Simulated microgravity accelerates alpha-synuclein aggregation and induces oxidative stress in an *in vitro* Parkinson's disease model

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Abstract

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the accumulation of alpha-synuclein aggregates and progressive neuronal loss in the substantia nigra, with aging being its primary risk factor. The current available models to study PD mechanisms are largely relying on genetic mutations to recapitulate PD typical hallmarks, such as increased alpha-synuclein aggregation. However, they do not model the aging features associated with the disease.

Microgravity, a condition experience by astronauts during space missions, is known to induce ageing-like modifications on both systemic and cellular physiology.

To replicate the aging-related stress observed in PD patients, we exposed SH-SY5Y and 3K-SNCA mutant cell lines to simulated microgravity.

Our findings revealed that simulated microgravity enhanced PD alterations, with a significant increase in misfolded and phosphorylated a-syn. This was accompanied by heightened oxidative stress, as evidenced by increased levels of reactive oxygen species, without a sufficient antioxidant response. These results suggest that simulated microgravity effectively mimics and accelerate the stress associated with aging in PD cell models, regardless of the presence of PD mutation. This study

highlights the potential of simulated microgravity as a tool for investigating aging processes in neurodegenerative diseases.

Introduction

Parkinson disease (PD) is the second most common neurodegenerative disorder and is characterized by both motor and non-motor clinical features [1–3]. PD is defined pathologically by the presence of alpha-synuclein (a-syn) aggregates in Lewy Bodies and neurites with associated loss of pigmented dopaminergic neurons in the substantia nigra parts compacta (SNc). A-syn is a member of a group of intrinsically disordered amyloid proteins that assemble into oligomeric and fibrillar aggregates [4–8].

The exact aetiology of PD is still unknown. The most important risk factor for developing PD is advancing age. Variants in genes like *SNCA, GBA1, PINK1, PRKN, and LRRK2*, contribute to the disease pathology, of which the most important risk factor are the mutations in the *GBA1* gene, found in 10-15% of PD patients [9–15]. *GBA1* is located on chromosome 1 and encodes the lysosomal hydrolase glucocerebrosidase (GCase, or acid-β-glucosidase) that catalyses the cleavage of glucosylceramide (GlcCer) and glucosylsphingosine (GlcSph) into glucose and ceramide, or glucose and sphingosine, respectively [16].

A-syn toxic accumulation has been associated with impairment in several cellular functions, involving mitochondria, proteasomes and lysosome, as well as endoplasmic reticulum (ER) stress, axonal transport deficiencies and, ultimately, impaired dopamine synaptic release [17].

Additionally, a-syn oligomers trigger apoptotic pathways via reactive oxygen species (ROS) production [18,19].

The lysosome plays a critical role in the clearance of a-syn; it is physiologically degraded by both the ubiquitin-proteasome system and the autophagy lysosomal pathway (ALP), using mainly chaperone-mediated autophagy (CMA) and macroautophagy [20–24]. Moreover, lysosomal proteins which are crucial for both macroautophagy and CMA and have been found to be defective in the post-mortem brains of PD patients [25]. Patients with *GBA1* mutations exhibit decreased lysosomal GCase enzyme activity in multiple brain regions [26]. Impairment of GCase activity leads to lysosomal dysfunction, obstructing the trafficking and processing of a-syn [27,28]. Conversely, lysosomal dysfunction can be induced by a-syn itself [29], mainly by interrupting the transport of lysosomal hydrolases from the ER to the Golgi apparatus and the lysosome in neurons [30–32]. Consequently, overexpression of a-syn reduces both GCase activity and protein levels, resulting in a reciprocal biochemical connection between a-synuclein and GCase [33–36].

A-syn accumulation can induce ER stress by disrupting protein trafficking and directly interacting with ER membranes. In addition, ER stress exacerbates a-syn toxicity, forming a feedback loop that contributes to neurodegeneration in synucleinopathies [37–39].

In addition to this, a-syn aberrant accumulation has been linked to increased oxidative stress, a cellular status characterized by an accumulation of ROS. The latter is defined by an imbalance in ROS production and the ability of the cell to increase its antioxidant response, causing cellular toxicity at multiple levels, ranging from altered mitochondrial function to membrane damage.

Microgravity, the condition that is naturally experienced by astronauts in space, has been shown to induce oxidative stress by increasing ROS productions and lipid peroxidation. Space agencies such as the National Aeronautics and Space Administration (NASA) and the European Space Agency (ESA) conduct experiments aboard space stations like the International Space Station (ISS) to explore phenomena not observable under terrestrial gravity (1g). Microgravity profoundly affects biological

systems, from single cells to entire organisms and cellular adjustment in these conditions can help the exploration of diverse pathophysiological processes [40–48].

Simulated microgravity (s-μg) environment also impairs cellular stress responses, including the unfolded protein response and protein turnover, which may, in turn, lead to the accumulation of misfolded protein.

These findings imply a potential induction of senescence across various cellular models, including cancer cell lines, stem cells, and differentiated cells [49–52]. While further investigation is necessary to fully understand the implications of microgravity on senescence, several researchers propose that exposure to microgravity triggers cellular aging, possibly through heightened oxidative stress resulting from mitochondrial dysfunction [44,53–58]. This process may initiate apoptotic pathways and alter cellular proliferation, differentiation, and growth [51,59].

In the present study, a systematic characterization of PD features under s-μg conditions using a random positioning machine (RPM) was performed, focusing on a-syn alterations, including the formation of aggregates, lysosomal physiology, and oxidative stress. We employed two neuronal cell line models, the SH-SY5Y and a-syn mutant 3K-SNCA line. Whilst the first cell line is widely used as a model for neurodegenerative disorders [60,61], the mutant line is designed to accelerate a-syn aggregation. Specifically, the familial PD-linked mutation E46K, which changes the a-syn repeat motif KTKEGV to KTKKGV in repeat 4, was amplified by inserting analogous mutations into the immediately adjacent KTKEGV repeat motifs (E35K+E46K+E61K), stabilizing membrane-associated helical a-syn, hence promoting the protein oligomerization and eventual aggregation [62,63].

Our study shows that s-μg conditions result in a-syn alterations and oxidative stress in mutated and wild type cells, indicating that s-μg exposure leads to increased a-syn accumulation and aggregation, independently of the presence of a-syn mutations.

100

101 **Methods**

102 ***Cell culture***

103 Human neuroblastoma cells SH-SY5Y (ATCC number: CRL-2266) and 3K-SNCA cells were cultured in
104 DMEM/F-12, GlutaMAX™ medium (10565018; Gibco) supplemented with 10% fetal bovine serum
105 (FBS) and 1x Penicillin/Streptomycin (Pen/Strep) at 37° C and 5% CO_2 in T-75 flasks.

106

107 ***Microgravity***

108 The Random Positioning Machine (RPM) was used as a microgravity simulator, while, although it
109 cannot be directly compared to the real condition of microgravity, it still serves as a valuable tool for
110 conducting preliminary tests [64]. It is composed by two frames that rotate independently. The
111 samples, which are placed close to the centre in the inner frame, are subjected to a mean gravity
112 force close to zero g, due to the continuous re-orientations and random movements of the axes.

113 The flasks containing the cells were carefully filled up with complete medium to avoid the shear
114 forces and to allow the cells to maintain a state of free-fall motion. For the experiment, the Random
115 Walk mode was used with a velocity of 60 deg/sec. Cells were left in the machine for different time
116 points (24 hours and 48 hours) and compared with the 1g control group placed in the static bar of
117 the RPM.

118

119 ***Bradford Assay***

120 Bradford assay was used as a method to quantify the concentration of proteins in samples using
121 bovine serum albumin as a standard according to manufacturer instruction (B6916; Sigma Aldrich).

122

123 ***Western Blot Analysis***

124 Cells pellets were lysed, and proteins were separated as previously described [65]. The primary
125 antibody were added at the following concentrations: rabbit anti a-syn 1:1000 (ab138501; abcam);
126 mouse anti-GBA1 1:500 (AP1140; Calbio Biochem); mouse anti β-Actin 1:10000 (ab6276; abcam);
127 mouse anti LAMP1 1:500 (611042; BD Trasduction), mouse anti p-Synuclein (Ser129) 1:1000
128 (ab51253; abcam), rabbit anti Catalase 1:1000 (ab209211; abcam), and mouse anti ATF6 1:500
129 (ab227830; abcam). The secondary antibody was added at a 1:2000 final concentration: Goat anti-
130 mouse HRP (P0447; Dako) and goat anti-rabbit HRP (P0448; Dako). The membrane was imaged prior
131 incubation with ECL reagent using the ChemiDoc Imaging System.

132

133 ***Immunostaining and Confocal***

134 Immunostaining was performed as previously described [60,66]. The primary antibodies were added
135 at the following concentrations: mouse anti a-syn antibody 1:750 (SIG-39730; BioLegend) and rabbit
136 anti-LAMP1 1:2000 (ab278043; abcam). The secondary antibodies were added at the following
137 concentrations: 1:2500 Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa
138 Fluor™ Plus 647 (A32733; Invitrogen) and 1:2500 Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed
139 Secondary Antibody, Alexa Fluor™ Plus 488 (A32723; Invitrogen). 1ng/ml of Hoechst solution (62249;
140 Thermo Scientific) was used to visualize the nuclei. The slides were imaged using the Nikon C2
141 Confocal Microscope.

142

143 ***ROS quantification***

144 ROS levels were quantified using Invitrogen™ carboxy-H2DCFDA (C400; Invitrogen) as a fluorescent
145 indicator with a final concentration of 20µM. The experiment was performed on live cells which

146 were prepared by adding 0.5µl of protease inhibitor and anti-phosphatase 1 and 2 to the cell pellets.
147 1µl of pellet was diluted in 1ml of PBS. H_2O_2 was used to prepare a standard curve for different ROS
148 concentrations, using PBS as a control for 0µM ROS. 100 µl of diluted samples and standards were
149 added to a black 96-well plate with 92µl PBS and 8µl carboxy-H2DCFDA probe. After a 1hour
150 incubation in the dark at 37°C with 5% CO_2, using a filter-based reader (fluorescence), samples were
151 analysed with an excitation of 488nm and emission of 530nm. Final values were normalised for the
152 total amount of protein, quantified using the Bradford assay.
153
154 ***MDA quantification***
155 The Cayman TBARS assay kit (10009055; Cayman) was performed to measure Malondialdehyde
156 (MDA) levels. In this kit, MDA and Thiobarbituric Acid (TBA) react under high temperature (90-100°C)
157 and acidic conditions, and their product is measured colorimetrically at 530-540nm. MDA standard
158 was diluted to create a stock solution of 125µM, and a standard curve was prepared with varying
159 MDA concentrations. Samples and standards were added to Eppendorf® tubes, along with the SDS
160 solution and the Color reagent (composed of Thiobarbituric Acid, Acetic Acid, and NaOH) according
161 to the manufacturer instructions. The mixture was heated for 1 hour at 100°C, cooled on ice for 10
162 minutes, centrifuged for 10 minutes at 1,600 x g at 4°C, and then loaded in a clear plate for
163 absorbance reading at 530-540 nm. The standard curve was plotted, and unknown values were
164 interpolated after subtracting the blank. Final values were normalised for the total amount of
165 proteins, quantified using the Bradford assay.
166
167 ***Total Antioxidant Capacity (TAC) quantification***
168 Cayman's Antioxidant Assay (709001, Cayman) is a versatile method for assessing the total
169 antioxidant capacity (TAC) of various biological samples. This assay evaluates the combined

170 antioxidant activities of a range of constituents, such as vitamins, proteins, lipids, glutathione, and
171 uric acid.

172 All the reagents and samples were prepared and diluted according to manufacturer instructions.

173 Trolox standard wells received 10µl of Trolox standard (from a concentration of 0µM to 0.33 µM as
174 suggested in the assay protocol), 10µl of metmyoglobin, and 150µl of chromogen per well. Similarly,
175 sample wells were prepared with 10µl of sample, 10µl of metmyoglobin, and 150µl of chromogen.
176 The reactions were initiated by adding 40µl of hydrogen peroxide working solution to all the wells.
177 It is crucial to add the hydrogen peroxide quickly (within one minute is recommended when using a
178 multichannel) to ensure uniform reaction initiation across wells. The plate was then covered and
179 incubated on a shaker for five minutes at room temperature to allow for the completion of the
180 reactions. After incubation, the plate cover was removed, and the absorbance was measured at 405
181 nm using a plate reader. The standard curve was plotted, and unknown values were interpolated
182 after subtracting the blank, values were then multiplied for the dilution factor. Final values were
183 normalised for the total amount of proteins, quantified using the Bradford assay.

184

185 ***DATA analysis***

186 Statistical comparisons and analysis were performed using GraphPad Prism 9.1 software. The
187 Shapiro-Wilk test was first used to determine whether the collected data was normally distributed.
188 In non-normally distributed data, Kruskal-Wallis test and Dunn's post-hoc analysis were used to
189 conduct multiple comparisons. The differences between three or more groups of normally
190 distributed samples were compared using the ANOVA test. Each experiment was conducted three
191 times. Each condition was carried out in triplicate in every experiment conducted. Results are

192 displayed as mean ± standard error of the mean. Results were considered statistically significant if
193 their p-value was less than 0.05.

194

195 **Results**

196 ***A-syn pathogenic species are modulated by s-μg exposure***

197 To study whether s-μg exposure influences a-syn protein behaviour, we quantified various species
198 of the protein. Initially, we questioned whether the total level of a-syn is affected by prolonged
199 exposure to s-μg. Thus, two different time points (24 and 48 hours) were assessed to monitor the
200 changes in a-syn levels. We then studied whether a change in total levels of a-syn would translate in
201 the different subspecies of the protein. We quantified aggregated forms of a-syn which are known
202 to impair lysosomal function, induce ER stress, and promote an aberrant UPR [26,67,68].

203 The initial investigation on total a-syn levels, using a western blot approach, did not reveal any
204 significant difference between cells exposed s-μg and those cultured in 1g, independently of the
205 SNCA mutation (Fig. 1 a-d). These findings suggest that the overall quantity of a-syn in cell cultures
206 in s-μg is not affected. However, confocal microscopy analysis of 3K-SNCA cells exposed to s-μg
207 revealed a significant increase in a-syn aggregates after just 24 hours of exposure (P=0.0007), with a
208 further marked increase following 48 hours of incubation in the s-μg gravity environment
209 (P=0.000006) (Fig. 2 a, b). To ensure that the observed effects were not due to a combined influence
210 of the 3K mutation and s-μg, we exposed the SH-SY5Y control cell line to s-μg. This experiment
211 revealed a significant increase in a-syn aggregates at both 24 hours (P=0.0076) and 48 hours of
212 exposure (P=0.0364) (Fig. 2 c, d). The detection of aberrant a-syn accumulation in the SH-SY5Y
213 control line confirms that the observed increase was indeed driven by s-μg and not an exacerbation
214 of an existing defect, such as the 3K-SNCA mutation. Following the identification of increased

215 presence of a-syn aggregates, we questioned whether these findings were driven by a constitutive
216 increase in the a-syn monomeric fraction, acting as a potential additional protein source and thereby
217 promoting the formation of aggregated forms. To investigate the levels of a-syn monomers, we
218 quantified the remaining a-syn signal that was not identified as an "aggregate" throughout the
219 confocal microscopy screening. This assessment revealed a significant depletion of a-syn monomers
220 in both 3K-SNCA cells (P=0.0145; P=0.0025) and SH-SY5Y cells (P=0.039; P=0.015) following s-μg
221 exposure at both time points analyzed (Fig. 2 e, f).

222 To understand whether the observed increase in aggregated a-syn following s-μg exposure was
223 accompanied an initial shift in pro-aggregation forms of the protein, we quantified the levels
224 phosphorylated form on Serine-129 of a-syn (pS219). This phosphorylated form is a well-established
225 marker of the aggregated state, as approximately 90% of a-synuclein in Lewy Bodies from PD brains
226 contains pS129. Moreover, pS129 has been shown to accelerate the rate of a-syn fibrillation in vitro,
227 suggesting that this specific phosphorylation event is closely linked to pathological aggregation.[69–
228 71].

229 Initially, we conducted a comparative analysis between 1g SH-SY5Y cells and 1g 3K-SNCA cells to
230 ascertain variations in pS219 presence. Our findings revealed a notably higher abundance of pS219
231 in 3K-SNCA cells compared to the wild-type model, substantiating and aligning with previous reports
232 on the literature (data not shown) [72]. Levels of pS129 were significantly elevated in both cell types
233 following exposure to s-μg, with this increase becoming more pronounced over the two time points
234 analyzed (Fig. 3 a-d). These findings suggest that under s-μg conditions a-syn shifts from a non-
235 aggregated status to phosphorylated and aggregated forms.

236

237 ***GCase is not increased in s-μg.***

238 Given that s-μg may impact cellular processes, the abnormal behavior of a-syn observed under these
239 conditions might be due to disrupted lysosomal function, leading to impaired clearance and
240 increased aggregation of the protein. Thus, we hypothesized that this could be mechanistically
241 related to lysosomal dysfunction. To investigate this, we quantified the levels of GCase upon
242 exposition of the cells to s-μg conditions using the RPM. GCase was selected as it is an important
243 lysosomal enzyme in PD and a-syn turnover. Our analysis revealed that GCase levels were not
244 significantly altered at 24 or 48 hours (Fig. 4 a-d). We also measured Lysosomal-Associated
245 Membrane Protein 1 (LAMP1) levels as a reflection of lysosomal mass. Quantification of LAMP1
246 punctae using confocal microscopy revealed a significant increase in LAMP1-positive organelles over
247 time under s-μg conditions. Specifically, SH-SY5Y cells exhibited a more than 2-fold increase at both
248 24 hours (P=0.039) and 48 hours (P=0.025) of s-μg exposure. The PD model line 3K-SNCA presented
249 more variable levels of LAMP1, with a significant increase only present in exposure to s-μg at 48
250 hours (*P*=0.003). Although requiring further investigation, these results suggest that LAMP1 increase
251 may not be followed by a simultaneous increase in lysosomal hydrolases like GBA1, potentially
252 resulting in an impaired lysosomal maturation in s-μg.

253

254 ***ROS levels are increased in s-μg conditions***

255 When subjected to the s-μg induced by the random motion of the machine, both the 3K-SNCA
256 overexpressing cells and the control SH-SY5Y cells exhibited an elevated quantity of both monomeric
257 and aggregated states. An increase in oxidative stress is seen in the brain in several
258 neurodegenerative diseases including PD and has been noted in microgravity cell models
259 previously[56,57]. To investigate oxidative stress in our a-syn models, we quantified ROS and lipid
260 peroxidation levels in SH-SY5Y and 3K-SNCA cells exposed to s-μg conditions at 24 hours and 48
261 hours.

262 SH-SY5Y cells showed a significant 3-fold (*P*=0.019) and 2-fold increase (*P*=0.02) in ROS at 24 hours

263 and 48 hours respectively. Similarly, 3K-SNCA cells showed a significant increase of ROS cellular

264 content at both time points analysed (*P*=0.015; *P*=0.031) (Fig. 6 a, b).

265 We also measured MDA levels as an indicator of lipid peroxidation. MDA is generated by the
266 peroxidation of membrane polyunsaturated fatty acids [73] as well as produced in the process of
267 prostaglandin synthesis [74]. As s-μg conditions are a source of oxidative stress, s-μg also increases
268 MDA levels [75]. We exposed 3K-SNCA and SH-SY5Y cells to s-μg conditions for 24 and 48 hours,
269 using standard gravity exposed-cells as a control. There was no significant change in MDA levels in
270 the SH-SY5Y cells at either time point. In the 3K-SNCA, however, there was a significant 3-fold
271 increase (P=0.025) at 24 but not 48 hours (Fig. 6 c, d). These data are consistent with previous studies
272 showing that microgravity results in an increased presence of oxidative stress markers within the
273 cells. The failure of MDA levels to rise in the SH-SY5Y cells and the reversal of increased levels
274 between 24 and 48 hours in the 3K-SNCA cells suggests that compensatory mechanisms are
275 modulated in response to the increased ROS. For this, we investigate the levels of Catalase,
276 responsible of H_2O_2 hydrolysis, along with the TAC. However, we did not find any change in both
277 Catalase (Fig. S1 a-d) and TAC (Fig. S2 a-b) in either cell at any time point suggesting a potential
278 failure of antioxidant response despite the increased presence of ROS following s-μg exposure.

279

280 ***Discussion***

281 Microgravity research is increasingly recognized for its potential across various scientific disciplines.
282 While further investigation is needed to fully understand cellular adaptation to space-like conditions,
283 exploring how cells adjust to microgravity offers substantial promise for revealing diverse
284 pathophysiological processes [44,54,78,79]. In this study, s-μg was employed to simulate accelerated
285 aging, providing insights into the underlying pathophysiological mechanisms of PD. We report that
286 s-μg increases a-syn aggregate levels in both SH-SY5Y and 3K-SNCA cell models. Notably, exposure
287 to s-μg shifts the a-syn ratio towards aggregated form and increased pS129 modification, both
288 favoring the potential for Lewy body-like pathology [56,57].

289 All these observations could be attributed to s-μg induced cell stress, conceivably leading eventually
290 to a diminished clearance of a-syn, particularly pronounced in 3K-SNCA overexpressing cells. For this
291 reason, it was essential to investigate whether the increase in a-syn relates to compromised protein
292 clearance mechanisms, specifically involving autophagy–lysosome system dysfunction.

293 We hypothesised that lysosomal biogenesis was upregulated in s-μg as compensation for
294 accumulated a-syn aggregates. To evaluate this, we quantified both GCase lysosomal hydrolase as
295 well as LAMP1 in both cell types. Our analyses revealed that GCase levels remains unchanged, whilst
296 LAMP1 increases over time in both cell types. Several studies have demonstrated that GCase
297 homeostasis is affected by a-syn accumulation [32] by causing ER fragmentation and compromising
298 ER folding capacity and facilitating the aggregation of lysosomal hydrolases in the ER. The failure for
299 GCase levels to rise in our study may reflect this effect of a-syn, even against a compensatory attempt
300 to increase the endo-lysosomal content [30,31,46]. Accumulation of both a-syn and immature
301 lysosome hydrolases in the ER leads to activation of ER stress, thus evoking UPR [74,76] and resulting
302 in loss of ER homeostasis. This potentially culminates in ER dysfunction (changes in ER calcium
303 concentration or dysregulation in the redox potential of the ER) [77] or indirectly lysosomal catabolic
304 malfunction or mitochondrial abnormalities, which in turn could results in harmful oxidative damage
305 to the cells, and further a-syn aggregation [78,79].

306 In addition to this, ROS levels were increased in both cell types under s-μg conditions. Both s-μg and
307 a-syn aggregates accumulation are known to increase oxidative stress [19,80–82]. Additionally, a-
308 syn has been reported to suppress catalase activity [83]. These results suggests that cells exposed to
309 s-μg fail to activate protective mechanisms against rising levels of ROS, which may contribute to an
310 endo-lysosomal impairment, promoting GCase lysosomal hydrolase aberrant homeostasis,
311 potentially further facilitating a-synuclein aggregation.

Our findings describe a senescence model of PD cell pathology induced by microgravity, with misfolded a-syn accumulation and oxidative stress. While the characteristics of the cell models used in our study offer valuable insights for specific experimental contexts, they do not fully replicate the complex cellular dynamics observed in PD. In contrast, differentiated cells, which more closely resemble the specialized neuronal populations, would provide a more accurate representation of PD pathological processes. These cells would better capture the scenario of neuronal dysfunction, including the impact of a-syn aggregation and oxidative stress on cellular health and will be used in future studies.

Conclusions

We report the first studies on the effect of s-μg on a-syn and show that it induces and enhances a-syn accumulation in neuronal-type cells, accompanied by an increase in oxidative stress. These findings provide valuable insights into cellular responses to s-μg, providing a basis for the usage of s-μg and space-based experiments to model complex neurodegenerative disorders and ageing features.

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Authors contributions

VL and GU contributed equally to this research. VL and GU conceived the study, designed and executed the analysis plan with supervision from GC, AP, and AHVS. GU, VL, AM, MAEF, and ND performed tissue culture work, immunostaining and data collection. VL and GU performed image analysis and conducted statistical analysis. AP and AHVS supported data interpretation. VL, GU, SLDP and AHVS wrote, designed, drafted and edited the manuscript with feedback and input from all authors. All authors read and approved the final manuscript.

Conflict of intertest

Nothing to report.

Data availability

The datasets in this study are not publicly available at the moment. They will be publicly available once the manuscript is accepted for publication.

Figures

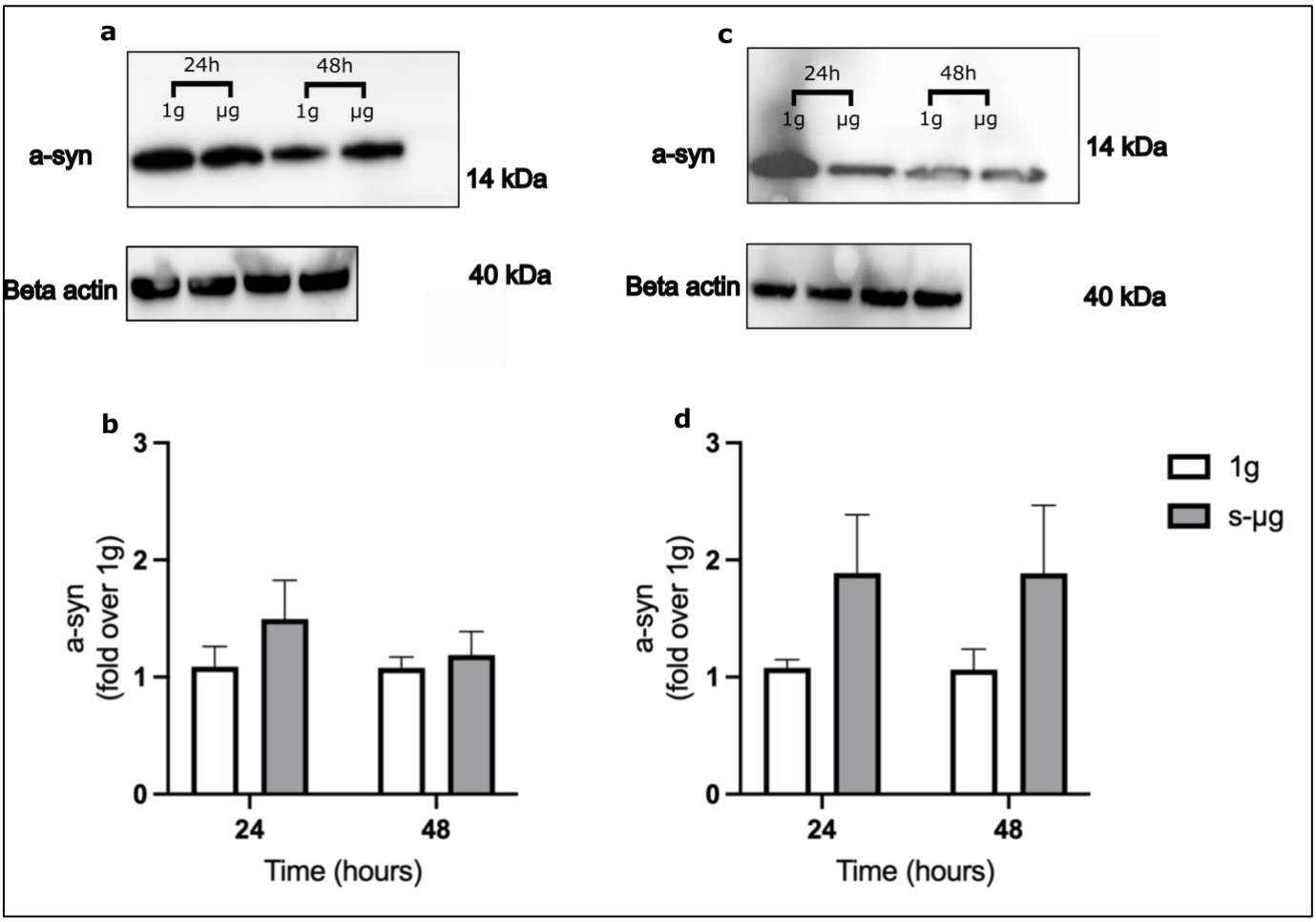

Figure 1. Total a-syn quantification in SH-SY5Y and 3K-SNCA cells exposed to s-mg conditions at different time points. a) Representative WB membrane image of a-syn and control protein beta-actin in SH-SY5Y cells. b) Quantification of a-syn levels in 1g and s-mg exposed SH-SY5Y cells. c) Representative WB membrane image of a-syn and control protein beta-actin in 3K-SNCA cells. d) Quantification of a-syn levels in 1g and s-mg exposed SH-SY5Y cells. Multiple unpaired t-tests were used to compare differences. Data are presented as mean±SEM of 3 independent experiments.

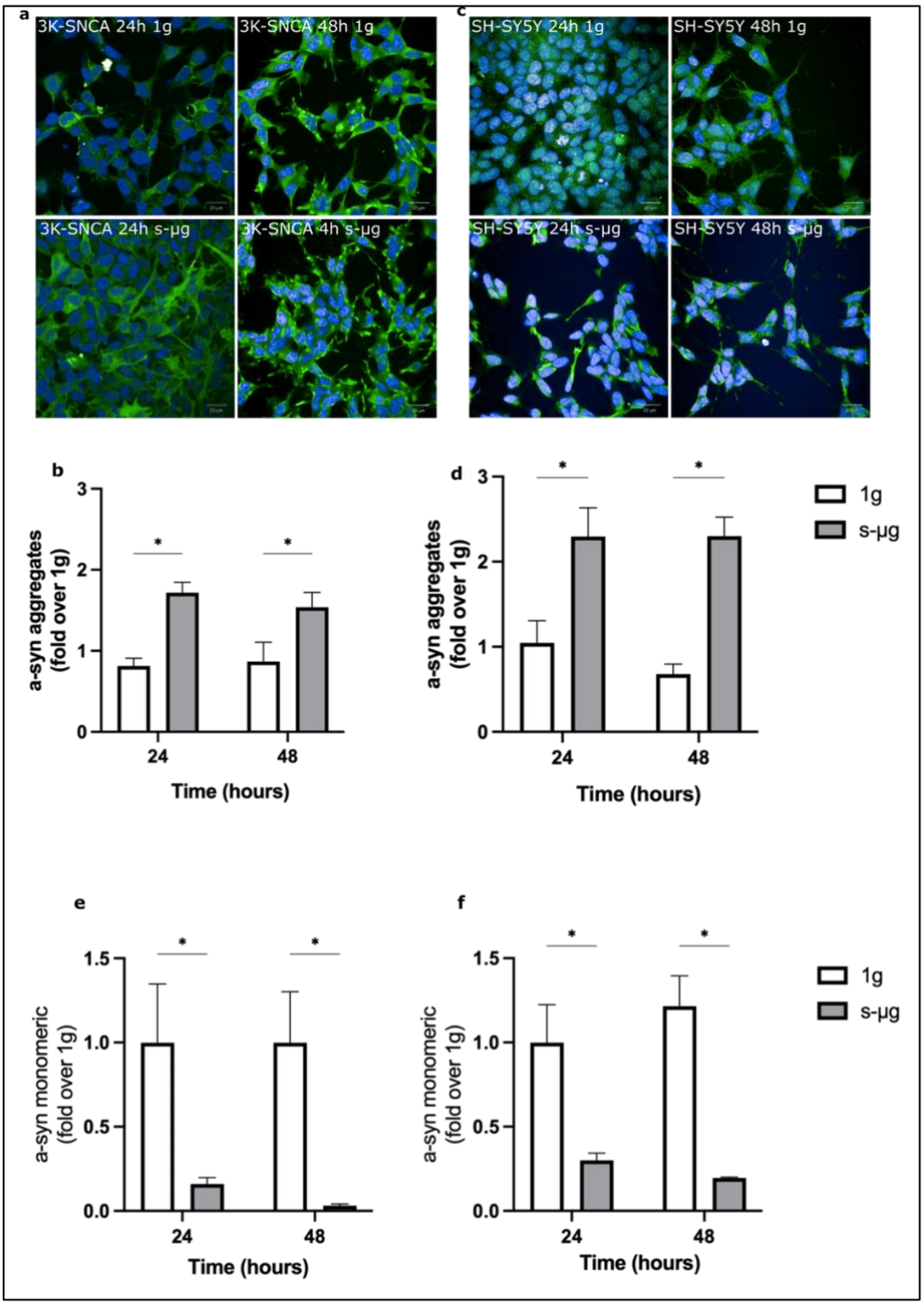

Figure 2. Quantification of a-syn aggregates via confocal microscopy in SH-SY5Y cells and 3K-SNCA cells. a-syn (green), Nuclei (blue). **a)** Representative confocal membrane images of a-syn aggregates in 3K-SNCA cells cultured in 1g and s-µg. **b)** Quantification of a-syn aggregates in 1g and s-µg exposed 3K-SNCA cells cultured in 1g and s-µg. **c)** Representative confocal images of a-syn aggregates in SH-SY5Y cells. **d)** Quantification of a-syn aggregates levels in 1g and s-µg exposed SH-SY5Y cells. **e)** Quantification of non-aggregated a-syn in 1g and s-µg exposed 3K-SNCA cells cultured in 1g and s-µg. **f)** Quantification of non-aggregated a-syn in 1g and s-µg exposed SH-SY5Y cells cultured in 1g and s-µg. Multiple unpaired t-tests were used to compare differences. Data are presented as mean±SEM of 3 independent experiments. *P<0.05

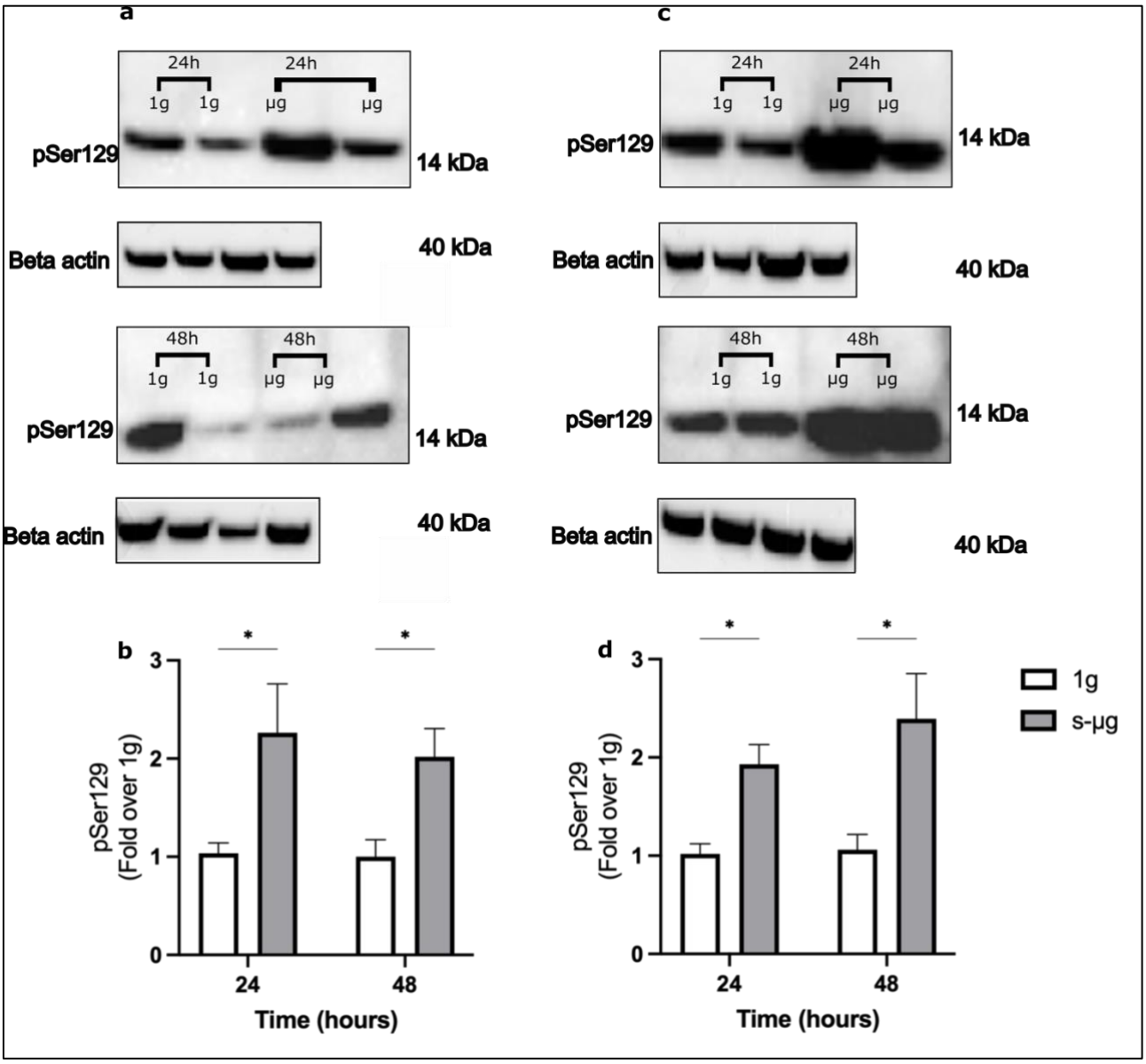

Figure 3. phosphorylated Serine 129 a-syn (pSer129) quantification in SH-SY5Y and 3K-SNCA cells exposed to s-µg at different time points. **a)** Representative WB membrane image of pSer129 and control protein beta-actin in SH-SY5Y cells. **b)** Quantification of pSer129 levels in 1g and s-µg exposed SH-SY5Y cells. **c)** Representative WB membrane image of pSer129 and control protein beta-actin in 3K-SNCA cells. **d)** Quantification of pSer129 levels in 1g and s-µg exposed SH-SY5Y cells. Multiple unpaired t-tests were used to compare differences. Data are presented as mean±SEM of 3 independent experiments. *$P<0.05$

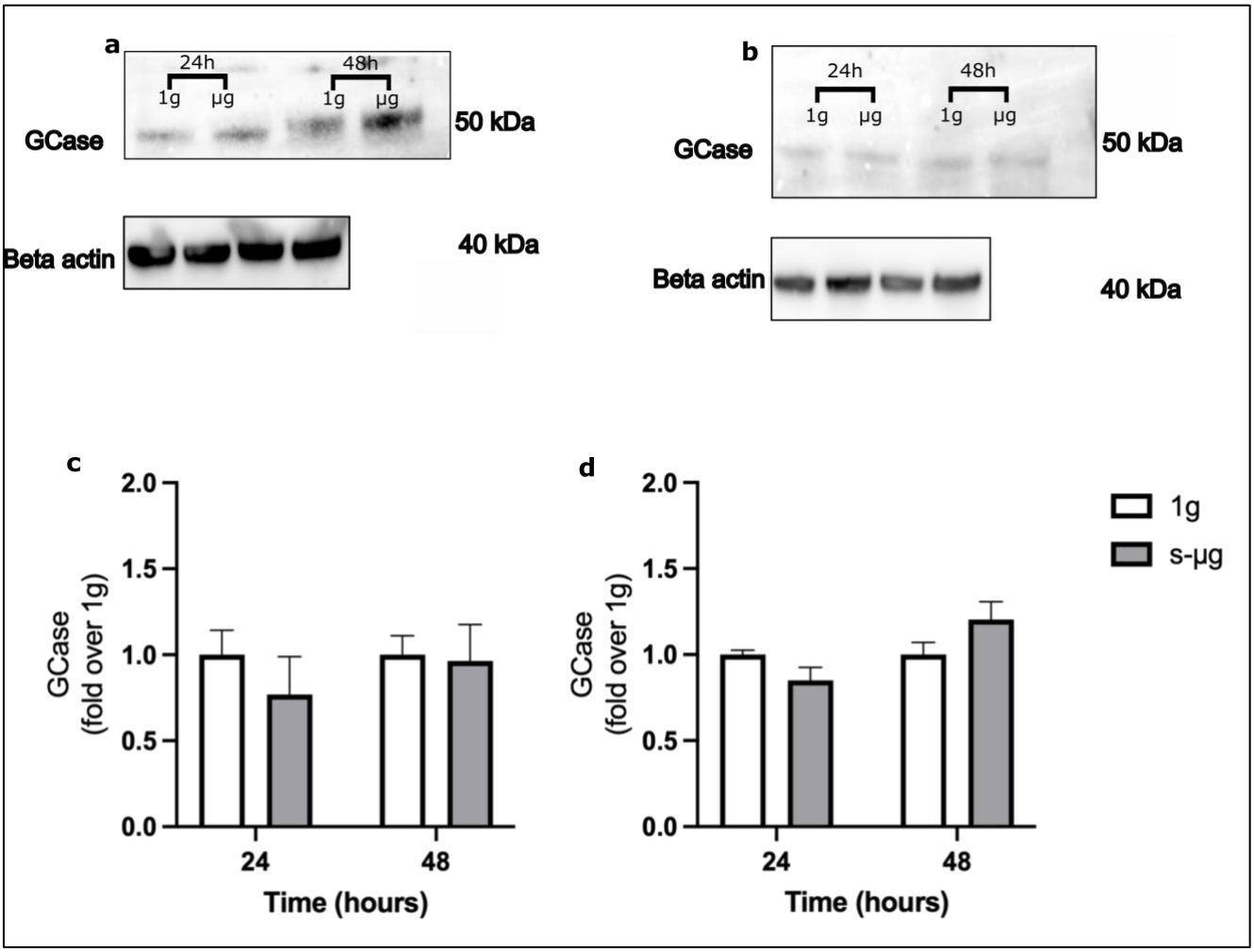

Figure 4. GCase WB quantification in SH-SY5Y and 3K-SNCA cells exposed to s-µg conditions at different time points. **a)** Representative WB membrane image of GCase and control protein beta-actin in SH-SY5Y cells. **b)** Quantification of GCase levels in 1g and s-µg exposed SH-SY5Y cells. **c)** Representative WB membrane image of GCase and control protein beta-actin in 3K-SNCA cells. **d)** Quantification of GCase levels in 1g and s-µg exposed SH-SY5Y cells. Multiple unpaired t-tests were used to compare differences. Data are presented as mean±SEM of 3 independent experiments.

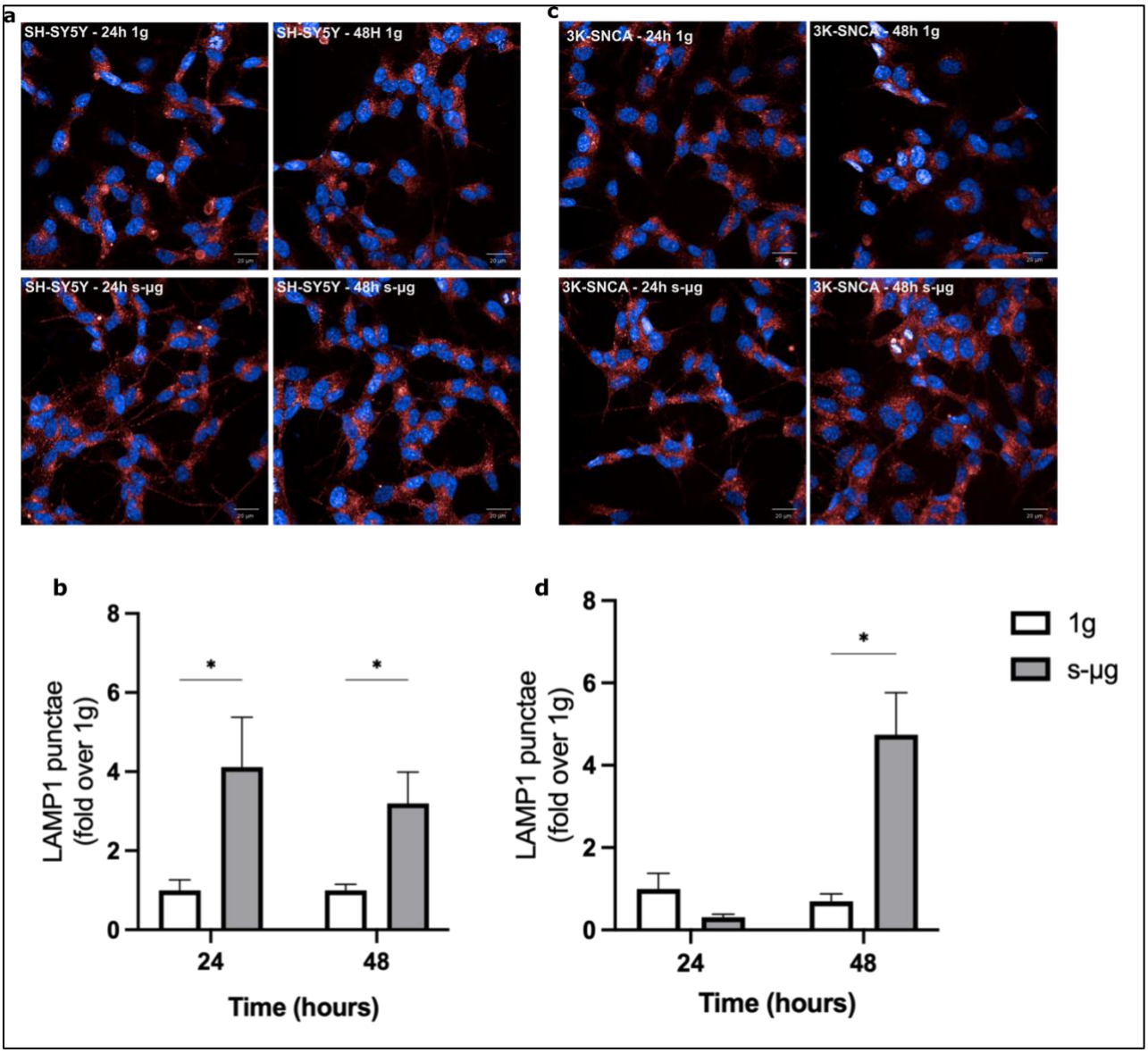

Figure 5. Quantification of LAMP1 punctae via confocal microscopy in SH-SY5Y cells and 3K-SNCA cells. LAMP1 (red), Nuclei (blue). **a)** Representative confocal membrane images of LAMP1 punctae in SH-SY5Y cells cultured in 1g and s-µg. **b)** Quantification of LAMP1 punctae in 1g and s-µg exposed SH-SY5Y cells. **c)** Representative confocal images of LAMP1 punctae in 3K-SNCA cells. **d)** Quantification of LAMP1 punctae levels in 1g and s-µg exposed 3K-SNCA cells. Multiple unpaired t-tests were used to compare differences. Data are presented as mean±SEM of 3 independent experiments. *$P<0.05$

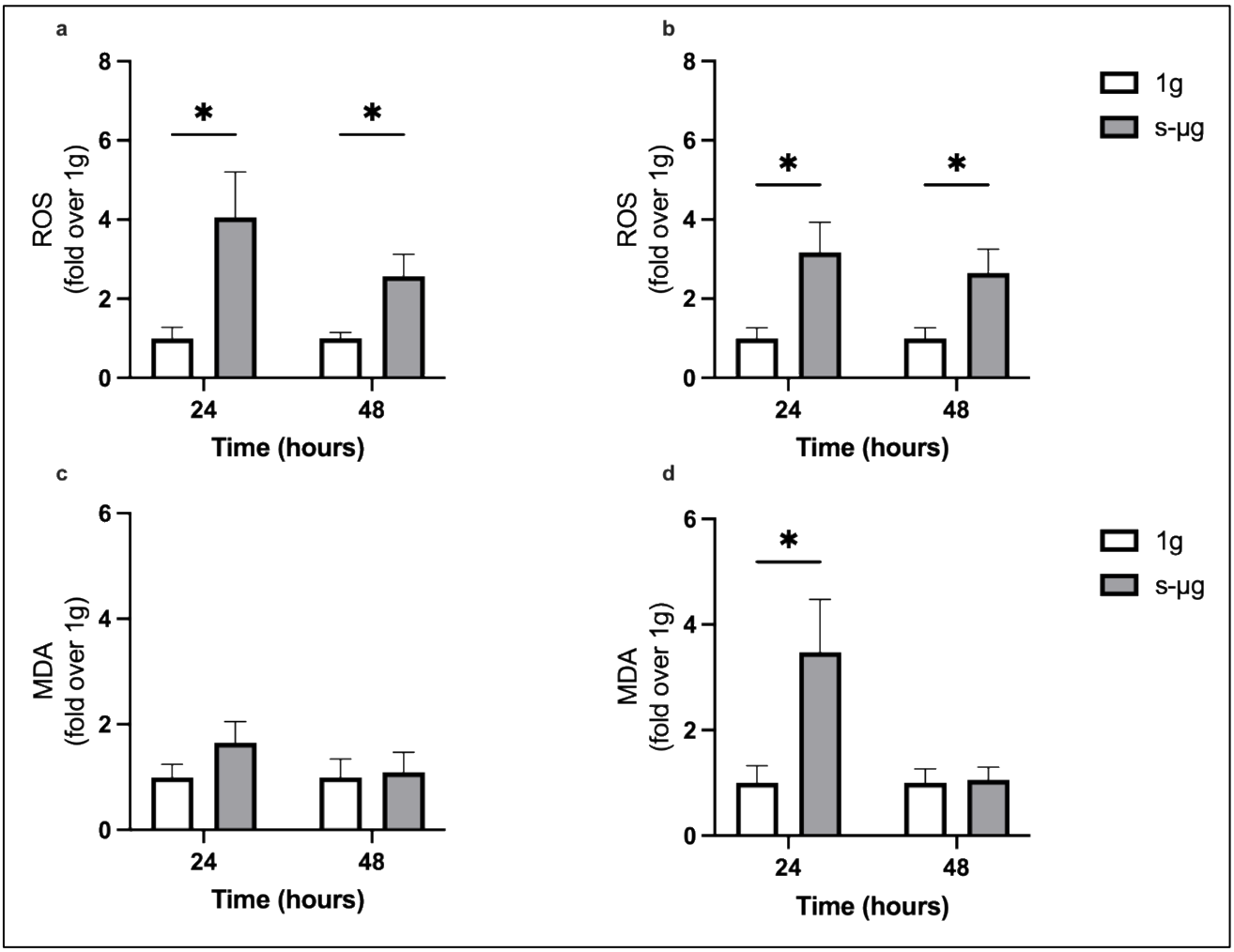

Figure 6. Investigation on oxidative stress marker in SH-SY5Y cells and 3K-SNCA cells exposed to s-µg. **a)** Quantification of ROS levels in SH-SY5Y cells cultured in 1g and s-µg. **b)** Quantification of ROS levels in 3K-SNCA cells cultured in 1g and s-µg. **c)** Quantification of MDA level in 1g and s-µg exposed SH-SY5Y cells. **d)** Quantification of MDA level in 1g and s-µg exposed 3K-SNCA cells. Multiple unpaired t-tests were used to compare differences. Data are presented as mean±SEM of 3 independent experiments. *$P<0.05$

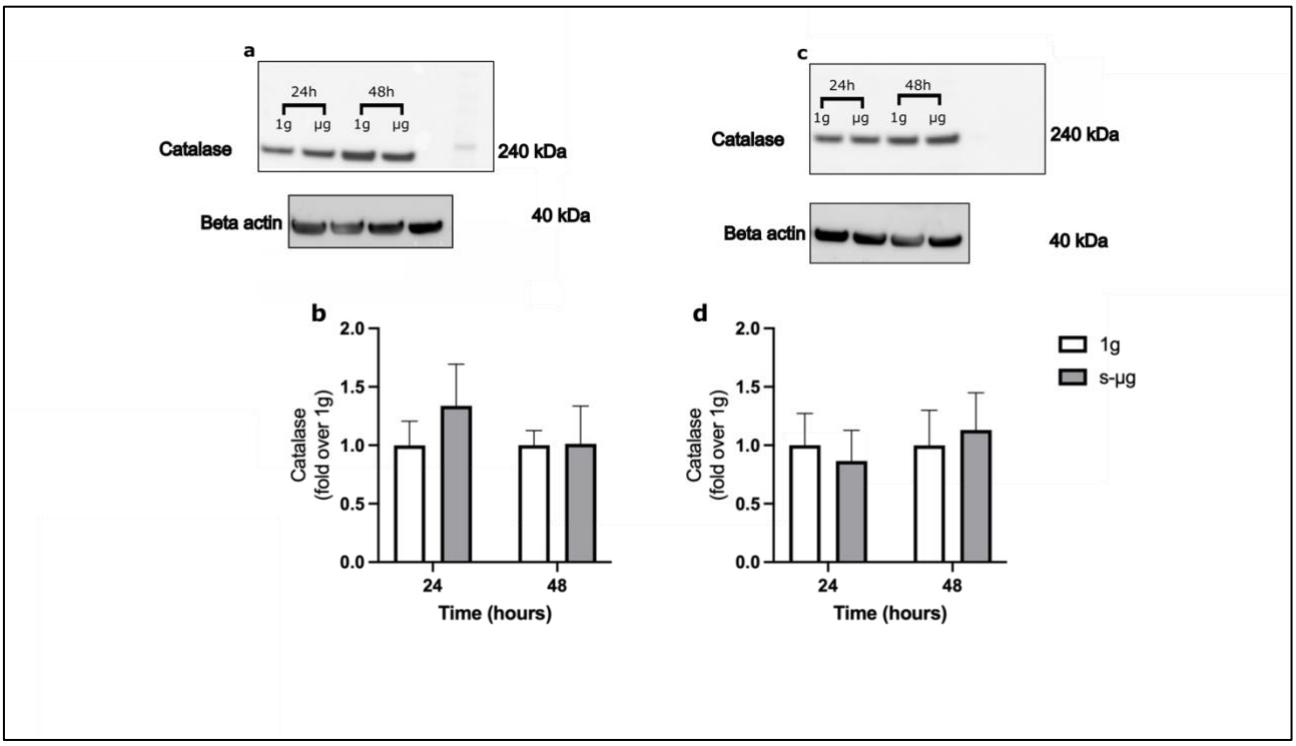

Figure S1. Catalase WB quantification in SH-SY5Y and 3K-SNCA cells exposed to s-µg at different time points. a) Representative WB membrane image of Catalase and control protein beta-actin in SH-SY5Y cells. b) Quantification of Catalase levels in 1g and s-µg exposed SH-SY5Y cells. c) Representative WB membrane image of Catalase and control protein beta-actin in 3K-SNCA cells. d) Quantification of Catalase levels in 1g and s-µg exposed SH-SY5Y cells. Multiple unpaired t-tests were used to compare differences. Data are presented as mean±SEM of 3 independent experiments.

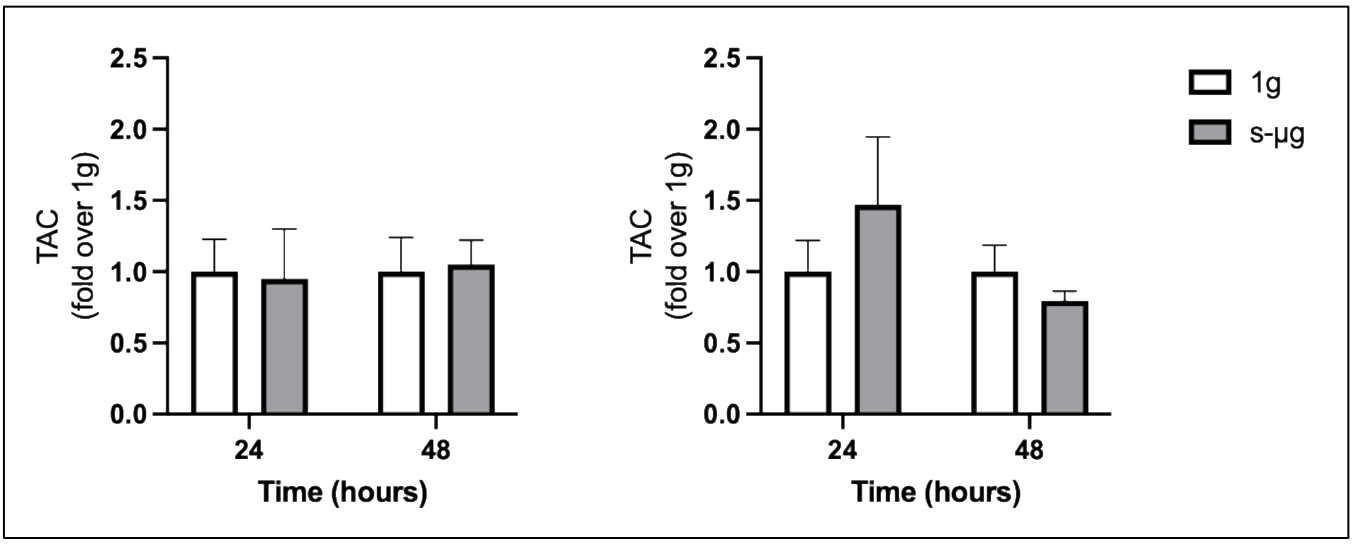

Figure S2. Investigation on total antioxidant capacity (TAC) in SH-SY5Y cells and 3K-SNCA cells exposed to s-µg. a) Quantification of TAC level in SH-SY5Y cells cultured in 1g and s-µg. b) Quantification of TAC level in 3K-SNCA cells cultured in 1g and s-µg. c) Quantification of TAC level in 1g and s-µg exposed SH-SY5Y cells. d) Quantification of TAC level in 1g and s-µg exposed 3K-SNCA cells. Multiple unpaired t-tests were used to compare differences. Data are presented as mean±SEM of 3 independent experiments.

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