Pre-Clinical Investigations of Re-Purposed and Novel Compounds that Reduce or Eliminate Synucleinopathy in a Human Neuronal Model of Parkinson's

Yvonne Clarke; Supervisor: Tilo Kunath (Scottish Centre for Regenerative Medicine) University of Edinburgh

Project Aim

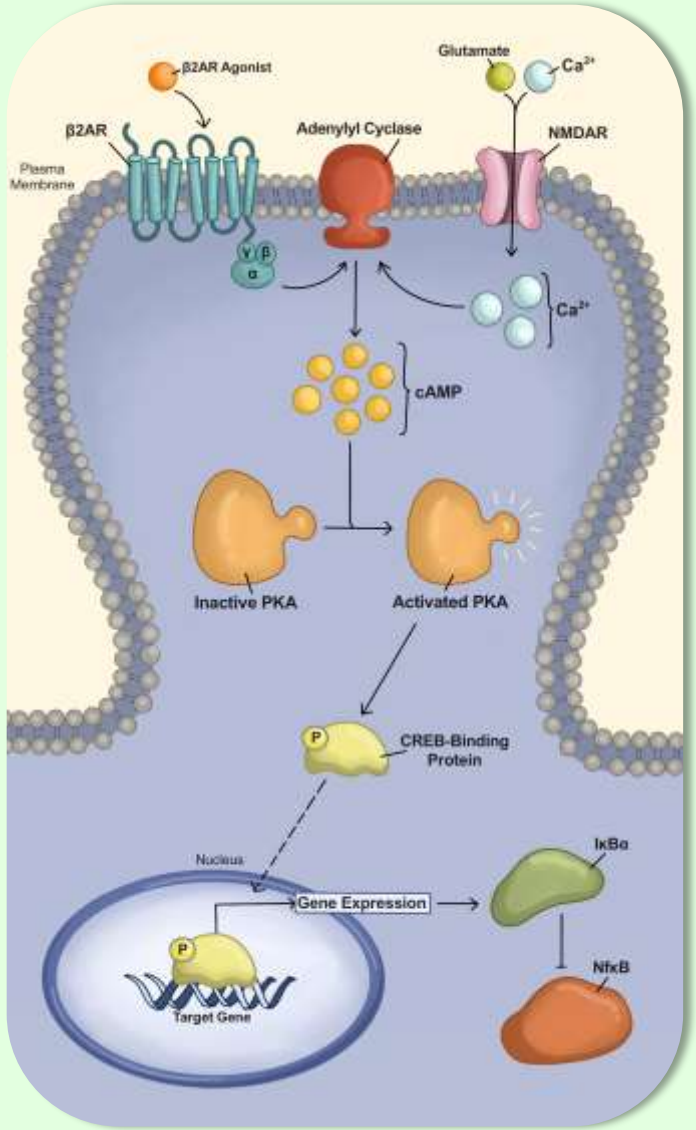
The aim of this ongoing project was to validate the ability of Clenbuterol and Riluzole to reduce α-synuclein transcription in a human neuronal model of Parkinson's with synucleinopathy. By comparing these drugs, we have attempted to validate data published by Mittal et al.*, of the most potent repurposed drugs for PD in an effort to identify existing compounds that can be used for this new therapeutic purpose.

Background

One hallmark of Parkinson's Disease (PD) is characterised by the dysfunctional aggregation of the protein α-synuclein within cells in the brains of sufferers. The α-synuclein gene locus, **SNCA**, when mutated or multiplied is sufficient to cause familial PD, with an amplification of SNCA mRNA and transcribed protein between 50-100%. The most significant risk from a genetic standpoint in terms of predisposition to PD is that of polymorphisms within and around the SNCA gene. However, the causes for sporadic PD have remained elusive, and as such it has been complicated to identify any one target to investigate. The one common link is this synucleinopathy.

As such, much drug development in PD has focused on clearance of α-synuclein protein, identifying a barrier that prevents its transformation into a toxic species or attempting to mediate its pathogenic downstream effects. As it has been shown that deletion of SNCA acts as a protective mechanism against PD-inducing agents, here, we proposed that the use of re-purposed drugs Riluzole (acting on the N-methyl-D-aspartate receptor) and Clenbuterol (acting on the β2-adrenoreceptor) – compounds which act to reduce SNCA transcription as opposed to targeting the protein itself – may make it possible to delay disease progression of PD.

Both the N-methyl-D-aspartate receptor (NMDAR) and the β2-adrenoreceptor (β2AR) are found on midbrain dopaminergic (mDA) neurons.



β2AR is stimulated by agonist

β2AR activates adenylyl cyclase

Activation of adenylyl cyclase induces intracellular increase of cyclic adenosine monophosphate

Increased cAMP concentrations lead to the activation of protein kinase A (PKA)

Activated PKA phosphorylates cAMP-response binding element (CREB)

CREB induces synthesis of protein IκBα, an inhibitor of NfκB

NfκB cannot translocate into nucleus and thus is prevented from inducing pro-inflammatory gene expression

*Original Paper: Mittal et al., 2017. β2-Adrenoreceptor is a regulator of the α-synuclein gene driving risk of Parkinson's disease. doi:10.1126/science.aaf3934

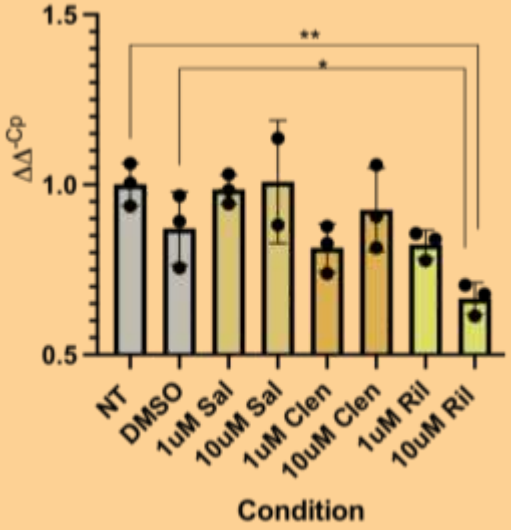
Analysis of SNCA Expression in Neuronal Cell Lines.

Methodology.

- The cell lines used in this investigation have been established to present with Lewy pathology.
- We treated the cell line SHSY-5Y and a midbrain dopaminergic neuronal (mDA) cell line with varying concentrations of drugs Riluzole, Clenbuterol and Salbutamol, using DMSO as a vehicle control, and synthesised cDNA after a 48 hour to 4 day period.
- We then quantified the levels of SNCA and ADRB2 expression using an RT-qPCR IDT analysis. Statistical analyses performed were One-way Anova and a students T-test in order to identify any significant decreases in SNCA transcription to be used as an indicator of the drugs potential.

SHSY-5Y Cells

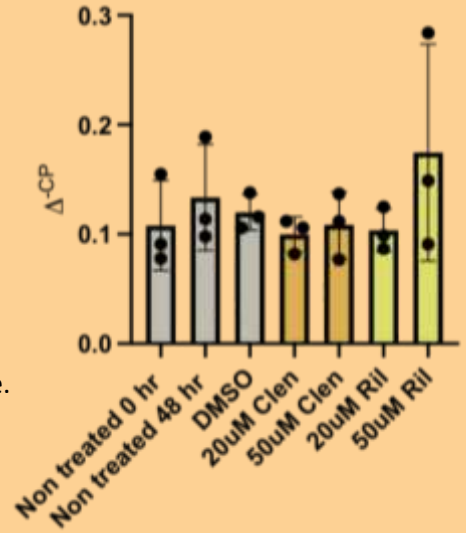
- SHSY-5Y cells showed no apparent difference between conditions, with no significant difference seen between non-treated and drug-treated samples.
- These results indicate that the drugs are not reducing SNCA transcription in these cells. When compared with the original paper with which we were attempting to validate, we found these results to corroborate theirs in that SNCA expression is not reduced in these cell lines upon drug treatment as the level of SNCA transcription is lower than in other cell lines.



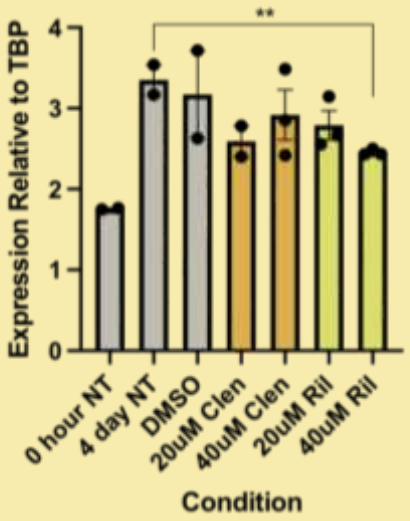
We hypothesised, based off the original paper, that incubation time correlates with increased SNCA reduction. As such, we increased the incubation time of all samples to a 4-day treatment period.

2-Day Treated Neurons.

- A significant reduction in SNCA transcription was observed between non-treated samples and the DMSO vehicle control with samples treated with 10µM Riluzole.

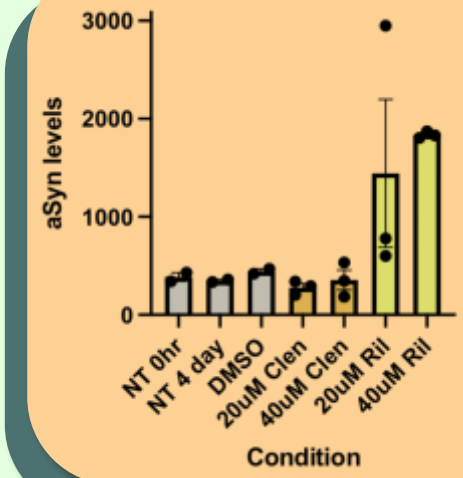


4-Day Treated Neurons.



- As in above experiment with mDA neurons, it was only when treated with Riluzole (in this case, **40µM Riluzole**) that the cells showed a significant decrease in SNCA transcription when compared to 4-days non-treated cells.
- A trend towards a decrease can also be seen in samples treated with 20µM of Clenbuterol, however this trend is not continued in 40µM Clenbuterol-treated cells.
- This is consistent with what is observed for both Clenbuterol- and Riluzole- treated samples in SHSY-5Y cells; this may be due to either cell stress induced by media deprivation, or cytotoxic effects of the drugs at too high concentrations.

Analysis of α-synuclein expression in mDA Neurons



- When investigating the levels of α-synuclein expression in mDA neurons, we saw that there is a large increase in the protein levels in both 20uM and 40uM – treated samples.
- On the contrary, we saw that both Clenbuterol-treated samples showed a decrease in α-synuclein expression.

This is interesting as it suggests two different mechanisms of action for the two drugs:

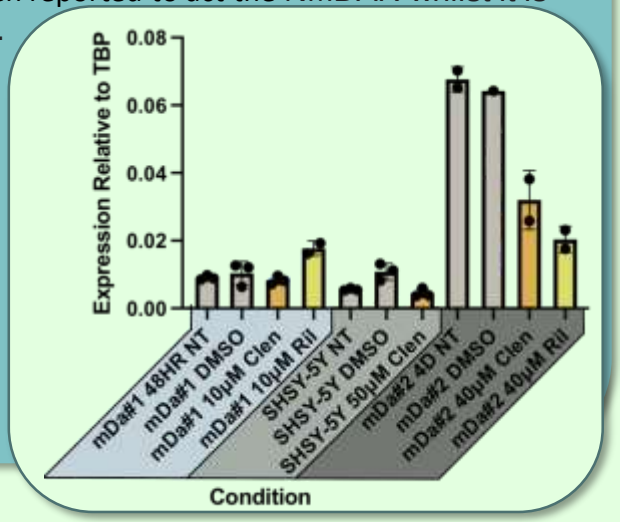
1. The β2AR may act to regulate transcription of SNCA and α-synuclein protein expression through H3K27 acetylation of promoters and enhancers within the SNCA locus. Agonists of the β2AR receptor, such as Clenbuterol and Salbutamol, have been shown to correlate with decreased H3K27 acetylation levels, which is reflected by a decrease in SNCA transcription, thus providing evidence that the β2AR is linked to α-synuclein transcription, and thus the risk of developing PD.

2. A mechanism of action has yet to be determined for Riluzole, however both drugs receptors - the β2AR and the NMDAR – are both inextricably linked by their pathway of activating CREB, and as such have been thought to act similarly. We found that Riluzole is actually reported to act through the NMDAR as an antagonist. By blocking NMDAR, Riluzole consequently blocks the transmission of glutamate through the channel and prevents re-uptake of gamma-aminobutyric acid (GABA), whilst also stabilising sodium channels. These effects all mitigate a neuroprotective action profile upon neurons when exposed to the drug.

Analysis of the β2AR expression levels

Following the findings of differential SNCA expression upon drug treatment, we decided to explore the expression levels of the β2-adrenoreceptor (β2AR).

- In the 2-day treated neurons (termed mDA #1), there is an **increase in receptor expression** from the non-treated samples to the **Riluzole- treated samples**.
- Although this appears to be consistent with the reduction in SNCA expression observed in the qPCR analysis, this result is puzzling as Riluzole has been reported to act the NMDAR whilst it is Clenbuterol that is presumed to act through β2AR.
- This contradicts what is seen in the 4-day treated neurons (mDA #2), however, with **clenbuterol-treated samples showing a higher level of β2AR expression** than those treated with Riluzole.
- An interesting point to mention is that **the drugs appear to reduce β2AR expression in the 4-day treated neurons**, which contradicts what is seen in the 2-day treated neurons in which β2AR expression appears to increase in Riluzole-treated samples. The reason for this is yet unclear and requires further investigation



Future Steps:

- To imitate how the drug would be used commercially, we intend to administer the drug over a period of 4 days as opposed to a 4 day incubation. This may act to ameliorate cytotoxic effects resulting from a heavy drug dose, as well as mitigate any efforts by the cell to remove the drug
- Compare intracellular and extracellular levels of α-synuclein in Riluzole-treated samples to investigate whether Riluzole acts to increase its secretion from the cell - would point to both whether it is this particular receptor through which Riluzole is acting, as well as give an indication to a mechanism of action for Riluzole as a therapeutic in PD.