Mens Sana in Corpore Sano

The effect of metabolic factors and the actions of *Triiodo-L-thyronine* on mitochondrial morphology

Chloe-Anna Hunt

Supervised by Dr Gayle H Doherty

School of Psychology and Neuroscience

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Abstract

Alzheimer's disease (AD) is characterised pathologically by mitochondrial dysfunction and oxidative damage. Aberrant levels of metabolic factors, such as homocysteine, copper and amyloid-β (Aβ), can damage neurons, setting the scene for the progression to dementia. *Triiodo-L-thyronine* (T3) is an active form of thyroid hormone that is a central regulator of mitochondrial activity. However, its effects with relation to sustaining the mitochondrial morphology after being treated with metabolic stressors commonly seen in AD remain largely unknown. To address this question, two well-defined models of AD were used: SH-SY5Y neuroblastoma cells and HT-22 hippocampal cells. Using a combination advanced microscopy and MitoRed staining techniques, it is evident that the stressor conditions induced mitochondrial stress which led to profound aberrations in mitochondrial morphology and apoptosis. The T3 typically had a modulating effect on all aspects of the mitochondrial morphology, however, longer exposures to T3 appeared to have detrimental effects on the mitochondria and mimicked the stressor conditions, suggesting that it may not be safe to use long-term. Therefore, this research concluded that T3 increases mitochondrial health, however excessive levels destroy the mitochondrial morphology.

1 | Introduction

Alzheimer's disease (AD) is the most common type of dementia, with approximately 55 million people having the disease worldwide ("Dementia", 2022). As population age and life expectancy increases, the prevalence of AD has dramatically increased, leading to significant social and economic implications on medical and social care costs. This degenerative disorder is defined by a loss of neurons in the limbic system, neocortex, and basal forebrain (Moreira et al., 2010). AD is marked by a constellation of cognitive disturbances and severe memory loss, the earliest and most prominent being episodic memory. It is characterised by intracellular neurofibrillary tangles and extracellular senile plaques in subcortical regions essential for cognitive function (Breijyeh & Karaman, 2020).

Mitochondria contributes toward maintaining cellular homeostasis and function. They are essential for the demands of diverse cellular processes such as regulating cell survival and energy production (Galluzzi et al., 2012; Safiulina & Kaasik, 2013). As a highly dynamic organelle, they frequently undergo structural and functional remodelling. Therefore fission and fusion processes are vital for maintaining their quality and typical cell function (Chan, 2020). These processes have widespread benefits for the mitochondrial population and controlling their size, number, and shape. However, changes in these proteins affect mitochondrial localisation in AD. Aberrant mitochondrial fission and fusion dynamics lead to altered infrastructure, damaging the energy supply by impeding mitochondrial function (Cheng et al., 2020), an essential factor in AD aetiology. Extensive evidence supports the contribution of this abnormal mitochondrial function to oxidative stress (Lin & Beal, 2006; Guo et al., 2013) and cellular energy depletion (Divya et al., 2013). This leads to individuals with AD displaying increased alterations in the mitochondria's shape, number, and enzyme activity (Swerdlow, 2018; Baloyannis, 2006).

Triiodo-L-thyronine (T3) is an active form of thyroid hormone and is a central regulator of mitochondrial activity. Significant amounts of T3 are internalised in cells, which could directly affect mitochondrial activity and energy metabolism (Sterling et al., 1984). Compelling evidence from laboratory rat studies highlights that T3 is fundamental for the growth, development and maintenance of basal forebrain cholinergic neurons typically involved in AD, as T3 deficiencies prompted marked retardation of the developmental patterns of the enzyme activity in the hippocampus (Mutvei et al., 1989; Calzà et al., 1997). This impact is mediated by T3-binding transcription factors, specifically the c-erbAα gene, which encodes these receptors. A depletion of c-erbAα T3 receptors is present in AD brains, specifically in the hippocampus, an area primarily affected by the disease (Sutherland et al., 1992). This, therefore, shows that the hormone may control apoptosis in the brain (Labudova et al., 1999; Sampaolo et al., 2005). Consequently, reduced T3 levels within the normal range could be associated with cognitive decline in AD.

The regulation of copper (Cu) in the mitochondria is critical for normal cellular physiology. This is done by two Cu-containing enzymes present in the mitochondria, cytochrome c oxidase (COX) and superoxide dismutase [Cu-Zn] (SOD1) (Cobine et al., 2021). Copper homeostasis is tightly regulated due to the high toxicity of Cu in excess, with homeostasis deficits or increased environmental Cu exposure leading to inappropriate protein binding or provocation of oxidative stress (Babaei et al., 2012; Sokol et al., 1993). Cu is a vital element in neuronal cells; however, elevated Cu concentrations in the brain, notably the hippocampus, are neurotoxic and can cause oxidative stress and mitochondrial reduction and dysfunction (Behzadfar et al., 2017; Kalita et al., 2017). It is found in high concentrations in the senile plaques in AD patients' brains (Dong et al., 2003; Smith et al., 1997). Cu is associated with the hallmark Alzheimer's proteins amyloid and tau in the brain, which appear as *amyloid plaques* and *tau tangles* and cause damage in AD patients' brains (Bagheri et al., 2018).

Similarly, abnormally high levels of homocysteine (Hcy) have been indicated as a strong risk factor for AD. Multiple mechanisms have been put forward for Hcy-induced oxidative stress, including activating oxidant systems and inhibiting antioxidant systems (Kumar et al., 2018). The link between Hcy-induced oxidative stress and mitochondrial dysfunction remains unclear, but studies suggest that Hcy deteriorates mitochondrial energy metabolism (Kaplan et al., 2020). Notably, the activation of glutamate N-methyl-D-aspartate receptors mediates Hcy-induced neuronal cell death (Lipton et al., 1997), and this induction of oxidative stress and apoptosis is a significant mechanism in Hcy-induced neurotoxicity. Extensive evidence highlights this induced cell death in hippocampal regions of mice and fruit flies (Zhao et al., 2012) and SH-SY5Y cells (Hirashima et al., 2010; Jang et al., 2016).

Increased amyloid-β (Aβ) leads to the onset of AD. This protein is renowned for localising mitochondrial membranes, increasing reactive oxygen species production, causing mitochondrial damage, and preventing neurons from regular functioning (Reddy & Beal, 2008). Studies show that Aβ peptides use ryanodine to enhance mitochondrial dysfunction, induce morphological changes and notably cause mitochondrial swelling (Mungarro-Menchaca et al., 2002). Likewise, Sirk et al. (2007) found that Aβ blocks the entry of nuclear-encoded proteins into mitochondria, which causes blockages leading to decreased mitochondrial membrane potential and altered mitochondrial morphology. T3 may have an inhibitory effect of T3 in regulating Aβ, a protein precursor that is a major component of amyloid plaques in AD patients' brains, counteracting these detrimental factors. There is compelling evidence that Aβ is responsible for synaptic mitochondrial damage and dysfunction in AD.

Therefore, in this study, I examined the effects of T3 on mitochondrial dynamics and morphology in Hcy treated SH-SY5Y human neuroblastoma cells and in copper treated and Aβ treated differentiated HT-22 hippocampal neuronal cells as AD models.

I predicted that cells co-treated with T3 would show improvements in mitochondrial health.

2 | Methods

2.1 | Cell Culture

The human neuroblastoma cell line SH-SY5Y and mouse hippocampal neuronal cell line HT-22 were examined. The thyroid hormone, *Triiodo-L-thyronine*, was used as a potential enhancer of metabolic activity, therefore being a treatment condition. Oxidative stress was introduced by exposing the undifferentiated HT-22 cells to either 10uM of copper chloride or 10uM of Aβ. The undifferentiated SH-SY5Y cells were subjected to 100uM of Hcy as a stress condition.

The cells were either pre-treated with T3 24 hours before administering the respective stress condition for 24 hours (PT3). Alternatively, the cells were treated with T3 alongside the stress condition for 24 hours.

2.2 | MitoRed Staining

MitoRed is a cell membrane permeable rhodamine-based dye that is used to stain active mitochondria. All the cells were incubated in MitoRed solution for 30-45 minutes, then fixed in neutral buffered formalin and mounted onto microscope slides and sealed with nail polish. The effects of the stressors on mitochondrial dysfunction were explored using the SH-SY5Y and HT-22 cells stained with MitoRed dye.

A Zeiss Axio MR2 microscope was used to image the cells, using an excitation wavelength of 495nm. The cell images were captured using the accompanying Zen software.

2.3 | Analysis

Mitochondrial Morphology-

The quantitative analysis of mitochondrial morphology was examined using ImageJ. A polygon shape was drawn around the edges of each individual cell, pre-processing the individual cells. Then, the software analysed the individual mitochondrial particles for the count, area, perimeter, and circularity. From these, various indices were calculated relating to changes in mitochondrial morphology. The index of fragmentation was calculated by the individual mitochondria count to the total mitochondrial area within the cell. The index of interconnectivity was calculated from the ratio of the mean area of the cell to the perimeter of the cell. The elongation was calculated by taking the reciprocal of the cell circularity.

Subjective image analysis was also carried out throughout analysis of the cells; in doing so, I was blinded to the condition and cell line. The cell images were interpreted and classified them based on their mitochondrial morphology. The Wappler method (Wappler et al., 2013) was used to do this, and the mitochondria were sorted into four distinct categories based on their appearance- normal/tubular, rounded, poorly labelled cells, and highly interconnected.

Statistics-
All statistical analyses and graphs were generated using GraphPad PRISM 5 (Graph Pad Inc.). There were no sample size calculations performed, and the data are expressed as mean ± SEM (standard error mean). Statistical analysis was performed using a one-way analysis of variance (ANOVA) with Tukey's post hoc test for comparisons between multiple groups.

3 | Results

3.1 | SH-SY5Y Neuroblastoma Cells treated with Hcy

The mitochondrial morphology of untreated control cells was long and tubular (figure 2a). Following treatment with Hcy, the mitochondria were rounded (figure 2f). However, most mitochondria co-treated or pre-treated with T3 alongside the Hcy retained their normal and tubular morphology (figure 2c/e).

To analyse this data quantitatively, I used macro analysis code used previous (Cheng et al., 2020) to assess mitochondrial number and morphology. Hcy treatment decreased the mean number of mitochondria per cell compared to the untreated control cells ($p<.05$, one-way ANOVA, figure 3a). There was a significant difference between the Hcy treated with T3 and PT3, compared to Hcy alone ($p<.001$, one-way ANOVA, figure 3a). The Hcy treatment significantly increased the mean size of the mitochondria ($p<.001$, one-way ANOVA, figure 3b). This difference was sustained when the cells were treated with Hcy alongside T3 and PT3.

It was demonstrated that treatment with Hcy increased the mitochondrial fragmentation ($p<.001$, one-way ANOVA, figure 3c). Treatment with PT3 and Hcy escalated this fragmentation, by significantly increasing the fragmentation index ($p<.001$, one-way ANOVA), compared to Hcy alone. The interconnectivity was significantly greater than the untreated control, following treatment with Hcy ($p<.001$, one-way ANOVA, figure 3d). T3 treatment prevented the increased index of interconnectivity induced by Hcy, with a significant difference observable ($p<.001$, one-way ANOVA). There was a significant decrease in the elongation of the cells following treatment with Hcy ($p<.001$, one-way ANOVA, figure 3e). Taken together, this indicates that Hcy alters mitochondrial morphology, and that thyroid restores morphology to control levels in SH-SY5Y cells.

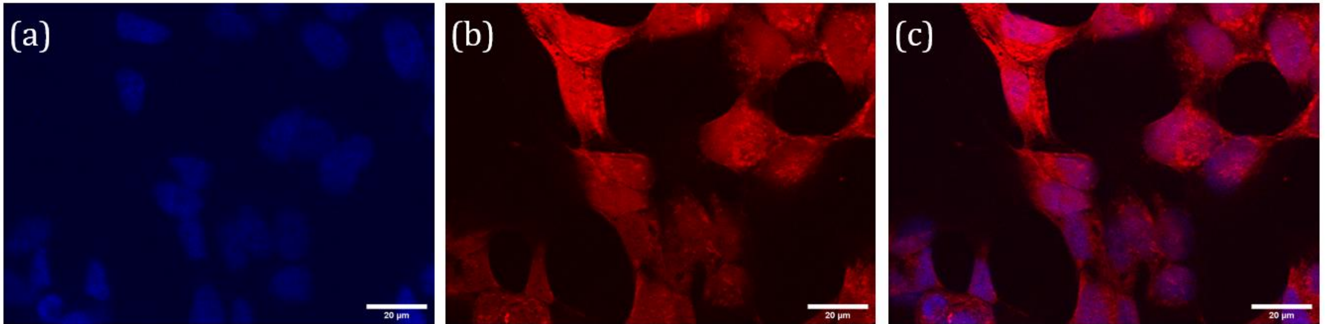

Figure 1 Representative images of untreated SH-SY5Y cells with DAPI staining (a), MitoRed staining (b), and an overlay of DAPI and MitoRed staining (c). The cells were imaged at x63 magnification, and the scale bar represents 20 μm.

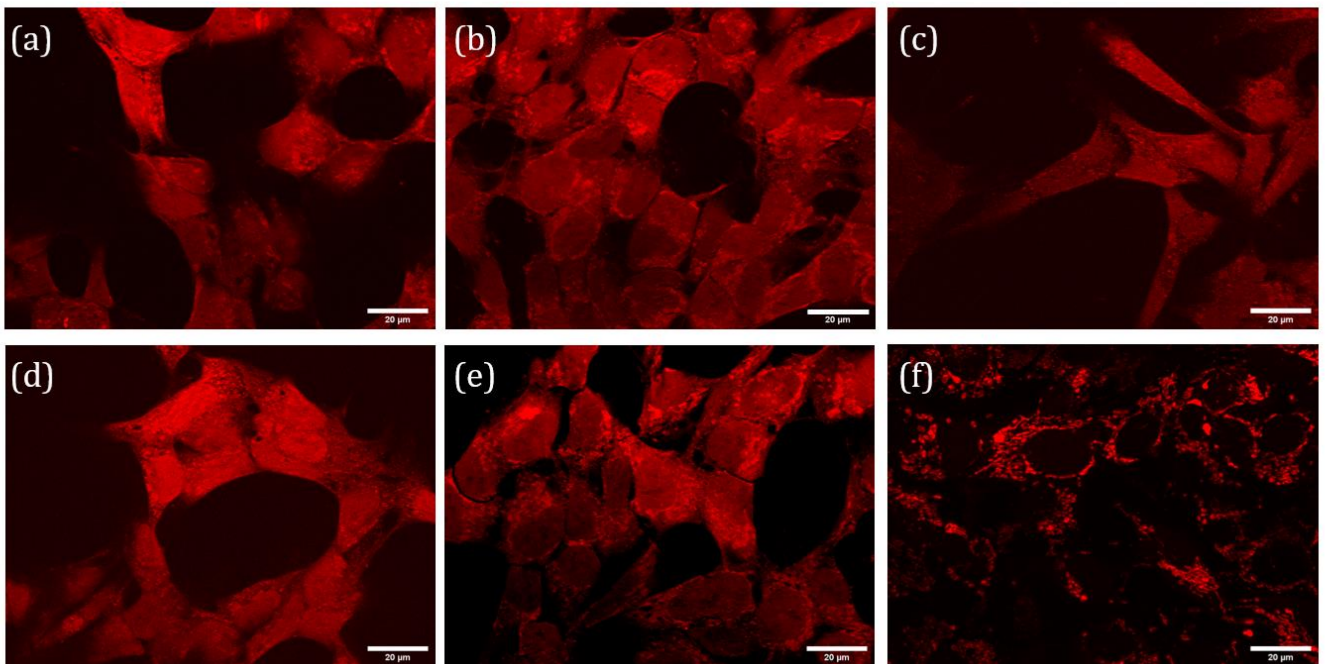

Figure 2 Representative images of SH-SY5Y cells with MitoRed staining after treatment, with different mitochondrial morphologies: untreated (a), T3 treated (b), T3+Hcy treated (c), PT3 treated (d), PT3+Hcy treated (e), Hcy treated (f). The cells were imaged at x63 magnification, and the scale bar represents 20 μm.

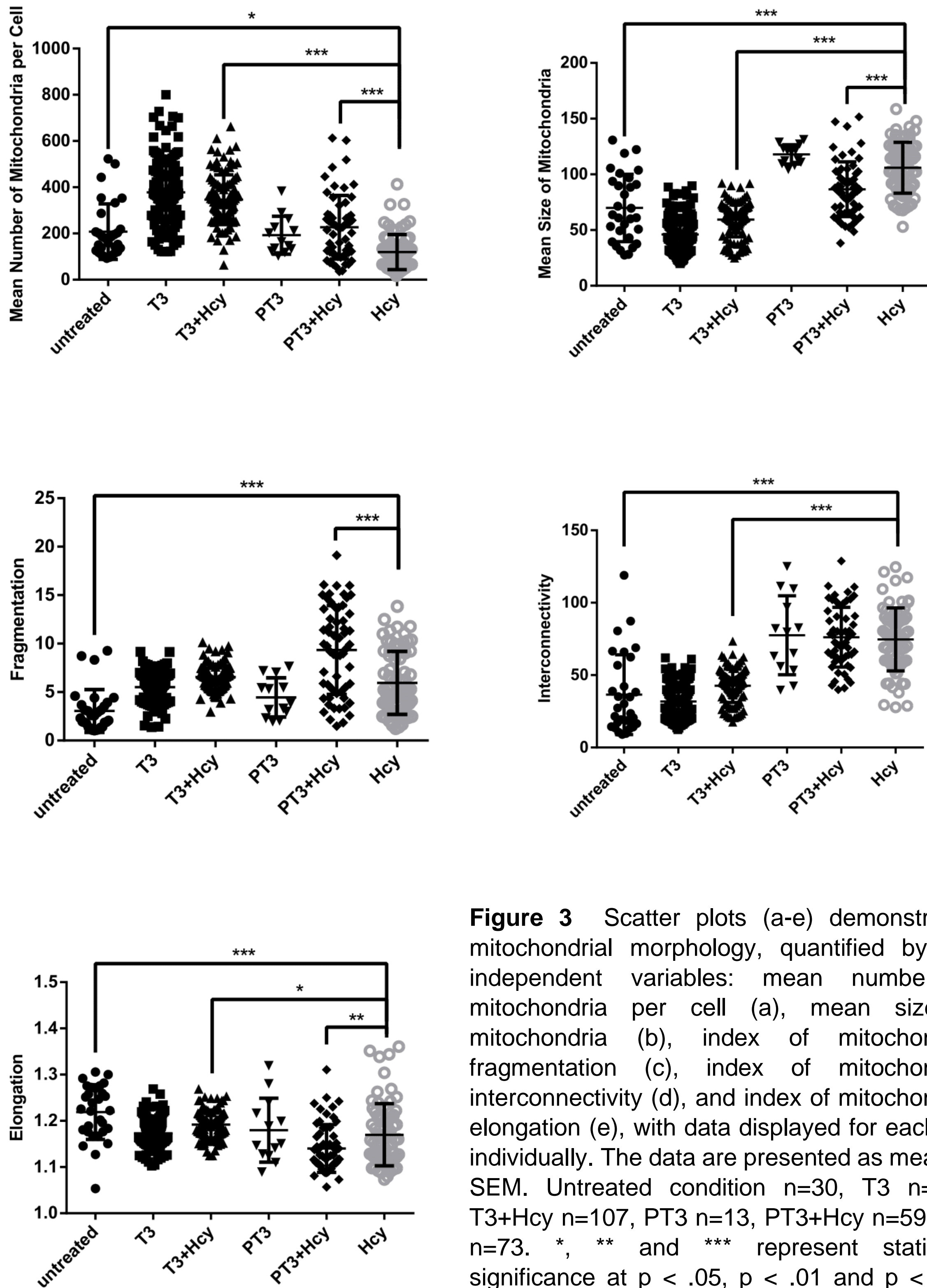

Figure 3 Scatter plots (a-e) demonstrating mitochondrial morphology, quantified by five independent variables: mean number of mitochondria per cell (a), mean size of mitochondria (b), index of mitochondrial fragmentation (c), index of mitochondrial interconnectivity (d), and index of mitochondrial elongation (e), with data displayed for each cell individually. The data are presented as means + SEM. Untreated condition n=30, T3 n=126, T3+Hcy n=107, PT3 n=13, PT3+Hcy n=59, Hcy n=73. *, ** and *** represent statistical significance at $p < .05$, $p < .01$ and $p < .001$ respectively.

3.2 | HT-22 Hippocampal Cells treated with Copper

In untreated HT-22 cells, the predominant mitochondrial morphology is normal and interconnected (figure 4a). Treating these cells with 10uM of copper chloride, resulted in a significant increase in the rounded mitochondria compared to the control (figure 4f). When treating the cells with copper alongside T3, the cells remained rounded (figure 4c), however when treating them with copper and PT3, they were somewhat normal yet slightly rounded (figure 4e).

From the macro analysis, there is a significant difference in the mean number of mitochondria per cell between untreated condition and copper condition. However, there is no significant difference present between the copper and thyroid conditions and the copper kill condition ($p<.01$, one-way ANOVA, figure 5a). Additionally, there are no significant differences in the mean size of the mitochondria between any of the conditions (figure 5b).

There is a significant decrease in mitochondrial fragmentation when comparing the untreated condition to the copper treated condition ($p<.001$, one-way ANOVA, figure 5c). Likewise, this difference is still significant between the copper and T3 condition when comparing it to the copper kill condition, however. The interconnectivity is significantly lower in the copper kill condition compared to the untreated HT-22 cells ($p<.001$, one-way ANOVA, figure 5d). Likewise, there the copper kill condition is also significantly less than the copper treated with T3. The elongation also disrupts the mitochondrial morphology, as there is significantly greater elongation in the copper condition compared to the untreated cells ($p<.001$, one-way ANOVA, figure 5e). Similarly, this is the case for the copper condition when comparing it to the HT-22 cells treated with copper with T3 and PT3.

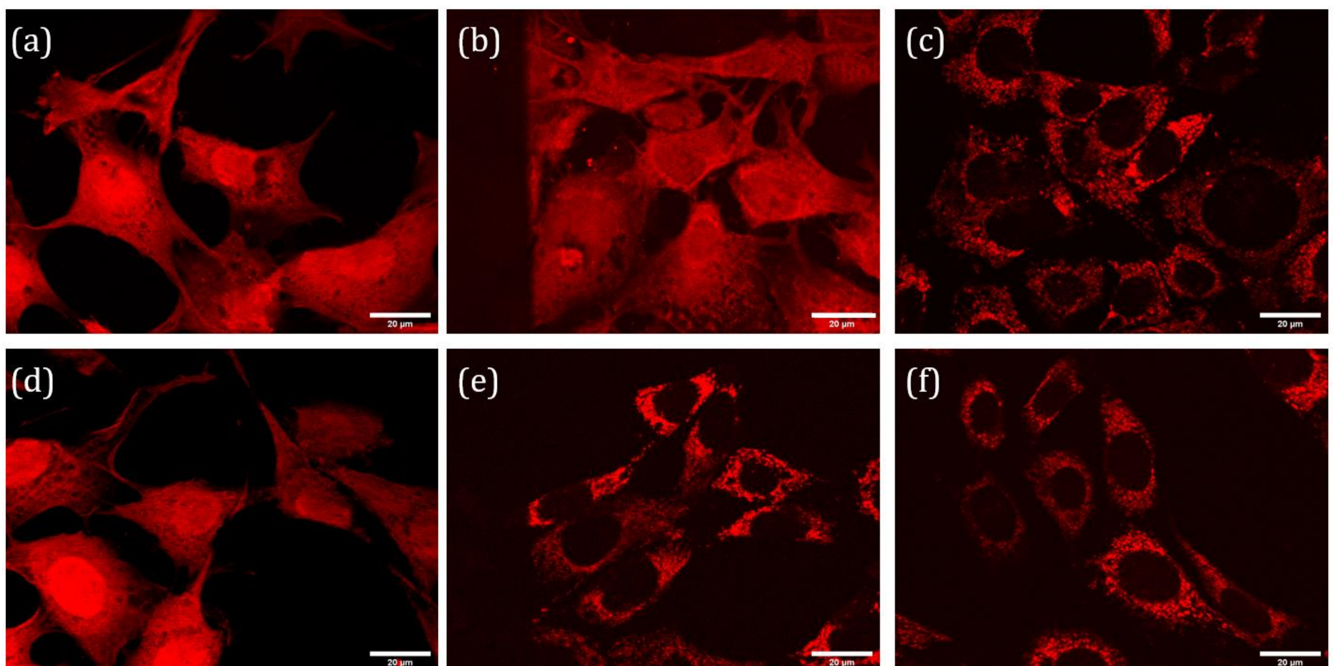

Figure 4 Representative images of mitochondrial morphology of MitoRed stained undifferentiated HT-22 hippocampal neuronal cells with/without the 10uM copper and T3 treatment are shown. Untreated cells (a), T3 treated (b), T3+$CuCl_2$ treated (c), PT3 treated (d), PT3+$CuCl_2$ treated (e), $CuCl_2$ treated (f). The cells were imaged at x63 magnification, and the scale bar represents 20 µm.

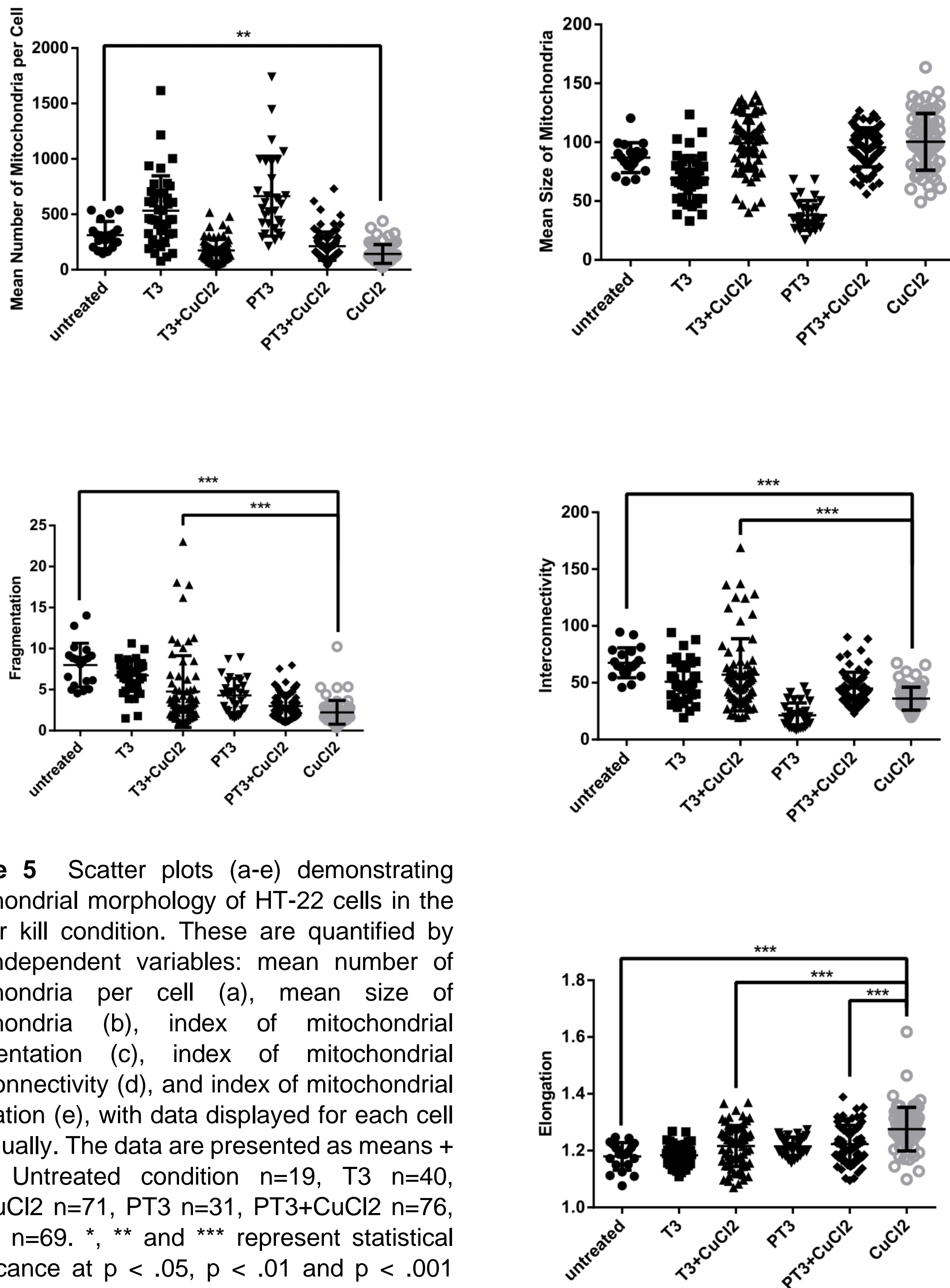

Figure 5 Scatter plots (a-e) demonstrating mitochondrial morphology of HT-22 cells in the copper kill condition. These are quantified by five independent variables: mean number of mitochondria per cell (a), mean size of mitochondria (b), index of mitochondrial fragmentation (c), index of mitochondrial interconnectivity (d), and index of mitochondrial elongation (e), with data displayed for each cell individually. The data are presented as means + SEM. Untreated condition n=19, T3 n=40, T3+CuCl2 n=71, PT3 n=31, PT3+CuCl2 n=76, CuCl2 n=69. *, ** and *** represent statistical significance at $p < .05$, $p < .01$ and $p < .001$ respectively.

3.3 | HT-22 Hippocampal Cells treated with Aβ

The untreated HT-22 cells retained a normal and tubular mitochondrial morphology (figure 6a). The kill condition, where cells were only treated with Aβ had large and

rounded mitochondria (figure 6f). The cells treated with T3 alongside the Aβ treatment were rounded, and the majority were interconnected (figure 6c). When the cells were pre-treated with T3 in conjunction with the Aβ, they retained their normal and tubular mitochondrial morphology, although they were slightly interconnected (figure 6e).

Untreated mitochondria are significantly larger than the mitochondria treated with Aβ ($p<.001$, one-way ANOVA, figure 7a). Also a significant difference between the HT-22 mitochondria treated with T3 alongside the Aβ, compared to being treated with Aβ alone ($p<.05$, one-way ANOVA**,** figure 7a). Furthermore, the cells treated with Aβ had significantly greater mean size of mitochondria, compared to the untreated control cells ($p<.001$, one-way ANOVA, figure 7b). Likewise, the cells treated with Aβ also had a significantly greater mean size of mitochondria compared to the HT-22 cells treated and pre-treated with T3 alongside the Aβ ($p<.001$, one-way ANOVA, figure 7b).

The fragmentation is significantly different between the untreated control cells and those treated with Aβ ($p<.05$, one-way ANOVA, figure 7c). This difference was also present between the cells treated with T3 alongside Aβ, and those treated with Aβ alone ($p<.01$, one-way ANOVA, figure 7c). Additionally, there are no significant differences in the interconnectivity of the mitochondria between any of the conditions (figure 7d). There were significant differences when comparing the cells only treated with Aβ, to the untreated cells, the cells treated with T3 alongside Aβ, and the cells pre-treated with T3 and Aβ ($p<.001$, one-way ANOVA, figure 7e).

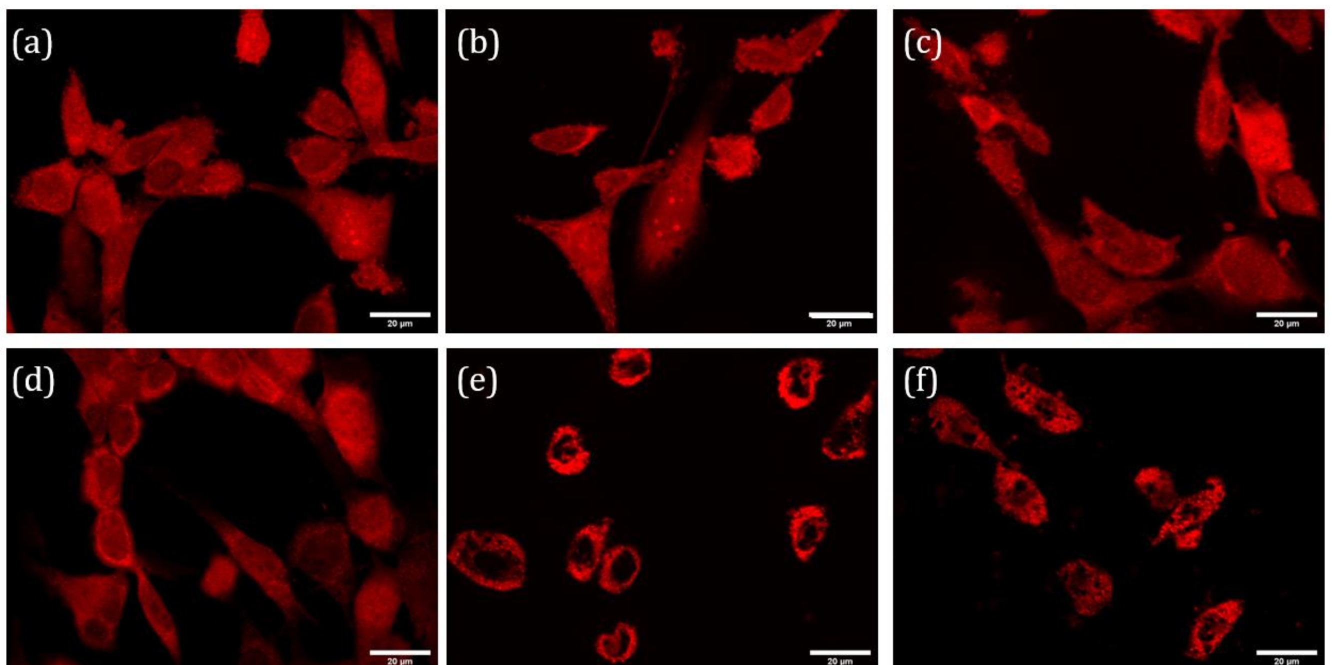

Figure 6 Representative images of the mitochondrial morphology of undifferentiated HT-22 hippocampal neuronal cells with MitoRed staining, with/without the 10uM Aβ and T3 treatment are presented and stained with DAPI and MitoRed: untreated (a), T3 treated (b), T3+Aβ treated (c), PT3 treated (d), PT3+Aβ treated (e), Aβ treated (f). The cells were imaged at x63 magnification, and the scale bar represents 20 μm.

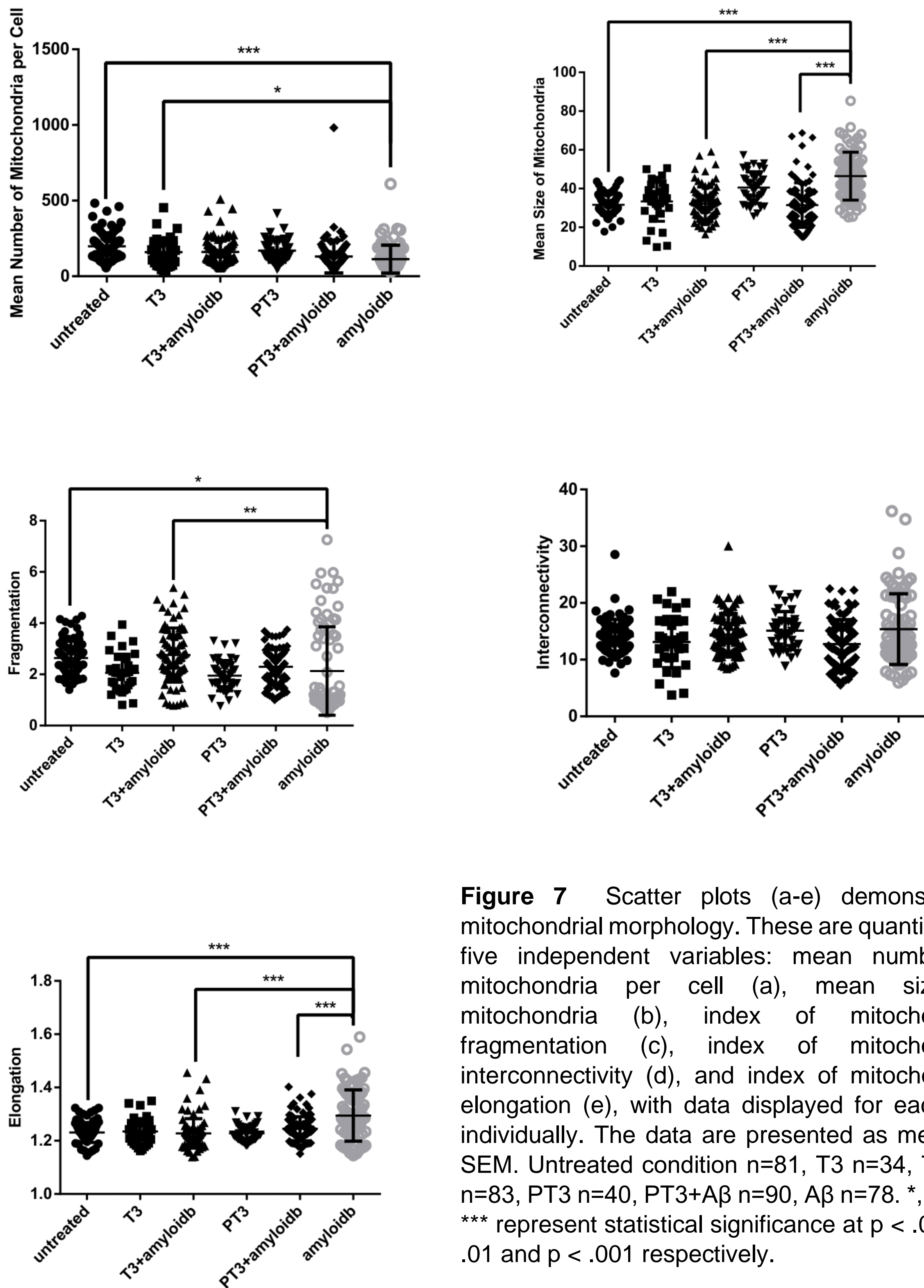

Figure 7 Scatter plots (a-e) demonstrating mitochondrial morphology. These are quantified by five independent variables: mean number of mitochondria per cell (a), mean size of mitochondria (b), index of mitochondrial fragmentation (c), index of mitochondrial interconnectivity (d), and index of mitochondrial elongation (e), with data displayed for each cell individually. The data are presented as means + SEM. Untreated condition n=81, T3 n=34, T3+Aβ n=83, PT3 n=40, PT3+Aβ n=90, Aβ n=78. *, ** and *** represent statistical significance at $p < .05$, $p < .01$ and $p < .001$ respectively.

4 | Discussion

Dementia and thyroid dysfunction are both prevalent conditions in the elderly population. In previous research, thyroid dysfunction has arisen as a potential risk factor for AD, with several studies linking it to hyperthyroidism (Tan, 2008). Mitochondria are deeply involved in producing relative oxygen species and are highly susceptible to oxidative stress generated by Hcy, copper, and Aβ (Lenaz, 1998). Evidence shows that T3 can combat this oxidative stress by stimulating the oxidative process and regulating antioxidant enzyme activity (Cioffi et al., 2022).

Given that the SH-SY5Y cells are highly active, they are sensitive to alterations in mitochondrial dynamics. The significant depletion in the mean number of mitochondria in cells treated with Hcy supports the theory that oxidative stress leads to mitochondrial death. T3 prevented this, sustaining a typical mitochondrial count; this agrees with other literature, which suggests that T3 stimulates mitochondrial replication (Jang et al., 2016). Hcy led to increased mitochondrial size, suggesting that the metabolite is causing damage by accumulating within the organelle and perturbing the critical fission and fusion dynamic (Ganapathy et al., 2011). T3 has a modulating effect on the mitochondrial size, however, cells pre-treated with T3 have the opposite effect of increasing the mitochondrial size to levels comparable to Hcy. This implies a toxic effect of having too much T3, hyperthyroidism, as it has similar implications to the stressor condition. However, the possibility of problems with imaging the pre-treated T3 and stressor condition cannot be discounted. Another explanation experimental repeats but only one set of replicates.

The Hcy exacerbates mitochondrial fragmentation, which could again link to the imbalance in fission and fusion dynamics, agreeing with broader literature (Kowluru & Mohammad, 2022). The data shows that T3 and pre-treated T3 could not control the fragmentation produced by Hcy. Notably, the cells pre-treated with T3 had more significant fragmentation than Hcy, suggesting that T3 promotes fragmentation, mimicking stressor characteristics. The data shows that Hcy significantly increased mitochondrial interconnectivity but significantly decreased elongation. Due to the potential disruption in fission and fusion dynamics, it could be implied that increased fusion favours the increased fragmentation and interconnectivity, and a lack of fusion could have led to the decreased mitochondrial elongation and inhibition of adequate energy production. On both occasions, the Hcy treated alongside the T3 had a rescuing effect, with the mitochondrial morphology remaining at the baseline level. Comparatively, the pre-treatment T3 condition presents a toxicity effect, having the same effect as the stress condition.

Research highlights that copper causes physical mitochondrial disruption, with the morphology being described as round due to the copper overload (Gonzalez-Ibanez et al., 2020; Hu et al., 2014). My subjective analysis corresponds with the rounded mitochondria appearing in all the copper conditions, suggesting that it provokes

failures that lead to mitochondrial destruction. The thyroid treatment and pre-treatment conditions increase the mean number, implying that the T3 could affect mitochondrial function by stimulating replication and energy production. The results show no overall effect of copper on the size of the mitochondria, which contradicts other literature that show copper produces smaller mitochondria (Jensen et al., 2019). Again, the T3 and pre-treatment conditions decreased the mitochondria size, but the pre-treated T3 led to a significantly larger reduction. This implies a somewhat destructive and toxic effect of cells being exposed to T3 for long periods.

The results highlight that copper increases the number and size of mitochondrial fragments, and decreases the interconnectivity of the mitochondrial network. Thus, this agrees with previous literature that the oxidative stress caused by copper overload may mediate radical-induced fragmentation (Yurkova et al., 2011). The mean fragmentation highlights that there is somewhat of a rescuing effect of T3 and no effect of the pre-treated T3, implying that it is inefficient at combatting the copper. Mitochondrial elongation increased with copper, supporting other research which states that copper produces oval and elongated mitochondria (Marín-García, 2010). The recovery effect of T3 also sustains the typical mitochondrial elongation, and there is somewhat of a rescuing effect of the pre-treatment T3. Therefore, this agrees with the idea that copper-induced mitochondrial stress has led to dysfunctions in mitochondrial morphology and apoptosis, which are standard features of neurodegeneration.

The data reveals that Aβ decreases the mean number of mitochondria per cell. This is coherent with wider literature examining Aβ triggering apoptotic cascades (LaFerla et al., 1995). The Aβ also significantly increased the size of the mitochondria, supporting the point that it progressively accumulates within the mitochondrial matrix (Chen & Yan, 2007) and implies that Aβ exposure may lead to organelle swelling. With this, there is a recovery effect of T3; contrastingly, there is no recovery in the number or size of mitochondria when the cells are pre-treated with T3 and Aβ. Interestingly, there is an additional negative effect in the pre-treated T3 and Aβ treated conditions on the mean size of the mitochondria, suggesting that thyroid overload is negatively affecting mitochondrial morphology by potentially accumulating within the mitochondria along with the Aβ. From this, it would be interesting to explore how different variations and concentrations of thyroid hormone affect the mitochondria and how this can directly influence cognitive performance.

Aβ causes a slight decrease in mitochondrial fragmentation, suggesting a lack of cellular stress and that increased fusion and decreased fission has occurred. This contradicts wider literature, which stated that extended exposure to Aβ in hippocampal neurons led to mitochondrial fragmentation and degradation (Zhang et al., 2018), showing differing alterations in the fission and fusion dynamics. Interestingly, there are no significant differences in interconnectivity between the control and the Aβ, T3 and pre-treated T3 conditions. This lack of changes signifies that there is still sufficient

transfer of mitochondrial DNA and balances in fusion dynamics. This may suggest that interconnectivity is immune to the oxidative stress produced by Aβ. Mitochondrial elongation is increased with Aβ, contradicting other literature that found no significant difference in mitochondrial length (Zhang et al., 2018). However, with elongation, thyroid hormone did have a rescuing effect, suggesting that it supports the regulation of mitochondrial growth.

To conclude, these findings provide compelling evidence that metabolites damage mitochondria, resulting in alterations to their morphology. These metabolites are found in AD, therefore similar events could be occurring in situ. There is a pattern of T3 influencing mitochondrial activity by modulating and sustaining typical mitochondrial morphology highlighting the protective role of thyroid hormone in regulating mitochondrial dynamics. However, the pre-treated T3 destroys the morphology, mimicking the stressor conditions. If longer exposures to T3 have detrimental effects on the mitochondria, it may not be safe to use long-term. Therefore, it would be beneficial to look at this in greater detail to ascertain this, potentially looking at different concentrations and exposure times of T3. Hyperthyroidism-like problems appear to be present in the three metabolite conditions. It is known that hyperthyroidism inflicts structural and functional damage to mitochondria, eventually leading to energy depletion and cardiac dysfunction, which is significantly associated with increased risk of AD (Maity et al., 2013; Qiu et al., 2006). Although further research is needed to fully understand the molecular links and mechanisms between the metabolites, T3, and mitochondrial dysfunction.

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