Modelling the unfolded protein response (UPR) in vitro

Abstract

This study investigates the IRE1 pathway activation within the unfolded protein response (UPR) by using SH-SY5Y neuroblastoma cells treated with Brefeldin A, Thapsigargin, and Tunicamycin. Qualitative PCR assessed the splicing of XBP-1 mRNA as an indicator of UPR activation, revealing that Brefeldin A and Tunicamycin substantially increase the relative intensity measure (RIM), indicative of heightened UPR activity. In contrast, Thapsigargin elicited a moderate response, suggesting a differential impact of ER stressors on UPR pathways. This investigation increases understanding of UPR's role in cellular stress and provides insight into the variable cellular responses to ER stress, with implications for diseases associated with protein misfolding.

Introduction

The UPR is a critical cellular mechanism activated in response to the accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER), a condition known as ER stress. To mitigate this stress UPR temporarily halts protein translation, degrades misfolded proteins, and activates genes involved in regulatory and cell survival processes through the ATF6, PERK, and IRE1 pathways (Markouli *et al.*, 2020). Among these, the IRE1 pathway plays a pivotal role in managing ER stress by splicing XBp-1 mRNA, thereby facilitating the expression of genes that restore normal cell function (Luo *et al.*, 2022). Despite significant advances in our understanding of UPR, questions remain regarding its dual role in promoting survival or contributing to pathology in diseases such as cancer, neurodegenerative and metabolic disorders (Siwecka *et al.*, 2021). Furthermore, the lack of comparative studies examining the effects of various er stress inducers withing a unified framework limits our understanding of nuanced responses elicited by different stressors as this is an ongoing research field (Chen and Cubillos-Ruiz, 2021).

This study aims to fill theses gaps by focusing on the IRE1 pathways activation in response to pharmacological stressors: Brefeldin A, Tunicamycin, Thapsigargin (Grandjean *et al.*, 2020). Therefore we hypothesise that as these compounds induce ER stress through the IRE1 pathways, the degree to which each treated cell does this will be evidenced by the increased splicing of XBP-1 mRNA. This experiment does not extend to other UPR pathways, nor does it address long-term physiological or pathological consequences of chronic UPR activation.

Methods

In this study, SH-SY5Y human neuroblastoma cells were propagated in DMEM/F12 medium enriched with 10% fetal bovine serum (FBS) and maintained at 37°C in a 5% CO2 atmosphere. To elucidate the cellular responses to ER stress, cells at 70% confluence were treated with predetermined optimal concentrations or ER stress inducers - Brefeldin A (5µg/mL), Thapsigargin (2µM), Tunicamycin (10µg/mL) – identified through dose-response experiments. Following treatment, cells were collected at various time intervals (0,2,4,6, and 8 hours) via trypsinisatuion and subsequently pelleted for analysis.

Total RNA was extracted using TRIzol reagent and quantified by NanoDrop spectrophotometry. A quantity of total RNA (1µg) was then reverse transcribed using SuperScript IV (50°C for 60 min) and oligo-dT primers, under conditions optimised for the generation of cDNA, which was later diluted to a tenth of its original concentration for polymerase chain reaction (PCR) assays.

The PCR amplifications were performed in a 25µL mixture containing Taq polymerase and XBP-1 specific primer that flank splice sites, under a cycling protocol that includes an initial denaturation at 95°C for 30 seconds, annealing (58°C for 30 seconds), and extension (72°C for 1 minute). The PCR products were then subjected to electrophoresis on a 2% agarose gel, visualised alongside a 100bp DNA ladder, and quantified via densitometry with normalisation against GAPDH expression levels.

For data analysis, the study was designed with three biological replicates and two technical replicates for each PCR, across all conditions and time points. Statistical analysis was conducted using a two-way ANOVA coupled with Tukey's multiple comparison test to assess the dynamics of XBP-1 splicing in response to ER stress over time. Additional analysis was

conducted in SPSS version 27, with an out pout of mean, median, maximum, minimum and range values. Graphs made in Excel.
The methodology outlined adheres to the highest ethical standards, with experimental protocols approved by the Manchester Metropolitan University review board, ensuring that all procedures were conducted responsibly and ethically.

Results

Quantitative analysis using two-way ANOVA revealed significant variations in the relative intensity measure (RIM) across SHSY5Y cells subjected to ER stress inducers ($F(3, 15) = 7.39$, $p<0.003$). Suggesting substantial differences and alterations in protein levels between the treated cells. Treatment with Brefeldin A resulted in a significantly elevated RIM expression level ($M=5029.70$, $SD=868.15$) in contrast to the control group ($M=1949.25$, $SD=473.87$). This heightened response was similarity in the Tunicamycin group, which exhibited the highest mean level of RIM ($M=5341.15$, $SD=1906.98$). Thapsigargin treatment displayed a moderate increase in expression ($M=3579.14$, $SD=1090.16$), intermediate between the control and Brefeldin A treatments, as shown in **Figure.1**. The data indicates the differential impact of ER stressors on cellular RIM levels and the distinct cellular mechanisms of ER stress induction.

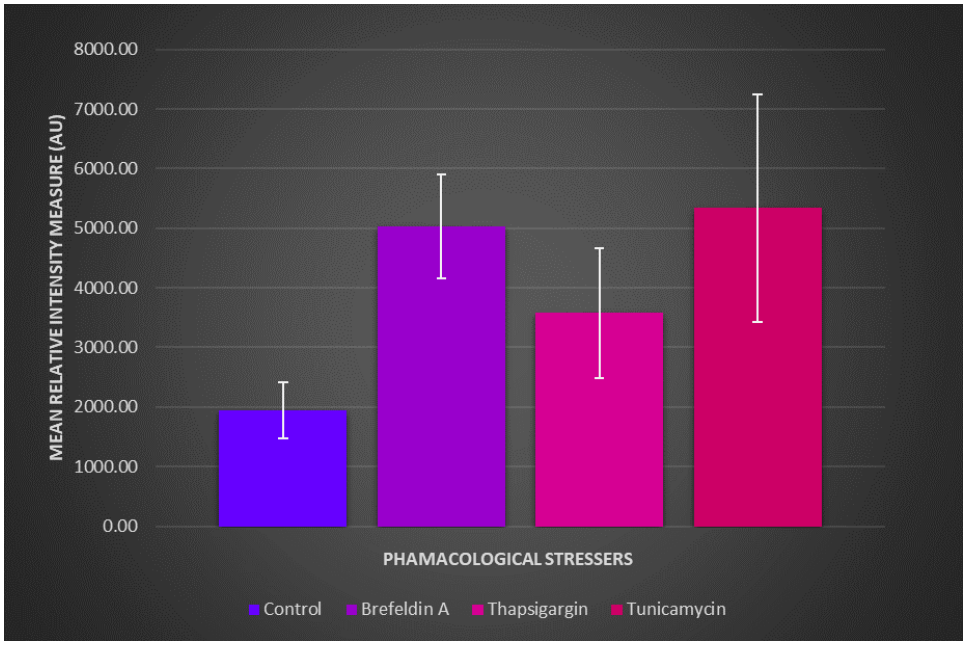

Figure.1 Bar graph showing mean cellular responses to ER stress in Arbituary Units (au) for untreated Control, Brefeldin A, Thapsigargin, and Tunicamycin. Error bars represet variability. The graph illustrates the relative UPR activation through the IRE1 pathway by each stressor.

Further analysis resulted in a boxplot shown in **Figure.2**, which presents a clear escalation in median RIM levels in response to the pharmacological agents, compared to the control group. The median for the RIM for the control was consistent with basal levels of UPR activity (Richardson, Kinkel and Kim, 2011) shown in **Table.1**.
Brefeldin A-treated cells exhibited a heightened median RIM implying a substantial UPR induction, while maintaining a narrow interquartile range (IQR). Thapsigargin treatment resulted in a closer median to the control with a wider IQR. Tunicamycin showed the greatest median and the broadest IQR, reflecting the most potent UPR activation and heterogeneity in cellular responses.

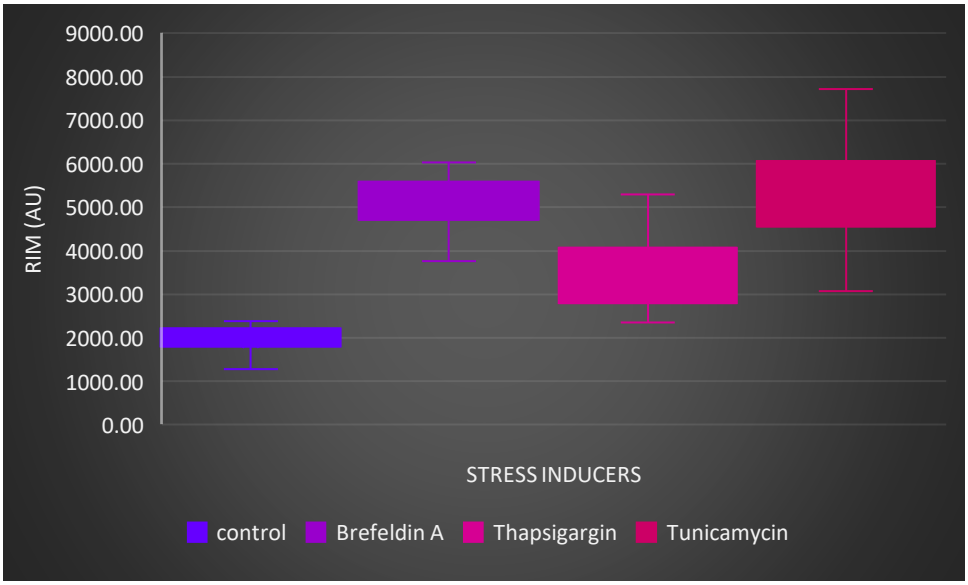

Figure.2 Boxplot of relative intensity measured in Arbitrary Units (au) indicating cellular response of SH-SY5Y human neuroblastoma cells to endoplasmic reticulum (ER) stress. Control (untreated) and cells treated with Brefeldin A, Thapsigargin, Tunicamycin are compared.

Table.1 Descriptive statistics of relative intensity measure in arbitrary units for cells under endoplasmic reticulum stress. Control and treatments with Brefeldin A, Thapsigargin, and Tunicamycin are compared, detailing, mean, median, maximum, minimum, standard deviation and range. This indicates variability in unfolded protein response activation.

Stress inducer	Mean (AU)	Median (AU)	Maximum (AU)	Minimum (AU)	Range (AU)	Standard Deviation
Control	1949.25	2063.35	2383.33	1286.96	1096.36	473.87
Brefeldin A	5029.70	5149.10	6028.35	3762.15	2266.19	868.15
Thapsigargin	3579.14	3337.46	5300.88	2348.33	2952.56	1090.16
Tunicamycin	5341.15	5283.06	7721.49	3076.98	4644.51	1906.98

Discussion

This study highlights the heterogeneous nature of UPR activations via the IRE1 pathway in SH-SY5Y cells by Brefeldin A, Tunicamycin, and Thapsigargin as known ER stress inducers where influence is indicated by an increase in XBP-1 mRNA splicing. Brefeldin A and Tunicamycin significantly enhanced the RIM intensifying the activation of UPR through transcription and translation of genes involved in protein folding, ER-associated degradation (ERAD), to mitigate ER stress which shows their potent roles in the UPR activation. this is especially true through inhibition of N-linked glycosylation. This results, while consistent with existing literature (**Figure.1**, **Table.1**) (Wu *et al.*, 2018), prompt further exploration into the selective activation of these pathways and their implications for cellular health.

Thapsigargin's moderate UPR induction, linked to ER Ca2+ ATPase (SERCA) inhibition and altered calcium homeostasis, suggests a nuance stress response that may not precipitate immediate cellular dysfunction but could have long-term implication, underlining the necessity of examining UPR's temporal dynamics. Unlike the more aggressive UPR triggers, Thapsigargin doesn't immediately compromise cellular functions but suggests a gradual, possibly accumulating stress effect. This indicates that Thapsigargin is a useful tool for exploring the early and adaptive phases of UPR, a perspective supported by {Sehgal, 2017}.

The potential therapeutic strategies inferred from modulating UPR highlight a promising avenue for preventing or treating diseases associated with ER stress. However, the feasibility of such interventions requires a comprehensive understanding of UPR's multifaceted roles in cell survival and pathology.

Potential limitations include inaccuracy when pipetting, dose-response relationship where cell sensitivity can influence generalisability. Overall, this experiment showed the varied degree of ER stress response and UPR activation depicted from the increase of levels of splicing, creating an in vitro model of the UPR response.

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Determination of the Receptor Types and Signal Transduction Mechanisms Involved in Purine Signalling in Glial Cells

Abstract
In this study, we investigated purine signalling in glial cells, focusing on the role of P2Y receptors in intracellular calcium dynamics. Ethically sourced mouse optic nerve tissues were treated with purine agents and assessed using Fluo-4 calcium imaging. The results demonstrate that P2Y receptor antagonism significantly attenuated calcium responses, confirming their central role in glial signalling. This aligns with existing literature on P2y receptors in physiological and pathological states. The differential effects of ATP analogues suggest varied receptor subtype activation, indicating multiple purinergic pathways in glial function. These findings contribute to further understanding of glial biology and have potential uses for therapeutic targets in CNS disorders.

Introduction
Glial cells, particularly astrocytes and microglia, indispensable roles in maintaining homeostasis and supporting neuronal function within the central nervous system (CNS) (Sofroniew and Vinters, 2010). Their involvement in inflammatory processes underscores their significance as indicators of neurological diseases. Purines, such as adenosine triphosphate (ATP) and adenosine, act as vital signalling molecules, modulating synaptic transmission by engaging glial cells to regulate activation and the release of inflammatory mediators (Fields and Burnstock, 2006; Ai *et al.*, 2023). Understanding the receptor types involved is crucial for elucidating the underlying mechanisms, offering insights into synaptic function plasticity, and potentially informing therapeutic strategies for neuroinflammatory conditions.
Despite recognising that purines exert their effects on glial cells through various receptors, the specific subtypes and their roles in different physiological and pathological processes remain incompletely understood. Additionally, there's a notable gap in research concerning the intracellular signalling cascades initiated by purines ('Purines 2018 Basic and Translational Science on Purinergic Signaling and its Components for a Healthy and Better World,' 2018). Hence, this research aims to identify and characterise the specific receptor types mediating purine signalling in glial cells, alongside uncovering the molecular pathway through which purines modulate glial function. The scope of this research will not include structural or biochemical characterisation of purine receptors, in vivo studies, or the development of therapeutic strategies.
Our hypothesis posits that purine signalling in glial cells involves distinct subtypes of P1 adenosine receptors (A1, A2A, A2B and A3) and P2 purinergic receptors (P2X and P2Y subtypes). Specifically, we predict that activation of A1 receptors inhibits cyclic adenosine monophosphate signalling (cAMP), while activation of A2A receptors stimulates cAMP production. Furthermore, we propose that P2X receptors mediate fast, ionotropic responses via calcium influx, whereas P2Y receptors initiate slower metabotropic responses through G-protein-coupled pathways.

Experimental Design and Ethical Considerations
In accordance with ethical guidelines and regulations for animal research, all procedures involving animal were conducted following the guidelines set forth by the Home Office of the United Kingdom under the Animals (Scientific Procedures) Act, 1986. Humane treatment of animals was ensured throughout the experiment.
Tissue samples of optic nerves were obtained from mice following euthanasia procedures. Optic nerves were isolated intact and immediately transferred to chilled oxygenated artificial cerebrospinal fluid (aCSF) containing: NaCl, 133; KCl, 3; CaCl2, 1.5; NaH2PO4, 1.2; MgCl2, 1.0; D-glucose. 10; HEPES buffer, 10; pH 7.3). anaesthesia was administered during tissue collection to minimise pain and distress in animals.

Fluorescent Calcium Imaging Setup
Fluo-4, calcium dye (4 Micro-Moles; Molecular Probes) was used for labelling glial cells and capturing changes in intracellular calcium levels. Optic nerves were incubated for an hour at room temperature (RT) in aCSF containing Fluo-4 dye. Dye loaded nerves were then placed in a chamber on an upright confocal microscope (Zeiss LSM 5 Pascal Axioskop 2) and continuously

perfused with aCSF solution. Excitation of fluo-4 was performed at 488nm using argon-ion later sources and fluorescent emission (green) of ca2+-bound Fluo-4 was detected at 525 nm using optical filter sets designed for fluorescein. (FITC).

Data Acquisition and Analysis

Changes in Fluo-4 fluorescence intensity in glial cell bodies were captured at 200-400 ms intervals. Regions of interest were selected, and changes in fluorescence intensity were measured relative to baseline using Zeiss LSM Image Examiner software V 4.0. consistency and reproducibility in data acquisition were ensured by standardising dye loading, image processing, and baseline normalisation procedures.

Peak response measurements and calculations were based on the maximum change in fluorescence intensity compared to baseline (expressed in arbitrary units). Statistical analysis methods such as analysis of variance (ANOVA), pairwise independent t-test, and Bonferroni correction for multiple comparisons were used to compare responses of different experimental conditions.

Drug Application and Experimental Procedures

Test agents (ATP, P2 receptor agonist and antagonist) were dissolved in aCSF and applied to optic nerve preparations at a concentration of 10microMoles. Proper control experiments, including ATP application for calcium response comparison and antagonist washout, were conducted to validate the specificity of drug effects.

Quality Control, Validation and Safety

To address potential sources of variability or bias in experimental procedures, measures such as replicating experiments, performing internal controls, and comparing responses across different experimental conditions were implemented. Validation of experimental results was ensured through rigorous quality control measures. All potential risks associated with experimental procedures were identified and addressed in accordance to the Manchester Metropolitan University's laboratory safety guidelines.

Results

The experiment to assess the impact of various agent on intracellular calcium response in glial cells, as indicated by changes in Fluo-4 fluorescence (DeltaF/F), revealed significant variations among treatments (ANOVA, $F(7,72) = 48.52$, $p<0.0001$. Subsequent t-test comparisons shown in **Figure.1** highlighted that the P2Y antagonist MRS2179 significantly reduced the calcium response compared to ATP (117.27, 31.22, $t(18) = 13.14$, $p<0.0001$), MeSATP (130.30, 37.60, $t(18) = 10.69$, $p<0.0001$), and BzATP (82.50, 24.31, $t(18) = 10.30$, $p<0.0001$) treatments. Thapsigargin, a SERCA pump inhibitor also elicited a distinct response (34.70, 25.70, $t(18) = 7.41$, $p<0.0001$). These differences were corroborated by Bonferroni-corrected post hoc analysis, maintaining statistical significance and reinforcing the notion of specific agent effect on calcium dynamics. **Table.1** summarises the t-tests with corrected p-values for all comparisons.

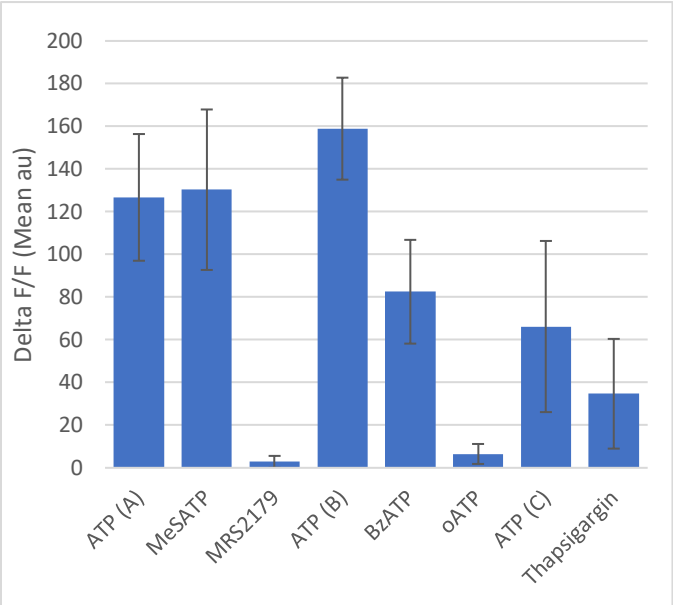

Figure.1. Shows the mean relative changes in fluorescence using agonists and antagonists for the examination of P2Y receptors, (agonist-ATP (A), P2Y agonist- MeSATP, P2Y antagonist-MRS2179). The examination of P2X receptors, (agonist ATP (B), $P2X_7$ agonist BzATP, $P2X_7$ antagonist oATP). Finally, the evidence that ATP stimulates Ca^{2+} release from intracellular stores shown by ATP (C) and thapsigargin. Standard error of mean was also calculated in Excel for each data point.

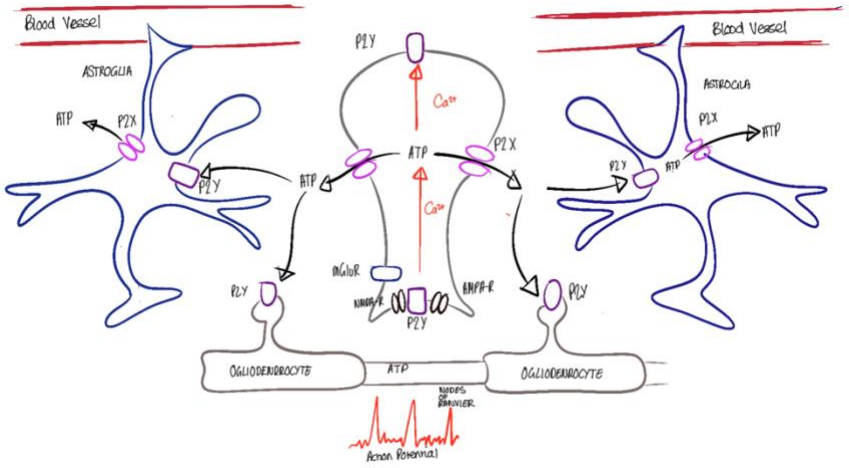

Figure.2. Illustrates the actions/role of ATP, receptors (P2Y, P2X), and pathways that are involved in calcium response. Diagram was drawn by student using software Procreate.

Discussion

This study identifies the role of P2Y receptors in mediating intracellular calcium responses within glial cells, as evidenced by significant variations among treatments (ANOVA, $F(7,72) = 48.52$, $P<0.0001$). P2Y antagonist MRS2179 significantly reduced calcium responses compared to ATP and its analogues, as highlighted in **Figure.1**, This suggestsRS2179 effectively blocks the activation of P2y receptors, which are crucial for mediating ATP-induced calcium signalling in glial cells showing the receptors critical involvement in calcium signalling (Puchałowicz *et al.*, 2014). Furthermore, Thapsigargin known to inhibit the SERCA pump and increase intracellular calcium by releasing calcium from ER stores, has shown a distinct response pattern. This mechanism contrasts with the receptor-mediated effects of other agents, which suggests an intricate interplay between extracellular ATP and intracellular calcium release, aligning with (Verkhratsky and Butt, 2013) findings on the importance of calcium signalling in astrocyte function and neuron-glia function communication.

Our findings, indicating differential engagement of P2 purinergic receptors by ATP and its analogues, resonate with the observation made by (Burnstock, 2007). Suggesting a complex receptor landscape within glial cells that warrants further investigation into the specific downstream pathways involved in calcium signalling dynamics.
However, the reliance on fluorescence changes as the sole measure of calcium responses may overlook other aspects of receptor activity and intracellular signalling cascades. Future research should aim to dissect the specific G-protein subunits, secondary messengers, and kinases activated following receptor engagement to provide a more comprehensive understanding of the mechanisms at play.

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