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Research Paper

# Reductive stress promotes protein aggregation and impairs neurogenesis

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ABSTRACT

Redox homeostasis regulates key cellular signaling in both physiology and pathology. While perturbations result in shifting the redox homeostasis towards oxidative stress are well documented, the influence of reductive stress (RS) in neurodegenerative diseases and its mechanisms are unknown. Here, we postulate that a redox shift towards the reductive arm (through the activation of Nrf2 signaling) will damage neurons and impair neurogenesis. In proliferating and differentiating neuroblastoma (Neuro 2a/N2a) cells, sulforaphane-mediated Nrf2 activation resulted in increased transcription/translation of antioxidants and glutathione (GSH) production along with significantly declined ROS in a dose-dependent manner leading to a reductive-redox state (*i.e.* RS). Interestingly, this resulted in endoplasmic reticulum (ER) stress leading to subsequent protein aggregation/proteotoxicity in neuroblastoma cells. Under RS, we also observed elevated Tau/α-synuclein and their co-localization with other protein aggregates in these cells. Surprisingly, we noticed that acute RS impaired neurogenesis as evidenced from reduced neurite outgrowth/length. Furthermore, maintaining the cells in a sustained RS condition (for five consecutive generations) dramatically reduced their differentiation and prevented the formation of axons ($p < 0.05$). This impairment in RS mediated neurogenesis occurs through the alteration of Tau dynamics *i.e.* RS activates the pathogenic GSK3β/Tau cascade thereby promoting the phosphorylation of Tau leading to proteotoxicity. Of note, intermittent withdrawal of sulforaphane from these cells suppressed the proteotoxic insult and re-activated the differentiation process. Overall, this results suggest that either acute or chronic RS could hamper neurogenesis through GSK3β/TAU signaling and proteotoxicity. Therefore, investigations identifying novel redox mechanisms impacting proteostasis are crucial to preserve neuronal health.

*Abbreviations:* CHOP, CCAAT-enhancer-binding protein homologous protein; DHE, Dihydro-ethidium; ER, Endoplasmic Reticulum; ERO1α, Endoplasmic reticulum oxidoreductase 1 alpha; ERP44/72, Endoplasmic Reticulum Protein 44/72; GCLC, γ-Glutamyl Cysteine Ligase Catalytic subunit; GCLM, γ-Glutamyl Cysteine Ligase Metabolic subunit; GPX1, Glutathione peroxidase 1, GRP78/94, Glucose-regulated protein 78/94; GSH, Reduced glutathione; GSK3β, Glycogen Synthase Kinase 3 beta; GSR, Glutathione-S-reductase; GSSG, Oxidized glutathione; $H_2O_2$, Hydrogen peroxide; MTT, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; NQO1, NADPH quinone oxidoreductase 1; Nrf2, Nuclear factor (erythroid-derived-2)-like 2; OS, Oxidative stress; PDI, Protein disulfide isomerase; PQC, Protein quality control; RA, Retinoic acid; ROS, Reactive oxygen species; RS, Reductive stress; SEM, Standard Error of Mean; SF, Sulforaphane; UPR, Unfolded protein response.

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## 1. Introduction

Neurodegenerative diseases including Alzheimer's, Parkinson's and Huntington's are a major health problem in aging population across the world. Neurological disorders contribute to 2% of the global burden of disease and disability-adjusted life years is projected to increase by ~12% (~103 million) in 2030 [1]. The identification of redox-regulated processes, such as regulation of biochemical pathways involved in the maintenance of redox homeostasis in the brain has provided deeper insights into mechanisms of neuroprotection and neurodegeneration [2]. Disruption of redox homeostasis shifting toward oxidative stress (OS) is often associated with neurodegeneration [3,4]. Although multiple studies have demonstrated a strong connection of OS with neurodegeneration at the end stages of the diseases [5,6], it is not clear whether the generation of reactive oxygen species (ROS) appears as a compensatory signaling response to a pathological trigger or is the trigger *per se*.

A previous study reported that an increased activity of glucose-6-phopshate dehydrogenase (G6PD) and elevated glutathione (GSH) levels (2.0-fold) in the brain of Alzheimer's patients than in healthy controls [7], suggesting a reductive compensation during pathogenesis. In addition, detection of abundant NADPH-Quinone Oxidoreductase 1 (NQO1) and Heme Oxygenase 1 (HO-1), key antioxidant enzymes, in post-mortem brains of Alzheimer's and Parkinson's patients, indicate changes in the redox metabolism and hyperactivity of these enzymes may contribute to pathogenesis [8–11]. Recently emerging reports suggest that antioxidant-induced RS has untoward consequences on brain microvasculature [12] and persons at risk of Alzheimer's during their young-adult stage [13]. Therefore, it warrants a closer investigation and further understanding of redox alterations and their impact on the onset and progression of neurodegenerative diseases.

Although the misfolding and aggregation of proteins are common events in different forms of neurodegenerative diseases, there are specific proteins (*i.e.* Tau and/or β-amyloid for Alzheimer's; α-Synuclein for Parkinson's etc.) driving proteotoxicity and cell death [14,15]. Indeed, the endoplasmic reticulum (ER), which is a key organelle in maintaining proteostasis and the unfolded protein response (UPR), is triggered in response to protein accumulation and ER stress [14]. Chronic activation of ER stress response in the brain, as well as in newly-generated immature neurons contribute to suppressed neuronal survival and neurogenesis [16]. However, the mechanisms associated with RS-proteotoxicity in the brain are unknown.

Formation of new neurons (neurogenesis), maturation (i.e. dendritic and axonal development) and integration into the entire neuronal network are central for gaining the functional plasticity [17,18]. While there are limited therapeutic options currently available for neurodegenerative diseases, healing chronically injured neurons is still challenging [19]. Therefore, pharmacological interventions to modulate proliferation, migration and differentiation of neurons are considered to be an effective strategy for neurodegeneration [20]. Given that, attempts using small molecular antioxidants to promote neurogenesis resulted in poor outcomes [21,22]. Although, correlations between the redox state and the neurogenesis exist, the role of a hyper-reductive redox setting (*i.e.* Reductive stress/RS) has not been investigated yet. Here, we test the hypothesis that RS abrogates oxidant signaling and impairs ERfunction, thereby promoting protein aggregation/proteotoxicity and diminishing neurogenesis.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Dulbecco's modified eagle medium (DMEM) and Opti-Minimal Essential Medium (OMEM) were procured from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). RNeasy kit (74106), QuantiTect SYBR Green PCR, and QuantiTect reverse transcription kit (205313) were purchased from Qiagen, Inc. (Valencia, CA). Protein assay reagent (500–0006) was procured from Bio-Rad, Inc. (Hercules, CA). Secondary antibodies (anti-rabbit and anti-mouse) for immunoblots (horseradish peroxidase-conjugated with IgG) were purchased from Vector Laboratories (Burlingame, CA). Primers for qPCR were designed using the Harvard Medical School PrimerBank website and purchased from integrated DNA Technologies (IDT) (Coralville, IA). Proteostat® was procured from Enzo Lifesciences (Farmingdale, NY, USA), and Dihydroethidium (DHE) was obtained from Molecular probes (USA). All other chemicals including L-Sulforaphane (SF), retinoic acid (RA), *meta*-phosphoric acid and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St, Louis, MO, USA), unless otherwise stated.

### 2.2. Cell culture and treatments

Neuroblastoma (N2a) cells were cultured in proliferation medium (1:1 DMEM: Opti-MEM containing 5% FBS) and treated with sulforaphane (1.0 and 5.0 μM) for 48 h. Sulforaphane-treated N2a cells were analyzed for: glutathione redox levels [23,24], ROS using DHE [25], and protein aggregation using Proteostat® [26]. Using qPCR/immunoblotting [23,24,27], we analyzed the antioxidant, ER stress markers and neuronal protein aggregation targets at genes and/or proteins levels. To induce differentiation, the N2a cells were shifted to a differentiation medium (DMEM: OMEM 1:1 containing 0.5% FBS) with or without retinoic acid (RA, 2.5 & 5.0 μM) [28] and sulforaphane (5.0 μM) for six days (day 0 – day 5). Then, we assessed differentiation through morphological and morphometry on neurite outgrowth and axon formation. For prolonged RS, N2a cells were treated with sulforaphane at 5.0 μM for 10 days (five consecutive generations) and then analyzed for the rate of protein aggregation and their impact on differentiation. For rescue experiments, RA and sulforaphane were used at the dose of 5.0 μM. Sulforaphane was removed by feeding fresh differentiation medium (without sulforaphane) at day 3 and then allowed to differentiate until day 6 (day 0 – day 6).

### 2.3. Immunofluorescence, image acquisition, and quantification

N2a neuroblastoma cells were cultured on a cover-slip pre-coated with 0.02% gelatin (in 6-well plates) and treated with sulforaphane for 48 h. In preparation for staining, cells were incubated with 4% paraformaldehyde for 15 min and washed thrice with PBS followed by permiabilization with 0.25% Triton X-100. Following three washes with PBS, the cells were incubated with DHE or Proteostat® in PBS in a light-protected chamber maintained at room temperature for 30 min [29]. For dual immunostaining, cells stained with Proteostat® was blocked with 5% goat serum and incubated with anti-Mouse phospho (S202, T205) TAU (#MN1020; Thermofisher, Waltham, MA, USA) overnight at 4 °C. Following incubation, the cells were washed thrice with PBS, and incubated with Alexa fluor 488 (A-21042; Thermofisher) conjugated secondary antibody for 1 h at RT. Then the cells were washed thrice with PBS and mounted with Fluoroshield/DAPI (ab104139; Abcam, Cambridge, MA). Images were acquired with an Olympus BX43 fluorescent microscope using a 20/40× objective lens. At least three to four images were taken per n (n = 3) and used to calculate the appropriate intensity using ImageJ (NCBI).

### 2.4. Quantification of neurite bearing cells and neurite length

Neurite bearing cells were quantified as described by Phan et al. (2013) [30]. Briefly, in five randomly chosen fields (100–200 cells/-field) neurite bearing cells were measured using Fiji (ImageJ, NCBI). The number of neurite outgrowths, defined as axon like extensions that were double or more than the length of the cell body diameter was recorded. The percentage of neurite bearing cells is the number of neurite bearing cells divided by the total number of cells in a field and then multiplied by 100% [30]. Quantification of neurite length was performed as described

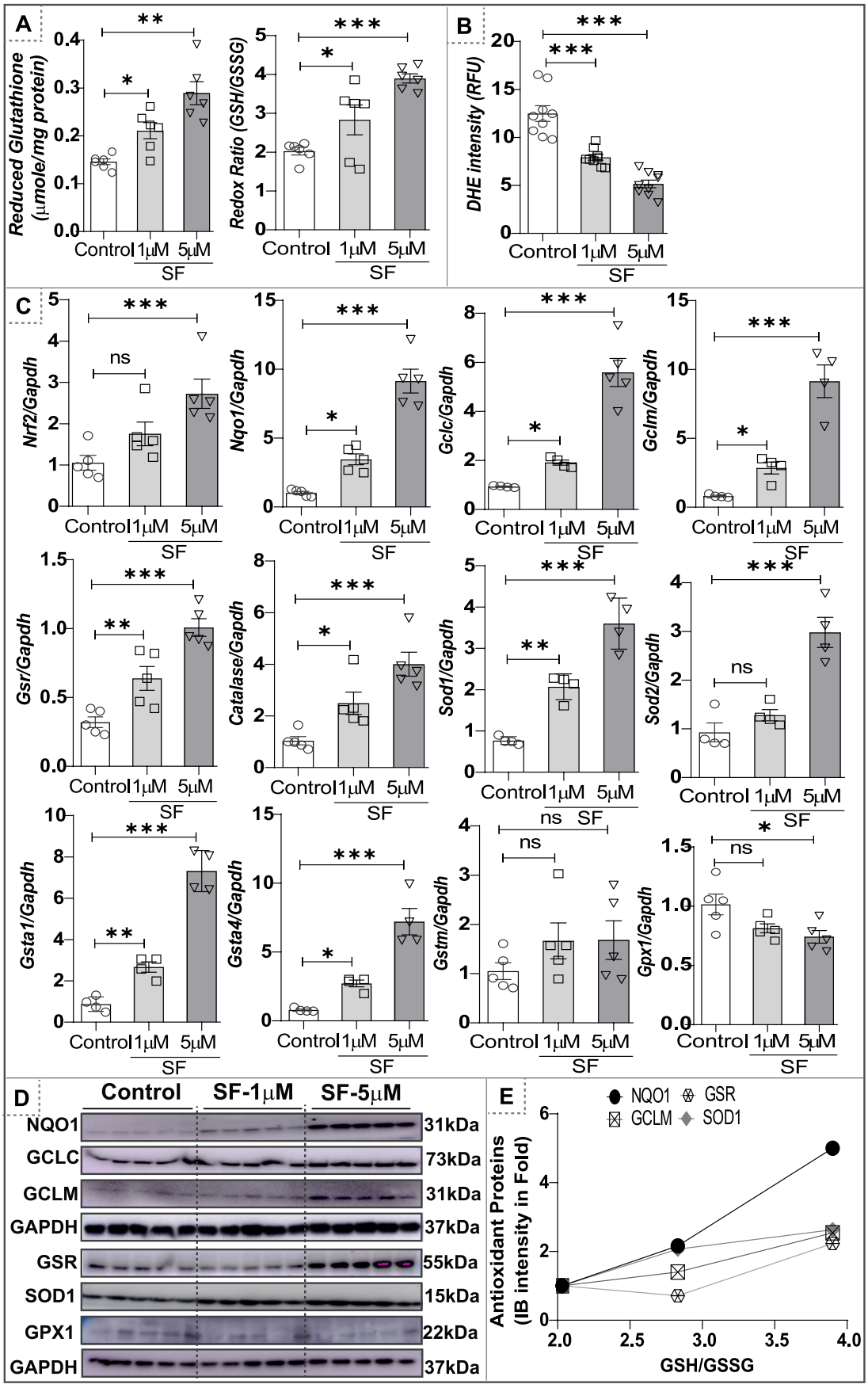


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**Fig. 1. Sulforaphane treatment establishes reductive stress in N2a neuroblastoma cells:** N2a neuroblastoma cells were cultured in proliferation medium for 48-h with or without sulforaphane. **(A)** Glutathione levels and its redox (GSH/GSSG) ratio in the N2a neuroblastoma cells upon sulforaphane treatment (1.0 & 5.0 μM) was measured using enzymatic recycling assay. The redox ratio (GSH/GSSG) was dose-dependently elevated upon sulforaphane-treatment when compared with untreated (control) cells (n = 6). **(B)** N2a cells were stained with dihydroethidium (DHE) after 48 h of sulforaphane-treatment. The fluorescence intensity of oxidized 2-hydroxyethidium was quantified using ImageJ and expressed as relative fluorescent units (RFU). ROS levels were markedly decreased in a dose-dependent manner upon sulforaphane treatment [n = 3, 3 images (different regions) per group]. **(C)** qPCR-based quantification of Nrf2 and Nrf2-driven antioxidant transcriptome (*Nqo1, Gclc, Gclm, Gsr, Catalase, Sod1, Sod2, Gsta1, Gsta4, Gstm, and Gpx1*) was performed in N2a cells treated with/without sulforaphane (n = 4–5). **(D)** Immunoblot analysis of antioxidant proteins (NQO1, GCLC, GCLM, GSR, SOD1 and GPX1) was performed in N2a cells treated with/without sulforaphane (n = 5). **(E)** Analyses between antioxidant protein abundance and redox ratio (GSH/GSSG) indicating a strong-positive correlation. Elevated redox ratio, gene/protein expressions of antioxidants along with declined ROS levels demonstrate the establishment of reductive stress (RS) in N2a neuroblastoma cells. Data are presented as Mean ± SEM (*p < 0.05; **p < 0.01; ***p < 0.001; ns - no significance).

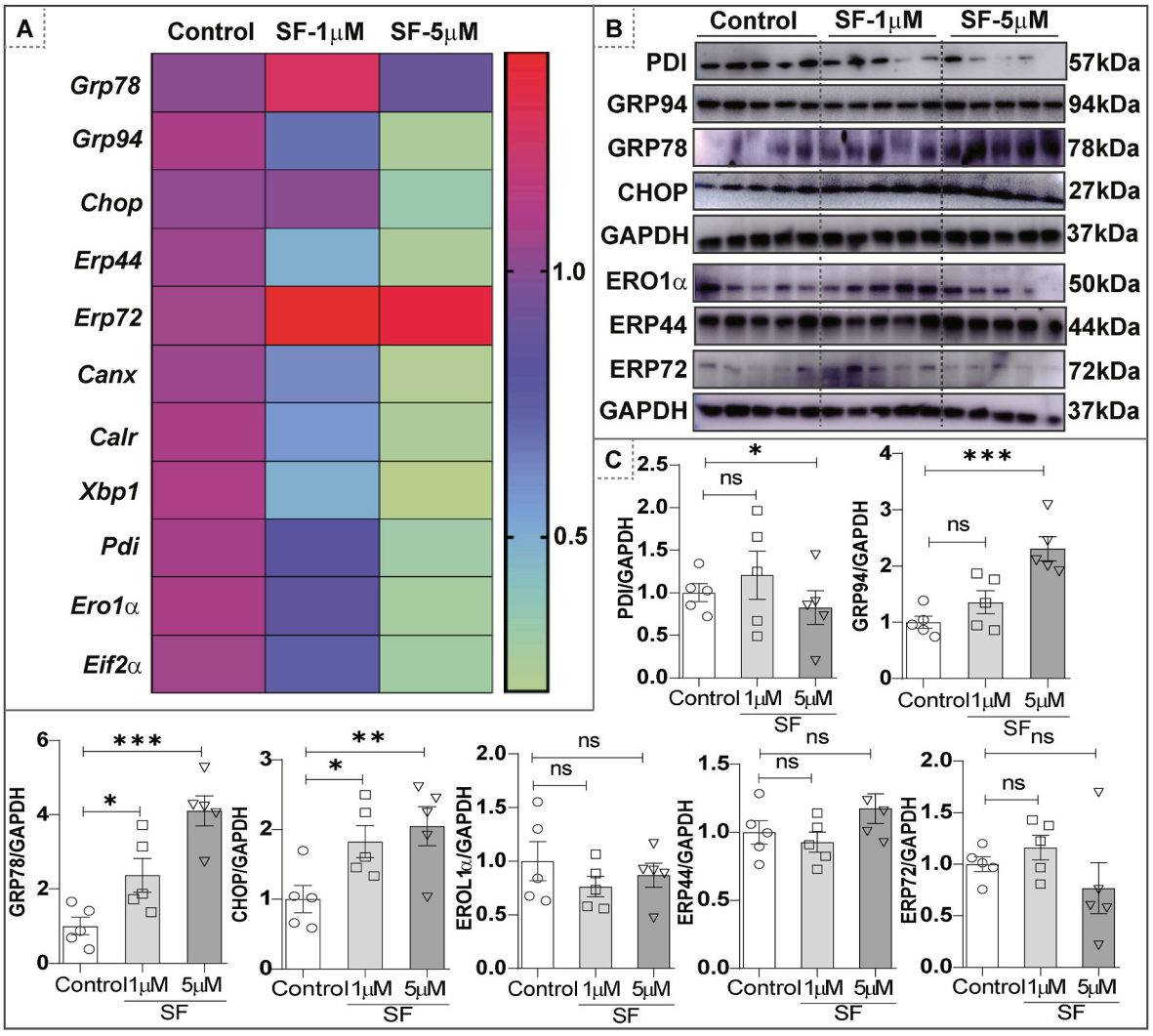


**Fig. 2. Reductive stress impairs endoplasmic reticulum/protein folding pathways in N2a-neuroblastoma cells: (A)** qPCR of endoplasmic reticulum (ER) stress/protein folding genes in N2a cells treated with sulforaphane (1.0 & 5.0 μM). Fold change (*i.e.* relative quantification, RQ) was normalized to endogenous *Gapdh* expression. Differential expression of ER stress/protein folding genes (*Grp78, Grp94, Chop, Erp44, Erp72, Calnexin, Calreticulin, Xbp1, Pdi, Ero1α, Eif2α)* is illustrated as a heat map (n = 4–5). **(B)** Immunoblot analysis of key proteins involved in ER stress/protein folding (PDI, GRP94, GRP78, CHOP, ERO1α, ERP44 and ERP72) signaling in N2a cells after sulforaphane 1.0 & 5.0 μM treatment (n = 5). **(C)** Respective densitometries of the immunoblots indicating significant changes in the levels of these proteins. Data are presented as Mean ± SEM (*p < 0.05; **p < 0.01; ***p < 0.001; ns - no significance).

by Pemberton et al. (2018) [31] using NeuronJ, a plugin in the Fiji (ImageJ, NCBI) and neurite length was expressed as μm. Other methods and techniques employed in this study are described in the supplemental section.

### 2.5. Statistical analysis

All data are represented as Mean ± Standard Error of the mean. One-way ANOVA with post-hoc Dunnett multiple comparison test was performed. Student's t-test was used to calculate statistical significance between two-groups. GraphPad Prism 8 was used for statistical analyses and to create graphs and heat-maps.

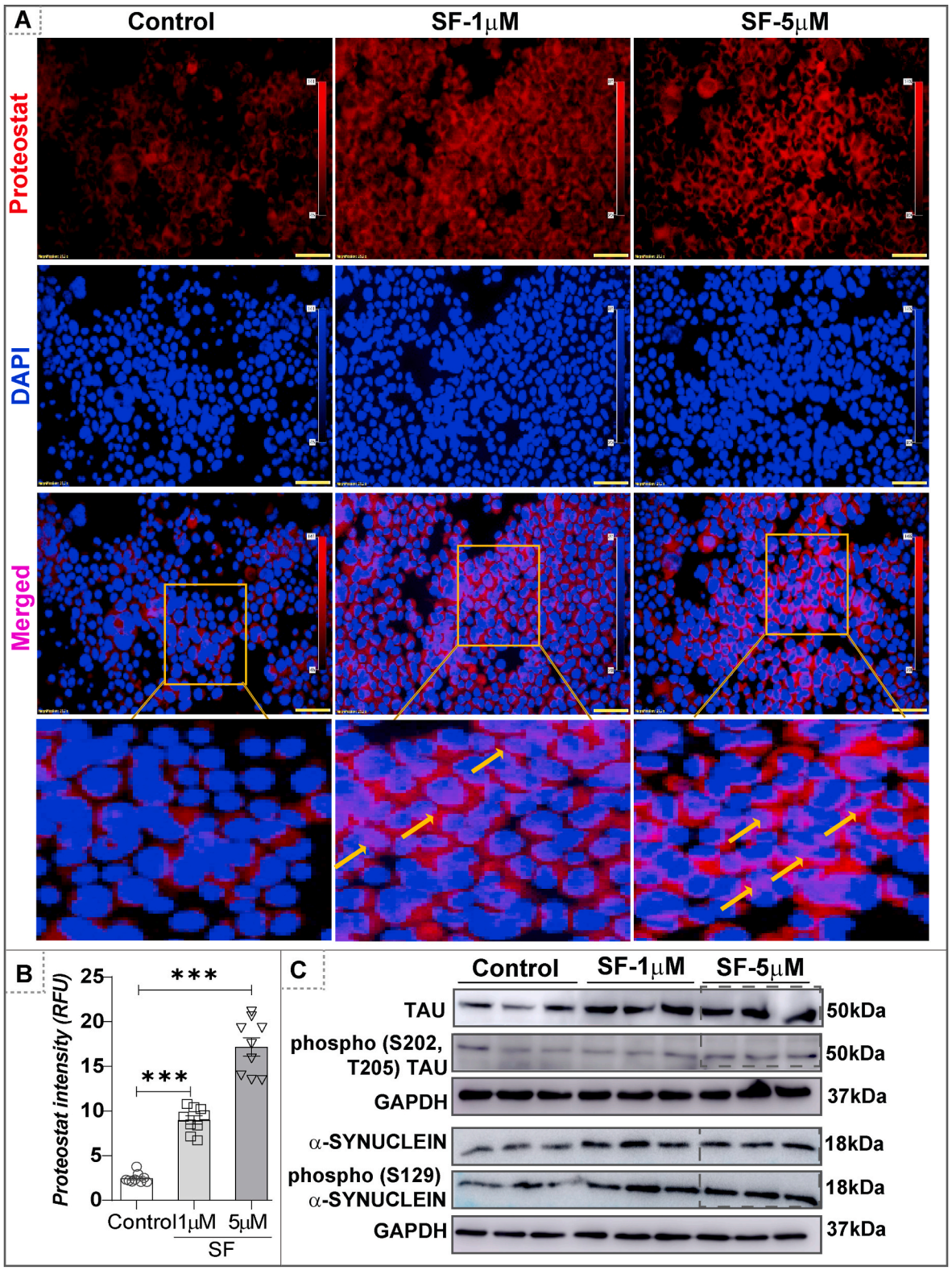


**Fig. 3. Reductive stress promotes protein aggregation in N2a neuroblastoma cells: (A)** N2a cells were stained with Proteostat® dye to determine the intensity of protein aggregation upon sulforaphane-induced RS. Diffuse cytoplasmic staining was observed with discrete punctuate staining in cells treated with sulforaphane (1.0 & 5.0 μM), indicating the aggregation of proteins, and the intensity of these aggregates was relatively more in the perinuclear space. **(B)** Quantification of the Proteostat® staining within cells revealed increased fluorescence intensity in the sulforaphane-treated ($p < 0.001$) than un-treated (control) cells [$n = 3$, 3 images (different regions) per group]. **(C)** Immunoblot analysis of neuronal aggregation markers (TAU, phospho S202/T205 TAU, α-SYNUCLEIN and phospho S129 α-SYNUCLEIN) in N2a cells after sulforaphane 1.0 & 5.0 μM treatment ($n = 3$). Data are presented as Mean ± SEM (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns - no significance).

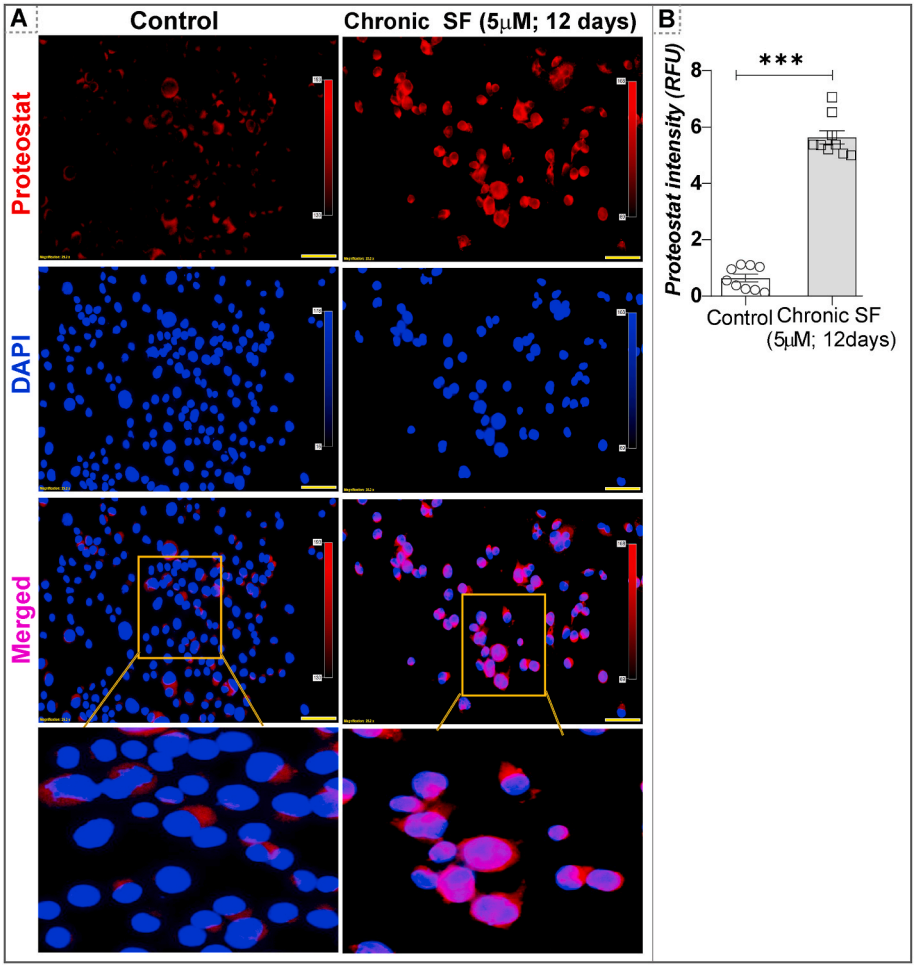


**Fig. 4. Chronic reductive stress promotes protein aggregation in N2a-neuroblastoma cells**: N2a neuroblastoma cells were cultured in proliferation medium supplemented with sulforaphane and passaged every 48 h. After five generations (48 h/generation), the cells were seeded on a cover-slip pre-coated with gelatin (in 6-well plates) and continued to undergo sulforaphane treatment for an additional 48 h (total sulforaphane treatment was achieved for 12 days). The chronic sulforaphane-treated cells and controls (without sulforaphane) were then stained with Proteostat® to measure the protein aggregation. **(A)** Fluorescence images of Proteostat® staining in control and chronic sulforaphane treated N2a cells. Following chronic sulforaphane-treatment, we observed discrete punctuate staining and an intensive accumulation of perinuclear/nuclear protein aggregates. **(B)** The fluorescence intensity of the Proteostat® dye was quantified using ImageJ and expressed relative fluorescent units (RFU) [n = 3, 3 images (different regions) per group]. Data are presented as Mean ± SEM. Significance: *p < 0.05; **p < 0.01; ***p < 0.001; ns - no significance.

## 3. Results

### 3.1. Establishing reductive stress in N2a neuroblastoma cells

To test whether reductive reprogramming contributes to neurodegeneration, we established a robust *in-vitro* model of reductive stress (RS) using sulforaphane, which activates Nrf2/ARE signaling and leads to antioxidant augmentation [32]. First, we validated cell viability using MTT assay. We noticed a dose-dependent decreased viability and 100% cell death occur at the concentration of 15.0 μM of sulforaphane (Fig. S1A). Treating N2a cells with sulforaphane (1.0 and 5.0 μM) resulted in a dose-dependent augmentation of reduced glutathione (GSH; ~1.45 & ~3.0 fold) and the redox ratio (GSH/GSSG; ~1.4 & ~2.5 fold; Fig. 1A) confirms RS [23,27,33]. DHE based fluorescence imaging [25] of the sulforaphane-treated N2a cells revealed a dose-dependent decline of both the nuclear and cytosolic ROS levels (Fig.S1B; Fig. 1B ~1.6 & ~2.5-fold). Next, we assessed the transcript/protein levels for Nrf2-and its targeted antioxidants in these cells. As expected, upregulation of Nrf2 and its target antioxidant genes (*i.e., Nqo1, Gclc, Gclm, Gsr, Catalase, Sod1, Sod2, Gsta1, Gsta4, and Gstm*) were observed in sulforaphane-treated N2a cells (Fig. 1C). Subsequently, protein levels for NQO1, GCLC, GCLM, GSR and SOD1 were also significantly ($p < 0.05$) increased in a dose-dependent manner (Fig. 1D & S1C). These results demonstrate that sulforaphane could promote the reductive environment in N2a cells (Fig. 1E). While most of the antioxidant genes/proteins were increased, GPX-1 was decreased dose-dependently upon sulforaphane-treatment in N2a neuroblastoma cells (Fig. 1D). This might be due to increased catalase levels, in response to sulforaphane, which could quench hydrogen peroxide ($H_2O_2$) and result in negative feedback on the transcription and translation of GPX1 in the N2a cells experiencing RS.

### 3.2. Reductive stress impairs endoplasmic reticulum/protein folding pathways in N2a neuroblastoma cells

The subcellular compartments within a cell exhibit unique redox environments [34]. Of note, while mitochondria, cytosol, and the nucleus experience a reductive redox milieu, the endoplasmic reticulum (ER) holds a relatively oxidative redox state [35–37]. As we established a RS condition in sulforaphane-treated N2a cells, we postulated that this may disrupt the ER function. Expression analysis was performed using qPCR for key genes involved in ER stress/protein folding pathways (*i.e. Grp78, Grp94, Chop, Erp44, Erp72, Calnexin, Calreticulin, Xbp1, Pdi, Ero1α,* and *Eif2α*). All these genes were dose-dependently down-regulated except *Erp72* (upregulated) and Grp78 (unaltered) upon RS (Fig. 2A). In addition, immunoblotting revealed a significant decrease in protein disulfide isomerase (PDI) ($p < 0.05$) and an increase in CHOP, GRP78/94 ($p < 0.001$) in 5.0 μM sulforaphane-treated N2a cells (Fig. 2B and C). These results highlight that ER/protein folding pathways are impaired in response to RS.

### 3.3. Reductive stress promotes protein aggregation in N2a neuroblastoma cells

We next determined whether impaired ER/protein folding mechanism could lead to misfolding and aggregation of proteins in these cells. To monitor this, we stained the N2a cells with Proteostat® dye, a molecular rotor that binds selectively to misfolded/aggregated proteins

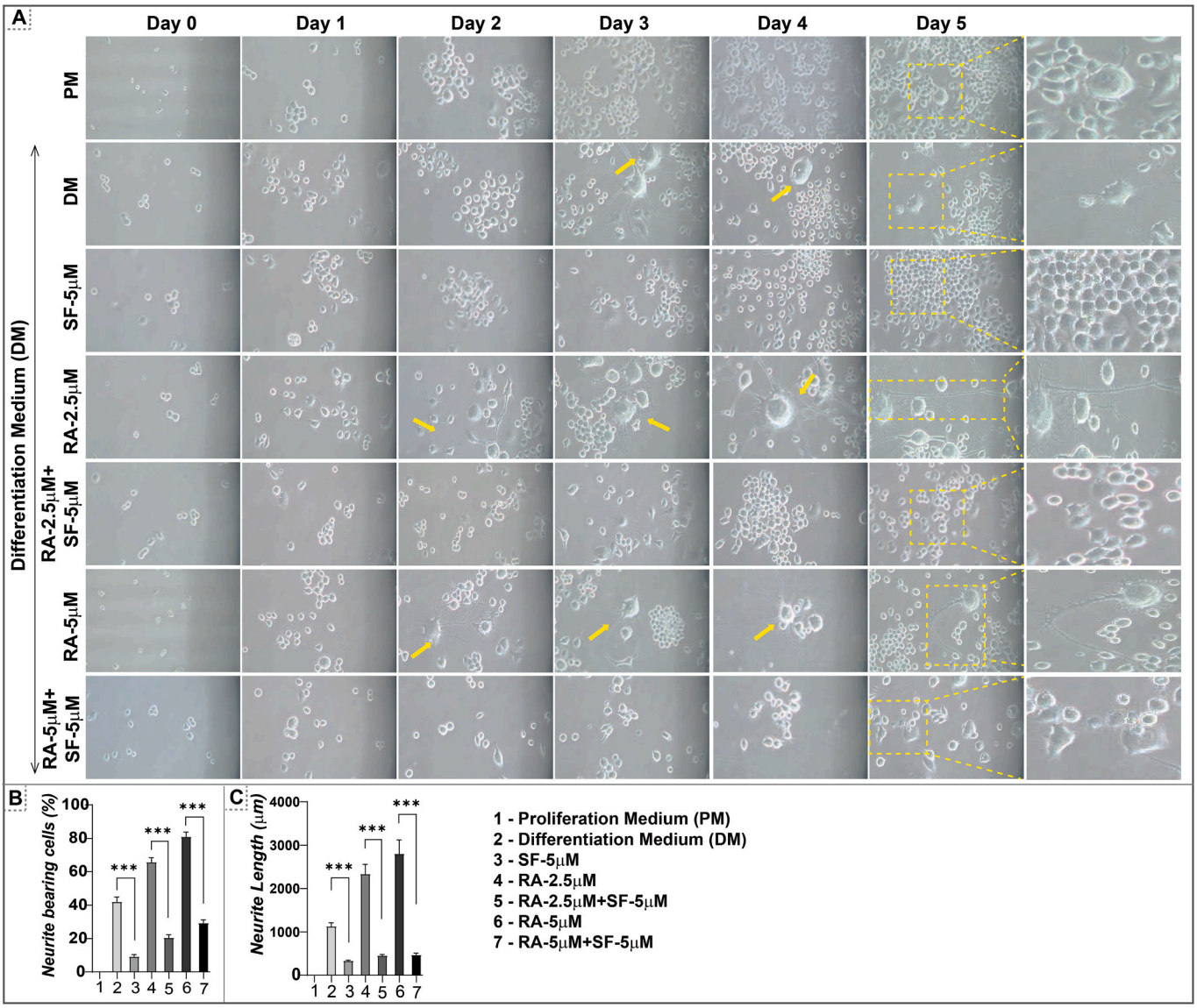


**Fig. 5. Reductive stress impairs neurogenesis in N2a neuroblastoma cells:** N2a neuroblastoma cells were cultured in proliferation medium to 70% confluence; then sub-cultured for differentiation studies. Following 24 h of proliferation, the cells were grown in differentiation medium (1:1 DMEM: OMEM comprising 0.5% FBS) containing retinoic acid (RA, 0, 2.5 and 5.0 μM) to induce differentiation up to 5 days with or without sulforaphane (5.0 μM). **(A)** Bright-field microscopic images of N2a neuroblastomas during proliferation and differentiation (day 0 to day 5). A dose-dependent increase for RA (2.5 & 5.0 μM) on the rate of differentiation was observed, but this was impaired upon co-treatment with sulforaphane (5.0 μM) (Magnification 20×). **(B)** The number of cells with neurite outgrowths, defined as axon-like extensions that were double the size than the cell body (in diameter) were counted (denoted in yellow-arrow markings). The percentage of neurite bearing cells is the number of neurite bearing cells divided by the total number of cells (50-cells) in a microscopic field and then multiplied by 100% [n = 4]. **(C)** The neurite outgrowth (length) was measured in all the groups using Fiji (ImageJ) with NeuronJ plug-in and the neurite length is indicated as μm [n = 4]. RA-driven neurogenesis was impaired in sulforaphane co-treated cells. Moreover, the telodendria and synaptic bouton developed during differentiation (RA; 0, 2.5 & 5.0 μM) were minimal or absent in sulforaphane (5.0 μM) co-treated cells. Data are presented as Mean ± SEM (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns – no significance).

[26]. Diffused cytoplasmic punctuate staining was observed in cells treated with sulforaphane (1.0 & 5.0 μM), indicating the aggregation of proteins, and the intensity of these aggregates was relatively more in the perinuclear space (Fig. 3A). Quantification of the Proteostat® staining revealed an increased fluorescence intensity in the sulforaphane-treated ($p < 0.001$) than untreated (control) cells, suggesting that RS promotes protein aggregation (Fig. 3B). Interestingly, RS elevated the expression of native, as well as the phosphorylated forms of TAU (S202/T205) and α-SYNUCLEIN (S129) (Fig. 3C & S2A), demonstrating misfolding or unfolding events that can trigger the formation of toxic oligomers and abnormal intracellular aggregates [38]. Next, we elucidated whether the elevated Tau and α-synuclein are localized within the protein aggregates, we performed a double-fluorescence staining of N2a cells with Proteostat® followed by immunostaining with phosphorylated and non-phosphorylated Tau and α-synuclein antibodies. The results indicate that the Tau/α-synuclein were localized within the protein aggregates (data not shown). A strong correlation between glutathione redox (GSH/GSSG) and protein aggregation (*i.e. Tau & α-synuclein*) (Fig.S2B) revealed that RS promotes proteotoxicity in neuroblastoma cells. Next, we determined the extent of protein aggregation in a chronic RS condition. Following prolonged sulforaphane-treatment (for five-consecutive generations), we observed discrete punctuate staining and an intense accumulation of perinuclear/nuclear protein aggregates in the N2a cells (Fig. 4A and B). Of note, the rate/intensity of nuclear protein aggregation was more prominent in the chronic vs. acute RS condition.

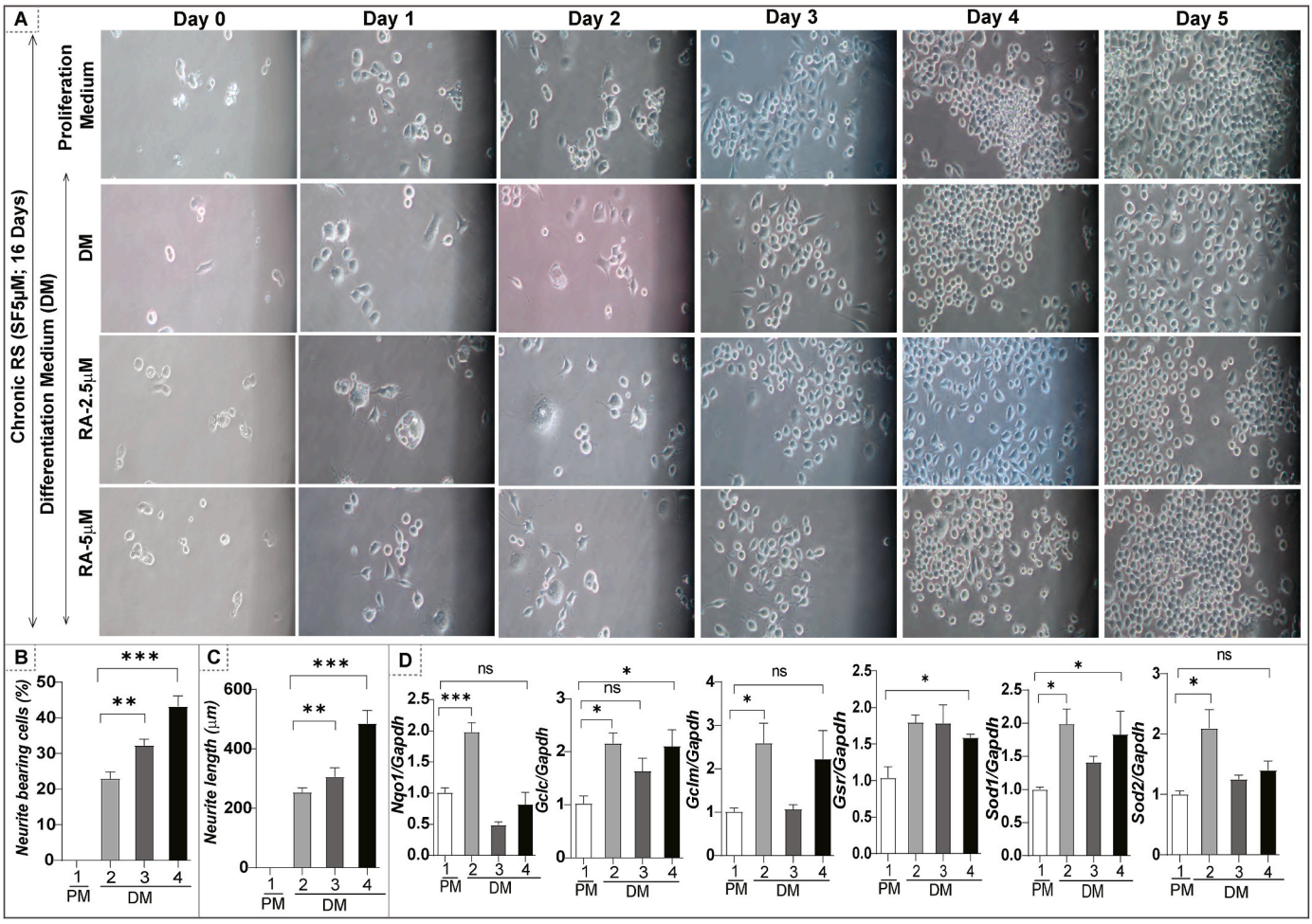


**Fig. 6. Effect of chronic reductive stress (cRS) on the differentiation of neuroblastoma cells:** N2a neuroblastoma cells were cultured in proliferation medium with sulforaphane and passaged every 48 h for up to five generations to establish chronic reductive stress (cRS). The cells were then subjected to differentiation using serum depleted medium (1:1 DMEM: OMEM comprising 0.5% FBS) containing retinoic acid (RA, 2.5 and 5.0 μM) and sulforaphane (5.0 μM) up to 6 days. **(A)** Bright-field microscopic images of N2a neuroblastomas during proliferation and differentiation (days 1–5). Chronic RS (sulforaphane 5.0 μM) impairs the differentiation/neurogenesis of N2a neuroblastoma cells (Magnification 20×). **(B)** The percentage of neurite bearing cells [n = 4, 2 images (different regions) per group]. **(C)** The neurite length was measured in all the groups using Fiji (ImageJ) with NeuronJ plug-in and expressed as μm [n = 4, 2 images (different regions) per group]. **(D)** qPCR validation of antioxidant genes *(Nqo1, Gclc, Gclm, Gsr, Sod1 and Sod2)* in N2a cells (experiencing cRS; n = 3–4) treated with RA (2.5 & 5.0 μM). Data are presented as Mean ± SEM. Significance: *p < 0.05; **p < 0.01; ***p < 0.001; ns – no significance.

### 3.4. Reductive stress impairs neurogenesis in N2a neuroblastoma cells

A balance between oxidants and reductants confer a steady state of physiological signaling required for neurogenesis [39]. Here, we hypothesized that shifting the redox towards RS would impair neurogenesis in N2a neuroblastoma cells. To understand whether RS dissuades neurite outgrowth, retinoic acid (RA), a known inducer of neurite outgrowth in N2a cells, was used [28]. First, a dose-dependent effect of RA (to induce differentiation) on the viability of N2a cells was determined by the MTT assay (Fig.S3A) and 2.5 and 5.0 μM was selected to induce differentiation. In response to RA treatment, we noticed a dose- and time-dependent increase in the differentiation of N2a cells into axons/dendrites. Furthermore, while neurite outgrowths were seen on day 1, the formation/maturation of neurons/axons began on day 3 in the RA (2.5 & 5.0 μM) treated cells (Fig. 5A). Fully grown neurons with axonal projections, dendrites, and synaptic clefts were apparent on day 5 (Fig. S3B). Of note, the development of telodendria and synaptic bouton were minimal or null in the N2a cells fed with differentiation medium containing sulforaphane ($p < 0.001$), as they exhibit RS (Fig. 5A). The morphologic and morphometric analyses show less number of cells with neurite outgrowth (axon-like extensions) and the decreased neurite length (at day 5) in sulforaphane (5.0 μM) co-treated cells when compared to RA-only-treated cells ($p < 0.001$) (Fig. 5B–C & Fig.S3B). After observing interesting outcomes in neurogenesis during acute RS, we then explicated the impact of chronic RS on neurogenesis through sulforaphane treatment for five successive generations prior to differentiation. We observed vigorous suppression of neurite outgrowth ($p < 0.001$) and differentiation upon chronic RS (Fig. 6A–C). This impairment in neurogenesis correlates with robustly augmented antioxidants (Fig. 6D) and intensely increased protein aggregation/proteotoxicity upon prolonged RS (Fig. 4A and B). These observations in acute and chronic RS conditions indicate a prerequisite of pro-oxidant signaling during neuronal differentiation.

### 3.5. Rescuing N2a cells from RS reactivates neurogenesis through suppressing the pathologic GSK3β/Tau cascade

To investigate the mechanism behind how RS impairs neurogenesis, we devised a rescue-experiment. Briefly, the N2a cells were subjected to differentiation using RA-5.0 μM and co-treated with sulforaphane (5.0 μM). In the rescue group, the sulforaphane was withdrawn from the differentiating cells after 3 days and continued the differentiation for

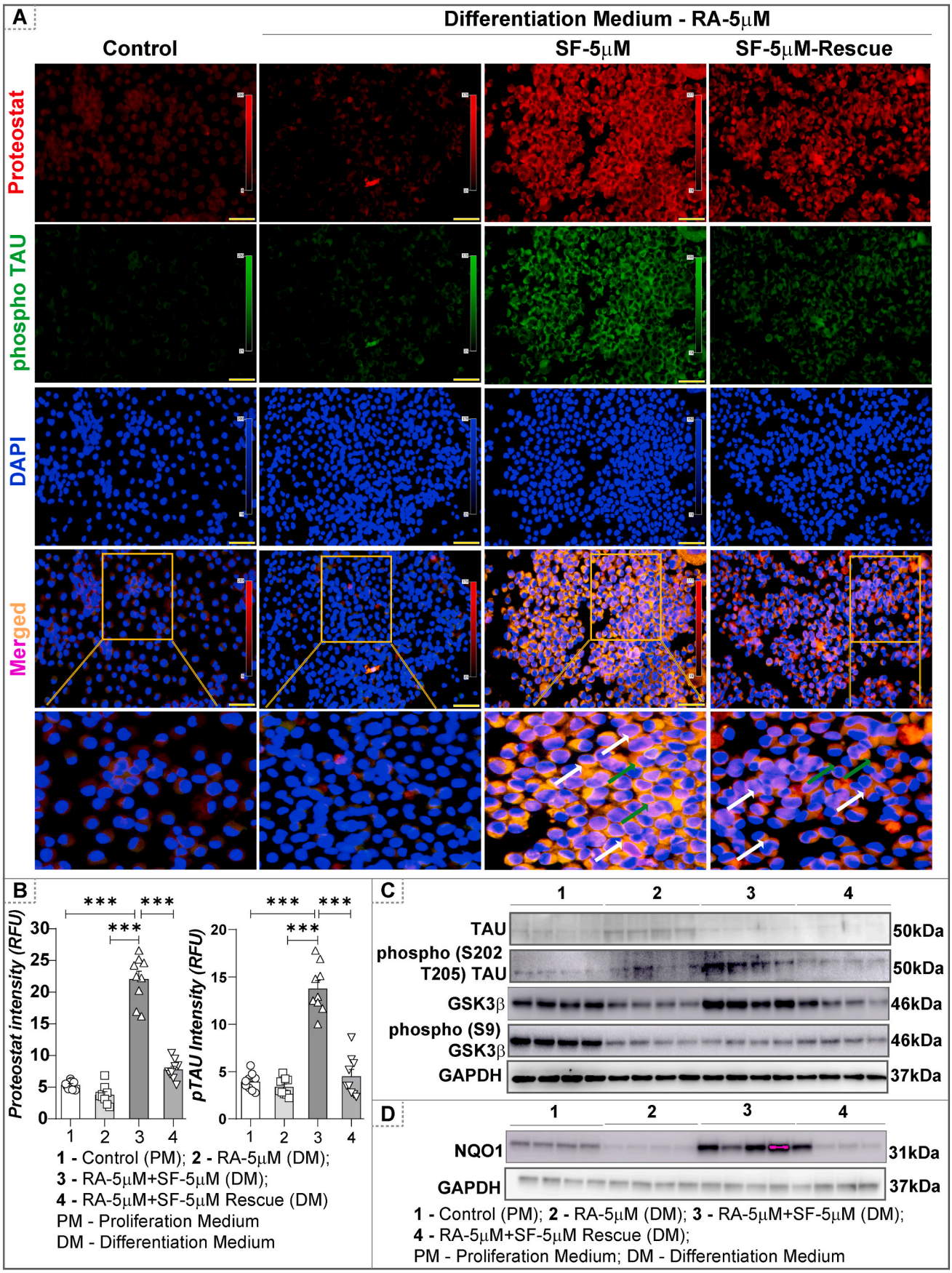


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**Fig. 7. Rescuing N2a cells from RS reactivates neurogenesis through suppressing the pathologic GSK3β/Tau cascade:** To examine the mechanistic link of how RS impairs neurogenesis, we rescued the differentiating cells from RS by withdrawing sulforaphane at the end of day 3 and allowed the cells to differentiate for additional three days (Total experimental period 6-days). **(A)** Fluorescence images of Proteostat® and phospho (S202, T205) TAU immuno-staining in control and differentiating N2a cells treated with/rescued from sulforaphane. Following sulforaphane-treatment, we observed discrete punctuate staining and an intensive accumulation of perinuclear protein aggregates along with elevated phospho TAU, whereas rescuing N2a cells from sulforaphane exposure declined the Proteostat® intensity. **(B)** Quantification of the Proteostat®/phospho TAU staining within cells revealed increased fluorescence intensity in the sulforaphane-treated ($p < 0.001$) than rescued cells [n = 3, 3 images (different regions) per group]. **(C)** Immunoblot analysis of TAU, phospho (S202, T205) TAU, GSK3β, phospho (S9) GSK3β and **(D)** NQO1 was performed in differentiating N2a cells treated with/rescued from sulforaphane (n = 4). Activation of GSK3β in sulforaphane treated cells which was partially declined upon the rescue of sulforaphane. Data are presented as Mean ± SEM. Significance: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns – no significance.

additional 3 days (Day 0 – Day 6). As expected, we noticed a dose- and time-dependent increase in the differentiation of N2a cells into axons/dendrites in response to RA treatment, and this was impaired in sulforaphane treated N2a cells (Fig.S4A). However, upon withdrawal of sulforaphane (rescue experiment) we observed significant reactivation of differentiation at day 4 (12-h after sulforaphane withdrawal) and fully matured/differentiated neurons on day 6 **(**Fig.S4A**)**. Next, we determined the extent of protein aggregation in these cells by Proteostat® and phospho TAU dual immunostaining. Of note, we observed an elevated deposition of protein aggregates and phospho (S202, T205) TAU ($p > 0.05$) in sulforaphane co-treated cells but this was partially reversed following sulforaphane withdrawal (Fig. 7A-B). Moreover, immunoblot analysis of native and phosphorylated forms of TAU and GSK3β revealed that sulforaphane favors the activation of GSK3β (dephosphorylation at S9 to native GSK3β), which induces TAU phosphorylation leading to proteotoxicity and impaired neurogenesis **(**Fig. 7C and S4B**)**. We also confirmed that the antioxidant signaling is reduced ($p < 0.05$) following sulforaphane withdrawal (*i.e.* NQO1 protein levels; Fig. 7D and S4B).

## 4. Discussion

Integrity of the proteostasis network is crucial for any living cell to maintain basal signaling and physiological functions [40,41]. In the recent past, we have demonstrated that shifting the redox towards a "reductive arm" exacerbated protein aggregation, which then induced pathological cardiac remodeling and heart failure [42,43]. Here, we investigated whether a reductive milieu (*i.e. RS*) could induce protein aggregation and impair neurogenesis. Our findings revealed that reductive stress (i) diminishes the ROS signaling along with profound increase in gene and protein expression of key antioxidants; (ii) impairs ER stress/protein folding signals; (iii) induces Tau and α-Synuclein phosphorylation/aggregation; (iv) impairs the axonal outgrowth in neuroblastoma cells; and (v) evokes GSK3β dependent signaling involved in Tau phosphorylation during neurogenesis. Altogether, our results suggest that RS impairs proteostasis, aggravates proteotoxicity and suppresses neurogenesis.

Intact ER function is crucial for protein conformation and biological activity in cells as well as for terminally differentiated cells like neurons [44]. Perturbations in ER function leads to dysfunction of synapses and axonal transport, thereby triggering neurodegeneration [40]. In this study, we observed that RS induces ER stress/dysfunction, leading to impaired protein folding and aggregation. While the transcript was unaltered, GRP78 protein was increased in response to RS, suggesting possible misfolding and its accumulation. Increased GRP78 and ER stress were consistent with Aβ plaque formation in postmortem brains of AD patients [45]. Of note, the PDI protein was decreased in N2a cells under RS, which could impair the disulfide exchange [46]. Inactivation of PDI through oxidation was also observed in AD patients [47]. Deregulation of protein thiol redox may impair protein stability and prolong the ER stress [48]. Moreover, excessive GSH will cause the ER microenvironment to be in a reduced state, accompanied by reductive stress, which triggers unfolded protein response (UPR) [49]. We postulate that augmentation of reductive-redox signaling, leading to impaired glutathionylation of oxidized proteins, may cause adverse changes in neuronal function as observed in the current study (Figs. 1A and Fig. 2A–C).

Many studies implicate that the impairment of protein quality control (PQC) mechanisms may lead to an abnormal accumulation of proteins and proteotoxicity [50–52]. We observe elevated phosphorylated forms of both the Tau and α-synuclein in neuroblastoma cells experiencing RS. Earlier studies have reported protein aggregation as a cellular response to OS placing ROS in the centerfield and attributing it to the cause and consequence of protein aggregation [41]. However, in our study despite diminished OS/ROS we observed enhanced protein aggregation. One reason might be due to impaired ER protein folding and subsequent ER stress observed in this reductive milieu which may promote proteotoxicity. In Alzheimer's brain, increased levels of ER stress markers were shown to closely correlate with the presence of Tau (native as well as the phosphorylated form) [53], suggesting that ER stress in neurons occurs at an early stage and that prolonged ER stress is involved in both Tau aggregation and neurodegeneration in Alzheimer's pathogenesis. Similar to that of the association between Tau and ER stress, an earlier report indicated that α-synuclein aggregation increases its interaction with GRP78 in SH-SY5Y neuroblastoma cells as well as in the SYN120 transgenic mice, suggesting sequestration of GRP78 in to aggregates [54].

Although it is believed that aggregation of Tau/α-Synuclein could trigger the antioxidant signaling [55] as a compensatory response, it is not clear whether this could sustain and lead to a reductive-condition. Hence, our novel model of RS in N2a cells (sulforaphane-induced Nrf2/antioxidant signaling) shown here, provide an evidence that a pre-existing RS could further impair the protein quality control, thereby exacerbating the aggregation of nascent proteins other than Tau/α-Synuclein. Moreover, it has been reported that sulforaphane treatment ameliorates OS induced aggregation of Tau and α-Synuclein by promoting antioxidant defense *via* Nrf2 [56,57]. These investigations were performed in a 3×Tg-Alzheimer's and rotenone-induced Parkinson's mice, respectively, wherein there was a well-established oxidative milieu and activating the Nrf2-antioxidant signaling in such-conditions might be protective or therapeutic. However, in the current study, activating the Nrf2-antioxidant signaling under basal-setting is detrimental to neurons as it promotes the aggregation of Tau and α-Synuclein.

Several reports indicate that neurogenesis is sensitive to OS [22,39,58] and perturbation in the redox balance might impair the neurogenic microenvironment and trigger pathological processes. Here, we found that either acute or chronic RS could suppress the neurogenesis. Interestingly, a decline in antioxidant signaling in RA-treated N2a cells may be attributed to pre-requisite of a pro-oxidative condition for differentiation. Nevertheless, upholding the antioxidant system through sulforaphane (*i.e.* RS) significantly inhibited differentiation. Earlier studies reported RA as a potent inhibitor of Nrf2-transcriptional activity [59] and pro-oxidative milieu as a vital factor for neurogenesis and neuronal development [22,58]. Although neurogenesis is regulated by various signaling pathways, the ability of GSK-3β to play an important role in adult neurogenesis has been reported [60,61]. This intrigued us to investigate whether RS impairs neurogenesis through GSK3β. Interestingly, we find that, there is a significant activation of GSK3β, resulting in enhanced phosphorylation of TAU and subsequent aggregation under RS. Pathological activation of GSK3β/Tau cascade in response to RS aggravates proteotoxicity in the N2a cell, thereby impairing their

regeneration. Previously, it has been reported that ischemic-injury based OS induces GSK3β phosphorylation in rats and human-induced pluripotent stem cell (iPS) derived neural stem cells [62,63]. Under RS-mediated proteotoxic stress, Tau hyper-phosphorylation might result in reduced differentiation due to impairment in its ability to act as a microtubule-associated protein [18]. Of note, rescuing the N2a cells from RS, reactivated the differentiation process (Fig. 7 and S4).

In conclusion, our results demonstrate that RS can be detrimental to neuronal structure and function. We report that acute, as well as chronic RS can impair neurogenesis through ER dysfunction/protein mis-folding signals and by activating pathologic GSK3β/TAU cascade, thereby promoting protein aggregation and proteotoxicity. Our data suggest that despite the association of OS and neuronal damage, RS (the extreme of OS) can play a crucial role in promoting proteotoxicity, and thereby lead to neurodegeneration. Moreover, this study adds to the emerging view that the regulation of redox homeostasis and its impact on diverse diseases is part of a complex process in which antioxidants are required only in response to an oxidative or toxic challenge in cells or organisms. Therefore, deciphering the exact mechanisms of how neurons sense and translate changes under RS into further downstream signaling events will be crucial to understand the early/late phases of neurodegenerative diseases.

## Translational statement

Our findings claim that previously reported preliminary clinical observations associated with reductive compensation during the progression of Alzheimer's disease might become detrimental due to a transition of an overcompensated hyper-reductive state to reductive stress.

## Declaration of competing interest

The authors declare that they have no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101739.

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