UNIVERSITY OF CALIFORNIA SAN DIEGO

Human in vitro model of neuro-inflammation

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Biomedical Sciences

by

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The Dissertation of Leah Anne Boyer is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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LIST OF ABBREVIATIONS

AA	ascorbic acid
AADC	amino acid decarboxylase
BDNF	brain derived neurotrophic factor
BMP	bone morphogenic protein
cAMP	cyclic adenosine monophosphate
CM	conditioned medium
CNS	central nervous system
COX	cyclooxegenase
CRE	cAMP response element
CREB	cAMP response element binding protein
CRTC	cREB regulated transcription coactivator
DA	dopaminergic
DMSO	dimethylsulfuroxide
EB	emrbyoid body
En	engrailed
FBS	fetal bovine serum
FGF	fibroblast growth factor
GDNF	glial cell derived neurotrophic factor
GFAP	glial fibrilary acidic protein
hESC	human embryonic stem cell
ICC	immunocytochemistry
IL	interleukin
hESC	human embryonic stem cell
LPS	lipopolysaccharide
MEF	mouse embryonic fibroblast
ml	milliliter
nM	nanomolar
NPC	neuronal progenitor cell
PCR	polymerase chain reaction
PD	Parkinson's disease

PET	positron emission tomography
qPCR	quantitative PCR
RA	retanoic acid
SHH	sonic hedgehog
SN	substantia nigra
TGF	transforming growth factor
TH	tyrosine hydroxylase
TLR	toll like receptor
TNF	tumor necrosis factor
uM	micromolar

LIST OF FIGURES

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ABSTRACT OF THE DISSERTATION

Human in vitro model of neuro-inflammation

by

Leah Anne Boyer

Doctor of Philosophy in Biomedical Sciences

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Parkinson's Disease (PD) is the most common neurodegenerative movement disorder. Historically, PD has been considered a strictly neuronal disease; however, clinical observations and evidence from animal models suggest inflammation may contribute to disease progression. It remains controversial whether glial activation, and the resulting inflammatory cascade, is a result or a cause of neuronal death.

Towards resolving this distinction, I established cultures of human embryonic stem cell derived dopaminergic neurons, and primary human

astrocytes and microglia. These neural cells are used to investigate the glial inflammatory response to extracellular insults, and the neuronal response to pro-inflammatory mediators.

I established that these cells are a robust model of neuro-inflammation, found that the glial derived pro-inflammatory response can be serially propagated between astrocytes and microglia following a single inflammatory insult, and that glial derived inflammatory factors are neurotoxic. Using these assays, I identified the anti-inflammatory and neuro-protective effects of flavonoid apigenin, an inducer of orphan nuclear receptor Nurr1, whose dual role as both neuro-protective and anti-inflammatory renders it an ideal target for therapeutic intervention in PD.

The purpose of this project is to elucidate the role of inflammation in PD and identify key molecular events involved at early stages in PD to exploit as potential targets for therapeutic intervention. These results are important because they establish an *in vitro* model that enables investigation into the inflammatory contribution to the pathological development of PD, and identification of novel therapeutic compounds.

CHAPTER 1.

Differentiation of human pluripotent stem cells into neural subtypes enriched for dopaminergic neurons.

ABSTRACT

The ability of human pluripotent stem cells to differentiate into clinically relevant cell types defines them as one of the most promising tools in biomedical research; however, many of these differentiation protocols remain less than ideal. Dopaminergic neurons are implicated in a variety of diseases, including Parkinson's disease and Multiple Systems Atrophy. Though many protocols have been published, they are generally undefined and have a very low yield. In addition to modulating plating density and maturation time, I have explored different paradigms and chemical inhibitors towards improving dopaminergic differentiation. I have compared two intrinsically distinct differentiation protocols: one monolayer paradigm based heavily on the inhibition of alternative pathways, and the other more traditional five step method based on neural rosette formation. Overall, our results indicate that the monolayer paradigm is more efficient and faster, and therefore well suited for studying neural development, while the traditional five step method is more well suited for disease modeling since it involves the generation of pure, multi-potent neural progenitor cell line, which facilitates comparison's between patients. Effective differentiation paradigms are critical for modeling neurodegenerative diseases in vitro and will

serve as a powerful tool for understanding the molecular mechanisms involved in disease pathogenesis and neural development. Directed differentiation of pluripotent cells into specific cell types is also a crucial step towards understanding human development and realizing the biomedical relevance of these cells, whether for replacement therapy or disease modeling.

BACKGROUND AND SIGNIFICANCE

Parkinson's disease (PD) is the most common movement disorder and the second most common neurodegenerative disorder, affecting an estimated four million people worldwide[1]. A recent epidemiological study of people over 65 in the United States reported an annual incidence of 0.16% (59,000 total) and 0.95% prevalence (349,000 total)[2]. That incidence rate rises to 3% when the sample population is limited to those 80 years of age and above[3]. Given that age is the most consistent risk factor, PD's prevalence is expected to rise with the aging worldwide population and increasing life expectancy. This increasing economic and social burden on society further strengthens the need to unravel the elusive mechanism of this complex disease. Pharmacological drug treatment is usually effective only in the early stages of the disease, surgical intervention, including deep brain stimulation, is highly invasive, and neither approach prevents further neuronal degeneration.

The first known description of the disease is "An Essay on the Shaking Palsy" written in 1817 by the British physician James Parkinson. Since then, the disease that bears his name has been extensively characterized as a disorder

stemming from reductions in the neurotransmitter dopamine. PD is clinically characterized by four cardinal motor symptoms: resting tremor, rigidity, bradykinesia, and postural instability. Furthermore, life quality of PD patients is often hampered by several non-motor symptoms such as olfactory deficits, cognitive impairments, depression, and autonomic dysfunctions even before the onset of motor symptoms. Probable PD is diagnosed by three cardinal motor symptoms and a positive response to dopamine replacement therapy; a definite PD diagnosis requires histological examination upon autopsy[4]. Upon autopsy 24% of clinically diagnosed PD cases lack the characteristic neuropathology[5]. The motor symptoms in PD are attributed largely to a loss of dopaminergic neurons in the substanti nigra (SN), and the resulting reduction of dopaminergic innervation to the striatum. In addition to the loss of dopaminergic neurons in the midbrain's substantia nigra pars compacta, the neuropathological hallmark of PD are cytoplasmic inclusions called Lewy bodies and threadlike inclusions in their axons and dendrites called Lewy neuritis in the remaining cells in both inherited and sporadic form of PD. These proteinacious aggregates contain primarily alpha-synuclein, but also ubiquitin, neurofilament protein, crystalline and other cytoskelletal components[6]. A pathogenic role of a-synuclein is confirmed by gene multiplications and mutations that promote aggregation and cellular dysfunction, resulting in early onset PD[7], though when combined the 16 loci identified for PD only represent 5% of all PD cases[8]. While many loci that confer susceptibility have been identified using genome wide association studies, the majority of PD cases are likely multigenic or environmental in origin[9].

Since most PD cases are sporadic, and environmental factors such as inflammation and aging have important roles in both onset and progression, disease development, course, and treatment paradigms are apt to vary. Typically animal models are employed to clarify, model, and identify compounds to treat human diseases, however no murine equivalent of PD exists. Genetic models consistently fail to recapitulate all the aspects of PD: non-motor symptoms, slowly progressing motor symptoms, loss of dopaminergic neurons in the SN and the formation of Lewy bodies[10]. Current mouse models do not display all of the cardinal features of PD, and the pathologies they do manifest are promoter dependent[11]. A novel human system to model diseases is required to fully understand the relationship between inflammation and the progression of PD pathology. Human brain samples can provide insight into the development and progression of PD, but the inability to sample live brain cells limits our knowledge of pathological abnormalities and neuro-inflammation. Our current understanding of PD in a human context is mostly generated from analyzing end-stage postmortem brain tissues, where concomitant testing of inflammatory markers is no longer possible. Specifically, human samples from the early course of the disease would be informative for understanding neuro-inflammation in PD, but they are rare.

Human embryonic stem cells (hESCs) were first isolated in 1998, almost two decades after their murine counterparts, and have since become a potential source of cells for cell replacement therapies and as a tool to model human development and disease[12]. They are defined by their ability to self renew while

maintaining pluripotency, and more specifically: are derived from an inner cell mass of a human blastocyst, can proliferate *in vitro*, have a normal karyotype, can differentiate into all three germ layers, express makers of pluripotency (most notably the transcription factor Oct4), and have telomerase activity. The recent success in reprogramming fibroblasts into inducted pluripotent stem cells (iPSCs)[13] offers the unique possibility of obtaining cells directly from patients with specific diseases, as has been demonstrated for a number of neurodegenerative diseases, including PD[14]. A set of transcriptional factors, OCT4, SOX2, KLF4, CMYC, has the ability to "jump start" a pluripotent cell fate. This technology allows the reprogramming of a terminally differentiated cell type into a pluripotent one with all of the above-mentioned characteristics. Since variability in growth properties and differentiation capacity have been reported for both types of pluripotent stem cells, for the purpose of this chapter I have chosen two hESC lines and one iPSC line towards establishing a robust differentiation paradigm with which to model of PD.

Towards using pluripotent stem cells to model neurodegenerative disease, they must be differentiated into the relevant cell type affected by that disease. While dopaminergic neurons are the most prominent cell type implicated in the etiology of Parkinson's disease (PD), they are not the only cell type affected[15], and therefore I sought a neural differentiation protocol which produced a variety of neural subtypes, but was enriched for dopaminergic neurons. Identifying and modulating the appropriate differentiation protocol requires an understanding of the development of these cells *in vivo.* Dopaminergic development in general is

regulated by signaling from the isthmus, at the junction of Otx2 and Gbx2 transcription factor expression, through signaling factors fibroblast growth factor 8 (FGF8), sonic hedgehog (SHH), Wnt1, and transforming growth factor (TGF-α/β)[16]. Dopaminergic precursors express Pitx3, Lmx1b, En1/2, Nurr1, which are crucial for the appropriate patterning, development, and survival of mature dopaminergic neurons[17]. Terminal differentiation and maintenance of adult dopaminergic neurons is orchestrated mainly by glial cell-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), and cyclic adenosine monophosphate (cAMP). Mature dopaminergic neurons express the enzymes, receptors, and transporters necessary for dopamine synthesis, storage, release and reuptake, including tyrosine hydroxylase (TH), amino acid decarboxylase (AADC), vesicular monoamine transporter (Vmat2), dopamine receptor 2 (D2r), and dopamine active transporter[18],[19].

DA neurons are present throughout the brain but are chiefly present in three distinct regions: two nuclei within the mesencephalon (SN pars compacta and ventral tegmental area), the hypothalamus and ventral thalamus in the diencephalon, and a small population within the olfactory bulb of the telencephalon[16]. Each group of DA neurons is involved in the control of a specific brain function, depending on its projected region, and can be subject to disregulation and degeneration. Dopamine dysfunction is implicated in a variety of disease, including hyperprolactinaemia (infertility), addiction, depression, schizophrenia, drug abuse and, most notably PD[20]. All but the first of these

disorders are linked to midbrain DA cells, which have, therefore, become a relevant cell type of interest for disease modeling and drug targeting.

Midbrain DA neurons (mDA) arise from progenitors in the floor plate region of the ventral midline where they are patterned by FGF8, required for establishing the midbrain-hindbrain organizer, and SHH secreted from the notochord. These diffusible morphogens activate key transcription factors, including Otx2, Lmx1a/b, Pitx3, and Msx1/2[21]. Early mDA progenitors express Ngn2, Lmx1a and Msx1, immature mDA express Nurr1, En1, Lmx1b, and Pitx3, and mature mDA also express Nurr1, TH, AADC, VMAT and DAT[22,23].

Towards identifying a robust, reliable, reproducible differentiation method for generating maximal amounts of DA neurons from pluripotent stem cells, we modified and tested existing protocols established in the Gage lab and from the literature. There are three prominent methods for in vitro differentiation of pluripotent cell types into DA: stromal coculture, 5-step EB-based methods, and adherent monolayer differentiation based on the chemical inhibition of alternate pathways. Neural induction of pluripotent cells with bone marrow-derived stromal cells (eg. PA-6 or MS5) can generate DA neurons (~15% of total cells[24]) without endogenous pattern factors, for reasons that remain unexplored; however, the inclusion of a second feeder cell line renders this paradigm more variable and undefined. The more traditional five-step method is the most classic strategy and, though time consuming, is a chemically defined way to consistently generate a high yield of DA neurons (~30% of total cells[25]). A more recently established protocol based on chemical inhibition of bone morphogenic protein (BMP) and

transforming growth factor beta (TGFβ) in adherent monolayer cultures, is a much shorter, less intensive paradigm and can generate ~30% DA, depending on the pluripotent cell line, since no selection step exists at the progenitor stage[26]. Additionally, over the years, many chemical enhancers of dopaminergic differentiation have been published and may be useful depending on the specific dopaminergic subtype desired and disease to be modeled[27], [28], [29].

In this chapter, I use multiple human pluripotent stem cell lines to test the different differentiation paradigms towards establishing a robust, reliable and reproducible method of generating DA neurons. I eschewed stromal-based protocols due to their inherent variability and absence of mechanism underlying their effects. The two protocols described here are chemically defined and represent two very different methods for generating DA neurons. My primary outcome measurement was TH immuno-reactive cell numbers, since TH is the rate-limiting enzyme in dopamine synthesis, and its expression is routinely used to identify dopaminergic neurons *in vitro* and *in vivo*. In upcoming chapters I will use these differentiation paradigms to model the cell autonomous and non-autonomous aspects of PD.

RESULTS

Traditional five step method for dopaminergic differentiation

This aggregate based five step paradigm is common in the literature describing the generation of a variety of neural subtypes, dictated by which growth factors are used to pattern the developing neuro-ectoderm. This paradigm

was first described with mouse embryonic stem cells over by the McKay lab at the NIH over two decades ago, and it involves the formation of embryoid bodies (EBs), is well established in the field, and has since been adapted and specialized for many different lineages including dopaminergic differentiation[25],[30]. In the Gage laboratory, this protocol has been used to generate a variety of neural subtypes. Patterning with FGF2 results in a generaly pan-neuronal population that is enriched for forebrain neurons, the majority of which are GABAergic[31]. When we pattern these cells with SHH and RA we derive a neural population enriched for motor neurons, which express markers including HB9 and ChAT[32]. For the purpose of this project, the progenitors were patterned with FGF8 and SHH, to enrich for dopaminergic subtypes (Figure 1.1a).

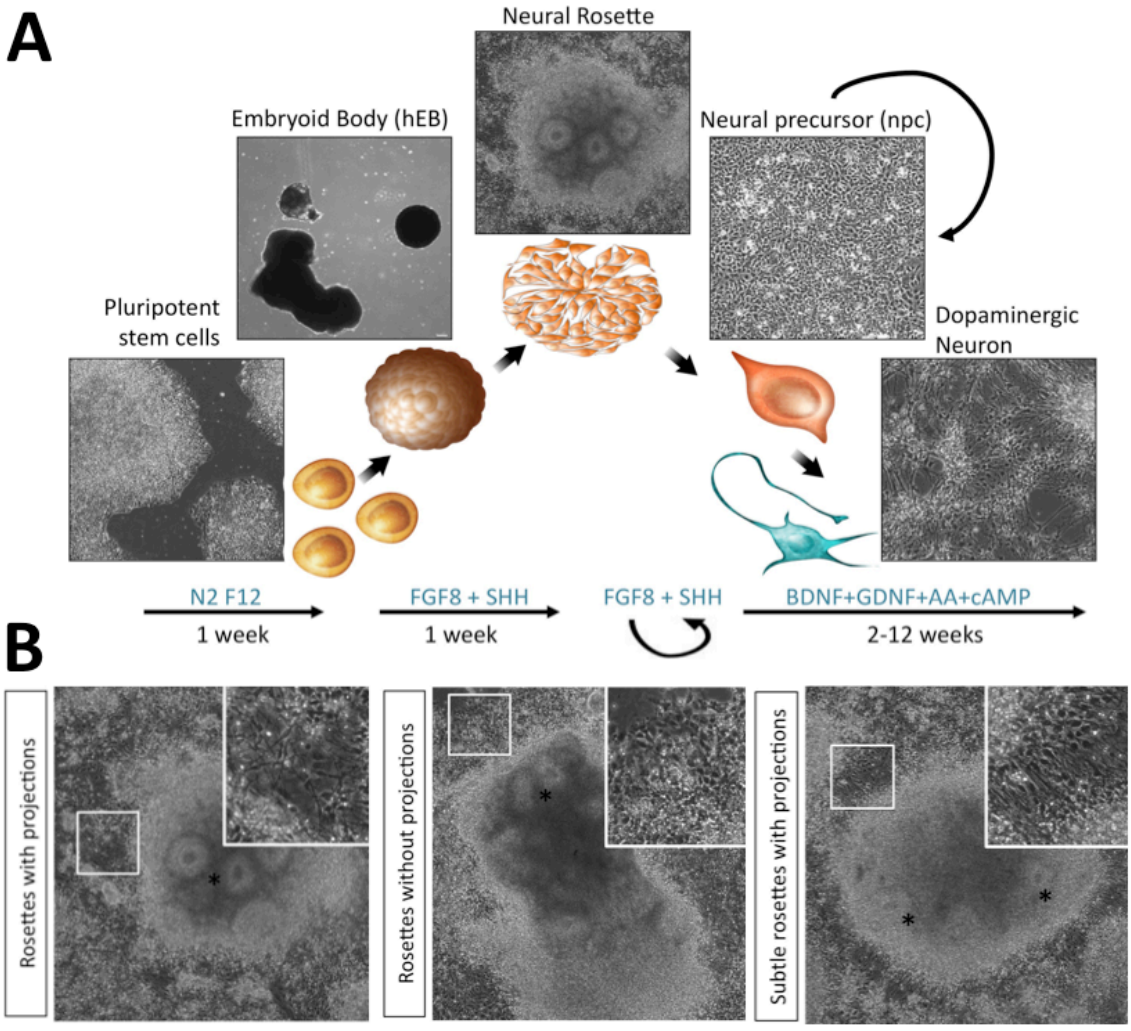

Figure 1.1 Traditional Paradigm for Dopaminergic Differentiation. A. Five-step dopaminergic differentiation paradigm. Pluripotent stem cells are grown as floating hEBs for one week, after which they are plated onto laminin for an additional week to allow for rosette formation, which are manually isolated and dissociated into a multipotent progenitor line that can differentiate into a mixed population of neural cells. Abbreviations: Ascorbic Acid (AA); Sonic Hedgehog (SHH). B. Brightfield phase contrast images of rosettes after one week on laminin. Insets highlight the morphology of the cells growing out of the hEBs, asterisks are adjacent to and below rosettes within the hEB.

For the traditional five-step paradigm, hESCs are pretreated with DMEM/F12 containing N2 and B27 supplements for two days prior to EB

formation, which increases their survival[30]. After whole colony dissociation of the pluripotent stem cells, the resulting EBs are grown as floating aggregates for one week, after which they are plated onto laminin for an additional week to allow for rosette formation and maturation. Rosettes are manually isolated and the resulting monolayer culture of neural progenitor cells (hNPCs) can be propagated, frozen, and easily genetically manipulated for up to 20 passages. The NPCs can be terminally differentiated into a mixed neural population of neurons and astrocytes for up to three months. Additionally, the NPCs can be plated onto astrocytes, which can expedite the maturation of the resulting neuronal population.

The most crucial step in this paradigm is rosette selection (Figure 1.1b). Neural rosettes are an in vitro representation of embryonic structure resembling the neural tube and are indicative of early neuro-ectoderm development. The rosettes generated in vitro vary based on their neurogenic potential and the neurogenic potential of the parentally pluripotent cell line from which they were derived. Generally, about a dozen rosettes form per adhered hEB and they can be judged based on the cells emanating from the rosettes. Some rosettes still contain immature, undifferentiated pluripotent cells, easily identified by their phase dark, tightly packed morphology; these rosettes should be avoided at all costs. Some rosettes are more glial in their potential, they radiate flat, cobblestone like cells, which tend to be positive for early glial markers like NG2 (Figure 1.1b). The rosettes best suited for neuronal differentiation radiate phase bright bipolar cells. Long projections from spontaneous differentiation are also

likely present, and is a good indicator that the rosettes contained within the hEB that have strong neurogenic potential (Figure 1.1b). In hEBs containing rosettes, the presence of bipolar neuronal cells containing projections is more important than the structure or quantity of the rosettes themselves.

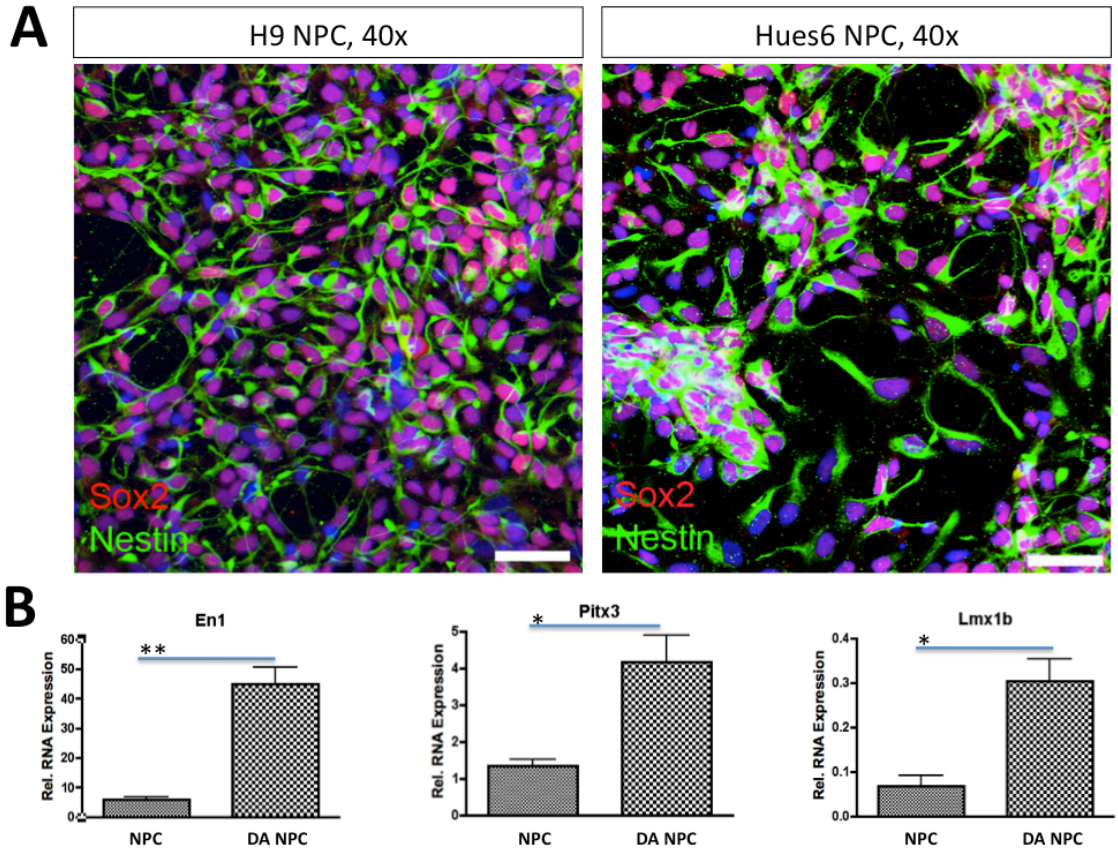

Figure 1.2 Characterization of NPC lines.
A. Fluorescent images of NPC lines generated from two independent hESC lines. 40x, Scalebar 50um. B. Quantitative PCR analysis of intermediate dopaminergic specific genes in H9 derived DA NPCs relative to pan-neuronal NPCs patterned with FGF2. All data represented relative to GAPDH expression. * = p < 0.05, ** 0.01, * 0.001**

The resulting NPC line should be homogeneous, consisting of small, bipolar cells with small processes. If the line appears heterogeneous, not much can be done, though differentiation is the only true test of a multipotent precursor

line. The monolayer of NPCs that result after dissociation of projection containing rosettes are validated using immunohistochemistry against markers Sox2 and Nestin (Figure 1.2a). An NPC line must contain at least 95% co-expressing cells, though ideally it is a pure population. While these markers are robust and well established in the literature, they are not subtype specific and therefore cannot relay any information as to the regional identity of the cells. Antibodies against markers important for dopaminergic development including Nurr1 and En1, seem to be unspecific in our hands. Therefore, we assessed the regional specificity of the immature and mature neurons by quantifying gene expression levels. Quantitative PCR was used to assess the levels of LMX1a, PITX3, and EN1 relative to cells generated using the pan-neuronal differentiation paradigm patterned with FGF2 described above (Figure 1.2b). We found that the NPCs patterned with FGF8 and SHH express significantly higher levels of these midbrain specific markers (Figure 1.2b).

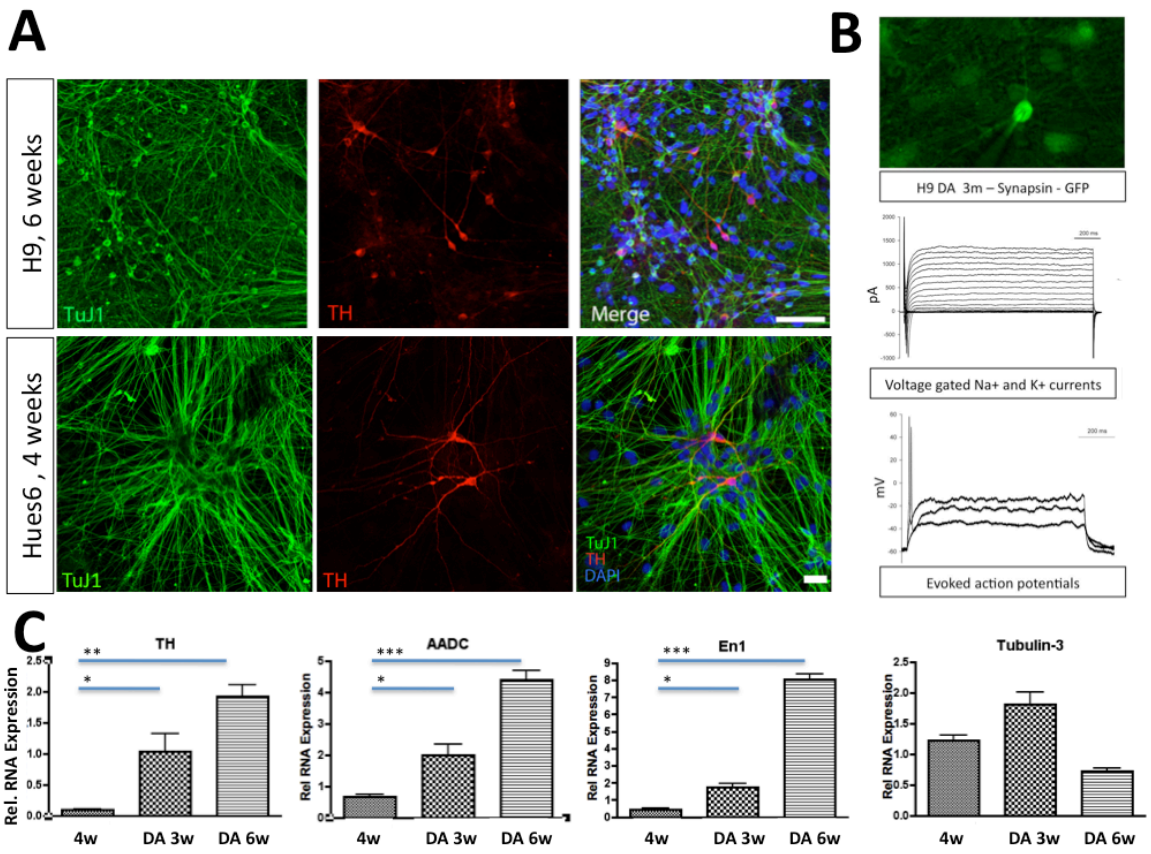

Figure 1.3 Terminal differentiation of NPCs into DA neurons.
A. Fluorescent images of neurons from two independent hESC lines that express TH and have mature, branched morphology. 40x, Scale bar 50um. B. Functional characterization of 3 month old H9 derived neurons expressing synapsin-GFP. C. Neurons are enriched for dopamine-specific genes relative to the general non-DA neurons, yet have similar levels of a pan-neuronal marker, β3-Tubulin. All data represented relative to GAPDH expression. * = p < 0.05, ** 0.01, * 0.001**

The neurons differentiated from the NPC population mature for minimally six weeks and up to three months in culture in the presence of BGNF, GDNF, ascorbic acid and cAMP. The regional identity of these cells was confirmed by immunostaining for tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, which is routinely used to identify these cells *in vivo* and *in vitro* (Figure 1.3a). Neural cultures differentiated from NPCs that were patterned

with FGF8 and SHH showed significantly higher expression levels of genes implicated in dopaminergic function (Figure 1.3c). The regional identity of the differentiated neural cultures was further confirmed by quantitative PCR for markers of mature midbrain dopaminergic cells, including TH, AADC and En1 (Figure 1.3c). These cells are also electrophysiologically active (Figure 1.3b). Three month old neural cultures were infected with a lenti-virus expressing GFP under the control of the synapsin promoter, to ensure that the cells being analyzed were neurons. Cells were then assayed for functional sodium and potassium channels and their ability to propagate action potentials upon current injection (Figure 1.3b).

Adherent Paradigm for Dopaminergic Differentiation

This adherent monolayer protocol is based on chemical inhibition of alternative pathways, rather than manual selection of rosettes, and can be used to generate a diversity of neuronal subtypes, utilizing the same battery of growth factors described above. This protocol, first established by the Studer lab at Memorial Sloan Kettering Cancer Center, has been optimized for maximal efficiency (Chambers, 2009). While it is likely possible to adapt this protocol to alternative neuronal lineages, in our hands it has only been employed to generate dopaminergic neurons.

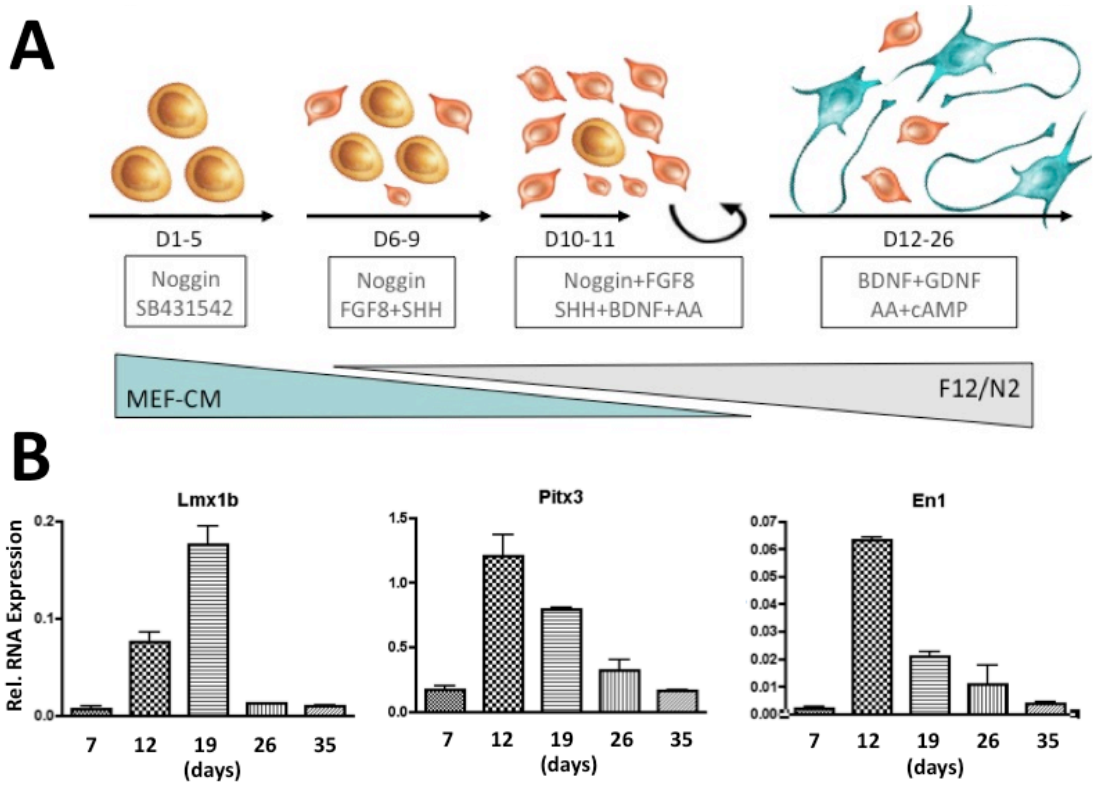

Figure 1.4 Adherent Protocol of Dopaminergic Differentiation
A. Monolayer based differentiation paradigm. Confluent monolayer of hESCs fed for five days with fibroblast conditioned medium (MEF-CM) with noggin and a TGFβ superfamily inhibitor (SB43152) are ventralized for one week before they are terminally differentiated for two weeks. B. Time course of expression of intermediate dopaminergic specific genes, using quantitative PCR analysis on H9 derived cells. All data represented relative to GAPDH expression.

Pluripotent stem cells are dissociated from colonies into single cells and re-plated onto a feeder free Matrigel surface. Once the cells have formed a monolayer the differentiation begins with five days of supplementing the cells with either fibroblast conditioned medium or defined stem cell medium in which the pluripotency factors have been removed and neuralizing factors Noggin (BMP antagonist) and SB431542 (TGFβ inhibitor) are added. Beginning on day six, the stem cell medium is gradually reduced by 25% daily, and replaced with a

neuralizing N2 and B27 supplement based medium. From days six to nine the cells are patterned with FGF8 and SHH in the presence on Noggin. The cells are then transitioned for two days into a terminal differentiation medium with the addition of BDNF and ascorbic acid. Finally, at day 12 the cells are fed a terminal differentiation medium, the same as that described for the traditional five step method to facilitate comparison (Figure 1.4a).

The adherent differentiation method requires a very high density of cells, and is therefore difficult to visualize using traditional light microscopy. Given how compact the cells become at high density, the progenitor stage was very difficult to asses using immunofluorescence for co-localization of Sox2 and Nestin. Though it appears all cells express Sox2, this is not a reliable marker without Nestin, since it is also expressed in pluripotent stem cells; instead, gene expression changes were used to monitor the differentiation (Figure 1.4b).

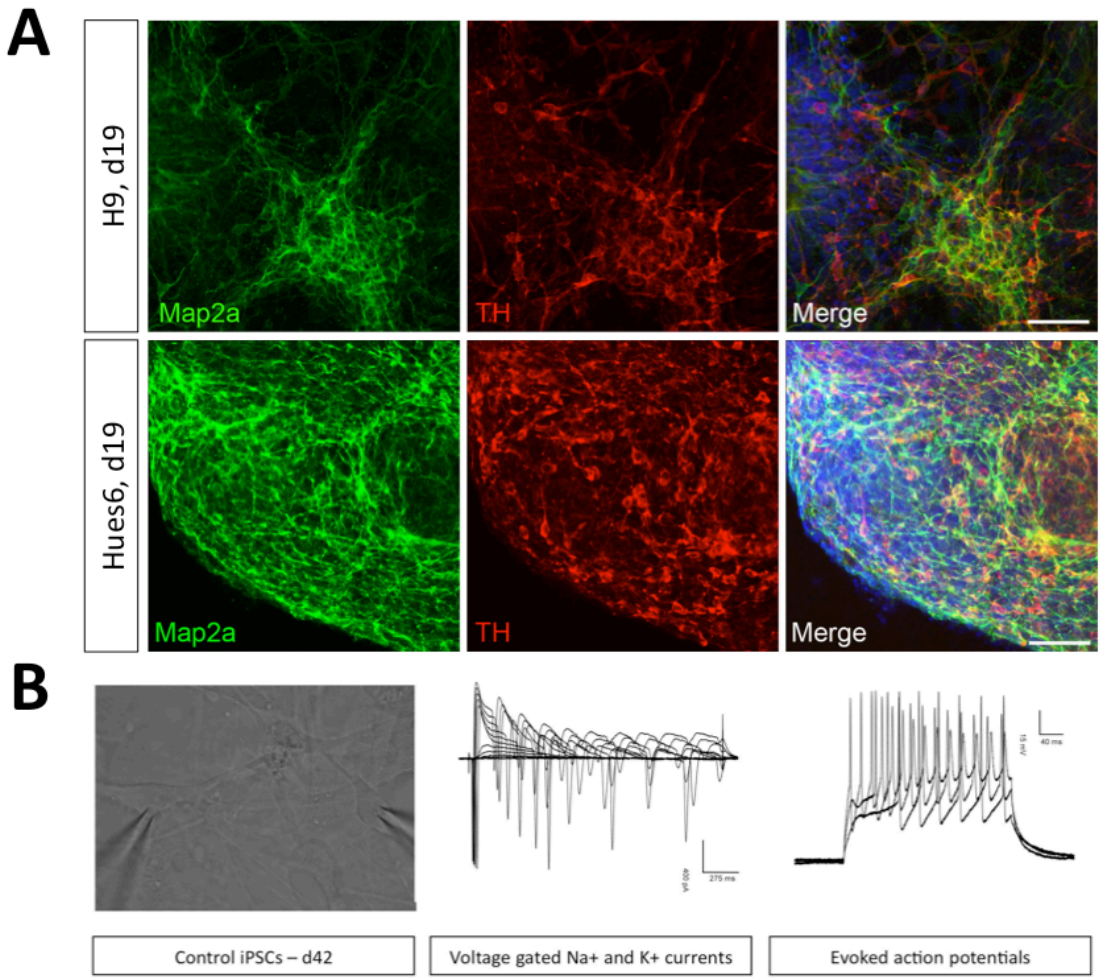

Figure 1.5 Mature neurons generated with adherent DA differentiation paradigm.
A. Fluorescent images of neurons from two independent hESC lines that express TH. 20x, scale bar 100um. B. Functional characterization of neurons expressing synapsin-GFP derived from an iPSC line differentiated on astrocytes for 42 days.

Neurons generated from this protocol express TH, though their morphology is a less mature than those differentiated using the traditional paradigm, with smaller processes and less branching (Figure 1.5a). The published version of this protocol suggests that the cells are fully differentiated after one week of terminal differentiation, i.e. after 19 days post pluripotency. In

our hands, intermediate markers were still high at day 19, therefore we chose to extend this protocol until day 35, at which point all intermediate markers were reduced as determined by quantitative PCR (Figure 1.4b). We also found that passaging the cells to laminin plates at d12 seemed to improve the efficiency of dopaminergic differentiation for unknown reasons (Figure 1.6). To achieve electrophysiological activity the cells needed to be split at day 12 onto astrocytes for terminal differentiation (Figure 1.5b), cells were assayed for functional maturity as described for the traditional differentiation paradigm.

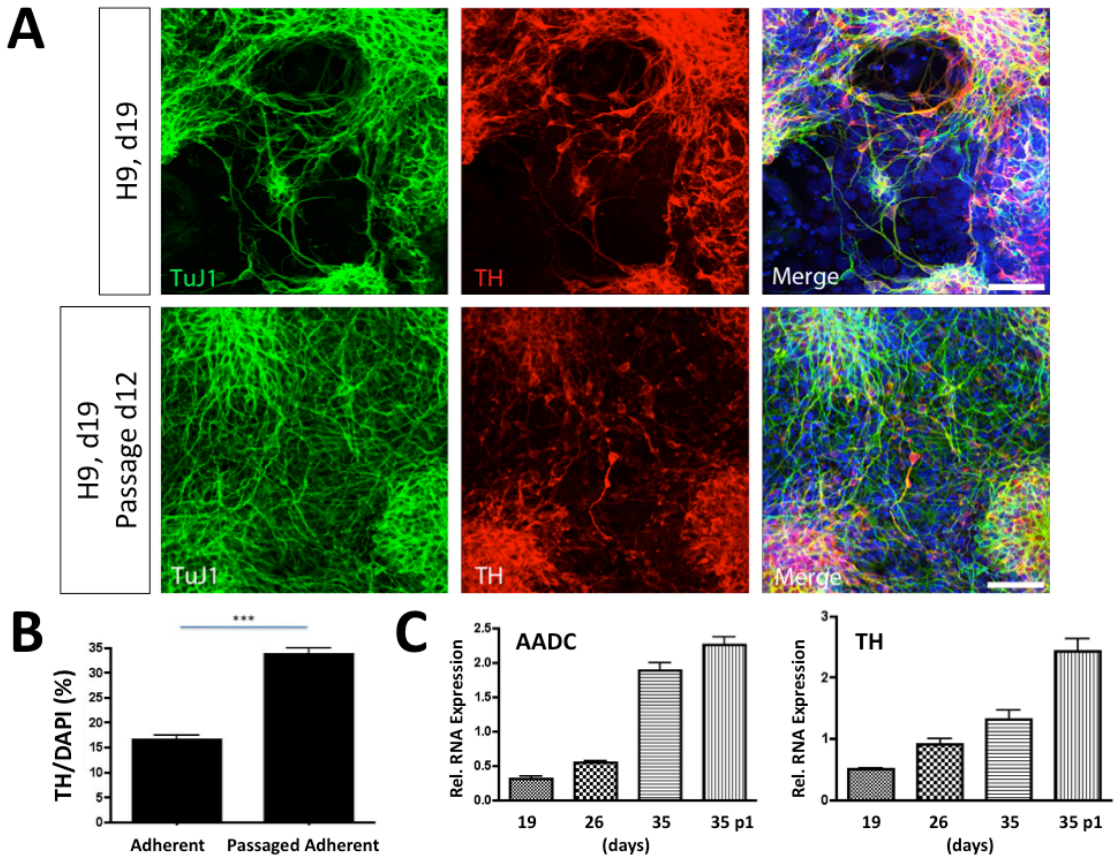

Figure 1.6 Passaging increases efficiency of dopaminergic differentiation in adherent paradigm.
A. Fluorescent images of neurons differentiated from H9 for 19 days, with or without an en bloc passage at day 12. B. Quantification of TH immuno-reactive neurons from A. n = 4. C. Time course of expression levels of terminal differentiation markers in H9 cells. All data represented relative to GAPDH expression. * = p < 0.05, ** 0.01, * 0.001**

Comparison of the traditional and adherent paradigms

Cells differentiated from both paradigms generate mature, functional dopaminergic neurons, they vary most in terms of timing and yield. In our hands the traditional protocol can generate around 10-15% dopaminergic neurons of the total cells in the population (Figure 1.5). The yield of the adherent paradigm is higher, closer to 15-20%, which is further raised to 30-35% if the cells are

passaged at day 12 (Figure 1.7a). Using quantitative PCR to determine gene expression changes reveals similar levels of TH expression from both protocols (Figure 1.7b).

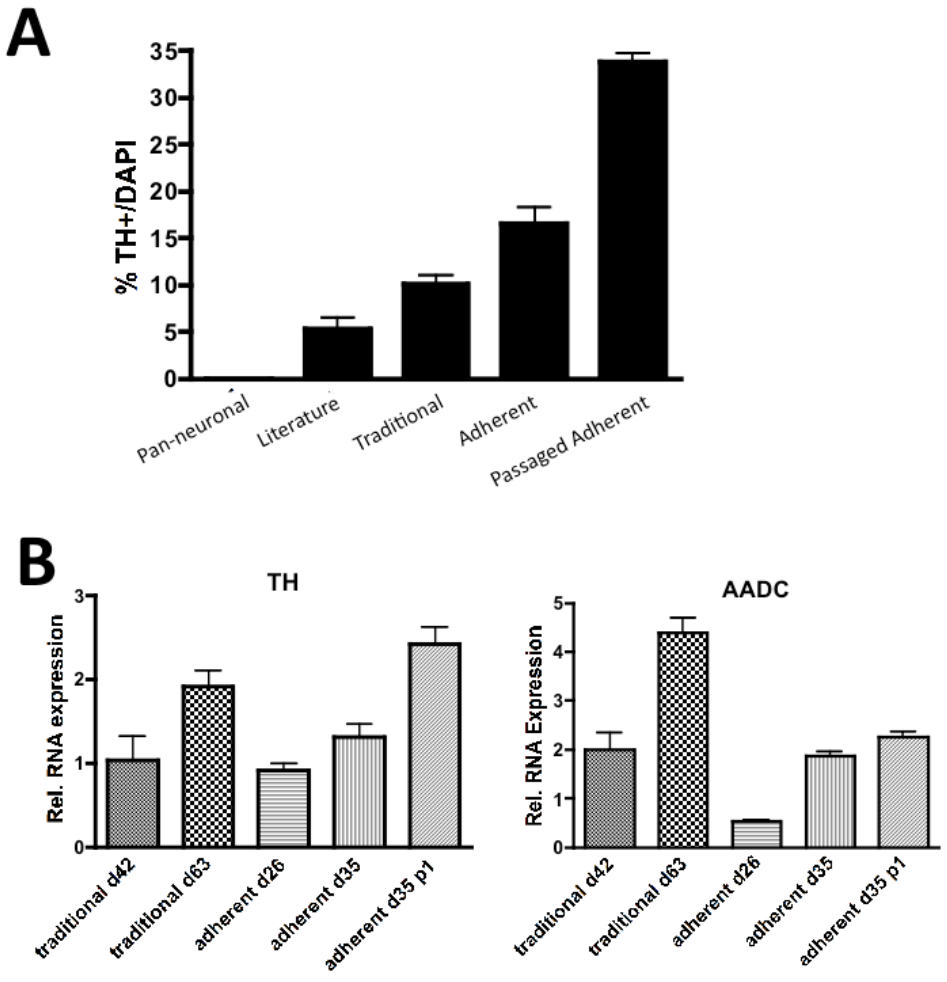

Figure 1.7 Comparison of differentiation paradigms.
A. Quantification of TH immuno-reactive cells in cultures differentiated in various conditions, including pan-neuronal patterned with FGF2, as described in the literature (Yan, 2005), optimized traditional paradigm, adherent, and adherent with a passage at day 12. B. Expression levels of terminal differentiation markers in methods previous described for dopaminergic differentiation. All data represented relative to GAPDH expression.

DA neurons are sensitive to inflammatory insults

To assess the functional applications of these cells we chose to go forward with the DA neurons generated using the traditional protocols. The cultures resulting from this differentiation paradigm are mixed with astrocytes and neurons, and are therefore best suited for modeling the inflammatory contribution to neurodegenerative disease. We treated these mixed cultures with IL1β and TNFα for one to four days, depending on the experimental readout, cyclohexamide (1ug/ml), an inhibitor of protein translation, was used as a positive control for cell death in these assays (Figure 1.8a). After four days of daily treatment with IL1β and TNFα we see a striking loss of DA neurons, as characterized by TH immuno-reactivity (Figure 1.8b). When quantified, we saw significant loss of TH positive neurons in both inflammatory and known cytotoxic conditions (Figure 1.8c). After four days, around half of the DA neurons in culture were lost (Figure 1.8c). When these cultures were analyzed for gene expression changes, we saw induction of both IL1β and cell death marker FAS, whose expression levels correlate with apoptosis[33] (Figure 1.8d).

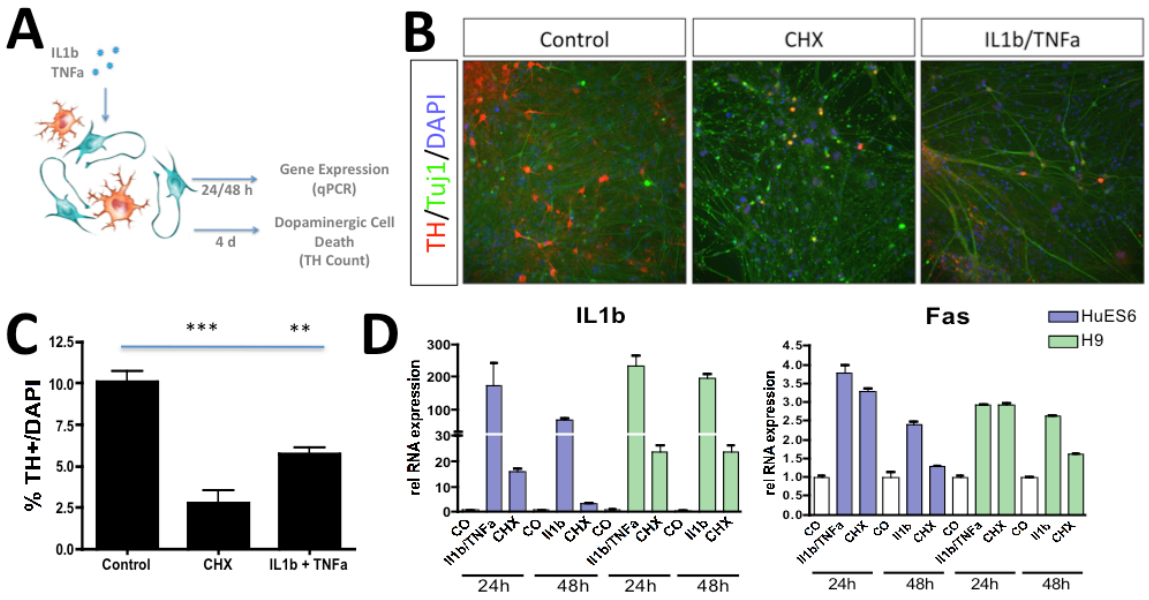

Figure 1.8 hESC-derived dopaminergic neurons are sensitive to immune insults.
A. Inflammation induced neurotoxicity paradigm. Briefly, hESC derived neurons at least two months post NPC differentiation were treated with IL1β (10ng/ml) and TNFα (50ng/ml). Cyclohexamide (CHX, 1ug/ml) was used as a positive control. B. 20x representative images of H9 mDA treated as described in A for four days prior to immunohistological analysis. C. Quantification of images in B. n = 4, four images per replicate. D. Gene expression data from H9 and Hues6-derived DA treated as described in A. All data relative to GAPDH expression. * = p < 0.05, ** 0.01, * 0.001**

CONCLUSION AND DISCUSSION

Both protocols described in this chapter are chemically defined methods of generating dopaminergic neurons, but vary in terms of their efficiency, timing, and application. The traditional paradigm is a longer protocol, requiring one week of standard cell culture, one week of floating hEBs and one week of attached neural rosettes prior to the formation of an multipotent neural precursor line and terminal differentiation (additional month at least). However, once the precursor line is generated it can be expanded and frozen, and is much easier to maintain

than a pluripotent culture. The adherent paradigm is much faster, requiring one week of standard cell culture, and then minimally 26 days of differentiation, but does not result in a multipotent precursor line and therefore requires the continual maintenance of pluripotent cells to feed into this differentiation paradigm.

The percentage of TH immuno-reactive neurons is highly variable in the adherent protocol due to the omission of the rosette selection step. The percent of DA neurons is directly proportional to the neural capacity of the pluripotent cell lines, which are highly variable. This variability in neural conversion is eliminated in the traditional five-step paradigm by the generation of a NPC line that has the same neurogenic potential as every other NPC line, regardless of any bias in the parental pluripotent cell line. This is less of a concern if only one pluripotent lines is being employed for a study, but rather problematic if multiple lines are to be compared, as required for effective disease modeling.

Though the reagents used in these protocols are chemically defined, the N2 and B27 supplements are still highly variable. The high density of cells required for the adherent paradigm makes the differentiation process very difficult to assess by light microscopy, immunofluorescence is required to effectively do so, whereas if reagent variability is affecting the differentiation it is very easily observed in the traditional paradigm.

The traditional paradigm results in a mixed neural population of neurons and astrocytes. We used multiple markers to document astrocytic differentiation in the adherent paradigm, but never observed this neural cell type, the most

common in the adult brain. While we did not fully investigate why this cell type is not present in these cultures, it could explain why they do not functionally mature without being passaged onto primary astrocytes. The absence of astrocytes is likely due to the short duration of this protocol, since astrocytes develop after neurons *in vivo*, or the prolonged exposure to Noggin. Noggin inhibits BMP signaling, which is crucial for astrocyte development. This might be advantageous for studying cell autonomous aspects of neural development and disease, but if cell non-autonomous aspects want to be investigated, the traditional protocol is the most appropriate protocol.

The two protocols described in this chapter are both chemically defined and yet represent very different methods for generating DA neurons. While the traditional protocol is more time consuming and contains more points at which variability can be introduced, it is also more established in the literature, and allows for the production of other neuronal lineages (including astrocytes and oligodendrocytes). The adherent protocol is faster and more efficient in our hands, but comparing lines using this protocol might be challenging if they vary in their neurogenic potential. The use of both protocols will enable effective disease modeling and the study of human development and disease pathogenesis.

For the purpose of this project, the mixed neural population that arises from the traditional differentiation paradigm is better suited. The presence of both astrocytes and neurons provides a useful platform for studying the non-cell autonomous aspects of neurodegeneration. The sensitivity of neuronal cells to pro-inflammatory mediators is well established in the literature, and the

experiment described in this chapter not only confirms those results, but extends them to a human system, which can serve as a platform for compound screening towards identifying new clinical candidates that can prevent inflammation induced neuro-toxicity.

MATERIALS & METHODS

Five-step differentiation of pluripotent stem cells. Pluripotent stem cells were grown in large colonies until confluent are lifted as whole colonies from the plate using Collagenase type IV (Sigma, 1mg/ml). Colonies are gently washed and then replated into Ultra-Low Attachment Plates (Corning) in DMEM/F12 with 1x N2 and 1x B27 Supplements (Invitrogen) for one week. They are then transferred to a laminin coated plate (Sigma, 1ug/ml) in the same media, supplemented with laminin (Sigma, 1ug/ml). The resulting neural rosettes are manually selected based on morphology and transferred to a new laminin coated well for a second round of selection. Once all isolated hEBs contain rosettes and projections, the culture is dissociated with TrypLE (1x, Invitrogen), and the resulting monolayer culture is cultured in DMEM/F12 with 1x N2 and 1x B27 Supplements (Invitrogen), with Fibroblast growth factor 8 (100ng/ml, Peprotech), Sonic hedgehog (200ng/ml, R&D) and split at a 1:6 ratio once a week. To differentiate the cells, they are enzymatically dissociated with TrypLE into single cells and plated on laminin coated plates at $2x10^5$ cells / cm^2 in medium containing Brain Derived Neurotrophic Factor (20ng/ml Peprotech), Glial Derived Neurotrophic

Factor (20ng/ml Peprotech), Ascorbic Acid (200nM, Sigma), Cyclic AMP (1mM, Sigma), for up to three months.

Adherent differentiation paradigm. Pluripotent stem cells were grown as a monolayer on Matrigel (1ug/50cm^2) until 95% confluent, at which point they were fed daily with a pluripotent stem cell medium in which the growth factors have been removed, either fibroblast conditioned medium or defined TeSR medium with out FGF2 and TGFb work well for this purpose. For the first five days, the cells are fed daily with this medium, supplemented with SB431542 (Tocris, 10uM) and Noggin (Peprotech, 500 ng/ml). From day 6-9 cells are transitioned into a DMEM/F12 with 1x N2 and 1x B27 Supplements (Invitrogen) based medium, 25% per day, in the presence of Fibroblast growth factor 8 (100ng/ml, Peprotech) and Sonic hedgehog (200ng/ml, R&D). Days 9-12 involve feeding with a pure DMEM/N2/B27 medium with Fibroblast growth factor 8 (100ng/ml, Peprotech), Sonic hedgehog (200ng/ml, R&D), Brain Derived Neurotrophic Factor (20ng/ml Peprotech), Ascorbic Acid (200nM, Sigma). From day 12 – 26 and on, cells are fed with a terminal differentiation medium containing Brain Derived Neurotrophic Factor (20ng/ml Peprotech), Glial Cell Derived Neurotrophic Factor (20ng/ml Peprotech), Ascorbic Acid (200nM, Sigma), and Cyclic AMP (1mM, Sigma).

Immunocytochemistry. Cells were fixed with 4% paraformaldehyde for 15-30 minutes, prior to permabilization and blocking with 0.1% Triton-X and 3% Donkey Serum. Cells were incubated in primary antibodies overnight at 4 degrees, and in secondary for two hours at room temperature, prior to a brief incubation with

DAPI (Sigma, 1ug/ml). Images were obtained on an Olympus IX51 fluorescent microscope.

Quantitative gene expression analysis. RNABee (ThermoFisher) was used to lyse and extract RNA per manufacturer's instructions. 2 ug of purified RNA is used per cDNA synthesis reaction using High Capacity cDNA reverse transcriptase kit (Applied Biosystems). The resulting cDNA was diluted to 200ng/ul and used for 384 qPCR reactions, using Taq Man probes (Applied Biosystems). qPCR was performed on an Applied Biosystems 7300 qPCR machine, per manufacturer's instructions. All data was analyzed using SDS v2.4, and four technical replicates were performed per sample.

Electrophysiology. Whole cell patch recording was performed on neurons at least two months old expressing Synapsing driving GFP. Methods as previously described [32].

Inflammatory neurotoxicity assay. Neurons differentiated using the traditional five step method, as described above, were treated with IL1β (10ng/ml, R&D) and TNFα (50ng/ml, R&D) in terminal neural differentiation medium for minimally six hours and up to four days depending on the assay. Quantification was performed using either gene expression or by manual counting of TH immuno-reactive neurons and DAPI stained nuclei using ImageJ (RSB, NIH). At least four images are quantified per replicate.

Statistical Analysis. All data were analyzed using Prism software. Data was analyzed using either a Student's T-test or ANOVA followed by a Bonferroni's posthoc test. $p < 0.05$ is considered statistically significant.

ACKNOWLEDGEMENTS

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CHAPTER 2.

Human in vitro model of glial based neuro-inflammation

ABSTRACT

Historically, neurodegeneration has been considered a strictly neuronal disease; however, recent evidence suggests a role for inflammation and the aging brain in neurodegenerative diseases including Alzheimer's Disease, Amyotrophic Lateral Sclerosis, and Parkinson's Disease. Both clinical observations and evidence from animal models suggest inflammation may contribute to disease progression. It remains controversial whether glial activation, and the resulting inflammatory cascade, is a result or a cause of neuronal death. Towards resolving this distinction, we have established cultures of primary human astrocytes and microglia. These glial cells enable investigation into the glial-derived inflammatory response to extracellular insults, and the neuronal response to pro-inflammatory mediators. Both the human astrocytes and microglia reliably recapitulate their in vivo counterparts by expressing the appropriate markers, having spontaneous calcium transients, and in their ability to mount an inflammatory response. We have found that the glial derived pro-inflammatory response can be serially propagated between astrocytes and microglia following a single inflammatory insult. Our human cell based model also establishes the neurotoxic response to a glial derived inflammatory cascade, allowing us to study the inflammatory contribution to the pathological

development of PD in a human system. The goal of this chapter is to establish a robust model of human glial derived neuro-inflammation, which will allow us to study the role of inflammation in PD and identify key molecular events involved at early stages in PD to exploit as potential targets for therapeutic intervention.

BACKGROUND AND SIGNIFICANCE

The human brain contains over 100 billion cells, trillions of synapses, and stands alone in its enormous cellular diversity in both form and function[34]. These neural cells can be divided into neurons and non-neuronal glial cells. The glial subtypes in the central nervous system (CNS) include astrocytes, microglia, oligodendrocytes, and ependymal cells; I'll be focusing on the first two for the purpose of this chapter, due to their well established contribution to neuro-inflammation. Astrocytes are the most abundant glial cell in the central nervous system (CNS) and provide metabolic support and respond to injury by clearing damaged cells and forming a glial scar[35]. Microglia are specialized macrophages that compose roughly 10% of the CNS and are the initial sensors of and responders to injury[36]. Upon stimulation, both of these cell types can undergo activation, which results in the release and amplification of cytotoxic and pro-inflammatory factors (ex. TNF-α, IL-1β, iNOS). Though neurons and glia exist in the human brain in equal proportion, the latter are traditionally overlooked when investigating brain disease[37].

Parkinson's disease (PD) is the most prevalent movement disorder worldwide and results in severely debilitating motor symptoms (bradykinesia,

resting tremor, rigidity, and postural instability). The motor symptoms in PD result largely from a deficiency in dopaminergic neurons in the substantia nigra (SN) pars compacta. The neuropathological hallmark of PD are Lewy bodies and Lewy neurites, intra and extracellular proteinacious clusters, thought to disrupt normal cellular function. Alongside Lewy bodies, reactive microglia positive for human leukocyte antigen HLA-DR and CD11b are found in the SN of PD patients and in other brain regions[38]. The prominent histological hallmark of PD is the resulting dopaminergic deficit within the nigrostriatal projection; therefore, a successful therapy for the motor symptoms will aim at preventing further degeneration and restoring dopaminergic neurotransmission. Pharmacological drug treatment is usually effective only in the early stages of the disease and cannot halt the progressive neuronal degeneration[39].

Though once considered a purely neuronal disease, recent findings have illuminated an inflammatory component to PD etiology and other neurodegenerative diseases (reviewed in [40]). Evidence from Amyotrophic Lateral Sclerosis (ALS) studies suggest that a sustained inflammatory response involving interplay between neurons and astrocytes can contribute to neurodegeneration[41]. Additionally, most neurodegenerative disease are associated with advanced age, as are increases in pro-inflammatory factors. The connection between the two was recently established in aged rats, which had increased levels of pro-inflammatory cytokines and oxidative stressors in their nigras, and were more sensitive to a chemical injury model of PD[42]. Giving the advancing age of the worldwide population, it is of utmost importance that we

identify a therapy that targets the wide array of dysfunction observed in neurodegenerative diseases.

Clinical observations, and post-mortem and epidemiological studies have implicated inflammation in PD pathogenesis. Clinicians have seen increases in dystrophic astrocytes and activated microglia in PD affected brains upon autopsy[43,44]. Activated microglia, as determined posthumously by HLA-DR expression or during disease progression by PK11195 binding in PET scans, are significantly increased in PD patients [38],[44]. Increases in dystrophic astrocytes are also associated with disease progression[43]. These data suggests that microglia and astrocytes are involved in PD, but not whether their activation correlates and contributes to PD onset and progression.

Microarrays performed on postmortem SN tissue have shown elevated expression of pro-inflammatory cytokines and mediators including IL1β, IL6, IL8, TNFα, iNOS, and COX2 in PD patients relative to controls[45,46]. Patients with PD also have elevated levels of pro-inflammatory cytokines in their cerebrospinal fluid (CSF) and serum[47]; some of which, including IL6, have been shown to correlate with severity of motor symptoms[48]. This finding is consistent with reports of polymorphic regions within the promoters of IL1β and IL8 that correlate with PD risk and onset[49,50,51]. In addition to pro-inflammatory cytokines, reactive oxygen and nitrogen species, and the genes that are responsible for their production, are strongly implicated in PD[45]. These data, taken together, strongly implicates glia and the inflammation that results upon their activation, as active players in PD pathogenesis.

In chemical and transgenic models of PD activation of glia seems to precede neuronal death (reviewed in [52]). Mice deficient in microglia and pro-inflammatory factors, or those treated with microglial inhibitors, have less neuronal death in chemically injury models of PD [52,53]. Additionally, rodents which chronically over-express IL1β show nigral degeneration[54], and in a separate study, those treated with dominant negative forms of the TNFα receptor have attenuated nigral degeneration[55]. Furthermore, an intranigral injection of lipopolysaccharide (LPS), which alone has no neuro-toxic effect, is sufficient to induce dopaminergic specific neurodegeneration in a glial model of PD[56]. Additionally, multiple epidemiological studies have found reduced risk of PD among non steroidal anti-inflammatory users[57,58]. Taken together, these data suggests that inflammation can play an active role in neurodegeneration by contributing to the progression, if not onset of these age related diseases.

The association between inflammation and PD is well established by clinical observations and murine studies[52]; however it remains controversial whether a pro-inflammatory stimulus contributes to disease progression in sporadic PD. Since most PD cases are idiopathic in origin, it is of utmost importance that we understand and target the potentially pathogenic contribution of glia to PD onset and progression. While inflammation and neurodegenerative disease correlate in the aging brain, we must look beyond clinical observations to animal models for indication of causality. The inability to sample live brain cells limits our knowledge of pathological abnormalities and neuro-inflammation in human PD. Our current understanding of inflammation in PD in a human context

is mostly generated from analyzing end-stage postmortem brain tissues, where concomitant testing of inflammatory markers is no longer possible. Specifically, human samples from early in the course of the disease would be informative for understanding neuro-inflammation in PD, but they are rare. We have therefore chosen human cells to model neuro-inflammation to resolve this distinction because rodent models do not sufficiently recapitulate neurodegeneration, and most compounds found in these studies fail FDA safety trials due most likely to intrinsically different neuro-chemistry and off-target effects[59]. Human stem and primary cells provide us with the unique opportunity to model the inflammatory component of PD *in vitro,* which allows us to further our understanding of the contribution of inflammation to PD and identify compounds that can slow pathogenic phenotypes while having a better chance of being safe and efficacious.

Several lines of evidence suggest that these inflammatory mediators, derived from non-neuronal cells including microglia and astrocytes, modulate the progression of PD [60],[61]. Therefore, modulating neuro-inflammation during the early stages of the disease might largely decrease disease severity and slow PD progression. In this chapter, I aim to model the non-cell autonomous aspects of PD, towards establishing a robust platform that can be employed to test the inflammatory potential of exogenous factors and serve as a targeted drug-screening platform.

RESULTS

Modeling neuro-inflammation with human microglia and astrocytes

Towards modeling neuro-inflammation *in vitro*, we established cultures of primary human microglia (Clonexpress, Gaithersburg, MD). These cells do not grow appreciably in culture, and therefore are used sparingly and only for terminal experiments. The cells display traditional macrophage like morphology and express appropriate markers including Iba1 (Figure 2.1a and 2.1b). Towards characterizing these human cells as a valid model of neuro-inflammation, the microglia were treated with lipopolysaccharide (LPS)[62], the immuno-reactive component of bacterial outer cellular membranes, also known as endotoxin. The cells were exposed to 0.1ug/ml of LPS, and samples of the medium were collected over a 48 hour period and assayed for cytokine content using an ELISA. We found that the microglia are capable of activation, as evidence by the pro-inflammatory factors secreted upon LPS stimulation, including IL1β, IL6, IL8, and TNFα, and reactive oxygen and nitrogen species, all of which have been implicated in PD pathogenesis (Figure 2.1c, 2.1d). Immortalized human microglial cell lines were also assayed, and while they expressed the appropriate markers, they failed to activate at the same magnitude as the primary ones, potentially because they already underwent activation during the immortalization process (data not shown).

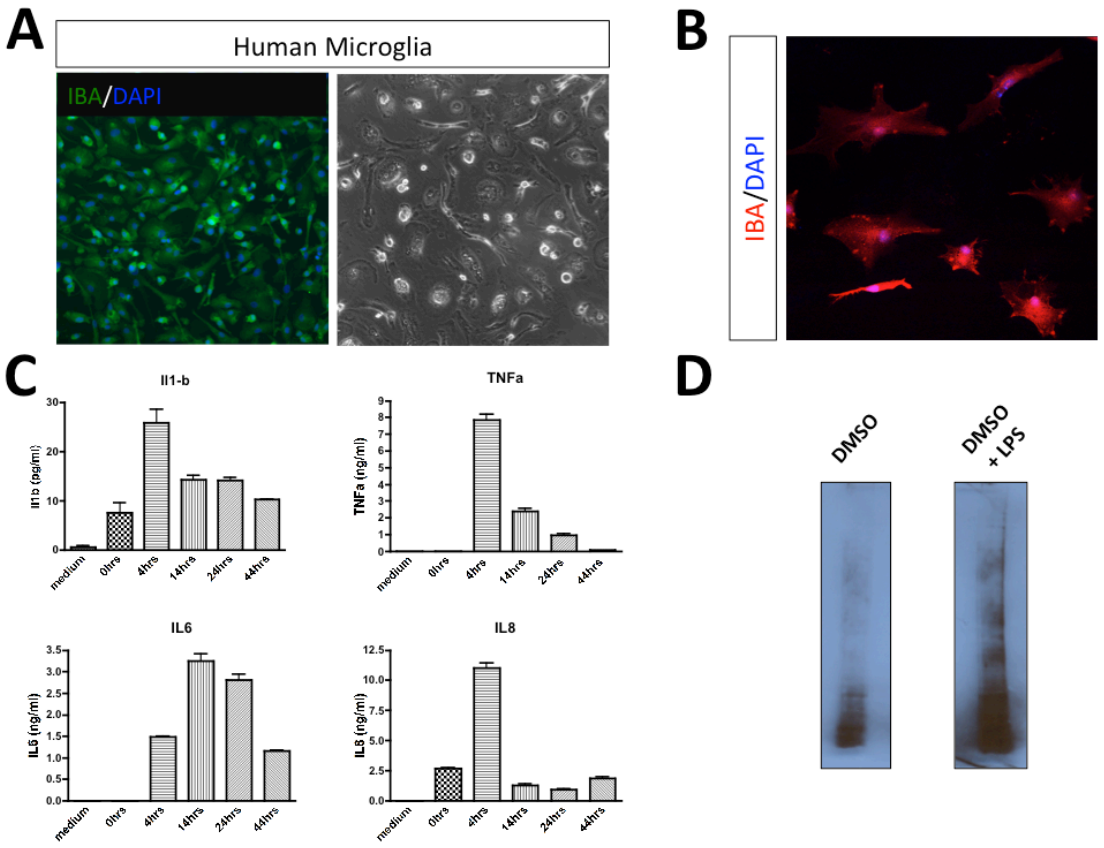

Figure 2.1 Primary human microglia express appropriate markers and undergo activation.
A. Immunofluorescence and bright-field images of human primary microglia, 20x. B. 40x fluorescent image. C. Pro-inflammatory cytokine protein levels determined through ELISA from cell supernatant of primary human microglia treated with LPS (0.1ug/ml) over 48 hours. D. Immunoblot indicative of reactive species in microglia four hours after LPS treatment.

We procured primary human astrocytes from a San Diego biotechnology company (Sciencell, Carlsbad, CA). These cells grow nicely in culture, senescing after about 10 passages or around 25 cell doublings. The human astrocytes have the expected star like morphology and express the appropriate markers, including GFAP, A2B5 and S100b (Figure 2.2a, 2.2b). They also have spontaneous calcium transients (Figure 3.7). The astrocytes secrete pro-

inflammatory cytokines upon stimulation with LPS, to confirm that they are capable of contributing to the immune response in the brain. The human astrocytes were treated with LPS (1ug/ml) and supernatant was collected over 48 hours and assayed for pro-inflammatory cytokines (Figure 2.2b).

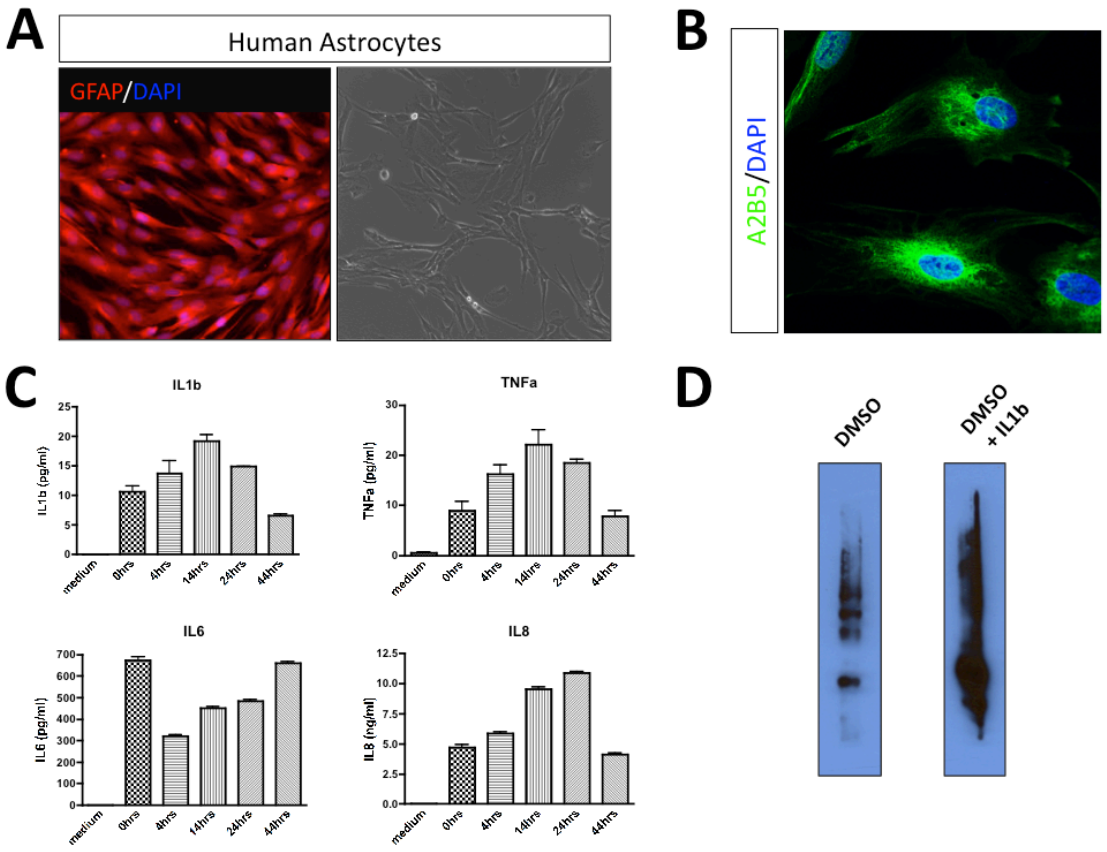

Figure 2.2 Primary human astrocytes express appropriate markers and undergo activation.
A. Immunofluorescence and bright-field images of human primary astrocytes, 20x. B. Higher magnification fluorescent image, 40x. C. Pro-inflammatory cytokine protein levels determined through ELISA from cell supernatant of primary human astrocytes treated with LPS (1ug/ml) over 48 hours. D. Immunoblot indicative of reactive species in microglia four hours after IL1b treatment.

Comparing immune response in human glia

In comparing the response to LPS generated by both glial cells types, it became apparent that microglia mount a much greater inflammatory response, which is consistent with the hematopoietic origin of the cells, and the notion that microglia are the primary initiators of an immune reaction in the brain. The peak in cytokine secretion also occurs earlier for microglia, at around six hours,

depending on the cytokine, while the astrocytes tend to peak around 12 hours (Figure 2.3a-d). Overall, the immune reaction to LPS is faster and greater in microglia than astrocytes (Figure 2.3), this is intuitive since LPS is a Toll-like receptor 4 (TLR4) agonist, and microglia are known to express higher levels of this receptor than astrocytes.

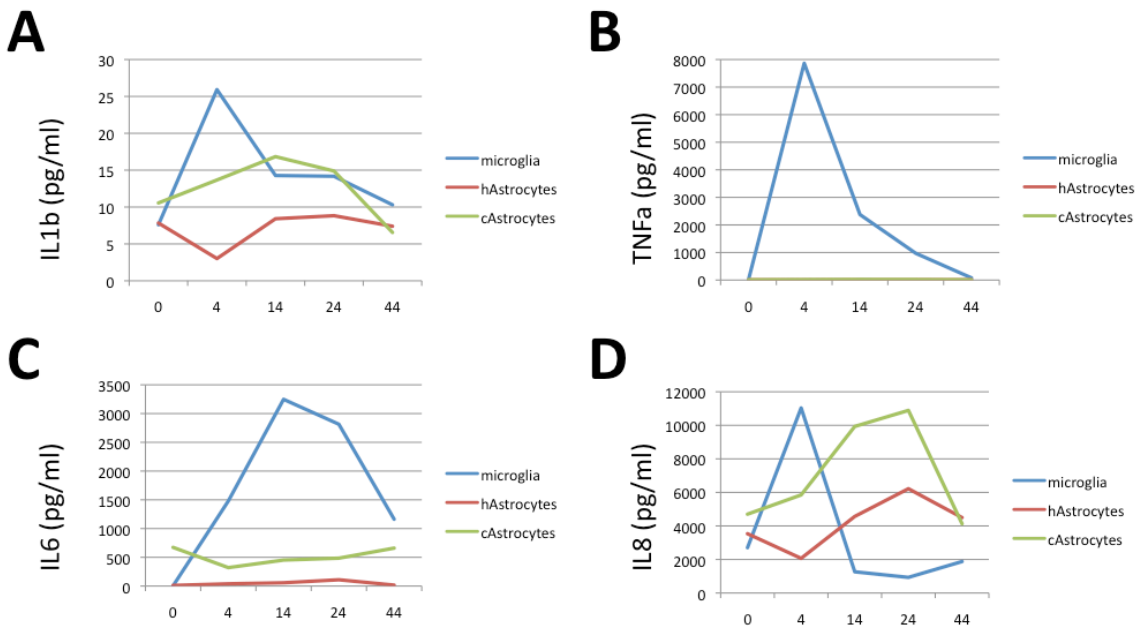

Figure 2.3 Human microglia have a faster and greater response than astrocytes to LPS.
A. Time course of IL1β protein levels after stimulation with LPS (0.1ug/ml microglia, 1ug/ml astrocytes). B. Time course of IL6 protein levels after stimulation with LPS (0.1ug/ml microglia, 1ug/ml astrocytes). C. Time course of IL8 protein levels after stimulation with LPS (0.1ug/ml microglia, 1ug/ml astrocytes). D. Time course of TNFα protein levels after stimulation with LPS (0.1ug/ml microglia, 1ug/ml astrocytes).

Since astrocytes do not respond as well to TLR4 receptor agonists, we reconsidered this activation assay in light of their perceived role in the brain as amplifiers of the immune response, rather than initiators. We therefore treated

the human astrocytes with pro-inflammatory mediators, which we confirmed were being secreted from the microglia upon activation, in comparison with LPS. The astrocytes were stimulated with IL1β (10ng/nl) and TNFα (50ng/ml) alone, and in combination over 48 hours. Media samples assayed for the secretion of pro-inflammatory cytokines IL6 and IL8 indicated that pro-inflammatory mediators are much more potent activators of astrocytes (Figure 4). This data establishes that the primary purpose of astrocytes in the human brain is not to respond to TLR4 agonists, but to amplify the signals secreted by other activated glia and potentially their own battery of activators for which they do have receptors. Given the strong immune potential of these cells, both in cytokine secretion and production of reactive oxygen and nitrogen species (Figure 2.2c and 2.2d), it is conceivable that they can act as primary responder to other immune insults independent of TLR4.

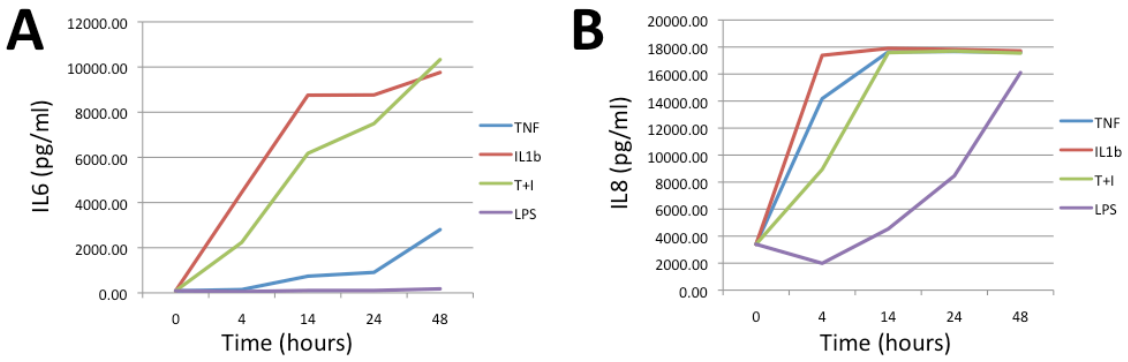

Figure 2.4 Pro-inflammatory cytokines are potent activators of astrocytes. A. Time course of IL6 protein levels after stimulation with either LPS (1ug/ml), IL1β (10ng/ml), TNFα (50ng/ml) or both IL1β and TNFα combined. B. Time course of IL8 protein levels after stimulation with either LPS (1ug/ml), IL1β (10ng/ml), TNFα (50ng/ml) or both IL1β and TNFα combined.

Glial cells propagate inflammatory signals

Towards establishing the role of non-neuronal cells in neurodegeneration, we tested whether these cells glial cells are capable of propagating pro-inflammatory signals in the absence of the initial insult. Having established that human microglia are the initiators of the immune response, we first stimulated them with LPS for one hour, washed away all remnants of this potent TLR4 agonist, and then allowed the pro-inflammatory factors, including cytokines and reactive oxygen and nitrogen species to accumulate for six hours prior to transferring that medium to astrocytes (Figure 2.5a). The astrocytes were incubated in the medium for 12 hours, a period determined in prior experiments as the maximal for astrocytic cytokine secretion (Figure 2.4), before being transferred to a fresh plate of microglia (Figure 2.5a). This was repeated for four cycles and medium samples were collected at each point and analyzed for cytokine content. We found that inflammatory signals can be propagated between glial cells in the absence of the initial insult due to the sustained immune response as indicated by sustained IL6 and IL8 levels (Figure 2.5b, 2.5c).

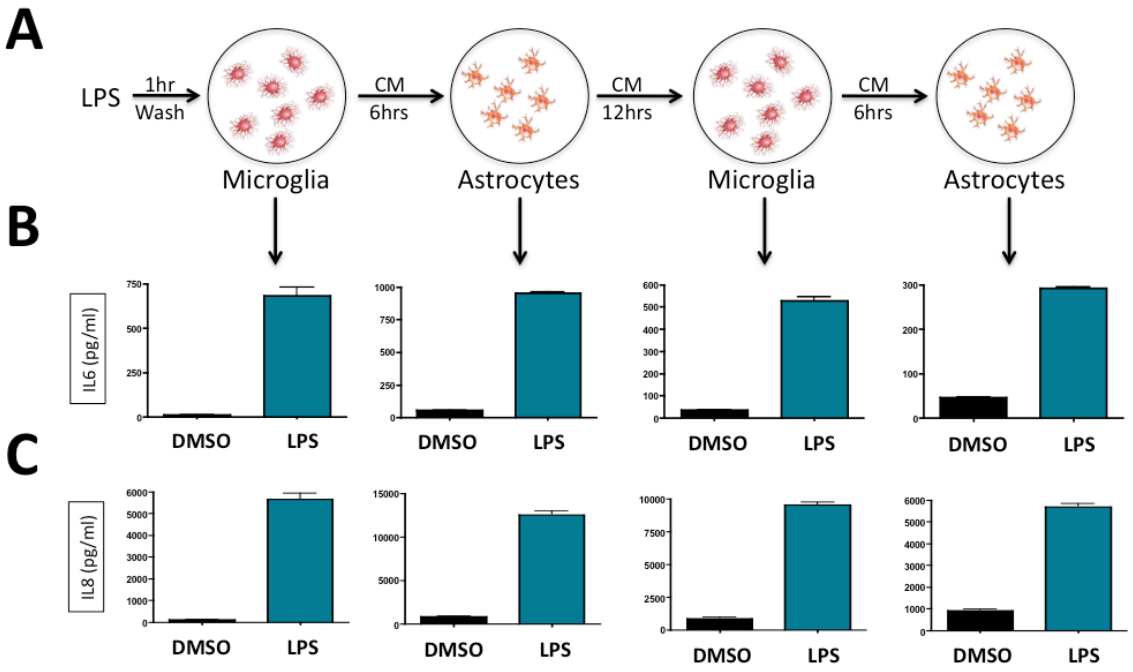

Figure 2.5 Propagation of inflammatory signals initiated by activated microglia.
A. Experimental paradigm. Briefly microglia were stimulated with LPS (0.1ug/ml) for one hour, washed thoroughly. Pro-inflammatory mediators were allowed to accumulate for six hours prior to transfer to the next glial culture. Media samples were removed at each transfer and assayed for cytokine levels. B. IL6 protein levels from each step of the propagation described in A. C. IL8 protein levels from each step of the propagation described in A.

This propagation assay can also be performed with activated astrocytes as the initiators of the inflammatory cascade (Figure 2.6a). While the astrocytes were activated with a mediator, IL1β, not an traditional TLR4 ligand, this experiment still provides proof of concept that inflammatory signals generated from astrocytes are capable of inducing a propagatable cytokine storm (Figure 2.6b, 2.6c). This is relevant for PD and other neurodegenerative diseases because it shows definitively that an initial, one time insult is sufficient to set off a cascade of inflammation that is self-perpetuating and persistent.

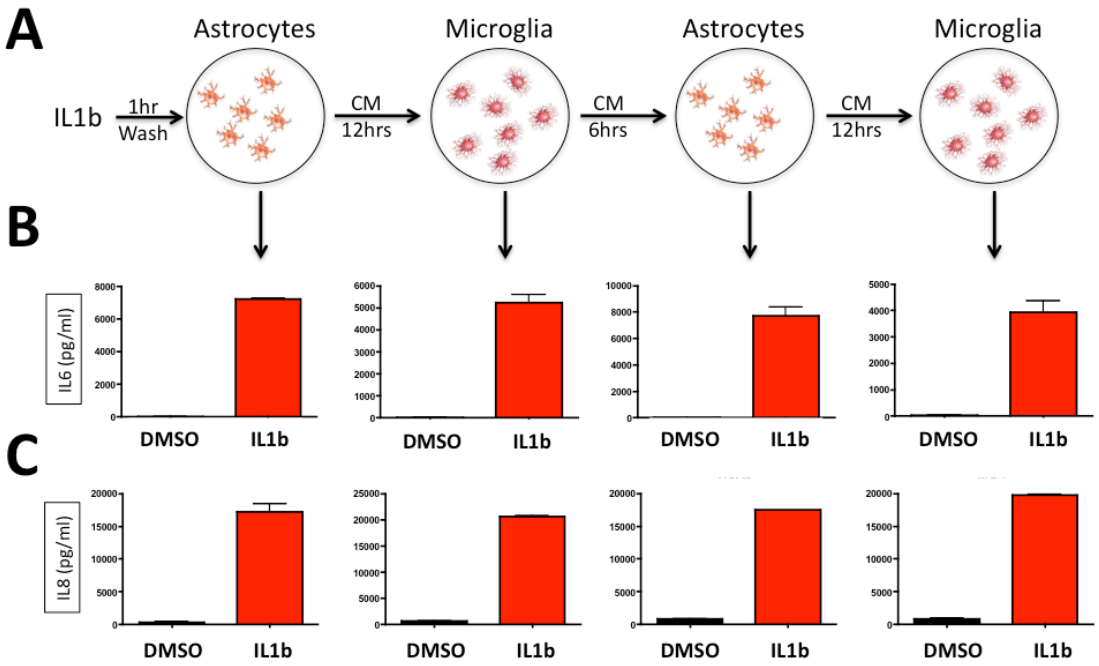

Figure 2.6 Propagation of inflammatory signals initiated by activated astrocytes.
A. Experimental paradigm. Astrocytes were stimulated with IL1β (10ng/ml) for one hour, washed thoroughly. Pro-inflammatory mediators were allowed to accumulate for 12 hours prior to transfer to the next glial culture. Media samples were removed at each transfer and assayed for cytokine levels. B. IL6 protein levels from each step of the propagation described in A. C. IL8 protein levels from each step of the propagation described in A.

CONCLUSIONS AND DISCUSSION

From the data generated in this chapter we can conclude that human glial cells can serve as a robust and biologically relevant platform for modeling neuro-inflammation. The primary human cells described in this chapter express the expected cellular markers and are functional contributors to inflammation, as indicated by their ability to undergo activation. We assessed this cellular conversion by quantitative biochemical means rather than by gross morphological changes, as is common *in vivo*. The reactive gliosis described in

the literature was not observed in our hands *in vitro*, potentially due to the absence of the complex *in vivo* environment. Greater rigor was not placed on this method of assessing activation since it is more subjective and qualitative, and it remains unclear what affect the morphological changes play in disease pathogenesis, whereas quantifiable measures including cytokine and ROS secretion have been directly implicated in PD progression. Since these cells are primary in origin, we were concerned about the donor to donor variability. Multiple primary lines were assayed per glial cell type, from multiple brain sources, isolated at various time points. We found that while there is more variability in the astrocyte populations, both cell types responded consistently to their respective activators, independent of donor source.

As expected the human microglia are faster and more potent responders to TLR4 mediated insults, which is intuitive since they are the brain's resident macrophage. However, astrocytes are still capable of activation in response to pro-inflammatory mediators, as determined through secretion of pro-inflammatory cytokines and ROS; they do so at a lower magnitude in response to TLR4 agonists. The astrocytes are amplifiers of pro-inflammatory mediators, including but not limited to IL1β and TNFα, both of which can play active roles in PD etiology. Signals generated from astrocytes in response to pro-inflammatory mediators are capable of eliciting a cytokine storm that can be propagated between glial cells, even in the absence of the initiating insult.

The ability for glial cells to propagate pro-inflammatory signals is of high relevance to any neurodegenerative disease in which inflammation is implicated.

This assay models the ability of a single inflammatory insult to initiate a perpetual cycle of inflammation that over time can contribute to neuronal cell death. Any traumatic injury, either physical or chemical, to the brain could initiate this pro-inflammatory cytokine storm and the propagation assay shows that such an injury, if it generates an immune response, is sufficient to cause further disease pathology in the form of glial activation and the resulting neuronal death.

In addition to informing us on the basic biology of pro-inflammatory signal transduction, the propagation assay will also serve as a valuable tool for drug screening. Any compound that can successfully dampen, or ideally prevent, the propagation of these signals will be a promising candidate for therapeutic applications. In the event that such a compound is found clinically safe and efficacious *in vivo*, it could be used not only for patients with early symptoms of PD, but also for individuals with traumatic brain injury, or who have been exposed to known chemical toxins. Early treatment with a compound that can prevent the initiation of a self-perpetuating inflammatory cascade will be of great clinical relevance.

Overall, the cells described in this chapter can serve as a robust *in vitro* model of neuro-inflammation. This model can be employed to assay the neuro-inflammatory response to extracellular insults, for example alpha-synuclein, the protein whose mis-folding is the pathological hallmark of PD, or to chemical insults including but not limited to oxidative stressors. This model can also act as a drug screening platform for compounds to determine their anti-inflammatory potential, or to eliminate compounds that might cause an adverse immune event.

Data generated from this model can also be applied to iPSC technology from which disease specific neural cells can be derived. The assays developed in this chapter can be used to assess the hyper-excitability of diseased cells relative to non-diseased controls. Taken together, this model is a powerful tool in the investigation of the contribution of inflammation to neurological disease.

MATERIALS & METHODS

Human astrocytes culture. Astrocytes were obtained from Sciencell (Carlsbad, CA), and cultured per manufacturer's instructions. Briefly, cells were expanded and frozen in a 10% DMSO solution for stocks at passage three. All experiments start with cells thawed from this stock into Astrocyte Medium (Sciencell), split at a 1:3 ratio whenever confluent and fed as necessary. For experimental purposes, cells were plated at $2x10^4$ cells/cm^2 and allowed to divide for two days prior to treatment.

Astrocyte activation assay. Cells prepared as described above were treated with 10ng/ml IL1β (R&D) or 50ng/ml TNFα (R&D) for at least one and up to six hours.

Human microglia culture. Microglia are purchased from Clonexpress (Gaithersburg, MD), do not grow appreciably in culture, and therefore are thawed directly into the format appropriate for the desired assay. Microglia are thawed into a DMEM/F12 based medium supplemented with 5% Fetal Bovine Serum (HyClone) and recombinant human macrophage colony stimulating factor

(rhMSCF, 10ng/ml, R&D). Media is exchanged every 2-3 days, and cells are assayed after two days and up to a week.

Microglia activation assay. Microglia are thawed as described above and treated with lipopolysacaride (LPS, 0.1ug/ml, Sigma) for at least one and up to six hours.

Signal propagation assay. Cells were stimulated with their respective activators for one hour, and thoroughly washed twice. Pro-inflammatory factors were allowed to accumulate in a DMEM/F12, 5% FBS medium, supplements with both MCSF and Astrocyte Growth Supplement (AGS, 1x, Sciencell), for either six or 12 hours for microglia or astrocytes respectively. At each transfer, half of the medium was frozen at -80 degrees for later analysis and half was supplemented with it's volume in the afore described medium before being transferred to the next cell type. At least four transfers were performed, using both types of glia as the initiating cell type.

Cytokine quantification. Pro-Inflammatory cytokines were measured using a multi-spot ELISA. The Human Pro-Inflammatory Type II kit from MesoScaleDiscovery (MSD, Gaithersburg, MD) was used per manufacturer's instructions and all data was read on the MSD Sector Imager. Data was validated using their MSD Sector program. These assays were always performed with three technical replicates.

Quantitative gene expression analysis. Refer to Chapter 1.

Statistical Analysis. All data were analyzed using Prism software. Data was analyzed using either a Student's T-test or ANOVA followed by a Bonferroni's posthoc test. $p < 0.05$ is considered statistically significant.

ACKNOWLEDGEMENTS

Chapter 2 contains material submitted for publication by Leah Boyer, Chris Tse, and Fred H. Gage. The dissertation author is the primary investigator and author of this material.

CHAPTER 3.

Apigenin is anti-inflammatory and neuro-protective

ABSTRACT

Inflammation is a well-established component of neurodegenerative diseases, however it has yet to be effectively targeted as a means of therapeutic intervention. We recently found that Nurr1, whose role in dopaminergic neuron survival is well established, also plays an essential role in both microglia and astrocytes as a transcriptional repressor of genes that encode pro-inflammatory, neuro-toxic factors. The dual role of this orphan nuclear receptor renders it an ideal target for therapeutic intervention in PD. In this chapter, I use cultures of human dopaminergic neurons, astrocytes and microglia as a platform for targeted screening of compounds that are both anti-inflammatory and neuro-protective. We screened many compounds and found the most effective compound to date is a flavonoid called apigenin, which significantly reduces glial activation and is neuro-protective. Apigenin is acting partially through MAPK mediated, PKA and PKC independent, activation of CREB, which is a well-established inducer of Nurr1 expression. This natural compound and the nuclear receptor that it targets, in combination with our human in vitro stem cell modeling platform, represent a highly innovative strategy to furthering therapeutic approaches to ameliorating PD. The purpose of this chapter is to employ human cell based models to identify novel therapeutic compounds.

BACKGROUND AND SIGNIFICANCE

Drug discovery for novel compounds to treat human disease is traditionally undertaken in animal models of disease. However, animal models for neurodegenerative diseases consistently fail to recapitulate all aspects of the disease. Additionally, compounds identified using these models have particularly high FDA failure rates for lack of safety, due to the large range of off target and side effects in the brain, and lack of efficacy, likely due to intrinsic inter-species differences, which are likely amplified in the central nervous system[59]. Towards addressing these concerns, we have established a targeted drug screening paradigm using the human cells established in Chapter 2. We believe that compounds identified as both efficacious in human cells, that have also been proven safe in animal models, will have a greater likelihood of being both safe and efficacious in human patients.

The activation paradigms and propagation assays described in Chapter 2 provide an ideal platform for compound testing. Compounds are initially assayed for their ability to prevent the activation of astrocytes, followed by microglia. The gene expression data for the most promising compounds are validated with an ELISA assay for reduction in secretion of pro-inflammatory cytokines. Remaining compounds are then tested in the propagation assay, to ensure they can effectively dampen the self-perpetuating cytokine storm that is implicated in the pathogenesis of PD.

Towards selecting a viable target for this compound screening, we focused on nuclear receptors since their activity has been widely implicated in

neuro-inflammation generally and PD specifically[63]. Within the nuclear receptor families, we identified a nuclear receptor whose activation is both neuro-protective and anti-inflammatory. Nuclear receptor related protein 1 (Nurr1, NR4A2) is an orphan nuclear receptor that serves many functions through its role as an intracellular transcription factor[64,65]. While most of the literature describing Nurr1's function, including work from our lab, is focused on its role in maintaining the dopaminergic system[66], Nurr1 is also expressed in non-neuronal cells including macrophages, in which it's expression can be induced by immune activation[67,68]. Reductions in Nurr1 function have been associated with a variety of degenerative and inflammatory diseases, including PD, schizophrenia, and rheumatoid arthritis[69,70,71]. Nurr1 belongs to the nuclear receptor 4A (NR4A) family, which it shares with two other members, Nur77 (NR4A1) and NOR1 (NR4A3); all three members have high homology and may serve redundant functions[72].

Deletion of the Nurr1 gene in mice results in a severe reduction in dopaminergic neurons and perinatal lethality[73], which is consistent with the gene's essential role in the development and maintenance of dopaminergic neurons. Reductions in Nurr1 levels also increases susceptibility of DA neurons to chemical injury models of PD[74]. Human mutations resulting in reduced expression of Nurr1 are associated with late-onset familial PD[75], indicating that Nurr1 may play a protective role for PD. A homozygous polymorphism in the Nurr1 gene was shown to be more frequently present in PD patients than in controls[76]. Nurr1 is also expressed in non-neuronal cell types and Nurr1

expression is induced by inflammatory stimuli, including LPS, in macrophages[67]. The role of Nurr1 in inflammation was strengthened by a recent study in the cardiovascular field correlating Nurr1 haplotypes with outcome following percutaneous coronary intervention[77]. Nurr1 inhibited proliferation of smooth muscle cells, and an association was found between complications including re-stenosis and Nurr1 haplotypes. In this case, Nurr1 is protective by inhibiting proliferation and inflammatory response following the intervention.

In collaboration with Dr. Glass' laboratory at UC San Diego, we ascertained that Nurr1 plays an essential role in both microglia and astrocytes as a signal-dependent transcriptional repressor of genes that encode pro-inflammatory, neuro-toxic factors[78]. We found that Nurr1 is induced by inflammatory signals in the brain and functions as a key component of a negative feedback loop in both microglia and astrocytes by recruiting CoREST co-repressor complexes to NF-κB target genes. Loss of Nurr1 function in extra-neuronal cells of the SN results in exaggerated and prolonged inflammatory responses that accelerate the loss of dopaminergic neurons in response to LPS[78]. These findings reveal a potent, repressive function of Nurr1 in glia to protect dopaminergic neurons from exaggerated production of inflammation-induced neuro-toxic mediators.

While the endogenous ligand for Nurr1 remains unknown, the cAMP response element binding protein (CREB) is a well-studied activator of its expression. CREB is a transcription factor that binds the cAMP response element (CRE), located in the promoter region of many target genes, to either enhance or

dampen transcription. CREB activity has been implicated in a variety of cellular processes including cell proliferation, differentiation and survival[79]. CREB is localized in the nucleus, where it can associate with its downstream target genes upon activation by phosphorylation. Its activation is induced by a variety of kinases, including the most traditional protein kinase A (PKA), which is activated by increases in intracellular cAMP, protein kinase C (PKC), which is activated by increases in intracellular calcium, and members of the MAP Kinase family, including ERK and JNK. Once activated, CREB associates with its binding partner, CREB binding protein (CBP), which also serves as a co-factor required for the activation of NF-κB, and activates or represses its target genes[80]. Over 10,000 CREB target sites have been identified in the promoter regions of human genes[81]. The effects of CREB activation in an immune context are generally anti-inflammatory[82]. CREB seems to dampen NF-κB activation by sequestering their shared cofactor, thereby preventing the induction of pro-inflammatory genes, and also induces the transcription of Nurr1 and IL10, two anti-inflammatory genes [80], [83].

CREB activity is modulated through an additional co-factor, CREB regulated transcriptional co-activator 2 (CRTC2)[84]. In a basal state, CRTC2 is localized to the cytoplasm in its inactive, phosphorylated state. CRTC2 becomes activated through the phosphatase calcineurin, after which it can translocate into the nucleus, where it aids CREB[85]. While CRTC2 is not required for CREB activity, it strengthens the association between CREB and CRE and helps confer specificity in target site association[86]. Given so many CRE target sites exist in the

genome, including in both pro- and anti-inflammatory genes, its co-regulators including CRTC2 are critical for determining functional specificity and appropriate tissue-specific response.

Apigenin (5,7,40-trihydroxyflavone) is a natural flavonoid that is found in most plants, but enriched in leafy greens including celery, parsley, and green tea[87]. Apigenin is a flavone, which is a chemical subclass of flavonoids and has been touted for having anti-cancer, anti-microbial and neurogenic properties[88]. Additionally, apigenin is anti-inflammatory.

Apigenin has been shown to reduce the LPS-induced secretion of NO and PGE2, through dampening iNOS and COX2 induction respectively in a murine macrophage cell line[89]. In an independent study, apigenin was shown to prevent the LPS-induced release of IL6 and TNFα in vitro (murine macrophages) and in vivo (murine serum)[90]. The secretion of those two pro-inflammatory cytokines was also inhibited by apigenin in primary murine macrophages activated with IFNγ[91]. The mechanisms of the anti-inflammatory properties of apigenin remain poorly characterized, though it has been shown that apigenin can block proliferation of murine microglia, thereby reducing pro-inflammatory cytokine production; however, since the microglia employed by this study are not very mitotically active, and the experimental time frames relatively short, this is unlikely the mechanism[92]. Other groups have shown in both mouse and human cells that apigenin blocks LPS induced secretion of pro-inflammatory cytokines by inhibiting IKKβ mediated phosphorylation of the p65 subunit of NF-κB[93]. The anti-inflammatory effects of apigenin, and its effects on CREB activation and

Nurr1 induction are previously undefined in human glial cells; these interactions and their effects will be explored in this chapter.

RESULTS

Apigenin is anti-inflammatory in human glia

We established a drug screening paradigm based on the cells and assays developed in Chapter 2 (Figure 3.1a). Since the astrocytes grow robustly in culture, all primary screens were performed on these cells. Briefly, primary human astrocytes are pretreated with the compounds for one hour, prior to activation with IL1β in the presence of the compound. After six hours, RNA is collected and qPCR is performed to determine the compounds ability to reduce the IL1β induced changes in gene expression in NF-κB downstream target genes, IL1β and TNFα. Known Nurr1 agonists are anti-inflammatory in this assay, as indicated by the reduction in both IL1β and TNFα response (Figure 3.1b). The primary readout from this screen is relayed in fold reduction of the IL1β and TNFα response (Figure 3.1c). Known Nurr1 agonists reduce the IL1β response by two fold; while this may be a clinically relevant reduction in inflammation, our lead compound, apigenin reduced the IL1β response by 500 fold (Agonist 3, Figure 3.1c).

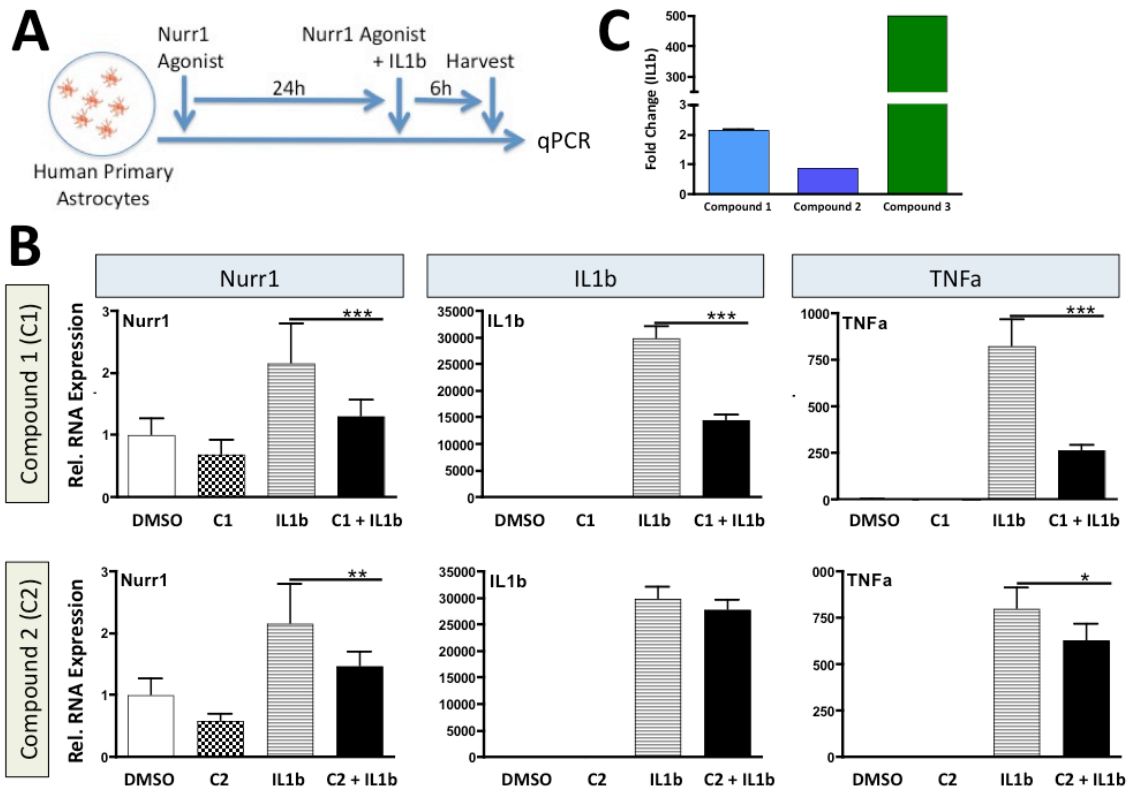

Figure 3.1 Known Nurr1 targeting compounds have an anti-inflammatory effect on human glial cells.
A. Paradigm of astrocyte-based assay of neuro-inflammation. B. Expression levels of Nurr1 and inflammatory genes after treatment with two known Nurr1 agonists using the paradigm described in A. All data relative to GAPDH. C. Fold expression changes in IL1β expression levels to relay anti-inflammatory effects of the Nurr1 agonists. * = p < 0.05, ** 0.01, * 0.001**

We continued to characterize apigenin and confirmed it had potent anti-inflammatory properties in human astrocytes (Figure 3.2). Using the activation assay described above we found that apigenin significantly induced Nurr1 expression, and that it could significantly decrease the induction of NF-κB pro-inflammatory downstream target genes including IL1β and Cox2 (Figure 3.2a). These changes in gene expression levels were then validated at the protein level. Astrocytes were pretreated for one hour with apigenin prior to stimulation

with IL1β in the presence of apigenin. After one hour, cells were washed thoroughly and the cytokines were allowed to collect in the media for 12 hours, as determined in Chapter 2, and then assayed using ELISA for protein content. We found that apigenin significantly reduces the secretion of IL6, IL8 and TNFα (Figure 3.2b). Despite striking reductions in IL1β at the transcript level, no significant reduction was observed at the protein level, potentially due to residual recombinant IL1β in the supernatant masking the effects of apigenin on the endogenously produced IL1β.

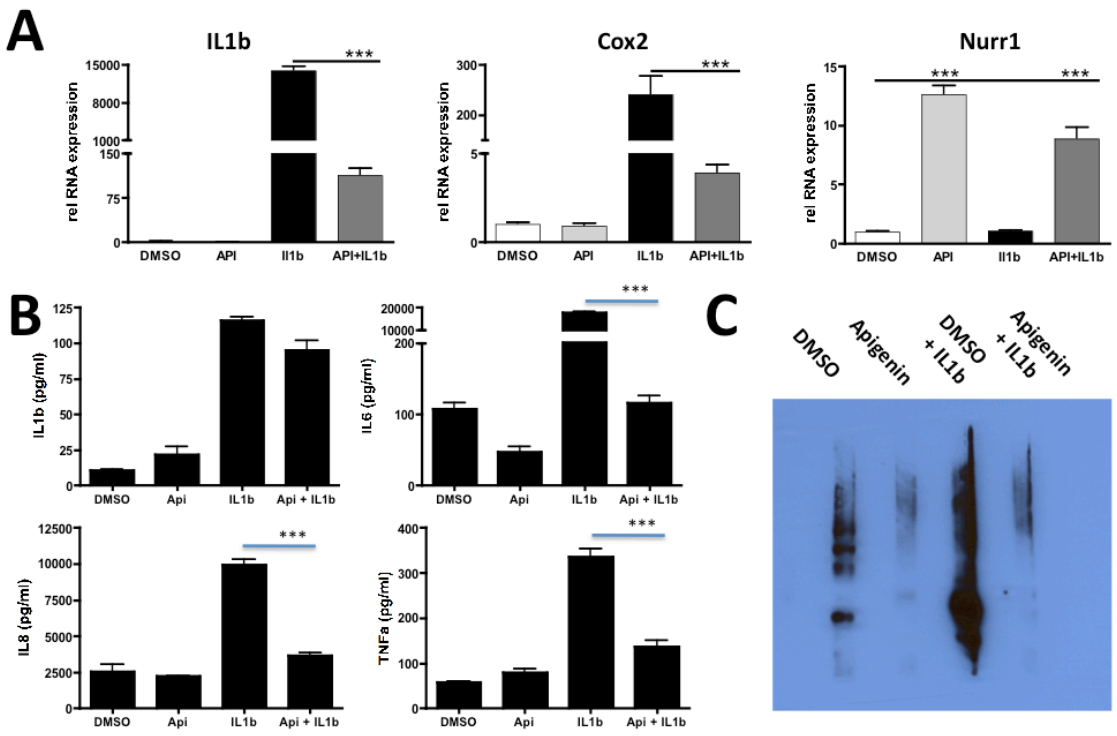

Figure 3.2 Apigenin is anti-inflammatory in human astrocytes.
A. Expression levels of inflammatory markers and Nurr1 after activation with IL1β (10ng/ml) in the presence of apigenin (20um). All data relative to GAPDH. B. Secretion of pro-inflammatory cytokines as measured by ELISA (MSD, Gaithersburg, MD) after activation with IL1β (10ng/ml) in the presence of Apigenin (20um). C. Immunoblot of astrocyte cell lysate treated activated with IL1β (10ng/ml) in the presence of Apigenin (20um) using OxyBlot (Millipore, Billerica, MA). * = p < 0.05, ** 0.01, * 0.001**

In addition to pro-inflammatory cytokines, we assayed for reductions in reactive oxygen and nitrogen species in activated astrocytes treated with apigenin. Using an assay designed to detect the carbonyl groups introduced into proteins by oxidative reactions with reactive oxygen and nitrogen species, we found that apigenin can block the production of these reactive species (Figure 3.2c). Briefly, astrocytes were treated with either IL1β, or IL1β in the presence of apigenin for four hours prior to cell lysis and immuno-blotting. Interestingly apigenin also reduced baseline reactive species in non-activated astrocytes (Figure 3.2c). Taken together, this data indicates that apigenin is a potent anti-inflammatory compound in astrocytes.

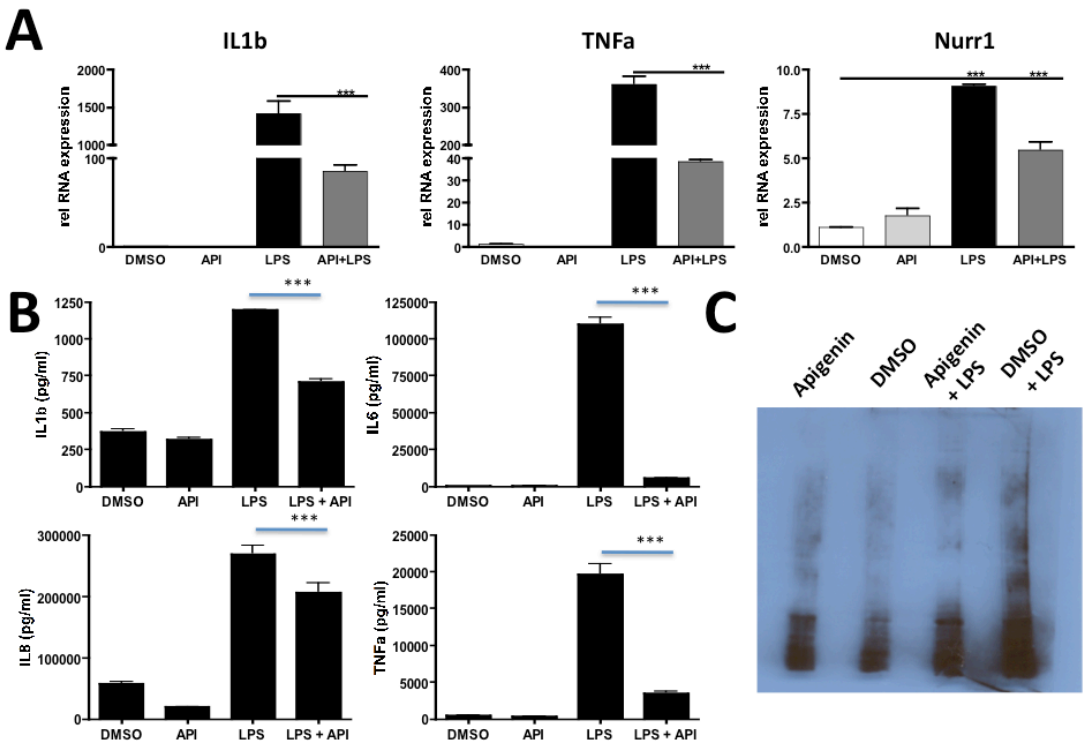

Figure 3.3 Apigenin is anti-inflammatory in human microglia.
A. Expression levels of inflammatory markers and Nurr1 after activation with LPS (0.1ug/ml) in the presence of Apigenin (20um). All data relative to GAPDH. B. Secretion of pro-inflammatory cytokines as measured by ELISA (MSD, Gaithersburg, MD) after activation with LPS (0.1ug/ml) in the presence of Apigenin (20um). C. Immunoblot of astrocyte cell lysate treated activated with LPS (0.1ug/ml) in the presence of Apigenin (20um) using OxyBlot (Millipore, Billerica, MA). * = p < 0.05, ** 0.01, * 0.001**

We then assayed for anti-inflammatory effects in the non-proliferating human primary microglia (Figure 3.3). We employed the activation assay described in Chapter 2. Briefly, human microglia were pretreated with apigenin prior to activation with LPS in the presence of apigenin. RNA was collected after four hours and assayed for decreases in expression levels of NF-κB pro-inflammatory downstream target genes in the presence of apigenin. We found that this flavone is capable of significantly inducing Nurr1 in human microglia and

reducing IL1β and Cox2 transcript levels (Figure 3.3a). This trend was maintained at the protein level. Microglia were stimulated with LPS for one hour, washed, and medium was allowed to collect for six hours, as determined in Chapter 2. We found highly significant reductions in the secretion of all four cytokines assayed (Figure 3.3b). Apigenin was also able to reduce the production of reactive oxygen and nitrogen species in human microglia four hours after LPS activation (Figure 3.3c).

Apigenin reduces the propagation of inflammatory signals in human glia

Towards further characterizing the therapeutic potential of apigenin, we assayed for its ability to reduce the propagation of pro-inflammatory signals. We used the propagation assay described in Chapter 2, with both the microglia and the astrocytes as the initiating cell type (Figures 3.4 and 3.5). Briefly, the initiating cell type was activated using its respective stimulant (astrocytes – IL1β, microglia – LPS) for one hour, after which the well was thoroughly washed and the cytokines allowed to collect in the medium for six hours for microglia and 12 hours for astrocytes (Figure 3.4a and 3.5a respectively). The resulting conditioned medium was then propagated from glial culture every 6 or 12 hours, depending on the cell type, with samples frozen at each step for later protein analysis with ELISA (Figure 3.5a). When human astrocytes initiated the signal we saw significant reductions in IL6 levels at every stage of propagation (Figure 3.5b).

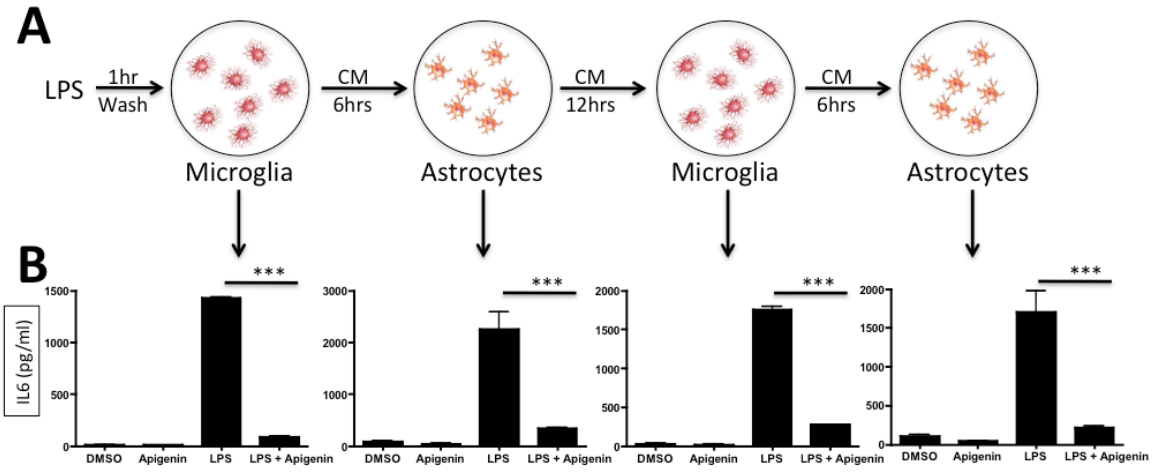

Figure 3.4 Apigenin reduces the levels of inflammatory signals in propagation assay initiated by microglia. A. Experimental paradigm. Briefly microglia pretreated with Apigenin (20um) were stimulated with LPS (0.1ug/ml) for one hour, washed thoroughly. Pro-inflammatory mediators were allowed to accumulate for six hours prior to transfer to the next glial culture. Media samples were removed at each transfer and assayed for cytokine levels. B. IL6 protein levels from each step of the propagation described in A. * = p < 0.05, ** 0.01, * 0.001**

Apigenin was also able to significantly decrease the propagation of pro-inflammatory signals in cascades initiated by human microglia (Figure 3.6b). This shows that apigenin is capable of dampening signals from both human astrocytes, and greatly increases its therapeutic applications.

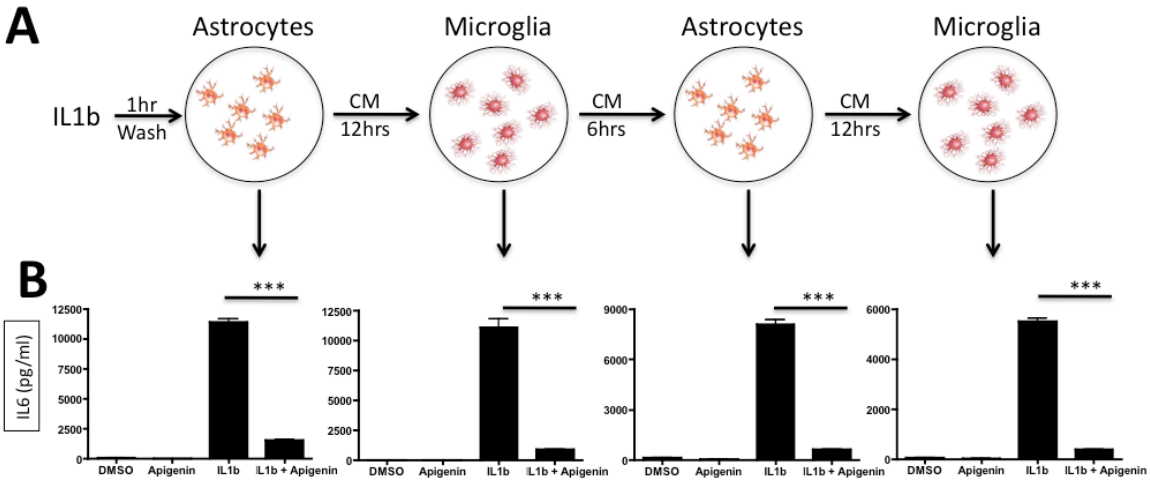

Figure 3.5 Apigenin reduces the levels of inflammatory signals in propagation assay initiated by astrocytes. A. Experimental paradigm. Briefly astrocytes pretreated with Apigenin (20um) were stimulated with IL1β (10ng/ml) for one hour, washed thoroughly. Pro-inflammatory mediators were allowed to accumulate for 12 hours prior to transfer to the next glial culture. Media samples were removed at each transfer and assayed for cytokine levels. B. IL6 protein levels from each step of the propagation described in A. * = p < 0.05, ** 0.01, * 0.001**

Apigenin's anti-inflammatory effects are partially CREB mediated

Towards understanding the mechanistic underpinnings of apigenin's anti-inflammatory properties, we investigated pathways known to activation Nurr1, including CREB and its co-activator, CRTC2. We treated human microglia and astrocytes with apigenin for 30 minutes, and found increases in phosphorylated forms of CREB, it's active form, with no change in total CREB levels (Figure 3.6a and data not shown). We confirmed these results in a time course of apigenin treatment on human astrocytes using a Western blot, in collaboration with the Montminy lab at the Salk Institute, which specializes in CREB based gene regulation. We saw a strong induction of phosphorylated CREB and dephosphorylated CRTC2, using Forskolin (FSK, 10um), a known CREB

mediated inducer of Nurr1, as a positive control (Figure 3.6b). We obtained a previously reported dominant negative form of CREB from the Montminy lab, to determine if apigenin's anti-inflammatory ability is in fact CREB dependent. Since this vector is unlabeled, we could not purify the infected population, and instead used FSK, a potent CREB activator, as a positive control. In our hands, FSK is also anti-inflammatory in our activation assay, resulting in a 3 fold decrease in IL1b induction (Figure 3.6c). Decreasing CREB levels reduced FSK's anti-inflammatory ability by around 50%, from a 3 fold to a 1.3 fold reduction in anti-inflammatory ability. Dominant negative ACREB decreased apigenin's efficacy by around 75%, by reducing the fold change in IL1β response from 400 to only a 100 fold reduction (Figure 3.6c). Similar results were obtained for CRTC2 knockdown. Since the CRTC2 knockdown is RNA interference mediated, using short hairpin RNA constructs (shRNAs), transcript level was used to quantify knockdown efficiency. With lenti-virus infection we were able to reach 85% knock down of the protein (Figure 3.6d). When CRTC2 levels were reduced, we saw about a 90% reduction in apigenin's ability to reduce the inflammatory response at the gene expression level in human astrocytes (Figure 3.6e). Taken together, this data indicates that apigenin's anti-inflammatory are partially mediated through CREB and its cofactor CRTC2.

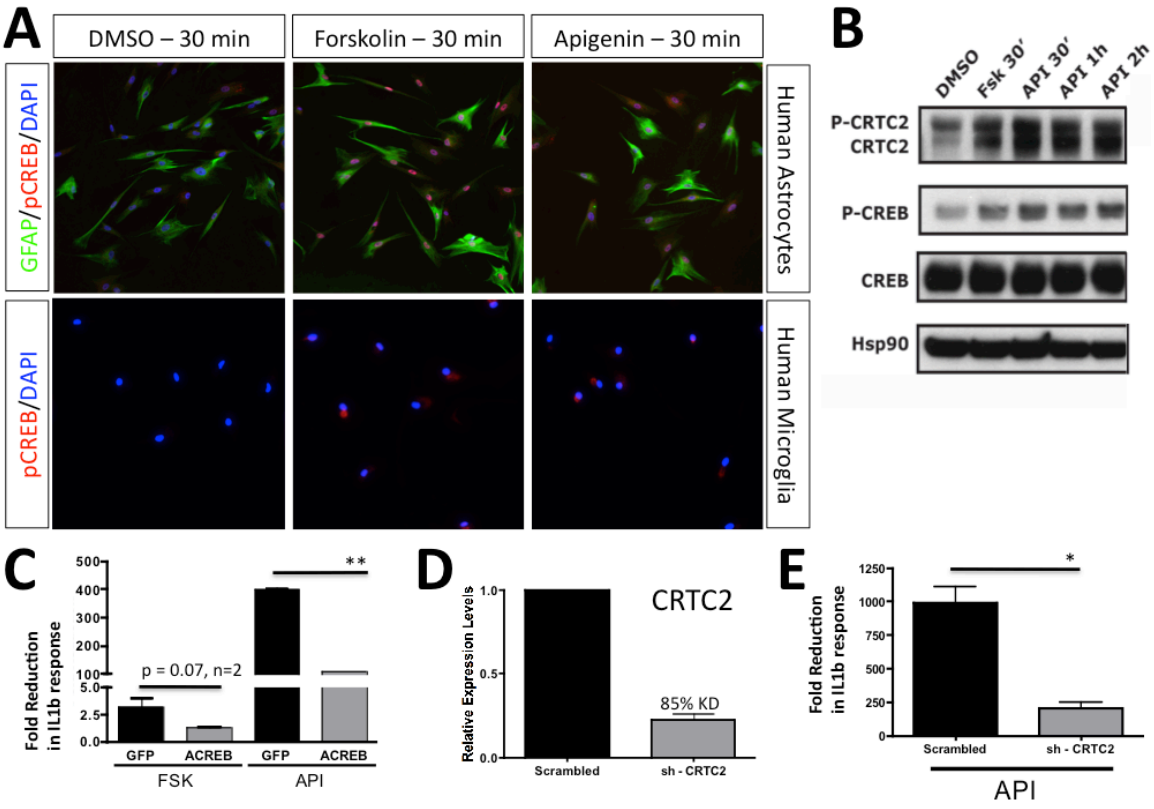

Figure 3.6 Apigenin activity is partially dependent of CREB and CRTC2. A. Immunofluorescent images of astrocytes and microglia treated with apigenin (20um) or Forskalin (FSK, 10um) for 30 minutes using an antibody against active forms of CREB. B. Immunoblot on protein extracted from astrocytes treated as described in A. C. Fold change in IL1β expression levels in astrocytes treated with a dominant negative CREB (ACREB) with treatment using either FSK or apigenin. D. Expression levels of CRTC2 after knock down using sh-RNAs, relative to a scrambled control. E. Fold change in IL1β expression levels in astrocytes treated with a sh-CRTC2 prior to activation in the presence of Apigenin. * = p < 0.05, ** 0.01, * 0.001**

Towards determining how CREB is becoming activated, we examined the most commonly described pathways, including PKA, PKC, and MAPK. First we used a PKA inhibitor, H89, which effectively blocked FSK activity, but was unable to dampen the effects of apigenin (Figure 3.7a). This data was confirmed by checking intracellular cAMP levels, which rose upon FSK treatment, but not

apigenin (Figure 3.7b). FSK was used as a positive control for these studies since it activates CREB through cAMP activation of PKA. We then used a PKC inhibitor, Calphostin C, which was also unable to reduce apigenin's efficacy (Figure 3.7c). We confirmed apigenin was not dependent on this pathway by using Fluo-4 to examine calcium activity, since Ca^{2+} is the second messenger responsible for PKC activation. Upon addition of apigenin to human glial cultures, no changes in intracellular calcium were observed (Figure 3.7d). Traces were quantified, and on average 2% of all cells show spontaneous calcium activity, and this percent did not increase upon apigenin treatment (Figure 3.7e). We then tried inhibitors of different MAPK pathways, including ERK and JNK. We found that inhibition of JNK, but not ERK, resulted in a decrease in apigenin's efficacy in both the IL1β and COX2 response (Figure 3.8a, 3.8b). This is consistent with reports from the literature that apigenin exerts it's anti-tumorgenic effects through JNK, and that JNK inhibition reduces apigenin's efficacy in caner models[94]. Taken together, this data indicates that MAP kinases likely play a role in apigenin's anti-inflammatory effects.

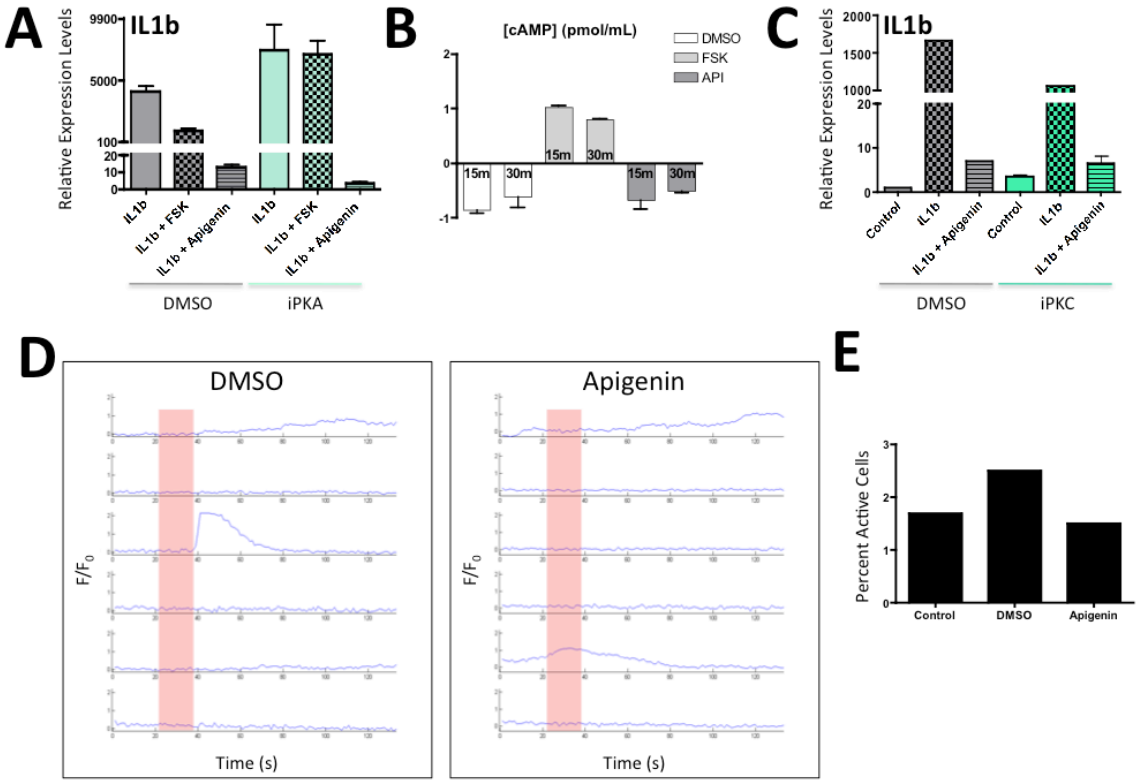

Figure 3.7 Apigenin activates CREB in a PKA, PKC independent mechanism.
A. Gene expression changes in human astrocytes after activation assay with apigenin in the presence of absence of PKA inhibitor, H89. B. Intracellular cAMP levels after treatment with apigenin and forskolin (API and FSK, respectively). C. A. Gene expression changes after activation assay on human astrocytes with apigenin in the presence of absence of PKC inhibitor, Calphostin C. D. Intracellular calcium changes after treatment with Apigenin, compared to DMSO treatment.

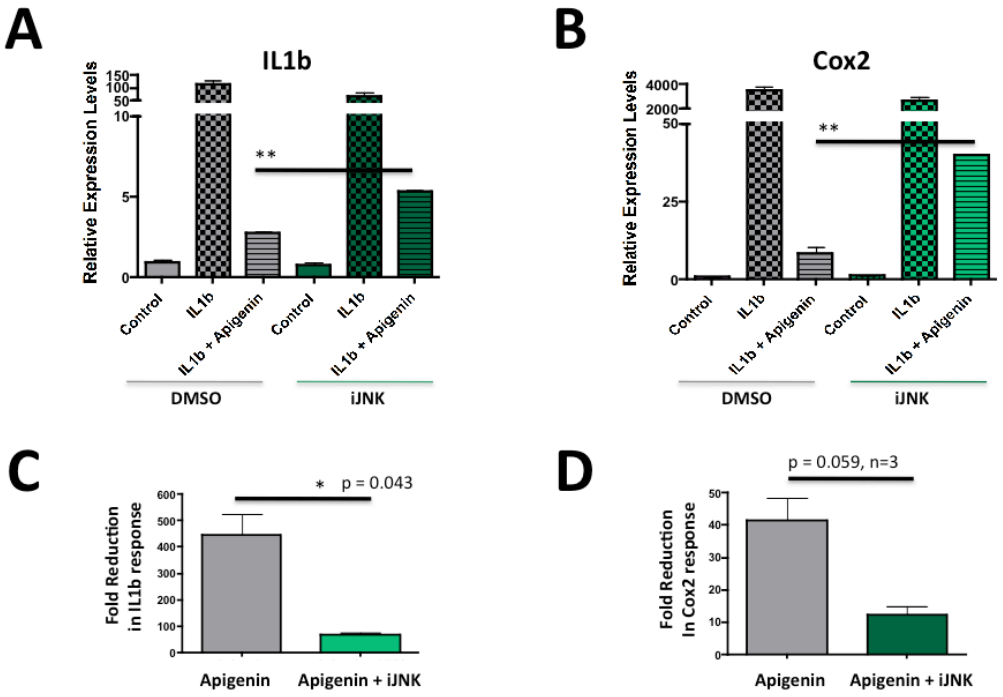

Figure 3.8 Inhibiting JNK activity dampens apigenin's efficacy. A. IL1β gene expression changes in human astrocytes after activation assay with apigenin in the presence of absence of JNK inhibitor, SP600125. B. COX2 gene expression changes in human astrocytes after activation assay with Apigenin in the presence of absence of JNK inhibitor, SP600125. C. Data represented as fold change of IL1β reduction. D. Data represented as fold change of COX2 reduction. * = p < 0.05, ** 0.01, * 0.001**

Towards determining if apigenin's anti-intflammatory effects are due activation of Nurr1, a well studied CREB target whose expression is induced upon apigenin treatment, we used two independent shRNAs to ablate Nurr1 by 90% and 95% (Figure 9a). Despite strong reductions in transcript level, the human astrocytes still responded to apigenin treatment, indicating that apigenin's effects are not mediated solely through Nurr1 (Figure 9b). We therefore determined the effects of apigenin on all NR4A family members and found that apigenin can significantly up-regulate all of them. The most modest effects were

on Nurr1, and the strongest effects were on Nur77, whose transcript level increased almost 150 fold (Figure 9c).

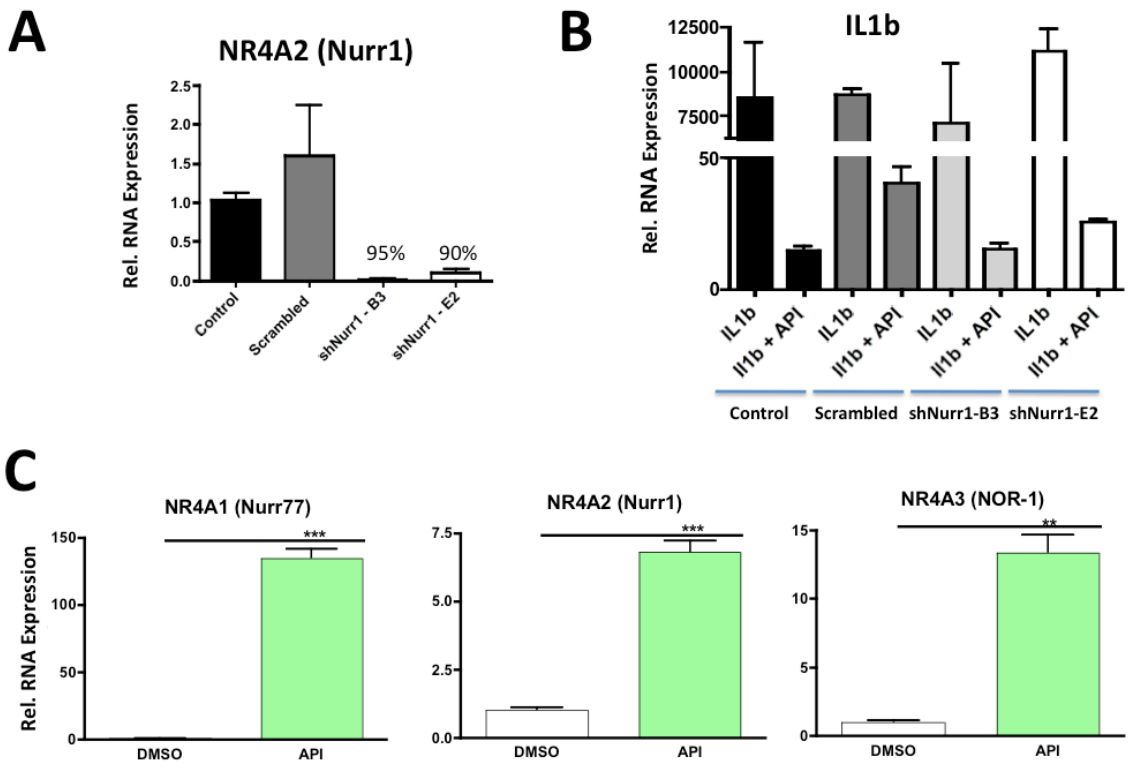

Figure 3.9 Apigenin's activity is not Nurr1 dependent.
A. Quantitative gene expression data assessing Nurr1 knockdown levels, in human astrocytes four days after infection with lenti-virus expressing a scrambled sh control, or two shRNAs targeting Nurr1. B. Quantitative gene expression levels of IL1b in human astrocytes in activation assay after Nur1 knockdown. C. Induction of all three NR4A family members six hours after apigenin treatment in human astrocytes. * = p < 0.05, ** 0.01, * 0.001**

Apigenin is neuro-protective

We then sought to determine if apigenin can protect DA neurons from the neurotoxic effects of pro-inflammatory factors, as described in Chapter 1. Briefly, we treated cultures of hESC-derived DA neurons with IL1β and TNFα in the presence or absence of apigenin for either 6 or 24 hours, depending on the

assay's readout (Figure 3.10a). After six hours of treatment, the RNA was harvested and assayed for markers of inflammation and cell death. In both Hues6 and H9-derived DA neural cultures we saw a significant reduction in both IL1β and Fas upon apigenin treatment, this effect was no longer present after 12 or 24 hours of treatment prior to RNA analysis (Figure 3.10b, and data not shown). We also performed TUNEL assay 24 hours after treatment as described in A. We saw significant reductions in cell death in Hues6-derived DA cultures, but no effect in this assay for H9-derived DA neurons, this discrepancy requires further resolution (Figure 3.10c, and data not shown).

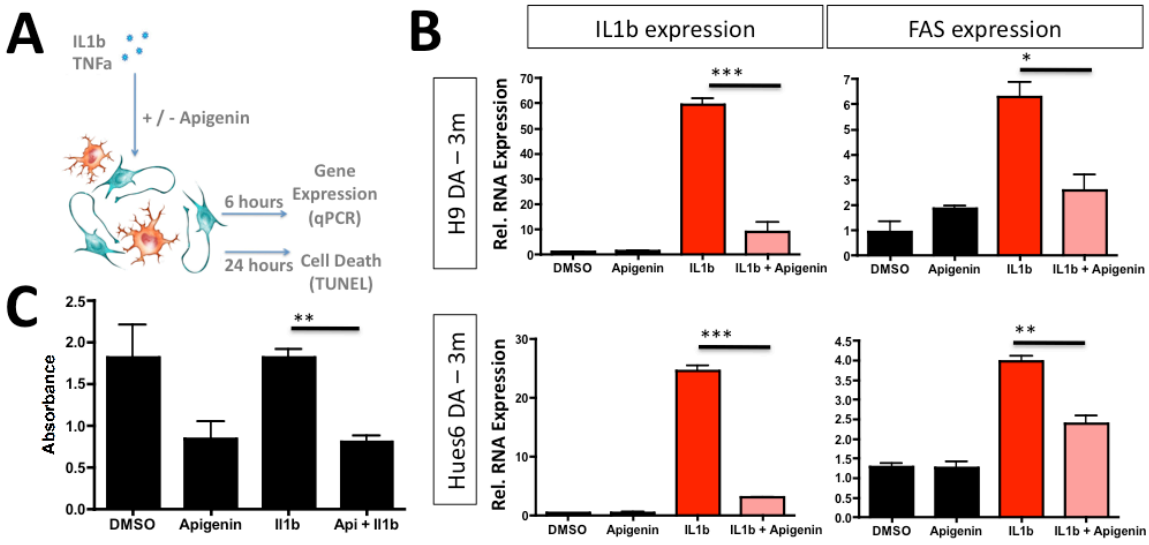

Figure 3.10 Apigenin is neuro-protective.
A. Inflammation induced neurotoxicity paradigm. Briefly, hESC-derived neurons at least two months post NPC differentiation were treated with IL1β (10ng/ml) and TNFα (50ng/ml) for either six or 24 hours prior to analysis. B. Gene expression levels in Hues6-derived dopaminergic neurons differentiated for three months, six hours after treatment as described in A. All data relative to GAPDH. C. Quantification of cell death in Hues6-derived DA neurons, two months after NPC differentiation, 24 hours after immune insult using TUNEL assay (Cell Death Detection, Roche), n = 2. * = p < 0.05, ** 0.01, * 0.001**

Towards investigating the DA neuron specific effect of apigenin, we counted TH immuno-reactive neurons in cultures treated as previously described for four days (Figure 3.11a). Immunohistological characterization revealed a loss of TH positive neurons, while the rest of the B3-tubulin cells remained unaffected (Figure 3.11b). While not significant, due to the large range of DA cell numbers in randomly chosen fields of view in these hESC derived cultures, cells treated with apigenin were closer in morphology and TH number to untreated, rather than IL1β and TNFα treated cells (Figure 3.11c). Overall, apigenin is not only anti-inflammatory, but also neuro-protective.

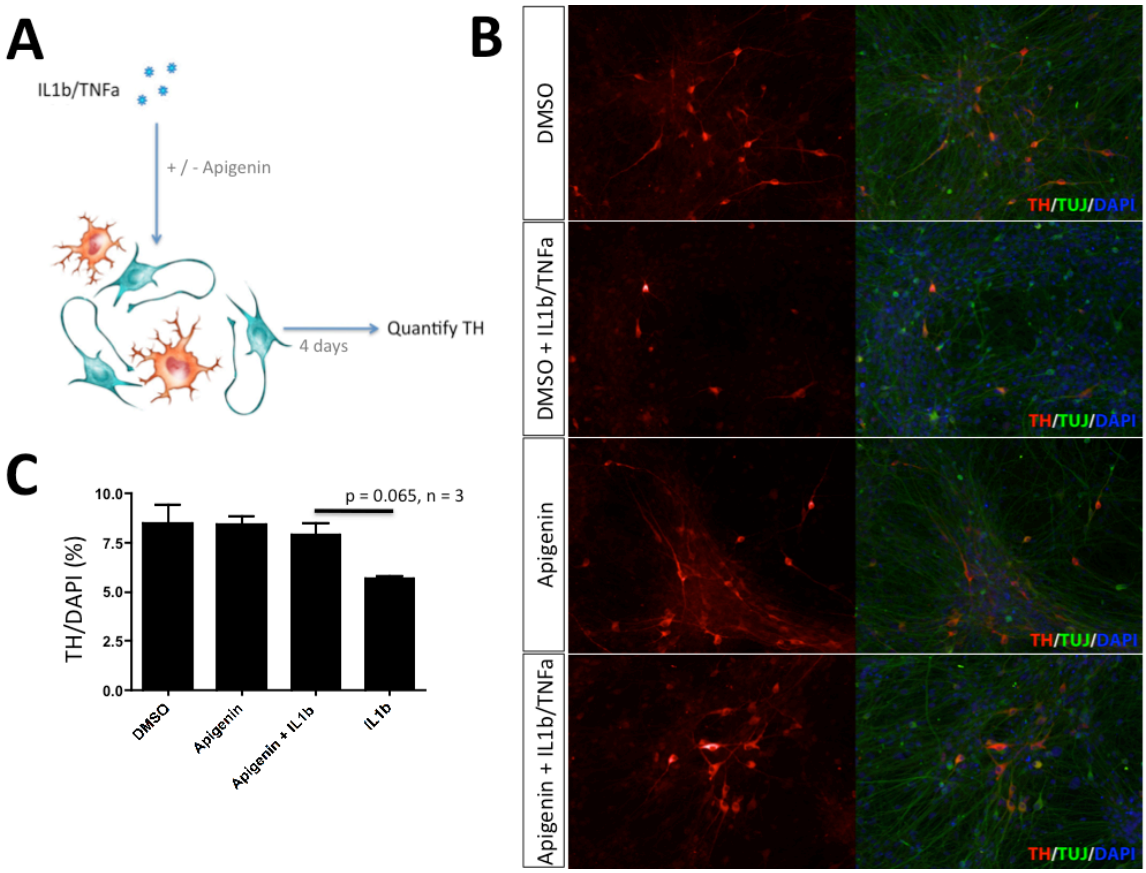

Figure 3.11 Apigenin rescues dopaminergic specific cell loss.
A. Inflammation induced neurotoxicity paradigm. Briefly, hESC-derived neurons at least 2 months post NPC differentiation were treated with IL1β (10ng/ml) and TNFα (50ng/ml) for four days prior to immunohistochemical analysis. B. 20x representative fluorescent images of H9 dopaminergic neurons differentiated for 2.5 months, treated as described in A. C. Quantification of total TH immuno-reactive cell numbers. n = 3, four images per well.

CONCLUSION AND DISCUSSION

From the data presented in this chapter, we conclude that apigenin is strongly anti-inflammatory in a human model of neuro-inflammation, and that these effects are partially mediated through JNK and CREB activation. Apigenin is also neuro-protective in an inflammation based neuro-toxicity assay.

In our hands apigenin is strongly anti-inflammatory in both human microglia and astrocytes, which confirms observations from both the hematopoietic lineage and animal studies. The anti-inflammatory effects of apigenin are the strongest and most robust that we have witnessed in our targeted chemical screen for Nurr1 agonists; compounds from pharmaceutical companies and other flavonoids have paled in comparison. Very little batch to batch variability was observed in preparations of the compound, and storage and handling are straightforward. Our data confirms an epidemiological study that showed increase consumption of flavonoids reduced the risk of developing PD[95]. Taken together, we believe that apigenin is an attractive candidate for future clinical applications in any disease in which inflammation may contribute to its progression.

Apigenin can significantly reduce the propagation of inflammatory signals between different glial cell types. It's ability to prevent this pro-inflammatory cycle indicates that it is an attractive treatment option for patients who have undergone events capable of producing neuro-inflammation, including but not limited to traumatic brain injury, neurodegenerative diseases, and intracranial surgery. Animal studies will have to be performed to assess the appropriate treatment regiment, but preliminary data indicates that apigenin significantly reduces acute in vivo inflammation in an *in vivo* murine, LPS-based model of neuro-inflammation. Further studies on the anti-inflammatory effects of apigenin in vivo are underway, and will further reveal the relevance of this compound for clinical application.

The anti-inflammatory effects of apigenin are mediated at least partially through CREB activation. Partial ablation of CREB drastically reduces apigenin's efficacy, even more so than known CREB activator FSK. This is consistent with a study showing that the beneficial effects of flavonoids in an Alzheimer disease model are CREB mediated[96]. Strong effects were also observed after the ablation of CREB cofactor CRTC2, which has been shown to direct strength and specificity of CREB binding in CRE target regions in Nurr1, and other CREB target genes. CREB activation is not mediated through traditional PKA or PKC phosphorylation, however, inhibitors of JNK, a MAP Kinase, effectively reduced apigenin's anti-inflammatory properties. This is consistent with reports in the literature, primarily from the cancer biology field, confirming interactions between apigenin and JNK[94]. Further studies are required to ensure that JNK inhibition directly prevents CREB phosphorylation in the presence of apigenin.

Nurr1 is a well-studied CREB target that plays roles in both neuro-protection and dampening inflammation, whose expression is induced upon apigenin treatment. We therefore knocked down Nurr1 and were surprised to discover that this reduction did not ablate apigenin's effects. Since there is high homology between members of the NR4A family, and they have all been implicated in neuro-inflammation[97], we investigated the effects of apigenin on NR4A1-3. We found that apigenin induces all three family members, and its effects on Nurr1 were the most modest, while Nur77 was strongly induced. This indicates that other nuclear receptors might be mediating the CREB induced

effects of apigenin, and future studies will include knocking down all three family members, individually and in concert.

Apigenin also has acute neuro-protective effects in hESC-derived neurons treated with neuro-toxic pro-inflammatory factors. These neuro-protective effects apply to whole DA cultures, but also seem to be specific to DA neurons. This selective neuro-protective effect indicates that apigenin is particularly well suited to protect against the DA cell loss responsible for the cardinal motor symptoms in PD. This is likely due to apigenin's ability to up-regulate Nurr1 expression, a known regulator of both inflammation and DA neuron maintenance and survival. Further studies are required to understand the mechanism of apigenin mediated neuro-protection, CREB and Nurr1 activation are likely candidates, since their activity is induced by apigenin, and neuro-protective effects have been shown in the literature[98]. Since we do not have pure cultures of DA neurons, it remains unclear whether apigenin's neuro-protective effects are a result of direct effects on neurons or by inhibiting the secretion of pro-inflammatory, neuro-toxic factors from astrocytes. Further studies involving conditioned medium from microglia are being performed towards clarifying this distinction.

While reductions in CREB levels significantly reduce apigenin's anti-inflammatory properties, it remains to be seen whether or not these effects are Nurr1 dependent. While Nurr1 is a well-studied CREB target, Nurr1 knock down in our astrocytes does not abrogate apigenin's effects. While surprising, there are many other CREB targets and potential mechanisms to explain apigenin's effects, including NF-κB inactivation through competitive inhibition of cofactors or

inhibition of phosphorylation of IKK, as suggested in the literature[93]. These alternative explanations will require further exploration. Additionally, Nurr1 shares it's nuclear receptor subfamily with two other members, whose expression is also induced by apigenin. It is possible that they play similar roles in neurodegeneration, and are compensating for the loss of Nurr1. We are currently exploring a triple knock out of the entire NR4A family to confirm this hypothesis. Further studies will help us fully understand the mechanistic underpinnings of the strong anti-inflammatory and neuro-protective effects of apigenin.

The effect of apigenin in human neural cells is surprisingly strong and highly reproducible. Taken together, apigenin represents an attractive target because it has both anti-inflammatory and neuro-protective effects in our human in vitro model; this two-pronged approach represents a novel treatment strategy for neurodegenerative diseases. Once mechanistic nuances have been resolved and the in vivo potential of apigenin is assessed, we believe this natural compound and it's chemical derivatives, will be a strong candidate for therapeutic interventions in a variety of neurodegenerative diseases, including PD.

MATERIALS AND METHODS

Glial culture and activation assay. Refer to Chapter 2.

Apigenin resuspension and handling. Apigenin was procured from the Mars corporation as a yellow powder and resuspended in DMSO to a stock concentration of 20mM, stored at -80 degrees in light protective tubes. This stock concentration was added to the appropriate media generally at 1000x for a final

concentration of 20uM, and this medium was then protected from light. Other Nurr1 agonists were obtained in a collaboration with Sanofi-Aventis, and were resuspended in DMSO according to their specifications.

Cytokine quantification. Refer to Chapter 2.

Quantitative gene expression analysis. Refer to Chapter 2.

Reactive oxygen and nitrogen species. Reactive species produced upon activation were investigated using the OxyBlot Protein Oxidation Detection Kit (Millipore) per manufacturer's instructions. Glial cells were lysed for blotting four hours after activation.

Western blotting. Immunoblots for CRTC2 and CREB activation were performed in the Montminy Lab (Salk Institute) following standard procedures on human astrocytes treated with apigenin (20uM) or forskolin (Sigma, 10uM) over a time course from 30 minutes to two hours.

CREB and CRTC2 knockdown. Dominant negative and shRNA constructs were obtained from the Montminy Lab (Salk Institute) and 293T cells were used to package them into third generation lenti-virus. Astrocytes were infected with these high titer viruses at an MOI of roughly 10, twice over two days for one hour each.

Nurr1 knockdown. shRNA constructs were obtained from Open Biosystems and lenti-virus was generated and astrocytes infected as described for CRTC2 knockdown above.

Chemical inhibitor treatment. All inhibitors were resuspended in DMSO and frozen in 1000x stocks at -80 degrees. H89 was used at a final concentration of

10uM, Calphostin C was used at a final concentration of 100nM, JNK inhibitor (SP600125) was used at a concentration of 5uM.

cAMP assay. To determine levels of intracellular cAMP, cells were treated with apigenin (20uM) or forskolin (10uM) for 15 and 30 minutes prior to analysis using Cyclic AMP EIA Kit, without Acetic Anhydride per manufacturer's instructions (Cayman Chemical).

Calcium signaling. Glial cells were plated onto coverslips (glass etched, $2cm^2$), and assayed for calcium activity two days after seeding. Cells were loaded with Fluo-4AM (5uM) for 30 minutes at 37 degrees and then placed onto the live imaging confocal microscope (Olympus, FV1000). A 40uM solution of Apigenin, or the equivalent concentration of DMSO (0.1%) was pressure applied to the cells (as opposed to whole bath addition), the final concentration was around 20um of Apigenin and 0.1% DMSO. Cells were imaged at 0.9Hz for up to three minutes. Images were analyzed in ImageJ and MATLAB was used to generate trace curves.

TUNEL assay. Cell death was quantified in neurons 24 hours after neurotoxicity assay using Roche Cell Death Detection ELISA, per manufacturer's instructions. Two technical replicates were performed per sample.

Statistical Analysis. All data were analyzed using Prism software. Data was analyzed using either a Student's T-test or ANOVA followed by a Bonferroni's posthoc test. $p < 0.05$ is considered statistically significant.

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CONCLUSION

From the data generated for this thesis, we conclude that human cells can recapitulate the neuro-inflammatory aspects of neurodegeneration. We have shown that inflammation can contribute to neurodegeneration and used human glial cells to identify a compound, apigenin, which can dampen both the inflammatory response and the resulting neuro-toxicity; this activity is partially dependent on CREB and MAPK signaling pathways. Preliminary in vivo studies, not included in the scope of this thesis, indicate that this compound is safe and effective in animal models. Future studies include further characterizing the effect of apigenin on human neurons mechanistically, and applying this model to other neurodegenerative disease. Taken together, this human in vitro model is a robust, relevant, and highly useful tool towards modeling the contribution of neuro-inflammation to neurodegeneration, which can both further our understanding of disease pathogenesis and enable identification of new compounds and clinical targets for therapeutic intervention in neurodegenerative diseases.

APPENDIX I. RECIPES FOR CELL CULTURE MEDIA

Media Name	Plate coating	Base media	Serum	Supplements	Growth factors
MEF	Gelatin	DMEM	10% FBS	L-glutamine	None
HEK293T	None	DMEM	10% FBS	L-glutamine	None
iPSC/hESC	Matrigel	TeSR	BSA	L-glutamine, NEAA, 2-ME	100 ng/mL bFGF
iPSC/hESC	MEFs	DMEM/F12	1X N2 supplement, 1X B27	L-glutamine, NEAA, 2-ME	10 ng/mL bFGF
NPCs	Matrigel	DMEM/F12	1X N2 supplement, 1X B27 supplement	L-glutamine	FGF8 100ng/ml, SHH 200ng/ml
Neurons	Laminin	DMEM/F12	1X N2 supplement, 1X B27	L-glutamine	600 nM dorsomorphin, 10 mM SB431542
Astrocytes	None	DMEM/F12	2% FBS	1x AGS	1x AGS
Microglia	None	DMEM/F12	5% FBS	L-glutamine	10ng/ml MCSF

APPENDIX II. PRIMER SEQUENCES FOR qPCR

Gene	Application	Orientation	TaqMan Reference
PITX3	qPCR	F+R	Hs01013935_g1
LMX1A	qPCR	F+R	Hs00892663_m1
EN1	qPCR	F+R	Hs00154977_m1
IL1β	qPCR	F+R	Hs01555410_m1
TNFa	qPCR	F+R	Hs00174128_m1
Nurr1 (NR4A2)	qPCR	F+R	Hs00428691_m1
COX2 (PTGS2)	qPCR	F+R	Hs00153133_m1
TH	qPCR	F+R	Hs00165941_m1
TUJ1	qPCR	F+R	Hs00964962_g1
FAS (CD95)	qPCR	F+R	Hs00236330_m1
CRTC2	qPCR	F+R	Hs01064500_m1

APPENDIX III. PRIMARY AND SECONDARY ANTIBODIES

Antibody	Conjugation	Species	Vendor	Dilution	Application
Sox2	Unconjugated	Rabbit	Cell Signaling	1/250-500	Immunocyto-chemistry (ICC)
Nestin	Unconjugated	Rabbit	Millipore	1/250-500	ICC
TH	Unconjugated	Rabbit	Pelfreez	1/250-500	ICC
Tuj1	Unconjugated	Mouse	Covance	1/500	ICC
GFAP	Unconjugated	Chicken	Millipore	1/1000	ICC
Iba1	Unconjugated	Rabbit	Wako	1/1000	ICC
CREB	Unconjugated	Rabbit	Montminy Lab	1/250	Western Blot (WB)
pCREB	Unconjugated	Rabbit	Montminy Lab	1/250	ICC & WB
CRTC2	Unconjugated	Rabbit	Montminy Lab	1/1000	WB
anti-mouse secondary antibody	Alexa Fluor 488	Donkey	Invitrogen	1/500	ICC
anti-mouse secondary antibody	Alexa Fluor 555	Donkey	Invitrogen	1/500	ICC
anti-rabbit secondary antibody	Alexa Fluor 488	Donkey	Invitrogen	1/500	ICC
anti-rabbit secondary antibody	Alexa Fluor 555	Donkey	Invitrogen	1/500	ICC

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