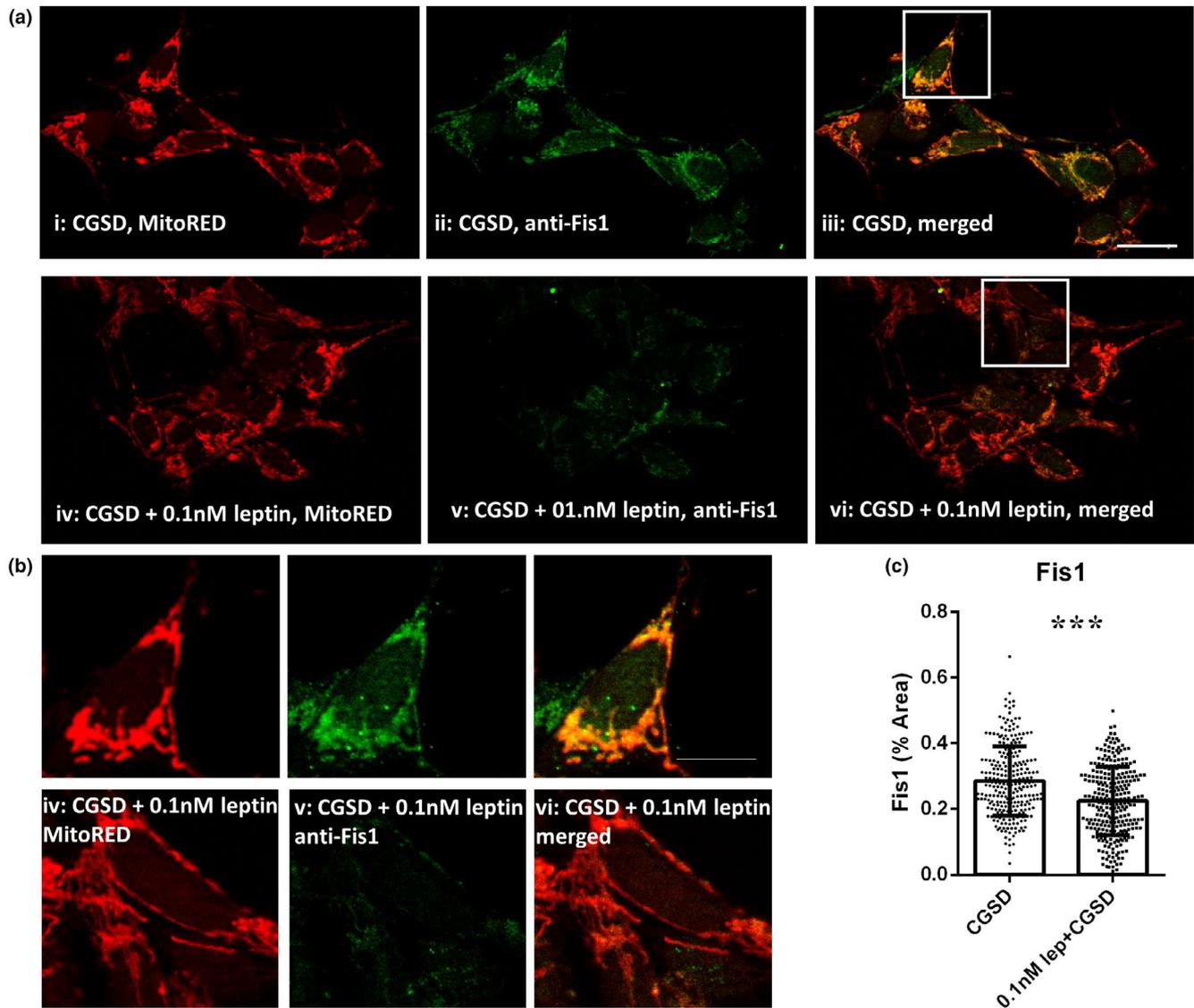


FIGURE 5 Leptin decreases Fis1 expression in combined glucose and serum deprivation (CGSD) SH-SY5Y cells. Representative photomicrographs of CGSD SH-SY5Y cells (ai–iii; bi–iii) and those also treated with 0.1 nM leptin (aiv–vi and biv–vi). Single channel images of MitoRed (shown in red) or Fis1 (shown in green) staining are presented along with the merged image and digitally expanded views (b). The merged views confirm that the Fis1 immunoreactivity is mitochondrial. The expanded areas are denoted by the white quadrangles on aiii and avi. Scale bar represents 20 μm with the scale bar on the expanded views representing 10 μm . Bar graph demonstrates the outcome of quantitative analysis Fis1 expression within the mitochondrial network (c). Individual data points are overlaid on the chart to allow visualisation of the distribution of individual data points. Data are presented as mean \pm SEM. $N \geq 200$ cells from three independent experiments and *** represents significance at $p < .001$

($p = .6996$, one-way ANOVA, $n \geq 4$, Figure 7g) were significantly altered. However, both MAO-A and MAO-B expression were increased significantly in $\text{A}\beta_{1-42}$ -treated hippocampal neurons, exhibiting around a 50% increase compared to untreated control neurons (Figure 7h–l). Co-treating $\text{A}\beta_{1-42}$ -treated hippocampal neurons with leptin for 24 hr, significantly reduced the increase in MAO-A ($p < .01$, one-way ANOVA, $n \geq 4$, Figure 7h) and MAO-B ($p < .01$, one-way ANOVA, $n \geq 4$, Figure 7i). These results indicate that leptin down-regulates enzymes involved in the production of ROS that may contribute to its protection against mitochondrial dysfunction in models of ischaemic stroke and AD in vitro.

3.6 | Leptin inhibits $\text{A}\beta_{1-42}$ -induced neuronal membrane permeability and prevents loss of neuronal viability

It is well established that leptin protects against CGSD in SH-SY5Y neural cells (Russo, Metaxas, Kobayashi, Harris, & Werther, 2004) and therefore our results can be interpreted to be part of the known neuroprotective actions of this hormone in these cells. In contrast, it has never been demonstrated that leptin protects against neuronal cell death in fully differentiated HT-22 hippocampal cells. Therefore we aimed to elucidate leptin's potential as a neuroprotectant in this model. Treating differentiated HT-22 mouse hippocampal neurons

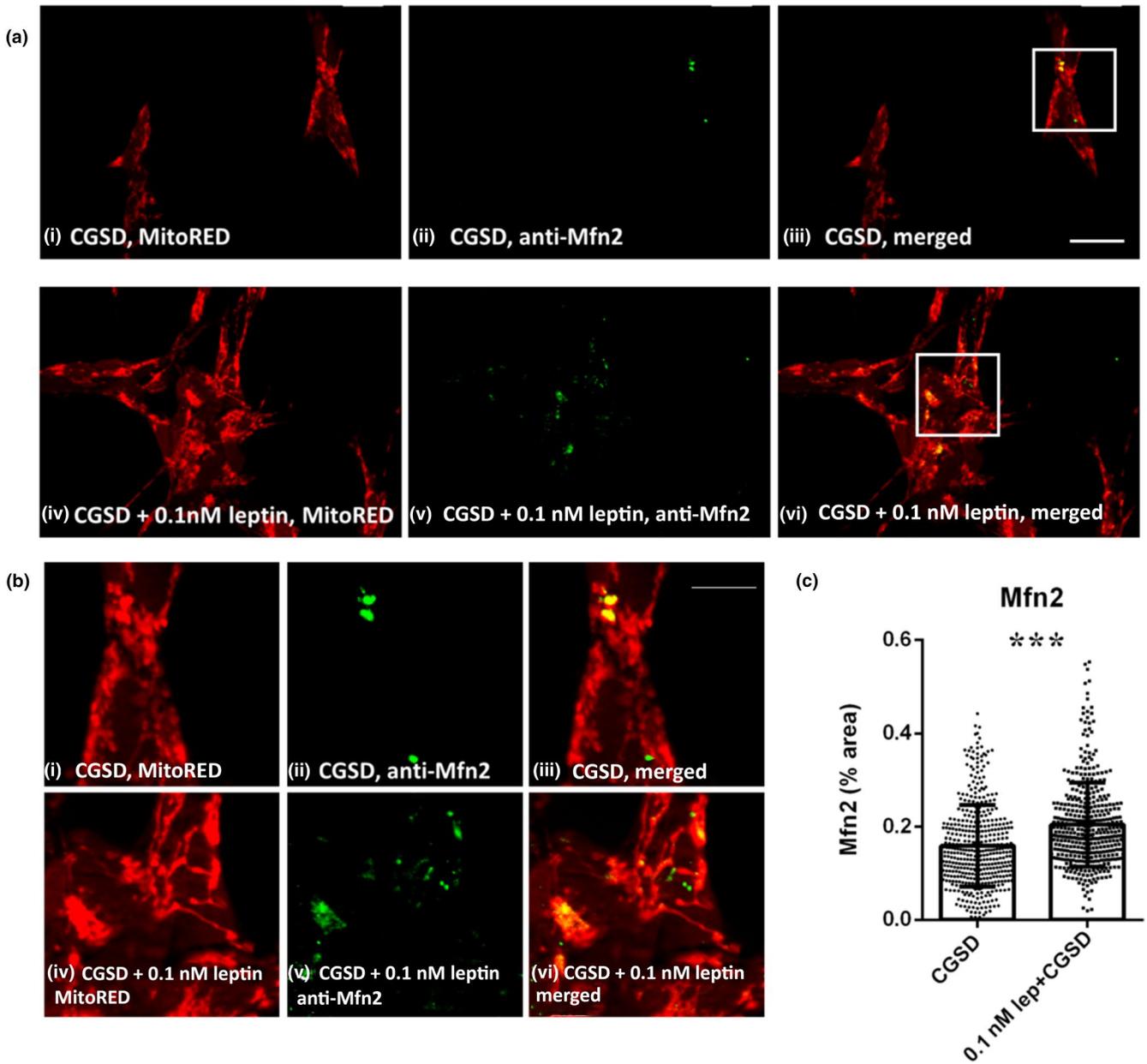


FIGURE 6 Leptin increases Mfn2 expression in combined glucose and serum deprivation (CGSD) SH-SY5Y cells. Representative photomicrographs of CGSD SH-SY5Y cells (ai–iii; bi–iii) and those also treated with 0.1 nM leptin (aiv–vi and biv–vi). Single-channel images of MitoRed (shown in red) or Mfn2 (shown in green) staining are presented along with the merged image and digitally expanded views (b). The merged views confirm that the Mfn2 immunoreactivity is mitochondrial. The expanded areas are denoted by the white quadrangles (aiii and avi). Scale bar represents 20 μm , with the scale bar on the expanded view representing 10 μm . Bar graph demonstrates the outcome of quantitative analysis of Mfn2 (c). Individual data points are overlaid on the chart to allow visualisation of the distribution of the data. Data are presented as mean \pm SEM. $N \geq 200$ cells from three independent experiments and *** represents significance at $p < .001$

with $\text{A}\beta_{1-42}$ (10 μM) results in the release of LDH, which indicates damaged membrane permeability. Leptin treatment at 10 and 0.1 nM significantly decreased the release of LDH induced by $\text{A}\beta_{1-42}$ significantly (Figure 8 one-way ANOVA, $p < .05$, $n = 6$). These data demonstrate that leptin ameliorates neuronal membrane permeability induced by $\text{A}\beta_{1-42}$. In addition, treating hippocampal neurons with $\text{A}\beta_{1-42}$ leads to 13.5% decrease of neuronal number, as determined by crystal violet assay, compared to an untreated control. Leptin treatment at both 10 and 0.1 nM significantly reduced the $\text{A}\beta_{1-42}$ -mediated decrease in cell number (Figure 8 one-way ANOVA,

$p < .05$, $n = 8$). Taken together, leptin ameliorates neuronal loss, inhibits abnormal membrane permeability and promotes hippocampal neuronal viability induced by treatment with $\text{A}\beta_{1-42}$.

4 | DISCUSSION

Leptin, known as an essential mediator of energy homeostasis, exerts a protective role in models of neurodegenerative diseases, including AD (Doherty, 2011; Li, Yan, Guo, & Wang, 2016). Epidemiological

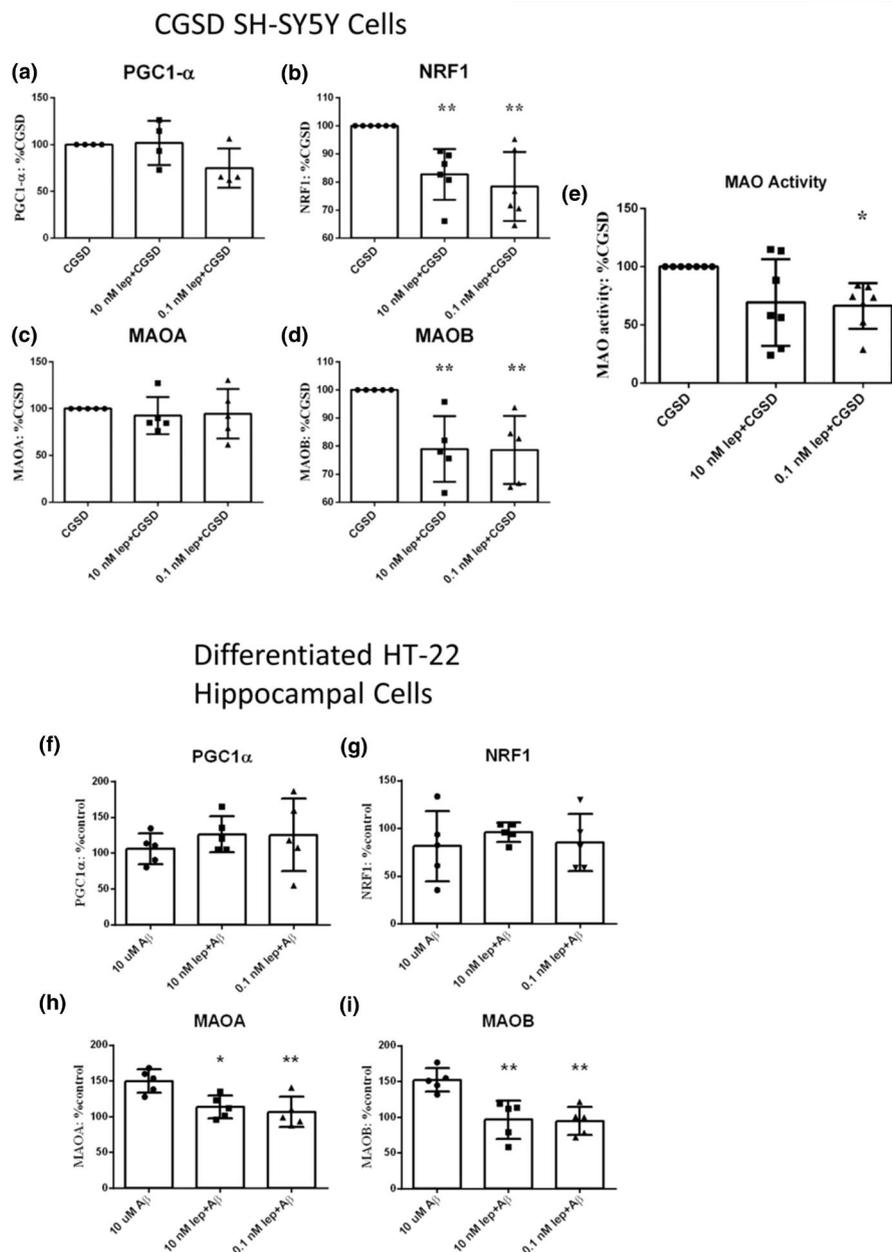


FIGURE 7 Leptin regulates the expression and activity of mitochondrial biogenesis enzymes and monoamine oxidase (MAO). Bar graphs (a–d) show ELISA data determining the effects of leptin administration on the expression levels of mitochondrial biogenesis proteins, PGC1 α (a) and NRF1 (b) and monoamine oxidases, MAOA (c) and MAOB (d) relative to the combined glucose and serum deprivation (CGSD) condition in SH-SY5Y cells. The effects of leptin administration on MAO activity in these cells is also presented (e). Corresponding bar graphs for the expression levels hippocampal cells (f–i) are also presented showing the effects of leptin administration on the expression levels of mitochondrial biogenesis proteins, PGC1 α (f) and NRF1 (g) and monoamine oxidases, MAOA (h) and MAOB (i) relative to the cultures treated with A β_{1-42} alone. Data are presented as means \pm SEM, with $n \geq 4$ experimental repeats for each protein. The distribution of the data derived from the individual experimental repeats is demonstrated by the individual data points on each bar. Each experimental data point represents the mean of three technical repeats for each sample. Statistical significance is denoted by * and **, representing $p < .05$, and $p < .01$ respectively

studies have revealed that higher serum leptin levels protect against cognitive impairment (Holden et al., 2009; Witte et al., 2016) and are associated with a reduced incidence of AD (Gilbert et al., 2018; Lieb et al., 2009). Expression of Ob-Rb is decreased and leptin signalling (Akt) disrupted in the hippocampus of APP/presenilin 1 mice (King et al., 2018) further implicating leptin signalling in AD pathogenesis. In addition, leptin treatment is capable of attenuating pathological

deposition of A β and phosphorylated tau in AD models (Guo et al., 2016; Liu, Zhang, Liu, & Yin, 2017; Marwarha, Dasari, Prasanthi, Schommer, & Ghribi, 2010). Furthermore, increased mitochondrial ROS and damaged $\Delta\Psi_m$ are observed in brains of diet-induced obese rats (Ma, Yuan, Yu, Xi, & Xiao, 2014) that lack efficient leptin signalling because of leptin resistance (Scarpace & Zhang, 2009). Mitochondrial degeneration in the blood–brain barrier has been

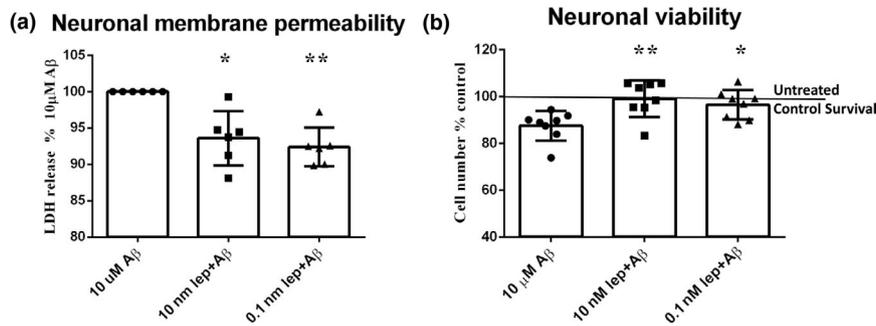


FIGURE 8 Leptin inhibits $A\beta_{1-42}$ -mediated increased neuronal membrane permeability and increases neuronal viability in hippocampal cells. Bar graphs showing effects of leptin administration on the release of lactate dehydrogenase (LDH) into cell culture relative to $A\beta_{1-42}$ treatment alone (a), and the output of a crystal violet assay for cell number demonstrating cell viability relative to that observed in control, untreated cultures (b), are presented. Data are presented as means \pm SEM. $N \geq 6$ independent experiments; * and ** represent statistical significance at $p < .05$ and $p < .01$ respectively. Individual data points have been overlaid onto the bar charts to reveal the distribution of the data gathered

detected in *db/db* mice with leptin receptor deficiency (Corem, Anzi, Gelb, & Ben-Zvi, 2019). It is well established that mitochondrial dysfunction presents as an early and predominant feature in the development of AD (Hauptmann et al., 2009), and promotes the pathophysiology of AD (Cheng & Bai, 2018). Thus the potential for leptin to benefit mitochondria has numerous potential clinical applications. However, the effect of leptin on mitochondrial function in the early and late stages of AD has not been examined.

Our study provides compelling evidence that leptin exerts its known neuroprotective role through balancing mitochondrial morphological dynamics and improving mitochondrial dysfunction in AD-linked cell models. Thus leptin ameliorates increased mitochondrial fragmentation through regulating the expression of mitochondrial fission and fusion proteins, and prevents the loss of mitochondrial membrane potential induced by $A\beta_{1-42}$.

The neurobeneficial effects of leptin have long been known, with the first reports revealing leptin's anti-apoptotic actions in SH-SY5Y neural cells in vitro (Russo et al., 2004). We have expanded on this to reveal that leptin protects differentiated HT-22 hippocampal neurons from $A\beta_{1-42}$ -mediated neurotoxicity. Thus in both our CGSD SH-SY5Y human neuroblastoma cells, an ischaemia stroke model (Lorenz et al., 2009) and in $A\beta_{1-42}$ -treated differentiated HT-22 hippocampal neuronal cells, an AD model, leptin protects against cell death. The concentrations of leptin that we have used in this investigation are within the low nanomolar physiological range (0.1–10 nM; Moulton et al., 2010). However, the data reveal that the lower concentration (0.1 nM) is more effective than 10 nM administration in mediating leptin's neurobeneficial actions. The possibility that there is endogenous secretion of leptin in the cultures, pushing the true concentration into a supraphysiological range with the higher concentration treatment cannot be discounted. Indeed HT-22 cells have recently been shown to up-regulate the leptin transcript following hydrogen sulphide administration (Zhu et al., 2019) and SH-SY5Y cells are known to express endogenous leptin (Marwarha, Dasari, & Ghribi, 2012). Nonetheless, in our study the effects of administering physiological doses of leptin on the mitochondrial network as clear.

Maintaining balanced mitochondrial dynamics is an essential process, which promotes mitochondrial distribution across axons into synapses and separates damaged mitochondrial constituents, to meet high neuronal energy demand and facilitate protective effects (Diaz & Moraes, 2008; Scott, Youle, Pike, Lee, & Yoon, 2016). Unbalanced dynamics is hypothesised to be an essential mechanism leading to synaptic and neuronal dysfunction in AD (Santos et al., 2010; Wang et al., 2009, 2014). In our study, we reveal that leptin inhibits mitochondrial fission induced by CGSD and $A\beta_{1-42}$, and promotes mitochondrial fusion balancing the morphological dynamics and improving mitochondrial dysfunction. In previous studies, it was found that over-expression of Fis1 and decreased levels of Mfn1, Mfn2, Drp1 and OPA1 led to the impaired balance of mitochondrial fission/fusion dynamics in an in vitro AD model (Wang et al., 2008, 2009). In agreement with these previous studies, up-regulation of Fis1 was observed in neuronal cells induced by AD-linked insults $A\beta_{1-42}$ and CGSD. Moreover expression of Drp1 was also increased in $A\beta_{1-42}$ -treated hippocampal neurons. A recent study supports the possibility that interaction of Drp1/Fis1 in mitochondria leads to excessive mitochondrial fission and neuronal injury in AD (Joshi, Saw, Shamloo, & Mochly-Rosen, 2018). We found that over-expression of Fis1 and Drp1 was linked to excessive mitochondrial fragmentation in $A\beta_{1-42}$ -treated neuronal cells. Leptin inhibited the over-expression of Fis1 in CGSD neural cells, and for both Drp1 and Fis1 the increased levels induced by $A\beta_{1-42}$ were decreased by leptin treatment. Thus leptin decreases the expression of mitochondrial fission proteins in our experiments.

Depletion of fusion protein Mfn2 results in excessive mitochondrial fission and cellular death in cortical neurons (Uo et al., 2009). Human Mfn2 mutations cause mitochondrial network fragmentation (Rocha et al., 2017), and leptin treatment increases the expression of Mfn2 and Drp1, modulating mitochondrial dynamics in MCF-7 breast cancer cells (Blanquer-Rosselló et al., 2015). Here we show that in addition to the known data from the breast cancer cell line, leptin also increases the expression of Mfn2 in CGSD neural cells. No significant increases in Mfn levels were discovered in the $A\beta_{1-42}$ -treated hippocampal neurons, but these cells have a less interconnected mitochondrial network in the untreated condition as



compared to the SH-SY5Y cell line and therefore a lesser degree of fusion may be required to maintain the normal mitochondrial morphology. In summary, our results show that leptin inhibits abnormal mitochondrial fission in AD models by down-regulating Fis1 and Drp1 and up-regulating Mfn2.

Furthermore, mitochondrial biogenesis, a process in which PGC1- α is a central inducer, contributes to the maintenance of the healthy mitochondrial population. PGC1- α also regulates the removal of ROS (Austin & St-Pierre, 2012), and increases the expression and activity of NRF1, a transcription factor that mediates production of nuclei-encoded mitochondrial genes coding for subunits of the oxidative phosphorylation system (Virbasius, Virbasius, & Scarpulla, 1993; Wu et al., 1999). However, we show here that mitochondrial biogenesis is not a powerful regulator in leptin-mediated mitochondrial dynamics balance, with no differences in the level of expression of proxisome proliferator-activated receptor γ coactivator 1 in either in vitro model. NRT-1 expression is decreased by leptin treatment in CGSD-treated cells but not in the A β_{1-42} -treated cells. As NRF-1-mediated changes in gene expression are linked to protection from oxidative stress (Hertel, Braun, Durka, Alzheimer, & Werner, 2002), it could be hypothesised that a protective agent might up-regulate this protein. Taken together we did not find any evidence that proxisome proliferator-activated receptor γ coactivator 1 and/or activation of NRF-1 contribute to the neuroprotective actions of leptin.

Because of the production of hydrogen peroxide and induced oxidative stress, excessive MAO activity is regarded as a risk factor in AD (Quartey et al., 2018). Indeed, inhibition of MAO has been proposed as a treatment target in AD (Hroch et al., 2017; Naoi & Maruyama, 2010). We provide here the first evidence that leptin inhibits the expression and activity of MAO in AD models, implying the involvement of leptin in protection against oxidative stress through modulation of this enzyme. In addition, MAO regulates mitochondrial fission in cardiomyocytes during aging (Vigneron, Guilbeau-Frugier, Parini, & Mialet-Perez, 2013). In our hands leptin significantly down-regulated MAOB in CGSD cultures and the A β_{1-42} -treated hippocampal neurons, with MAOA down-regulated in the latter population as well. The large volume of protein that can be derived from the SH-SY5Y cultures allowed us to further investigate this and determine that there is a functional consequence of this down-regulation of protein expression with a marked decrease in MAO activity detected in the MAO activity assay. Unfortunately the nature of the differentiated hippocampal cultures is such that it is not possible to harvest the highly concentrated protein samples required for the assay at sufficient volume and therefore we could not confirm this finding in these cells. Nonetheless, we demonstrate a robust down-regulation of expression of MAO enzymes in A β_{1-42} -treated hippocampal cells revealing that leptin can target the MAO system, a previously proposed therapeutic goal for AD treatment (Hroch et al., 2017; Naoi & Maruyama, 2010).

The maintenance of a normal $\Delta\Psi_m$ is important for optimal mitochondrial function (Zorova et al., 2017). In recent developments of mitochondria-targeted therapeutics for neurodegenerative disease,

antioxidant drugs such as MitoQ and peptide SS take advantage of the $\Delta\Psi_m$ to accumulate in mitochondria and implement their protective role (Birk, Chao, Bracken, Warren, & Szeto, 2014; Snow et al., 2010). However, two facts should be taken into consideration. Dysfunctional mitochondria tend to have disrupted $\Delta\Psi_m$, which may limit the intake of drugs. Excessive uptake of drugs driven by $\Delta\Psi_m$ may result in a depressed $\Delta\Psi_m$ (Wang & Chen, 2016). Here we show that leptin regulates enzymes that protect against oxidative stress and protects mitochondrial function without depending on or damaging $\Delta\Psi_m$. However, it does restore a depleted $\Delta\Psi_m$ thereby benefitting mitochondrial function, further underpinning the beneficial effects of leptin.

Our data reveal that leptin is a potential therapeutic target for disorders, such as AD, where mitochondrial dysfunction has been intricately linked to pathogenesis. It is likely that leptin-based therapeutics would not be suitable for all patients as it is known that some individuals exhibit leptin resistance which would inhibit their responsiveness to a class of drugs based on this hormone (Liu, Yang, Yu, & Zheng, 2018). Nonetheless, given the proven reduced circulating leptin levels in AD patients (Lieb et al., 2009) and the emerging knowledge of compounds such as celastrol that can sensitise individuals to leptin's effects (Chellappa, Perron, Naidoo, & Baur, 2019), this remains a crucial pathway to explore in the search for anti-degenerative therapeutics. Leptin is a large molecule and is hard to administer, requiring subcutaneous injections. Pharmaceutical work on leptin mimetics has been undertaken and promising bioactive fragments such as leptin 116–130 that retain the CNS actions of leptin have been uncovered (Malekizadeh et al., 2017). Further development of this region of the molecule into an orally deliverable preparation, [D-Leu-4]-OB3, that crosses the blood–brain barrier (Anderson, Jacobson, Novakovic, & Grasso, 2017) suggest that overcoming the administration difficulties of the full length molecule offer an exciting avenue in development of leptin as a therapeutic. Further work to investigate whether these small fragments of the leptin molecule mirror its beneficial effects on the mitochondrial network are therefore an area of research that warrants future exploration.

In conclusion, these findings provide compelling evidence that leptin protects against early mitochondrial dysfunction and against A β_{1-42} -induced aberrant mitochondrial dynamics in two established AD-related cellular models. This further underlines the essential role of mitochondrial dysfunction in AD and also identifies a novel protective role of leptin in the regulation of mitochondrial dynamics.

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All experiments were conducted in compliance with the ARRIVE guidelines.

CONFLICT OF INTEREST

The authors confirm that they have no conflicts of interest relating to this work.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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