Characterisation of the immunogenicity of stem cell derived neural cells in response to an inflammatory stimulus and flavonoid treatment.

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Abstract

Neurodegenerative diseases (NDD) such as Alzheimer’s Disease (AD) and Parkinson’s disease (PD) are debilitating conditions characterised by the progressive degeneration of neurons within the central nervous system (CNS). These diseases currently have no known cure; however extensive research is being carried out into potential therapies to help alleviate or delay the onset of symptoms. Inflammation in the CNS is a common known pathology of both AD and PD respectively, and is modulated by different neural cells within the CNS. Furthermore, flavonoids are naturally occurring compounds found in plants and other food products, and have been widely researched for their anti-inflammatory properties in many models of disease. By looking at flavonoids mechanisms of neuroprotection, and by understanding the mechanisms of neuroinflammation it could be possible to find new novel treatments for NDD.

The aim of this experiment is to characterise the immunogenicity of human embryonic stem cell (hESC) derived neural stem cells (NSC), astrocytes, and neurons in response to an inflammation induced by interferon gamma (IFN-γ). Furthermore, the anti-inflammatory effects of the flavonoid apigenin will be assessed by monitoring the expression of tumour necrosis factor alpha (TNF-α). The success of apigenin to modulate cytokine release could be of further use in treatments of chronic inflammatory conditions such as AD and PD.

The current study tests the viability of NSCs, astrocytes and neurons to increasing concentrations of IFN-γ in order to gain an insight into the cell tolerance for this inflammatory cytokine. Moreover, an enzyme-linked immunosorbent assay (ELISA) was carried out to monitor the expression of TNF-α in response to inflammatory insult from IFN-γ, and further analyse the effects of adding the flavonoid apigenin.

The results of the present study show that IFN-γ was unable to elicit a significant pro-inflammatory effect on NSCs, astrocytes and neurons viability, with potential questions left on the concentration-dependency of this effect. Furthermore, ELISA shows that again, IFNy did not increase the cellular expression of TNF-α, and apigenin did not show a significant ability to modulate the inflammatory cytokines present.
1. Introduction

Neurodegenerative diseases (NDD), such as Alzheimer’s disease (AD) and Parkinson’s disease (PD) are characterised by the progressive loss of neurons in the central nervous system (CNS) (Amor et al., 2014; Niikura et al., 2006; Frisardi et al., 2016). Over the last 25 years, extensive research into possible treatments for NDD has been occurring, and neural stem cells have shown great potential in advancing the understanding of the pathophysiology of these different diseases (Darbinyan et al., 2013). Currently there are no available cures, so the need for research into novel therapeutic methods for preventing and/or treating these diseases is increasing. It has been previously described that neural cell cultures could provide this essential step in learning about mechanisms of neurodegeneration, by modelling different disease conditions in vitro, and there are a number of ongoing clinical trials that use neural stem cells as potential treatments for CNS disorders (Bang, 2016). However, there are a lack of human studies available in this area of research and most previous studied cell cultures are derived from rodents (Ray et al., 2014).

Neural stem cells (NSC) have been widely researched for potential therapeutic uses in diseases such as AD and PD (Rossignol et al., 2014; Svendsen et al., 2012; Gage et al., 2013; Löser et al., 2010), and current advances in stem cell research have led to the discovery that NSCs can be used to replace lost or damaged neurons, help restore function, and promote neuroplasticity in different models of NDD (Björklund et al., 2000); this could prove beneficial to human sufferers of NDD if the research is translated into clinical practice.

NSCs can be derived by a number of different methods highlighted in Figure 1. Human embryonic stem cells (hESC) are obtained from early blastocysts, and are pluripotent cells that can be cultured in vitro and can differentiate into any somatic cell type (Moon et al., 2006). For the purpose of this study, the NSCs used were derived by this method.
Figure 1. Schematic diagram showing the potential ways to derive neural stem cells (NSC), for this study, NSC were derived from the blastocyst of human embryonic stem cells (hESC) (Jones et al., 1998 Casarosa et al., 2014).

A considerable advantage of these NSCs is that they can differentiate into cells of the neural lineage, including functional neurons, astrocytes and oligodendrocytes (Wichterle et al., 2002); this gives rise to the potential to provide therapeutic benefit to diseases where these cells are lost or damaged, such as reduction in substantia nigra dopamine neurons observed in PD (Hirsch et al., 2012).

Inflammation is one of the major pathological mechanisms that leads to death of neural cells, and is known to have considerable implications in NDD (Glass et al., 2010; Siebert et al., 2015). Much of the understanding of how the brain responds to inflammation is credited to studies on AD and PD respectively, with inflammation observed in AD as a consequence of amyloid β peptides, and formation of neurofibrillary tangles (Akiyama et al., 2000); and post mortem PD brains have showed activated microglia along with increased expression of pro-inflammatory cytokines (Hirsch et al., 2012). It is these microglia, described as the resident immune cells of the brain, that are known to mediate inflammation; and activation of these cells can result in neuronal damage as a consequence of cytokine release (Thameem Dheen et al., 2007; Walter et al., 2006). Pro-inflammatory cytokines elicit an inflammatory response that can cause damage to cells,
examples of which include Interleukin-6 (IL-6), interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNFα) (Benveniste et al., 1990). Studies have shown that in post mortem AD brains, increased levels of microglial activation is associated with increased levels of inflammatory cytokines and chemokines, mainly IFN-γ, TNF-α and IL-6 (Akiyama et al., 2000; Skokowa et al., 2006), more importantly these cytokines have been seen to reduce levels of insulin degrading enzyme, which is a key amyloid beta (Aβ) degrading protease, which could have considerable consequences in the progression of AD (Akiyama et al., 2000; Skokowa et al., 2006). However, microglia are not the sole mediators of inflammation in the brain, neurons and astrocytes also play a role by upregulating the expression of pro-inflammatory cytokines.

This study concentrates on how NSCs, astrocytes and neurons respond to the inflammatory stimuli IFN-γ by monitoring the release of the inflammatory cytokine TNF-α. IFN-γ is known to cause inflammation via a number of different signalling mechanisms, one of which being activation of the Janus kinase (JAK) signal transducer and activator of transcription (STAT) signalling pathway; consequently, this activation promotes inflammation-associated gene expression. Specifically, the isoform STAT1 is phosphorylated via JAK2 when activated by IFN-γ, and the role of STAT1 is to encourage cell death (Nicolas et al., 2013). By knowing the mechanisms of action for these cytokines, and their detrimental effects on the brain it is possible to look at ways to prevent these pathological mechanisms.

Flavonoids are bioactive compounds generally found in plants and other food products. These compounds have been linked with reducing the risk of a number of different diseases (Szostak-Wegierek et al., 2014; Horáková, 2011; Dryden et al., 2006). Furthermore, the neuroprotective ability of flavonoids is well researched (Khanum et al., 2012; Tillekeratne et al., 2013; Beart et al., 2016), specifically describing anti-inflammatory and anti-oxidant properties. Apigenin is a naturally occurring flavonoid that has shown potential in many different disease conditions including cancers (Shukla et al., 2010), type 2 diabetes (Ren et al., 2016), and chronic neuroinflammation in AD (Münch et al., 2015). This study uses apigenin as it has previously shown to supress the production of TNF-α by inhibiting phosphorylation of STAT1 induced by IFN-γ (Rezai-Zadeh et al., 2008), this could prove significant in regards to inflammatory conditions of
the CNS. And in other studies, apigenin has shown to inhibit collagenase activity seen in rheumatoid arthritis, as well as suppressing lipopolysaccharide (LPS)-induced nitric oxide production in RAW 264.7 macrophages (Lee et al., 2007), giving apigenin a profound reputation as an anti-inflammatory agent. Additionally, apigenin has proven to be able to readily cross the blood brain barrier in animal models (Popović et al., 2013); with these abilities, as well as being cheap, non-invasive and easy to administer, this product could provide a novel therapeutic benefit to chronic inflammatory conditions such as AD and PD.

This study set out to characterise the immunogenicity of human embryonic stem cell-derived NSCs, astrocytes and neurons in response to exposure to the pro-inflammatory agent interferon gamma (IFN-γ); and further investigate the anti-inflammatory effects of flavonoid treatment using apigenin on the release of the inflammatory cytokine tumour necrosis factor alpha (TNF-α).
2. Materials and Methods

2.1 *in vitro* expansion of cells.

Human H9 Neural Stem Cells (NSC) (GIBCO®) were used in this study. The cells were maintained in growth media containing KnockOut™ DMEM/F-12, FGF Basic Recombinant Human (20ng/ml), EGF Recombinant Human (20ng/ml), and StemPro® Neural Supplement, with media being changed every two days for a total of 8 days. Proliferation was monitored and on the 8th day of feeding the cells were visualised via microscopy and determined as confluent enough to split.

In order to split cells into two separate 12 well plates, adherent cells were detached from the wells using 0.5ml Human Recombinant Trypsin (Tryple, GIBCO®), and cells were triturred within the medium to distribute. NSC media was added to neutralise the reaction, and tubes were then centrifuged. The medium was aspirated and discarded, then re-suspended with phosphate buffered saline (PBS). Re-suspended cells were prepared for a cell count by trypan blue method, 10µl of cell suspension was added to 40µl of media and were diluted according to Figure 1.

![Sample well contained 20µl total. 10µl Trypan blue 10µl 1:250 diluted cell suspension.](image)

Figure 1. Schematic diagram detailing the dilution process from the retrieval of the cell pellet after re-suspension of cells when sub-cultured. A 1:250 dilution was the final concentration for use in the cell count.

The final sample was mounted onto a haemocytometer and observed under a Nikon Eclipse T100 inverted microscope with GT Vision hiChrome software, to carry out the cell count; as shown in Figure 2.
Cells were counted in the outermost four quadrants marked by an X.

Figure 2. Image details the use of a haemocytometer (left side of image), using the dye Trypan blue it is possible to visualise the cells using the counting grid (right side of image), the cells are counted in the four outermost quadrants of the counting grid (Parhizkar et al., 2014).

It was determined that the cells were on average 65,000/ml, using the equation \[ \text{Cell Count} / 5 \times 10 \times 250 = \text{cells/µl x Volume = Total Cell no.} \]

2.2 Differentiation of NSC into Astrocyte and Neuron culture.

Information provided by the trypan blue cell count meant that cells could be split and seeded at 65,000 cells/ml. 1 x 12 well plate was differentiated into astrocytes, and 1 x 12 well plate differentiated into neurons. This was achieved by supplementing the original NSC growth media with D-MEM, N-2 supplement, GlutaMAX™-I, and Foetal Bovine Serum (FBS) for astrocytes. Differentiation into neurons was achieved by supplementing the growth media with Neurobasal® Medium, 2% B-27® Serum-Free Supplement, and GlutaMAX™-I Supplement. Subsequently, both the NSC, astrocyte and neuron plates were fed every two days with their respective growth medium for 6 days to increase confluence.

2.3 Addition of inflammatory stimuli.

In order to monitor differences between cells and their response to inflammatory stimuli, Interferon Gamma (IFN-γ) was used to induce an inflammatory response. 10µl of IFN-γ with a stock concentration of 15ng/ml, was diluted 1:66 with Phosphate Buffered Saline (PBS) and added to wells 2 and 4 of the NSC, Astrocyte and Neuron plates.
2.4 Addition of flavonoid treatment.

Apigenin (Sigma-Aldrich™, United Kingdom) was the flavonoid that was used in this study. This flavonoid was added in concentrations of 50µM/ml and 5µl was added directly into wells 3 and 4.

Figure 3. Schematic representation of the three cell culture plates, NSC (left), Astrocyte (middle) and Neuron (right). The diagram shows the positioning of the wells in which IFNγ was added in order to initiate an inflammatory response, while the other wells only contained the respective cells at this point.

Figure 4. Schematic representation of the cell culture plates containing all the additional components. The diagram shows the positioning of the IFN-γ, the Flavonoid (apigenin) and the wells that contain both.
2.7 Viability assay.

A viability assay was carried out in order to determine the percentage of cells that were living in comparison to the dead cells or debris within the samples, this assay further provided information on the cell tolerance to different concentrations of inflammatory stimuli. The dye propidium iodide (PI) was used for this assay. IFN-γ was used to assess cell tolerance in concentrations of 1, 10 and 100ng/ml, and the stock solution of 100mg/ml was provided by PeproTech, USA. 0.5ml of cell sample was added to 12ml falcon tubes (in triplicate) and centrifuged at 250RCF for 5 minutes to remove drug/stimulant. One supernatant sample was retained and frozen at -80°C for the ELISA analysis, the other supernatants were discarded, ensuring the cell pellet remained intact, and cells were washed with 1ml Dulbecco’s Phosphate Buffered Saline (PBS) and centrifuged again for 5 minutes at 250RCF. Again, the supernatant was removed and discarded, and samples were suspended in 100µl of FACS buffer (dPBS containing 3% FBS, 1% sodium azide). 10µl of propidium iodide (PI) was then added to each tube, and the contents were briefly vortexed and incubated in the dark for 15 minutes. Finally, 0.5ml FACS was added to each tube, and cell viability was tested using the BD Accuri C6 flow cytometer.

2.6 ELISA analysis

96 well ELISA plates (R&D Systems) were prepared and prior coated with the capture antibody (mouse anti-human TNF-α), the plates were stored at room temperature for 48 hours, and were washed at the halfway point of 24 hours using 0.05% Tween® 20 in PBS, pH 7.2-7.4 (R&D Systems). The supernatant that was previously frozen from the viability protocol was thawed and ELISA was carried out according to the DuoSet® ELISA Development System protocol. Reagent diluent was formulated using 1ml 4% BSA diluted 1:40 with 39ml PBS, then 7.4µl of reconstituted standard was used to construct the standard curve. 7 tubes were prepared by serial dilution for the standard curve, in 1000, 500, 250, 125, 62.5, 31.3 and 15.6pg/ml. The 96 well plate was split to contain the standards, NSC, neuron and astrocyte samples. The detection antibody (Biotinylated Goat anti-human TNF-α) was added after 2 hours, a 1:60 dilution was carried out by mixing 167µl to 10ml reagent diluent. After a further 2 hours’ streptavidin-HRP B was added in 100µl amounts per well, and incubated for 20 minutes in the dark. The plate
was washed and 100µl of substrate solution was added per well (1:1 mixture of Colour Reagent A (H₂O₂)) and Colour Reagent B (Tetramethylbenzidine) from R&D Systems, then 50ul of ELISA stop solution (2N H₂SO₄) was added to end the reaction. The results were recorded using an ELISA plate reader.

2.8 Statistical Analysis.

Statistical analysis in the form of Single-Factor ANOVA was carried out on both the viability assay data, and the ELISA data using the Microsoft Excel® software. ANOVA was used to assess the relationship between astrocytes, neurons and NSCs viability both individually at different concentrations (1, 10 and 100ng), as well as in comparison to each cell type. Similarly, the ELISA data was analysed looking at the mean florescence between the groups cells only, stimulus only, flavonoid only, and stimulus and flavonoid, as well as between the different cell types.
3. Results

This study looked to characterise the immunogenicity of three different stem cell-derived neural cells (NSC, Astrocytes, Neurons) in response to an inflammatory stimulus (IFN-γ) and flavonoid treatment (apigenin). The results of the experiments carried out are as detailed below.

3.1 characterisation of NSC, astrocyte and neuron phenotype.

Over an 8-day duration GIBCO® Human H9 Neural Stem Cells were grown in vitro and images were taken at different stages of proliferation. Once confluent enough, the cells were provided with differential media in order to promote differentiation into an astrocyte and neuron culture respectively in Figures 5a, 5b and 5c.

![Figure 5a](image.png)

Figure 5a. Image taken using Nikon Eclipse T100 inverted microscope with GT Vision hiChrome software, image shows NSCs just before being split on the 8th day of growth. At this point the cells were deemed confluent enough to split.
Figure 5b. Image taken using Nikon Eclipse T100 inverted microscope with GT Vision hiChrome software, image shows neuron culture on day 6 post-differentiation from NSC. At this point these cells were deemed confluent enough to undergo further analysis in the form of ELISA.

Figure 5c. Image taken using Nikon Eclipse T100 inverted microscope with GT Vision hiChrome software, image shows astrocyte culture on day 6 post-differentiation from NSC. At this point these cells were deemed confluent enough to undergo further analysis in the form of ELISA.
3.2 Viability assay results.

A viability assay was carried out to investigate the tolerance of NSCs, astrocytes and neurons to a changing concentration of inflammatory stimuli (IFN-γ). This assay uses propidium iodide (PI) to measure the florescence and therefore provide details of the amount of viable or non-viable cells within the samples. The concentrations used for this assay are noted on the X axis in Figures 6a, 6b, 6c and 6d.

From Figure 6a, it can be observed that the majority of the cells within each of the sample groups are viable. Comparison between groups however is variable, showing that 1ng of IFN-γ decreases the NSC cell number in comparison to the NSC only control, but 10ng shows an increase, and with a concentration of 100ng IFN-γ there is a decrease in mean florescence and viable cells. Single factor ANOVA was carried out on both the viable and non-viable cell groups respectively. With a P value of 0.3807 in the viable cell group, and 0.5851 in the non-viable cell group it must be noted that the data shows no statistical significance (P>0.05).

![Viability assay graph](image)

**Figure 6a.** Viability assay graph detailing the number of NSCs present within the sample at different concentrations of IFNγ (1, 10, 100ng), non-viable cells P>0.05, viable cells P>0.05, so the data proves statistically insignificant. Error bars were plotted +/- SEM.
Viability was also investigated within the astrocyte sample; Figure 6b represents the results obtained. This cell type also displayed a majority of cells in the viable group. Within this sample, it can be seen from the graph that 1ng IFNγ causes a decrease in cell number in comparison to the cells only control group. Further increasing the concentration to 10ng causes a further decrease in cell number in comparison to the 1ng IFNγ group, however in the 100ng concentration group an increase in cell number is observed to a level higher than that of the cells only group. A single factor ANOVA was carried out on both the viable and non-viable cell groups and showed a P value of 0.5836 and 0.2849 respectively, thus indicating that there is no statistical significance between the groups (P>0.05).

Figure 6b. Viability assay graph showing the number of stem cell derived astrocytes at different concentrations of IFNγ (1, 10, 100ng). Non-viable cells P>0.05, and viable cells P>0.05, so the data proves statistically insignificant. Error bars were plotted +/- SEM.
In addition, viability was investigated in the neuronal cell culture. Figure 6c details the number of cells present in the samples at different concentrations of IFN-γ. Similarly, a majority of the sampled cells were viable. The neuronal group with 1ng IFN-γ displayed an increased cell number in comparison to that shown in the cells only control group. And interestingly the cell number continued to increase in the 10ng and 100ng concentration groups respectively. However showing no statistical significance between the groups with a P value of 0.4033 in the viable cell group, and 0.6505 in the non-viable cell group (P>0.05).

Figure 6c. Viability assay graph showing the number of stem cell derived neurons at different concentrations of IFNγ (1, 10, 100ng). Non-viable cells P>0.05, and viable cells P>0.05, so the data proves statistically insignificant. Error bars were plotted +/- SEM.
In order to compare the number of viable cells present within the different cell types, Figure 6d was constructed. The graph shows the compiled data from the three different cell types. It can be observed that there is variability between the mean fluorescence of each cell type, and astrocytes and neurons producing varied data across the groups for the different concentrations of IFNγ. It can be noticed that NSCs show the lowest cell number overall, with the neuronal sample containing the highest number of viable cells in the groups 1ng, 10ng and 100ng respectively; furthermore, astrocyte data shows varied fluctuations. A single factor ANOVA was carried out to assess the relationship between the different cell types and P > 0.05 so no statistical significance was observed between the different cell types (P = 0.9428).

Figure 6d. Viability assay graph showing the number of the three different cell types (NSC, neurons and astrocytes) at different concentrations of IFNγ. Non-viable cells P > 0.05, and viable cells P > 0.05, so the data proves statistically insignificant. Error bars were plotted +/- SEM.
3.3 ELISA.

To investigate the immunogenicity of NSCs and stem cell-derived astrocytes and neurons an ELISA was carried out. The cells were exposed to an inflammatory stimulus (IFN-γ), and using specific antibodies, the expression of TNF-α was monitored. Apigenin was added in order to test its anti-inflammatory abilities, allow comparison between the inflammatory cytokines released in cells only, and flavonoid treated cells.

The results collected for NSC production of the inflammatory cytokine TNF-α are detailed in Figure 7a, the graph shows that the average absorbance at 450nm was 0.04787 within the cells only group, and showed a reduction in absorbance (0.04503) when the inflammatory stimuli was added (IFN-γ). For the well that contained flavonoid treatment (apigenin) only, the absorbance decreased further (0.04490). Finally, an overall decrease (0.04150) was observed in the flavonoid + stimulus group. However, no statistical significance was observed between these groups with a P value of 0.3746. P>0.05 therefore the null hypothesis is rejected.

![Graph showing NSC production of TNF-α](image)

**Figure 7a.** Cells were divided into four categories (Cells only, Flavonoid only, Stimulus only and Flavonoid and Stimulus). This graph shows the amount of the inflammatory cytokine TNF-α that was released when NSCs were provided with different well contents. Error bars are plotted +/- SEM.
The resulting analysis for astrocytes is represented in 7b. The cells only group for astrocytes produced a mean absorbance at 450nm of 0.03693. In the stimulus only group, the mean absorbance was seen to increase (0.03863), and treatment with apigenin in the flavonoid only group showed an increase in absorbance (0.04550). Finally, a decrease of absorbance was observed within the stimulus + flavonoid group (0.03980). However, again, with a P value of 0.6940, P>0.05 so the data shows no statistical significance between the different groups, therefore the null hypothesis is rejected.

Figure 7b. This graph shows the resulting absorbance at 450nm for stem cell-derived astrocytes when provided with different conditions (Cells only, Flavonoid only, Stimulus only, Flavonoid and Stimulus). Error bars are plotted +/- SEM.
Stem cell-derived neurons were also analysed by ELISA and their production of TNF-α was recorded in Figure 7c. The mean absorbance at 450nm for the cells only group was 0.04703. The stimulus only group displayed a decrease in absorbance when compared to the cells only group (0.04683). The flavonoid only group saw a lower mean absorbance than the stimulus only group (0.04533), When both the flavonoid and the stimulus were present the mean absorbance was at its highest (0.04897). Furthermore, the single-factor ANOVA gave a P value of 0.7567, so there was no statistical significance demonstrated between the groups. P>0.05 so the null hypothesis is rejected.

Figure 7c. This graph shows the absorbance at 450nm for stem cell derived neurons with provided with different conditions (Cells only, Flavonoid only, Stimulus only, Flavonoid and Stimulus). Error bars are plotted +/- SEM.
Figure 7d is a collaboration of the three previous figures that consider Figures 7a, 7b and 7c, it can be seen that for each different cell type there is a considerable difference in the data produced and this is consistent with the individual graphs generated. However, a single factor ANOVA was carried out to compare the data between the different cell types and the P value was 0.9322. This indicates that there is in fact no statistical significance between the data produced by the different cell types (P>0.05).

Figure 7d. This graph contains information regarding all three different cell types (NSC, astrocyte and neuron) and their corresponding ELISA absorbance value at 450nm when provided with different well contents. Error bars are plotted +/- SEM.
Discussion

With the ever-growing stress of finding a cure for NDD such as AD and PD, and with inflammation being a known pathology of several NDD; this study set out to characterise the immunogenicity of hESC derived NSCs, astrocytes and neurons in response to an inflammatory stimulus (IFN-γ); and further investigate the possible ways that flavonoid treatment with apigenin can modulate cytokine release, in the hope that flavonoid treatment could potentially inhibit inflammation.

IFN-γ is a pleotropic pro-inflammatory cytokine usually involved in the innate immune response, and is produced by natural killer cells (NK) and T-lymphocytes respectively (Lee et al., 2007). However, with these cells inability to cross the blood brain barrier, IFN-γ is usually undetectable within the CNS (Lee et al., 2007). IFN-γ has been described to negatively affect cells by increasing expression of inflammatory cytokines and apoptotic mediators in different cell lines respectively (Hashioka et al., 2009; Molina-Holgado et al., 2001), however this was not seen in the present study. The results of the cell viability assay shown in Figure 6d show a considerable difference in cellular responsiveness of NSC, astrocytes and neurons to the increase in concentration of inflammatory stimuli, which suggests that viability was affected by IFN-γ, however with no trend in the data being observed, IFN-γ failed to produce a significant effect at different concentrations. This was also apparent between the different types of neural cells (P>0.05). This is not consistent with the literature available for the effects of IFN-γ on cell viability. Previous studies have hypothesised that increasing the concentration of an inflammatory stimulus, causes an increase in damage to cells, or even cell death (Luna-Medina et al., 2005); and this is not observed in Figure 6d. A possible explanation to the uniformity observed in the results could be that the concentrations chosen for IFN-γ (1, 10 and 100ng) were not high enough to elicit a change in cell viability. In order to gain a more accurate representation of the cell tolerance of NSCs, astrocytes and neurons to an increasing concentration of IFN-γ, future studies should investigate a wider range of concentrations, notable higher than 100ng.

Moreover, questions have been raised in other studies regarding the immunocompetence of human NSCs, astrocytes and neurons. Although these neural cells present the receptors for major histocompatibility complex (MHC) class I and II
molecules, which are essential for the immune system to mount an immune response; Akesson et al., states that in a transplant study neither NSCs, nor NSC-derived astrocytes or neurons can initiate an allogenic lymphocyte response, instead they exhibited an inhibitory effect on the allogenic immune response (Akesson et al., 2008). This potentially suggests that rather than failure of the inflammatory stimuli to induce inflammation in Figure 6d, the cells failed to mount an immune response to the insult provided in this study.

The ability of IFN-γ to increase the production of the pro-inflammatory cytokine TNF-α, was investigated using ELISA, furthermore apigenin was added to provide an idea of how flavonoids can be used to modulate inflammatory cytokine release. The capture antibody mouse anti-human TNF-α, and the detection antibody Biotinylated Goat anti-human TNF-α were chosen for this study as they are specific to human TNF-α; this provides the opportunity to monitor the expression of the inflammatory cytokine, and furthermore provide a comparison between the TNF-α expression in the cells only group, and the flavonoid treated group. It can be observed in Figure 7d, that although there was a noticeable change in the ELISA absorbance at 450nm, suggesting that IFN-γ and apigenin were causing a change in TNF-α expression, there was no significant variability within the cells response to the inflammatory stimuli (P>0.05). Other studies have shown that IFN-γ is a potent inducer of TNF-α gene expression in neural cells (Hanisch, 2002), and that NSCs, astrocytes and neurons can secrete TNF-α in response to IFN-γ (Olmos et al., 2014); but this was not observed in the present study. IFN-γ acts upon cells by binding to specific cell surface receptors that consist of two ligand-binding subunits: Interferon Gamma Receptor 1 (IFNGR1) and IFNGR2 (Hu et al., 2008), and cellular expression of TNF-α is largely controlled by several different signalling pathways. As previously mentioned, IFN-γ utilises the Jak STAT signalling pathway, the specific protein tyrosine kinases involved in IFN-γ signal transduction are Jak1 and Jak2, these bind to IFNGR1 and IFNGR2 respectively (Hu et al., 2008). The interaction with STAT1 is initiated by the phosphorylation of an essential tyrosine residue on IFNGR1, and consequently STAT1 phosphorylation mediates IFN-γ transcription, and promotes cell death (Matsumoto et al., 1999), which has considerable implications in NDD. With the knowledge that NSCs, astrocytes and neurons all possess Jak STAT receptors (Wang et
al., 2014), there could potentially be another underlying reason why the neural cells responded per Figure 7d. IFN-γ also has the capacity to enhance macrophage responses to other inflammatory stimuli such as Toll-Like Receptor (TLR) ligands, which in turn leads to the production of pro-inflammatory mediators such as TNF-α (Hu et al., 2008), however, previous research has shown that low concentrations of IFN-γ can fail to activate cells (Taniguchi et al., 2001), and it is unknown whether the concentration used in this study (15ng/ml) is enough to elicit a response.

Unexpectedly, Figure 7d also displays uniformity in the cellular response to apigenin. An abundance of research has been carried out on apigenin and its anti-inflammatory abilities (Balez et al., 2016; Gupta et al., 2001; Münch et al., 2015), and previous studies have shown that apigenin can inhibit TNF-α production through inhibition of IFN-γ-induced STAT1 phosphorylation (Rezai-Zadeh et al., 2008). This is valuable knowledge as it not only suggests that apigenin should be able to directly block IFN-γ interaction with STAT1, but it makes the transcription factor a potential target for therapy in different inflammatory conditions and provides a link between the pro-inflammatory effects of IFNγ, and the anti-inflammatory effects of apigenin. With this information, it could be possible to utilise apigenin in the clinical setting. Furthermore, the ability of apigenin to prevent IFN-γ-induced TNF-α production is not exclusive; multiple studies have shown that apigenin can significantly inhibit TNF-α production in LPS-induced inflammation respectively (Smolinski et al., 2003; Wang et al., 2014; Zhang et al., 2014). Although there is evidence to show apigenin as a successful anti-inflammatory agent, this study proved inconsistent as apigenin failed to produce a statistically significant effect when used in ELISA (P>0.05). Some studies have shown that apigenin inhibits pro-inflammatory cytokines in a concentration-dependent manner (Zhang et al., 2014), however none of the studies eluded to in this paper described any failure to inhibit inflammatory cytokine release. Possible causes of this effect include ineffective concentration of flavonoid (50µM), and/or errors within the ELISA protocol such as too little incubation time of samples.

There are several conclusions that can be drawn from the results of this study. Cell viability did not show any significant change in response to different concentrations of IFN-γ, Similarly, the IFN-γ used in the ELISA failed to produce a significant change in the
amount of TNF-α within the samples, this could be due to the inability of 15ng/ml IFN-γ to initiate an inflammatory response in the NSC, astrocytes and neurons respectively. Furthermore, apigenin was not seen to significantly inhibit the release of the pro-inflammatory cytokine TNF-α, a potential explanation is that apigenin is ineffective in doses of 50µM, or potentially that IFN-γ failed to upregulate expression of TNF-α, therefore preventing apigenin from carrying out its known neuroprotective abilities. These conclusions should consider that the present study is a small-scale study performed once, over a short period of time; so, generalisations cannot be made in regards to the results. This study also used just one pro-inflammatory stimulus IFN-γ and just the flavonoid apigenin; given the ability to repeat this study without limitations may have produced different results.

There were a considerable set of limitations that arose during this study. The laboratory time allocated for this study was approximately 6 weeks, whereas the duration of many other studies is not pre-decided. With more time in the lab it could have allowed for more robust testing, more repeats to add validity to the data, and even further tests to be carried out. Furthermore, due to technical errors with the equipment, this experiment failed to run a flow cytometry assay. In future experiments, it may prove beneficial to carry out this protocol in order to provide a more accurate representation of the amount of inflammatory cytokine (TNF-α) within the cell suspension. This, combined with ELISA results would provide another set of data that could be compared to previous studies, and increase validity and accuracy of the study. Cost was another limitation, as the materials used and methods carried out were expensive, budgets limited the amount of repeatability available for this study. Ideally it would have been beneficial to provide more repeats at a range of different concentrations of IFN-γ and apigenin to gain a more accurate insight into how neural cells respond to inflammatory stimuli and flavonoid treatment. Finally, cells were split and seeded at 65,000 cells per millilitre, whereas the GIBCO® guidelines for using these cells states seeding should provide 150,000 cells/ml, and due to the timing and cost limitations previously mentioned, it was not possible to perform a repeat of the cell culture procedure, so 65,000 cells/ml sufficed.
Much more research in this area is necessary before all the answers can be provided for the questions raised in this study. It has been mentioned that both concentrations of IFN-γ and apigenin in this study failed to elicit the expected response; in future studies, wider concentration ranges would provide a more accurate representation of how NSCs, astrocytes and neurons respond to inflammatory insult provided by IFN-γ, and the neuroprotective ability of apigenin could be further investigated. Advancing from that, further studies could investigate a wider range of pro-inflammatory cytokines such as members of the interleukin family (IL-1, IL-1β, IL-6, IL-17) (Turner et al., 2014), as these cytokines have been seen to upregulate the expression of inflammatory cytokines and chemokines (Glass et al., 2010) involved in NDD. Moreover, the area of research into nutraceuticals is ever-growing, and apigenin has been widely researched and shown success in many animal models of disease, future research should aim to translate this to clinical practice for use in human models. Considering this, there are many other flavonoids that exhibit neuroprotective abilities, including luteolin, epigallocatechin-3-gallate and resveratrol. (González et al., 2011; Guo et al., 2012; Weinreb et al., 2009), these, as well as other studied flavonoids have seen to protect neurons against neurotoxins, suppress inflammation and further alleviate memory loss, and promote cognitive function (Vauzour et al., 2008). In the wider picture, extensive research into these compounds could advance therapeutic and regenerative medicine, and with the current available drugs being known to produce serious long term side effects (Balez et al., 2016), flavonoids could be the future therapy of choice for sufferers of NDD.

Word Count: 4955.
Appendix

Table 1. The raw data obtained directly from the ELISA plate reader, this data was used to construct the standard curve in figure 8. The standard curve data in this table is located within columns 1, 2, and 3.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
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<td>1.4842</td>
<td>1.4835</td>
<td>0.0678</td>
<td>0.0561</td>
<td>0.0512</td>
<td>0.0509</td>
<td>0.0504</td>
<td>0.0423</td>
<td>0.0011</td>
<td>-0.0028</td>
<td>0.0046</td>
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<tr>
<td>B</td>
<td>0.8052</td>
<td>0.8291</td>
<td>0.7904</td>
<td>0.0649</td>
<td>0.0537</td>
<td>0.0596</td>
<td>0.0485</td>
<td>0.0424</td>
<td>0.0438</td>
<td>0.0049</td>
<td>0.0026</td>
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<tr>
<td>C</td>
<td>0.4294</td>
<td>0.4226</td>
<td>0.4223</td>
<td>0.0504</td>
<td>0.0552</td>
<td>0.0474</td>
<td>0.0412</td>
<td>0.0445</td>
<td>0.0494</td>
<td>0.0021</td>
<td>0.0038</td>
<td>0.0072</td>
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<tr>
<td>D</td>
<td>0.25</td>
<td>0.2521</td>
<td>0.2367</td>
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<td>0.0539</td>
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<td>0.0007</td>
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<td>0.1513</td>
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<td>0.0457</td>
<td>0.0496</td>
<td>0.0413</td>
<td>0.0382</td>
<td>0.0313</td>
<td>0.0033</td>
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<td>0.0002</td>
</tr>
<tr>
<td>F</td>
<td>0.1161</td>
<td>0.1083</td>
<td>0.1053</td>
<td>0.0497</td>
<td>0.0435</td>
<td>0.0428</td>
<td>0.0375</td>
<td>0.0655</td>
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<td>-0.0032</td>
<td>-0.0006</td>
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<tr>
<td>G</td>
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<td>0.089</td>
<td>0.0838</td>
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<td>0.0407</td>
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<tr>
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<td>0.0482</td>
<td>0.0443</td>
<td>0.0392</td>
<td>0.0359</td>
<td>0.0024</td>
<td>0.0073</td>
<td>0.0052</td>
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Figure 8. The standard curve was plotted using the data provided in Table 1. This standard curve was used as reference for the absorbance at 450nm for NSC, astrocyte and neuronal samples respectively.
**Figure 9a.** The data collected from the cell viability assay for NSCs, plot 3 is reference to the number of living cells within the sample, and plot 2 is in reference to the number of dead cells/debris. A01/2/3 detail the NSC sample that contained no propidium iodide as a control sample. B01/2/3/4 contains PI, and C01/2/3/4 are repeats.

<table>
<thead>
<tr>
<th></th>
<th>Plot 2 (FL2-A)</th>
<th></th>
<th>Plot 3 (FL2-A)</th>
<th></th>
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<tr>
<td></td>
<td>This Plot</td>
<td>Mean FL2-A</td>
<td>This Plot</td>
<td>Mean FL2-A</td>
</tr>
<tr>
<td></td>
<td>This Plot</td>
<td>% of All</td>
<td>This Plot</td>
<td>% of All</td>
</tr>
<tr>
<td>A01 NScs no PI</td>
<td>716.21</td>
<td>34.95%</td>
<td>1,174.36</td>
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<tr>
<td>A02 1ng IFN no PI</td>
<td>820.55</td>
<td>36.44%</td>
<td>4,434.22</td>
<td></td>
</tr>
<tr>
<td>A03 10ng IFN no PI</td>
<td>683.35</td>
<td>39.57%</td>
<td>1,685.10</td>
<td></td>
</tr>
<tr>
<td>A04 100ng IFN no PI</td>
<td>676.34</td>
<td>39.18%</td>
<td>1,558.50</td>
<td></td>
</tr>
<tr>
<td>B01 NSc with PI</td>
<td>10,398.77</td>
<td>40.76%</td>
<td>107,663.18</td>
<td></td>
</tr>
<tr>
<td>B02 1ng IFN with PI</td>
<td>14,781.49</td>
<td>29.46%</td>
<td>115,427.15</td>
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<tr>
<td>B03 10ng IFN with PI</td>
<td>12,891.55</td>
<td>39.16%</td>
<td>139,393.55</td>
<td></td>
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<tr>
<td>B04 100ng IFN with PI</td>
<td>10,837.53</td>
<td>38.20%</td>
<td>126,910.14</td>
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<tr>
<td>C01 NScs with PI</td>
<td>13,187.84</td>
<td>37.96%</td>
<td>143,777.53</td>
<td></td>
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<tr>
<td>C02 1ng IFN with PI</td>
<td>11,254.56</td>
<td>38.53%</td>
<td>125,092.70</td>
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<tr>
<td>C03 10ng IFN with PI</td>
<td>13,298.42</td>
<td>37.06%</td>
<td>158,708.44</td>
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<td>C04 100ng IFN with PI</td>
<td>11,298.14</td>
<td>37.42%</td>
<td>136,743.72</td>
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</table>

**Figure 9b.** The data collected from the cell viability assay for neurons, plot 3 is reference to the number of living cells within the sample, and plot 2 is in reference to the number of dead cells/debris. E01/2/3 detail the NSC sample that contained no propidium iodide as a control sample. E01/2/3/4 contains PI, and G01/2/3/4 are repeats.

<table>
<thead>
<tr>
<th></th>
<th>Plot 2</th>
<th>Plot 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>E01 Neurons no PI</td>
<td>26.57%</td>
<td>2.36%</td>
</tr>
<tr>
<td>E02 Neu 1ng IFN no PI</td>
<td>25.14%</td>
<td>2.98%</td>
</tr>
<tr>
<td>E03 Neu 10ng IFN no PI</td>
<td>23.43%</td>
<td>3.63%</td>
</tr>
<tr>
<td>E04 Neu 100ng NO PI</td>
<td>25.10%</td>
<td>3.59%</td>
</tr>
<tr>
<td>F01 Neurons with PI</td>
<td>26.73%</td>
<td>2.69%</td>
</tr>
<tr>
<td>F02 Neu 1ng IFN with PI</td>
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<td>2.77%</td>
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<tr>
<td>F03 Neu 10ng IFN with PI</td>
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<td>2.70%</td>
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<td>G01 Neurons with PI</td>
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<td>G02 Neurons 1ng IFN with PI</td>
<td>26.53%</td>
<td>3.49%</td>
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<tr>
<td>G03 Neurons 10ng IFN with PI</td>
<td>24.64%</td>
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<td>G04 Neurons 100ng with PI</td>
<td>26.21%</td>
<td>3.37%</td>
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</table>
Figure 9c. The data collected from the cell viability assay for astrocytes, plot 3 is reference to the number of living cells within the sample, and plot 2 is in reference to the number of dead cells/debris. E01/2/3 detail the NSC sample that contained no propidium iodide as a control sample. F01/2/3/4 contains PI, and G01/2/3/4 are repeats.
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Apigenin and naringenin regulate glucose and lipid metabolism, and ameliorate


