Stress granule formation dynamics and its link to α-synuclein aggregation in A53T *SNCA* mutant SH-SY5Y cell line

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ABSTRACT

RNA granules are transient, non-membrane bound, cytoplasmatic RNA-protein condensates that play an essential role in post-transcriptional regulation of RNAs. Stress granules (SGs) a subset of RNA granules, are induced in response to environmental stress providing a cellular mechanism for coping with acute conditions. These granules have unique dynamics which are based on liquid-liquid phase separation, with the endoribonuclease RasGAP SH3-binding protein (G3BP) being a key effector of the process. Such dynamics, when impaired may lead to pathological accumulation of protein aggregates, making SGs a relevant and intriguing target in neurodegenerative disease investigation. Accordingly, in this study we sought to characterize SG formation dynamics and its association with phosphorylated α-synuclein (α-syn) aggregates in response to D-sorbitol induced hyperosmotic stress. We did this in a mutant SH-SY5Y cell line overexpressing A53T-α-syn tagged with green fluorescent protein (GFP), which provides an *in-vitro* model for the study of Parkinson's disease (PD). First, we calibrated the experimental set up and optimized the protocol, and then we used Immunohisto-fluorescence staining and confocal laser scanning microscopy for imaging. We were surprised to find no apparent α-syn aggregates in general, nor clear SG formation in the stress-induced cells as expected. In line with these results, we elaborated and recommended new possible directions and tools that could improve our ability to obtain more informative findings, and thus advance our understanding of the complex pathological mechanism underlying PD.

INTRODUCTION

RNA granules are self-assembled predominantly spherical, non-membrane bound condensates of RNAs packed with RNA-binding proteins (RBP), that provide functional compartmentalization within cells [1]. They also contain various ribosomal subunits, translation factors, decay enzymes, helicases, and scaffold proteins. Thus, they control the localization, stability, and translation of RNA cargo, and are considered post-transcriptional regulators [2]. RNA granules are formed via multivalent macro-molecular interactions that transition the physiochemical properties of proteins, to induce membranelles structures exhibiting liquid characteristics, in the cellular cytoplasm [3]. This liquid-liquid phase separation (LLPS) allows the granules to dynamically condense, or dissolve based on environmental conditions [1].

There are several classes of RNA granules. Such include germ granules or neuronal RNA transport granules which are highly specialized and form in a specific subset of cells, whilst others such as processing bodies (PB) and stress granules (SG) are much more ubiquitous [1]. SGs are induced rapidly (15-30 min) in the cellular cytoplasm when translation initiation is limited, usually as a result of environmental stress (such as heat-shock, oxidative conditions, UV irradiation, hypoxia, endoplasmic reticulum (ER) stress or viral infections [1-2, 4]) in attempt to prioritize translation of proteins that are necessary for the cell's survival. This is done by selective recruitment of "house-keeping" mRNA transcripts to the SGs where they are transitionally-repressed, allowing enhanced translation of excluded molecular chaperones and enzymes involved in damage repair, at the cytoplasm [2]. After the cells recover from the stress, the SGs dissolve and their content can once again be translated [1].

One key component, and regulator of SG formation is the endoribonuclease RasGAP SH3-binding protein (G3BP), which activates upon phosphorylation at several different sites [5]. It was proposed that G3BP undergoes an RNA-induced conformational rearrangement which regulates its available valence for phase separation, promoting condensates assembly. Namely, under non-stress conditions G3BP dimerizes and adopts an autoinhibited compact state, defined by intramolecular interactions. Upon stress, unfolded RNA molecules released from polysomes outcompete these interactions, thus inducing the expansion of G3BP and its transition to a multivalent state where intermolecular interactions dominate. This results in the assembly of G3BP clusters that crosslink RNA molecules to form inhomogeneous condensates of low protein density [6]. Hence, high expression levels of G3BP induce SG formation, and this is done in a dose-dependent manner [7].

Mutations in proteins that increase the formation or decrease the clearance of SGs, may cause aberrant LLPS and lead to pathological, non-reversible accumulation of aggregates that share components with SGs [8]. These are leading hallmarks in several neurodegenerative disease including α-synuclein (α-syn) aggregation into Lewy bodies in Parkinson's disease (PD) [3]. It has been proposed that the α-syn toxic aggregation is enhanced by the phosphorylated form of α-syn at Ser^{129} residue, a post-translational modification prevalent among PD patients [9]. Along with other disease mechanisms,

these aggregates lead to accelerated neuronal death of primarily dopaminergic neurons, resulting in both motor impairments (such as bradykinesia and tremor) and a range of non-motor features (like depression, constipation, and disturbed sleep) [10]. One convenient and broadly used *in-vitro* cell-based model for mimicking pathological α-syn aggregation in PD is the SH-SY5Y human catecholaminergic, proliferative neuroblastoma cell line carrying the A53T point mutation in the *SNCA* gene encoding α-syn. This missense mutation is found in familial forms of PD [11].

Affected RNA granule dynamics in PD are further demonstrated in two recent studies. The first proposed that the N-terminus of the α-syn protein, but not of other synucleins, dictates mutually exclusive binding to PB components, with high affinity towards a key region of the Edc4 decapping module protein. Thus, pathologically increasing levels of α-syn disturb the interactions of Edc4 with other core decapping proteins, directly modulating PB complexes and mRNA stability in the cytosol [12]. The second study found that the RBP DJ-1 has specific interaction with a subset of mRNAs that localize to SGs upon hyperosmotic shock in SH-SY5Y cells. This suggests that mutations in *PARK7* gene encoding DJ-1, which are associated with the familial forms of PD, may pathogenically modulate RNA machinery contributing to neurodegeneration [13].

In light of the currently available knowledge and due to the fact that RNA granules have only gained traction as potential mediators of neurodegeneration in recent years, in this study we aimed to further observe and elucidate SG formation dynamics in response to D-sorbitol induced hyperosmotic stress in the *SNCA* mutant SH-SY5Y cell line, overexpressing A53T-α-syn, GFP tagged. Initially we calibrated the experimental setup in terms of cell seeding protocol, stress treatment intensity and duration, required recovery period and fixation procedure. Once all the parameters were defined, we preformed Immunohisto-fluorescence (IHF) staining in order to visualize SGs' formation via the pattern of G3BP expression and colocalization with phosphorylated α-syn aggregates. We hypothesized that the expression levels of G3BP in the stress-induced cells would be higher than in the control group.

MATERIALS AND METHODS

Cell Culture. SH-SY5Y cells overexpressing the mutant A53T-α-syn and GFP were thawed from -200˚C liquid nitrogen freezer. The cells were cultured in 10ml growth medium (GM; 175ml Roswell Park Memorial Institute (RPMI) medium, 175ml Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12), 8ml Sodium Bicarbonate 7.5%, 40ml Fetal Bovine Serum (FBS), 4ml GlutaMAX, 4ml Penicillin/Streptomycin) and incubated in 37˚C. GM was changed 24 hours after thawing, and later on based on cell consumption. Cells were passaged every 4-5 days depending on confluency levels.

Hyperosmotic-induced Stress Treatment. Cells were seeded on sterile 2D-well plate bottom glass coverslips coated with entactin-collagenIV-laminin (ECL) or poly-D-Lysine (PDL) cell attaching matrices. D-sorbitol was dissolved in GM to yield 0.1, 0.2

or 0.4 Molar (M) concentrations. The recovery from the stress treatment was carried out with GM.

Cell Fixation. After the experiment, cells were carefully washed with 1x phosphate-buffered saline (PBS) for 5 minutes and fixated with 4% Paraformaldehyde (PFA) (in 1x PBS) for 10 minutes or with 8% PFA + 8% sucrose (in 1x PBS) for 15 minutes at room temperature. Then the cells were washed again with 1x PBS for 5 minutes, three times, and left in 2ml PBS for storage. The plate was sealed with parafilm, covered with aluminum foil (to protect from light), and placed in a 4˚C fridge.

Immunohisto-fluorescence Staining. ***Day 1***. Cells were washed with 1x PBS for 5 minutes and permeabilized in 0.25% Triton-X (in 1x PBS) for 10 minutes. Then the cells were washed again with 1x PBS for 5 minutes, three times and 1ml blocking solution was added (0.1% Bovine Serum Albumin (BSA) in 1xPBS solution, with 5% Normal Goat Serum (NGS)) for 2 hours, light protected at room temperature. Afterwards the glass coverslips were placed in 350µl primary antibody buffer (anti-mouse to phosphorylated α-syn 1:500 [BLG: #849202] and anti-rabit to G3BP 1:500 [Abcam: #ab181150] in block solution) overnight and light protected at 4˚C fridge. ***Day 2.*** The glass coverslips were washed with 1x PBS for 5 minutes, three times. The cells were than incubated with 250µl secondary antibody buffer (goat anti-mouse Alexa Flour-based AF647 1:500 [Abcam #ab150115] and goat anti-rabbit Cyanine-based fluorescent CF568 1:500 [Biotium #20801] in block solution) per-well, for 3 hours at room temperature and light protected. Next, the cells were washed with 1x PBS for 5 minutes, three times and mounted onto microscope glass coverslips with Vectorshields DAPI (4′,6-diamidino-2-phenylindole) mounting medium, a blue-fluorescent double strand DNA stain for nuclear segmentation.

Confocal Laser Scanning Microscopy Analysis. The microscopy analysis was performed using Olympus FV3000 confocal laser scanning microscope. Regions of interest (ROIs) were imaged in sequential mode, using a 60X magnifying lens, with a line average of 4. The emission laser ranges were set to capture the following used wavelength: DAPI (excitation 405nm) a nucleus marker, GFP (excitation 488nm) reporter for the expression of the A53T *SNCA* mutation, AF647 a marker for phosphorylated α-syn and CF568 a marker for the SG-associated G3BP. Each ROI was imaged in a bright field (BF) as well.

Image Processing. Image processing and quantification were carried out with 'ImageJ' software. Standardized brightness and contrast values were applied to all representative images in each of the five channels (DAPI, GFP, phosphorylated α-syn, G3BP and BF). For quantifying the SG formation, an automatic standardized threshold was applied in the G3BP channel and the mean fluorescence intensity per ROI was calculated.

Statistical Analysis. The statistical test and graph illustration were carried out with 'Prism9' software. A two tailed, unpaired t-test with Welch's correction was conducted. P-values were considered significant if smaller than 0.05.

RESULTS

Experiment 1. In the first experiment 24-well plate bottom glass coverslips were coated with 250µl of 0.8% ECL (in 1x PBS) for 1 hour. Approximately 200,000 SH-SY5Y cells (in 500 µl) were seeded on each coverslip. Based on previous research [13], 0.4M D-sorbitol treatment inducing hyperosmotic shock was applied to the experimental wells, while regular GM was applied to the control wells. After a varying period of time (2-3 hours), the D-sorbitol solution/control treatment was aspirated and replaced with GM for another period of time (0.5-2 hours) to allow the cells to recover. This was done according to three experimental conditions: 2 hours stress + 0.5-hour recovery; 2 hours stress + 1 hour recovery; 3 hours stress + 2 hours recovery (Fig. 1A). After the recovery period, the medium was pipetted from the cells, and they were washed with 1x PBS for 5 minutes and fixated with1 ml of 4% PFA (in 1x PBS) for 10 minutes.

Treating SH-SY5Y cells with 0.4M D-sorbitol for 2 hours resulted in massive cell death regardless of the recovery time provided (Fig. 1B), and even more so for the 3 hours treated group.

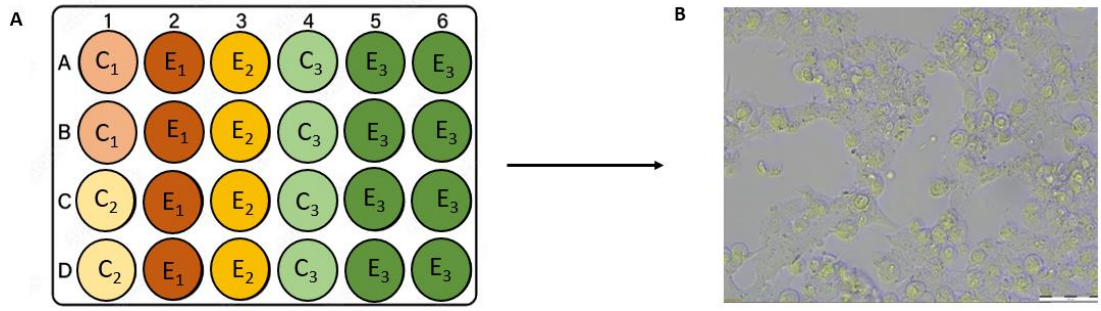


Figure 1. First Experiment's design and representative results. ***(A)*** *Schematic 24-well plate based experimental design: E_1 - 2 hours stress + 0.5-hour recovery, C_1 - 2 hours GM + 0.5-hour recovery; E_2 - 2 hours stress + 1 hour recovery, C_2 - 2 hours GM + 1 hour recovery; E_3 - 3 hours stress + 2 hours recovery, C_3 - 3 hours GM + 2 hours recovery.* ***(B)*** *Representative light microscope image of cell's death extent after 2 hours of 0.4M D-sorbitol treatment inducing hyperosmotic shock.* ***Scale bar = 100µm.***

Experiment 2. Next, we wanted to check whether a milder treatment (reduced D-sorbitol concentration and shorter durations) would promote the formation of SGs without killing the cells. For this purpose, 6-well plate bottom glass coverslips were coated with 250µl of 0.8% ECL (in 1x PBS) for 1 hour. Based on cell-stock size, about 100,000 SH-SY5Y cells (in 200 µl) were then seeded on each coverslip. This time 0.2M D-sorbitol treatment (also previously used [13]) was applied to the experimental wells, and again regular GM was applied to the control wells. After shorter treatment/control GM periods than in experiment 1 (1-2 hours), the medium was aspirated and replaced with GM for the cells to recover (1-2 hours). This was done, again, according to four experimental conditions: 1 hour stress + 1 hour recovery; 1 hour stress + 2 hours recovery; 2 hours stress + 1 hour recovery; 2 hours stress + 2 hours recovery (Fig. 2A). After the recovery period, the medium was pipetted from the cells, and they were washed with 1x PBS for 5 minutes and fixated with 1ml of 4% PFA (in 1x PBS) for 10 minutes.

After the treatment, and mainly after the fixation we found that many cells have detached from the glass coverslips and died (Fig. 2B). There was no observed difference in the death extent of cells between the two recovery times, after 1 hour of 0.2M stress induction. Consequently, we assumed that the treatment has to be even more subtle and that a longer recovery period (of 2 hours) is not necessarily more effective or advantageous. Further, we speculated that the coating should be changed to better attach the SH-SY5Y cells to the glass coverslips, and that the fixation process should be more gradual and gentler considering the post-stress sensitivity of the cells.

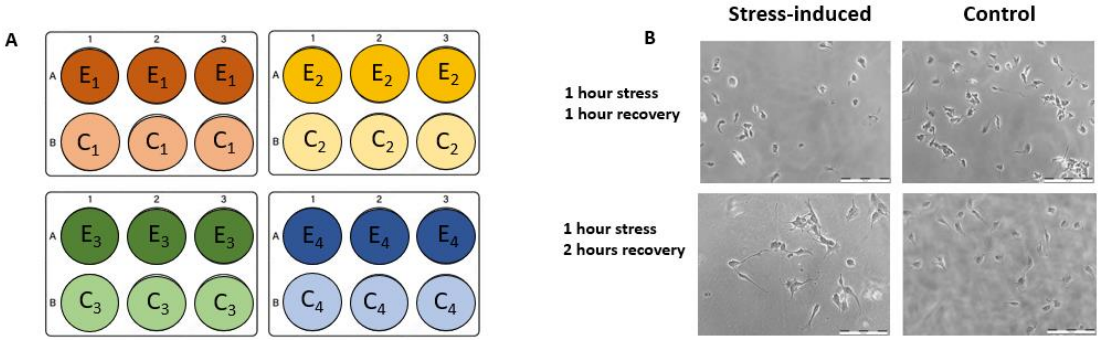


Figure 2. Second Experiment's design and representative results. ***(A)*** *Schematic 4X6 well-plate based experimental design: E_1 - 1 hour stress + 1 hour recovery, C_1 - 1 hour GM + 1 hour recovery; E_2 - 1 hour stress + 2 hours recovery, C_2 - 1 hour GM + 2 hours recovery; E_3 - 2 hours stress + 1 hour recovery, C_3 - 2 hours GM + 1 hour recovery; E_4 - 2 hours stress + 2 hours recovery, C_4 - 2 hours GM + 2 hours recovery.* ***(B)*** *Representative light microscope images of cell's death extent after 1 hour of 0.2M D-sorbitol treatment inducing hyperosmotic shock.* ***Scale bar = 100µm****.*

Experiment 3. The results above prompted us to conduct a third session with new adjustments. In attempt to improve the coating layer and make it more appropriate for SH-SY5Y cells, we coated 6-well plate bottom glass coverslips with 1ml of 0.2% PDL (in 1x PBS) for 1 hour, like recommended in the literature [14-15] instead of 250µl of 0.8% ECL. Then, we seeded an average of 70,000 cells (in 200 µl) on each coverslip. Regarding the D-sorbitol stress treatment, in this session we retested the concentration used in the last experiment (0.2M) and also added a reduced concentration condition (0.1M). The treatment/control GM durations were kept as before (1-2 hours), while the recovery periods were equally set to 1 hour for all groups. After the recovery period, instead of pipetting all the medium from the cells and washing with 1x PBS for 5 minutes like we did in the first two sessions, we pipetted out half of the medium (1ml) and added to the remaining 1ml medium, 1ml of 8% PFA + 8% sucrose solution. This way we managed to reach 4% PFA fixation (left for 15 minutes), in a more delicate and gradual manner.

The adaptations made in this experiment enabled the cells to undergo and survive the hyperosmotic stress treatment and fixation process (Fig. 3B). The calibrated parameters were set into a defined protocol (that is the attaching matrix's type, D-sorbitol concentrations, treatment's duration, recovery period, and fixation procedure) and we could move on to the IHF staining.

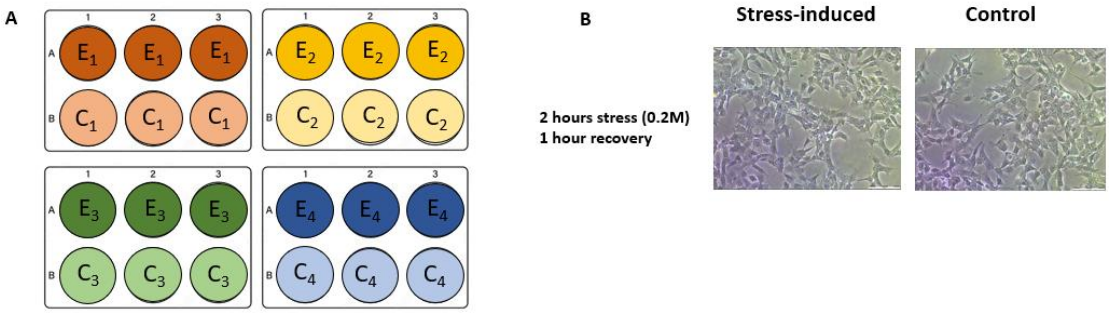
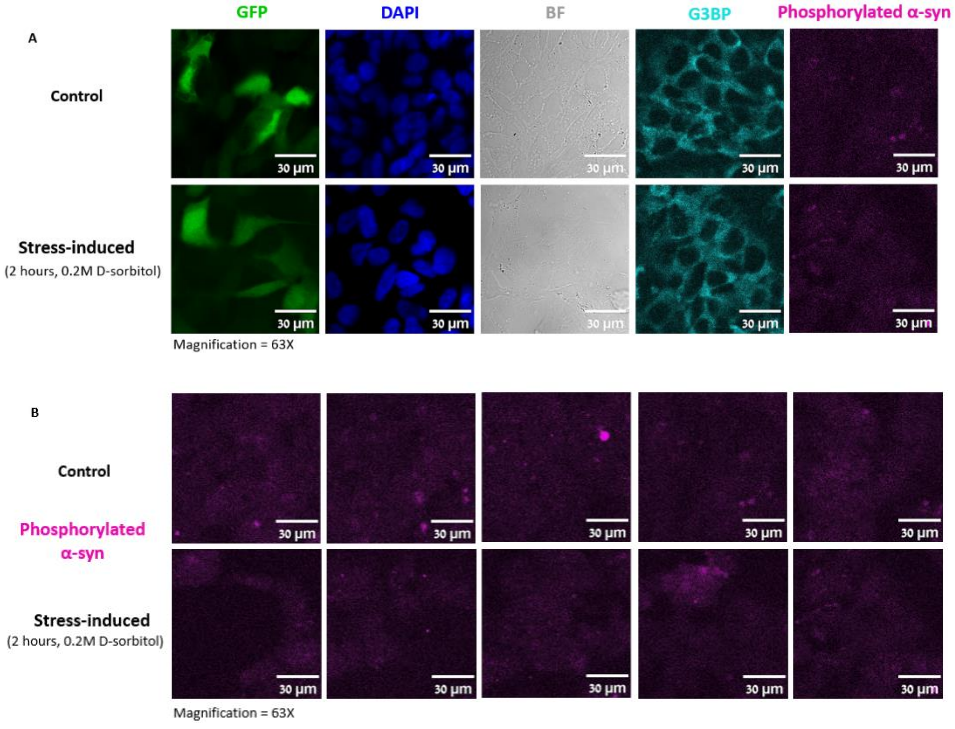


Figure 3. Third Experiment's design and representative results. (A) *Schematic 4X6 well-plate based experimental design: E_1 - 1 hour stress (0.2M), C_1 - 1 hour GM; E_2 - 2 hours stress (0.2M), C_2 - 2 hours GM; E_3 - 1 hour stress (0.1M), C_3 - 1 hour GM; E_4 - 2 hours stress (0.1M), C_4 - 2 hours GM. Recovery period was set to 1 hour for all conditions.* ***(B)*** *Representative light microscope images of cell's viability and confluency after 2 hours of 0.2M D-sorbitol treatment inducing hyperosmotic shock.* ***Scale bar = 100µm.***

Following IHF, cells were imaged in confocal laser scanning microscope in five channels (DAPI, GFP, phosphorylated α-syn, G3BP and BF) (Fig. 4A). All cells showed a strong GFP signal indicating that the A53T *SNCA* mutation was expressed in them. The phosphorylated α-syn signal on the other hand, appeared weak and not specific, thus we referred to it as background noise (further demonstrated in Fig. 4B).

The expression of G3BP (Fig. 4C) was quantified (mean fluorescence intensity was calculated per ROI) and compared between the stress-induced group (0.2M, 2 hours) and the control group (GM, 2 hours) (Fig. 5). Contrary to our expectations, we observed a trend in which the G3BP signal in the control group (M = 378.7 A.U) was stronger than in the stress-induced group (M = 324.6 A.U). Additionally, neither of the groups showed a granular G3BP expression indicating SG formation, despite expected granulation in the stress-induced group. For this reason, we did not move on to analyzing the milder stress-induced conditions (0.2M, 1 hour; 0.1M, 1-2 hours).



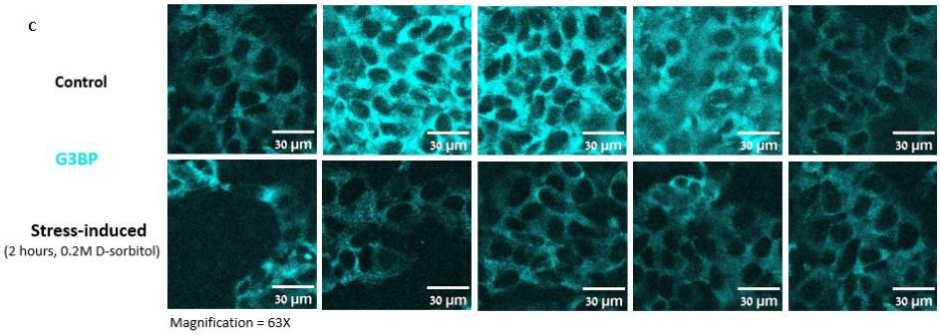


Figure 4. Representative results from confocal laser scanning microscopy imaging. *Untreated SH-SY5Y cells (top row in each panel) compared to cells treated with 0.2M D-sorbitol for 2 hours + 1 hour recovery (bottom row in each panel).* ***(A)*** *Representative ROI displayed in five channels.* ***(B)*** *Phosphorylated α-syn expression in 5 representative ROIs.* ***(C)*** *G3BP expression in 5 representative ROIs.*

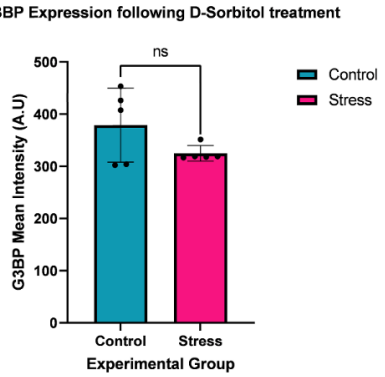


Figure 5. G3BP expression following 2 hours of 0.2M D-sorbitol treatment + 1 hour recovery. *Analysis with unpaired parametric, two tailed t-test with Welch's correction; equal SDs not assumed; N=5; *(p≤0.05).*

DISCUSSION

RNA granules, the transient membranelles cytoplasmic structures that play an essential role in post-transcriptional regulation of RNAs, have recently gained traction as potential mediators of neurodegeneration. Respectively, in this study we aimed to experimentally induce SGs in order to validate an assay through which we can investigate the link between their formation dynamics and α-syn aggregation. To achieve this, we used D-sorbitol treatment inducing hyperosmotic stress in A53T-α-syn overexpressing SH-SY5Y cell line. We then used IHF staining and confocal laser scanning microscopy to characterize phosphorylated α-syn and SG-associated G3BP expression patterns.

Surprisingly, we were not able to observe phosphorylated α-syn in the stress-induced cells nor in the control untreated ones. It was previously shown that A53T *SNCA* mutant SH-SY5Y cells do express significantly increased levels of phosphorylated α-syn at Ser^{129} compared with basal expression in WT [16]. But it was also shown that the A53T *SNCA* mutation solely does not always lead to increased formation of inclusions in the cells and sometimes an additional trigger such as cell differentiation, together with $FeCl_2$ (and H_2O_2) treatment or Hsp70 blockage is required [11]. This might imply that in our case the phosphorylated form of α-syn was not properly induced and aggregates were not assembled. A different explanation would suggest that the phosphorylated

α-syn expression is intact, however the staining was not successful. This could happen due to impairments issuing the specific primary phosphorylated α-syn antibody or the secondary AF647 conjugated antibody that we used.

Unfortunately, we were not able to observe the formation of SGs that we tried to stimulate through hyperosmotic stress induction. This could be due to several reasons. First, it is possible that the cells we used were resistant to G3BP-induced granule formation like was suggested in a study where some cells expressing G3BP even at high doses did not contain SGs [7]. This is not very likely though since previous research did manage to capture SG formation in SH-SY5Y cells after D-sorbitol treatment [13].

Another possible explanation involves the recovery period that we provided the stressed cells with. Research in the field proposed that SG's disassembly process occurs in two steps. First, the larger complexes break into smaller foci within a narrow time window (poorly defined). Second, the smaller foci disassemble or get cleared out by autophagy, depending on the context of the specific stress induced [17]. They become microscopically undetectable within a similar time scale as required for their initial detection during assembly (~15 minutes) [8]. Accordingly, there is a possibility that the 1-hour recovery period we defined was too long for SGs to persist after hyperosmotic stress inducing treatment. If so, another calibration experiment with 0.2M D-sorbitol and recovery periods ranging between 0-1 hours, could be useful and informative. To further improve our understanding and certainty we could try to use live-cell imaging to determine whether SGs were formed and disassembled or never formed in the first place.

A different direction for investigating SGs in SH-SY5Y cells would suggest using other environmental stress-inducing manipulations such as heat-shocks, oxidative stressors, UV irradiation, induction of hypoxia or viral infections that were previously shown to induce SG formation [1-2, 4].

The use of the proliferative ontogenically transformed SH-SY5Y cell line with catecholaminergic rather than exclusively dopaminergic properties has limitations. Therefore it is recommended to use another cell line with neuronal phenotype in parallel during the experiment. For example the hybrid MN9D immortalized dopaminergic neuronal cell line, the pheochromocytoma cell line PC12, the Lund human mesencephalic cells (LUHMES), or the rat immortalized neuronal progenitor cell line CSM14.1 [18]. Together with the SH-SY5Y cell line, these represent the basic first step in experiments related to PD that must be followed by confirmation of the results through more complex and physiologically relevant models.

Indeed, this study was a preliminary step towards using SH-SY5Y cells in their differentiated dopaminergic neuronal phenotype and further on using rodents mesencephalic or cortical primary neurons. The most physiologically reliable *in-vitro* cell base-model that we could strive to work with involves patient-derived induced pluripotent stem cells (iPSCs) differentiated into dopaminergic neurons [11].

In this study we attempted to investigate SG dynamics in the context of PD *in-vitro*. Although we were unable to obtain informative results concerning SG formation patterns or its association with disease-related α-syn aggregation, we did manage to calibrate D-sorbitol treatment that preserves cells' viability. With future validation of the hyperosmotic stress induction in the cells as well as improvements in the assay, we may be able to obtain clearer results and gain better understanding of the relationship between SG formation and PD. This is important for elucidating the impact of PD-related mechanisms on RNA translation dynamics, that may potentially lead to neurodegeneration.

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