

Investigating Selective Neuronal Vulnerability in Alzheimer's and Parkinson's Disease with Induced Pluripotent Stem Cells



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Abstract

Neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) are enormous, growing, global challenges. However, owing to long prodromal periods, disease complexity, and the inaccessibility of the brain, their causes remain elusive. Nevertheless, the protein aggregation and cell loss which are the cardinal features of these diseases occur with a highly stereotyped and well-defined spatiotemporal progression, where only some cell populations are susceptible, while others remain resilient. Therefore, in this thesis, I present a framework for how this phenomenon of selective neuronal vulnerability might give insight into the mechanisms underlying disease.

Comparison of diverse vulnerable and resilient cell types in the brain, in the manner of a natural experiment, can identify underlying divergent phenotypes might predispose cells to disease. Large, excitatory, glutamatergic neurons in the hippocampus and cortex are selectively vulnerable in Alzheimer's, but relatively resilient in Parkinson's. Conversely, midbrain dopaminergic neurons are highly vulnerable in Parkinson's, but relatively spared in Alzheimer's disease. Postmortem studies have associated many different phenotypic pathways with the vulnerability of these cell types to protein aggregation and neurodegeneration. However, these studies cannot clearly determine which pathways are causative and cannot examine early-stage dysfunction in live cells.

Therefore, in this thesis, I use human induced pluripotent stem cells (hiPSC) to generate differentially vulnerable neuronal subtypes, to establish a system for the investigation of selective neuronal vulnerability in a live human cell model. I differentiate hiPSCs from patients with PSEN1 AD and LRRK2 PD into both cortical and midbrain dopaminergic neurons (CNs, mDANs), and examine their divergent phenotypes. To assess their differential vulnerability, I

employ an alpha synuclein pre-formed fibril insult paradigm to seed protein aggregation in these cells.

I present experiments demonstrating robust formation of pathology in both CNs and mDANs in a morphological and cell type-dependent pattern that reflects Lewy pathology in the brain, since mDANs were much more vulnerable to aggregate accumulation. Tyrosine hydroxylase expressing cells in mDAN cultures were especially affected, and aggregation was also related to alpha synuclein expression and culture density. These are the first experiments to examine alpha synucleinopathy in an Alzheimer's cell model, and I observed that PSEN1-Int4del CNs were highly susceptible to aggregate formation. This was related to cell type-dependent deficits in autophagy and calcium homeostasis. PFF insult also led to synapse loss, aggregation independent changes in nuclear and neuronal morphology in all genotypes, and neurite outgrowth impairment in PSEN1 and LRRK2-G2019S CNs.

This is the first study to compare cell type-dependent disease phenotypes across multiple diseases. This further validates the hiPSC neuronal model for the examination of selective neuronal vulnerability and confirms a role for cell autonomous mechanisms driving disease. The hiPSC-derived models presented here are the first to show robust inclusionopathy without co-insult or overexpression, and the first to report nuclear alterations. The results here also support a role for PSEN1 in autophagy and calcium regulation. Collectively, this thesis presents the framework of selective neuronal vulnerability and its examination in live human cell models as an exciting means of understanding the fundamental mechanisms of neurodegenerative disease.

Declaration

Except where explicitly stated, the entirety of the work presented in this DPhil thesis, including research design, data collection, data analysis and interpretation of results is entirely my own independent effort within the framework of the University of Oxford's policies and regulations. The work reported in this thesis has not been submitted for any other degrees at the University of Oxford or elsewhere.

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Colour Schemes

Category	Colours				
Disease / Genotypes	Healthy Controls	Alzheimer's Disease	Alzheimer's Disease Corrected Controls	Parkinson's Disease	Parkinson's Disease Corrected Controls
Pairwise Comparisons	Alzheimer's Disease PSEN1 Intron-4-Deletion		Alzheimer's Disease PSEN1 Intron-4-Deletion Corrected Control		
Cell Types	Cortical Neurons		Midbrain Dopaminergic Neurons		Rat Astrocytes
Pairwise Treatments	Vehicle			Treatment	
PFF Insult Dose	Vehicle	2.5 ug/mL	5 ug/mL	10 ug/mL	10 ug/mL + Chloroquine
Time	10 Days	20 Days	30 Days	40 Days	

Chapter 1.

Introduction

Precis

Neurodegenerative diseases are among the most complicated conditions known to humankind. However, they contain a feature that could help uncover their fundamental causes. In Part I of this chapter, I introduce the phenomenon of selective neuronal vulnerability and lay out a theoretical framework for how it may give insight into underlying neurodegenerative disease mechanisms. I focus on Alzheimer's and Parkinson's disease, the two most common neurodegenerative diseases, and provide an overview of their pathological hallmarks. I then review the differentially susceptible cell types associated with this pathology, and their associated divergent phenotypes that might underly this, with a focus on proteostasis. From this, I identify induced pluripotent stem cell-derived neurons as an untapped opportunity to further examine and confirm cell autonomous mechanisms.

While many studies have examined disease in cell models, different cell types are rarely compared to model selective vulnerability. In Part II of this chapter, I thus outline the strategy of this thesis: to use alpha synuclein pre-formed fibrils in Alzheimer's cell lines from patients with PSEN1 mutations, and Parkinson's cell lines from patients with LRRK2 mutations, and compare the differential vulnerability of these human induced pluripotent stem cells (hiPSCs) as cortical and dopaminergic neurons. As well as providing background and rationale for these components of the thesis, I also introduce autophagy, a cellular waste degradation pathway that may logically underly differential vulnerability to protein aggregation. Finally, I outline the aims of the following chapters, and this thesis.

Introduction Part I: Selective Neuronal Vulnerability as a Theoretical Framework

1.1. Neurodegenerative Disease

1.1.1. The Problem Posed by Neurodegenerative Diseases

Neurodegenerative diseases are the leading causes of dementia, disability, and death in the world. The number of people with dementia is expected to nearly triple over the next 25 years with aging populations, and the burden of these diseases on individuals, carers, and economies is immense (Feigin et al. 2020; Nichols et al. 2022).

There is only a limited understanding of the mechanisms that may drive cell loss, the core feature of these diseases. This is substantially because disease initiation occurs decades before symptom onset, encased in the skull, within one of the most complex structures in the known universe. Since the initial conditions of these diseases have been inaccessible to observation, it remains uncertain what causes these diseases, and as such, disease modifying therapies remain few and largely ineffective. Nevertheless, *postmortem* examination and brain imaging have associated a broad platter of pathologies and perturbations throughout brain regions and pathways, with these diseases. These most notably include protein aggregation, as well as neurovascular dysfunction, lipid dysregulation, and neuroinflammation, which are variably associated with synapse loss, and neurodegeneration (Dugger and Dickson 2017; Boyle et al. 2018; Robinson et al. 2021; Coukos and Krainc 2024).

What is most striking about these diseases is that the cell loss and associated pathologies in the brain develop with well defined, highly stereotyped spatiotemporal patterns, distinctive to each disease (Fu, Hardy, and Duff 2018; Kampmann 2024). This phenomenon, known as selective neuronal vulnerability, identifies that while some cells, pathways, and regions in the

brain are highly vulnerable to pathology and neurodegeneration, other regions remain remarkably resilient to the ruination wrought elsewhere.

It is unclear why the cellular pathologies arise when and where they do, and we currently lack the technology and ability to observe the cellular conditions at the time and place in which they begin. *Postmortem* examination, by definition, examines late-stage disease, and analyses of incidentally discovered early-stage disease cannot be certain about how disease may have progressed (Burke, Dauer, and Vonsattel 2008). Neuroimaging of patients lacks sufficient resolution to gain the insights into cellular and molecular changes. Animal models of neurodegenerative disease are relatively artificial and lack the genetic context and circuit anatomy of these apparently uniquely human diseases (Drummond and Wisniewski 2017). Examination of living brain tissue from patients undergoing surgery is typically confounded by other neurological conditions such as epilepsy or cancer.

How, then, can the initial drivers of these diseases be determined? Pedigree analysis, genetic sequencing, and genome wide association studies (GWAS) have provided a wealth of insight into pathways which are either causative of rare familial forms of neurodegenerative disease, or confer higher levels of risk, and are therefore likely involved in disease processes (Klein and Westenberger 2012; Escott-Price and Hardy 2022; Andrews et al. 2023). However, the live, cellular context of disease initiation in the human brain is both opaque to us and not amenable to experimental intervention. Furthermore, it is not clear that there even is a definable 'moment' where these diseases begin. Rather, given the progressive nature of these conditions, it is more likely that a particular set of initial conditions, or an accumulation of small stochastic changes, leads to disease over time (Saxena and Caroni 2011; Engelender and Isacson 2017). How can we gain insight into what these initial conditions that predispose disease might be? How do we understand which of the many changes in the brain that occur

over the lifespan are crucial to developing disease? One framework to consider more closely as offering such insights is selective neuronal vulnerability.

1.1.2. Examining Disease Mechanisms Through Selective Vulnerability

Examining vulnerable and resilient cells in comparison can indicate what cellular factors are crucial for driving these diseases in humans. The strikingly stereotypic differential vulnerability of cells and circuits in neurodegenerative diseases offers a natural, controlled experiment to understand what molecular, biochemical, and cellular interactions lead to the development of dysfunction, disease, and death, and what cellular pathways therefore should be therapeutically targeted.

This is by way of analogy to natural experiments in economics. This method of empirical investigation, for which David Card won the 2021 Nobel Prize, uses random variations or policy divergences among otherwise similar populations to create treatment and control groups in circumstances not otherwise amenable to experimental interventions (Card and Krueger 2000). These experiments have allowed causal insight into important economic questions, by, for example, comparing similar adjacent populations on either side of a border in the wake of a policy change in one region. By analogy, comparing otherwise similar populations of cells in adjacent brain regions which are differentially vulnerable to pathology may help identify differences that lead to pathogenesis.

Since we cannot sufficiently or ethically access and experimentally intervene in the human brain, the next best way of understanding what causes neurodegeneration may require understanding what divergent phenotypes drive the pattern of differential vulnerability and resilience in the brain. Therefore, this thesis and introduction will examine selective neuronal

vulnerability in neurodegenerative disease, with a view to understanding the differential vulnerability of cells and what may underlie this.

In this following section, I outline a framework for considering neuronal vulnerability, and then examine the key pathological hallmarks of the most common neurodegenerative diseases, Alzheimer's and Parkinson's disease. I then examine their spatiotemporal development, before considering why these patterns are present.

1.1.3. Considerations for Examining Selective Neuronal Vulnerability

1.1.3.1. Defining Vulnerability

Vulnerability is a nebulous term. A starting definition of vulnerability in neurodegenerative disease might simply be vulnerability to cell death. However, cells may also become dysfunctional without dying or significant discernible pathology (Milber et al. 2012; Busche and Konnerth 2015; Cramb et al. 2023). Defining vulnerability by this relatively extreme and late-stage outcome may preclude earlier, subtler dysfunction, that may be crucial to the experience of disease and understanding underlying mechanisms.

Many reviews also consider vulnerability as vulnerability to the development of disease associated pathology (Fu, Hardy, and Duff 2018; Z.-T. Wang et al. 2020). The distinctive patterns of cell loss that drives neurodegenerative conditions are frequently associated with and apparently preceded by neuropathological changes. These include neuroinflammation, vascular pathologies, sclerosis, lipid and metal deposits, and protein aggregation (Dugger and Dickson 2017; Boyle et al. 2018). Of these, proteinaceous aggregates are the most prominent and best studied of these pathological changes.

The development of these inclusions unites and defines a wide spectrum of neurodegenerative diseases (Dugger and Dickson 2017; Fu, Hardy, and Duff 2018; Forrest and Kovacs 2022). The

relatively distinctive identities and patterns of inclusions are used to neuropathologically define neurodegenerative diseases, and the proteins composing these inclusions have also been increasingly used for fluid and neuroimaging biomarkers of disease.

As with the loss of cells, the appearance of these protein pathologies through the brain typically occurs with a disease-specific stereotypic distribution, and likely marks underlying dysfunction. This apparently orderly and progressive recruitment of neuronal populations into the protein aggregate-laden phase of the disease is often associated with the pattern of cell dysfunction and loss, especially for certain proteins like tau and alpha synuclein (Dugger and Dickson 2017; Balusu et al. 2023).

Therefore, it makes sense, in an examination of selective vulnerability, to consider vulnerability as vulnerability to the development of pathology, especially protein aggregates, as well as vulnerability to cellular dysfunction and death, which is often but not always associated with proteinaceous pathology (Gómez-Isla et al. 1997).

1.1.3.2. Selective Vulnerability Has Broad Potential Across Neurodegenerative Diseases

Among the most common of the misfolding and aggregating proteins are amyloid beta (A β), tau, and alpha synuclein (ASyn), though there are several more, such as TDP-43 and huntingtin. Plaques composed of A β and neurofibrillary tangles (NFTs) composed of tau are defining features of Alzheimer's disease (AD), but tau pathology also occurs in many other diseases (Kovacs 2015). ASyn aggregates are a defining feature of Parkinson's Disease (PD), but similarly, synucleinopathy is also present in many other diseases, including AD (Dugger and Dickson 2017; Koga et al. 2021; Robinson et al. 2021). Across the range of tauopathies, synucleinopathies, and other neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and Huntington's, selective vulnerability is a hallmark phenomenon (Fu, Hardy, and Duff 2018; Kampmann 2024). This framework therefore has the potential to be applied to understanding a diverse array of diseases.

1.1.3.3. Heterogeneity of Vulnerable Cells Between Diseases

While there is substantial overlap between diseases in terms of which proteins pathologically manifest, their spatial distribution in the brain, stereotypic for a given disease, is highly variable between diseases (Fu, Hardy, and Duff 2018; Kampmann 2024). As such, it may be fruitful to study selective vulnerability in multiple diseases, since a cell type that is vulnerable in one disease may be resilient to another.

Another important consideration in the study of selective vulnerability is, thus, to examine its opposite. Few studies on selective vulnerability go further to examine why similar or other populations of cells may be resilient instead, and fewer consider why a population that is so vulnerable to one disease may be resilient to another disease, or to a different pathological hallmark. Having these resilient 'control' populations of neurons, however, is what gives the framework its power to reveal divergent phenotypes and thus underlying mechanisms. The brain presents diverse populations of differential vulnerability, an ideal opportunity to understand what differences drive vulnerability.

It is thus useful to examine a broad array of cells, both stricken and spared by disease, and to consider a broader range of disease contexts. While the full scope of neurodegenerative disease is regrettably beyond the scope of this thesis, here, I focus on AD and PD. These are two most common neurodegenerative diseases and share several major proteinopathies and convergent putative mechanisms (Cherubini and Wade-Martins 2018; Gan et al. 2018; Robinson et al. 2021). Importantly for this investigation, while they share some vulnerable brain regions, they also oppositely affect other brain regions.

1.2. Alzheimer's and Parkinson's Disease Pathology and Staging

Before examining selective vulnerability in AD and PD, it is first essential to properly introduce these diseases, hallmark pathologies, and the links between them. Then, their pathological distribution throughout the brain may be delineated. Since the experimental portion of this thesis focuses on A β aggregates, I will introduce that in more depth.

1.2.1. Alzheimer's Disease

1.2.1.1. Overview

AD is the most common neurodegenerative disease and most common cause of dementia, characterised by the progressive loss of cognitive function, with memory deficits particularly prominent (Masters et al. 2015; Scheltens et al. 2021). AD is predominantly a sporadic disease, though GWAS have found a number of risk variants, most notably the E4 allele of the ApoE gene (Y. Chen et al. 2021; Scheltens et al. 2021; Talyansky et al. 2023). There also exist rare forms of the disease, where autosomal dominant inherited mutations lead to early-onset familial AD (fAD) (Van Cauwenberghe, Van Broeckhoven, and Sleegers 2016). These include either mutations in or duplications of the amyloid precursor protein (APP) gene, which encodes for the protein from which the A β peptide is derived, one of the hallmarks of AD (Sleegers et al. 2006). More commonly, mutations are also seen at various locations in the presenilin genes, PSEN1 and PSEN2, which encode for proteins that cleave APP to produce A β peptides (Masters et al. 2015; Selkoe and Hardy 2016; Kelleher and Shen 2017).

1.2.1.2. Alzheimer's Disease Pathological Distribution & Staging

Protein aggregates in AD are tightly linked to disease mechanisms, and comprise its defining pathologies (Jack et al. 2011; 2018; DeTure and Dickson 2019). The first of these is the

accumulation of A β peptides into extracellular plaques, implicated in disease pathogenesis by the genetics of fAD. The second of these is the aggregation of tau into neurofibrillary tangles.

While mutations in the MAPT gene which encodes the tau protein are not associated with AD, they are sufficient to cause other tauopathies, directly linking tau aggregation to neurodegeneration (Crary et al. 2014; Kovacs 2015). The spatiotemporal distribution of NFT pathology in the AD brain also identifies tau as integral to the disease process in AD, since it is closely associated with the pattern of cell death and is related to the domain and degree of cognitive impairment (Jellinger 2020; Tanner and Rabinovici 2021).

Animal models (over)expressing human or mutant versions of these proteins also develop AD-like aggregates and cognitive impairment, further linking these proteins to disease mechanisms (Drummond and Wisniewski 2017; Zhong et al. 2024). The emergence of tau and A β pathologies is thus likely an integral part of AD pathogenesis, and likely indicative of underlying disease-related malfunction. Therefore, understanding the selective vulnerability of different cells and brain regions to these aggregates should yield insights into these processes driving disease.

1.2.1.3. Tau Staging

The staged development of NFT pathology in the AD brain is most famously described by Braak staging. In this six-stage scheme, NFTs first appear in the superficial transentorhinal cortex and apparently spread to anatomically connected brain regions throughout the hippocampal formation, and then upwards into the temporal and frontal association cortical areas, only reaching the occipital lobes and brainstem later in disease, and largely sparing primary cortical areas and the cerebellum (H. Braak and Braak 1991; 1996; Heiko Braak et al. 2006).

While these foundational pathological surveys were limited in being inherently cross sectional, recent longitudinal neuroimaging data has validated aspects of the staging pattern (Schöll et al. 2016; Ossenkoppele et al. 2019; Leuzy et al. 2022), although the initial sites of pathology

are also contested. Increasing evidence, including from updated Braak schema, implicate the locus coeruleus as the earliest site of tau pathology in AD (Heiko Braak et al. 2011; Matchett et al. 2021). While some patients exhibit atypical subtypes of AD (Murray et al. 2011; Crutch et al. 2012; Jellinger 2020), as much as 86% of subjects match typical Braak-like patterns (A. J. Schwarz et al. 2016).

1.2.1.4. Amyloid Staging

Amyloid plaque development is described by both Thal and Braak staging as beginning neocortically in the frontal and cingulate regions of the brain, then appearing in allocortical regions, then subcortically in the diencephalon and striatum, then brainstem nuclei, and finally in the cerebellum (H. Braak and Braak 1991; Dietmar R. Thal et al. 2002; Mrdjen et al. 2019)

As with tau, recent longitudinal neuroimaging data has similarly shown a progressive accumulation along predictable patterns that match neuropathological data (Collij et al. 2020; Jelistratova, Teipel, and Grothe 2020; Zammit et al. 2021). One such study identified only 2% of 667 individuals from the ADNI cohort who deviated from a 4-stage scheme (Grothe et al. 2017).

While amyloid appears to be associated with default mode network structures and likely has consequences for early stage cellular dysfunction (Palop et al. 2007), it is tau pathology that best correlates with the patterns of neurodegeneration and development of clinical symptoms in the disease (Gómez-Isla et al. 1997; Heiko Braak et al. 2006; Josephs et al. 2008; Jellinger 2020), and upon which toxicity of amyloid appears to depend (Ng et al. 2024).

1.2.1.5. Alpha Synuclein and Other Co-pathologies

While amyloid and tau are defining hallmarks of AD, and either genetically or functionally linked to the disease, it would be a mistake to consider only these as the protein aggregates that constitute AD pathology. A study of over 1000 brains found fewer than 10% of AD cases

have "pure" AD pathology (plaques and tangles) (Boyle et al. 2018). In addition to plaques and tangles, the AD brain frequently features a mixture of other proteinaceous pathologies.

Two of the most common of these are cerebral amyloid angiopathy (CAA) and TDP-43 depositions (Josephs et al. 2014; Boyle et al. 2018). These also take on a stereotypical staged accumulation pattern (Dietmar Rudolf Thal et al. 2003; Josephs et al. 2014; P. T. Nelson et al. 2019; Robinson et al. 2021). Important for this thesis, however, is the accumulation of ASyn into aggregates known as Lewy bodies (LBs) and other Lewy Pathology (LP). Since it is typically associated with PD, where it is a defining hallmark, AD is often overlooked as, by prevalence, the most common synucleinopathy.

Many studies report that more than half of AD cases include LP as a copathology (Twohig and Nielsen 2019; Jin et al. 2022). In one study of 25 patients with PSEN1 or PSEN2 mutations, 24 had LB pathology centred on the amygdala (Leverenz et al. 2006). LP is typically observed in an amygdala predominant pattern, without substantial involvement of the brainstem or cortex until later stages (Leverenz et al. 2008; Twohig and Nielsen 2019; Robinson et al. 2021). However, it is also frequently reported that in AD, LBs are negligible in the brainstem, and largely affect the limbic system (Toledo et al. 2016; Twohig and Nielsen 2019). The severity and distribution of co-pathologies such as CAA, TDP-43, and LP has been noted to be related to AD pathological stage and especially tau pathology (Twohig and Nielsen 2019; Robinson et al. 2021), suggesting that these also reflect disease relevant mechanisms, though the role of these pathologies is poorly understood (Almeida et al. 2023).

1.2.2. Parkinson's Disease

1.2.2.1. Overview

PD is the second most common and fastest growing neurodegenerative disease, featuring progressive autonomic dysfunction, motor impairment, and often developing dementia (Kalia

and Lang 2015; Dorsey et al. 2018; Hayes 2019; Bloem, Okun, and Klein 2021). It most notably involves degeneration of midbrain dopaminergic neurons (mDANs) of the substantia nigra pars compacta (SNPC), leading to its characteristic parkinsonian motor symptoms, and is characterised by LP (Parkkinen et al. 2011; Kalia and Lang 2015; Dugger and Dickson 2017).

Like AD, it is predominantly sporadic, although there are some rare familial versions of the disease too (Billingsley et al. 2018; Blauwendraat, Nalls, and Singleton 2020). There is possibly a closer link between familial and sporadic PD, as onset, symptoms, progression, and neuropathology are very similar, and GWAS of sporadic PD (sPD) turn up many of the same loci that are mutated in familial PD (fPD) (Edwards et al. 2010; Nalls et al. 2019; Blauwendraat, Nalls, and Singleton 2020). Many of these genes are linked to protein homeostasis, as well as *SNCA* itself, which encodes ASyn. Point mutations and copy number variants of the *SNCA* gene are also sufficient to cause PD, genetically linking ASyn to disease pathogenesis (Singleton et al. 2003).

This genetic evidence, combined with the misfolding and aggregation of ASyn into LP (Spillantini et al. 1997; Lashuel 2020), deeply implicates ASyn in PD pathogenesis, and warrants a deeper examination of ASyn itself.

1.2.2.2. Alpha Synuclein

The ASyn protein itself is a small, ~14 kD, intrinsically disordered protein, giving it great conformational flexibility (Babu et al. 2011; Albert et al. 2022). It typically exists as a monomer or in membrane bound form and is expressed widely throughout the body and brain (Human Protein Atlas). It is typically presynaptically localised, where it plays a role in synaptic vesicle release, though it may play a role in mRNA regulation (Bendor, Logan, and Edwards 2013; Burré, Sharma, and Südhof 2015; Kalia and Lang 2015; Schaser et al. 2019; K. Yamamoto 2021; Hallacli et al. 2022; Estaun-Panzano, Arotcarena, and Bezard 2023).

Beyond genetics, there is strong evidence to suggest that it plays an important role in PD pathogenesis (Bendor, Logan, and Edwards 2013; Flagmeier et al. 2016; Surmeier, Obeso, and Halliday 2017b; Dettmer 2018). The misfolding and aggregation of ASyn has been shown to disrupt cell functions, especially in mDANs. ASyn can aggregate into oligomers, which are thought to be highly neurotoxic, as well as into large beta-sheet containing fibril structures (Lashuel et al. 2013; Dettmer 2018; Zambon et al. 2019; Mahul-Mellier et al. 2020; Fares, Jagannath, and Lashuel 2021; B. J. Ryan et al. 2021; Lam et al. 2024). Animals that overexpress ASyn develop parkinsonism, and therapeutic techniques targeting ASyn in these models have ameliorated conditions (Jackson-Lewis, Blesa, and Przedborski 2012; Janezic et al. 2013; Fields, Bengoa-Vergniory, and Wade-Martins 2019).

1.2.2.3. Parkinson's Disease Pathological Staging

The crucial link between ASyn and PD pathogenesis is inclusion of ASyn in LP, the defining hallmark of PD (Spillantini et al. 1997). These pathological inclusions include large, highly structured cytoplasmic inclusions known as Lewy bodies (LBs), and long, thread like structures known as Lewy neurites (LNs). The spatiotemporal emergence of these has also been described by Braak staging (Heiko Braak et al. 2003).

This describes pathology originating first in the olfactory bulbs and/or dorsal motor nucleus of Vagus (DMNV), in the caudal brainstem, before apparently spreading rostrally through autonomic and neuromodulatory nuclei of pons (such as the Locus Coeruleus, LC) and midbrain (such as the SNPC), before affecting limbic and cortical structures in later stage patients. However, these studies also rely on cross-sectional data to infer temporal sequence.

1.2.2.4. Complications with Braak Staging of Lewy Pathology in PD

1.2.2.4.1. Variable Temporal Sequence, Conserved Spatial Distribution

Unlike in AD, this caudal-rostral pattern of pathological development is not nearly as consistent a staging schema. Various studies, for example, have found LB pathology in the

SNPC, LC, and/or amygdala, without involvement of the DMNV (Kalaitzakis et al. 2008; Jellinger 2019; Borghammer et al. 2021). Only 53% of 71 cases in one study were consistent with the Braak sequential distribution (Kalaitzakis et al. 2008).

Further investigations suggest two patterns of development. In around two-thirds of cases, there is caudo-rostral spread, with relatively more pathology in the brainstem than cerebrum (Raunio et al. 2019). In around one third of cases, there is an amygdala-centred pattern, with the pathological 'peak' in the centre of the brain and limbic structures (Borghammer and Van Den Berge 2019; Borghammer et al. 2021). LP without clinical deficits, termed incidental Lewy Body Disease (iLBD), has also been observed (Parkkinen et al. 2005; Attems et al. 2021).

Unfortunately, there remain no good PET tracers for ASyn to longitudinally validate these staging patterns (Korat et al. 2021). However, Horsager et al. (2020) found neuroimaging evidence to support a dual subtype model of PD, through imaging dopamine. Nevertheless, regardless of order, many of the same brain regions are repeatedly afflicted by LP, including the SNPC in 100%, nucleus basalis in 98.5%, and DMNV in 93% of cases in the same study, clearly indicating a preferential vulnerability of certain neuronal populations in PD (Kalaitzakis et al. 2008). While various authors argue against Braak staging, these are predominantly concerns with temporal development, not selective vulnerability (Burke, Dauer, and Vonsattel 2008). Even in iLBD, the same vulnerable populations of the olfactory bulbs amygdala, DMNV, SNPC, and LC are routinely implicated (Beach et al. 2009; 2010; Raunio et al. 2019; Borghammer et al. 2021). This suggests these cells have an intrinsic phenotype prone to PD pathology.

1.2.2.4.2. Lewy Pathology is Highly Variable and Possibly Regionally Distinct

Related to the above complications is the emerging evidence of the polymorphic nature of LP. Ultrastructural characterisation of LBs, among other evidence such as their affinity for different dyes, has revealed that in different parts of the brain, these structures display diverse structural arrangements and compositions (Shahmoradian et al. 2019; Lam et al. 2024). Some

recent evidence suggests not all LP contains ASyn, and that other structures including membranes and organelles form more important components of this pathology, though this has been argued against and many inclusions nevertheless predominantly contain ASyn (Shahmoradian et al. 2019; Lashuel 2020; Trinkaus et al. 2021).

A spectrum of ASyn aggregation likely occurs across the brain, including granular or punctate forms, which may represent oligomeric ASyn, as well as larger 'pale bodies', and more compact LBs, which themselves seem to include a spectrum of staged development (Kuusisto, Parkkinen, and Alafuzoff 2003; J. Alegre-Abarrategui et al. 2008; Fares, Jagannath, and Lashuel 2021; Lam et al. 2024). This spectrum also appears to be regionally heterogeneous, with divergent cortical and brainstem LB subtypes. These are not well understood but point to intrinsic cellular characteristics leading to regionally-dependent disease processes.

1.2.2.4.3. Does Lewy Pathology Lead to Neuronal Loss?

Another complication of PD is the nebulous link between neuropathology and cell loss. LBs, rather than pathological markers, may be interpreted as markers of surviving, relatively resilient neurons (Javier Alegre-Abarrategui et al. 2019). In some fPD patients with the LRRK2-G2019S mutation, patients still experience cell loss but without LP (Surmeier, Obeso, and Halliday 2017b; Henderson et al. 2019). Furthermore, in early stages of disease, neuronal cell loss in the SNPC precedes and exceeds the development of LP in the SNPC, and precedes cell loss in other medullary and pontine regions that exhibited LP (Milber et al. 2012). Similarly, in the hypothalamus, profound cell loss can be observed in the supraoptic nucleus without significant pathology, while the LB-laden mammillary bodies do not atrophy (Ansorge, Daniel, and Pearce 1997). Halliday et al. (2005) also show substantial loss of motor neurons in the pre-SMA cortex, but rare LBs.

Nevertheless, it has also been argued that the development of LBs, drawing in proteins, lipids, and membranous organelles, is a toxic process in itself (Mahul-Mellier et al. 2020), and shown

that they can be neurotoxic (Osterberg et al. 2015), and that they might be a source of other toxic species (Bengoa-Vergniory et al. 2017; Javier Alegre-Abarrategui et al. 2019; Cascella et al. 2021). Furthermore, the formation of LBs also serves as a marker of disturbances in normal cell homeostasis (Javier Alegre-Abarrategui et al. 2019).

As such, despite the variable temporal sequence of regional involvement in pathological changes, the diverse phenotypes of those pathological changes, and the uncertain link between cell loss and aggregation, the cell types vulnerable in PD remain remarkably selective, especially mDANs of the SNPC.

1.2.3. Selective Vulnerability to Tau and Alpha Synuclein - linked proteins and Clues to the Pathogenesis of AD and PD

Therefore, genetic, functional, and histological evidence all present a role for the aggregation of proteins such as A β , tau, and ASyn in these diseases. As such, it is likely that even in sporadic cases, proteins like A β , tau, and ASyn are all either directly involved in or markers of pathogenic cellular environments. What remains uncertain is what the pathogenic conditions are that give rise to these pathologies.

It is interesting to briefly note the similarities between tau and ASyn in this regard. Both are widely expressed, natively unfolded proteins, with normal functions in the axosynaptic component of the neuron (Babu et al. 2011; Barbier et al. 2019). Recent emerging evidence also posits a role for both in regulation of gene expression (Hallaccli et al. 2022; Abasi et al. 2024). Both become hyperphosphorylated, misfold, and seed aggregation in an apparently prion-like manner, eventually forming large intracellular inclusions, as well as other potentially toxic species such as oligomers (Walker and Jucker 2015; Bengoa-Vergniory et al. 2017; Colom-Cadena et al. 2023; Meloni et al. 2023). Synergistic interactions between ASyn and

classical AD pathology have also been noted, as well as the presence of tauopathy in PD and synucleinopathy in AD (Wray and Lewis 2010; J. L. Guo et al. 2013; Irwin, Lee, and Trojanowski 2013; Henderson et al. 2019; Twohig and Nielsen 2019; Bassil, Brown, et al. 2020; Bassil, Meymand, et al. 2020; Jin et al. 2022; Lim et al. 2023).

It is perhaps unsurprising that this pair comprise the most common proteinopathies. Their overlapping characteristics also lead to the hypothesis that some shared mechanisms may govern their conversion from normal to pathological function. However, it is not clear what causes this conversion, what forms of the proteins are most toxic, whether toxicity is exerted through loss of normal function or gain of toxic function, or both, or even whether these protein aggregates reflect a protective mechanism.

However, the question of concern here is not whether or how these pathologies exert neurotoxicity, but why they occur at all. Irrespective of whether aggregates are harmful or helpful *per se*, their formation is nevertheless an indication of underlying failures of normal cellular homeostasis.

The protein pathologies may have their own toxicity, but they are not themselves the root cause of the disease. Rather, they result from upstream processes gone wrong. Since these proteins and their aggregation are apparently key aspects of these diseases, the upstream pathways leading to their accumulation should be among those that cause the disease. These malfunctioning pathways may thus be thought of as the true locus of disease and need to be identified. In this sense, pathological hallmarks provide biomarkers of where to locate cells with and without the fundamental cellular processes that underpin these neurodegenerative diseases.

Therefore, as discussed, examining cells differentially vulnerable and resilient to the development of these protein aggregates, as well as cell death, should reveal these fundamental mechanisms that drive pathology. The different brain regions that are notably

vulnerable and resilient in AD and PD to the development of protein pathology and/or neuronal dysfunction and death, and the mechanisms associated with their vulnerability and resilience are hence examined here.

1.3. Vulnerable and Resilient Cells and Brain Regions in AD and PD

The existence of sequential staging patterns for pathology in AD and PD suggests that not only are there regions that are selectively vulnerable to disease, but also that some regions are differentially vulnerable. I.e. Some regions consistently become pathologically involved much earlier in disease, and are relatively vulnerable, while others are only affected later, and are relatively resilient. Here, I summarise cell populations involved early, late, and not at all, over the course of pathology, and some of their relevant phenotypes.

1.3.1. Alzheimer's Disease

The hippocampus and entorhinal cortex (EC) have classically been thought of as among the most vulnerable and earliest structures affected by cell loss and neurofibrillary pathology in AD. Within these structures, specific populations of cells are selectively vulnerable, such as pyramidal neurons in cornu ammonis region 1 (CA1) and CA3, and pre-alpha projection neurons in the superficial EC, while others such as dentate gyrus granule cells (DGGCs) and CA2 neurons are more resilient (Hyman et al. 1984; H. Braak and Braak 1985; Morrison and Hof 2002; Leng et al. 2021). More recent evidence has highlighted the noradrenergic LC as another early vulnerable region (Heiko Braak et al. 2006; Matchett et al. 2021; Evans, Defensor, and Shamloo 2022; Bueichekú et al. 2024). From these apparently initial sites, various parts of the cortex become heavily involved, especially layers II and III in associational regions, rather than primary cortical areas (Pearson et al. 1985; H. Braak and Braak 1996; Ossenkoppele et al. 2019; Leuzy et al. 2022; S. Chen, Chang, et al. 2022; Zwang et al. 2023).

In general, large, excitatory, projection neurons tend to be vulnerable to neurofibrillary pathology, while small, inhibitory interneurons are relatively spared of pathology, though evidence suggests these are also lost (Kilimann et al. 2014; Fu, Hardy, and Duff 2018; Mrdjen

et al. 2019; Ruden, Dugan, and Konradi 2021; Deng et al. 2022; Mathys et al. 2023). Interestingly, while some large, neuromodulatory nuclei such as the LC and basal forebrain cholinergic nuclei (BFCN) show substantial vulnerability (Heiko Braak et al. 2006; Kilimann et al. 2014). However, mDANs remain relatively resilient to both tau and amyloid neuropathic changes until late in disease, though cell loss in the ventral tegmental area (VTA) has been observed in some models (Dietmar R. Thal et al. 2002; Heiko Braak et al. 2006; 2011; Nobili et al. 2017; Fu, Hardy, and Duff 2018). Even ASyn pathology is largely confined to the amygdala and limbic structures in early AD (Raunio et al. 2019; Borghammer et al. 2021; Robinson et al. 2021). Key regions of interest and their characteristics are summarised in full in Appendix Table 1.

1.3.2. Parkinson's Disease

If not consistently first, the DMNV and olfactory bulbs are affected early and frequently in PD (Heiko Braak et al. 2003; Kalaitzakis et al. 2008). Many nuclei in the brainstem, especially neuromodulatory nuclei with extensive, diffuse projections, are particularly vulnerable. Most notable here are mDANs in the SNPC, but also serotonergic neurons in the Raphe nuclei, noradrenergic neurons in the LC, and cholinergic neurons of the pedunclopontine nucleus (PPN) and basal forebrain (G. M. Halliday et al. 1990; Heiko Braak et al. 2003; Surmeier, Obeso, and Halliday 2017b). Interestingly, while still affected, mDANs in the VTA are relatively spared compared to the SNPC, and within the SNPC, gradients of vulnerability exist, providing valuable control populations (Fearnley and Lees 1991; Damier et al. 1999; Monzon-Sandoval et al. 2020; Kamath et al. 2022). The amygdala and limbic structures are also heavily implicated in PD, especially cells connected to the olfactory system (Harding et al. 2002; Borghammer et al. 2021; Y.-J. Guo et al. 2022).

By contrast, cortical regions, especially associational regions rather than primary cortical areas, are affected later in PD, with LBs predominantly in excitatory deeper layer projection neurons, and LNs in more superficial layers (Heiko Braak et al. 2003; Beach et al. 2009; Surmeier, Obeso, and Halliday 2017b; Borghammer et al. 2021; Goralski et al. 2024). Interestingly, both in the cortex and brainstem nuclei such as the PPN, GABAergic inhibitory neurons are relatively spared of pathology (Kingsbury et al. 2010; Double 2012; Goralski et al. 2024). These regions and their pathological characteristics in PD are summarised in Appendix Table 2.

1.4. The Pathways and Mechanisms Associated with Selective Vulnerability

Having identified populations of cells which are differentially vulnerable in AD and PD, these can be compared, to examine what differences may exist that may facilitate or resist disease. Through many decades of mostly neuropathological examination correlating cell vulnerability with molecular markers and functional characteristics of neuronal populations, a picture has slowly built up of potential pathways that may be responsible for the selective vulnerability of particular cell types.

Hof and Morrison (2004), and many others since, break these down into cell autonomous and non-autonomous mechanisms. This is not always such a clean division, since many pathways involve neither the cellular microenvironment, nor intracellular biochemical pathways alone, but an interaction of the two. Pathways have been extensively reviewed in AD, PD, and across neurodegenerative disease by several authors (Double 2012; Surmeier, Obeso, and Halliday 2017b; Fu, Hardy, and Duff 2018; Grothe et al. 2018; Javier Alegre-Abarrategui et al. 2019; Mrdjen et al. 2019; Z.-T. Wang et al. 2020; Leng et al. 2021; Kampmann 2024).

A full accounting of these pathways is, unfortunately, beyond the scope of this thesis. However, a summary of the key mechanisms, theories and the evidence for them is outlined in Appendix Table 3, for reference. These begin with pathways relating to the environment around vulnerable cells and insults or stressors they may be variably exposed to (non-autonomous pathways), and transition towards intrinsic features of cell function that might drive differential vulnerability (cell autonomous pathways). The quality and depth of evidence varies greatly, with some mechanisms being substantially more speculative, and other speculated mechanisms being more substantiated.

Briefly, non-autonomous pathways include the role of glial cells and myelination, how much exposure to toxins cells might have, either through vasculature or through exposure to the

external environment, and connectivity and spreading mechanisms. While the spreading of pathology along anatomical pathways is a contested concept, the weight of evidence suggests that while prion-like mechanisms are likely at play, they are not sufficient to explain the pattern of differential vulnerability in the brain (Surmeier, Obeso, and Halliday 2017a).

Given this, a role for the cell autonomous mechanisms in driving vulnerability might be postulated too. The inherent characteristics of a cell may include the neurotransmitters it produces and their toxic potential, the size, activity, and consequent energy and transcriptional demand of that neuron, the capacity of the cell to manage Ca^{2+} flux, and the balance of proteostatic pressures it faces.

1.4.1. A Focus on Proteostatic Differences Driving Differential Vulnerability

How cells regulate protein levels is of particular interest in neurodegenerative diseases, and this thesis. GWAS and familial variants frequently implicate genes involved in proteostasis, identifying this as a fundamental disease mechanism (Yamamoto, Zhang, and Mizushima 2023). Moreover, the prevalence of protein aggregate pathologies across diseases implies underlying dysregulation of proteostasis as a common feature of neurodegenerative disease, warranting further examination. This dysregulation might involve an imbalance in uptake, expression, clearance, or release, in many associated pathways.

With regards to protein expression levels, genetic copy number variants and overexpression models of AD and PD posit a causative role for increased protein concentrations in these diseases (Singleton et al. 2003; Sleegers et al. 2006; Blauwendraat, Nalls, and Singleton 2020; Andrews et al. 2023). While this alone does not explain differential vulnerability, larger neurons with longer axons and more synapses might be expected to express higher levels of tau and

ASyn. Direct measurement of the protein in individual cells in the brain is technically challenging, since they are large with diverse processes, and mRNA alone may not be a good measure of protein levels. However, it has been suggested that these ‘metastable’ proteins, whose concentrations are high relative to their solubility, are enriched in vulnerable cells (Ciryam et al. 2013; Freer et al. 2016; Taguchi et al. 2016; Fu, Hardy, and Duff 2018; Grothe et al. 2018). As well as endogenous expression, increased protein uptake may also lead to increased intracellular concentrations. LRP1 has recently been identified as a mediator of both tau and ASyn uptake, though its differential expression has not been closely examined (Rauch et al. 2020; Chen et al. 2022).

Elevated protein concentrations likely converge with impaired clearance and other homeostatic mechanisms which fail to maintain protein solubility. These include the endolysosomal pathway, autophagy, and ubiquitin-proteasome system. These are complex systems that are differentially regulated across the brain, with many potential points of failure (Nixon and Yang 2011; Nixon 2013; Szabo et al. 2022). Studies have shown differential autophagy and vesicle trafficking phenotypes between dorsal and ventral neurons in the human SNPC (Gonçalves et al. 2016; Monzon-Sandoval et al. 2020), between cortical and SNPC neurons in a PD rodent model (Wallings, Connor-Robson, and Wade-Martins 2019), and in TH+ compared to TH- neurons in midbrain hiPSC DAN cultures with glucocerebrosidase (*GBA*) mutations (Fernandes et al. 2016; Lang et al. 2019). Additionally, in LRRK2 knock out (KO) mice, there is age dependent increase in autophagic vacuoles and degeneration in SNPC and LC but not cortex and cerebellum (Giaime et al. 2017). These studies point to differential regulation of autophagy in vulnerable and resilient cell types that may lead to pathology in PD.

Many studies also show changes in expression of proteostasis genes in aggregate laden cells, such as nucleus basalis cells with tau pathology (Tiernan et al. 2016), cortical neurons with tau or ASyn dysfunction (He et al. 2020; Goralski et al. 2024), or CA1 HC neurons in early AD (Bordi

et al. 2016). While these studies point to a relationship between pathology and impaired proteostasis that may enter a vicious spiral, such studies cannot tell cause from effect.

By contrast, a particularly striking study by Freer et al. (2016) examined expression of tau and amyloid co-aggregating proteins across different brain regions in healthy brains, without pathology. Here, using tissue microarray, they show the expression patterns of proteins that promote aggregation matched the distinct brain regions vulnerable to developing pathology in both AD and PD. Meanwhile, proteins associated with protection from aggregation were enriched in resilient tissues. By examining healthy tissue across multiple brain regions, this paper provides strong evidence for intrinsic cellular qualities driving vulnerability and resilience to aggregation. However, it is unclear whether the co-aggregates actively promote pathology or are simply co-aggregates because they are the proteins expressed in the cells where tau pathology develops.

Thus, it is likely that proteostatic differences between cell types predispose certain populations to certain pathologies under certain genetic and environmental conditions. These lead to pathology, or in resilient cells, mitigate accumulating damage and dysfunction when combined with other factors such as Ca^{2+} buffering, mitochondrial function, energy demand, toxin exposure, and pathological spreading.

1.4.2. Perspectives and Shortcomings of the Pathways Associated with Selective Vulnerability

From a wider examination of the pathways associated with vulnerability, it is clear that no one mechanism is responsible. Many overlap and interact significantly, and their synergy has deleterious effects when they converge on certain neuronal populations. For example, the confluence of high energy burden from tonic synaptic activity over highly arborised,

unmyelinated axons, along with significant Ca^{2+} transients, limited Ca^{2+} buffering proteins, and reactive neurotransmitters, make dopaminergic neurons of the SNPC selectively and distinctively vulnerable compared to their similar but more resilient VTA neighbours, even without considering the development of LP in these cells (Surmeier, Obeso, and Halliday 2017b; 2017a). Despite their extreme intrinsic vulnerability, however, they are nevertheless relatively resilient in AD, where concomitant LB pathology has a shifted distribution (Toledo et al. 2016; Robinson et al. 2021).

It is therefore also useful to study this phenomenon within the context of multiple diseases, not only because of the differential vulnerability of cells in different disease contexts, but also because it highlights the substantial overlap in pathological mechanisms between diseases (Noori et al. 2021). Many proposed drivers of cellular vulnerability apply to both AD and PD, which presents broader therapeutic opportunities. AD and PD are in fact especially interesting to study in comparison. Some parallels between the biochemical properties of tau and A β have already been discussed, but it also appears they may share some pathogenic mechanisms and can cross seed pathology (Uemura et al. 2020; Williams et al. 2020). Tau and A β are frequently co-pathologies in AD, and even in PD, especially associated with LRRK2 mutations (Robinson et al. 2018; 2021; Henderson et al. 2019). Meanwhile, the main AD genetic risk factor ApoE4 exacerbates A β pathology in rodent models and is a risk factor for DLB (Zhao et al. 2020; Jin et al. 2022).

One difficulty is assessing the relative contribution of different mechanisms in different diseases and cell types. For example, the balance between prion-like spreading and other connectivity mechanisms versus cell autonomous mechanisms is highly contested, though both are likely involved. On the one hand, even with a significant role for pathological spreading, pathology must originate somewhere, and furthermore, synaptically connected regions to sites of pathology are not always affected in proportion to their connectivity

(Surmeier, Obeso, and Halliday 2017a; Henrich et al. 2020; Henderson et al. 2022). The alternative to a prion spreading model - the Threshold Theory for PD or Stressor-Threshold theory for AD (Saxena and Caroni 2011; Engelender and Isacson 2017; Mrdjen et al. 2019) - is a purely stochastic model. It holds that, rather than propagating between cells, the potential for developing pathology exists in most if not all cells, and stochastic insults affect cells with different thresholds for different stressors determined by their intrinsic phenotype.

This rules out any role for connectivity or spreading mechanisms, which seems improbable (Mutlu et al. 2017; Surmeier, Obeso, and Halliday 2017a; Ossenkoppele et al. 2019; Zarkali et al. 2020; Guo et al. 2022). This model also fails to explain why cell populations such as SNPC mDANs, which exhibit a confluence of many of the strongest mechanisms driving vulnerability, do not frequently degenerate in diseases other than PD, and are not even necessarily an early site of pathology in PD. Several authors have suggested that cell autonomous mechanisms drive primary vulnerability, while secondary vulnerability is mediated by a mixture of connectivity and autonomous factors (Surmeier, Obeso, and Halliday 2017a; 2017b; Fu, Hardy, and Duff 2018; Wang et al. 2020).

A major methodological advance in deciphering mechanisms driving vulnerability has come with the advent of single cell and especially spatial transcriptomic approaches to studying post mortem tissue (Balusu et al. 2023). Single cell studies have permitted a broad understanding of cell types enriched for genes and pathways identified through GWAS. Spatially defined transcriptomic approaches such as tissue microarrays (Freer et al. 2016), laser-capture microdissection (Monzon-Sandoval et al. 2020), and purpose built spatial transcriptomic platforms (Chen et al. 2021; Chen et al. 2022; Goralski et al. 2024) have also driven substantial understanding of the molecular environment in specifically vulnerable and resilient cells.

For example, single cell transcriptomics of the SNPC and VTA has delineated differentially expressed genes (involved in proteostasis and calcium buffering) between vulnerable ventral

and resilient dorsal tier neurons and identified specific subpopulations of SOX6+AGTR1+ dopaminergic neurons that are selectively vulnerable and enriched for PD risk loci (Monzon-Sandoval et al. 2020; Kamath et al. 2022). Studies examining healthy brain tissue such as those by Freer et al. (2016) and Monzon-Sandoval et al. (2020) are particularly powerful for identifying intrinsic characteristics and discrepancies between cell types known to be differentially vulnerable. However, spatial approaches to pathology laden brains can also identify specific subpopulations of cells vulnerable to pathology, such as superficial RORB+ neurons in the AD EC (Chen et al. 2022; Caramello et al. 2023) and deeper Layer 5 SATB2+ neurons in the PD cortex (Goralski et al. 2024), and examine for broader transcriptomic differences between cells bearing pathology and those without (Tiernan et al. 2016; Piwecka et al. 2024).

However, to truly discern the relative contribution of these identified pathways, and, indeed, to show they are causative, not merely correlational, they need to be isolated in a system amenable to experimentation. A repeated shortcoming of the present literature examining selective vulnerability is the reliance on *postmortem* tissue for human data. Neuropathological and single cell transcriptomic studies have been crucially important, but are inherently cross sectional, unable to examine how pathology develops over time, and typically based on late-stage pathology, where the cells remaining may be considered resilient, rather than vulnerable (Alegre-Abarrategui et al. 2019). The framework of selective vulnerability allows for the identification of associated disease mechanisms, but further confirmation is necessary. Most importantly, while many correlates of vulnerability can be established with post mortem analysis comparing vulnerable and resilient tissues, causality remains hard to determine (Balusu et al. 2023).

Furthermore, postmortem analysis limits research to coarse measures of vulnerability such as neuronal loss and proteinaceous pathology. These are visible to immunolabelling and histochemical techniques but are ultimately crude, and ignore changes in live cellular

functioning, synaptic connections, gene expression, and electrical properties that may contribute to disease at earlier stages, and substantially change our understanding of what regions we should consider to be vulnerable (Roberts, Wade-Martins, and Alegre-Abarrategui 2015; Fu, Hardy, and Duff 2018; Balusu et al. 2023).

Attempts to examine differential vulnerability in rodent models allow for some examination of early pathological development. One particularly elegant study altered the axonal growth of SNPC mDANs, to show that their arborisation drove vulnerability (Giguere et al. 2019). However, these models suffer from the gulf between rodent and human neuroanatomy, with differences in cell populations, structures, gene expression patterns, and even uniquely human vulnerable cell populations, such as Von Economo neurons (Jackson-Lewis, Blesa, and Przedborski 2012; Drummond and Wisniewski 2017; Gefen et al. 2018; Lin et al. 2019). Furthermore, many rodent studies to date have largely been postmortem neuropathological assessments too, and there remains substantial scope for experimental interventions that upregulate or downregulate aspects of different pathways that may underlie differential vulnerability, to build up a picture of vulnerability pathways with a better sense of causality.

Such interventional studies would also give a clearer sense of the relative importance of different pathways in driving cellular vulnerability, which is currently lacking. Ultimately, while postmortem and rodent studies have been important for our understanding of selective neuronal vulnerability, the mechanisms proposed by this framework need to be confirmed in a living, human model.

Introduction Part II: Models Applied in This Thesis to Examine Selective Vulnerability

1.5. hiPSC-Derived Neurons for Studying Selective Vulnerability

1.5.1. Advantages of the hiPSC model

One strategy that might be employed for studying selective neuronal vulnerability in a live model is to use human induced pluripotent stem cell (hiPSC)-derived neurons. The advent of hiPSC technology has allowed the *in vitro* examination of live human brain cells from patients with neurodegenerative diseases (Badger et al. 2014; Essayan-Perez et al. 2019; C. Lee, Willerth, and Nygaard 2020; Valadez-Barba et al. 2020). One advantage of this model is the employment of human cells and genetics, which are substantially different to those in rodents, and may have more translational validity than animal models for neurodegenerative disease (Delenclos et al. 2019; Drummond and Wisniewski 2017; Essayan-Perez et al. 2019; Jackson-Lewis, Blesa, and Przedborski 2012). Rodent models typically require transgenic expression of human genes in rodent cells, and resultant phenotypes may depend more on the expression level or promoter used (Polinski et al. 2018; Balusu et al. 2023).

The particular advantage of this model for studying selective vulnerability is the opportunity to create multiple different cell types from the same individual, enabling direct comparison of vulnerable and resilient cell types. Ordinarily, a reductionist *in vitro* model might be a limitation, lacking cell diversity and brain complexity. However, for this purpose, this is an advantage of the hiPSC model, which isolates any cell autonomous factors that might be driving neuronal vulnerability and resilience. A demonstration of differentially vulnerable hiPSC-derived neurons would, in effect, prove a role for cell autonomous mechanisms as an essential component of neurodegenerative diseases.

While hiPSC-derived neurons are thought to epigenetically reflect developing rather than aged cells (C. Lee, Willerth, and Nygaard 2020), here, it is the intrinsic differences in cell phenotypes that are of interest, rather than the effect of aging. The findings of Freer et al. (2016) suggest that the vulnerability of specific tissues to pathology is a feature of younger, healthy brains. Interestingly, hiPSC-derived mDANs were even shown to have substantial transcriptional overlap with mature midbrain dopaminergic neurons obtained from postmortem brains by laser capture microdissection (Sandor et al. 2017). Thus, the hiPSC neuron model remains an exciting opportunity to isolate the cell autonomous features of vulnerable and resilient cell types, in a live model of early stage dysfunction in a human genetic and cell context (Essayan-Perez et al. 2019; Balusu et al. 2023).

1.5.2. Stem Cell Examinations of Selective Neuronal Vulnerability

While there is a rapidly growing literature examining cellular mechanisms of AD in hiPSC cortical neurons (CNs), and PD in mDANs, there remain very few studies that compare these vulnerable cell types in each disease to resilient cells, to examine selective neuronal vulnerability. The first to do so was Muratore et al. (2017), where hiPSCs from two APP-V717I patients were differentiated into loosely defined 'caudal/hindbrain' and 'ventral/forebrain' populations of neurons. They found that the forebrain neurons had higher expression of APP, phospho-tau, and total tau. A higher A β 42:40 ratio and 40:38 ratio was also observed in the forebrain neurons. In response to brain homogenates from AD patients, caudal neurons responded no differently than controls, but ventral neurons saw increased levels of phospho-tau.

A second study examined two cell lines with *SNCA*-duplication, differentiated into both CNs and mDANs (Brazdis et al. 2020). Here, western blotting revealed higher levels of ASyn oligomers in mDAN control and PD lines compared to CNs. Furthermore, they also observed

elevated oxidative stress in PD lines compared to controls in mDANs, but no difference in CNs. Oosterveen et al. (2021) similarly found differences in rotenone sensitivity in mDANs guided towards a resilient VTA-type fate, compared to a vulnerable SNPC-type fate. These differentially vulnerable cell types were generated through alterations in Wnt signalling or through lentiviral transcription factor expression, and interestingly, cell lines from an SNCA-triplication patient expressed more ASyn in the SNPC-type than VTA-type cells.

Similarly, Fernandes et al. (2016) and Lang et al. (2019) found that perturbations of autophagy related proteins in heterogenous hiPSC mDAN cultures from GBA-N370S patients were selectively in TH+ and not TH- cells. Meanwhile, in an ALS context, Held et al. (2023) find ALS-associated genetic changes in hiPSC-derived motor neurons but not sensory neurons or CNs.

These studies, mostly from the last few years, collectively provide proof of concept that hiPSCs grown into differentially vulnerable cell types should reflect the relative vulnerability of their brain counterparts. These provide evidence for the role of cell autonomous mechanisms such as endogenous protein expression and secretion regulation in determining selective neuronal vulnerability, but still leave open questions.

First, while the above studies demonstrate differential vulnerability, few elaborate on underlying mechanisms for this, and only to a limited degree. Second, neurons in these studies are frequently not well defined (or entirely uncharacterised), important for understanding which cellular phenotypes predispose disease. Third, the above studies examine selective vulnerability in the context of a single disease, and in a very narrow range of cell lines, missing the opportunity to address whether the vulnerable cells of one disease remain vulnerable in the genetic context of a different disease. Finally, most of the above studies examine intrinsic properties of these cells, but do not examine their propensity to develop pathology, exhibit functional deficits, or die. While the divergent phenotypes of these cells are of great interest,

their differential susceptibility to disease relevant pathology and dysfunction is key to validating the model, yet remains largely underexamined.

This is, in part, due to a limitation of the hiPSC model alone, which, without years of pathological development, typically lacks any overt disease pathology, even with severe genetic alterations such as *SNCA*-triplication. The hiPSC neurons merely model the underlying pathways that make cells differentially susceptible to developing pathology. This makes hiPSC neurons an excellent system for understanding what these pathways are, manipulating them, and examining how they contribute to pathogenesis, so long as an appropriate model is used to induce pathology in these cells.

1.6. The Alpha Synuclein PFF Model

1.6.1. Overview

In 2009, Luk and colleagues first demonstrated how recombinant monomers of ASyn, aggregated into fibrils, could be used to induce endogenous proteins into insoluble inclusions in SH-SY5Y neuroblastoma cells (Luk et al. 2009). This pre-formed fibril (PFF) model has been used extensively since to model aggregate pathology in cell and animal models. Unlike toxin models, which do not develop aggregates, and unlike genetic or overexpression models, which typically do not develop filamentous inclusions, PFF insults seed disease-appropriate pathology in the endogenous proteins, within the physiological conditions of the cell (Jackson-Lewis, Blesa, and Przedborski 2012; Polinski et al. 2018). This, along with the spatiotemporal control over pathological induction, make it an ideal insult paradigm for examining the differential propensity of cells to develop or cope with protein aggregation (Polinski et al. 2018).

In theory, fibrils and oligomers of tau, A β , and ASyn may be produced. However, tau PFFs typically utilise truncated or mutant versions of the protein, in order to achieve the aggregation normally associated with post translational modifications *in vivo*, which is not physiological to AD (Basheer et al. 2024). ASyn, however, readily aggregates as a wild-type, full length, recombinant protein and unlike A β , is relevant to both PD and AD. Thus, while a comparison to tau is of great interest, this thesis therefore focuses on using ASyn PFFs.

1.6.2. ASyn PFFs in Cell and Rodent Models

Studies examining ASyn PFFs in cell models such as SH-SY5Y, PC12, and LUHMES are summarised in Albert et al. (2022). These find that PFFs are internalised by macropinocytosis and lead to some cytotoxicity and inflammatory responses, but not substantial aggregation

without SNCA overexpression. The majority of existing PFF studies have been conducted in *in vivo*, and *in vitro* rodent models.

Injection studies into rodents and primates are reviewed by several authors (Gómez-Benito et al. 2020; Y.-J. Guo et al. 2022). These show seeded aggregate pathology, mDAN degeneration, synaptic dysfunction, neuroinflammation, and glial senescence, among other phenotypes (Abdelmotilib et al. 2017; Paumier et al. 2015; Thomsen et al. 2021; D. K. Verma et al. 2021). However, of interest for selective vulnerability, the pattern of aggregate deposition typically matches known vulnerable PD regions, such as the amygdala, SNPC, striatum, and hippocampus. Furthermore, injection into the dorsal striatum leads to more pathology in the SNPC than VTA and motor impairments in both ASyn overexpressing and WT mice (Luk, Kehm, Carroll, et al. 2012; Luk, Kehm, Zhang, et al. 2012). Intracerebral injection of ASyn PFFs followed by live imaging found the progressive development of LNs at 2.5 months post injection, LB-like inclusions after four months, and loss of inclusion-bearing cells after seven months (Osterberg et al. 2015). These suggest that, in the rodent brain, ASyn PFFs preferentially affect vulnerable cell populations, leading to disease-relevant pathology and deficits.

Extensive protocols for using PFFs have also been developed for rodent primary cell cultures, and there is a substantial literature characterising this model (Luk et al. 2009; Polinski et al. 2018; Volpicelli-Daley, Luk, and Lee 2014; Volpicelli-Daley et al. 2011). As in the brain, PFF-insulted primary neurons develop neuritic and somatic Lewy-like inclusions, sometimes within days, dependent on dose and the endogenous expression model used (Luk et al. 2009; Mahul-Mellier et al. 2020; Sacino et al. 2017; Tapias et al. 2017; Volpicelli-Daley, Luk, and Lee 2014; Volpicelli-Daley et al. 2011; X. Wang et al. 2019). Aggregates are retrogradely transported in axons (Volpicelli-Daley et al. 2011) and resultant functional deficits have also been observed in these studies, including synaptic degeneration, loss of neuronal activity synchronisation,

axonal transport, autophagy and mitochondrial deficits, oxidative stress and neurodegeneration.

Aggregation depends on the expression and recruitment of endogenous ASyn, and occurs in HC, cortical and midbrain primary cultures (Luk et al. 2009; Volpicelli-Daley, Luk, and Lee 2014). Neurons with aggregates typically exhibit more cell death than those without, and treatment to *SNCA*-KO cells is usually non-toxic (Mahul-Mellier et al. 2020), though one study has shown mitochondrial deficits independent of aggregation in an *SNCA*-KO model (Denning et al. 2022). This is also the only study that has directly compared primary cultures from different brain regions from the same animal, where TH+ mouse primary neurons were more vulnerable to ASyn oligomers, paraquat, and rotenone, than cortical or hippocampal neurons. One of the studies with the longest incubation periods, 21 days, saw formation of LNs at 4 days, more LP-like pathology by 14 days, but much more somatic, nucleus adjacent, filamentous, ordered LB-like structures by 21 days, with characteristic sequestration of organelles and lipids (Mahul-Mellier et al. 2020).

One downside of the rodent model, other than lacking the human genetic context, is that the cultures only last 3-4 weeks (Mahul-Mellier et al. 2020; Volpicelli-Daley, Luk, and Lee 2014). This constrains the capacity for longer term observations, important considering how time dependent pathology appears to be. The use of hiPSC-derived neurons addresses both issues, and various studies have been conducted examining the effect of ASyn PFFs in these human cells.

1.6.3. ASyn PFFs in hiPSC-derived Neurons

A range of studies have examined various forms of cellular toxicity in hiPSC neurons. Some only examine functional deficits such as mitochondrial function, synapse loss, network

criticality state changes, and increased ROS, without observing (or examining) aggregate formation (Deas et al. 2016; Prots et al. 2018; Stephens et al. 2023; Valderhaug et al. 2021). Cascella et al. (2021) also note that the toxicity of PFFs is likely mediated by release of oligomers from these larger fibrils, and show uptake into SH-SY5Y and hiPSC mDANs. However, most hiPSC PFF studies observe seeded ASyn inclusions, which are often spatially and morphologically alike to LBs, with several studies conducting EM characterisation and examining hallmark markers too. These are detailed in Table 1.

Table 1.1. Summary of Studies Demonstrating Lewy-like Pathology in hiPSC-derived Neuronal Models with ASyn PFF Insults. Cell types and genotypes studied are reported under model, as noted in the study. Differentiation methods and markers used to characterise cells are also noted, with quantification or estimates where available. If cells were shown to be electrophysiologically active, this is noted as Ephys active. HC=Healthy control. GFG=Growth factor guided. Yellow rows indicate studies in CN models. Purple rows indicate studies in midbrain dopaminergic models.

Ref.	Model	Methods & Markers	Insult & Dosage	Time of Insult	Duration to Assay	Key Observations
Gao et al. (2019)	"Neural Stem Cells" HC (N=1)	GFG PAX6- Nestin- MAP2+ B3T+	PFFs 2-5 µg/mL	D7	3,6,9,12 days	• Transient, punctate, cytoplasmic inclusions, pASyn-S129+, p62+ • Peak at 6 days, dose dependent • pASyn-S129 sensitive to chloroquine and rapamycin and AMPK activators. • No toxicity at 5 µg/mL.
Bieri et al. (2019)	"Neurons" HC (N=1) LRRK2-G2019S (N=1) Isogenic Corr. (N=1) LRRK2-KO (N=1)	NGN2 Expression NeuN+ MAP2+ B3T+ Tau+ ASyn+ Synaptophysin+ PSD95+ Homer1+	PFFs 6.5 ug/mL	14-21 days	14 days	• Serpentine like pASyn-S129 labelling at 6-10 days in HCs • Aggregates colocalise with ubiquitin, SDS resistant • LRRK2-G2019S has more aggregation than isogenic control or LRRK2 KO • LRRK2 KO has less SNCA expression, less pASyn-S129 • G2019S neurons have 2-fold more pASyn-S129 aggregation after 3 weeks than isogenic controls • No loss of synaptic proteins
Gribaudo et al. (2019)	CNs in Microfluidics HC (N=2)	GFG (Nicoleau et al. 2013) B3T+ MAP2 (90%) GFAP (<3%) GABA (20%) VGlut1 (75%) ASyn+ Synaptophysin+ PSD95+ CUX2 (40%) BRN2 (~5%) TBR1 (~4%) CTIP2 (~4%)	PFFs (fibrils vs ribbons) 0.5 uM (~7 ug/mL)	D30	1,7,15,21 days Also 30 days for seeing inclusion s	• Uptake within 1 day by receptor mediated endocytosis • Time, dose, and conformer strain dependent Neuritic and LB-like aggregates after 30 days • Ribbons more potent than fibrils • Spread along cortico-cortico network in microfluidic chambers • mitochondrial fragmentation and Ca2+ oscillation frequency changes, related to neuronal dysfunction
Vajhøj et al. (2021)	CNs HC (N=1) with Dox-inducible SNCA-A53T	GFG (Shi-based) PAX6~ Ki67~ Nestin~ B3T+ MAP2+ Tau+ Doublecortin+ Synapsin I+	ASyn fibrils91	D46	14 days	• No increase in pASyn S129 intensity without A53T mutation • See dose dependent effect of Dox i.e. A53T endogenous ASyn on seeded aggregation

		GABA+ GFAP~ Ephys active, Glutamate responsive				
Kapucu et al. (2024)	CNs on MEA microfluidics HC (N=1)	GFG (Shi-based) B3T+ MAP2+ PSD95+ Synapsin I+ Synaptophysin+ vGLUT1+ Asyn+ Ephys Active	PFFs 10 ug/mL	DIV49 or 56	3,6,13 days	• Some small pASyn-S129 LN like structures , time dependent • Loss of presynaptic proteins at 3-6 days (synaptophysin+synapsin I) • Diminished neuronal activity • Diminished mitochondrial motility • No toxicity • Propagation between compartments
Lam et al. (2024)	CNs <i>SNCA</i> -Trip. (N=1) <i>SNCA</i> -A53T (N=1) Transgenic overexpressi on Isogenic & <i>SNCA</i> -KO controls	NGN2 Expression TUJ1+ MAP2+ vGLUT1+ CUX1+ TBR1-	PFFs 10 ug/mL	D11	7, 14, 21 days	• Inclusions mostly threadlike along neurites. • Neuritic and somatic inclusions in <i>SNCA</i>-Trip. > other cell lines • Inclusions at 14 days in A53T overexpressing line. • Triton insoluble, SDS soluble. • No clear perturbation of mitochondrial respiration, lysosomal flux • At 7 days, ~80% of somatic inclusions in <i>SNCA</i>-A53T were ubiquitin(+) (DIV18), whereas only a minority (~30%) were p62(+) • At 21 days, 80% of inclusions p62+ • Lipid/membrane rich somatic inclusions were more toxic
Kim et al. (2018)	mDANs HC (N=2) GBA-N370S (N=2 clones + 1)	Chambers et al. (2009) (Kriks- based) TH+ TUJ1 (80%) MAP2 (80%) FOXA2 (80%) NURR1 (60%) PITX3+ (70%) Ephys active	PFFs 5 ug/mL	DIV 60	10-14 days	• Increased pASyn-S129 intensity ~two-fold, dependent on endogenous free monomer • Two-week treatment induced neuronal toxicity by LDH and AlamarBlue
Jones- Tabah et al. (2024) Preprint	iPSC DANs HC (N=1) Isogenic <i>SNCA</i> KO	Embryoid body- based (Jefri et al. 2020)	PFFs Unknown	14 days	21-28 days	• PFF degradation is potentiated by cathepsin B activation and aggregation is enhanced by cathepsin B inhibition. • See pASyn inclusions after 3-4 weeks

	Isogenic CTSB KO					
Tanudjojo et al. (2021)	mDANs HC (N=3) SNCA-Trip. (N=3 clones) SNCA-A53T (N=2)	GFG (Kriks-based) TUJ1+ TH+ FOXA2+ Ephys Active	PFFs (Fibrils vs Ribbons) 1 uM (~15 ug/mL)	DIV45	7, 14, 21, 28 days	• Neuritic, speckled, and LB-like inclusions colocalised with p62, in HCs and SNCA-Trip. • Significant increase vs baseline in HCs and SNCA-Trip. • Significantly more pASyn-S129 in SNCA-Trip. vs HCs and SNCA-A53T, and in SNCA-A53T vs HC at 2 weeks • Time and dose dependent • Ribbons more potent than fibrils • DJ-1 KO increased aggregate induced toxicity • See neurotoxicity in SNCA-Trip. by week 2-3
Iannielli et al. (2022)	mDANs and CNs SNCA-Trip. isogenic correction series	mDAN: GFG (Kriks-based) (Kirkeby, Nelander, and Parmar 2013) MAP2+ TH+ (~70%) FOXA2+ (~75%) CX: GFG (None)	ASyn fibrils91 0.5 ug/mL	21 days	21 days	• In SNCA-Trip., 25% CNs vs 34% mDANs are pASyn-S129+ • In normal SNCA expressing lines, 3% CNs vs 8% mDANs are pASyn-S129+ (high variability) • No aggregation in SNCA-KO • Fibril density ~3x higher in mDANs • Aggregates were ~300 um diameter in CNs, ~600 um diameter in mDANs • Only see LBs in mDANs, not so much in SNCA-Trip, not so much at normal expression.
Bayati et al. (2023) (BioRxiv Preprint)	mDANs and CNs HCs (N=3)	mDAN: GFG (Jefri et al. 2020) MAP2+ TH+ DAT+ GIRK2+ TUJ1+ CX: GFG, Forced NGN2 Expression MAP2+ PSD95+	PFFs 1 ug/mL, 48 hrs + Immune co-insults: IFN γ IL-1 β Activated microglia	Unclear	2-14 days Up to 30 days for some mDANs	• Punctate, neuritic, and some LB-like pASyn-S129 • mDANs form PFF-seeded inclusions only with immune challenge (IFNγ or interleukin-1β, or coculture with activated microglia) • CNs from the same cell lines did not form inclusions • IFNγ exposure impairs autophagy, specifically lysosomes • EM characterisation of LB-like inclusions with mitochondria, filamentous ASyn, • Compact, filamentous, organelle filled, membrane enclosed inclusions by D30 • CNs have large dark lysosomes but fail to package these into LB-like inclusions • Use a SNCA-Trip. vs WT vs KO isogenic series to see that aggregation depends on SNCA expression, in mDANs • Basal LAMP1 and 2 expression levels were higher in CNs than

						mDANs, but are not abrogated by PFF+IFN γ like in mDANs • CellROX and MitoSOX show general oxidative stress levels are similar in CX and DA, but superoxide species were much higher in treated CX neurons • Higher lysosomal pH after PFF treatment and lower number of lysosomes
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Almost all of these studies utilise *SNCA*-A53T, *SNCA*-triplication, or other overexpression models, which are more aggregation prone (Baena-Montes, Avazzadeh, and Quinlan 2021; Oh 2019). This is likely for ease of obtaining results quickly. Indeed, most studies examine pathology within 2-3 weeks of seeding PFFs, and none more than 30 days. Interestingly, while some studies grow undefined or loosely characterised cell types, most studies examine CNs, with less examining mDANs, despite the relevance of this cell type to PD.

Two, however, are particularly notable for examining both CNs and mDANs from the same patient lines. Ianielli et al. (2022) use ASyn fibrils P91 and largely focus on mDANs from *SNCA*-triplication patients. In comparing to CNs in one experiment, they find that 25% of CNs but 34% of mDANs exhibited pathology three weeks after insult. mDANs exhibited a higher fibril density and much larger aggregates, with LB-like pathology emerging only in *SNCA*-triplication mDANs, and minimal LB formation in lines corrected to normal *SNCA* expression. A second study is detailed in a preprint from Bayati et al. (2023), where ASyn PFFs are delivered to healthy hiPSC-derived CNs and mDANs. Here, they found mDANs only developed PFF-seeded inclusions, including well-characterised LBs, following an immune challenge, while CNs did not form inclusions at all, and that the double insult ASyn PFF+IFN γ impaired autophagy selectively in mDANs.

These provide strong proof of principle for using hiPSC-neurons to examine selective vulnerability and strengthen the case for cell autonomous mechanisms such as autophagy.

However, these studies collectively leave much to be desired in examination of susceptibility to aggregation and other functional deficits in physiological, non-overexpressing models. The characterisation of the cells being studied is also often overlooked, especially when multiple cell types are being compared, though this is important for understanding what divergent phenotypes that might lead to their differential susceptibility. Finally, no studies consider ASyn aggregation across models from different diseases.

While several of the studies in rodent models and hiPSC-neurons note effects of PFFs on mitochondria and autophagy, little is known about how intrinsic cellular phenotypes in this regard might predispose cells to PFF vulnerability in different cell and disease contexts.

1.7. Autophagy as a Pathway of Interest Underlying PFF Vulnerability

1.7.1. Overview

Autophagy is crucial for breaking down and recycling waste and defective organelles, proteins, and other cellular cargos (Fleming et al. 2022; Pohl and Dikic 2019). As such, it is a critical pathway with regards to aggregate formation and has already noted as being potentially of interest to determining selective neuronal vulnerability. Autophagy is especially important in neurons, which, as post mitotic cells, rely on autophagy to clear waste and support normal neuronal function. Thus, they are highly vulnerable to deficits in autophagy (Cai and Ganesan 2022; Fleming et al. 2022; Nixon and Yang 2011; Sidibe, Vogel, and Maday 2022).

Autophagy includes several pathways. The most relevant of these, the core macroautophagy pathway (hereafter, referred to as autophagy), involves a complex and multistep process, reviewed in detail by several authors (Nixon and Yang 2011; Cai and Ganesan 2022; Fleming et al. 2022; Yamamoto, Zhang, and Mizushima 2023). Induction of autophagy is highly robust in neurons, and involves the recruitment of several key proteins to initiate formation of a phagophore. As the phagophore is elongated and enclosed around autophagic cargo for disposal, cytosolic LC3 (LC3-I) is recruited to the phagophore membrane and becomes conjugated to phosphatidylethanolamine (PE). LC3-PE (LC3-II) becomes a structural protein and thus marker of autophagosomes (Cai and Ganesan 2022). Autophagosomes produced distally are retrogradely transported towards the soma, where mature lysosomes are primarily located.

Lysosomes are cytoplasmic, membrane bound organelles containing hydrolytic enzymes that are active in the acidic conditions maintained in the lysosome (Cai and Ganesan 2022). In addition to pH regulation, lysosomes are also dependent on local Ca^{2+} release for fusion with autophagosomes, which allows the breakdown and clearance of autophagic cargos into the

autophagolysosome (R. Wallings, Connor-Robson, and Wade-Martins 2019; R. L. Wallings et al. 2019). Lysosomal function declines with age, and defects here or anywhere else along the potential points of failure mean that diverse aetiologies may converge on this system (Nixon and Yang 2011). Indeed, failures of autophagy seem to unify many neurodegenerative diseases that consequently feature the buildup of misfolded, aggregating proteins, including AD and PD (Ciechanover and Kwon 2015; Fleming et al. 2022).

1.7.2. Autophagy in AD

In AD, GWAS of patient populations and single cell analyses of vulnerable cell populations implicates many autophagy genes across diverse peoples (Gharesouran et al. 2014; Uddin et al. 2018; Szabo et al. 2022; Andrews et al. 2023; Mathys et al. 2023; Reitz et al. 2023; H. Yamamoto, Zhang, and Mizushima 2023). Complementing this, enlarged autophagic vacuoles (AVs, collectively autophagosomes and lysosomes) accumulate in *post-mortem* tissue and cell models (Cai and Ganesan 2022; Cataldo et al. 2000; Hung and Livesey 2018; Nixon 2013; Nixon et al. 2005; Udayar et al. 2022). A β and tau are also both autophagic substrates, and both are found in AVs, implicating autophagic deficits in their accumulation (Cai and Ganesan 2022; Terron et al. 2023; Tian et al. 2011).

Furthermore, lysosomal deficits and autophagic flux impairment are observed in AD vulnerable regions (Bordi et al. 2016; Cai and Ganesan 2022; He et al. 2020; Hung and Livesey 2018). Interestingly, enlarged AVs are evident in vulnerable neocortical pyramidal neurons during early-stage AD, when pathology remains restricted to the EC, suggesting these changes precede and are not just the result of pathological deposition (Cataldo et al. 2000; 1997). AD and other similar neurodegenerative conditions like PD have even been compared to lysosomal storage disorders, which cause severe neurodegeneration in infants, and often feature AD-like pathology, as a result of deficiency in lysosomal proteins (Lloyd-Evans and

Haslett 2016; Nixon 2013; Nixon and Yang 2011). Autophagy deficits are also observed in fAD (Cataldo et al. 2004; Hung and Livesey 2018), and PSEN1 is thought to important for lysosomal function and acidification (Chong et al. 2018; J.-H. Lee et al. 2015; 2010).

1.7.3. Autophagy in PD

Genes with alterations in fPD and other GWAS-identified risk genes similarly implicate autophagy in PD (Burbulla et al. 2019; Cai and Ganesan 2022; Coukos and Krainc 2024; Hou et al. 2020; Madureira, Connor-Robson, and Wade-Martins 2020; Nalls et al. 2019). Aberrant autophagy, including reduced autophagosome biogenesis, reduced lysosomal enzyme activity, and buildup of LC3-II+ autophagosomes is observed in PD brains and models, especially in nigral DA neurons (Nixon 2013; R. L. Wallings et al. 2019; Hou et al. 2020; Cai and Ganesan 2022; Udayar et al. 2022).

Autophagy is also particularly relevant in PD and to this thesis because it mediates clearance of accumulated ASyn (Cuervo et al. 2004; Desplats et al. 2009; MacIsaac et al. 2020), and ASyn accumulation itself can impair autophagy (Luna et al. 2018; Tanik et al. 2013). In hiPSC mDANs, ASyn impairs autophagy by disrupting lysosomal hydrolase trafficking, which can be restored by lysosomal enzyme activation (Mazzulli, Zunke, Isacson, et al. 2016a; Mazzulli, Zunke, Tsunemi, et al. 2016b; Stojkovska et al. 2021). Given the pronounced role of dysfunctional mitochondria in PD, especially in SNPC neurons, defects in autophagy leading to impaired mitophagy are also especially relevant. A review by Minakaki et al. (2020) highlights the convergence of ASyn, mitochondrial, and lysosomal pathways driving vulnerability in the SNPC mDANs.

1.7.4. Autophagy in hiPSC Models

Various studies have examined autophagy deficits in hiPSC models of AD and PD (Fernandes et al. 2016; Hung and Livesey 2018; Jiménez-Moreno et al. 2017). Some show that modulating autophagy in cell models, primary neurons, and hiPSC-neurons, either using small molecules or knock down of key genes such as Cathepsin B can ameliorate or exacerbate PFF-induced aggregation and dysfunction (Bayati et al. 2023; Gao et al. 2019; Israel et al. 2012; Jones-Tabah et al. 2024). Interestingly, several studies have also shown that constitutive perforation of lysosomal membranes leads to intracellular tau and ASyn pathology seeding (Polanco et al. 2021; Sanyal et al. 2024).

These studies collectively imply an important role for autophagy in AD and PD, both in disease and in cell models with PFF insults. Few, however, examine cell type-dependent basal differences in autophagy, or other proteostatic mechanisms, that might give rise to selective vulnerability.

1.8. Finding a Disease Model: Autosomal Dominant AD and PD

In examining the role of autophagy and other mechanisms in *in vitro* models, cell lines carrying genetic forms of disease are typically employed (Essayan-Perez et al. 2019; Jiménez-Moreno et al. 2017). While some deficits and pathology may be observed in sporadic cell lines, these typically require more statistical power than is practical to clearly discern effects of polygenic risk, absent any environmental factors that may contribute to the sporadic condition (C. Lee, Willerth, and Nygaard 2020). On the other hand, autosomal dominant forms of disease have, by definition, cell-autonomous mechanisms driving dysfunction, that can be directly examined and corrected *in vitro*.

In this thesis, I employ fAD cell lines with PSEN1 mutations, and fPD cell lines with LRRK2 mutations, both of which are known to drive autophagy deficits, but have not been previously examined under a differential vulnerability paradigm. Phenotypes associated with mutations in these two proteins are reviewed here.

1.8.1. Autosomal Dominant AD: PSEN1

1.8.1.1. Overview

To model AD, in this thesis, I examine cell lines with mutations in presenilin-1 (PSEN1), since these are the most common cause of autosomal dominant fAD and are associated with autophagic deficits (Kelleher and Shen 2017). PSEN1 is a widely expressed transmembrane protein that is, most famously, the catalytic subunit of the gamma secretase enzyme, which cleaves APP to produce A β peptides (De Strooper et al. 1998; Selkoe and Hardy 2016). Over 100 mutations in PSEN1 can cause fAD, as well as mutations in APP and PSEN2, many of which are amyloidogenic, in that they lead to an increase in the ratio of A β 42:40, with the longer peptides thought to be more hydrophobic and aggregation prone (Essayan-Perez et al. 2019;

Hardy and Selkoe 2002; Kelleher and Shen 2017; C. Lee, Willerth, and Nygaard 2020; Muratore et al. 2014). Where mutations do not produce a higher A β 42:40, some have argued that these fAD causing mutations are not solely amyloidogenic (Kelleher and Shen 2017; Shen and Kelleher 2007; Sun et al. 2017). However, others have shown that these mutations can lead to an elevation of A β 43, another aggregation prone peptide, supporting the amyloid hypothesis (Veugelen et al. 2016; Szaruga et al. 2017).

Nevertheless, beyond APP processing, PSEN1 has also been shown to be essential for learning and memory, and PSEN1-KO leads to neurodegeneration of hippocampal and CNs in rodents (Kang and Shen 2020; Saura et al. 2004; Wines-Samuelson et al. 2010). Other studies have proposed a role for presenilins in lysosomal acidification and as ER Ca²⁺ leak channels, with fAD mutations altering this function (Bezprozvanny 2013; Lee et al. 2010; Mustaly-Kalimi et al. 2022; Nelson et al. 2010; 2007). This has led to two non-exclusive views of PSEN1 mutations - that they lead to amyloidogenic toxic gain of function, and to neurodegenerative loss of function (Kelleher and Shen 2017; Selkoe and Hardy 2016)

PSEN1 fAD cases have a similar clinical presentation to sAD patients, including disease progression rate, biomarkers, clinical outcomes, and pathology. However, while sAD has a mean age of onset of 80 years, fAD cases are much earlier onset, typically around age 45 (Masters et al. 2015; N. S. Ryan et al. 2016). It is unclear if sAD stems from exactly the same aetiology as fAD, though there is undoubtedly some overlap (Foveau et al. 2019). Various transgenic rodent models have been made with APP/PSEN1 mutations, and these develop extensive plaque and other amyloid pathology (Drummond and Wisniewski 2017; Zhong et al. 2024). Rodent primary neurons with these fAD mutations also expressed altered A β ratios, are more vulnerable to A β aggregates, and have more oxidative stress than controls (Abdul et al. 2006).

1.8.1.2. *PSEN1 in hiPSC Studies*

Yagi et al. (2011) were among the first to show aberrant A β 42 in hiPSC-derived neurons with PSEN1/2 mutations. Since then, many more studies have demonstrated increased A β 42:40 in hiPSC-derived neurons from patients with a variety of PSEN1 mutations, typically by relatively long timepoints (D70-100) (Arber et al. 2020; 2019; Armijo et al. 2017; Essayan-Perez et al. 2019; Hung and Livesey 2018; Kondo et al. 2017; Q. Liu et al. 2014; Moore et al. 2015; Ochalek et al. 2017; Sproul et al. 2014; J. Yang et al. 2016). Many studies also show increased 42:40 levels in APP mutant lines, typically about 2-3x from D70-100 (Arber et al. 2020, 201; Foveau et al. 2019; Kondo et al. 2017; 2013; Moore et al. 2015; Muratore et al. 2014). Some studies even observe elevated pTau in PSEN1 neurons (Israel et al. 2012; Ochalek et al. 2017; Raja et al. 2016; J. Yang et al. 2016, 201), and APP mutant neurons (Israel et al. 2012; Muratore et al. 2017; 2014; Raja et al. 2016).

1.8.1.3. *PSEN1 in Autophagy*

PSEN1 and APP models also display autophagy deficits, and present evidence for a role for PSEN1 in lysosomal function. Enlarged early endosomes were observed across a large panel of fAD hiPSC-neurons (Kwart et al. 2019). This was also observed in APP, but not PSEN1 hiPSC-neurons by Hung and Livesey (2018), who also found lysosomal number and size was elevated in several APP and PSEN1 lines, and cathepsin D, a lysosomal enzyme, was diminished. Enlarged autophagosomes and impaired autophagic flux was also observed early in neurons, suggesting they were a primary effect of the mutation, and not a downstream effect of protein aggregation.

Supporting this, PSEN1-KO leads to accumulation of AVs and impaired flux in mice neurons (J.-H. Lee et al. 2010; Nixon and Yang 2011), implying a direct and underappreciated role for the protein in autophagy function. Lee et al. (2010) found that the targeting of the vATPase V0a1 subunit from the endoplasmic reticulum (ER) to the lysosome requires PSEN1, and that failure

of this results in alkalinisation and reduced proteolysis. However, this is contested by Zhang et al. (2012), who find no effect of PSEN1 on lysosomal function, cathepsins, vATPase trafficking, or expression of autophagy genes in HEK cells or murine fibroblasts or neurons. However, PSEN1-KO in mice did alter genes involved in lysosomal biogenesis.

More recent evidence from hiPSC neurons, and mice, however, backs up the idea that the folding in the ER of the V0a1 subunit of the vATPase lysosomal acidification pump, and its subsequent trafficking and assembly at the lysosome is regulated by ER-localised PSEN1 (Mustaly-Kalimi et al. 2022). Here, an A246E mutation altered PSEN1 function to increase ER Ca^{2+} release and reduce expression of the V0a1 subunit, leading to reduced acidification and degradative capacity at the lysosome. This was rescued by Ryanodex treatment, which restored ER Ca^{2+} levels and autophagic function.

This is consistent with the theory that presenilins function as low conductance Ca^{2+} leak channels in the ER, and this function is disrupted by some fAD mutations, leading to elevated ER Ca^{2+} and excessive release (Bezprozvanny 2013; O. Nelson et al. 2010; 2007). Chong et al. (2018) additionally show that PSEN1-KO in hiPSC neurons leads to suppressed autophagy through reduced autophagosome formation and lowered expression of autophagy related genes.

As such, there is some overlap between autosomal dominant mutation driven fAD and sAD in terms of pathology and dysregulated autophagy. While PSEN1 cell lines have been extensively examined as hiPSC-derived CNs, almost none compare these phenotypes to other cell types, so there is ample opportunity to examine cell type-specific deficits in autophagic function that might lead to selective vulnerability.

Since there are many mutations associated with PSEN1, I employ a range of these, in addition to one APP patient line. Known phenotypes associated with the specific mutations examined in this thesis are summarised in Table 1.2.

Table 1.2. AD PSEN1 and APP Mutations and Associated Phenotypes. The characteristics of mutations causing fAD in patient cell lines employed in this thesis are summarised here, including age of onset noted in different studies, associated neuropathology, and phenotypes observed in other studies employing hiPSC-derived neurons. Shading to contrast rows.

Mutation	Age of Onset	Remarks	Neuropathology	iPSC Neuron Data (CNs, unless otherwise noted)
APP-V717I	• 57 (Goate et al. 1991) • 55 (G. Zhang et al. 2017) • 52 (N. S. Ryan et al. 2016)	• One of the most common APP mutations (De Jonghe et al. 2001)	• AD pathology, CAA, cortical and brainstem LBs (Lloyd et al. 2020) • No LB pathology in a different family (Goate et al. 1991) • Frontal cortical neurodegeneration (Marinero et al. 2020)	• Increased pTau (Moore et al. 2015; Muratore et al. 2014) • Increased Aβ42:40 (Arber et al. 2020; Muratore et al. 2014) • Impaired autophagic flux, lysosomal function (Hung and Livesey 2018)
PSEN1-M146I	• 48 (N. S. Ryan et al. 2016)	• Variably altered PSEN1 levels may suggest altered protein stability (Arber et al. 2020)	• Frontal cortical neurodegeneration (Marinero et al. 2020) • Typical AD pathology, CAA (Willumsen et al. 2023)	• Elevated Aβ42:40 (Moore et al. 2015; Whiten et al. 2020; Willumsen et al. 2023) • Chronic TNF exposure leads to Aβ and ASyn-containing extracellular aggregates (Whiten et al. 2020) • Impaired autophagic flux, lysosomal function (Hung and Livesey 2018) • Premature neurogenesis (Arber et al. 2021)
PSEN1-A79V	• 75 (Day et al. 2016) • 60 (Petit et al. 2022)	• Presents similarly to sAD (Day et al. 2016)	• Cortical AD pathology, CAA, SNPC LBs in 1/5 cases (Day et al. 2016)	• No effect on ER Ca²⁺ leak channel function in mice primary neurons (O. Nelson et al. 2007)
PSEN1-M139V	• 40 (Petit et al. 2022; N. S. Ryan et al. 2016)	• Mutational hotspot in area of the protein likely of functional importance (Larner and Du Plessis 2003)	• In one case study, extensive cortical AD pathology, sparse Lewy-like pathology in the amygdala only (Larner and Du Plessis 2003)	• Elevated Aβ42:40 (Arber et al. 2020; O. Nelson et al. 2010; Petit et al. 2022) • Impaired ER Ca²⁺ leak function (O. Nelson et al. 2010)
PSEN1-E280A	• 48 (Petit et al. 2022)	• The most common PSEN1 mutation, near 100% penetrance (Fuller et al. 2019; Reitz et al. 2023)	• Typical AD pathology, cortical thinning, neuronal hyperactivity (Fleisher et al. 2015; Fuller et al. 2019)	• pTau, oxidative stress, apoptosis, Ca²⁺ dysregulation in cholinergic-like neurons (Soto-Mercado et al. 2020) • pTau and mitochondrial membrane impairment in cholinergic neurons (Mendivil-Perez et al. 2023)

PSEN1-Intron 4 Deletion (Int4Del)	• 37 (Janssen et al. 2000) • 38 (N. S. Ryan et al. 2016)	• Produces three alternately spiced transcripts. Two are truncations, with part or all of exon 4 deleted. Third is a threonine insertion, and responsible for elevated Aβ42 (De Jonghe et al. 1999) • Relatively severe reduction in gamma secretase activity, located near substate docking domain (Arber et al. 2020)	• Frontal cortical neurodegeneration (Marinero et al. 2020) • AD pathology in cortex, NBM, and HC, depigmented LC and SNPC, brainstem NFTs, brainstem and cerebellum spared of atrophy. Some LB pathology in SNPC, LC, and cortex of one case (Janssen et al. 2000) • Increased Aβ42:40 in brain homogenates (Janssen et al. 2000)	• Allelic series from KO to homozygous gets 'dose' response increase in Aβ peptide length ratios (Arber et al. 2019) • Elevated Aβ42:40 (Arber et al. 2020; Moore et al. 2015; Whiten et al. 2020) • No elevation in pTau (Moore et al. 2015) • Chronic TNF exposure leads to Aβ and ASyn-containing extracellular aggregates (Whiten et al. 2020) • Autophagic flux and lysosomal function not impaired (Hung and Livesey 2018)
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1.8.2. Autosomal Domainant PD: LRRK2

1.8.2.1. Overview and Functions

To model PD, in this thesis, I examine cell lines with mutations in leucine rich repeat kinase 2 (*LRRK2*), since this is the most common cause of fPD, implicated in sPD, and mutations are associated with autophagic deficits (Paisán-Ruiz, Lewis, and Singleton 2013). *LRRK2* is mainly expressed in glial and immune cells (Schapansky et al. 2018) although it is ubiquitous and relatively abundant in the brain, especially in striatal and cortical regions (Madureira, Connor-Robson, and Wade-Martins 2020; Mancini et al. 2020; Melrose et al. 2007).

Mutations in *LRRK2* are implicated in as much as 40% of PD cases in some populations (Klein and Westenberger 2012), though are weakly penetrant (Paisán-Ruiz, Lewis, and Singleton 2013; Schapansky et al. 2018; Trinh et al. 2014). *LRRK2* polymorphisms are also associated with increased sPD risk (Heyne Lee et al. 2020; Nalls et al. 2019), and patients with *LRRK2* mutations have a clinical presentation indistinguishable from sPD, with similar age of onset, progression, and neuropathology (Madureira, Connor-Robson, and Wade-Martins 2020; Paisán-Ruiz, Lewis, and Singleton 2013; R. L. Wallings et al. 2019). As such, *LRRK2* models have the potential to reflect many pathways relevant to PD (Rocha et al. 2022).

LRRK2 is a large, multidomain protein, with a Roc-GTPase that is essential for the activation of its kinase domain (Outeiro et al. 2019; Paisán-Ruiz, Lewis, and Singleton 2013). It has been postulated to be monomeric as a cytosolic protein, and homodimeric as a membrane bound protein, where it autophosphorylates and becomes kinase active (James et al. 2012; Mancini et al. 2020). It has roles in a vast array of cellular functions, many of which are relevant to PD and neurodegenerative diseases (Madureira, Connor-Robson, and Wade-Martins 2020; Mancini et al. 2020; Myasnikov et al. 2021). These have been extensively reviewed, and include synaptic vesicle trafficking and dopamine homeostasis (Biskup et al. 2006; Pan et al. 2017; Maximilian Sloan, Alegre-Abarrategui, and Wade-Martins 2012), mitochondrial regulation (Mancini et al. 2020; Singh, Zhi, and Zhang 2019), regulation of synaptic and mitochondrial Ca^{2+} (Bedford et al. 2016; Mancini et al. 2020), autophagy (Giaime et al. 2017; Ramonet et al. 2011; R. L. Wallings et al. 2019), microtubule dynamics and neurite outgrowth (Civiero et al. 2018; MacLeod et al. 2006; Maximilian Sloan, Alegre-Abarrategui, and Wade-Martins 2012), and interactions with both tau and ASyn.

LRRK2 directly phosphorylates tau (Kawakami et al. 2012), and mutant or LRRK2 overexpression models often have tau accumulation (Henderson et al. 2019; Li et al. 2009; MacLeod et al. 2006; Melrose et al. 2010; Santpere and Ferrer 2009). LRRK2 has also been suggested to play a role in the phosphorylation and synaptic targeting of ASyn (Brzozowski et al. 2021; Mancini et al. 2020). Overexpression of LRRK2 exacerbates ASyn pathology (Lin et al. 2009), and it is found in as many as 77% of LBs (Alegre-Abarrategui et al. 2009; Perry et al. 2008; Vitte et al. 2010). LRRK2 is thought to have an inhibitory effect on autophagy induction, in addition to being implicated in lysosomal function and recruitment of autophagic proteins (Alegre-Abarrategui et al. 2009; Wallings et al. 2019). *LRRK2*-KO mice also display neurodegeneration in the SNPC and LC, with sparing of the cortex and cerebellum, and increased AVs (Giaime et al. 2017).

1.8.2.2. *LRRK2 Mutations*

The two most common *LRRK2* mutations are the G2019S and R1441C mutation. G2019S is a gain of function mutation which increases kinase activity and is the most common genetic determinant of PD (Bieri et al. 2019; Ferreira and Massano 2017; Madureira, Connor-Robson, and Wade-Martins 2020; Monfrini and Di Fonzo 2017; West et al. 2005). Increased *LRRK2* kinase activation is also seen in sPD, with kinase inhibitors thought to be protective (Bieri et al. 2019; Di Maio et al. 2018; Rocha et al. 2022). R1441C, which reduces GTPase activity, is the second most common, and thought to be more severe (Madureira, Connor-Robson, and Wade-Martins 2020; Monfrini and Di Fonzo 2017; R. L. Wallings et al. 2019; West et al. 2005).

Most *LRRK2* patients have typical PD LP, often with substantial cortical pathology (Kalia et al. 2015; Pouloupoulos et al. 2012). Some studies have found that many *LRRK2*-G2019S cases do not experience LB pathology - as many as 50% in Henderson et al (2019). This study only examined 10 brains however, with almost all cases still exhibiting at least some LB pathology in the midbrain, and brains without substantial LBs still exhibited other Lewy-like inclusions and pASyn-S129.

1.8.2.3. *LRRK2 in Autophagy*

LRRK2 plays a major role in several parts of the autophagy process (Madureira, Connor-Robson, and Wade-Martins 2020; Wallings et al. 2019). *LRRK2* mutations affect autophagic targeting of cargo at initiation (Kalogeropoulou et al. 2018) and impair autophagic flux in hiPSC mDANs and rodent models (Ramonet et al. 2011; Sánchez-Danés et al. 2012; Wallings, Connor-Robson, and Wade-Martins 2019). *LRRK2*-G2019S and R1441C are also associated with lysosomal alkalinisation, enlarged lysosomes, impaired degradation, increased lysosomal Ca^{2+} , and decreased Ca^{2+} release (essential for autolysosome fusion) in rodent primary neurons, human tissue and hiPSC mDANs (Obergasteiger et al. 2020; Sánchez-Danés et al. 2012; Schapansky et al. 2018; Wallings, Connor-Robson, and Wade-Martins 2019;

Wallings et al. 2019). Interestingly, aged rats bearing LRRK2-G2019S mutation show elevated LC3 puncta in SNPC mDANs, but not in CNs (Wallings, Connor-Robson, and Wade-Martins 2019), so these effects may be cell type dependent.

LRRK2's role in autophagy has important implication for ASyn aggregation, and other neuronal functions such as neurite outgrowth. In mice brains, primary neurons, and hiPSC neurons, LRRK2-G2019S leads to higher levels of pASyn-S129 following ASyn PFF treatment (Volpicelli-Daley et al. 2016; Y. Zhao et al. 2020), though Henderson et al. (2018) find little effect in mouse primary mDANs. Others, however, do find an effect of LRRK2-G2019S, compared to isogenic, WT, and KO controls in mice primary neurons and hiPSC neurons, with LRRK2 knockdown being protective in SH-SY5Y cells following PFF insult (Bieri et al. 2019; MacIsaac et al. 2020). This suggests a link between LRRK2 activity and pathology. Accordingly, *Snca*-A53T overexpressing mice crossed with *Lrrk2* overexpressing mice have synergistic pathology, whereas *Snca* overexpressing mice crossed with *Lrrk2*-KO mice have attenuated pathology (Lin et al. 2009).

Another autophagy dependent function regulated by LRRK2 is neurite outgrowth. LRRK2 overexpression impairs neurite outgrowth *in vivo* and in primary culture and is rescued by kinase inhibition (Civiero et al. 2018; Sloan, Alegre-Abarrategui, and Wade-Martins 2012). Similarly, Macleod et al. (2006) found abnormal elongation of processes in *Lrrk2*-KO cells, but ectopic expression of LRRK2-G2019S led to increased pTau, neurite retraction, reduction in branching, and associated lysosomal dysfunction, in rat primary cortical and midbrain cultures. LRRK2-R1441C also led to diminished process length, while a LRRK2-K1906M that reduces kinase activity increase process length.

A plethora of studies reviewed in Civiero et al. (2018) demonstrate, at varying expression levels, that LRRK2-G201S causes neurite shortening in SH-SY5Y, primary cortical hippocampal and midbrain cultures, and hiPSC mDAN neurons. In PC12 cells, LRRK2-G2019S also reduces

neurite length compared to WT LRRK2, with LRRK2-R1441C presenting an intermediate phenotype (Heo, Kim, and Seol 2010). BAC transgenic LRRK2-G2019S mice primary neurons had unaffected axons, but slower dendritic growth and arborisation (Dächsel et al. 2010; Sepulveda et al. 2013), while in the brain, these mice have reduced dentate gyrus neurogenesis, with new neurons growing reduced dendritic arbors with fewer spines (Winner et al. 2011).

Further studies have also linked autophagy deficits to neurite outgrowth impairment in differentiated SH-SY5Y cells transfected with LRRK2-G2019S compared to WT LRRK2 or kinase dead LRRK2 (Plowey et al. 2008), and in hiPSC mDANs with LRRK2-G2019S or sPD lines compared to isogenic or healthy controls (Reinhardt et al. 2013; Sánchez-Danés et al. 2012). These studies find reduced autophagic flux and increased autophagosomes is associated with neurite shortening, while Su and Qi (2013) rescued LRRK2-G2019S mitochondrial fission deficits to restore neurite outgrowth.

A complication of LRRK2 studies is the (over)expression model used in rodent models, and transgenic expression of human protein. Nevertheless, the wide array of animal and cell models employed with a range of different expression models all pointing in the same direction suggest a strong role for *LRRK2* mutations leading to impaired autophagic function, associated with ASyn accumulation and neurite shortening. Despite the range of studies, *LRRK2* mutants have never been examined in hiPSC CNs, an important comparator to mDANs in considering whether and how these cell types are differentially susceptible.

1.9. Aims

1.9.1. Overview

In this introduction, I have outlined a core issue in the understanding of neurodegenerative disease. This is that these progressive conditions with long prodromal periods have aetiologies inaccessible to observation or experimental intervention. This means that our knowledge of the origins of neurodegenerative diseases has largely depended on genetic associations, observations of end stage pathology and patterns of cell loss, and experimentation in rodent and cell models.

A striking phenomenon evident across these diseases, however, is the stereotypic spatiotemporal patterns in which neuronal dysfunction, pathology, and neurodegeneration arise in the brain. This phenomenon of selective neuronal vulnerability offers the opportunity to compare the differential vulnerability and resilience of populations of cells in the brain, variously affected by disease. In doing so, an understanding of how the underlying phenotypes of these cells differ, and how these differences give rise to their selective vulnerability or resilience to disease, should help to elucidate the fundamental disease mechanisms that lead to dysfunction, pathology, and death, and identify pathways for therapeutic intervention.

Examinations of selectively vulnerable and resilient populations in AD and PD note that SNPC mDANs are routinely vulnerable to dysfunction, ASyn pathology, and degeneration in PD, but are largely spared in AD. By contrast, large excitatory cortical and hippocampal pyramidal neurons, especially in more superficial layers, are highly vulnerable in AD to amyloid and tau pathology, and even LB pathology in many cases, though these are relatively resilient in PD.

Comparison of the phenotypic differences between these vulnerable and resilient cells suggests a role for both cell non-autonomous and cell autonomous pathways, such as proteostatic qualities intrinsic to the cell type, that might underlie this differential vulnerability

and thus drive disease. However, most previous studies of selective vulnerability have been *postmortem* examinations, without an ability to examine early stage live cell functioning, and without an ability to distinguish cause and correlation. Furthermore, despite remarkable overlap and parallels in disease processes and pathology, few studies examine AD and PD in comparison, and no studies also consider why cells which are vulnerable in one disease context may be resilient in another.

In this thesis, I therefore set out to investigate selective neuronal vulnerability in live human stem cell-derived neuronal models from AD and PD patients. While there is some proof of concept for this in the literature for AD, PD, and ALS, no studies examine multiple diseases in comparison, and very few examine differential susceptibility to protein aggregation. PFF models have been employed to model aggregation and examine pathways modulating inclusion formation in rodent, cell, and hiPSC models, but few have studied how the different intrinsic cellular phenotypes of different cell types might affect the response to PFF insult. Similarly, while autophagy is known to play a substantial role in mediating the clearance of A β and other neurodegenerative disease-related aggregates, and autophagic deficits have been investigated in both AD APP, AD PSEN1, and PD LRRK2 models, no studies examine whether or how these deficits differ between cell types.

Given these gaps in understanding and the experimental opportunities offered by the hiPSC-neuron model for studying cell autonomous drivers of selective neuronal vulnerability, the experimental aims of this project and structure of the remainder of the thesis are thus outlined below. Figure 1.1 presents a schematic of the broad experimental paradigm, and a vulnerability matrix depicting the expected vulnerability of different cell and genotype combinations.

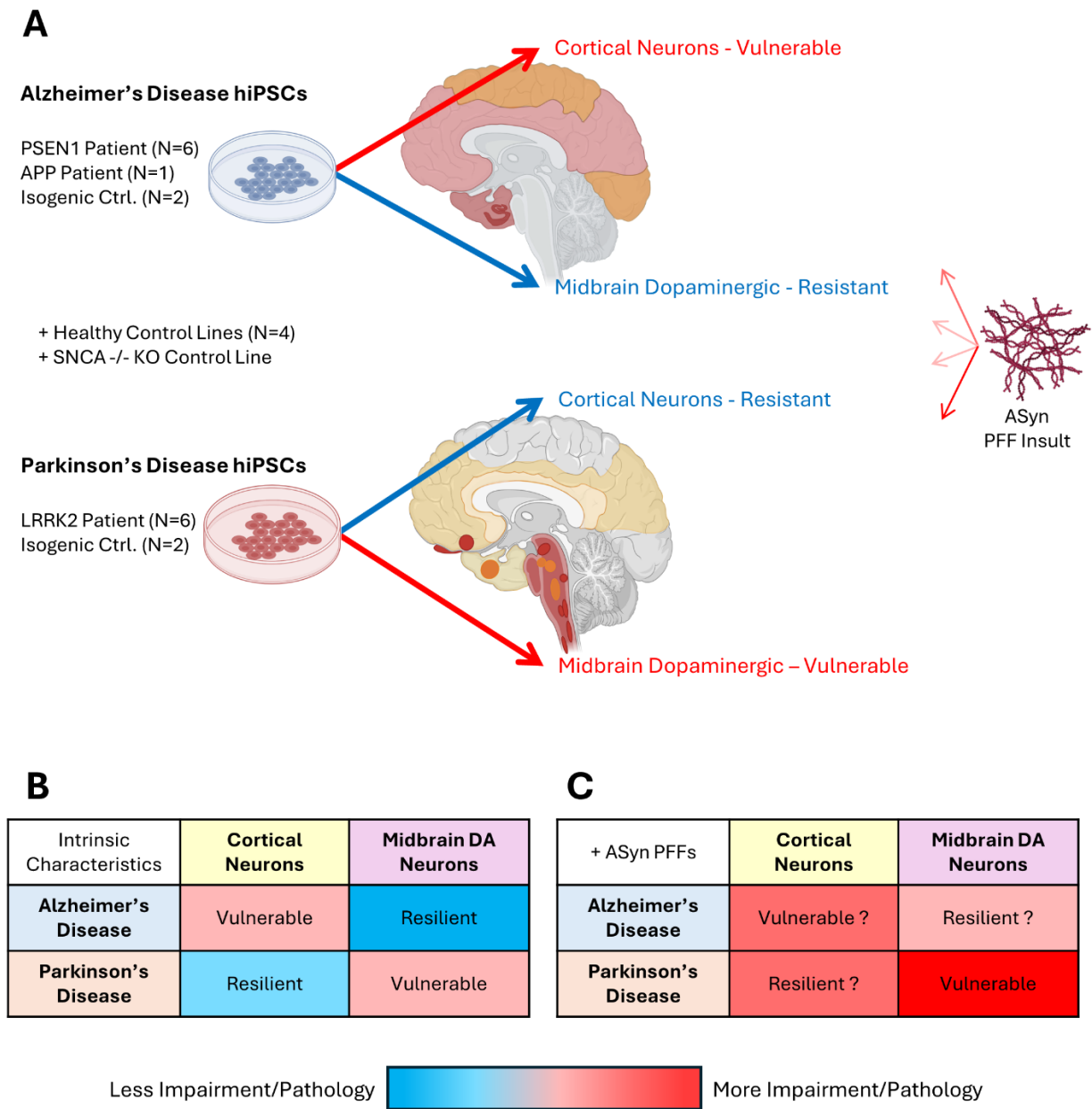


Figure 1.1. Experimental Design and Expected Vulnerability Hypotheses Schematic. (A) Schematic for overall chiastic study design, depicting differentiation of AD and PD hiPSCs into CNs and mDANs, each oppositely vulnerable in each disease. A PFF insult is applied to each of these four conditions, as well as healthy and corrected controls (not depicted). (B-C) Vulnerability matrices depicting expected relative vulnerability of each cell type under each disease condition. (B) The hypothesised relative vulnerability to intrinsic cellular dysfunction under basal conditions. (C) The hypothesised response to ASyn PFF insult.

1.9.2. Aims & Outcomes

Aim 1: Differentiate hiPSCs from healthy individuals and patients with different disease genetics into differentially vulnerable neuronal types (Chapter 2).

In Chapter 2, I detail 22 cell lines that will be employed in this thesis, including cells from healthy controls, AD PSEN1, and PD LRRK2 patients. I also detail methods used to differentiate these hiPSCs into comparable cultures of cortical glutamatergic neurons, and midbrain dopaminergic neurons, characterise the resultant neurons, and develop a rationale for the time course of PFF insult studies.

Aim 2: Examine whether hiPSC-derived neurons reflect the differential vulnerability to protein aggregate pathology of cells and genotypes in the brain (Chapters 3-4, 6).

In Chapters 3 and 4, I implement ASyn PFF insult regimes in cortical (Chapter 3) and midbrain dopaminergic (Chapter 4) neurons and aim to examine the degree to which they are vulnerable to developing pASyn-S129+ aggregates. Using a time course longer than any other in the literature, I find that both CNs and mDANs develop Lewy-like pathology with striking morphological similarity to that observed in the brains of synucleinopathy patients. In CNs, low levels of aggregation dependent on endogenous ASyn were present, except in PSEN1-Int4Del cell lines, which were highly vulnerable to aggregation. In mDANs, all cell lines, regardless of genotype, were highly vulnerable to pASyn-S129 accumulation and Lewy-like inclusion formation, especially in TH+ neurons. PFF treatment also had an impact on cell and nuclear morphology.

In Chapter 6, I focus on CNs, and examine how ASyn PFFs and disease genotypes impair other aspects of cellular function, beyond propensity to develop aggregate pathology, such as synaptic density and neurite outgrowth. I find that PFF insult leads to a severe decline in synaptic density, with some selectivity towards loss of presynaptic puncta, across genotypes. Neurite outgrowth was unimpaired in CNs by the various disease-related mutations, but AD-

PSEN1 and PD-LRRK2-G2019S cell lines were typically impaired in neurite outgrowth by ASyn PFF insult, while healthy, corrected controls, and LRRK2-R1441C CNs remained resilient.

Aim 3: Examine pathways underlying observed differences in vulnerability to ASyn PFF insult, such as differences in proteostasis and autophagy (Chapter 5).

In Chapter 5, I directly compare the aggregate pathology observed in CNs and mDANs in Chapters 3-4, finding much higher levels of accumulation in mDANs, but a striking effect of the PSEN1-Int4Del mutation in CNs that is absent in mDANs. To investigate the differences in vulnerability between cell types and genotypes, I focus on the PSEN1-Int4Del cell lines, and find that while mDANs do not express substantially higher levels of endogenous ASyn at the point of PFF insult, mDAN cultures are denser, and cell density has a dose dependent effect on aggregation propensity. To examine the specific vulnerability of the PSEN1-Int4Del CNs, I use live cell lysosomal and Ca^{2+} assays to show that PSEN1-Int4Del CNs have impaired autophagic flux, a buildup of LAMP1+ lysosomes, and excessive lysosomal and ER Ca^{2+} release in a cell type dependent manner, since mDANs from the same cell lines do not exhibit these deficits.

Summary

Collectively, these experiments demonstrate (1) that hiPSC-derived neurons have cell type-dependent phenotypes, which (2) are associated with differential vulnerability to disease-relevant pathology and (3) reflect cell and genotype-based differences in vulnerability that are observed in the brain. This highlights the role of cell autonomous mechanisms leading to disease, and the utility of the hiPSC model system to investigate these. The PFF insult paradigm employed here is the first to demonstrate robust ASyn inclusions in hiPSC neuronal models without ASyn overexpression, mutation, or additional insults. These are also the first experiments to examine the effect of ASyn PFFs in AD cell models, and to demonstrate severe pathology in PSEN1-Int4Del, adding to our understanding of the role of PSEN1 outside of

amyloid processing. This is also the first study to examine AD cell lines as mDANs, underscoring the intrinsic vulnerability of this cell type, and the first to examine neurite outgrowth in response to Asyn PFFs in PD hiPSC-derived CNs, emphasising the relative resilience of this cell model. In sum, these experiments emphasise the value of examining multiple cell types and disease models in tandem, and further validate the hiPSC-neuronal model and framework of selective neuronal vulnerability as a means of insight into the fundamental nature of neurodegenerative diseases.

Chapter 2.

Materials, Methods, & Characterisation

2.1. Introduction

In this chapter, I outline the cell lines used in this thesis, and the differentiation protocols used to generate cortical neurons (CNs) and midbrain dopaminergic neurons (mDANs) for ASyn PFF insult. I also detail the immunocytochemical (ICC) and immunoblotting methods used to characterise these cells and their response to PFF insult in the coming chapters.

In this thesis, I use differentiation protocols adapted from previous studies with modifications to make the two more analogous and comparable. Specifically, primary rat cortical astrocytes (rAstros) are grown in coculture with CNs as a technical aid, to enhance the maturation and synaptic development of the cultures, and to assist in maintaining adherence and morphology (Das et al. 2024; Hedegaard et al. 2020; Ng et al. 2022). These were thus added to the mDAN differentiation protocol too.

It is important that the cellular identities and functional characteristics of these cells are well understood for the purposes of studying selective neuronal vulnerability. This has often been overlooked in previous studies.

I thus aim to:

- 1) Confirm cortical and midbrain dopaminergic identities of the two cell models, and ensure the coculture with rAstros does not compromise mDAN differentiation,
- 2) Confirm that the neurons express ASyn and are electrophysiologically active,
- 3) Establish that the CN and mDAN models are appropriate and comparable for examining selective neuronal vulnerability under the PFF insult paradigm, and
- 4) Develop an empirical rationale for the timepoints of PFF insult in the CN and mDAN models, to be employed in subsequent chapters.

2.2. Materials & Methods

2.2.1. Reagents & Equipment

General reagents and chemicals used in this thesis are listed in Table 2.1, or with specific media compositions. All notable equipment used in this thesis are listed in Table 2.2.

Table 2.1. General Reagents.

Name	Supplier
Matrigel	Scientific Laboratory Supplies
Geltrex	Life Technologies
Poly-L-Ornithine	Merck
Poly-D-Lysine	Merck
Poly-L-Lysine	Merck
Laminin	Merck
Biolaminin	Biolamina
Fibronectin Bovine Plasma	Merck
mTeSR	STEMCELL Technologies
PBS	Thermo
dPBS++	Thermo
dPBS--	Thermo
EDTA	Thermo
TrypLE™ Express Enzyme	Thermo
Accutase®	Thermo
Dispase®	Merck
Y-27632 (Rock Inhibitor)	Tocris
FGF2	Merck
Puromycin	Sigma-Aldrich (Merck)
AraC	Sigma-Aldrich (Merck)
Mitomycin C	Abcam
Chloroquine	Merck
Lipofectamine™	Thermo
P3000	Thermo
Opti-MEM™	Thermo
BCA Reagents	Thermo
Pierce TM Bicinchoninic Acid (BCA) Protein Assay Kit	Thermo
MagicMark™ Protein Standard	Thermo
BLUeye Prestained Protein Marker	Jena Bioscience
Skim Milk Powder	Millipore
Immobilon® Western Chemiluminescent HRP Substrate	Millipore
DAPI	Insight biotechnology
NaCl	Merck
Tween-20	Merck
Saponin	Merck
Hydrochloric Acid	Merck

Table 2.2. Notable Equipment.

Name	Supplier
Costar 6 Well Clear TC-Treated Plates	Scientific Laboratory Supplies
Costar 12 Well Clear TC-Treated Plates	Scientific Laboratory Supplies
96 Well Half-Area Tissue Culture Treated Plates	Greiner Bio-One
T75 Flasks	Scientific Laboratory Supplies
Polyethersulfone 0.22 um pore membrane	Millipore
Cellulose acetate membrane 0.45 um pore	Millipore
Rotana 460R Centrifuge	VWR
Centrifuge 5424 R	Eppendorf
HeraCell 240i CO2 Incubator	Thermo
PHERastar FSX	BMG Labtech
T100™ Thermal Cycler	BioRad
Orion Star A111 pH Meter	Thermo
Trans-Blot® Turbo™ Transfer System	BioRad
ChemiDoc™ MP Imaging System	BioRad
MEA Maestro Pro™	Axion Biosciences

2.2.2. Cell Lines

Healthy control (HC), PD LRRK2 patient, and *SNCA*-KO hiPSC lines were obtained through Oxford STEMbancc. AD PSEN1-M146I and APP-V717I patient lines were obtained from Colin Ackerman and PSEN1-Int4Del patient lines from Selina Wray and Charlie Arber (Arber et al. 2019). Additional PSEN1 cell lines were obtained from the iNDI consortium/Jackson Laboratories. These are introduced point mutations on the HC background, KOLF2.1J (Pantazis et al. 2022). Lines and patient characteristics are listed in Table 2.3.

All hiPSC lines are regularly karyotyped, mycoplasma tested, and SNP tested, and have been well characterised as noted in Table 2.3.

2.2.3. hiPSC Culture

iPSCs were grown on Matrigel or Geltrex®, fed with mTeSR media with 1% penicillin/streptomycin (P/S), changed daily, and colony passaged using EDTA once approaching confluence. All cell cultures were maintained in a humidified incubator at 37°C with 5% CO₂.

Table 2.3. Cell Lines Employed in This Thesis. HC=Healthy Control. RV=Retrovirus. AD-Ind.= Alzheimer's Disease mutation introduced onto healthy background. Corr.=Isogenic Corrected Control. NA = Not Available. ApoE genotypes have been collated from cited literature or provided by Charmaine Lang, obtained from OPDC Neurochip screening.

Disease Status	Mutation	Sex	Age at Biopsy	ApoE Type	Source	Vector	Line Name	Reference
HC	-		55-57	3/3	iNDI / JAX	Sendai	KOLF2.1J	(Pantazis et al. 2022)
HC	-	M	65	NA	StemBancc	Sendai	SFC065-03-03	
HC	-	M	72	NA	StemBancc	Sendai	SFC067-03-01	(Lang et al. 2019)
HC	-	M	75	NA	StemBancc	Sendai	SFC156-03-01	(Lang et al. 2019)
HC	-	F	78	3/4	StemBancc	Sendai	SFC856-03-04	(Haenseler et al. 2017)
HC	SNCA-KO	F	75-79	3/4	StemBancc	-	SFC856-03-04-12E	
AD	APP-V717I	M	58	4/4	StemBancc	Sendai	SFC809-03-04	(Arber et al. 2020)
AD	PSEN1-M146I	F	38	3/3	StemBancc	Sendai	SFC803-01-01	(Arber et al. 2020)
AD	PSEN1-Int4Del	F	47	3/3	StemBancc	Sendai	SFC808-03-06	(Arber et al. 2019)
AD	PSEN1-Int4Del	F	47	3/3	StemBancc	Sendai	SFC808-03-04	(Arber et al. 2019)
AD-Corr.	PSEN1-Int4Del Corr.	F	47	3/3	StemBancc	-	SFC808-03-04-WT	(Arber et al. 2019)
AD-Ind.	PSEN1-A79V		55-57	3/3	iNDI / JAX	-	KOLF2.1J-B05	(Pantazis et al. 2022)
AD-Ind.	PSEN1-M139V		55-57	3/3	iNDI / JAX	-	KOLF2.1J-Het2	(Pantazis et al. 2022)
AD-Ind.	PSEN1-E280A		55-57	3/3	iNDI / JAX	-	KOLF2.1J-B10	(Pantazis et al. 2022)
PD	LRRK2-G2019S	M	49	2/3	StemBancc	RV	JR036-1	(Connor-Robson et al. 2019)
PD	LRRK2-G2019S	M	57	2/3	StemBancc	Sendai	SFC855-03-06	(Connor-Robson et al. 2019)
PD	LRRK2-G2019S	F	77	3/4	StemBancc	Sendai	SFC832-03-06	(Connor-Robson et al. 2019)
PD-Corr.	LRRK2-G2019S Corr.	F	77	3/4	StemBancc	-	SFC832-03-06-C47	
PD	LRRK2-R1441C	F	71	3/3	StemBancc	RV	JR207-03	(Connor-Robson et al. 2019)
PD	LRRK2-R1441C	M	32	NA	StemBancc	Sendai	SFC870-03-05	(Connor-Robson et al. 2019)
PD	LRRK2-R1441C	F	56	NA	StemBancc	Sendai	SFC869-03-01	(Connor-Robson et al. 2019)
PD-Corr.	LRR2-R1441C Corr.	F	56	NA	StemBancc	-	SFC869-03-01-A7	

2.2.4. Lentiviral Generation

Lentiviral particles for cortical differentiation were produced as described previously (Ng et al. 2024). Briefly, HEK293T cells were grown in virus generation media (Table 2.4.), passaged with TrypLE™, and were seeded at 80,000 cells/cm² per T75 flask (1,000,000 cells/flask). 24 hours later, Lipofectamine™ 3000 (40 µL) was mixed with P3000 (50 µL) in Opti-MEM™ (1 mL) for minimum 5 minutes. Separately, both packaging plasmids were mixed with one of the other plasmids (rtTA, NGN2, or NGN2-GFP; one per flask) in an equimolar ratio in 1 mL Opti-MEM™ media. Plasmids are listed in Table 2.5. Plasmids were then added to the Lipofectamine™ dropwise for a total volume of 2 mL, and then this mixture was added to the HEK cells, now growing in virus generation media (13 mL) without P/S.

Table 2.4. Lentiviral Generation Media.

Virus Generation Media	Concentration	Supplier
DMEM/F12	Base	Thermo
FBS	10%	Thermo
L-Glutamine	2 mM	Thermo
NEAA	100 uM	Thermo
Penicillin-Streptomycin	50 U/mL	Thermo

Plasmids were incubated overnight before changing to fresh, regular P/S-containing media the next day. 48 hours after this media change, media containing lentivirus was collected, spun at 400g for 5 minutes to remove any cells, filtered through a cellulose acetate membrane (0.45 µm pore size), snap-frozen on dry ice, and stored at -80°C.

Table 2.5. Plasmids Used for NGN2 Differentiation.

Plasmid Name	Addgene Code	Mass	Function
psPAX2	#12260	10 ug	Second generation lentiviral packaging plasmid
pMD2.G	#12259	5.65 ug	Second generation lentiviral packaging plasmid
rtTA (FUdeltaGW-rtTA)	#19780	9.6 ug	Express Transactivator
Ngn2 (pTet-O-Ngn2-puro)	#52047	9.6 ug	Doxycycline Inducible Expression of NGN2
Ngn2-GFP (pLV-TetO-hNGN2-eGFP-Puro)	#79823	9.6 ug	Doxycycline Inducible Expression of GFP

2.2.5. CN Differentiation

For differentiation of CNs, a previously reported protocol (Ng et al. 2024; Hedegaard et al. 2020) was employed with some modifications. This protocol combines the small molecule/growth factor guided approach based on Shi, Kirwan, and Livesey (2012) with transcription factor directed differentiation as reported in Zhang et al. (2013) as outlined in Figure 2.1, to achieve a highly replicable, scalable, and relatively pure glutamatergic cortical phenotype across all lines. Media formulations used are detailed in Table 2.6.

iPSCs were passaged onto Geltrex™-coated 6-well plates and, once confluent, were washed in PBS and then media changed to NIM. This was D0, and media was changed daily for dual SMAD inhibition. On D12, cells were passaged with 1 mg/mL Dispase® (added to culture media), leading to a sheet of cells detaching. This was lightly triturated and transferred to a tube with 10 mL NIM/well. Cells settled with gravity and were washed twice with 10 mL NIM, before plating 1:2 in aggregates onto 15 µg/mL fibronectin coated 6-well plates, where they formed rosette structures.

Full media changes with 20 ng/mL FGF2-supplemented NMM were conducted on D13 and D15, before a second Dispase® passage on D17. Cells were washed twice with NMM+FGF2, and then split 1:2 onto Poly-L-Ornithine/Laminin (PO/L) coated 6-well plates. Plates were coated overnight with Poly-L-Ornithine (PLO), and then overnight with laminin (10 µg/mL). From D18, media was changed every 2 days with NMM up to D25, at which point neural precursor cells (NPCs) were lifted with Accutase® and cryopreserved in freezing media and stored in liquid nitrogen.

Cells were treated for different experiments, harvested for western blot, fixed for ICC, as per Figure 2.2.

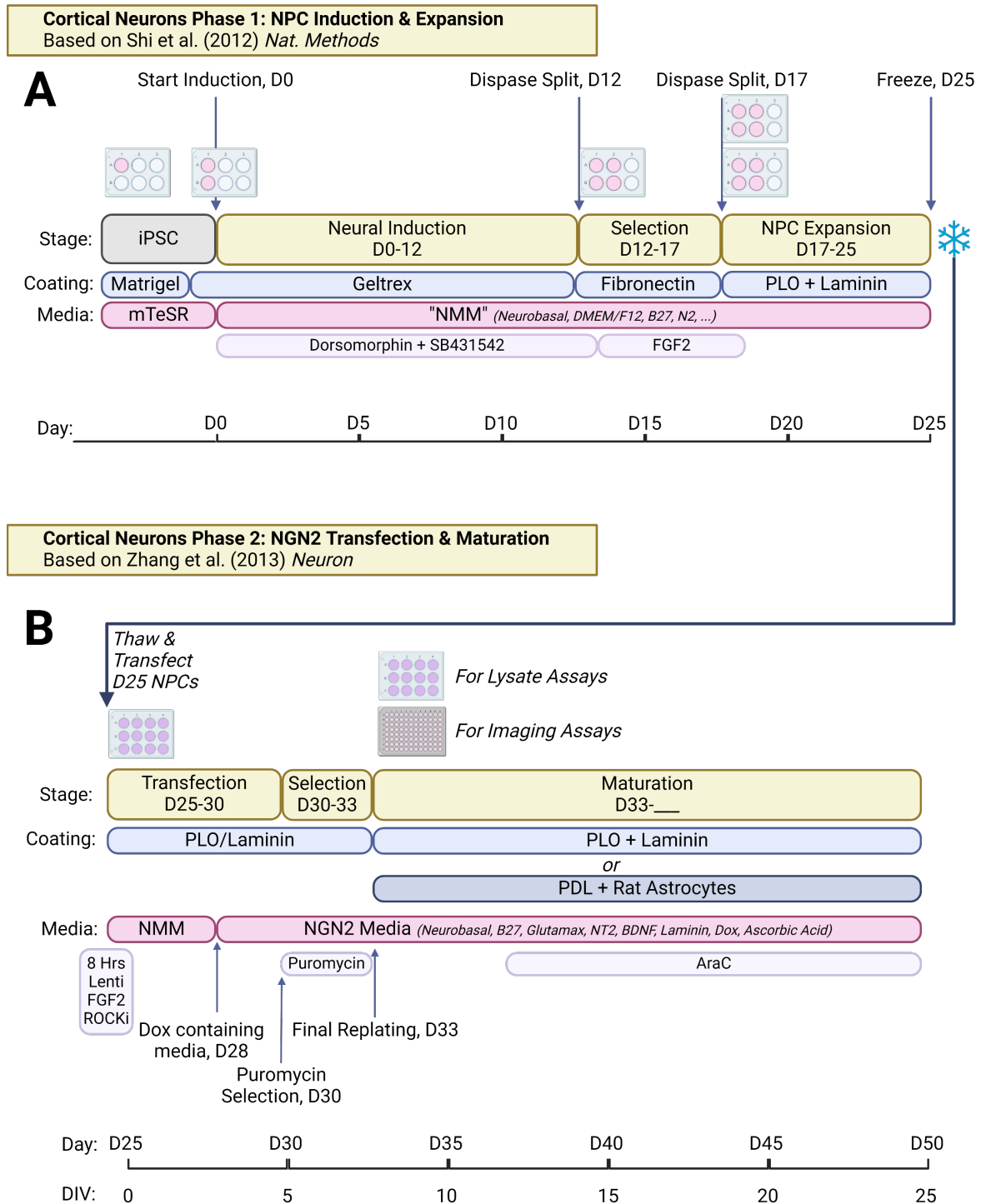


Figure 2.1. Cortical Neuron Differentiation Protocol Overview. Cortical neurons were differentiated using a 2-phase process. **(A)** Phase 1 was based off Shi et al. (2012) and involved growth factor and small molecule guided differentiation and expansion. **(B)** Phase 2 was based on Zhang et al. (2013) and involved lentiviral forced expression of NGN2.

Table 2.6. Media Compositions for CN Differentiation.

Media Component	Concentration	Source
Neuronal Maintenance Media (NMM)		
DMEM / F12 : Neurobasal	Base Media 1:1	Thermo/Thermo
N2	1X	Thermo
B27 with Vitamin A	1X	Thermo
GlutaMAX	1X	Thermo
Penicillin-Streptomycin	25 U/mL	Thermo
Human Insulin	2.5 ug/mL	Sigma
Non-essential amino acid	50 uM	Thermo
2-Mercaptoethanol	50 uM	Thermo
Sodium Pyruvate	1 mM	Thermo
L-glutamine	2 mM	Thermo
Neural Induction Media (NIM)		
NMM	Base Media	
SB-431542	10 uM	Tocris
Dorsomorphin	1 uM	Sigma-Aldrich (Merck)
Freezing Media		
NMM	Base Media	
FGF2	20 ng/mL	Sigma-Aldrich (Merck)
Dimethyl sulfoxide (DMSO)	10%	Sigma-Aldrich (Merck)
NGN2 Media		
Neurobasal™	Base Media	Thermo
B27™ with Vitamin A	1X	Thermo
GlutaMAX™	1X	Thermo
Penicillin/Streptomycin	50 U/mL	Thermo
Doxycycline	1 ug/mL	Sigma-Aldrich (Merck)
Brain-derived neurotrophic factor	10 ng/mL	Peprtech
Neurotrophin-3	10 ng/mL	Sigma-Aldrich (Merck)
Laminin	200 ng/mL	Sigma-Aldrich (Merck)
Ascorbic Acid	200 uM	Sigma-Aldrich (Merck)

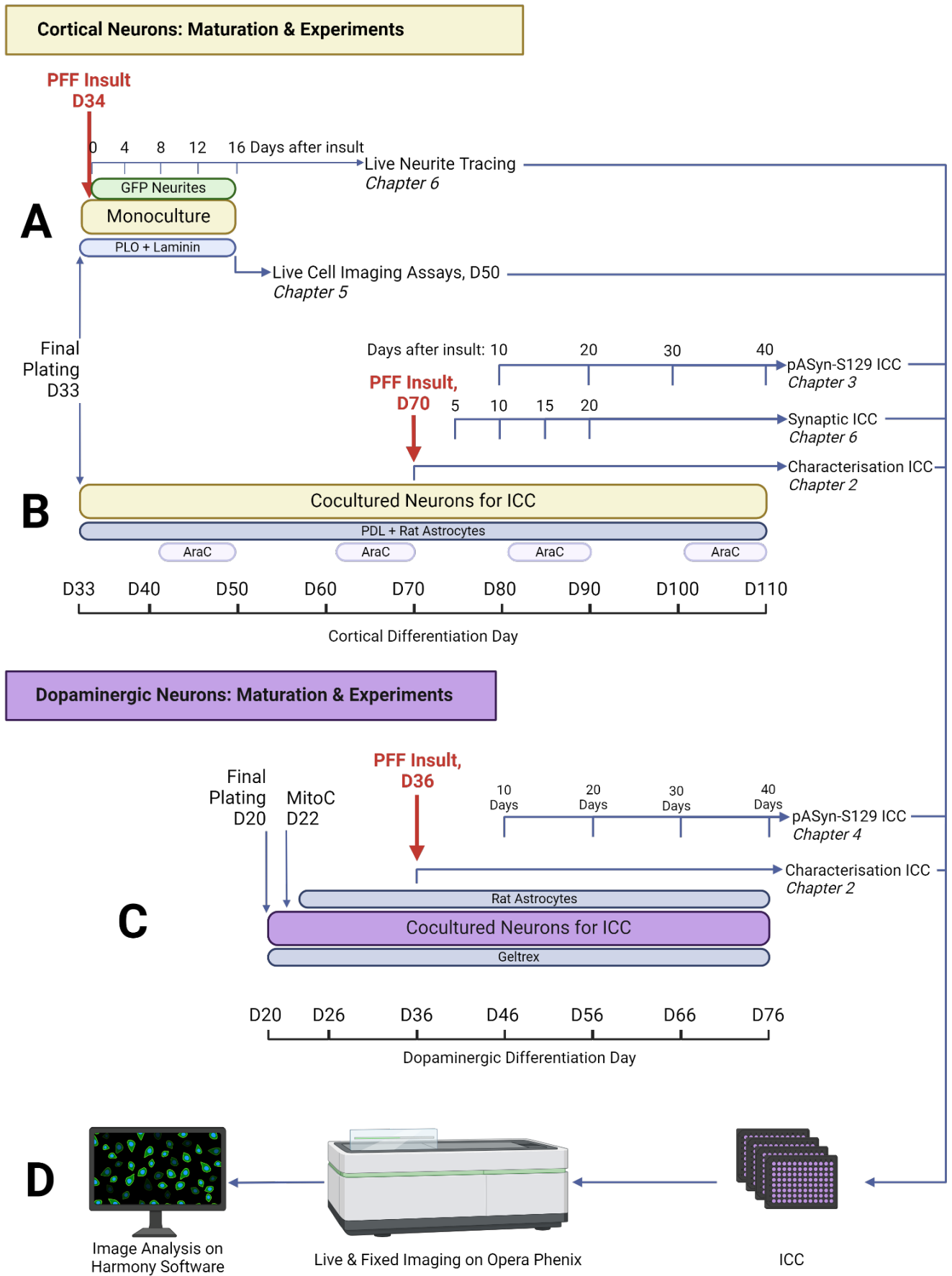


Figure 2.2. Cortical Neuron and Midbrain Dopaminergic Neuron Maturation and Insult Timelines. Cortical neurons were replated **(A)** in monoculture, for live cell assays performed on D50, and for live neurite outgrowth imaging, where PFF insult was performed on D34 and cells imaged every 4 days until D50, or **(B)** in coculture with rAstros, for ICC characterisation at D70, or PFF insult, with cells being harvested every 10 days until D110 for pASyn-S129 labelling, or every 5 days until D90 for synaptic density loss. **(C)** mDANs were replated into final well formats on D20 and cocultured with rAstros from D24 for ICC characterisation at D36 or PFF insult. **(D)** Cells for ICC were fixed, immunolabelled, and then imaged on Opera Phenix™. Images were analysed on harmony software.

2.2.6. Dopaminergic Neuron Differentiation

Dopaminergic differentiation was based off Krik's growth factor guided (2011) with an added expansion phase (Fedele et al. 2017), combined as previously described (Williamson et al. 2023). Here, rAstros were also incorporated to make the cultures more comparable to the CNs (Figure 2.3). Media components are listed in Table 2.7.

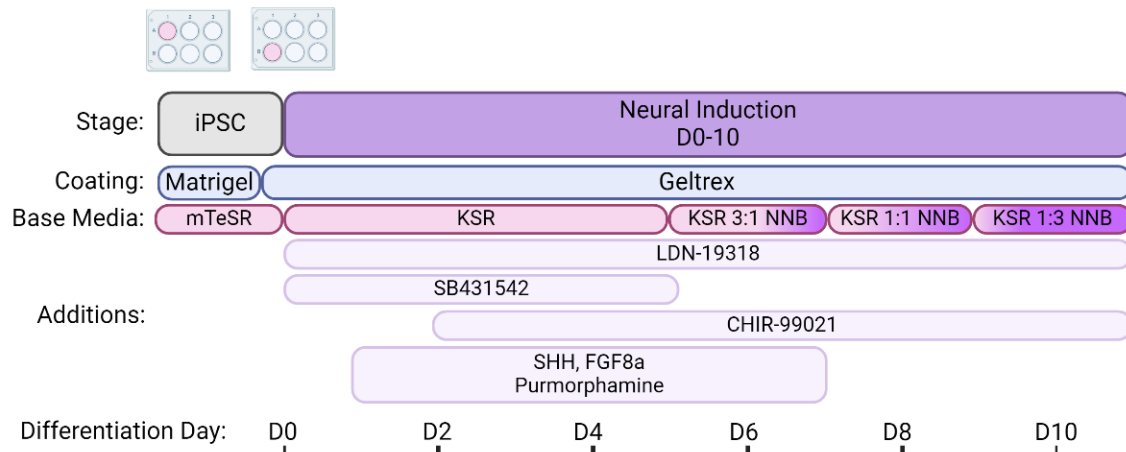
Singularised hiPSCs were seeded onto Geltrex™-coated plates and grown until confluent, then washed in PBS and changed to KSR media with LDN-19319 and SB431542. This was D0. Neural floorplate induction and ventral midbrain patterning was performed as per Figure 2.2, with alternating daily full and half media changes for 10 days.

On D11 (=Expansion Day E0), NPCs were split 1:2 with Accutase® onto Geltrex™-coated 6-well plates, then expanded in D10 media for 3 weeks, with further Accutase® splits every 7 days (E0, E7, E14), and feeding cells daily with alternating half and full media changes. Split cells were seeded in media containing 10 µM Y-27632, with full media change the following day. After 21 days of expansion, NPCs were lifted with Accutase®, cryopreserved in D10 media with 10% DMSO, and stored in liquid nitrogen.

For differentiation and maturation of NPCs, thawed cells were plated (5 million/well) onto Geltrex™-coated 6-well plates in D10 Media and maintained for four days, before transitioning to maturation media with CHIR-99021 for two days (D11-12) and then maturation media for a further 8 days before replating at Day 20. Media was changed daily with alternating half and full changes.

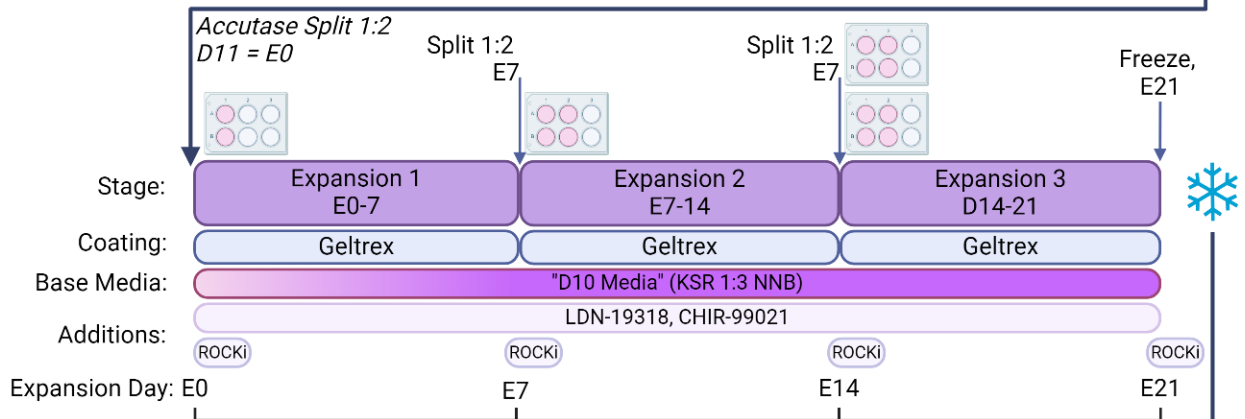
Dopaminergic Neurons Phase 1: NPC Induction

Based on Kriks et al. (2011) *Nature*



Dopaminergic Neurons Phase 2: NPC Expansion

Based on Williamson et al. (2023) *Hum Mol Gen*



Dopaminergic Neurons Phase 3: Differentiation & Maturation

Based on Kriks et al. (2011) *Nature*

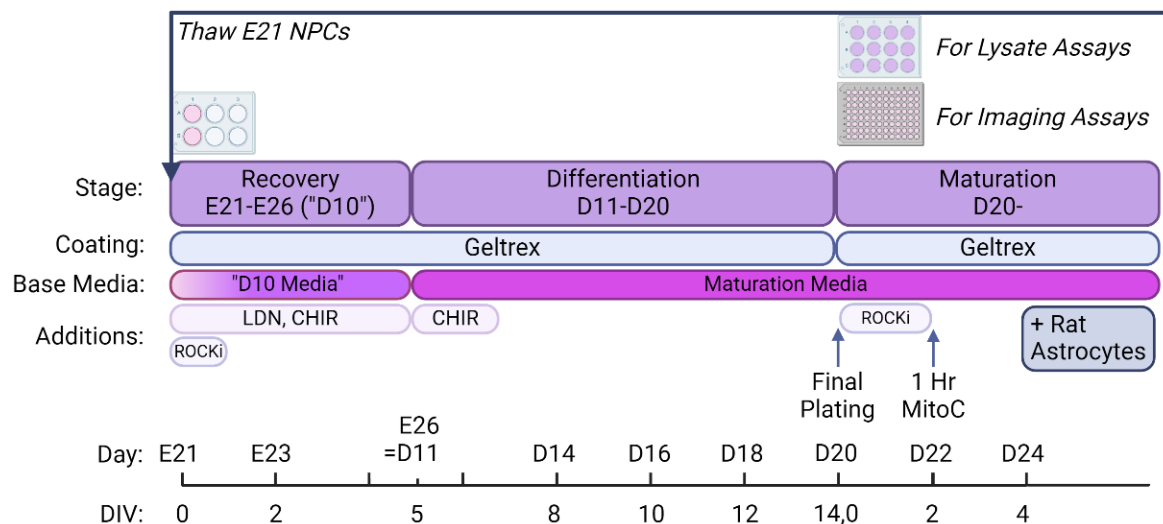


Figure 2.3. Midbrain Dopaminergic Neuron Protocol Overview. mDAN differentiation was conducted in three phases. Neural induction in Phase 1 involved dual SMAD inhibition and an evolving cocktail of other growth factors and medias. NPCs were expanded in Phase 2, with cells kept in “Day 10 Media”, before freezing down after 3 weeks of expansion. In Phase 3, thawed NPCs were differentiated in maturation media and replated into final well formats at D20, before MitoC treatment on D22 and addition of rAstros on D24.

Table 2.7. Dopaminergic Neuron Differentiation Media Components.

Reagent	Concentration	Supplier
KSR Media		
KO DMEM	Base Media	Thermo
Knock Out Serum Replacement	15% V/V	Thermo
2-Mercaptoethanol	10 μ M	Thermo
L-Glutamine	2 mM	Thermo
NEAA	1%	Thermo
NNB Media		
Neurobasal	Base Media	Thermo
N2	1X	Thermo
B27	1X	Thermo
L-Glutamine	2 mM	Thermo
Patterning Growth Factors (D0-10)		
SB-431542	10 μ M	Strattech Scientific
LDN-193189	100 nM	Merck
SHH	100 ng/mL	Bio-Techne
Purmorphamine	2 μ M	Bio-Techne
FGF8	100 ng/mL	Cambridge Bioscience
CHIR-99021	3 μ M	Bio-Techne
D10 Media		
KSR Media, NNB Media	1:3 Base Media	
LDN-19318	100 nM	Merck
CHIR-99021	3 μ M	Tocris
Maturation Media		
Neurobasal		Thermo
B27™ Without Vitamin A	1X	Thermo
L-Glutamine	2 mM	Thermo
BDNF	20 ng/mL	PeprroTech
GDNF	20 ng/mL	PeprroTech
TGF β	1 ng/mL	PeprroTech
DAPT	10 μ M	Abcam
Ascorbic Acid	200 μ M	Merck
db-cAMP	0.5 mM	Merck

On D20, cells were lifted with Accutase® and replated into final well formats on plates coated overnight sequentially with Poly-L-Ornithine and Geltrex™ (PO/G) with 10 µM Y-27632. Cells were then treated with 1 µg/mL MitoC for 1 hour on D22 to remove any remaining proliferating cells, washed once in neurobasal, and then returned to maturation media.

On D24, mDAN cultures were also supplemented with rAstros (32,000 cells/cm²) as required for certain assays, or simply half fed regular maturation media if not. From D26, cells were fed with maturation media in half changes every 2 days. Cells were harvested or fixed at D36 for characterisation, or insulted with PFFs at D36, as per Figure 2.2.

2.2.7. Rat Astrocyte Culture

rAstros (Gibco, N7745100) were used in some CN differentiation experiments as they help neuronal growth and maturation, and are essential for maintaining morphology and attachment through long term cultures (Lam et al. 2017; Hedegaard et al. 2020; Ng et al. 2024). For comparability, I also introduced rAstros into the mDAN protocol.

rAstros were thawed and expanded in T75 flasks as per manufacturer's protocols, in astrocyte growth media (Table 2.8).

Table 2.8. Rat Astrocyte Growth Media (AGM).

Component	Concentration	Supplier
High Glucose DMEM	Base Media	Sigma
Foetal Bovine Serum	15%	Thermo
L-Glutamine	2 mM	Thermo
Penicillin-Streptomycin	50 U/mL	Thermo

For CNs, final format plates were coated overnight in poly-d-lysine and washed three times in PBS before rAstros between P3 and P6 were seeded and allowed to grow to confluence (approximately 32,000 cells/cm²) before fully changing to NGN2 media and plating CNs on top

at D33. For mDANs, an equivalent number of astrocytes were seeded onto mDAN cultures at D24, to avoid MitoC treatment.

2.2.8. ASyn PFF Aggregation and Insults

PFFs were generated by Dr. Nora Bengoa-Vergniory, as previously described (Bengoa-Vergniory et al. 2020). Recombinant full length ASyn monomer (rPeptide, S-1001-2), suspended in a solution with 20 mM Tris and 0.1 M NaCl, pH 7.4, was spun to remove any aggregated material before shaking the supernatant at 250 RPM for 7 Days at 37°C. Fibrils were then sonicated for 10 minutes at 20% 0.25 duty cycle. Aliquots were stored at -80°C at 1 mg/mL. Aggregation was confirmed via electron microscopy. Cells were insulted with PFFs as per Figure 2.2. On the day of insult, a full media change to 30 μ L was made, and then 30 μ L media containing PFF insult at 2X final concentration was then added. Vehicle was added at equivalent concentration to the highest PFF concentration. Cells were left for 4 days before continuing half media changes every 4 days for CNs and every 2 days for mDANs. For chloroquine treated cells, chloroquine was included in the PFF insult media for a final concentration of 20 μ M.

2.2.9. Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 5 minutes for synaptic markers or 20 minutes otherwise and then left to rest for at least 1 day at 4°C in PBS. Cells were permeabilised using a relatively gentle 0.05% saponin made up in Tris-buffered saline (TBS) for 20 mins for synaptic markers, or 0.05% saponin made up in Tris buffered saline with 0.1% Tween-20 (TBST) for 30 minutes, which was sufficient to visualise targets while preserving membrane bound proteins. Cells were then blocked with 10% normal goat or donkey serum in TBST for 30 mins, and incubated in primary antibodies in 1% serum in TBST overnight at 4°C. All antibodies used are

detailed in Table 2.9 Cells were then washed 3-4 times in TBS, incubated in secondary antibodies for 1 hour, and then washed once in TBS before 15-minute incubation with 1 µg/mL DAPI. Cells were finally washed 2-3 more times before storage at 4°C, protected from light.

Table 2.9. Antibodies.

Target	Host	Application	Dilution	Manufacturer	Cat. Number
MAP2	Ck	ICC	1:800	Abcam	ab92434
B3T	Rb	ICC	1:1500	Abcam	ab78078
CUX2	Rb	ICC	1:200	Abcam	ab130395
CTIP2	Rt	ICC	1:500	Abcam	ab18465
“Human Nuclear Antigen” (HuNu)	Ms	ICC	1:200	Abcam	ab191181
vGlut1	Rb	ICC	1:500	Synaptic Systems	135303
GAD65/67	Gt	ICC	1:200	Santa Cruz	Sc-7531
Asyn 4D6	Ms	ICC	1:1000	Abcam	ab1903
Asyn MJRF	Rb	WB	1:5,000	Abcam	ab138501
TH	Sp	ICC	1:400	Millipore	ab1542
LMX1A	Rb	ICC	1:300	Abcam	ab139726
FOXA2	Gt	ICC	1:250	R&D Systems	AF2400
Synapsin I/II	Gp	ICC	1:250	Synaptic Systems	106004
Homer1	Rb	ICC	1:500	Synaptic Systems	160003
GFAP	Ck	ICC	1:500	Abcam	ab4674
S100B	Ms	ICC	1:500	Sigma	SAB4200671
pASyn-S129	Rb	ICC	1:250	Abcam	ab51253
LRP1	Rb	ICC	1:250	Abcam	ab92544
LRP1	Rb	ICC	1:500	Cell Signalling	64099
Beta Actin (HRP Conjugated)	Ms	WB	1:100,000	Abcam	ab49900
LAMP1	Ms	WB	1:500	Santa Cruz	sc-20011
pLRRK2-S935	Rb	ICC	1:500	Abcam	ab133450
LC3b	Rb	WB	1:5000	Sigma	L7543
IgG Negative Controls					
Rb IgG	Rb	ICC	Matched	Cell Signalling	2729S
Ms IgG	Ms	ICC	Matched	Abcam	ab37355
Sp IgG	Sp	ICC	Matched	Invitrogen	31243
Gt IgG	Gt	ICC	Matched	Abcam	ab37373
Secondary Antibodies					
Anti-Mouse IgG Alexa Fluor 488/555/647	Dk	ICC	1:800	Life Technologies	A21202 A31570 A31571
Anti-Rabbit IgG Alexa Fluor 488/555/647	Dk	ICC	1:800	Life Technologies	A21206 A31572 A31573

Anti-Chicken IgY Alexa Fluor 488/555/647	Dk	ICC	1:800	Life Technologies	A78948 A78949 A78952
Anti-Rat IgG Alexa Fluor 488	Dk	ICC	1:800	Life Technologies	A11006
Anti-Rat IgG Alexa Fluor 647	Gt	ICC	1:800	Life Technologies	A21247
Anti-Mouse Alexa Fluor 488/555/647	Gt	ICC	1:800	Life Technologies	A11001 A32727 A21236
Anti-Rabbit Alexa Fluor 488/555/647	Gt	ICC	1:800	Life Technologies	A11008 A21428 A21245
Anti-Chicken IgY Alexa Fluor 488/555	Gt	ICC	1:800	Life Technologies	A11039 A21437
Anti-Guinea Pig IgG DyLight® 488	Gt	ICC	1:800	Abcam	ab96959
Anti-Mouse IgG (HRP Conjugated)	Gt	WB	1:5000	Biorad	170-6516
Anti-Rabbit IgG (HRP Conjugated)	Gt	WB	1:5000	Biorad	170-6515

2.2.10. Microscopy and Image Analysis

High-content confocal imaging was performed on a Perkin Elmer Opera Phenix™, using a 40x magnification water immersion objective. Typically, 20-30 images were captured per well, and 3-4 wells were analysed per differentiation per cell line. For synaptic markers, a binning value of 1 was used, at a single focal plane of +1 μm . For other markers, a binning value of 2 was used.

Images were analysed using Harmony analysis software (Version 5.2). Custom analysis pipelines are detailed with results.

2.2.11. Western Blotting

Buffers used are detailed in Table 2.10. Cells were washed with PBS and then lysed on ice in cold radiomunoprecipitation assay (RIPA) buffer with protease inhibitor cocktail. Cell lysates

were then transferred to tubes on ice for at least 30 minutes with intermittent vortexing, then centrifuged at 20,000g for 30 mins at 4°C. Protein containing supernatant was then collected and stored at -80°C.

Table 2.10. Western Blotting Buffers.

Buffer Constituent	Concentration	Source
RIPA Buffer		Thermo
cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail	1 Tablet / 10 mL RIPA	Roche
Laemmli Buffer (4X)	BioRad	
Running Buffer		
Tris	25 mM	VWR
Glycine	192 mM	Promega
Sodium dodecyl sulfate	0.1%	Cambridge Bioscience

Protein concentration in the lysate was quantified using a BCA assay kit, following manufacturer's instructions. Briefly, 12.5 µL BSA standards or lysates were added per well in a 96-well plate in triplicate, and then 100 µL BCA reagent mixture. The assay was incubated at 37 °C for 30 mins, and then read using a PHERAstar® microplate reader at absorbance of 562 nm. Protein concentration was determined against a BSA standard curve and diluted to desired loading amount in RIPA. Laemmli buffer was added, and samples were boiled at 95 °C for 10 minutes.

Gel electrophoresis was performed in precast 4-15% gradient polyacrylamide gels in Running Buffer at 140 V for 90 minutes. Protein lysates were run alongside MagicMark™ protein standard and BLUeye Prestained Protein Marker before semi-dry transfer via a Trans-Blot® Turbo™ Transfer System at 1.3 A for 11 minutes to a 0.2 µm polyvinylidene fluoride membrane. Membranes were typically cut to separate proteins of interest and washed once in TBST.

For ASyn blots, membranes were then fixed in 4% PFA for 30 minutes, before washing in MilliQ water, then TBST. All washes and incubations were performed on a rocker. Blocking was

performed in 5% milk powder in TBST for 1 hr at RT, before incubation in primary antibodies diluted in 1% milk powder in TBST overnight at 4°C in.

Blots were then washed three times in TBST before incubation with secondary antibodies diluted in 1% milk powder in TBST for one hour at RT. Blots were finally washed a further three times in TBST before imaging. Blots were visualised using Immobilon® Western Chemiluminescent HRP Substrate and imaged in ChemiDoc™ Touch Gel Imaging System (Bio-Rad).

Densiometric quantification of blots was analysed using ImageLab v6.1 software (Bio-Rad).

2.2.12. Multielectrode Array (MEA) Recordings

All recordings were performed in 48-well MEA plates (Axion Biosciences), using a Maestro Pro™ reader.

For CNs, wells were coated overnight with PO/L, with laminin at 20 µg/mL, in 8 µL droplets. At D33, 15,000 CNs were seeded in a 5 µL droplet onto the PO/L coated surface, and approximately 30 minutes later, 5000 rAstros in 3 µL were seeded on top. Cells were left to settle for 1-2 hours before flooding the well with additional NGN2 media. Subsequently, a normal feeding regime was followed.

For mDANs, plates were coated overnight with 8 µL droplets of PLO, then 25 µg/mL biolaminin in dPBS++. On D20, 30,000 neurons were seeded onto the coated surface in a 5 µL droplet and allowed to settle. The well was flooded 1-2 hours later. The culture then underwent MitoC treatment on D22, and on D24, all media was removed, and 5000 rAstros seeded on top of neurons in a 6 µL droplet, before flooding the wells 1-2 hours later, and resuming a regular pattern of half media changes every two days.

All MEA recordings were at least 10 minutes long and were conducted at least 24 hours after a media change. Recordings were commenced after 15 minutes of settling in the temperature and CO₂ controlled recording chamber.

Raw recording files were analysed by AxIS software (Axion Biosystems). Firing rate was defined as the number of electrical spikes in a 1 ms window per recording length, >6 standard deviations above noise.

2.2.13. Statistical Analyses

Data is presented as raw values, normalised to a baseline, or normalised to an average across differentiations, as noted in each figure. For normalising across differentiations, the average values of cell lines used across all differentiations was calculated, and the mean fold change of these cell lines from this average calculated for each differentiation. Then all values for each differentiation were normalised by this mean fold change from the average.

All statistical analyses and graphical representations were produced in GraphPad Prism 10. Statistical comparisons are detailed for each figure. Typically, for comparisons between genotypes, a Kruskal-Wallis test was used. Where treatments were involved, 2-Way ANOVAs with multiple comparisons were used with Bonferroni correction. For these, N counted as biological (cell line) replicates per genotype, averaged across differentiations. For pairwise comparisons between two individual lines, Wilcoxon's signed rank test was used with independent differentiations constituting matched data points.

2.3. Results:

2.3.1. Cortical Neuron Characterisation

At D70, CNs cocultured with rAstros readily expressed MAP2 and β 3T. Cells had a wide range of morphologies and developed extensive dendritic arbours thin axonal networks (Figure 2.4).

To confirm cortical identity, cells were labelled with a human nuclear marker (HuNu), to distinguish human neuronal nuclei from rAstro nuclei, CUX2, an upper layer CN marker, and CTIP2, a deeper layer CN marker. ~80% of neurons produced were CUX2+, whereas only ~20% were CTIP2+, and this was consistent across genotypes.

I also labelled cells with vGLUT1 to confirm whether the CNs were excitatory (Figure 2.6). Interestingly, while all cell lines displayed some vGLUT1 labelling, puncta density varied substantially between cell lines, with high expression in APP-V717I, PSEN1-Int4Del, and one LRRK2-R1441C cell line (207), though this difference was not significant. Immunolabelling for GAD65/7 found no positive signal distinct from IgG negative control (Figure 2.7).

Immunolabelling also revealed that alpha synuclein (ASyn) was robustly endogenously expressed by the CNs at D70, though not in the *SNCA*-KO control line, and not in the rAstros. There was also no MAP2+ immunolabelling in rAstros. Quantifying puncta across cell lines using the same method as for vGLUT1 found no significant differences between genotypes (Kruskal-Wallis Test with Dunn's Correction).

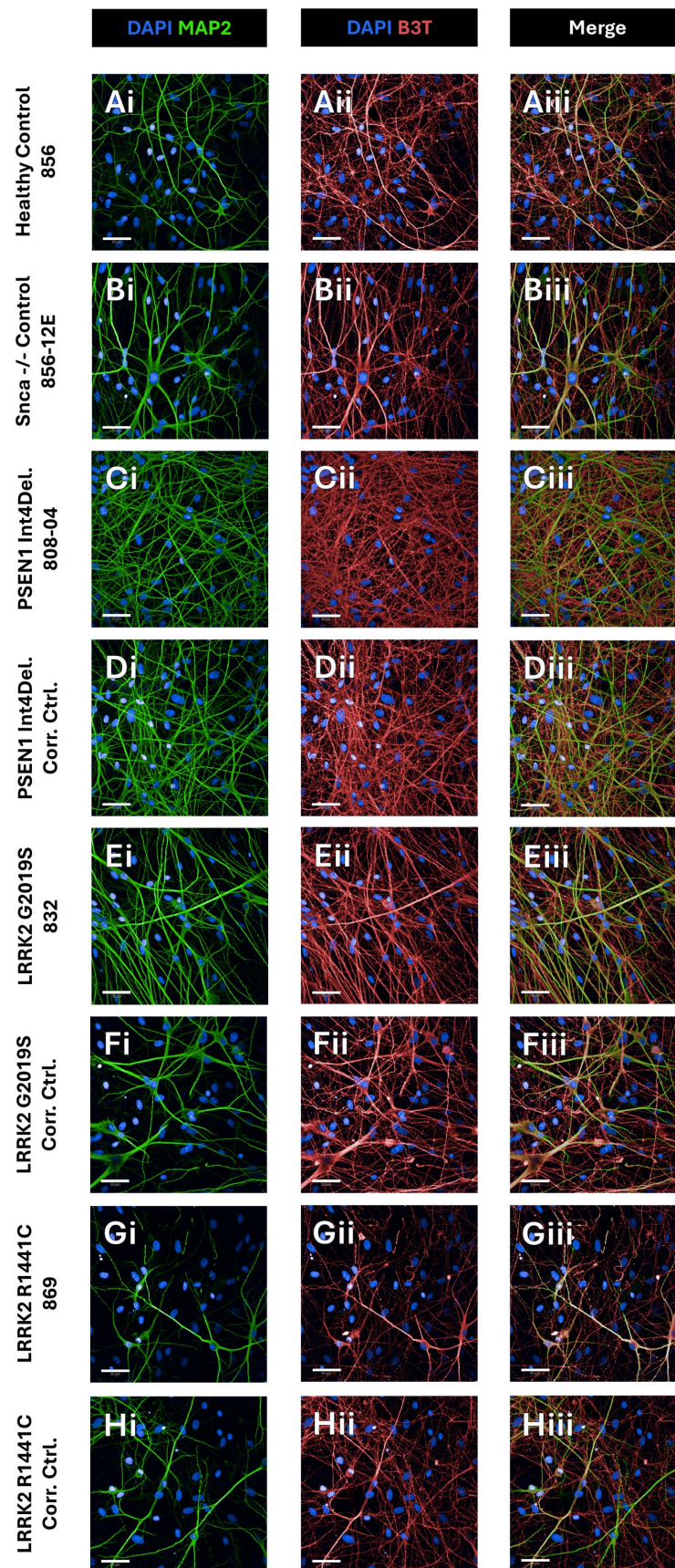


Figure 2.4. Cortical Neurons Express MAP2 and β 3T Neuronal Markers. Cortical neurons fixed at D70 were immunolabelled with (i) MAP2 and (ii) β 3T. Scale bars represent 50 μ m.

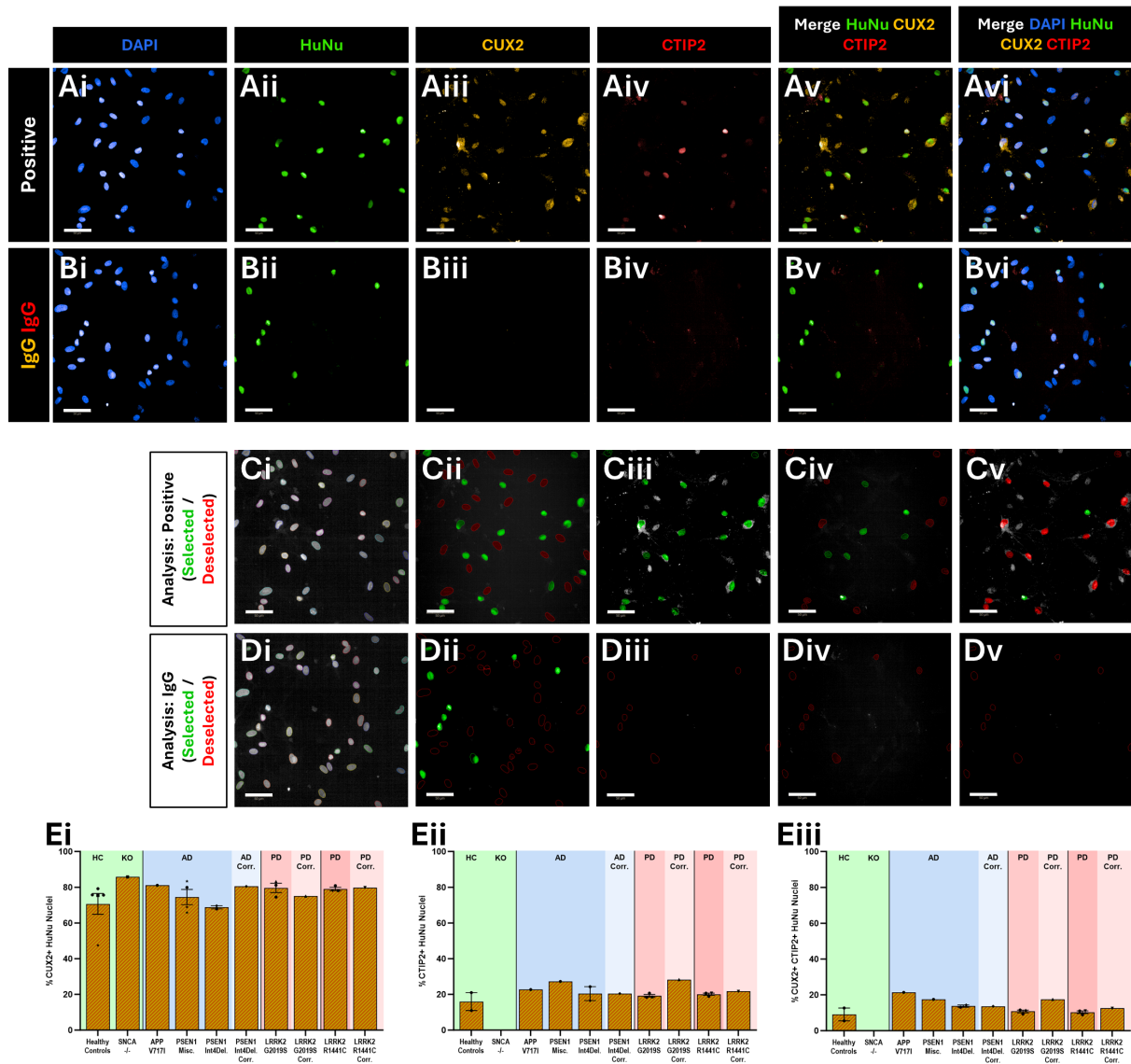


Figure 2.5. CNs Express Upper Layer Marker Cortical Marker CUX2, and CTIP2. (A) Representative images of CNs fixed at D70 stained with (i) DAPI, and immunolabelled with (ii) human nuclear (HuNu) antigen, to distinguish between human neuron and rat astrocyte nuclei, (iii) upper layer marker CUX2, and (iv) deeper layer marker CTIP2. (B) No immunoreactivity was observed for IgG negative control labelling of CUX2 (rabbit IgG) or CTIP2 (rat IgG). (C) Image analysis pipeline in Harmony software was conducted as follows: (Ci) DAPI nuclei were identified using the Find Nuclei function, Method B. (Cii) DAPI nuclei were selected (green) or deselected (red) as HuNu+ using fluorescence intensity. (Ciii-iv) CUX2+ and CTIP2+ HuNu+DAPI nuclei were selected for using fluorescence intensity thresholds set against IgG negative control labelling. (Cv). CUX2+CTIP2+ double positive HuNu+DAPI nuclei were selected using fluorescence intensity. (D) An equivalent pipeline was also run on IgG negative control wells to ensure no non-specific signal was captured by the analysis. IgG images were used to set thresholds for fluorescence intensity. (E) The proportion of (Ei) CUX2+, (Eii) CTIP2+ and (Eiii) CUX2+CTIP2+ HuNu+ Nuclei was quantified. No significant differences were found between genotypes using a Kruskal-Wallis test with Dunn's correction. Data points represent individual cell lines averaged across 3-4 differentiations for CUX2, and 1-2 differentiations for CTIP2. Isogenic pairs are noted with shared point shape. Healthy Control 856 and its SNCA -/- are square points. iNDI KOLF lines are represented by stars. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and iNDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as "PSEN1 Misc." Scale bars represent 50 µm.

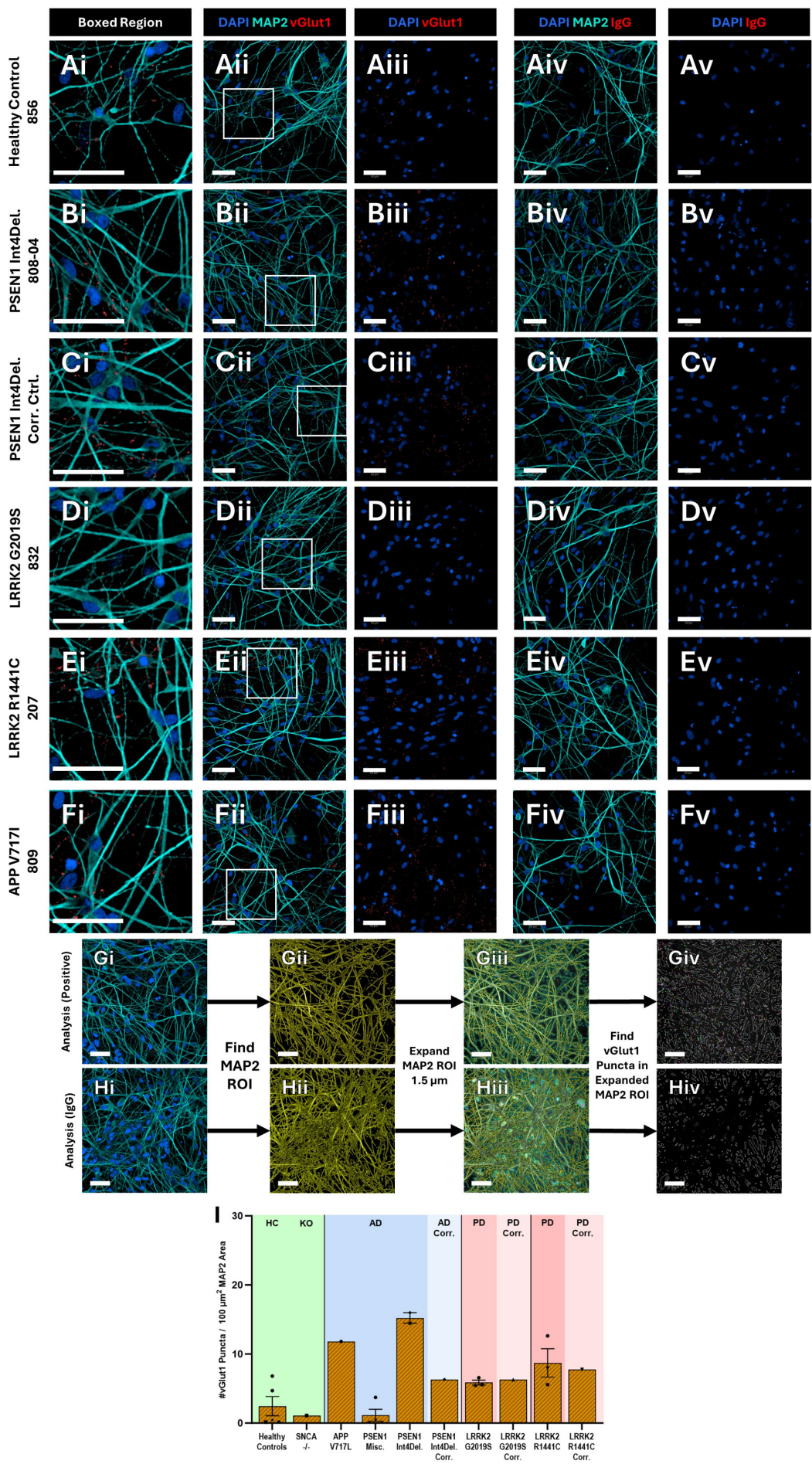


Figure 2.6. CNs Express vGLUT1. (A-F) Representative images of CNs from different genotypes fixed at D70 stained with **(i-iii)** MAP2 and vGLUT1, or **(iv-v)** MAP2 and rabbit-IgG negative control. **(G)** Image analysis pipeline in Harmony software was conducted as follows: **(Gii)** The MAP2 area was defined as an ROI using the Find Image Region function and common threshold. **(Giii)** Since vGLUT1 was largely presynaptically localised and therefore typically marked by puncta immediately outside MAP2, the MAP2 ROI was expanded by 1.5 μm . **(Giv)** vGlut1 puncta were found and counted within the expanded MAP2 ROI using the Find Spots function (Method A). **(H)** An equivalent pipeline was also run on IgG negative control wells to ensure no non-specific signal was captured by the analysis. IgG images were used to set thresholds for spot finding. **(I)** The number of vGlut1 puncta per 100 μm^2 MAP2 area was quantified. No significant differences were found between genotypes using a Kruskal-Wallis test with Dunn's correction. Data points represent individual cell lines averaged across 3 differentiations. Isogenic pairs are noted with shared point shape. Healthy Control 856 and its SNCA -/- are square points. iNDI KOLF lines are represented by stars. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and iNDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as "PSEN1 Misc." Scale bars represent 50 μm .

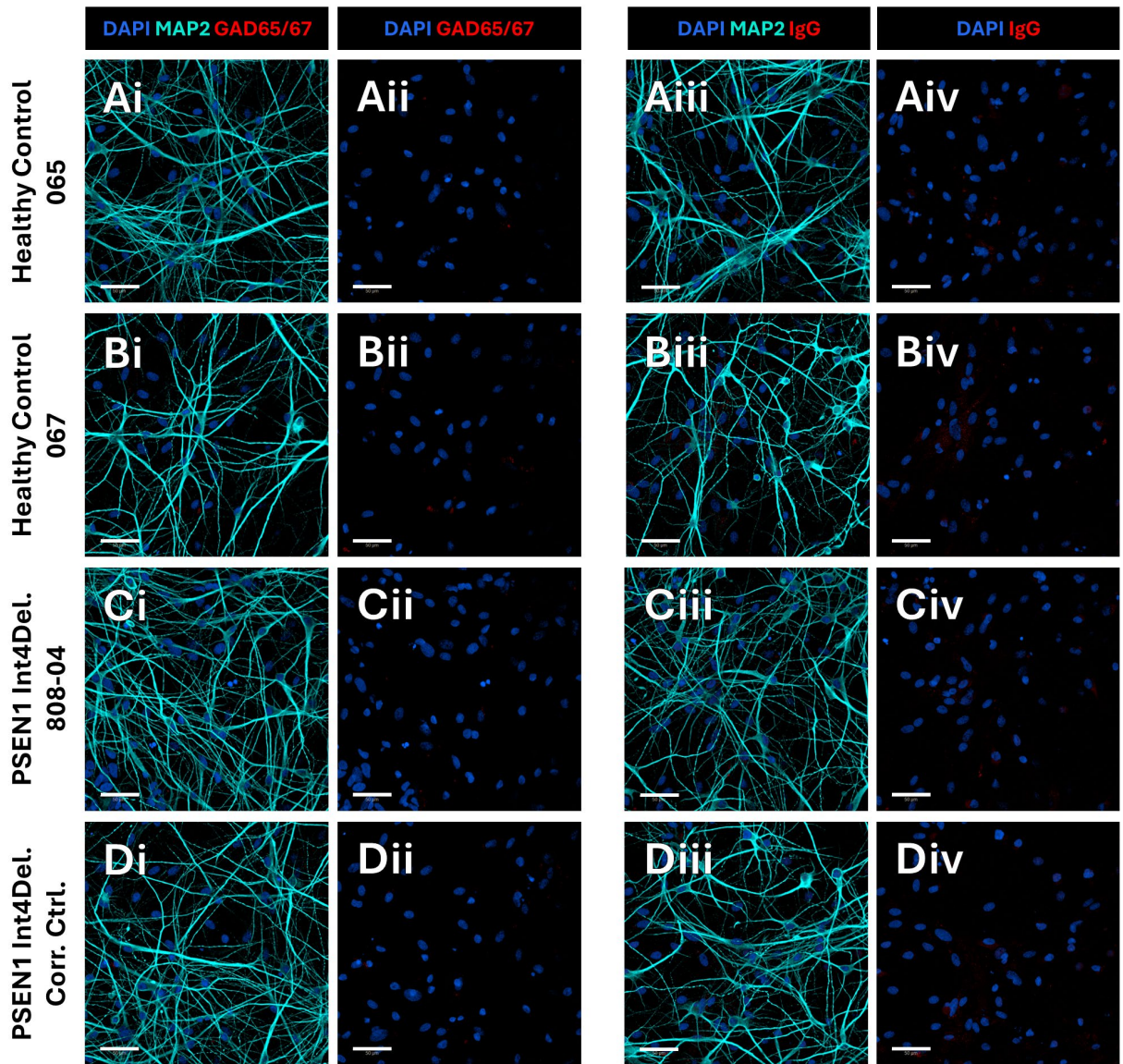


Figure 2.7. No GAD65/67 Labelling in CNs. (A-D) Representative images of CNs from various genotypes fixed at D70 stained with (i) MAP2 and GABAergic marker GAD65/67. (ii) GAD65/67 labelling shown without MAP2. (iii-iv) MAP2 and IgG negative control. Scale bars represent 50 μ m.

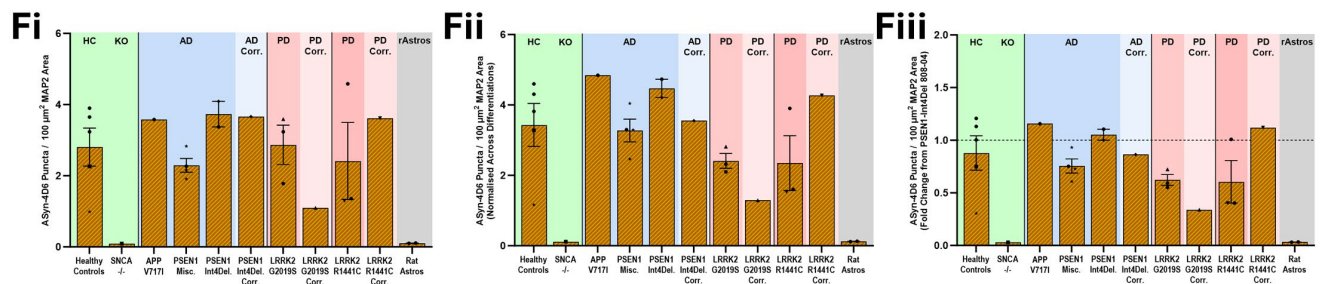
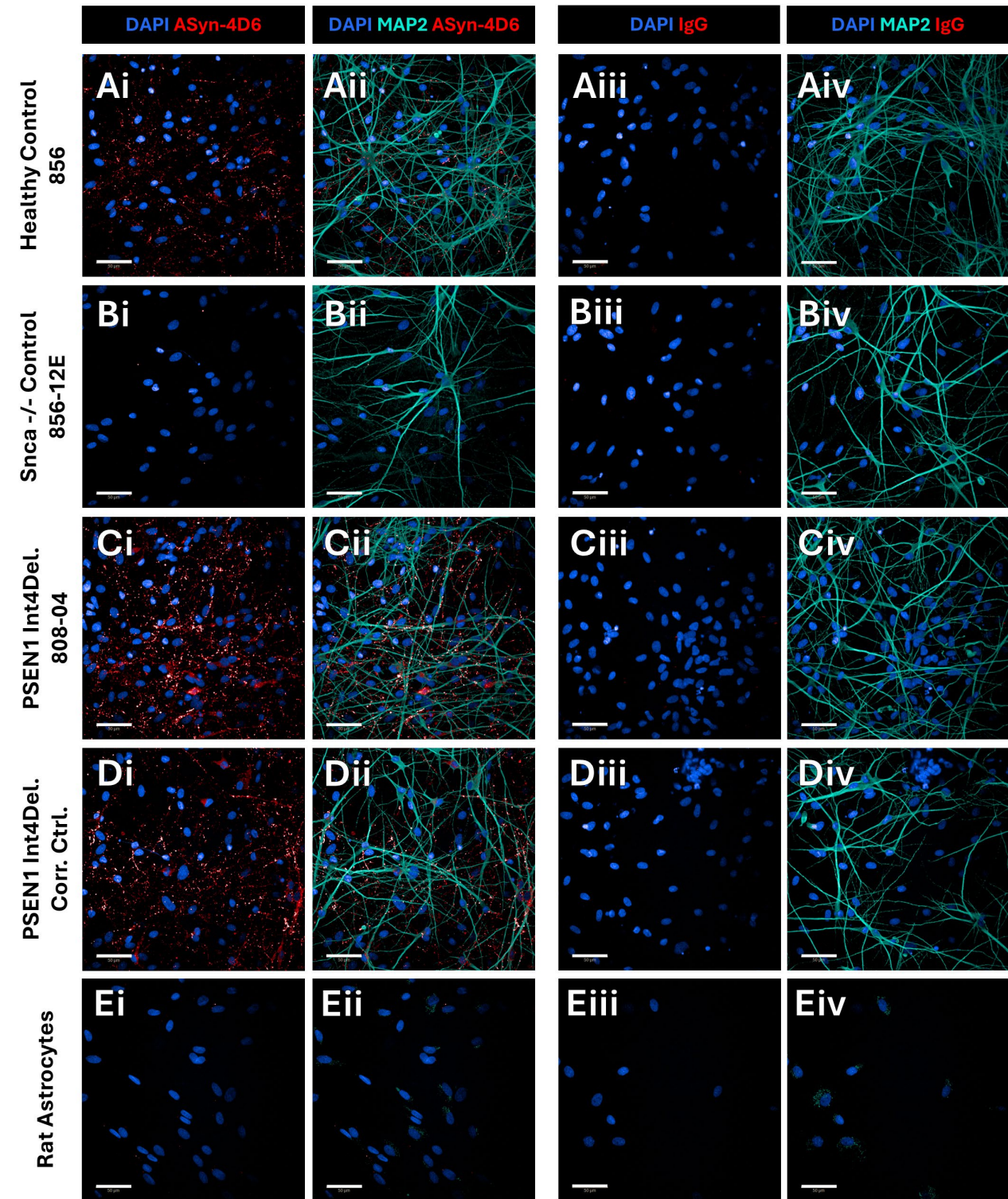


Figure 2.8. Alpha Synuclein is Expressed by CNs, not by rAstrocytes. (A-E) Representative images of CNs from different genotypes or rAstros grown in parallel in monoculture, fixed at D70 stained with **(i)** ASyn, **(ii)** merge of ASyn and MAP2, or **(iii-iv)** Rabbit-IgG negative control and MAP2. **(F)** Images were analysed in Harmony software and puncta counted as per vGlut1 in Figure 2.6. Quantified puncta are presented as **(Fi)** raw values, **(Fii)** values normalised to the average across differentiations, and **(Fiii)** fold change from PSEN1-Int4Del 808-04, which was included in each differentiation. No significant differences were found between genotypes using a Kruskal-Wallis test with Dunn's correction. Data points represent individual cell lines averaged across 2-4 differentiations. Isogenic pairs are noted with shared point shape. Healthy Control 856 and its SNCA -/- are square points. iNDI KOLF lines are represented by stars. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and iNDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as "PSEN1 Misc." Two points for rAstros represent cells grown in either NGN2 media or AGM. Scale bars represent 50 μ m.

2.3.2. Dopaminergic Neuron Characterisation

To confirm the midbrain dopaminergic cultures still readily produced mDANs while being cocultured with rAstros, I performed TH immunolabelling and saw robust expression of TH across cell lines (Figure 2.9). To quantify TH⁺ cells, labelled alongside HuNu and MAP2, I used two independent strategies, detailed in Figure 2.10. These two methods were well correlated (Spearman $r=0.8046$), and quantification indicated that the protocol produced cultures around 40-70% TH⁺ in most cell lines.

To confirm the ventral midbrain identity of these cultures, I examined LMX1A and FOXA2 expression in HuNu⁺ cells (Figure 2.11). To quantify this labelling, I used an analogous pipeline to that for CUX2 and CTIP2 in CNs. Both FOXA2 (~60-80%) and LMX1A (~80-90%) were well expressed in most cell lines and there were no significant differences between genotypes.

To confirm that these midbrain dopaminergic cells also endogenously expressed ASyn, I performed immunolabelling with the ASyn-4D6 antibody alongside MAP2 and TH cell markers and quantified the number of ASyn puncta within all MAP2⁺ cells and within TH⁺ cells specifically (Figure 2.12). ASyn was robustly expressed in the mDAN cultures by D36, with no significant differences between genotypes and minimal expression in the *SNCA*-KO cell line. Interestingly, the puncta density was slightly higher in TH⁺ neurons as compared to all MAP2⁺ cells.

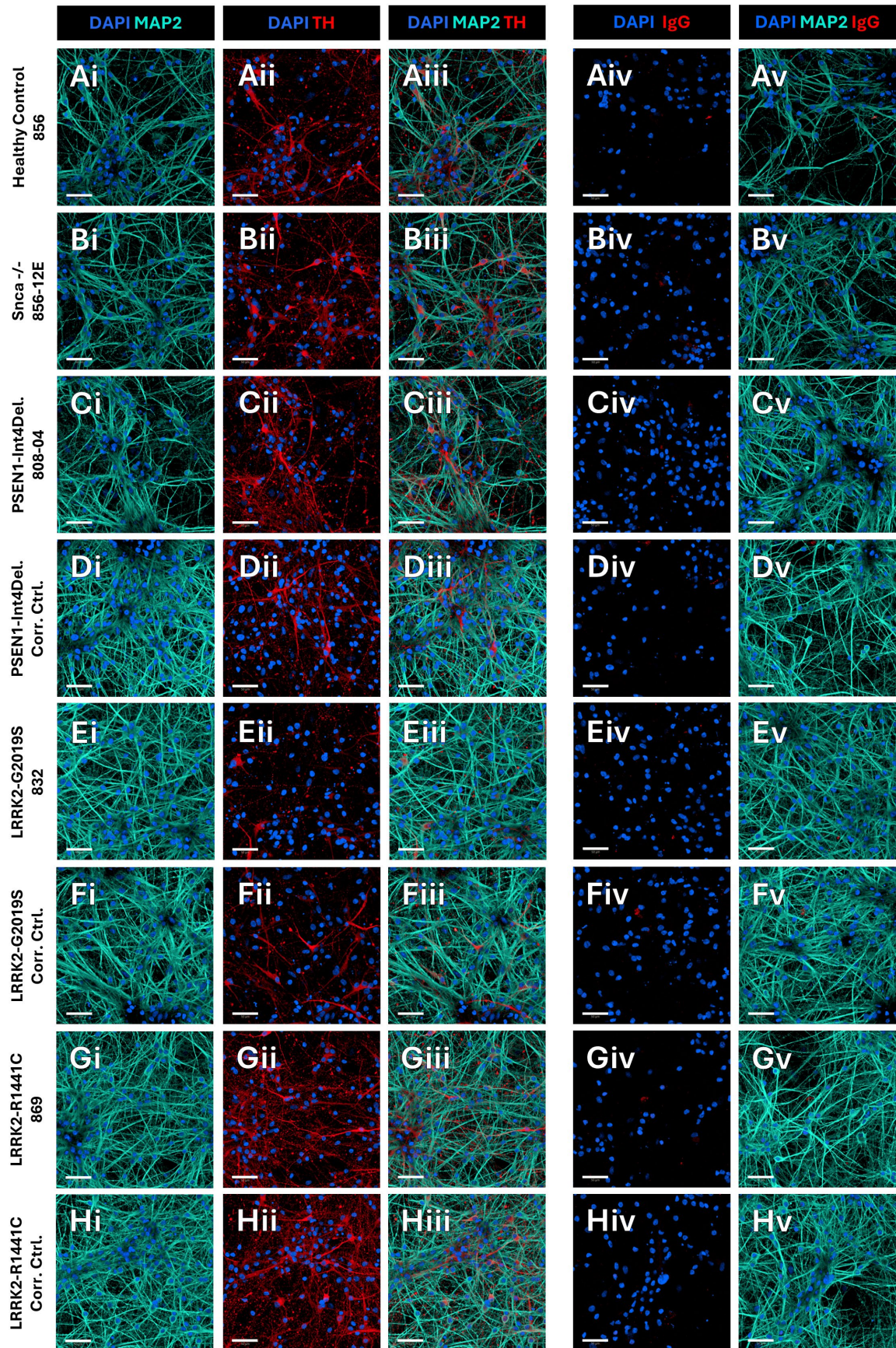


Figure 2.9. mDANs Express MAP2 and TH. mDANs fixed at D36 were immunolabelled with (i) MAP2 and (ii) TH. (iii) Merge of positive labelling. (iv-v) Sheep-IgG negative control shown against (iv) DAPI and (v) MAP2. Scale bars represent 50 μ m.

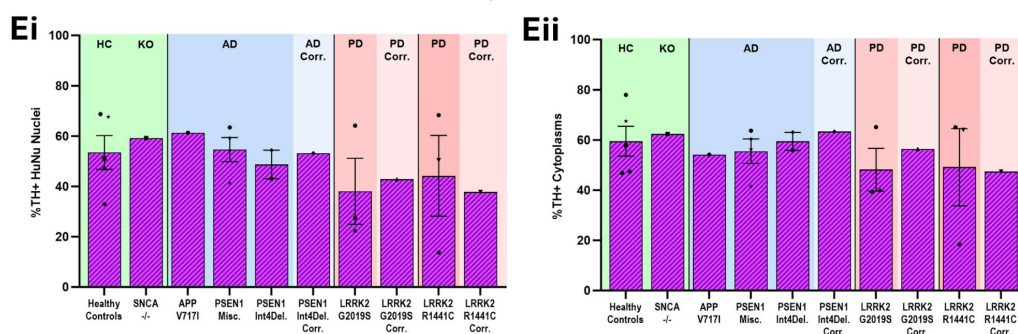
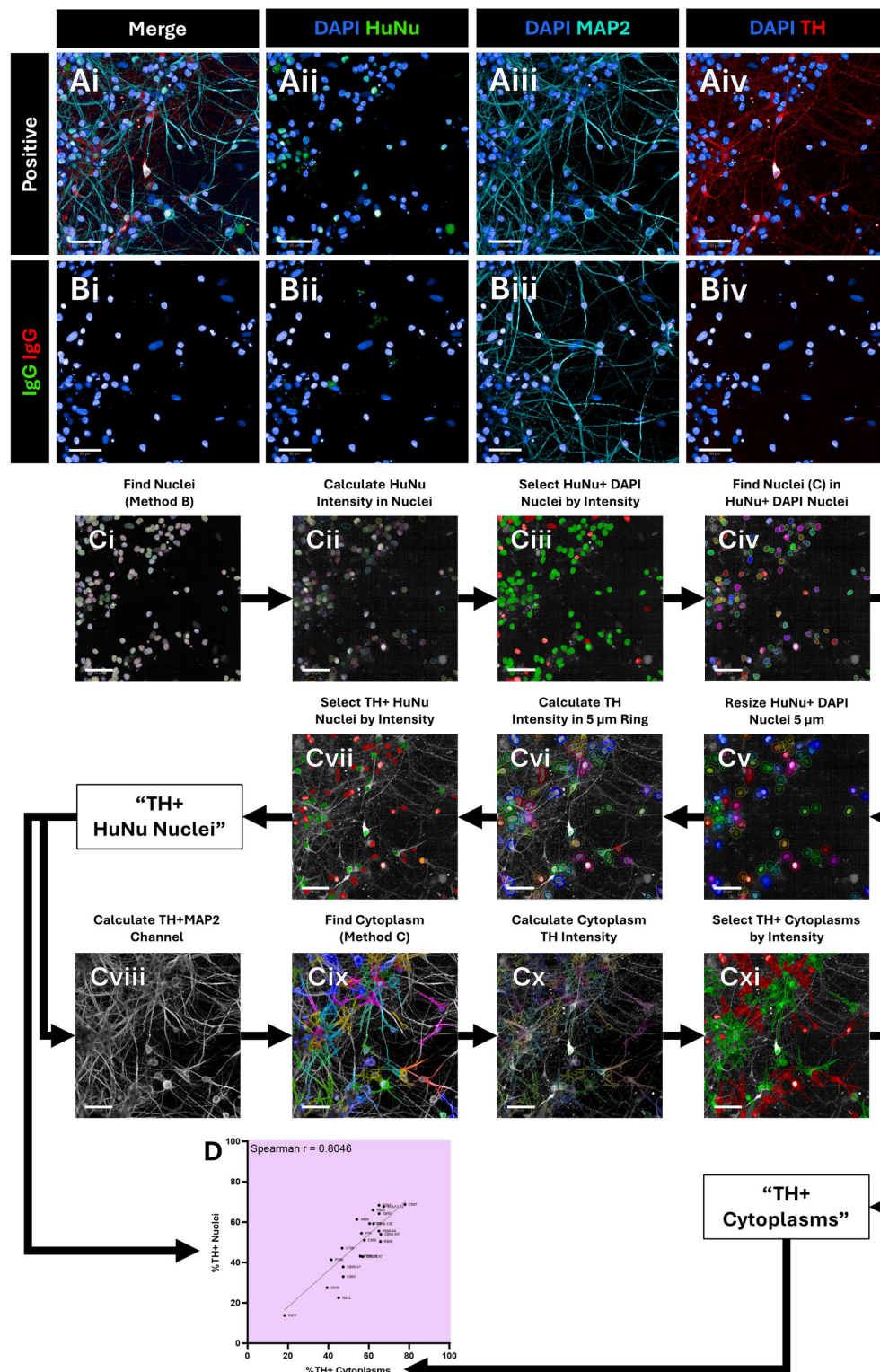


Figure 2.10. Quantification of TH+ Cells in mDAN Cultures. **(A)** Representative image of mDANs fixed at D36 stained with DAPI and **(Aii)** HuNu, **(Aiii)** MAP2, and **(Aiv)** TH. **(B)** Equivalent labelling was performed with mouse and sheep IgG antibodies for negative control labelling for HuNu and TH. **(C)** Two independent strategies to quantify the number of TH+ cells were used, with a representative pipeline showing image analysis on **(A)** in Harmony software, with steps as follows: **(Ci)** DAPI nuclei were identified using the Find Nuclei function Method B. **(Cii-iii)** HuNu+ DAPI Nuclei were selected using intensity thresholds set against IgG negative control labelling. A HuNu+DAPI ROI was created from this. **(Civ)** HuNu+ Nuclei were identified within the established ROI, using the Fund Nuclei Function Method C, which was better at distinguishing clusters of nuclei. **(Cv)** A new HuNu+DAPI nuclei ROI was expanded 5 μ m, and the HuNu+ DAPI nucleus subtracted to create a 5 μ m perinuclear ring. **(Cvi)** TH intensity was calculated in this perinuclear ring, and **(Cvii)** TH+ HuNu nuclei were selected by intensity threshold set against IgG negative control labelling. These were counted as “TH+ HuNu Nuclei”. In parallel to this, **(Cviii)** an image channel was calculated as the merge of the TH and MAP2 images. **(Cix)** individual cells in this merge channel were identified using the Find Cytoplasm function (Method C). **(Cx)** the TH intensity of each cytoplasm was measured, and **(Cxi)** TH+ cytoplasms were selected by intensity threshold, set against IgG negative control labelling. These were counted as “TH+ Cytoplasms”. **(D)** Non-parametric Spearman correlation was calculated for the measures of TH+ HuNu Nuclei and TH+ Cytoplasms, with $r=0.8046$ ($p<0.0001$). **(E)** Quantified proportions of TH+ neurons are presented per genotype as raw values. No significant differences were found between genotypes using a Kruskal-Wallis test with Dunn’s correction. Data points represent individual cell lines averaged across 3 differentiations. Isogenic pairs are noted with shared point shape. Healthy Control 856 and its SNCA -/- are square points. iNDI KOLF lines are represented by stars. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and iNDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as “PSEN1 Misc.” Scale bars represent 50 μ m.

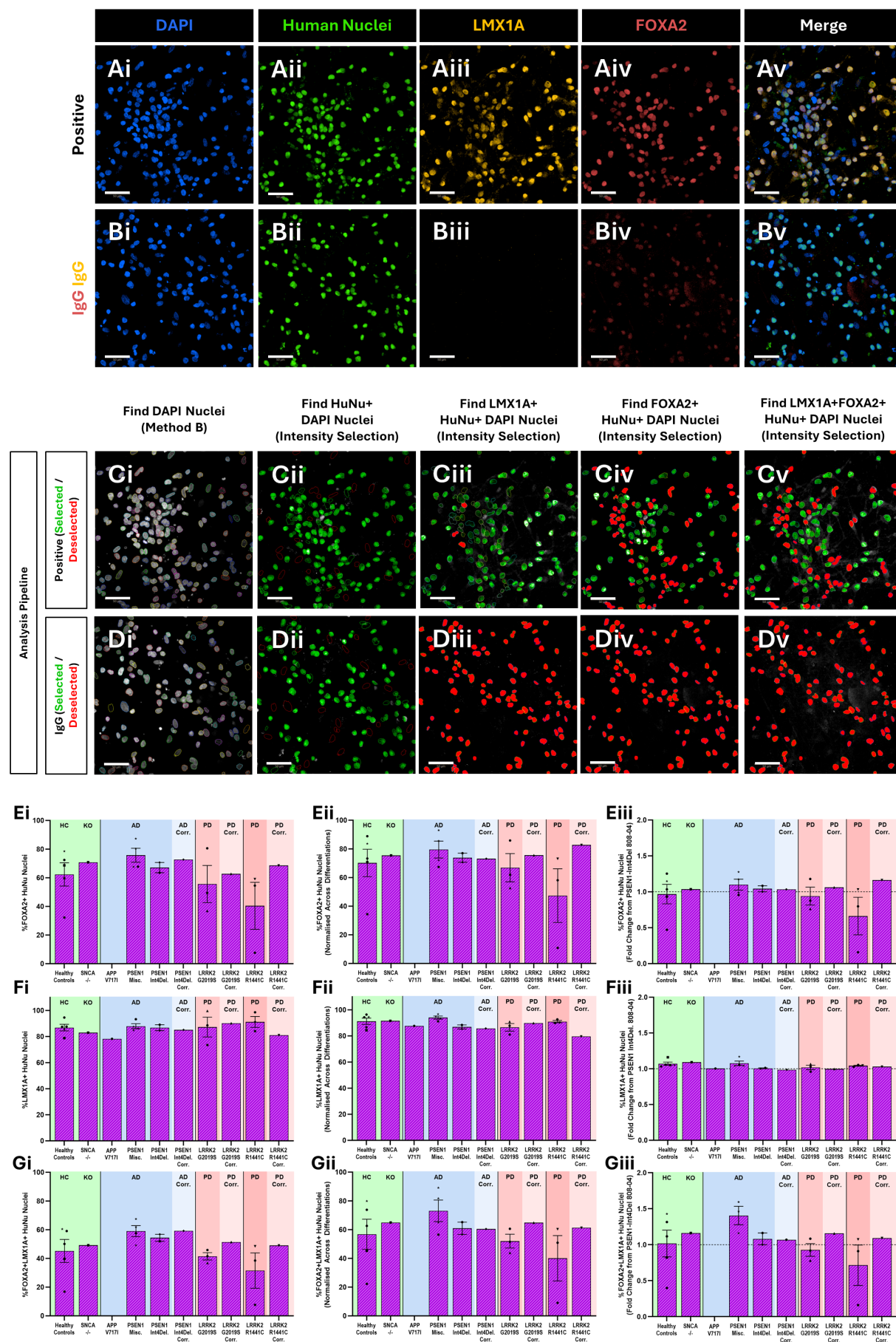


Figure 2.11. mDANs Express Midbrain Markers LMX1A and FOXA2. (A) Representative images of mDANs fixed at D36 stained with (Ai) DAPI, and immunolabelled with (Aii) HuNu, to distinguish between human neuron and rAstro nuclei, (Aiii) LMX1A and (Aiv) FOXA2. (B) No immunoreactivity was observed for IgG negative control labelling of (Biii) LMX1A (rabbit IgG) or (Biv) FOXA2 (goat IgG). (C) Image analysis pipeline in Harmony software was conducted as follows: (Ci) DAPI nuclei were identified using the Find Nuclei Function Method B. (Cii) DAPI nuclei were selected (green) or deselected (red) as HuNu+ using fluorescence intensity. (Ciii-iv) LMX1A+ and FOXA2+ HuNu+DAPI nuclei were selected for using fluorescence intensity. (Cv) LMX1A+FOXA2+ double positive HuNu+DAPI nuclei were selected using fluorescence intensity. (D) An equivalent pipeline was also run on IgG negative control wells to ensure no non-specific signal was captured by the analysis. IgG images were used to set thresholds for fluorescence intensity. (E-G) The proportion of (E) FOXA2+, (F) LMX1A+ and (G) FOXA2+LMX1A+ HuNu+ Nuclei was quantified. Data are presented as (i) raw values, (ii) values normalised to the average across differentiations, and (iii) fold change from PSEN1-Int4Del 808-04, which was included in all differentiations. No significant differences were found between genotypes using a Kruskal-Wallis test with Dunn's correction. Data points represent individual cell lines averaged across 3-5 differentiations for LMX1A, 2-3 differentiations for FOXA2 and FOXA2+LMXA1+. Isogenic pairs are noted with shared point shape. Healthy Control 856 and its SNCA -/- are square points. iNDI KOLF lines are represented by stars. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and iNDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as "PSEN1 Misc." FOXA2 labelling was not conducted in APP-V717I. Scale bars represent 50 μ m.

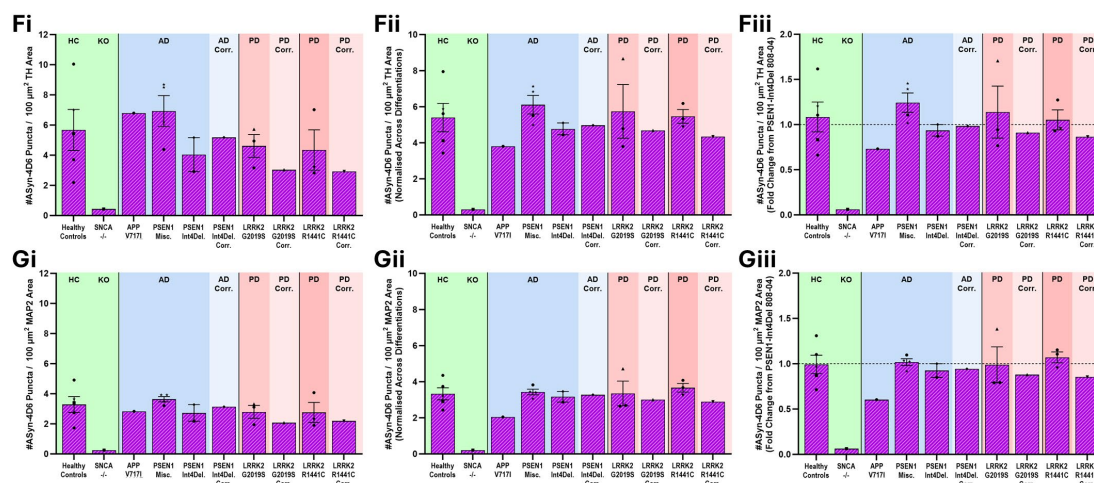
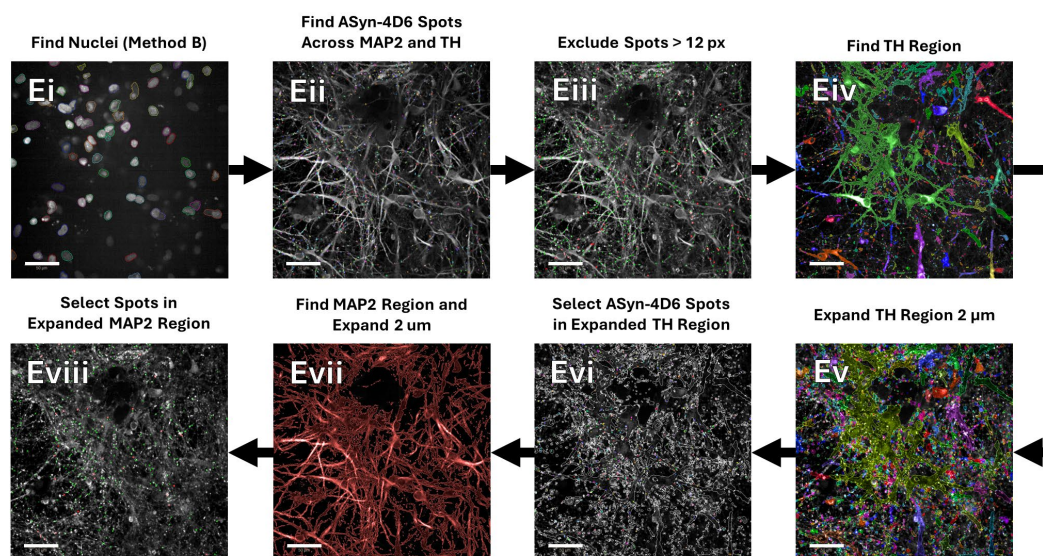
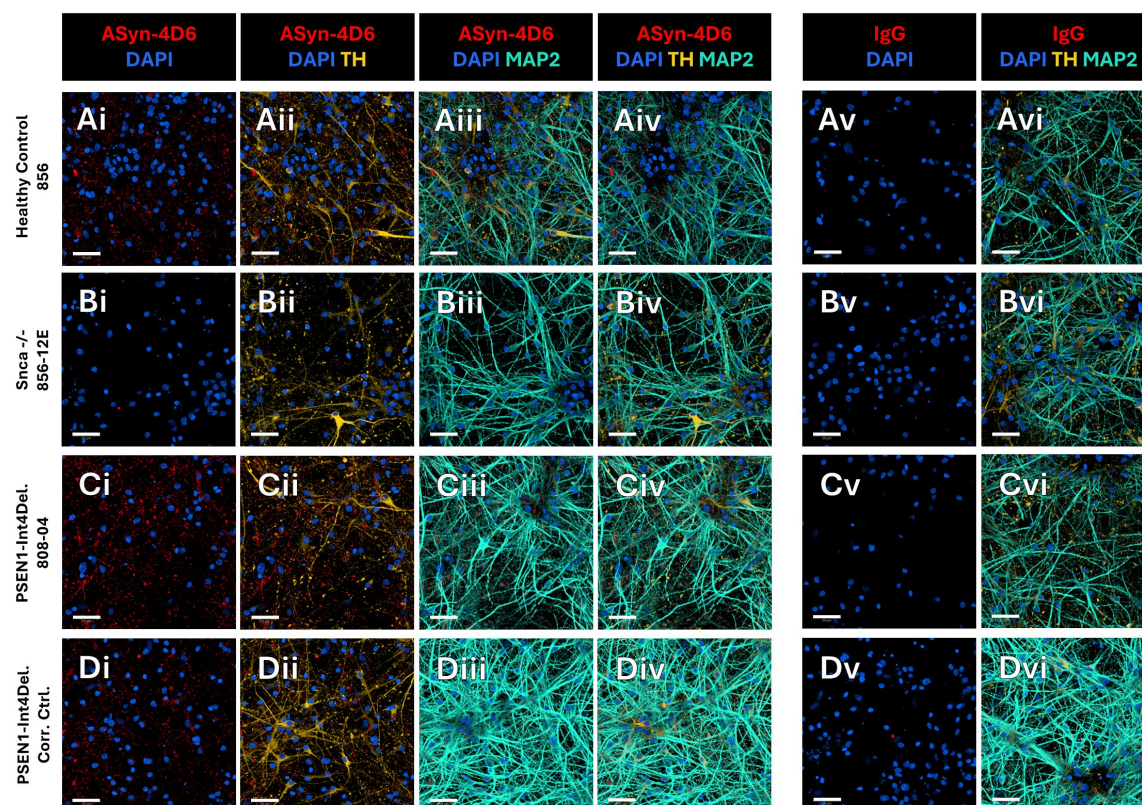


Figure 2.12. Alpha Synuclein is Expressed in TH+ Cells in mDAN Cultures. (A-D) Representative images of mDANs from different genotypes, fixed at D36 stained with **(i)** ASyn, **(ii)** merge of ASyn and TH, **(iii)** merge of ASyn and MAP2, **(iv)** merge of ASyn, TH, and MAP2, or **(v)** Rabbit-IgG negative control for ASyn against TH and MAP2. **(E)** Images were analysed in Harmony software under a custom pipeline using the following steps: **(Ei)** DAPI nuclei were identified using the Find Nuclei function Method B. **(Eii)** The Find Spots function (Method A) was used to identify ASyn puncta, and **(Eiii)** puncta greater than 12 px were excluded. **(Eiv)** The Find Image Region function was used to establish a TH+ cell ROI and, **(Ev)** since ASyn puncta were found predominantly as presynaptic puncta adjacent to TH and MAP2 boundaries, the TH ROI was expanded 2 μ m, and **(Evi)** ASyn puncta within this ROI were selected as ASyn puncta in TH area. **(Evii-viii)** a similar process was used to calculate the number of puncta across all MAP2+ cells. **(F-G)** Quantified puncta within **(F)** TH Cell area, and **(G)** MAP2 Cell Area, are presented as (i) raw values, (ii) values normalised to the average across differentiations, and (iii) fold change from PSEN1-Int4Del 808-04, which was included in each differentiation. No significant differences were found between genotypes using a Kruskal-Wallis test with Dunn's correction. Data points represent individual cell lines averaged across 3-4 differentiations. Isogenic pairs are noted with shared point shape. Healthy Control 856 and its SNCA -/- are square points. iNDI KOLF lines are represented by stars. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and iNDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as "PSEN1 Misc." Scale bars represent 50 μ m.

mDAN cultures showed no immunoreactivity for vGLUT1 (Figure 2.13A), and only non-specific labelling not within nuclei for CUX2 (Figure 2.13B). Negative sheep-IgG labelling for the TH antibody is also shown in Figure 2.13Biv. mDAN cultures did express presynaptic markers Synapsin I/II (Figure 2.13C), though this was not extensively surveyed across all lines.

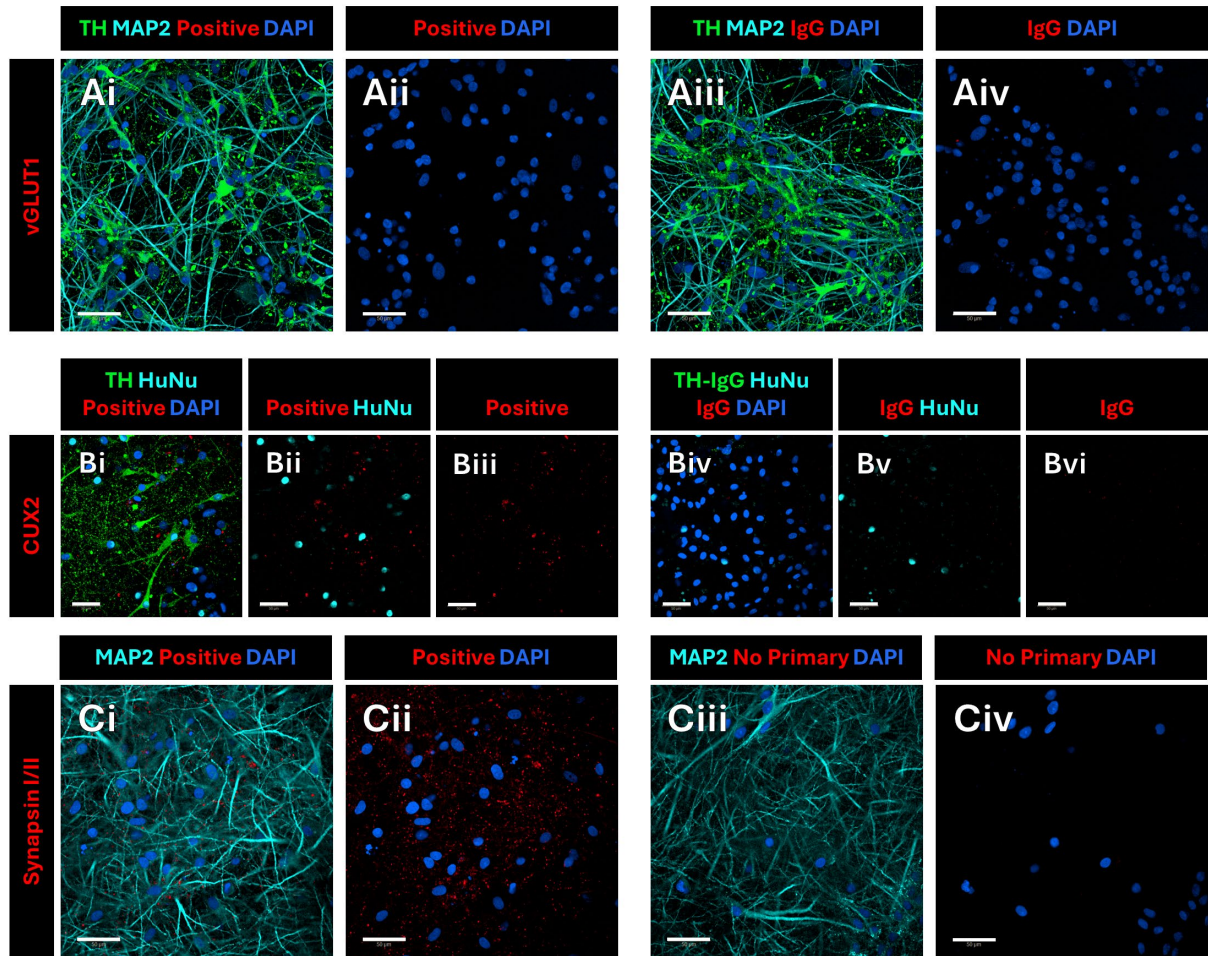


Figure 2.13. Synaptic, but no Excitatory Cortical Markers in mDAN Cultures. (A) Representative images of mDAN culture labelled for (Ai-ii) vGLUT1 or (Aiii-iv) rabbit-IgG negative control. (Ai,iii) show vGLUT1 or IgG labelling merged with TH and MAP2. (B) Representative images of mDAN culture labelled for (Bi-iii) CUX2 or (Biv-vi) rabbit-IgG negative control for CUX2 and sheep-IgG negative control for TH. (Biii,vi) show CUX2 or IgG labelling alone, and (Bii,v) merged with HuNu, and (Bi,iv) merged with HuNu and TH/Sheep-IgG. Scale bars represent 50 μ m. (C) Representative images of mDAN culture labelled for (Ci-ii) Synapsin I/II or (Ciii-iv) No primary control. (Ci,iii) show Synapsin I/II or No Primary labelling merged MAP2. Scale bars represent 50 μ m.

MEA Recordings

To gain insight into the electrophysiological activity of these cells, I grew a subset of cell lines as CNs and mDANs on MEA plates (Figure 2.14-15).

In CNs, while some spontaneous action potentials were evident as early as D40-50, robust firing did not take place until D60 (Figure 2.14A-B). Spike frequency and amplitude continued to increase over time, and by D70, the continuous noisy spiking activity became more organised into discrete, coordinated bursts, suggesting the development of functional synaptic networks in the culture. Across 2-3 independent differentiations, I reliably observed spontaneous activity that increased in frequency over time in all cell lines tested. However, the level of activity and differences between cell lines differed substantially between differentiations. Cells grown separately in 96-well plates labelled for presynaptic and postsynaptic markers showed development of synapses (Figure 2.14C-D).

Similarly, in mDAN cultures, I observed consistent but low frequency spontaneous activity as early as D36 (Figure 2.15A-B). This rapidly increased in frequency to bursts with as much as 400 Hz firing by D50, and slowly increased after this. Interestingly, mDAN cultures displayed a slightly different activity pattern to the cortical cultures, with tonic levels of low amplitude, lower frequency spikes, and regular short bursts of spikes occurring on top of these, that became more defined over time, again suggestive of the development of functional synapses.

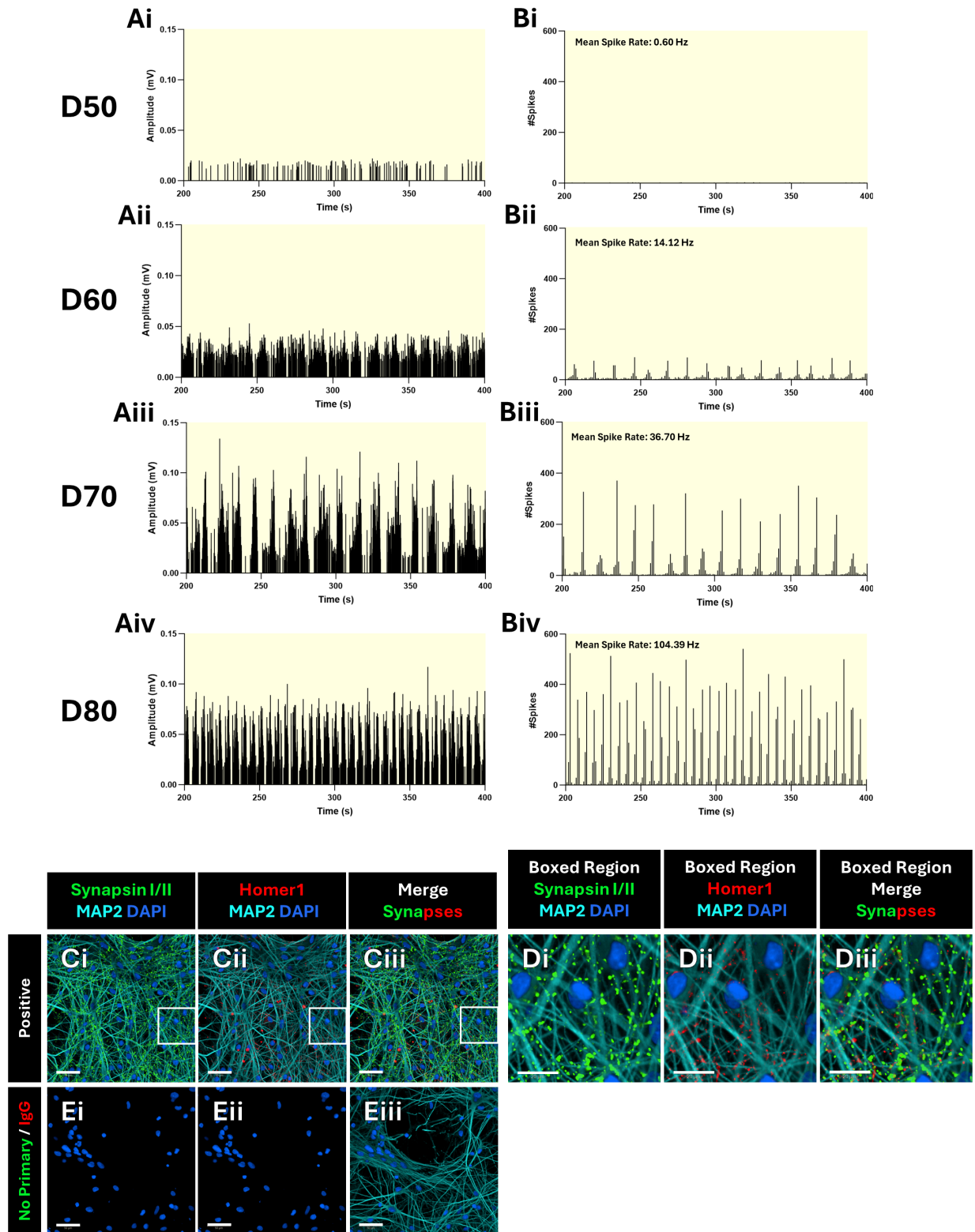


Figure 2.14. CNs are Electrophysiologically Active and Express Synapses by D70. (A-C) CNs grown on multielectrode array (MEA) plates were recorded for 15 minutes every 10 days from D50 of differentiation. (A-B) show, for a representative 200s period of a recording, (A) amplitudes of individual recorded spikes, and (B) a histogram of the number of spikes per one second bucket. (C-D) Representative immunolabelling of synapses in CN cultures grown on 96-well plates, fixed at D70, showing (i) presynaptic marker Synapsin I/II and (ii) postsynaptic marker Homer1 (iii) in apposition on MAP2+ neurites. (F) IgG negative control labelling for Homer1 and No primary control labelling for Synapsin I/II. Scale bars represent 50 μ m.

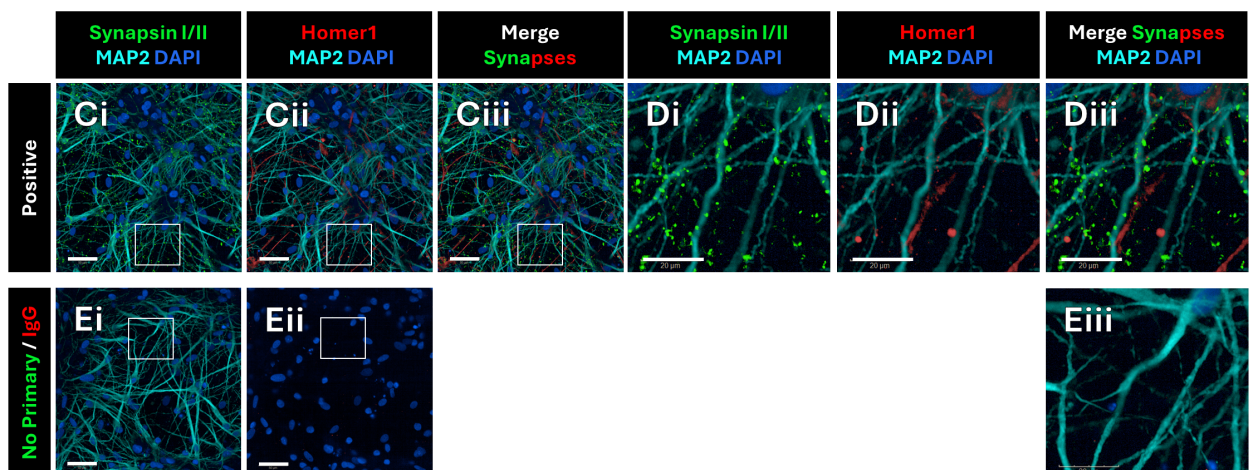
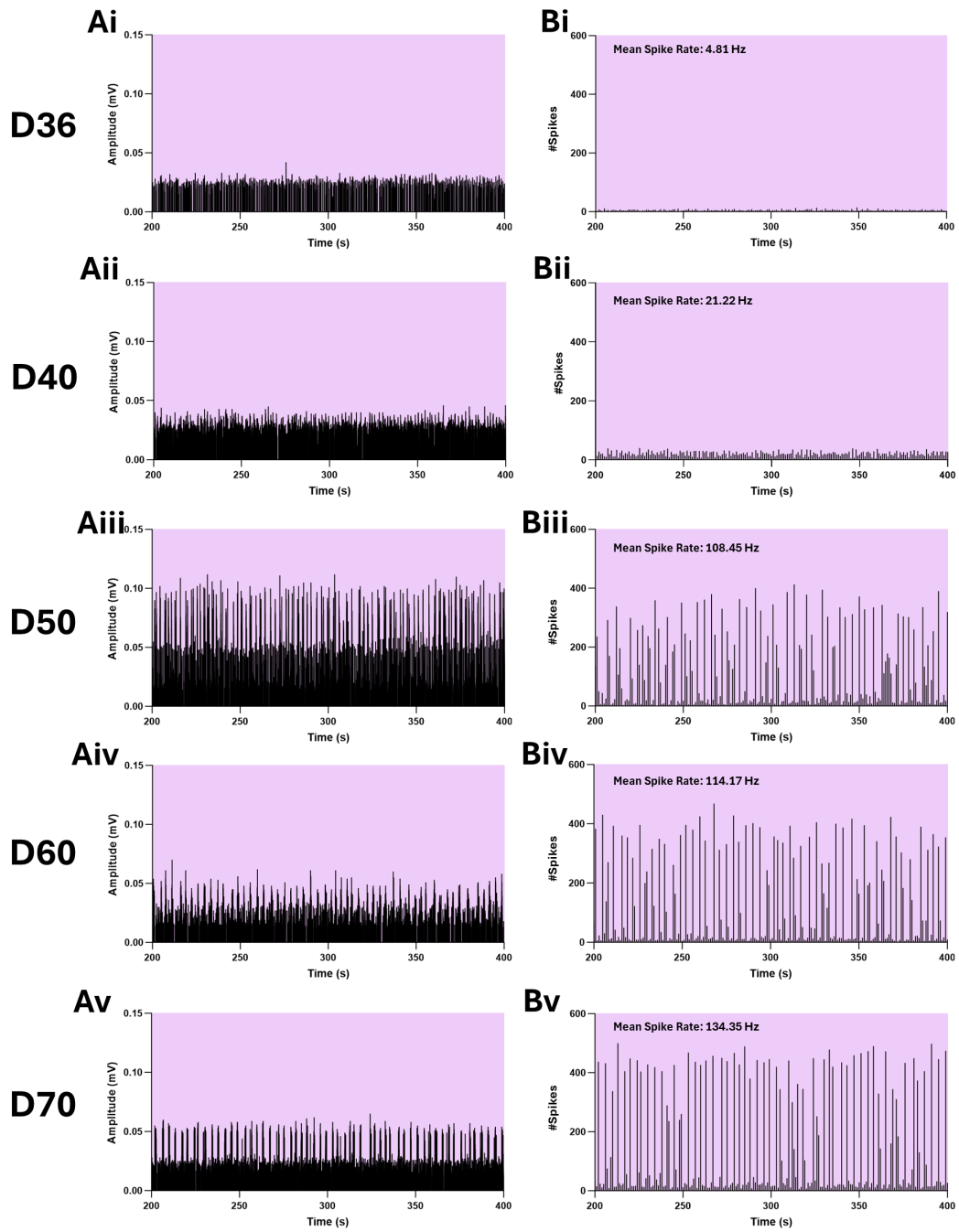


Figure 2.15. mDANs are Electrophysiologically Active by D50 and Express Synaptic Markers by D36. mDANs grown on multielectrode array (MEA) plates were recorded for 15 minutes on D36 and then every 10 days from D40 of differentiation. **(A-B)** show, for a representative 200s period of a recording, **(A)** amplitudes of individual recorded spikes, and **(B)** a histogram of the number of spikes per one second bucket, recorded from a representative well **(C-D)** Representative immunolabelling of synaptic markers in mDANs cultures grown on 96-well plates, fixed at D36, showing (i) presynaptic marker Synapsin I/II and (ii) postsynaptic marker Homer1 (iii) in apposition on MAP2+ neurites. **(F)** IgG negative control labelling for Homer1 and No primary control labelling for Synapsin I/II. Scale bars represent 50 μm .

2.3.3. ASyn PFFs

PFFs were generated and imaged under electron microscopy by Dr. Nora Bengoa-Vergniory.

These images showed that ASyn successfully aggregated into PFFs, and these included a mixture of conformers and lengths, including many around 50 nm.

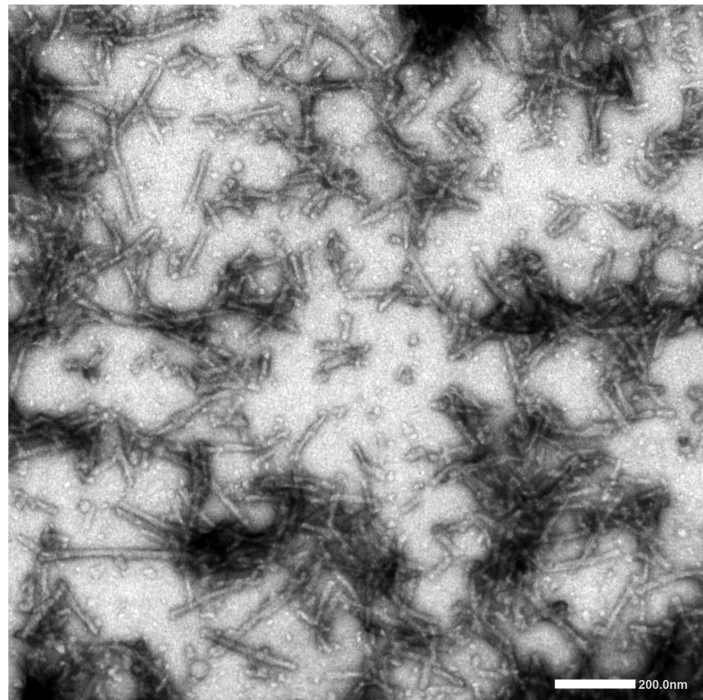


Figure 2.16. Electron Microscopy Image of Alpha Synuclein Pre-Formed Fibrils. Image captured by Dr. Nora Bengoa-Vergniory, showing ASyn PFFs generated after sonication with a range of lengths, including many around 50-100 nm. Scale bar represents 200 nm.

2.4. Discussion:

2.4.1. Results Summary

In this chapter, I outlined the protocols for generating CN and mDAN cultures and performing ASyn PFF insults, and characterise these. These characterisation experiments had the aim of confirming the identity of the cell cultures and informing a rationale for PFF insult timing. CN cultures differentiated using a three-phase process were MAP2+, β 3T+, and vGLUT1+, and mostly CUX2+, representing excitatory upper layer cortical projection neurons. mDAN cultures differentiated using an analogous three phase process were MAP2+ and TH+, and midbrain markers LMX1A and FOXA2 were also highly expressed across all cell lines.

As markers of relatively mature neurons, both CN and mDAN cultures were robustly ASyn-4D6+, exhibited synapses, and showed spontaneous electrophysiological activity on MEA that showed signs of functional synapse development and distinct neuronal firing patterns. Finally, PFFs generated showed a mixture of fibrils of different sizes (most around 50 nm), and limited oligomer structures.

2.4.2. Cortical Neuron Differentiation & Characterisation

In addition to generic neuronal markers MAP2 and β 3T, I sought to use cortical and excitatory neuron markers to specify the identity of cells generated by the CN protocol. CUX2 is mostly expressed in excitatory CNs (RNA Human protein atlas), and is a marker of upper layer CNs, which are typically the more vulnerable layers in AD (Heiko Braak et al. 2006; Fu, Hardy, and Duff 2018; Kampmann 2024).

Many single cell analyses of hiPSC-derived CNs produced through NGN2 expression find high expression of markers matching excitatory, glutamatergic cortical projection neurons (Meijer

et al. 2019; Lin et al. 2021; Das et al. 2024) which concurs with observations here, and marks these cells as a good model for those vulnerable in AD. Das et al. (2024) show more robust expression of vGLUT2 than vGLUT1, which may explain the low levels seen in some cell lines in this study.

One study also showed the generation of peripheral sensory neurons through NGN2 differentiation, alongside cortical phenotypes (Lin et al. 2021). Here, rather than directly inducing differentiation from hiPSCs using NGN2, I used an initial pharmacologically guided phase before forced expression of NGN2. This makes the protocol substantially more scalable, important for the size of the experiments conducted here. Alongside the extended use of AraC compared to other studies, these aspects of the protocol may prove beneficial to the purity of the final neuronal phenotype relative to other protocols (Yingsha Zhang et al. 2013; Meijer et al. 2019; Das et al. 2024).

A future approach might use DAPT as per Meijer et al. (2019) to promote formation of excitatory forebrain neurons (Qi et al. 2017) and to synchronise neural induction, because the NGN2 conversion is not as efficient at high densities. Here, CNs were grown at quite low densities, which did not impair differentiation, but enabled higher quality imaging and analysis of ASyn pathology performed in Chapter 3.

2.4.3. Use of Rat Astrocyte Coculture

In this protocol, CNs are cocultured alongside rAstros. This was found to be essential for maintenance of neuronal morphology and attachment beyond D50, in line with other studies using similar protocols (Meijer et al. 2019; Hedegaard et al. 2020; Ng et al. 2024). Thus, cells grown in monoculture for western blot or live assays were harvested at D50, before substantial

cell loss, and all ICC was conducted on neurons grown in coculture, since fixation and washing steps resulted in lost cells without astrocyte supported adherence.

Several studies have shown the importance of astrocytes for synaptic development (Pfrieger 2009; Eroglu and Barres 2010). As such, there is now substantial precedent for the coculture of astrocytes (either rodent primary, human primary, or hiPSC-derived) alongside hiPSC-derived CNs for *in vitro* modelling, including NGN2 CNs. Many studies have consequently found that astrocytes promote efficient maturation and synaptic connectivity (Lam et al. 2017; Prots et al. 2018; Meijer et al. 2019; Hedegaard et al. 2020; Das et al. 2024; Ng et al. 2024).

I used *rat* astrocytes to more easily distinguish between human neuronal and rat astrocytic cells through use of the human nuclear marker in ICC, and so that any ASyn that may be expressed by the rAstros would likely not cross seed with human ASyn PFFs (Luk et al. 2016; Polinski et al. 2018).

While the use of rAstros has numerous technical advantages, it does complicate the comparison of two different cell types for the purpose of examining selective neuronal vulnerability and cell autonomous mechanisms. To circumvent this for PFF-insult studies in Chapters 3-4, rAstros were also included at the same density in mDAN cultures, so that any differences observed in response to PFFs would be on account of the neurons. I also perform further experiments using live cell assays on cells in monoculture in Chapters 5-6, to ensure cell autonomous phenotypes. Other controls employed are detailed in Chapter 3.

2.4.4. Dopaminergic Neuron Differentiation & Characterisation

The use of primary rat cortical astrocytes has rarely been reported for mDAN differentiation and maturation protocols (Sánchez-Danés et al. 2012), and it was not clear how this would

impact differentiation efficiency. Therefore, I sought to quantify midbrain dopaminergic markers in these cultures.

Expression of TH, FOXA2, and LMX1A at D36, with coculture with rAstros, was found to be broadly in line with or higher than other studies using similar protocols at a similar time point (Zambon et al. 2019; Williamson et al. 2023). Beyond the use of rAstros, the dopaminergic protocol and phenotypes observed here are similar to what has been reported elsewhere, especially protocols employing an expansion step, which also makes the protocol more conceptually analogous to that for CNs (Fedele et al. 2017; Jonikas et al. 2018; Hiller et al. 2021; Beccano-Kelly et al. 2023). mDANs also developed spontaneous tonic activity characteristic of those in the ventral midbrain (Chung et al. 2016).

One potential downside of this model, compared to the CNs, however, is the relative heterogeneity of the culture. Not all cells in the culture are dopaminergic, and one study has shown not all the mDANs produced by Kriks-based protocols are of SNPC phenotype, with a more dominant VTA-type proportion of cells in the culture (Oosterveen et al. 2021). While this may be more physiological than a pure homogenous culture, it makes examining cell types of interest for selective vulnerability harder. Nevertheless, the heterogeneity of this model proves to be an asset in Chapter 4, where TH⁺ cells can be compared to TH⁻ cells in ICC.

Sheta et al. (2022) recently reported a forced NGN2 expression combined with dopaminergic patterning protocol for generation of mDANs, which might be a useful future strategy for improving the efficiency and purity of mDAN differentiation, and Oosterveen et al. (2021) outlines lentiviral transduction strategies and Wnt signalling modifications that can shift populations towards a purer SNPC phenotype.

2.4.5. Do the CNs and mDANs Appropriately Model Vulnerable Cells in AD and PD?

This leads to the key question for this thesis, which is whether these cell models of cortical and dopaminergic neurons are appropriate and sufficiently differentially vulnerable for the examination of selective neuronal vulnerability.

While the protocols have some conceptual differences, such as one being entirely growth factor guided while the other is a mixture of growth factor and transcription factor induced, they also have many parallels. These include utilising an initial dual SMAD inhibition step, and a substantial expansion phase and pause points, which are technically important to enable the large-scale assays conducted here.

However, while CNs are relatively vulnerable in AD, they are not the most vulnerable cell type. The hippocampal formation and noradrenergic locus coeruleus are the initial sites of much of the neurofibrillary and neurodegenerative lesions in the AD brain (Heiko Braak et al. 2006; 2011; Matchett et al. 2021; Bueichekú et al. 2024). While the vulnerable cells of the superficial entorhinal cortex and pyramidal cell layer in the HC do constitute excitatory, glutamatergic projection neurons, they do not express high levels of CUX2 (Human Protein Atlas), relative to other parts of the cortex. Nevertheless, while the neocortical layers which CUX2+ neurons represent are not involved at the earliest stages of AD, they are certainly more vulnerable than mDANs which, even in the case of concomitant ASyn pathology in AD, are affected less than excitatory glutamatergic projection neurons in the amygdala and cortex (Borghammer et al. 2021; Gcwensa et al. 2024; Jin et al. 2022; Leverenz et al. 2006; Raunio et al. 2019; Robinson et al. 2021; Toledo et al. 2016; Twohig and Nielsen 2019). Raunio et al. (2019) in fact find that ApoE4 status and AD pathology is more associated with cortico-limbic patterns of ASyn deposition, in contrast to brainstem predominant patterns.

Conversely, while mDANs are often not the first cells affected in PD, they are nevertheless consistently among the most vulnerable to dysfunction and ASyn aggregation. While it is SNPC dopaminergic neurons in the ventral tier that have attracted attention as the most vulnerable of these mDANs, LP is seen through the SNPC and even in VTA, which, though less vulnerable than SNPC, might be still considered as more vulnerable than CNs in PD. Furthermore, Braak et al. (2003)'s initial report states that cortical LP never appears to exist without brainstem involvement. Though the temporal procedure of ASyn pathology in PD and related synucleinopathies is contested, even later studies such as Borghammer et al. (2021)'s analysis of brain-first versus body-first patterns of synucleinopathy in DLB show that brain-first patterns still centre on the amygdala, entorhinal cortex, and SNPC, with relatively less involvement of the neocortical regions. These thus thoroughly implicate the midbrain and brainstem as being relatively vulnerable to LP, with CNs being relatively resilient, in PD.

Finally, as a practical consideration, many protocols for generating CNs and mDANs already exist and are amenable to modification, whereas protocols for differentiating olfactory bulb, DMNV, or even hippocampal neurons from hiPSCs are yet to be developed, and LC noradrenergic neurons have only been shown recently (Tao et al. 2023). This could be a useful line of future research.

Additionally, many of these regions are vulnerable in both PD and AD, especially the locus coeruleus. Examination of the CNs and mDANs here in opposition allows for a balanced experimental set up, where cells vulnerable to one disease and resilient to another are compared in cells from those two diseases.

2.4.6. KOLF2.1J Cell Lines

In an effort to examine phenotypes among a wider range of PSEN1 cell lines than those from StemBancc, we also sourced additional isogenic PSEN1 mutant cell lines with point mutations introduced into the healthy KOLF2.1J background (Pantazis et al. 2022), and these were included in later experiments. In characterisation of these lines, however, it appeared that the KOLF2.1J CNs behaved somewhat differently from other healthy controls, with lower CUX2, lower ASyn, and low vGLUT1 expression, compared to most other lines.

It has been recently reported that, despite extensive quality control from the supplier, the KOLF2.1J line does contain some variations associated with neurodevelopmental disorders (Gracia-Diaz et al. 2023). While no issues were noted in characterisation of KOLF2.1J mDANs, results from these cell lines are interpreted with some caution.

2.4.7. PFF Characterisation

For PFF generation, best practices with regards to pH, salt concentration, shaking times and temperatures, and concentrations outlined in Polinski et al. (2018) were adhered to. Unlike most protocols however, PFFs here were sonicated prior to freezing, and upon thawing, applied to cells without further sonication.

Sonication of fibrils results in a range of species, and it remains unclear whether oligomers, short fibrils, or longer structures are the most important seed for pathogenesis. Some studies have shown sonication must result in most fibrils at 50 nM or smaller in order to be maximally effective at seeding pathology, (Tarutani et al. 2016; Polinski et al. 2018), which we achieve here.

2.4.8. Rationale for PFF Insult Timepoints

The characterisation experiments performed here thus provide the data to develop a rationale for the PFF insult paradigm used in this thesis. It is thought that neuronal activity may drive ASyn phosphorylation, aggregation, and release (Yamada and Iwatsubo 2018; Wu et al. 2020; Ramalingam and Dettmer 2023; Ramalingam et al. 2023) and the energy demand of highly active neurons likely contributes to their vulnerability (Roselli and Caroni 2015; Fu, Hardy, and Duff 2018; Kampmann 2024). Having neurons exhibit electrophysiological activity and functional synapses, to be most physiologically relevant, is also desirable, and the importance of time in differentiation to achieve this has been emphasised (Beccano-Kelly et al. 2023). It is also known that endogenous expression of ASyn is essential for PFF-seeded aggregation (Volpicelli-Daley, Luk, and Lee 2014; Polinski et al. 2018), and that PFF-seeded aggregates appear in a time dependent manner (Gribaudo et al. 2019; Lam et al. 2024; Mahul-Mellier et al. 2020; Polinski et al. 2018; Tanudjojo et al. 2021; Tapias et al. 2017). Therefore, it is essential that cultures express ASyn at the point of insult, and ideally can subsequently be monitored for an extended period afterwards, with experimental timelines still being practical.

In balancing these considerations, I selected D70 to perform the PFF insult in CNs. This is slightly earlier than other studies using similar protocols with amyloid-beta insults (Ng et al. 2022; 2024). However, by D70, CNs nevertheless are highly electrophysiologically active, appear to have functional synapses, robustly express ASyn, and are well defined as CUX2+, with extensive dendritic arbours and networks. Furthermore, by coculture with rAstros, these cells could be reliably monitored for 40 days following insult, permitting longer insult timelines than has been examined previously.

Similarly, at D36, close to where many other studies perform experiments in similar models, despite this being a much shorter timeframe than CNs, mDANs nevertheless express similar, even slightly higher levels of ASyn than CNs, and are otherwise well defined as mDANs, lacking

in cortical markers. While they are not particularly electrically active at this point, Jonikas et al. (2018) find that Kriks mDANs are electrophysiologically active by D45. Here too, they do indeed rapidly become more active after D36, likely faster than in other studies due to the enhanced maturation with rAstros. The D36 timepoint also enabled a similarly long reliable monitoring period of 30-40 days after insult. Furthermore, at 16 DIV, D36 provided a similar point of comparison to D50 (DIV17) CNs grown in monoculture, for experiments in Chapter 5 and 6. Further optimisation of the coating and astrocyte coculture paradigms may enable even longer-term culture in the future.

For the purposes of this thesis, however, D70 CNs and D36 mDANs generated under the described protocols made for ideal points of insult for comparison, with similarly robust ASyn expression, synaptic formation, and distinct neuronal identities. The well-defined and distinct neuronal identities are particularly important for the purpose of considering selective neuronal vulnerability, and, as noted in the introduction, is often overlooked.

In the following two chapters, I thus apply this PFF insult regime to the CNs and mDANs in turn and compare results and underlying pathways in Chapter 5.

Chapter 3.

Alpha Synuclein PFF-Seeded Pathology in Cortical Neurons

3.1. Introduction

In this chapter, I examine the vulnerability of cortical neurons (CNs) to pathology seeded by alpha synuclein (ASyn) pre-formed fibrils (PFFs). The cortex in the brain, especially more superficial layers, are thought to be relatively resilient to ASyn pathology (Borghammer et al. 2021; Heiko Braak et al. 2003; Surmeier, Obeso, and Halliday 2017b), specifically Lewy Bodies (LBs) and Lewy Neurites (LNs), collectively referred to here as Lewy Pathology (LP). The few prior examinations of ASyn PFFs in hiSPC CNs typically find minimal LP inclusions, and often rely on cell lines with *SNCA* mutations and/or overexpression (Bayati et al. 2023; Gribaudo et al. 2019; Iannielli et al. 2022; Lam et al. 2024; Vajhøj et al. 2021).

Very little is known about the effects of ASyn PFFs in longer term CN cultures, or in AD *PSEN1* and PD *LRRK2*, most of which have not been examined before. PFF-seeded pathology is known to be time and dose dependent, and rely on the expression of the endogenous protein (Polinski et al. 2018). Therefore, since the cell lines under examination here express WT, physiological ASyn, to maximise the chance of observing pathological changes in these relatively resistant cells, I grew cells out to D70 of differentiation, when ASyn expression was robust, and monitored cells for up to 40 days after insult. This is 10 days longer than has been reported elsewhere in the literature (Gribaudo et al. 2019), and at least 19 days longer than most studies.

The aims of this thus relatively exploratory chapter are thus to:

- 1) Demonstrate an inclusionopathy model in hiPSC-derived for comparison with mDANs.
- 2) Determine the degree to which hiPSC CNs are vulnerable to ASyn PFF seeded pathology in the various AD and PD genotypes examined.
- 3) Measure other effects of ASyn PFFs and resultant pathology on cell and nuclear morphology, as indicators of vulnerability.
- 4) Confirm that seeded pathology occurs in neurons, not in cocultured rat astrocytes.

3.2. Methods

The PFF insult paradigm used in the CNs is described in Chapter 2. Briefly, cells grown in 96-well plates are insulted at D70, and then half media changes resume on D74. PFFs are not washed off after this long incubation time, consistent with other studies, in order to maximise uptake and resultant pathology (Gao et al. 2019; Lam et al. 2024; Vajhøj et al. 2021). As a positive control, in some cases, I also include chloroquine, an autophagy blocker, alongside PFF insult (Mauthe et al. 2018). Similarly, this is also not washed out, but diluted over time.

Fujiwara et al. (2002) first observed that ASyn undergoes phosphorylation at the S129 residue (pASyn-S129) when recruited into Lewy-type inclusions. This has since been extensively reported, and pASyn-S129 is thus used as a standard immunological marker of LP, and is likely an early pathological indicator, occurring before insoluble aggregates (Bartscher et al. 2020; MacIsaac et al. 2020; Mahul-Mellier et al. 2020; Moors et al. 2021; Volpicelli-Daley et al. 2011).

In these experiments, I thus use immunocytochemical labelling of pASyn-S129 in PFF-insulted cells as a primary outcome. Immunological and imaging methods are described in Chapter 2, and image analysis pipelines are described alongside results.

3.3. Results

3.3.1. Qualitative Observations of pASyn-S129+ Pathology in CNs

CNs cocultured with rat astrocytes were insulted with ASyn PFFs at D70, and subsequently fixed 10, 20, 30, or 40 days later. Fixed cells were then immunolabelled for pASyn-S129, as well as cell markers MAP2 and β 3T, and nuclei were stained with DAPI (Figure 3.1). Remarkable pASyn-S129+ pathology presented as early as 10 days after insult in some cell lines, and at a variety of different dosage conditions. Mostly, these seeded aggregates were observed in PSEN1-Int4Del CNs (Figure 3.2). Pathology included punctate labelling, long Lewy neurite (LN)-like structures, and round, perikaryal Lewy body (LB)-like structures. Though these were not ultrastructurally characterised, for simplicity, these are referred to as LNs and LBs.

In some cases, pASyn-S129+ pathology, especially some of the longer thread-like structures appeared to be outside of MAP2+ neurons. Co-labelling with neuronal marker β 3T revealed that these inclusions were typically in β 3T+ neurites, presumed to be axons (Figure 3.3). Often, the presence of pASyn-S129+ neuritic pathology was associated with weaker labelling of the cell marker (MAP2 or β 3T, as in Figure 3.3C). Nevertheless, it appeared that all pASyn-S129+ labelling is confined to identifiably neuronal structures.

Examining vulnerability to aggregation across cell lines from different genotypes at 30 days after insult, the PSEN1-Int4Del clonal pair exhibited a high pathological burden (Figure 3.4C-D), while other genotypes, including their corrected control, LRRK2 cell lines, and healthy controls exhibit a lower level of accumulation (Figure 3.4). Except for the *SNCA*-KO line, where no pASyn-S129+ labelling was observed, CNs of all genotypes displayed various forms of pathology, including punctate, neuritic, and perikaryal inclusions. However, pathology appeared to manifest with higher frequency and severity in the PSEN1-Int4Del cell lines, especially the -04 clone. By contrast, no pASyn-S129+ pathology was observed in rAstros

grown alone in the same NGN2 media after the same duration following PFF insult (Figure 3.4K).

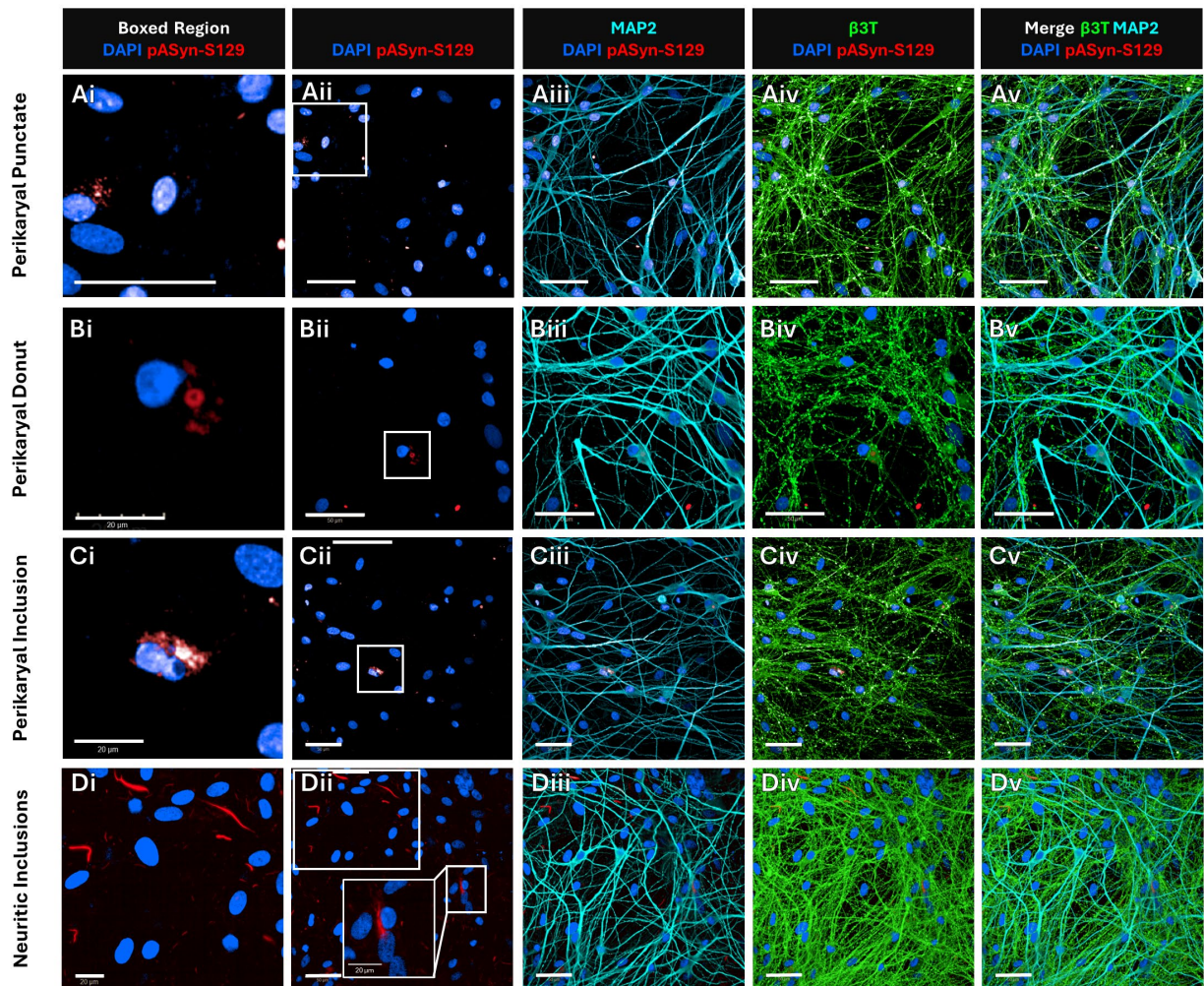


Figure 3.1. Examples of pASyn-S129+ Labelling in MAP2+ β 3T+ Cortical Neurons. Inclusions with a variety of morphologies were observed in PFF-insulted cells after 30-40 days, including **(A)** punctate perikaryal labelling, **(B)** donut-like inclusions adjacent to nuclei, **(C)** larger, more amorphous structures concentrated on one side of nuclei, and **(D)** neuritic inclusions, either within **(iii)** MAP2+ cells and dendrites, or **(iv)** β 3T+ axons. **(Dii)** also shows a perikaryal inclusion extending into the apical dendrite. These images are from PSEN1-M146I and LRRK2-R1441C cell lines. Scale bars in (i) are 20 μ m. Scale bars in (ii-v) are 50 μ m.

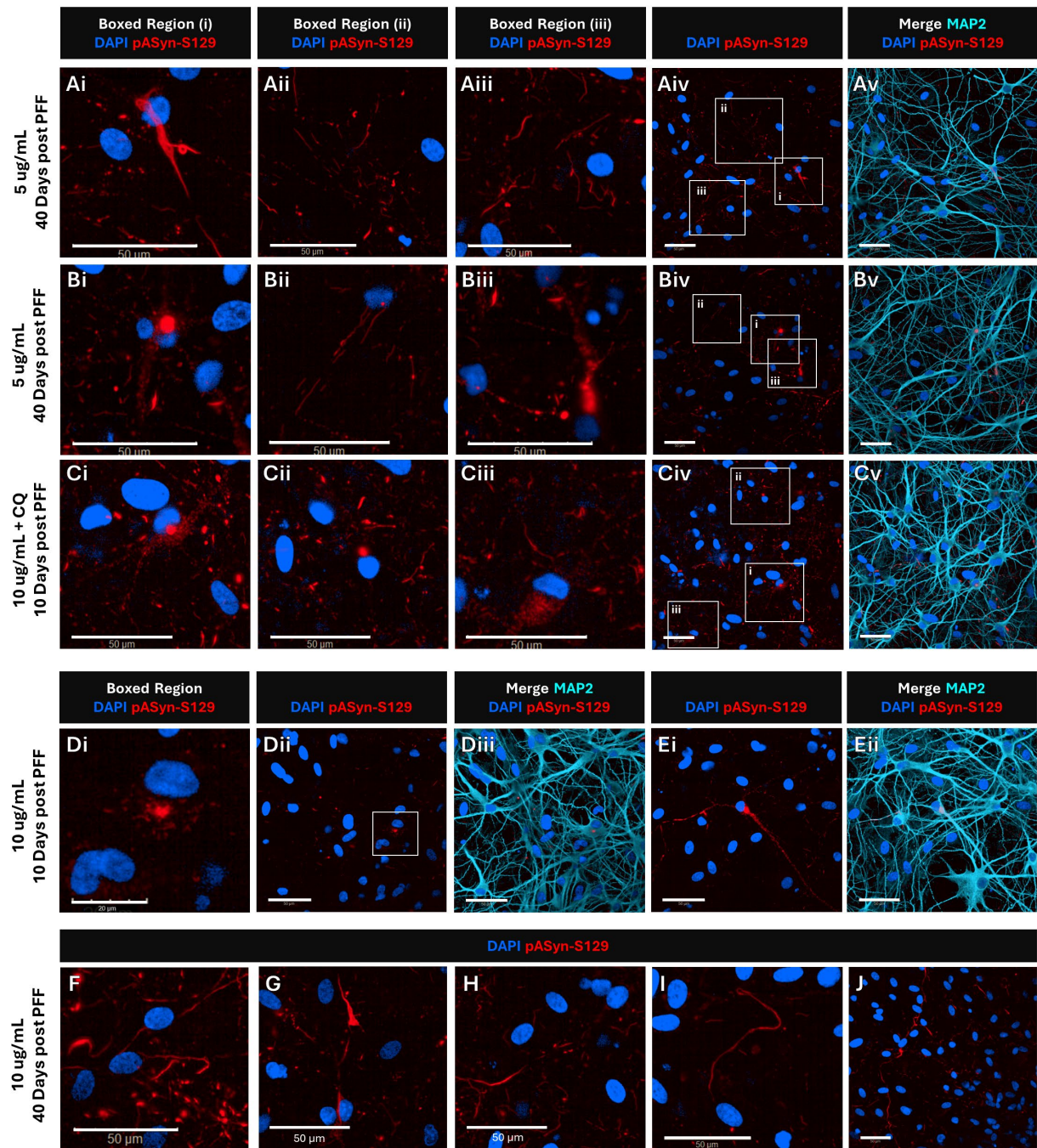


Figure 3.2. Examples of Large Somatic Lewy Body-Like, and Lewy Neurite-Like Labelling in PSEN1-Int4Del Cortical Neurons. A wide range of inclusion morphologies were observed in PSEN1-Int4Del cell lines by 40 days (A-B, F-J), but as early as 10 days (D-E), and especially at 10 days with chloroquine (CQ) treatment (C). **(A-C)** Structures included neuritic inclusions of varying lengths (A, B-Cii) which frequently reached into the soma (Ai, Bii); punctate pASyn-S129+ labelling of various sizes in the soma that extended out into dendrites and coalesced into large, round, nuclei adjacent inclusions (B-Ci); large punctate and/or globular inclusions extending between soma and apical dendrites (Biii); large, round, Lewy Body-like inclusions adjacent to the nucleus (Cii), and dense, punctate, pale body-like labelling filling the soma adjacent to nuclei (Ciii). **(D)** Punctate somatic inclusions in an apparent distention of the neuronal cell body. **(E)** Punctate labelling extending throughout the apical and basal dendrites of a pyramidal neuron, including some longer Lewy neurite-like inclusions in the basal dendrites. These appear to coalesce into a large somatic inclusion at the base of the apical dendrite, similar to B-Cii. **(F-J)** Further exemplars of Lewy neurite-like labelling, including hook-like shapes (F), large ‘flame-like’ aggregates extending from soma into dendrites (G), shorter, thinner fibrils, ~10-50 µm (H-I), and extremely long structures extending 100s of µm (J). Scale bars are 50 µm, except Di, 20 µm.

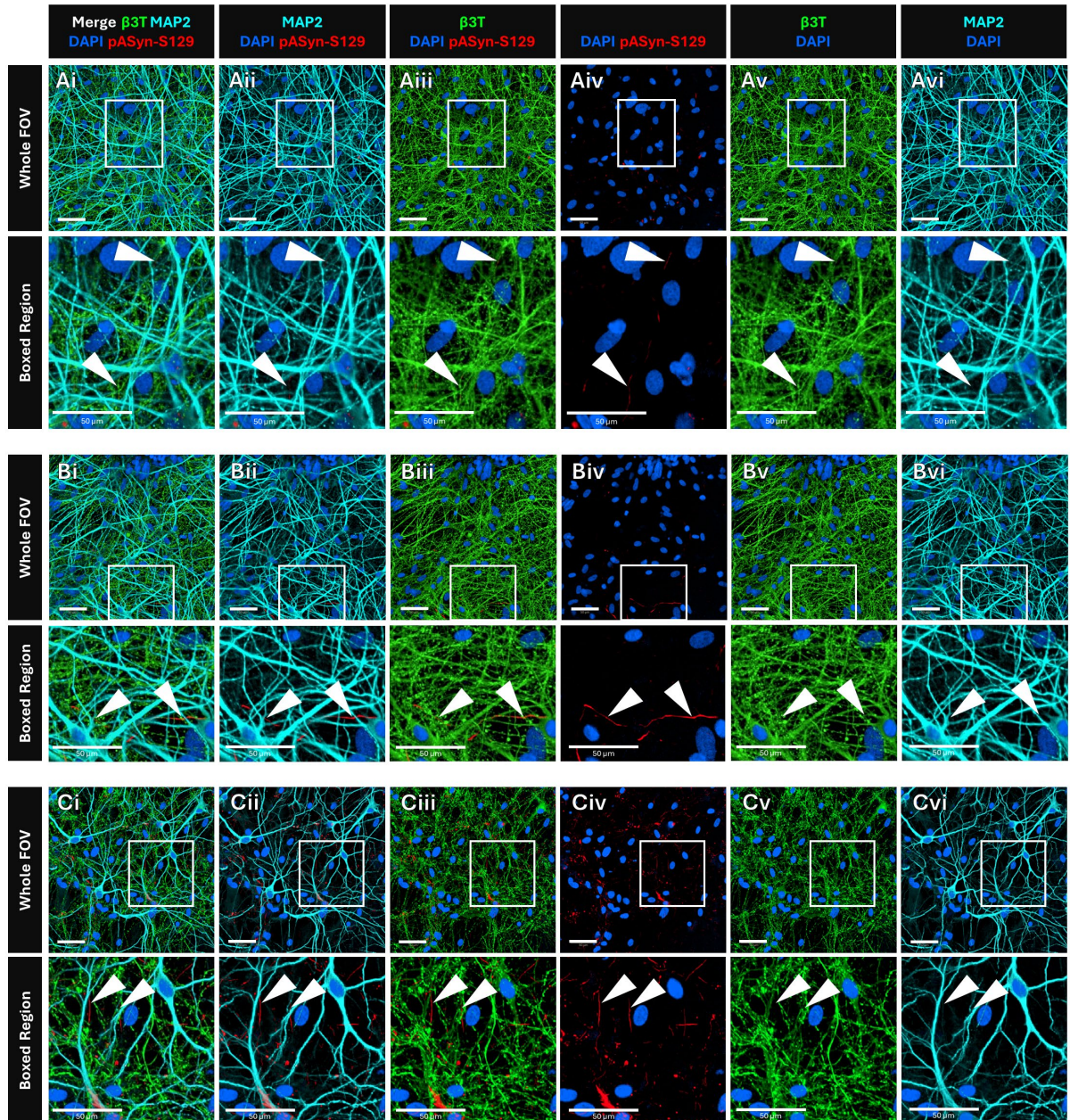


Figure 3.3. MAP2- Neuritic pASyn-S129+ Labelling is in β 3T+ Axons. Some neuritic inclusions (arrow heads) appeared to be outside MAP2+ cells. Here, Lewy neurites that are not within MAP2 structures are shown to typically colocalise with β 3T structures. Merge images are shown in (i), with pASyn-S129+ labelling shown against MAP2+ labelling only (ii) or β 3T+ labelling only (iii). Then, pASyn-S129+ labelling alone (iv), β 3T+ labelling alone (v), and MAP2+ labelling alone (vi) are shown, with DAPI for context. In some cases, as in (C), pASyn-S129+ neuritic inclusions appear to obscure β 3T+ labelling. For example, no β 3T labelling is visible for the left inclusion (Cv), though a faint MAP2+ process is (Cvi). Scale bars are 50 μ m.

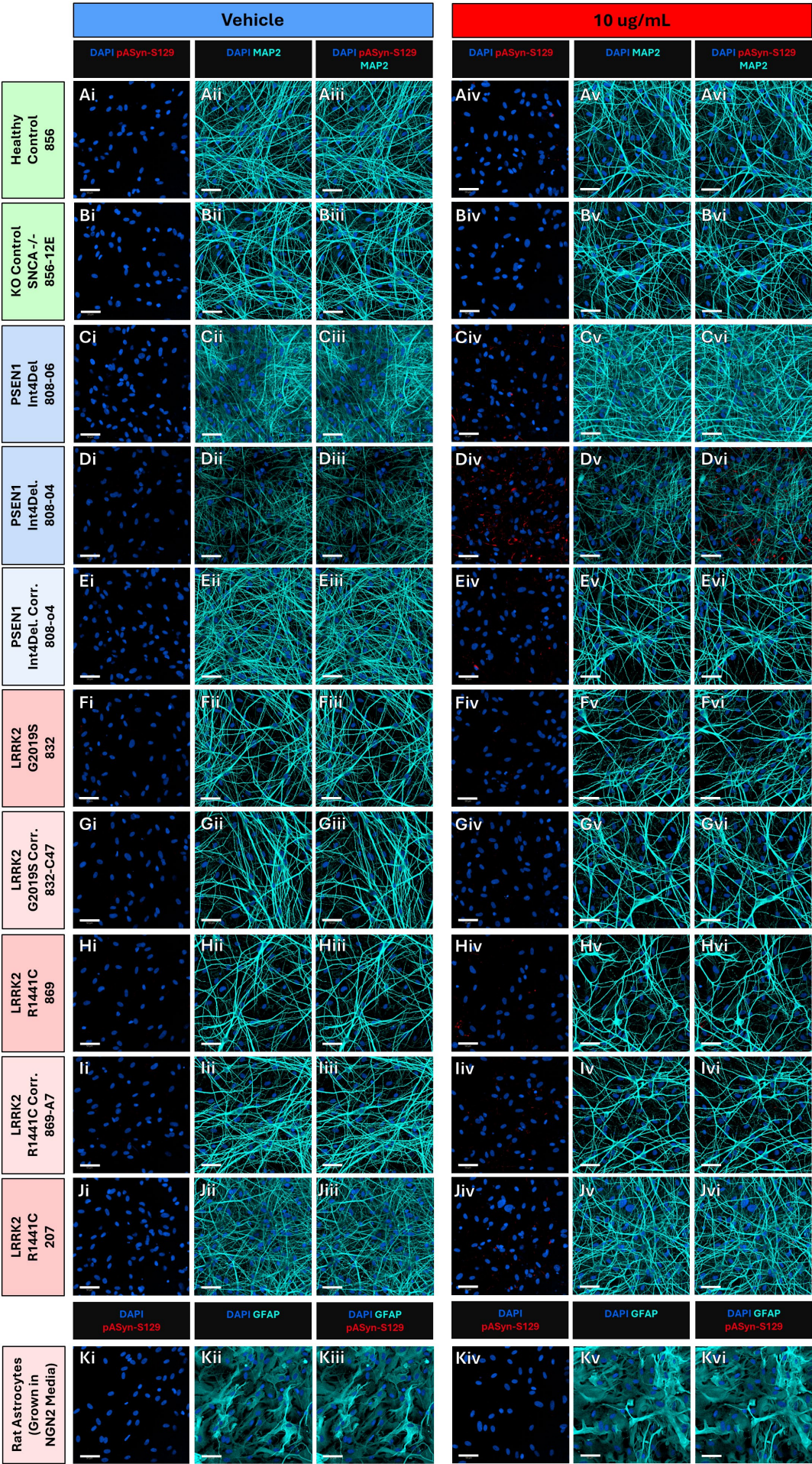


Figure 3.4. pASyn-S129+ Labelling Evident in All Cell Lines Except SNCA-KO and rAstrocytes. Representative images from cells 30 days after 10 µg/mL PFF insult from a range of different genotypes are shown, with **(i-iii)** vehicle treated cells on the left, and **(iv-vi)** PFF-insulted cells on the right. With the exception of SNCA-KO, all genotypes, including healthy and corrected controls, exhibited some neuronal pASyn-S129+ labelling, though typically not as severe as in PSEN1-Int4Del (D-E) cell lines. **(J)** LRRK2-R1441C 207 also developed marked inclusions. **(K)** No pASyn-S129+ labelling was observed in rAstros grown without neurons in NGN2 media and insulted with ASyn PFFs in parallel to neurons. Scale bars are 50 µm.

pASyn-S129+ labelling in the PSEN1-Int4Del cell lines (Figure 3.5) and its corrected control line (Figure 3.6) show that pathological development appeared to be both dose and time dependent. In the PSEN1-Int4Del cell line, profound neuritic pathology was observed as early as 10 days, including some larger perinuclear structures at higher doses, and these became exacerbated in size and frequency over time (Figure 3.5). In the corrected control line, some smaller neuritic pathology is evident at 10 days, with limited perinuclear aggregation at higher concentrations, and this also appears to increase over time in size and frequency, though appearing to reach a far lower overall pathological load (Figure 3.6).

No pASyn-S129+ labelling could be observed in the vehicle treated condition for either PSEN1-Int4Del or corrected control line, beyond levels of non-specific labelling in the IgG isotype negative control, which was minimal. Labelling patterns in the corrected control line were typical of all cell lines other than the PSEN1-Int4Del clonal pair and SNCA-KO.

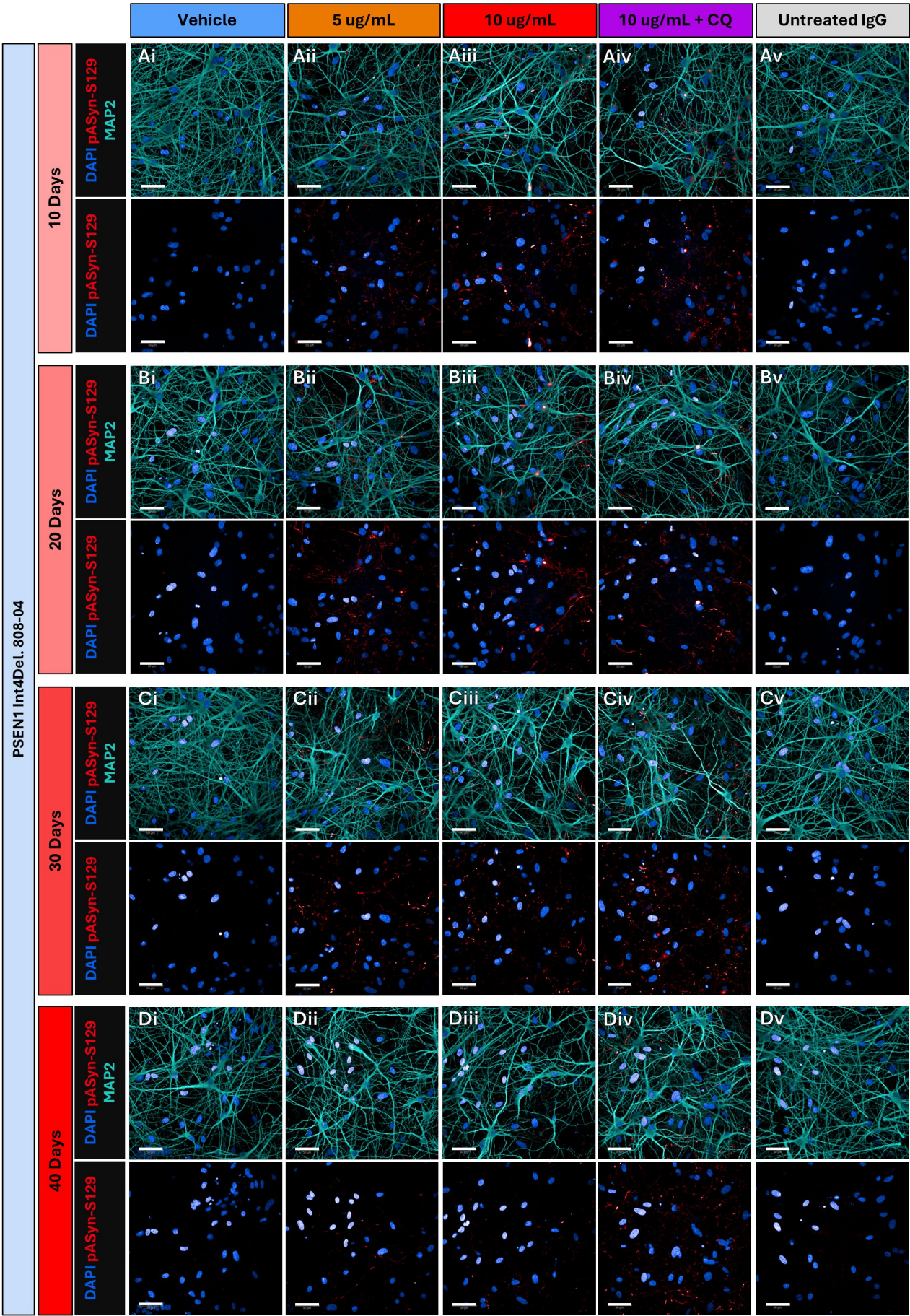


Figure 3.5. pASyn-S129+ Labelling Across Dosage and Time in PSEN1-Int4Del. The most labelling was observed in PSEN1-Int4Del cell lines, and representative images from the 808-04 cell line are depicted here. Cells were harvested at **(A)** 10, **(B)** 20, **(C)** 30, and **(D)** 40 days after PFF insult, with pASyn-S129+ inclusions becoming more severe over time. Cells were treated with **(i)** vehicle **(ii)** 5 µg/mL, **(iii)** 10 µg/mL, or **(iv)** 10 µg/mL with 20 µM chloroquine, an autophagy blocker, and a dose dependent effect was evident. **(v)** Minimal labelling was apparent in the PFF-untreated, IgG negative control labelled cells. Scale bars are 50 µm.

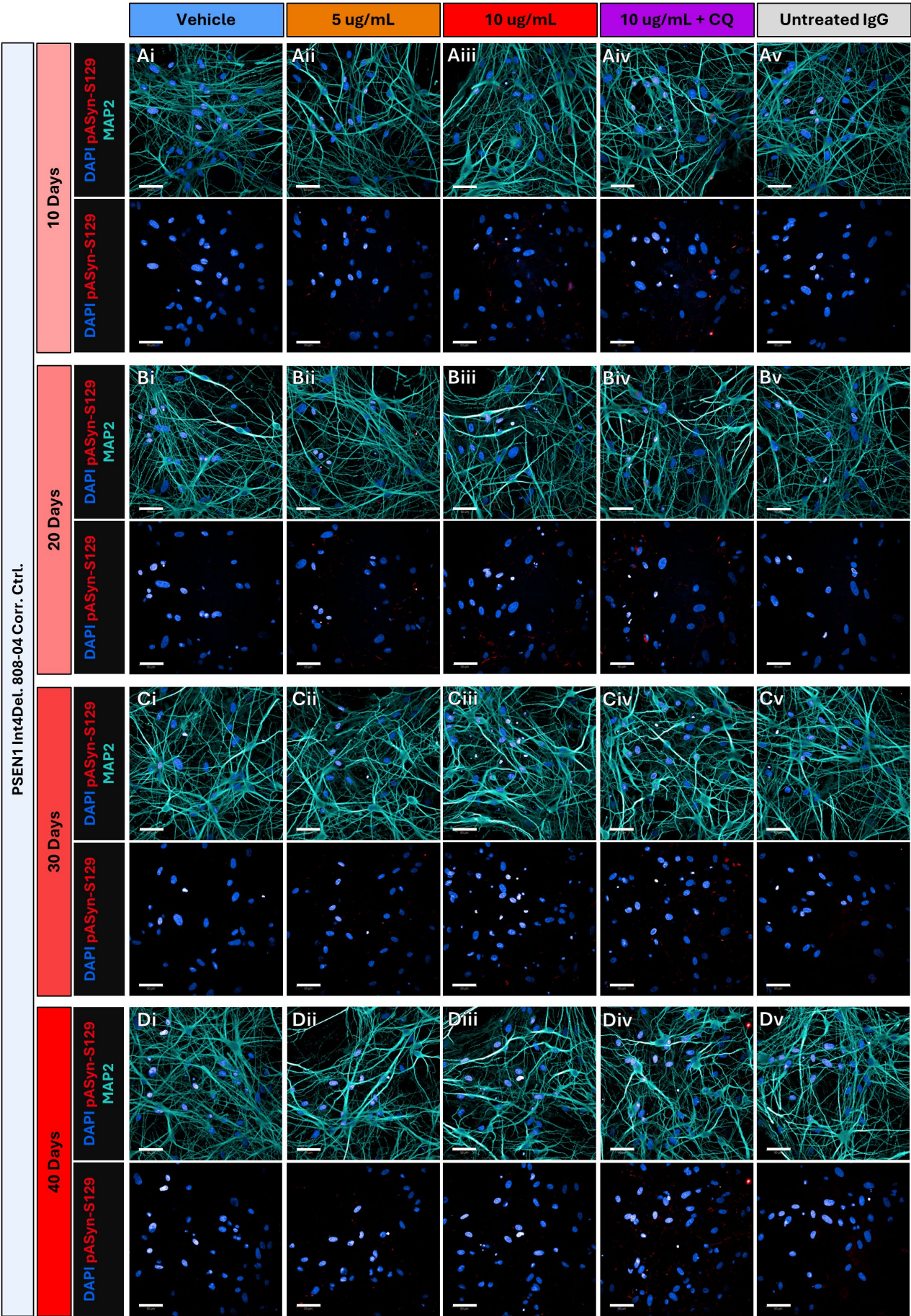


Figure 3.6. Dose and Time Dependent pASyn-S129+ Labelling Still Evident in PSEN1-Int4Del Corrected Control. While the most labelling was observed in PSEN1-Int4Del cell lines, pASyn-S129+ labelling was still apparent in its isogenic corrected control, though much less, and representative images from this are depicted here, in a paired panel with Figure 3.5. Some somatic and LB-like inclusions are present as early as 10 days, especially with CQ treatment. Scale bars are 50 μ m.

3.3.2. Quantitative Assessment of pASyn-S129+ LP in CNs

To quantify the changes to cells in these images, a custom pipeline was designed in Harmony 5.2 software (Figures 3.7-9).

Images were split into separate channels to create compartmentalised ROIs, as per Figure 3.7, within which to localise pathology. These included the total cell area, somatodendritic region (MAP2+), and axonal area (MAP2- β 3T+). DAPI nuclei were also separated out, and a series of filters were applied to select for only neuronal nuclei, ignoring rAstro nuclei, as outlined in Figure 3.8. These could be used as a definitional landmark for nearby pathology.

To quantify the pASyn-S129+ pathological burden in the cells, I categorised labelling patterns into three main categories: a broad LP to capture labelling of all morphologies, mostly punctate; then LNs and LBs. This is described in Figure 3.9.

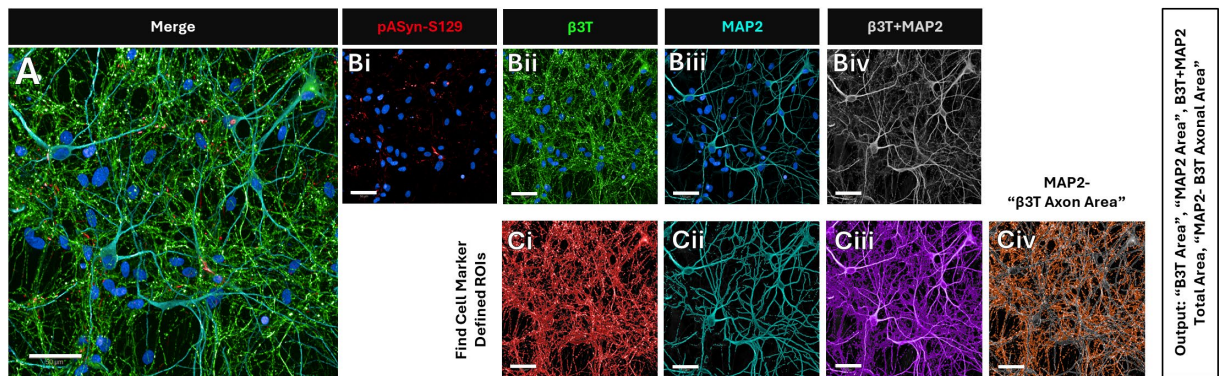


Figure 3.7. Analysis Pipeline for Neuronal Morphology Labelling and ROI Construction in Cortical Neurons. To analyse pASyn-S129+ labelling in MAP2 and β 3T-labelled neurons, neuronal ROIs were defined. **(A)** Shows a representative image with pASyn-S129+ labelling of various punctate, neuritic and somatic inclusions in β 3T+ and MAP2+ neurons. **(Bi-iii)** These channels are shown separately. **(Biv)** A new channel, merging the MAP2 and β 3T channel was created. **(C)** From the MAP2 and β 3T channels, four ROIs were created, using the Find Image Region function with common threshold. **(Ci)** β 3T Area, **(Cii)** MAP2 Area, **(Ciii)** β 3T+MAP2 “Total” Cell Area, **(Civ)** “ β 3T Axonal Area” created by subtracting MAP2+ soma and dendrites from the total cell area, thus leaving only β 3T+ axons. These ROIs were used to spatially compartmentalise pASyn-S129+ labelling, and their area was calculated. Scale bars are 50 μ m.

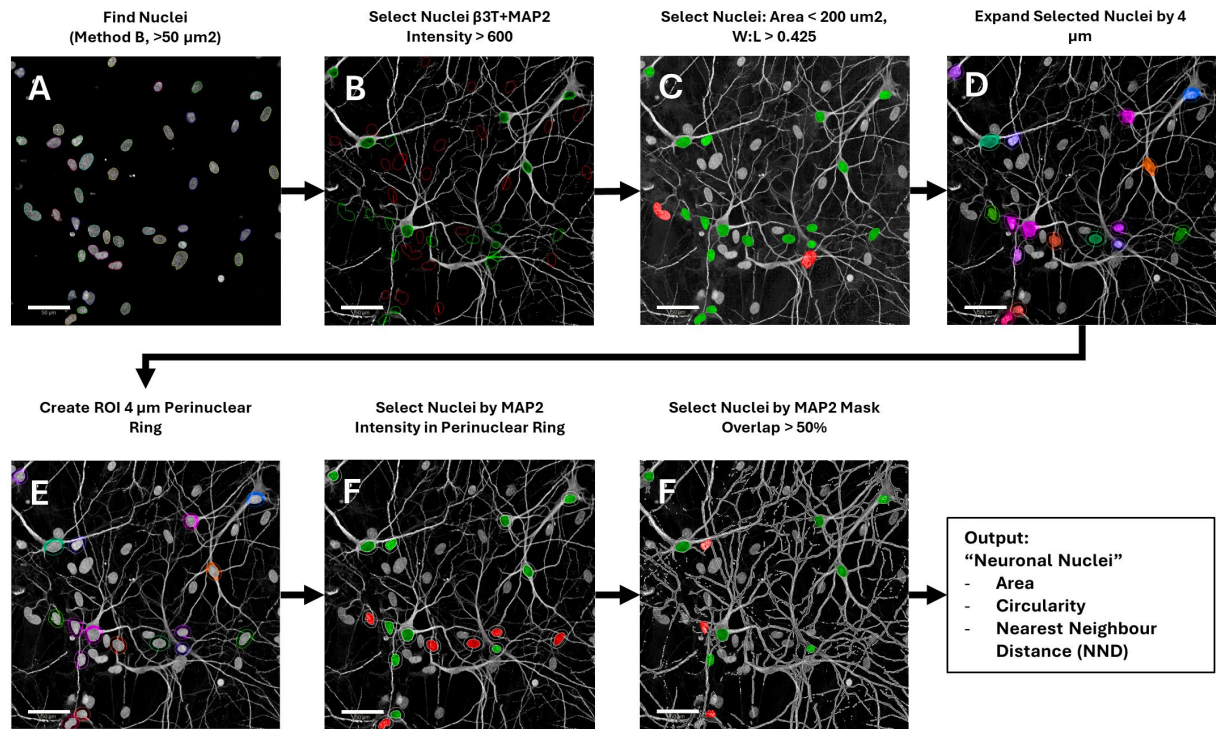


Figure 3.8. Analysis Pipeline for Neuronal Nuclei Labelling in Cortical Neurons. To distinguish human neuronal nuclei from rat astrocyte nuclei, since the HuNu antibody was not used here, a series of filters were used to progressively screen out non-neuronal (not clearly in a MAP2+ cell body) nuclei. **(A)** All DAPI-labelled nuclei were identified using the Find Nuclei Function (Method B). Nuclei were defined as $> 50 \mu\text{m}^2$ to exclude most pyknotic nuclei. **(B)** A nuclear ROI was created, and nuclei below a set threshold intensity in the $\beta\text{3T}+\text{MAP2}$ channel were excluded. **(C)** Nuclei larger than $200 \mu\text{m}^2$ or with a width:length ratio of < 0.425 were excluded. **(D-E)** A perinuclear ring ROI was then created by expanding the nuclear ROI by $4 \mu\text{m}$ and subtracting the DAPI+ nucleus itself. This captured more intense MAP2+ labelling around the nucleus, since labelling colocalised with DAPI was much weaker. **(F)** Nuclei with subthreshold MAP2+ labelling in the perinuclear ring were excluded. **(G)** Nuclei with $< 50\%$ overlap with the MAP2 mask were excluded. The remaining nuclei were termed Neuronal Nuclei, and their number, area, circularity, and distance to nearest other neuronal nucleus (nearest neighbour distance, NND) measured. In these images, DAPI and MAP2 labelling are shown in greyscale, with green highlights for selected objects and red highlights for excluded objects. Scale bars are $50 \mu\text{m}$.

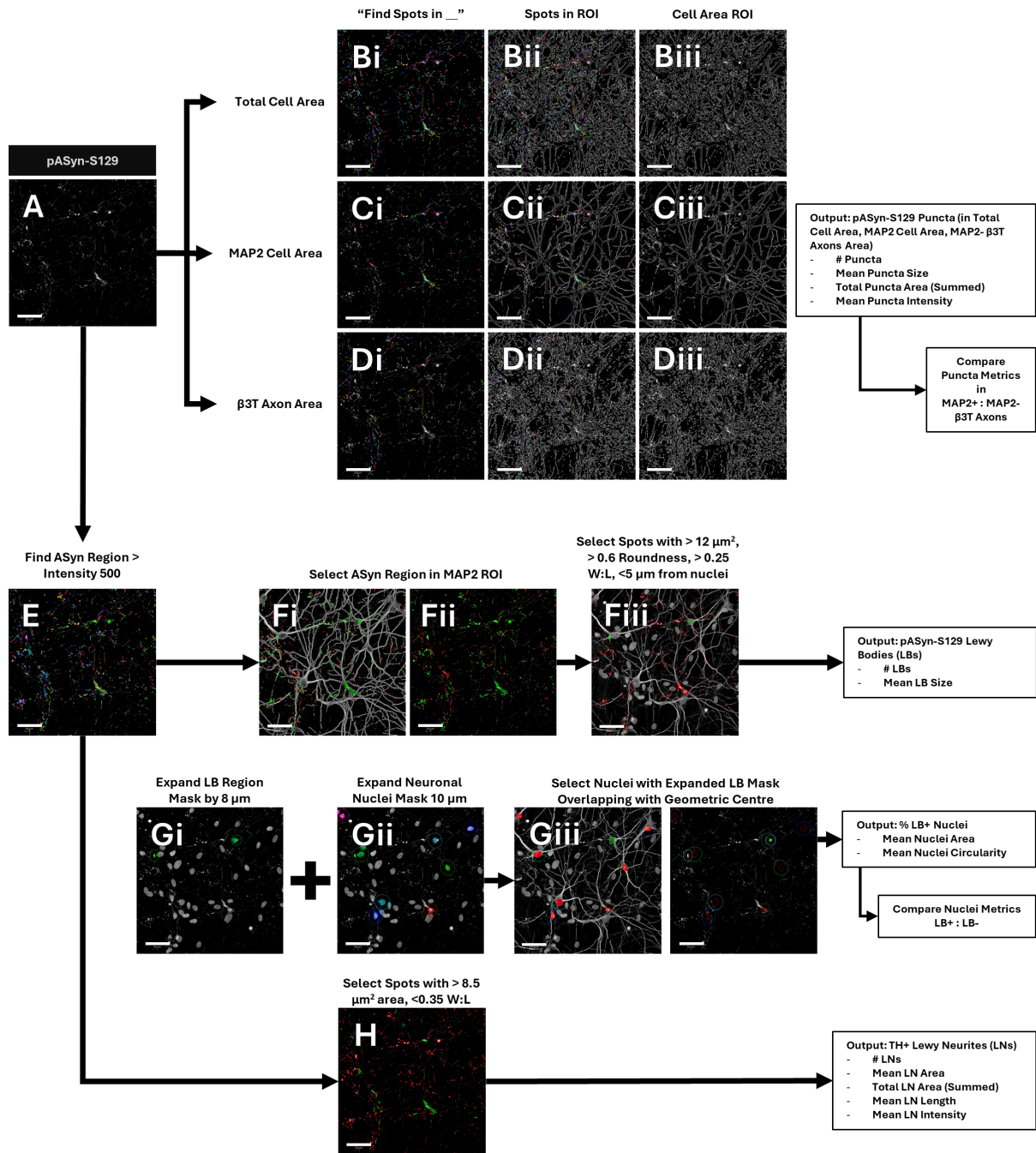


Figure 3.9. Analysis Pipeline for pASyn-S129+ Labelling in Cortical Neurons. pASyn-S129 labelling in cortical neurons was classed into three broad categories: punctate labelling, Lewy Bodies (LBs), and Lewy Neurites (LNs). **(A)** Depicts pASyn-S129+ labelling in the input image (same as for Figures 3.7-3.8). **(B-D)** Punctate labelling was analysed using the Find Spots function (Method A) and restricted to **(B)** the total cell ROI, **(C)** the MAP2 ROI, or **(D)** the β 3T Axonal Area ROI. (i) Shows just the puncta selected for that ROI, (ii) shows the ROI boundaries and puncta together, and (iii) shows the ROI boundaries alone. From the same input image, **(E)** high intensity pASyn-S129+ labelling was first selected using the Find Image Region function and an absolute intensity threshold. From these objects, **(F)** Lewy bodies were selected by **(Fi-ii)** selecting objects only within the MAP2 ROI, and then **(Fiii)** selecting objects that were >12 μm^2 , >0.6 circularity, >0.25 width:length ratio, and <5 μm from a neuronal nucleus. (Fi) shows MAP2 labelling and ROI for context, while (Fii) is the same image without the MAP2 channel or ROI, only showing pASyn-S129 labelling and selected/excluded objects. **(G)** Nuclei in cells containing Lewy bodies were identified by **(Gi)** expanding the Lewy body mask by 8 μm , and **(Gii)** expanding the neuronal nuclei mask by 10 μm , and **(Giii)** selecting nuclei whose geometric centre overlapped with the expanded

Lewy body mask. **(H)** To find Lewy neurites, high intensity pASyn-S129+ objects from (E) within the MAP2+ β 3T “total” cell area were selected $>8.5 \mu\text{m}^2$ and <0.35 W:L ratio. In (F-H), selected objects are highlighted in green, excluded objects are highlighted in red. All scale bars are $50 \mu\text{m}$.

Quantifying LP of all sizes and morphologies across the MAP2 ROI revealed pASyn-S129+ puncta density was significantly higher in PSEN1-Int4Del lines than in vehicle treated controls from as early as 10 days and increased over time (Figure 3.10A). The puncta density was also elevated in LRRK2 cell lines but not more than healthy controls, and did not substantially increase with time, except in the case of the LRRK2 R1441C 207 line. There was ~3-fold more accumulation in PSEN1-Int4Del than other lines with significant accumulation, and no substantial accumulation in the rAstros, SNCA-KO cell line, or other AD lines. The proportion of cell area that was pASyn-S129+ exhibited a similar pattern (Figure 3.10C). The mean size of individual puncta in the PFF treated condition also appeared to increase over time from around $6\text{-}8 \mu\text{m}^2$ at 10 Days to as much as $1.5 \mu\text{m}^2$ at 40 days (Figure 3.10B).

These metrics of pASyn-S129+ LP, as well as mean puncta intensity, were compared between MAP2+ and β 3T+MAP2- ROIs, to compare the relative pathological burden in the somatodendritic to the axonal compartment of the cell, over time (Figure 3.11). This analysis was performed in the genotypes which exhibited significantly elevated levels of punctate labelling. No notable disparity in the level of pathology between different compartments was observed. Puncta density (Figure 3.11A) and intensity (Figure 3.11B) suggests a slight preference for somatodendritic region at 10 days in the pathological process, which shifts to becoming more axonally weighted in days 20-30, and then moves back towards neutral or somatodendritic. High intensity pASyn-S129+ pathology appeared to be particularly weighted towards the somatodendritic region in PSEN1-Int4Del cell lines at D40 (Figure 3.11Biv), which may reflect the particular burden of LB pathology in these cell lines.

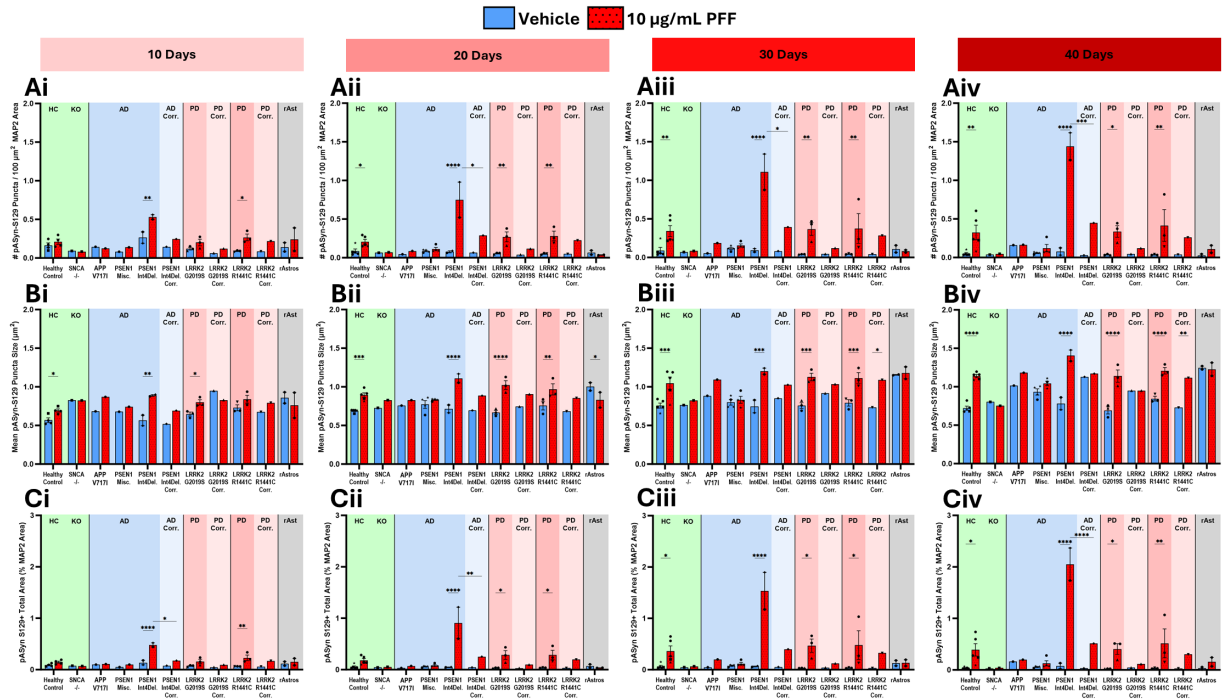


Figure 3.10. pASyn-S129+ Labelling in MAP2+ Cortical Neurons Increases with Time and is Elevated in PSEN1-Int4Del Cell Lines. Overall pASyn-S129+ LP labelling within the MAP2 ROI was quantified in three ways. **(A)** Puncta density, calculated as the number of puncta per 100 μm^2 MAP2 area. **(B)** The mean area of each individual puncta. **(C)** The summed area of all puncta, represented as a percentage of the total MAP2 ROI area. PSEN1-Int4Del cell lines had significantly elevated pASyn-S129+ labelling compared to their vehicle condition and the PFF-insulted corrected control as early as **(i)** 10 Days after PFF insult, and the severity of accumulation appeared to increase at **(ii)** 20 days, **(iii)** 30 days, and **(iv)** 40 days after PFF insult. Accumulation was also elevated in healthy controls, LRRK2-R1441C, and LRRK2-G2019S. Minimal labelling was detected in SNCA-KO cell lines or rat astrocytes, even after 40 days. Data points represent individual cell lines, averaged over 3-6 independent differentiations $\pm$ SEM. In each differentiation, 1000s-10,000s of puncta were measured per well, or 10s-100s in SNCA-KO and rAstros, across 3-4 wells per cell line. Isogenic pairs are noted with shared point shape. Healthy control 856 and its SNCA-KO are square points. INDI KOLF lines are represented by stars and were not examined at 10 days. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and INDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as “PSEN1 Misc.” Statistical comparisons are 2-Way ANOVA with Bonferroni correction for multiple comparisons.

The skew in puncta size (Figure 3.11C) and pASyn-S129+ area fraction (Figure 3.11D) towards the somatodendritic region may also reflect a tendency of larger aggregates to form in or close to the MAP2+ cell body. The lower values for PSEN1-Int4Del here (Figure 3.11Dii-iii) may also reflect a higher burden of axonal neuritic pathology in these lines, before developing more LB pathology at D40, while pathology remains substantially somatodendritic in other genotypes.

In quantifying LN pathology in Figure 3.12, it became clear that LNs were significantly elevated only in PSEN1-Int4Del lines at 10 days but were relatively scant at this timepoint. However, they substantially increased with time by 20 days and largely plateaued after 30 days. Other cell lines exhibited very few to no LNs, even at later time points, except for the cell line LRRK2 R1441C 207. There was, in the end, a ~6-fold LN burden in PSEN1-Int4Del lines compared to others.

LN mean area and length were also measured and are presented for a subset of genotypes that exhibited the most LN structures (Figure 3.13). There was a trend towards an effect of time in the PSEN1-Int4Del cell lines, though not so much in the PD or healthy controls, suggesting that most LN structures were around 20 μm^2 and 10 μm long, and that the increases with time observed in Figure 3.12 were mostly an effect of additional structures being seeded, rather than extant structures growing in size.

LB pathology was also quantified at all four timepoints (Figure 3.14). Again, LB pathology was significantly elevated only in PSEN1-Int4Del lines at earlier time points and continued to increase in burden over time through to 40 days. Other genotypes also developed LB pathology, particularly by D30 and D40, but not more than healthy or corrected controls, with around 3-4-fold greater LB burden in the Int4Del lines than others. LBs also showed a steady increase in size over time, and typically trended towards being larger in the PSEN1-Int4Del CNs than in other genotypes at a given timepoint (Figure 3.15). Intriguingly, when examining the nuclei of specifically PSEN1-Int4Del CNs, and comparing those of untreated cells with those of PFF-treated cells with and without adjacent LB pathology, there was a notable trend of PFF treatment and LB burden that seemed to exacerbate with time and diminished the size and roundness of the nucleus (Figure 3.16).

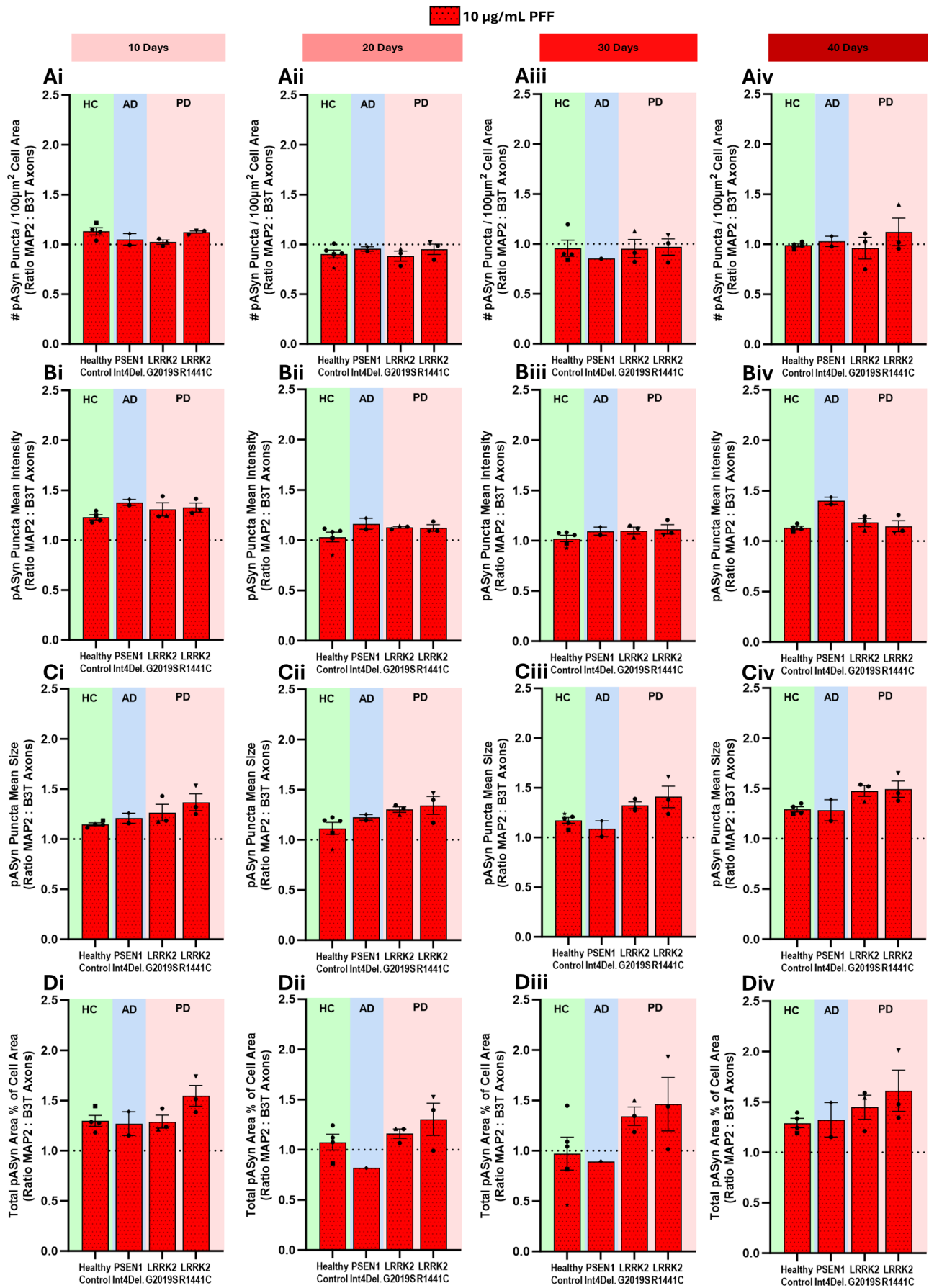


Figure 3.11. Somatodendritic versus Axonal Compartmentalisation of pASyn-S129+ Labelling Does Not Change Between 10 and 40 Days. Where pASyn-S129+ pathology developed, to examine whether the axonal compartment or somatodendritic compartment was more involved and how this changed with duration post insult, the ratio of various measures of punctate pathology in the MAP2+ ROI to β 3T+ axonal ROI was calculated. Only 10 μ g/mL PFF insulted values for cell lines in the four genotypes with significantly elevated punctate pathology (healthy controls, PSEN1-Int4Del and LRRK2 genotypes) are shown. Values above 1 indicate more pathology in MAP2. Punctate pathology was measured within each ROI as **(A)** Puncta density within the relevant ROI, **(B)** mean puncta intensity **(C)** mean puncta size, and **(D)** total summed puncta area as a proportion of the relevant ROI. No significant differences between genotypes were found (Kruskal-Wallis test with Dunn's correction). The system of denoting specific cell lines with different shaped points was retained from earlier figures.

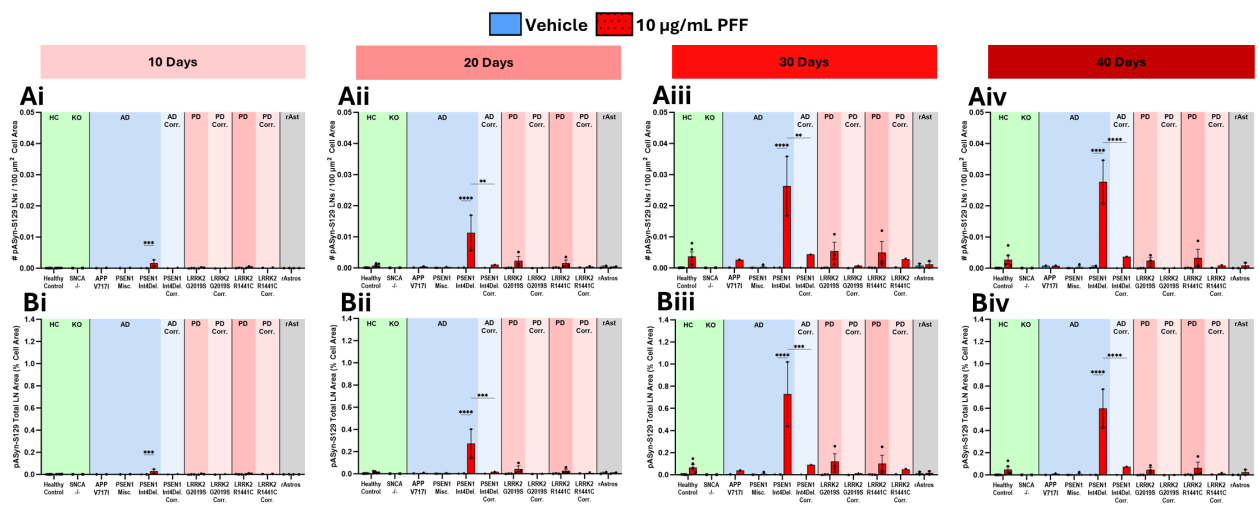


Figure 3.12. The Number of Lewy Neurite-like pASyn-S129+ Structures in Cortical Neurons Increases with Time and is Elevated in PSEN1-Int4Del Cell Lines. pASyn-S129+ Lewy Neurites (LNs) were quantified in two ways. **(A)** LN density, calculated as the number of LNs per 100 μ m² total cell area. **(B)** The area fraction, calculated by the summed area of all LNs, represented as a percentage of the total cell ROI area. PSEN1-Int4Del cell lines had significantly elevated pASyn-S129+ labelling compared to its vehicle condition as early as **(i)** 10 Days after PFF insult, and the severity of accumulation appeared to increase at **(ii)** 20 days, **(iii)** 30 days, and **(iv)** 40 days after PFF insult. Accumulation trended towards elevation in healthy controls, PSEN1-Int4Del corrected control, LRRK2-R1441C, and LRRK2-G2019S lines. Minimal labelling was detected in SNCA-KO cell lines or rat astrocytes, even after 40 days. Data points represent individual cell lines, averaged over 3-6 independent differentiations $\pm$ SEM. In each differentiation, 10s-100s of LNs were measured per 3-4 wells, and 1000s for PSEN1-Int4Del at 30-40 days. Isogenic pairs are noted with shared point shape. Healthy control 856 and its SNCA-KO are square points. INDI KOLF lines are represented by stars, and were not examined at 10 days. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and INDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as “PSEN1 Misc.” Two points for rAstros represent cells grown in either NGN2 media or AGM, alongside two differentiations. Statistical comparisons are 2-Way ANOVA with Bonferroni correction for multiple comparisons.

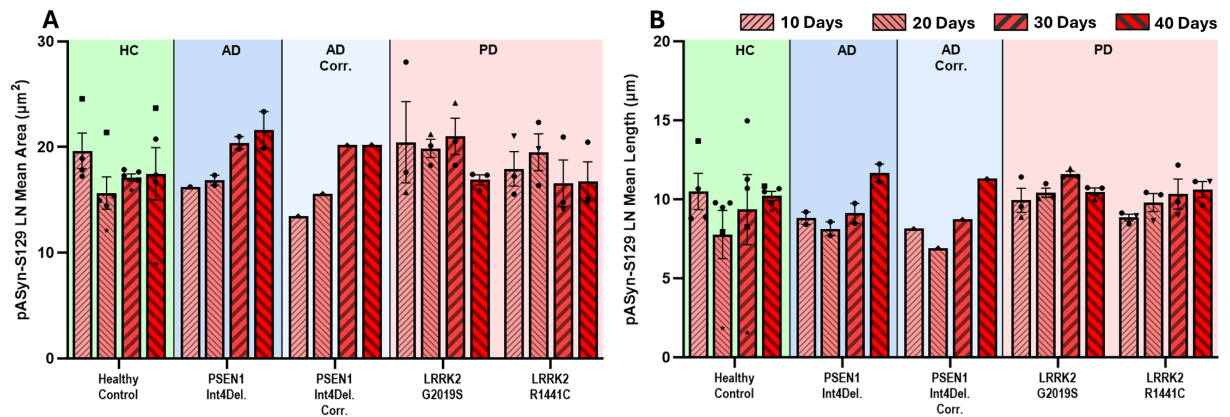


Figure 3.13. Lewy Neurite Length and Area Do Not Differ by Genotype in Cortical Neurons. The (A) Mean Area, and (B) Mean Length of each Lewy Neurite (LN) was measured and is represented for genotypes that developed the most pathology across each time point. No effect of genotype or time was found (two-way ANOVA with Bonferroni correction for multiple comparisons). Data points represent individual cell lines averaged across 3-6 differentiations +/- SEM. In each differentiation, 10s-100s of LNs were measured per 3-4 wells, and 1000s for PSEN1-Int4Del at 30-40 days. The system of denoting particular cell lines with different shaped points was retained from earlier figures.

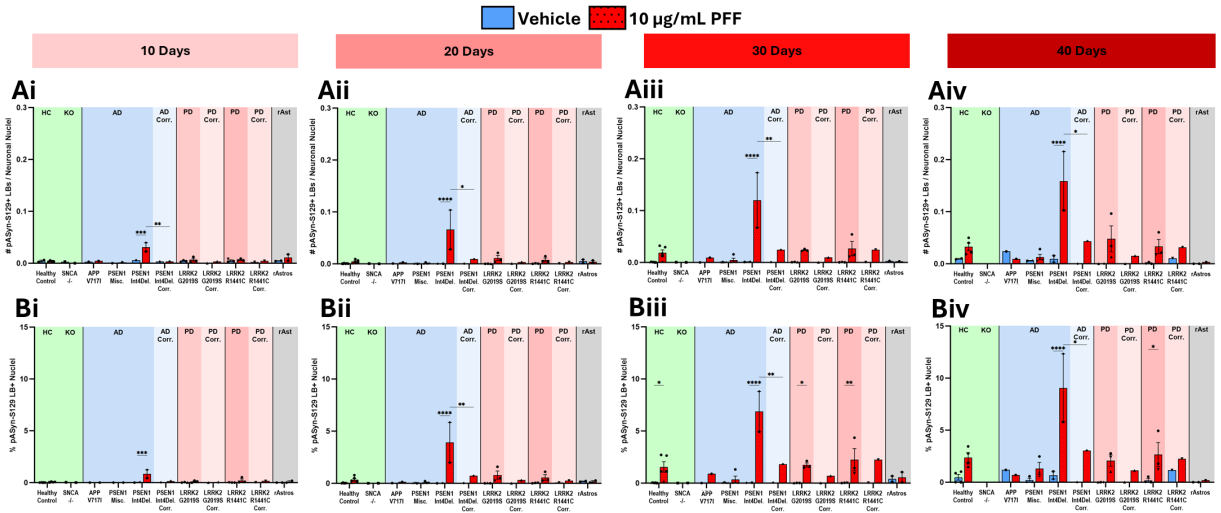


Figure 3.14. The Number of Lewy Body-like pASyn-S129+ Structures in Cortical Neurons Increases with Time and is Elevated in PSEN1-Int4Del Cell Lines. pASyn-S129+ Lewy Bodies (LBs) were quantified in two ways. (A) The number of LBs per neuronal nuclei. (B) The number of nuclei with adjacent LBs, calculated semi-independently from (A). PSEN1-Int4Del cell lines had significantly elevated pASyn-S129+ labelling compared to its vehicle condition and isogenic corrected control as early as (i) 10 Days after PFF insult, and the severity of accumulation appeared to increase at (ii) 20 days, (iii), 30 days, and (iv) 40 days after PFF insult. Accumulation trended towards or was significantly elevated in healthy controls, PSEN1-Int4Del LRRK2 corrected control, LRRK2-R1441C, and LRRK2-G2019S lines. Minimal labelling was detected in SNCA-KO cell lines or rat astrocytes, even after 40 days. Data points represent individual cell lines, averaged over 3-6 independent differentiations +/- SEM. In each differentiation, 1s-10s of LBs were measured per 3-4 wells, and 100s for PSEN1-Int4Del at 30-40 days. Isogenic pairs are noted with shared point shape. Healthy Control 856 and its SNCA-KO are square points. iNDI KOLF lines are represented by stars and were not examined at 10 days. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and iNDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as "PSEN1 Misc." Two points for rAstros represent cells grown in either NGN2 media or AGM, alongside two differentiations. Statistical comparisons are 2-Way ANOVA with Bonferroni correction for multiple comparisons.

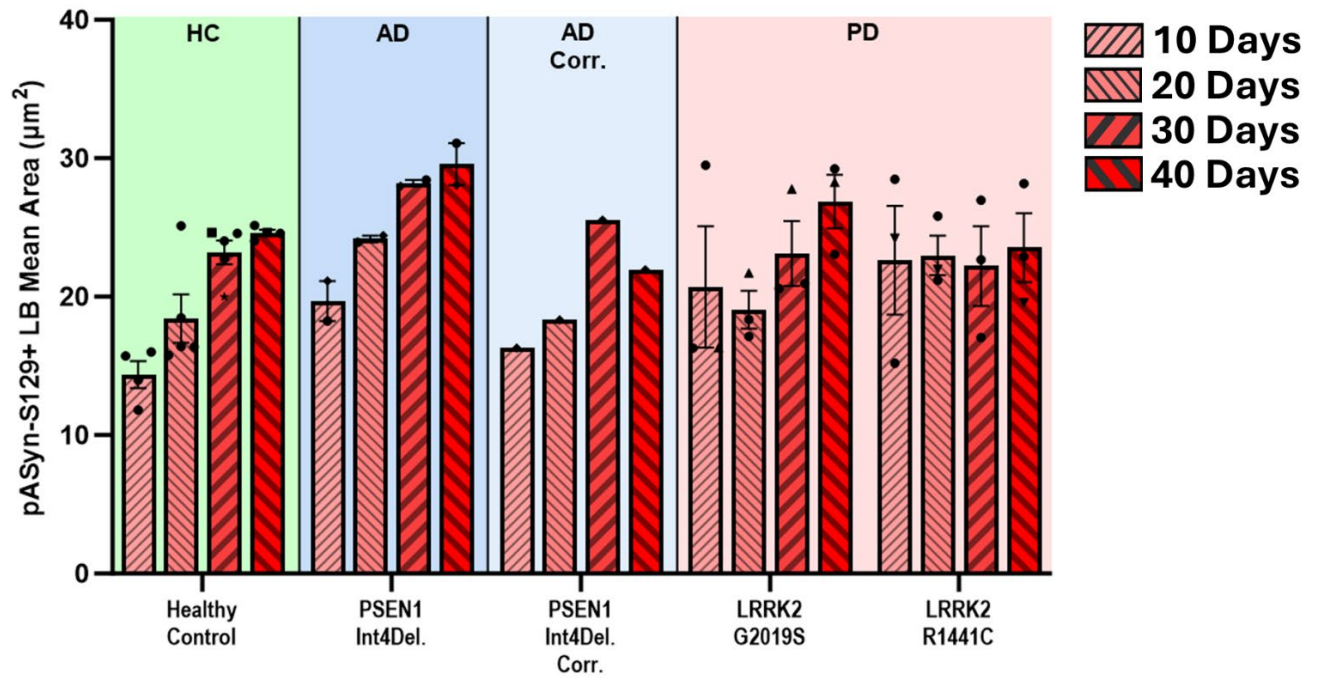


Figure 3.15. Lewy Body Area Does Not Differ by Genotype in Cortical Neurons. The mean area of each Lewy body (LB) was measured and is represented for genotypes that developed the most pathology across each time point. No effect of genotype or time was found, though there was a trend towards an effect of time ($p=0.0878$) (two-way ANOVA with Bonferroni correction for multiple comparisons). Data points represent individual cell lines averaged across 3-6 differentiations $\pm$ SEM. In each differentiation, 1s-10s of LBs were measured per 3-4 wells, and 100s for PSEN1-Int4Del at 30-40 days. The system of denoting particular cell lines with different shaped points was retained from earlier figures.

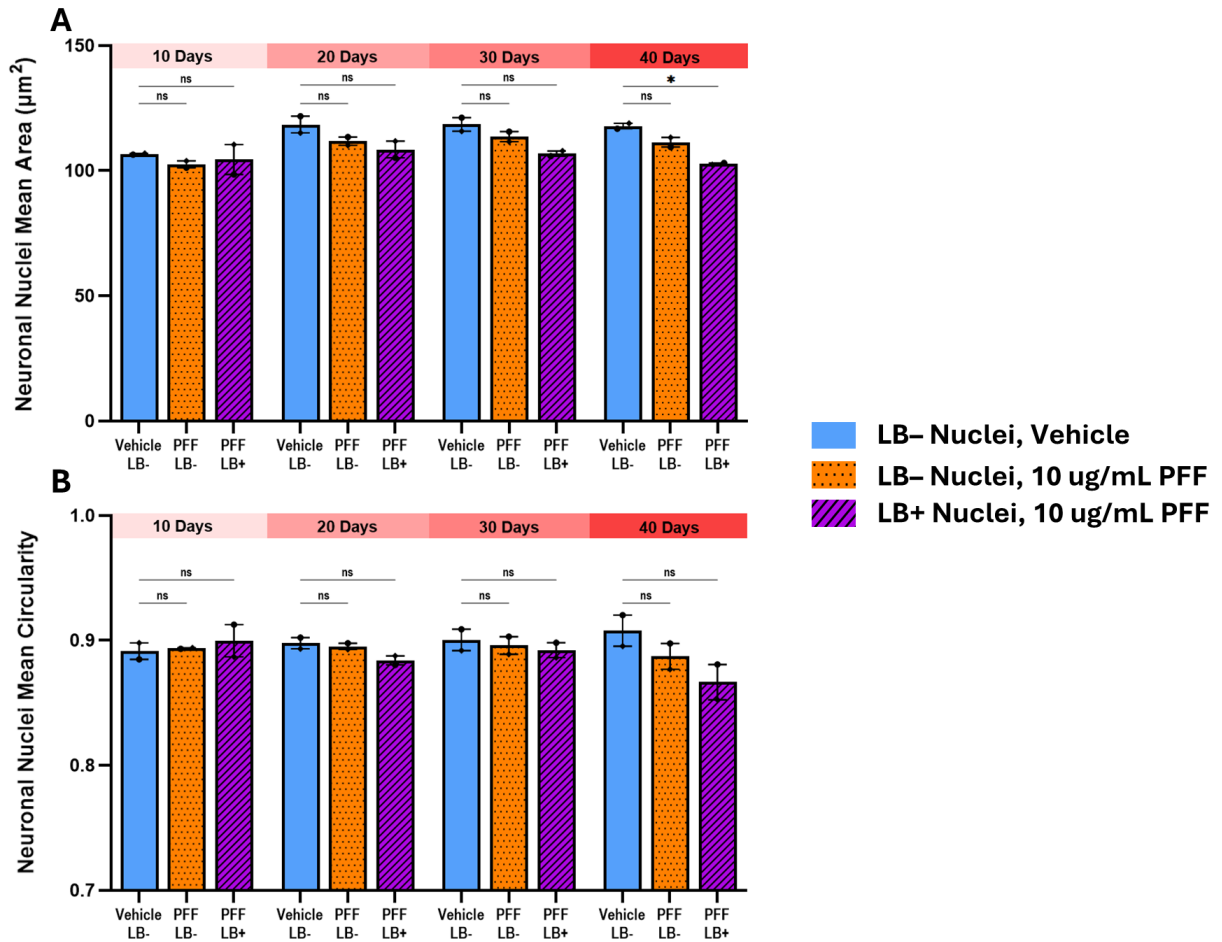


Figure 3.16. Lewy Bodies Negatively Impact Neuronal Nuclei Morphology in PSEN1-Int4Del Cortical Neurons. For PSEN1-Int4Del cell lines, which developed the most Lewy-Body (LB) pathology, the morphology of neuronal nuclei in vehicle-treated wells (thus, without Lewy bodies) (blue bars), was compared to nuclei in 10 $\mu\text{g/mL}$ PFF treated conditions without (orange bars with dots) and with (purple bars with stripes) LBs (repeated measures two-way ANOVA with Geisser-Greenhouse correction for sphericity and Dunnett's correction for multiple comparisons). **(A)** For nuclei area, there was a significant effect of time ($p=0.0173$) and a trend towards reduction in nuclear area with PFF and LB load, becoming significant by 40 days ($p=0.0408$). **(B)** For nuclear circularity (perfect circularity = 1), there was a trend towards reduction with pathology load at later time points, but no significant effects were found. Data points represent individual cell lines averaged across 4-6 differentiations $\pm$ SEM. In each differentiation, 10s of nuclei were measured in each of 3-4 wells (typically <50 at D10-20, >50 D30-40).

3.3.3. Effects of PFF Insult on CN Nuclear and Cellular Morphology

In addition to examining seeded aggregate pathology, I also sought to examine what effect the PFF insult had on cell health more broadly. Neuronal nuclei and morphology were examined in this regard.

Neuronal nuclei number was unaffected by PFF treatment, although declined across genotypes over time (Figure 3.17A). However, in most cell lines, there was a significant decrease in nuclear area as early as 10 days, including in the *SNCA*-KO cell lines, that appeared to attenuate slightly over time (Figure 3.17B), matching observations from Figure 3.16. This change in size was typically not accompanied by a change in nuclei circularity (Figure 3.17C).

Despite no apparent toxicity to nuclei number, there was nevertheless a relatively consistent 10-20% reduction in MAP2 area by PFF insult as early as 10 days, which worsened in most cell lines over time (Figure 3.18A-B). There was a similar but delayed reduction in β 3T+, axonal, and total cell areas (Figure 3.18C-E). This trended towards being more profound in PD cell lines and their controls, although these trended towards having lower MAP2 area to begin with.

In any case, changes to nuclear and cell region morphology appeared to be aggregation independent, since they do not correlate with pASyn-S129 pathological burden and affected the *SNCA*-KO line too.

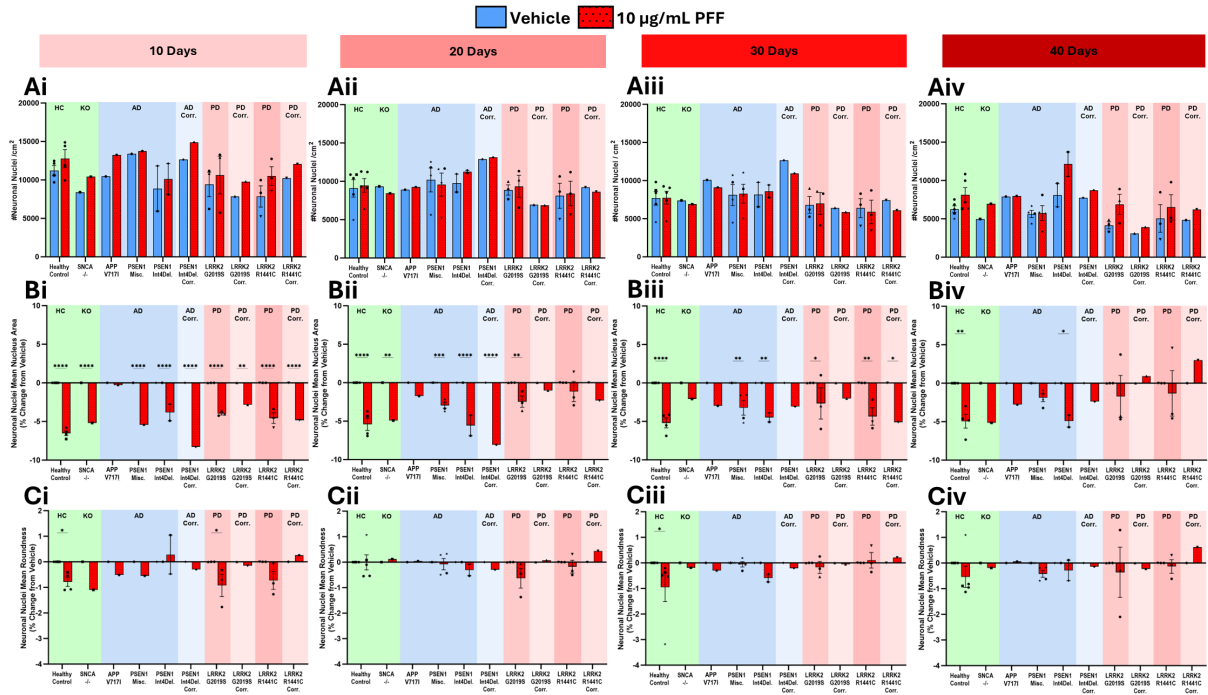


Figure 3.17. PFF Insult Does Not Impact Cortical Neuronal Nuclei Number but Does Depress Neuronal Nuclei Area Independent of Aggregation. (A) The number of neuronal nuclei, represented as a density per cm^2 is shown for each cell line across time points, showing no effect of PFF insult or genotype on cell number, and a decline in cells surviving in long term culture, consistent between treatment conditions. (B) The mean area of all neuronal nuclei was measured and compared between vehicle-treated and PFF-insulted cells. Many genotypes exhibited a ~5% reduction in nucleus area, which in most cases ameliorated over time. (C) The mean circularity of all neuronal nuclei was measured and compared between vehicle-treated and PFF-insulted cells. There was evidence of a small reduction in circularity in some genotypes, inconsistently. KOLF2.1J was noted to behave differently to other healthy controls. Data points represent individual cell lines, averaged over 3-6 independent differentiations $\pm$ SEM. In each differentiation, 100s of nuclei were measured per 3-4 wells. Isogenic pairs are noted with shared point shape. Healthy Control 856 and its SNCA-KO are square points. iNDI KOLF lines are represented by stars and were not examined at 10 days. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and iNDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as “PSEN1 Misc.” Statistical comparisons are 2-Way ANOVA with Bonferroni correction for multiple comparisons.

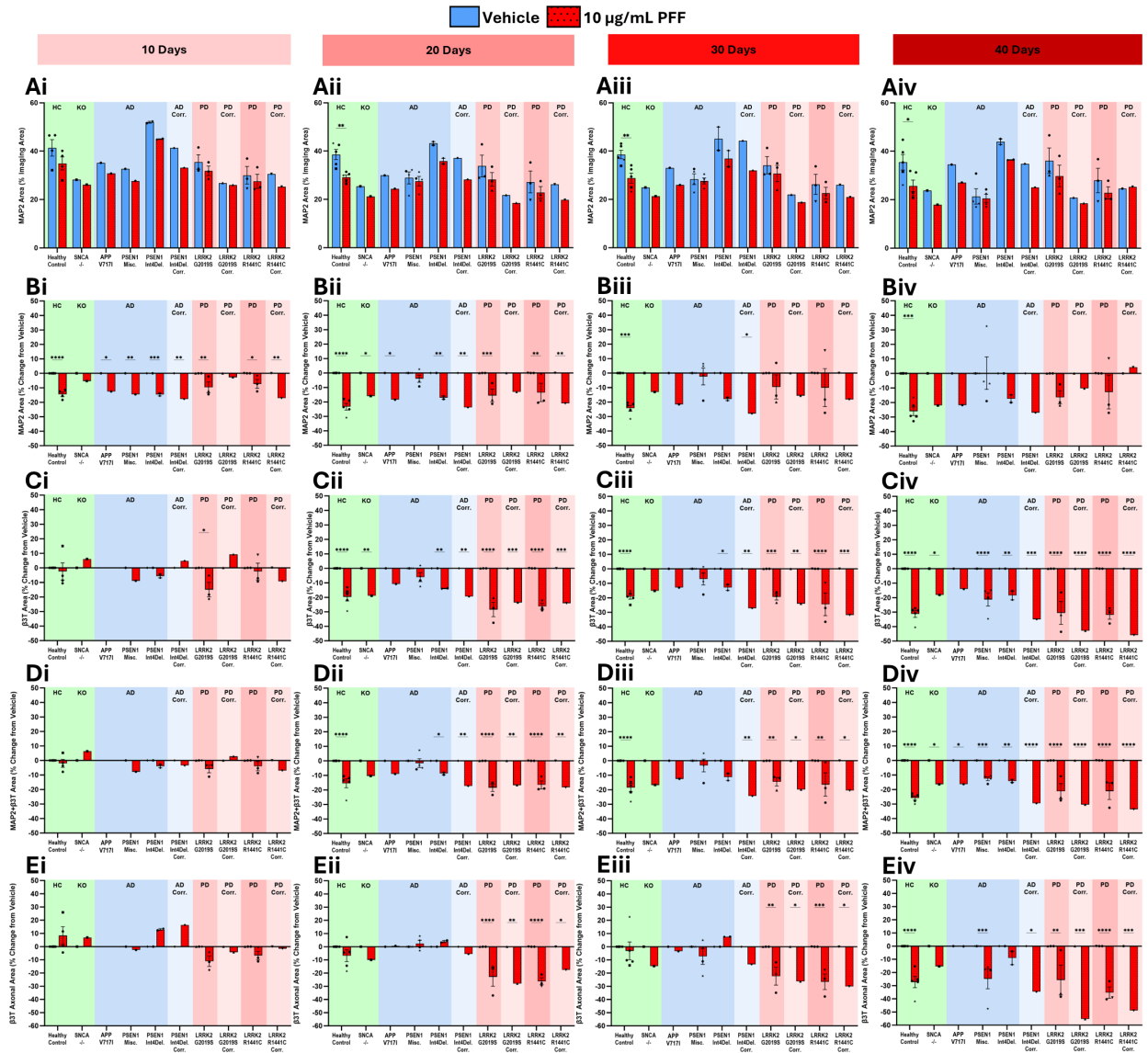


Figure 3.18. PFF Insult Depresses Neuronal Area, Independent of Aggregation. (A) The MAP2 cell area, represented as a percentage of the total imaged area in each well, is shown for each cell line across time points, showing no significant differences between cell lines. The cell area is also compared between vehicle treated and PFF-insulted conditions for raw MAP2 area values in (A), and then for the percentage change from baseline for (B) MAP2 area, (C) β 3T area, (D) total cell area, and (E) β 3T axonal area. In these cases, a slight depression in neuronal area was observed in most cell lines as early as 10 days for MAP2 and typically by 20 days for other ROIs and became more pronounced over time. Significant differences between vehicle and PFF-treated pairs are shown. By 30 days, there was a significant effect of treatment but no significant effect of genotype. Data points represent individual cell lines, averaged over 3-6 independent differentiations $\pm$ SEM. In each differentiation, 3-4 wells were measured. Isogenic pairs are noted with shared point shape. Healthy Control 856 and its SNCA-KO are square points. iNDI KOLF lines are represented by stars and were not examined at 10 days. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and iNDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as “PSEN1 Misc.” Statistical comparisons are 2-Way ANOVA with Bonferroni correction for multiple comparisons.

3.3.4. Effects of PFF Dose and Chloroquine Co-Treatment

In a subset of lines, across two differentiations, I examined to what extent the PFF insult was also dose dependent, using the same range of metrics as described above (Figures 3.19-22).

For overall LP (Figure 3.19), there was a clear dose dependent effect of PFF insult for PSEN1-M146I and PSEN1-Int4Del Corr Ctrl. Cell lines, but not as clearly for the PSEN1-Int4Del -04 clone, where 5 $\mu\text{g/mL}$ PFF insult also generated a substantial pASyn-S129+ response. Interestingly, there was less effect of CQ co-treatment with 10 $\mu\text{g/mL}$ PFF in PSEN1-Int4Del on pASyn-S129+ levels as it did in the other two cell lines, especially at D10 for PSEN1-M146I, where CQ led to 4-6-fold more punctate labelling (Figure 3.19Ai, Ci). CQ treatment did not have a substantial effect of puncta size (Figure 3.19B).

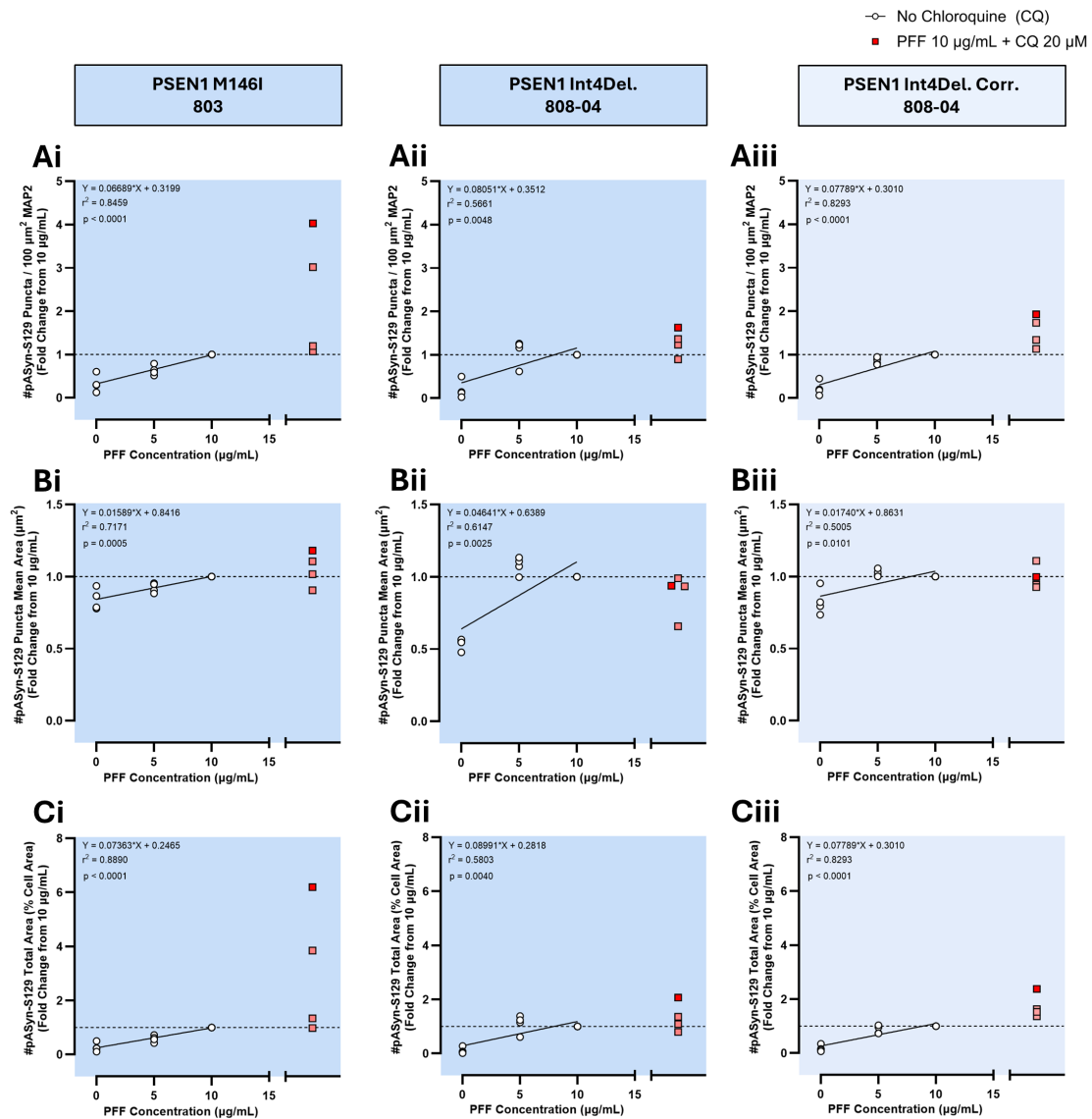


Figure 3.19. Effect of PFF Dose and Chloroquine on pASyn-S129+ Puncta. In a subset of cell lines, (i) PSEN1-M146I, (ii) PSEN1-Int4Del 808-04, and (iii) PSEN1-Int4Del 808-04 Corrected Control, the effect of lower dose and cotreatment with chloroquine (CQ), an autophagy blocker, was examined on the various measures of overall pASyn-S129+ pathology: (A) puncta density, (B) mean puncta size, (C) summed total puncta area, as a proportion of cell area. White circles represent cells treated with 0 (i.e. vehicle), 5 or 10 μg/mL PFF, without CQ. Red squares are plotted on an adjacent axis, representing cells insulted with 10 μg/mL PFF with 20 μM CQ. Data points represent time points (10, 20, 30, 40 days) averaged over two differentiations. Dark red squares indicate 10 days. Linear regression lines were fitted, and equations, r^2 values, and p values for whether lines were significantly different from zero plotted. For all cell lines and measures, there was a significant effect of dose, but typically much higher greater effect of CQ in the PSEN1-M146I lines, especially at D10.

While there was similarly no substantial increase in LN pathology at higher doses (Figure 3.20), CQ treatment had a striking ~80-130-fold increase in the burden of LN pathology in the PSEN1-M146I and PSEN1-Int4Del isogenic corrected line, but only a ~10-fold increase in the PSEN1-

Int4Del CNs, at 10 days (Figure 3.20A). Similarly, there was no clear effect of PFF dose on LB frequency or size, but CQ co-treatment had a much larger fold effect on the isogenic corrected control CNs (Figure 3.21iii) than the PSEN1-Int4Del CNs (Figure 3.21ii).

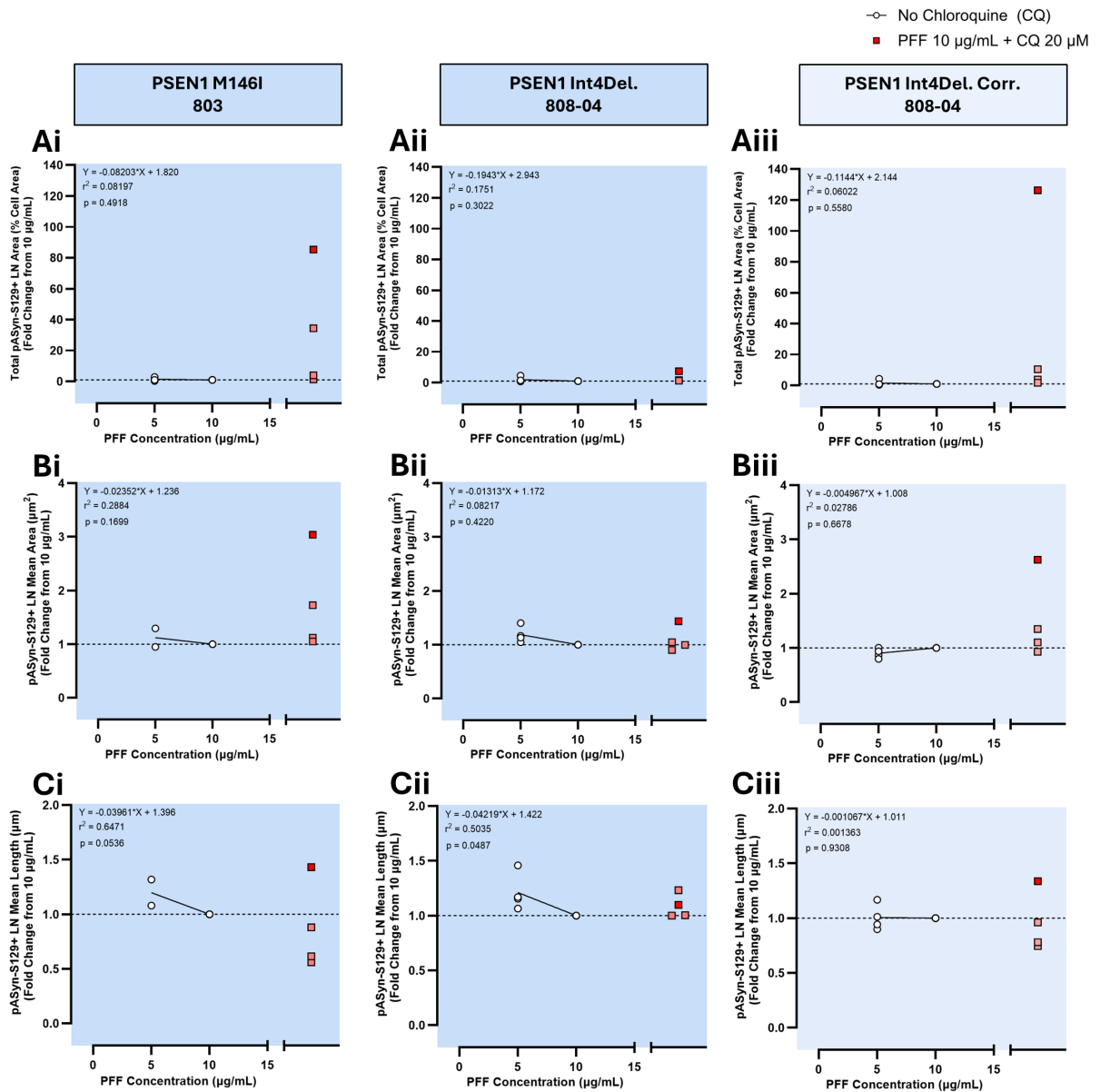


Figure 3.20. Effect of PFF Dose and Chloroquine on Lewy Neurites. In a subset of cell lines, (i) PSEN1-M146I, (ii) PSEN1-Int4Del 808-04, and (iii) PSEN1-Int4Del 808-04 Corrected Control, the effect of lower dose and cotreatment with chloroquine (CQ), an autophagy blocker, was examined on the various measures of Lewy neurites (LNs): **(A)** the cellular area fraction that was LN+, **(B)** mean LN size, **(C)** mean LN length. White circles represent cells treated with 5 or 10 µg/mL PFF, without CQ. Red squares are plotted on an adjacent axis, representing cells insulted with 10 µg/mL PFF with 20 µM CQ. Data points represent time points (10, 20, 30, 40 days) averaged over two differentiations. Dark red squares indicate 10 days. Linear regression lines were fitted, and equations, r^2 values, and p values for whether lines were significantly different from zero were plotted. For all cell lines and measures, there was no particular difference between LN pathology at 5 and 10 µg/mL. However, while CQ treatment had a substantial impact on LN area fraction, area, and length for PSEN1-M146I and the PSEN1-Int4Del corrected Control, mainly at 10 days, there was little effect in PSEN1-Int4Del 808-04.

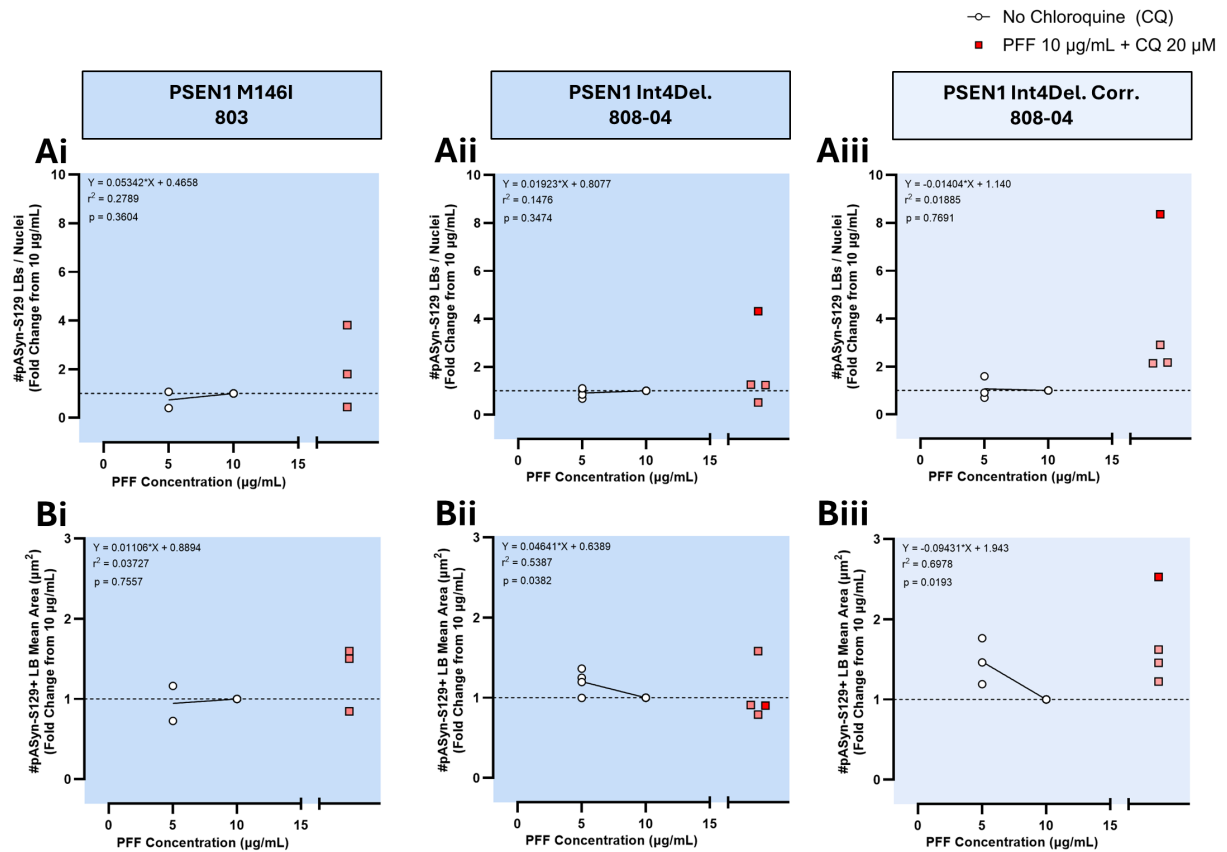


Figure 3.21. Effect of PFF Dose and Chloroquine on Lewy Bodies. In a subset of cell lines, (i) PSEN1-M146I, (ii) PSEN1-Int4Del 808-04, and (iii) PSEN1-Int4Del 808-04 Corrected Control, the effect of lower dose and cotreatment with chloroquine (CQ), an autophagy blocker, was examined on the various measures of Lewy Bodies (LBs): (A) the number of LBs per nuclei, and (B) mean LB area. White circles represent cells treated with 5 or 10 µg/mL PFF, without CQ. Red squares are plotted on an adjacent axis, representing cells insulted with 10 µg/mL PFF with 20 µM CQ. Data points represent time points (10, 20, 30, 40 days) averaged over two differentiations. In some early time points for PSEN1-M146I, there are no LBs. Dark red squares indicate 10 days. Linear regression lines were fitted, and equations, r^2 values, and p values for whether lines were significantly different from zero plotted. For all cell lines and measures, there was no clear effect of dose on LB formation. However, while CQ treatment had a substantial impact on LB number and area for PSEN1-M146I and the PSEN1-Int4Del corrected control, mainly at earlier timepoints, there was less or little effect in PSEN1-Int4Del 808-04.

PFF dose and CQ had little effect on nuclei number (Figure 3.22A) or roundness (Figure 3.22C), indicating limited toxicity, though there was a trend towards diminished nuclear area (Figure 3.22B), consistent with Figures 3.16-17. Similarly, there was a significant effect of PFF dose on reduction in MAP2 area (Figure 3.22D), though no discernible effect of CQ on MAP2 area or β 3T axonal area (Figure 3.22E).

Chapter 3. ASyn PFFs in Cortical Neurons

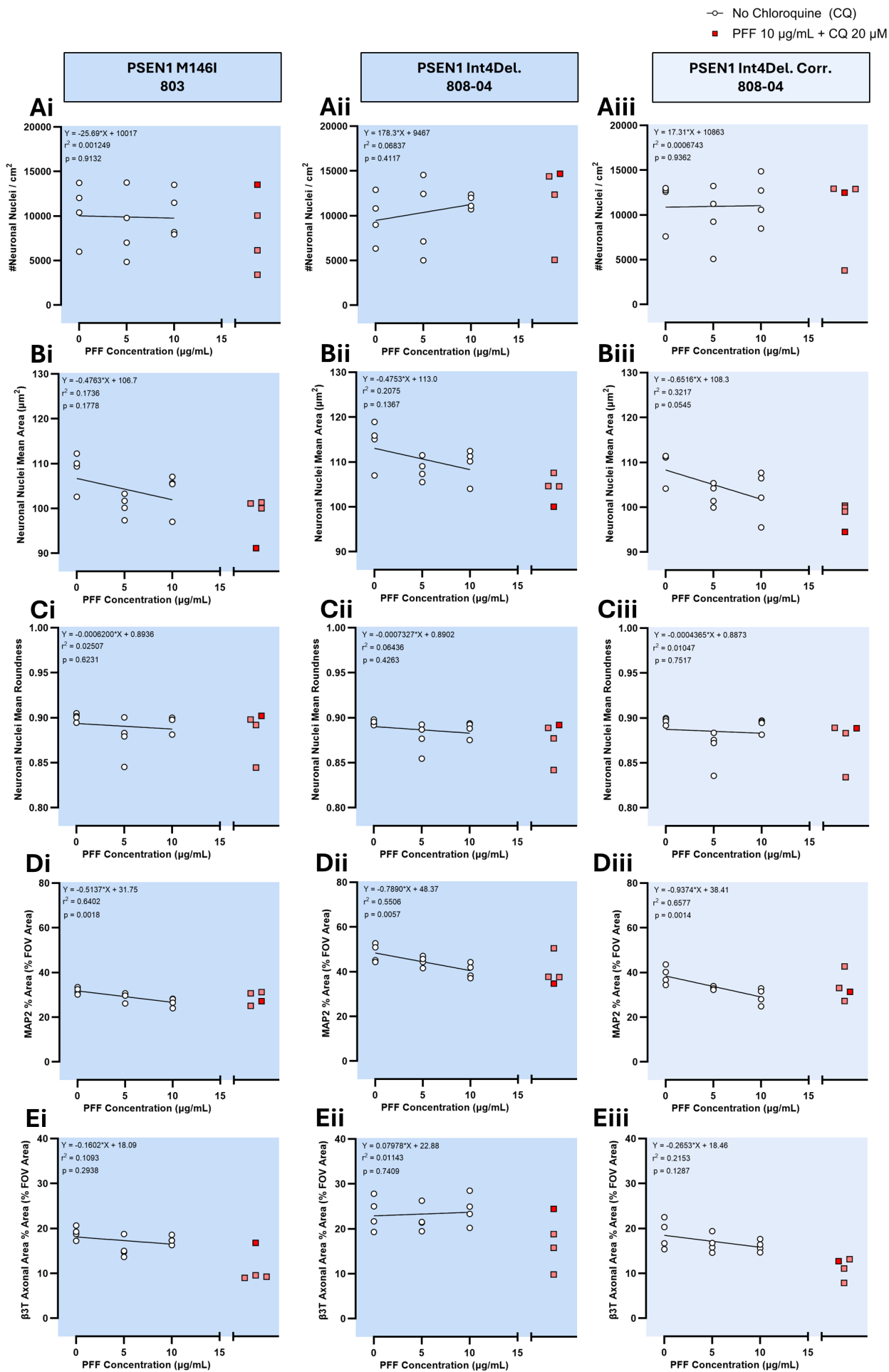


Figure 3.22. Effect of PFF Dose and Chloroquine Nuclear and Cell Morphology. In a subset of cell lines, (i) PSEN1-M146I, (ii) PSEN1-Int4Del 808-04, and (iii) PSEN1-Int4Del 808-04 Corrected Control, the effect of lower dose and cotreatment with chloroquine (CQ), an autophagy blocker, was examined on the various measures of nuclear and cellular morphology and health: **(A)** the number of nuclei, **(B)** mean nuclear area, **(C)** mean nuclear roundness, **(D)** total MAP2 area, and **(E)** total β 3T axonal area. White circles represent cells treated with 0 (vehicle), 5 or 10 μ g/mL PFF, without CQ. Red squares are plotted on an adjacent axis, representing cells insulted with 10 μ g/mL PFF with 20 μ M CQ. Data points represent time points (10, 20, 30, 40 days) averaged over two differentiations. Dark red squares indicate 10 days. Linear regression lines were fitted, and equations, r^2 values, and p values for whether lines were significantly different from zero plotted. For all cell lines and measures, there was no clear effect of dose except on MAP2 area, and a trend towards effect on neuronal nuclei area. Similarly, CQ had no discernible effect on nuclei number or roundness or cell area, though potentially had an effect on neuronal nuclear area, in each cell line.

3.3.5. Effects of PFF Insult on Rat Astrocytes

To ensure all the pASyn-S129+ pathology observed was in neurons and not rAstros, I also grew rAstros in monoculture in NGN2 media, and performed identical PFF treatments in parallel to the neurons in coculture. rAstros were labelled with GFAP, and at D40 with both GFAP and S100 β (Figure 3.23A-D). Even after 40 days, no pASyn-S129+ labelling distinct from vehicle-treated or IgG-labelled controls was evident in these cells. There were also minimal changes to rAstro nuclei and cell morphology and intensity, suggesting a lack of toxicity and no overt inflammatory reaction (Figure 3.23E-Q).

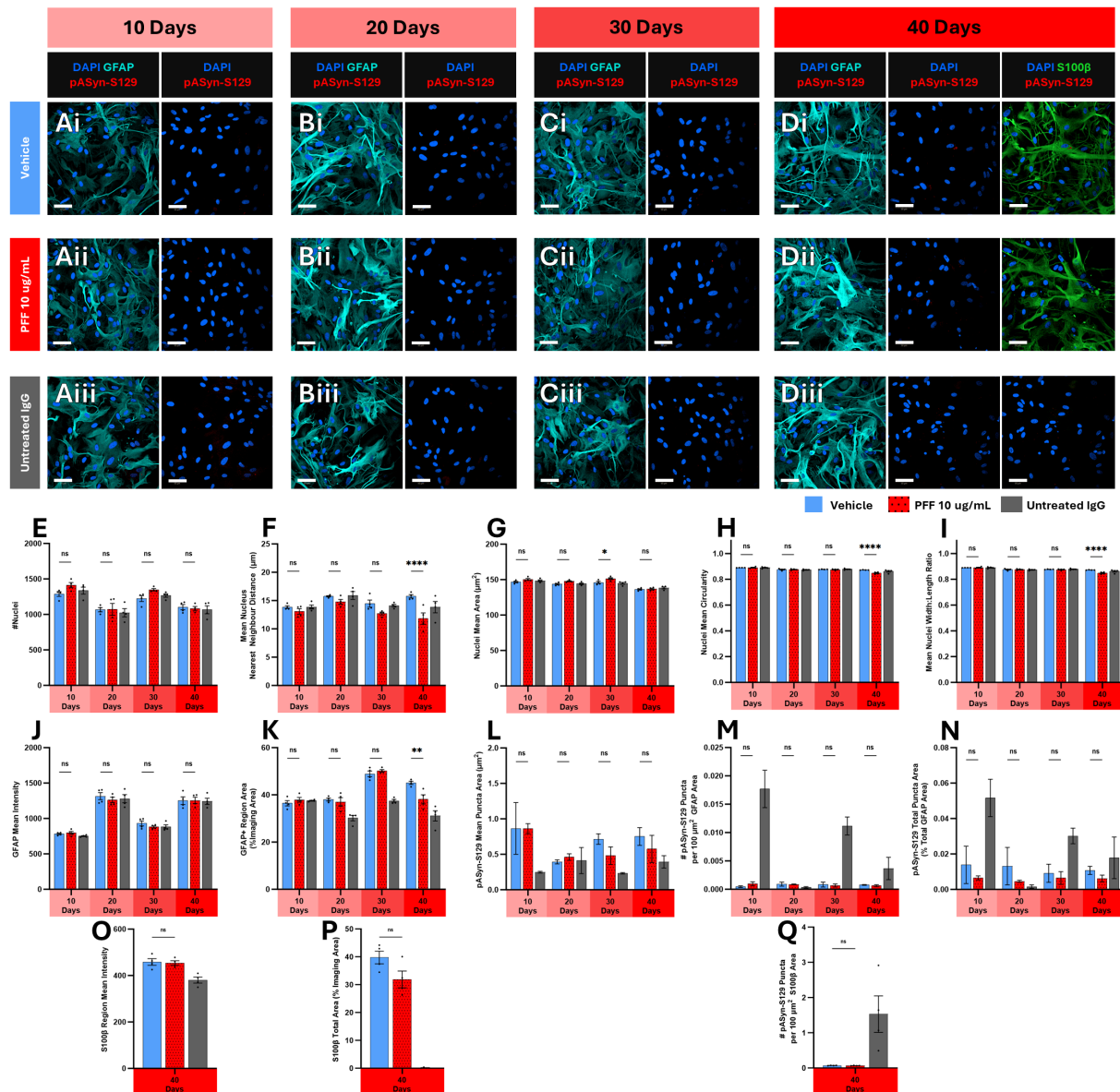


Figure 3.23. No pASyn-S129+ Labelling in rAstrocytes Grown Alone. rAstros were grown in NGN2 media on the same plates in parallel with cortical neurons during a differentiation and underwent the same insult paradigm as cocultures. (A-D) Images show no pASyn-S129+ labelling in (i) vehicle treated, (ii) 10 ug/mL PFF treated, or (iii) untreated IgG negative control labelled GFAP+ astrocytes, at (A) 10 Days, (B) 20 Days, (C) 30 Days, and (D) 40 Days after PFF insult. Scale bars are 50 μm. (E-Q) Quantification of image analysis in these images found that there was no consistent effect of PFF on (E) nuclei number, (F) nuclei clumping, except at 40 days, (G) nuclei area, except at 30 days, (H) nuclei circularity until 40 days, (I) nuclear width:length ratio until 40 days, (J) GFAP mean intensity, (K) total GFAP area until 40 days, (L) pASyn-S129+ puncta area, (M) pASyn-S129+ puncta density, or (N) pASyn-S129+ area fraction. Statistical comparisons are 2-way ANOVA with Bonferroni correction. Data points represent individual wells in a single differentiation, bars represent mean $\pm$ SEM. (D) S100β was also co-labelled in 40 day cells, and (O-Q) analysis was also performed using this ROI in D40 cells. No effect of PFF insult was found on (O) S100β mean intensity, (P) S100β mean area, or (Q) puncta density in S100β. Mann-Whitney test for statistical comparison between vehicle and PFF-insulted.

To examine for any interaction effects between rAstros and neurons in coculture, I also labelled S100 β + astrocytes in coculture with MAP2+ neurons and pASyn-S129+ pathology (Figure 3.24A-D). Here, it was evident that the neuritic and punctate pathology that did not neatly correspond to MAP2+ neurites also did not correspond to S100 β + astrocytes, and therefore likely represented axonal pathology in neurons, as per Figure 3.3.

Examining S100 β + labelling in the rAstros cocultured with a wide range of cell lines demonstrated no change in either intensity or cell area (Figure 3.24-E-F), suggesting pathological changes occurring in the neurons did not have a substantial effect on the rAstros, nor was there a substantial inflammatory response from the rAstros to the PFF insult in coculture. Here, there was also no significant pASyn-S129+ labelling in the IgG negative control condition, despite these cells also having been treated with 10 μ g/mL PFF, demonstrating that other instances of positive labelling were not antibodies non-specifically binding to PFF.

Separately, I also performed western blotting for alpha synuclein and found no detectable protein levels in rAstros under basal conditions (Figure 3.24G).

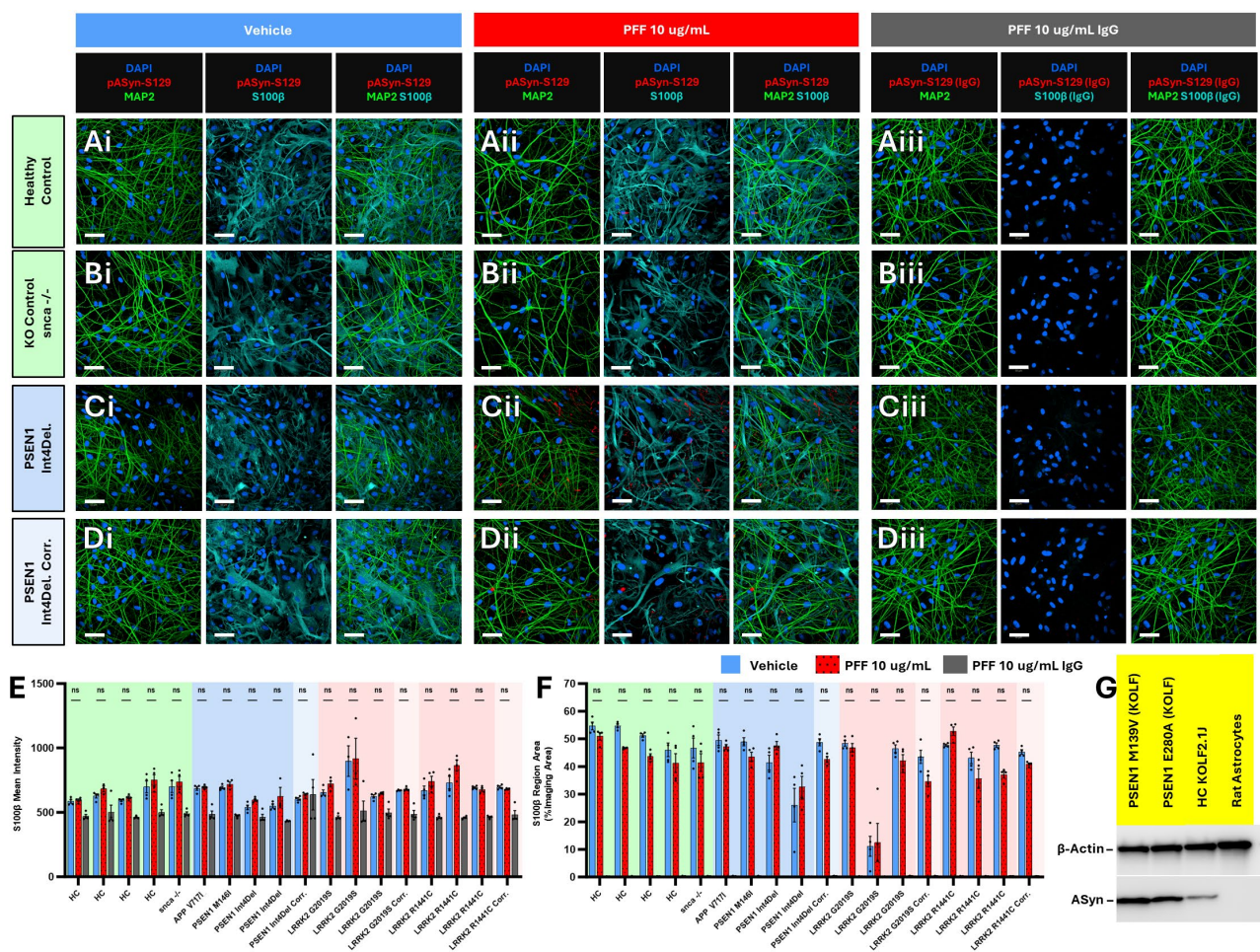


Figure 3.24. No pASyn-S129+ Labelling in S100β+ rAstrocytes Grown in Coculture after 40 Days. To confirm labelling outside of MAP2+ neurons was not within rAstros, (A-D) MAP2+ neurons were labelled alongside S100β for rAstros, in (i) vehicle treated, (ii) ASyn PFF-insulted wells, and (iii) ASyn PFF treated wells labelled with IgG negative control, to visualise both cells together. pASyn-S129+ labelling in (ii) did not appear to conform to any S100β + structures or processes. (E-F) Image analysis to quantify images found no effect of PFF insult on (E) S100B intensity, or (F) S100β area. Data points represent individual wells from a single differentiation, bars represent mean +/- SEM. Statistical comparison is 2-way ANOVA with Bonferroni correction. (G) Western blot of 10 µg loaded protein lysates from cortical neurons grown in monoculture and rat astrocytes, showing no ASyn expression in rAstros.

3.4. Discussion

3.4.1. Results Summary

In this chapter, I sought to examine the response of CNs to ASyn PFF insult. I found that CNs insulted at D70 develop pASyn-S129+ inclusions that are dependent on dose, time, and endogenous ASyn expression. These inclusions comprise an array of morphological structures including punctate labelling, LN-like threads, and round LB-like inclusions. By 10 days after PFF insult, overall pathology appeared to develop evenly across β 3T+ axonal and MAP2+ somatodendritic compartments. PFF insult had a minimal effect on nuclei number even after 40 days, though had a substantial time dependent effect on nuclear morphology, as well as diminishing MAP2+ and β 3T+ cell area across genotypes, in an apparently aggregation independent manner. While all cell lines, except the *SNCA*-KO, developed some pASyn-S129+ inclusions, the PSEN1-Int4Del clonal pair developed significantly higher levels of accumulation than their corrected control, and other genotypes, and CQ treatment did not substantially affect aggregation formation in these lines as it did for other genotypes. No pathology was observed in rAstros grown alone or in coculture with neurons, nor was any substantial change in morphology or GFAP/S100 β immunoreactivity evident.

3.4.2. Patterns of Inclusion Formation Across Cells Concur with Other PFF Studies

pASyn-S129+ inclusions observed in this study are largely in line with what has been reported previously in rodent slice and primary cultures, and hiPSC CNs, in being dose and time dependent, and dependent on endogenous ASyn expression (Iannielli et al. 2022; Lam et al. 2024; Mahul-Mellier et al. 2020; Polinski et al. 2018; Roux et al. 2020; Volpicelli-Daley, Luk, and Lee 2014). However, while almost all previous studies in rodent and human models have

employed ectopic *SNCA* overexpression, *SNCA* copy number variations, or *SNCA* mutations that enhance aggregation proclivity (Baena-Montes, Avazzadeh, and Quinlan 2021; Dettmer 2018; Iannielli et al. 2022; Lam et al. 2024), here, I observe significantly elevated levels of pASyn-S129+ pathology in LRRK2 and PSEN1 mutant lines, and even healthy controls, with endogenous, physiological levels of ASyn expression.

Only Bieri et al. (2019) observe serpentine-like pASyn-S129+ labelling by 6-10 days in healthy control NGN2 CNs, exacerbated by LRRK2-G2019S, and Gribaudo et al. (2019) observe LB and LN-like inclusions in healthy control growth factor guided CNs only 30 days, not 15 days following similar concentrations of PFF insult (~6.5 ug/mL). However, Bayati et al. (2023) observe no inclusionopathy in CNs after 14 days, even with concomitant immune insult, though at a lower dose of 1 ug/mL, and Vajhøj et al. (2021) explicitly do not observe any pathology without induction of an *SNCA*-A53T mutation in the endogenous pool of ASyn.

A combination of factors is likely at play here, to successfully bring about an inclusionopathy model in hiPSC CNs. First, at 10 ug/mL, the dose used is relatively high, though 5 ug/mL insults were also sufficient to seed pathology, not just in the PSEN1-Int4Del cell lines (Figure 3.1), and these are still within the range of what has been reported previously (Lam et al. 2024). Second, the duration post insult employed here, longer than any other study, also likely contributed to observation of more severe LB and LN-like inclusions, as these were more time dependent, though pASyn-S129+ pathology was evident as early as 10-30 days, in line with other studies. Third, the robust electrophysiological activity of these neurons at the time of treatment is likely important in driving pathology (Ramalingam and Dettmer 2023; Roselli and Caroni 2015; Wu et al. 2020; Yamada and Iwatsubo 2018). Other studies perform insults at much earlier timepoints, and do not employ astrocyte coculture, which has been shown to enhance maturation (Das et al. 2024; Hedegaard et al. 2020).

Fourth, the more mature state of the neurons may also contribute to higher ASyn expression. While endogenous ASyn here was unperturbed, it is not clear quantitatively how it compares to expression levels in other models, or even in the brain. Lam et al. (2022), using an NGN2-CN model, find that the ratio of ASyn to β 3T in their cell model is equivalent to that in the human cortex, though it is not clear this is a good measure of brain ASyn expression, since most ASyn would be expressed at the synapse rather than in the cell body itself, and the brain sample would contain a heterogeneous mixture of cells.

A final factor that might enhance the vulnerability of the cells may be ApoE genotype, which is thought to influence the risk of concomitant LP in the AD brain, and exacerbate ASyn seeding (Jin et al. 2022). Most of the lines employed here were 3/3 status, indicating relatively neutral risk, though it has not typically been reported what genotype other studies employed.

As such, a confluence of factors has enabled a CN model of ASyn pathology formation that, morphologically, resembles patterns of pathology not just in other cell models but also in the brain.

3.4.3. Correspondence Between Inclusions in the hiPSC CN Model and the Brain

In these experiments, I observed a range of pASyn-S129+ inclusions, especially in PSEN1-Int4Del. These included punctate labelling, long, thin, neuritic inclusions, which resembled LNs, and large, round, perikaryal inclusions, which resembled LBs, in line with reports from rodent primary cultures (Luk et al. 2009; Mahul-Mellier et al. 2020; Volpicelli-Daley, Luk, and Lee 2014; Volpicelli-Daley et al. 2011) and hiPSC neurons (Bieri et al. 2019; Gribaudo et al. 2019; Iannielli et al. 2022; Lam et al. 2024). In addition, I also observed a range of labelling patterns, such as cytoplasmic pale body-like inclusions, donut-like structures, and diffuse

cytoplasmic punctate labelling coalescing onto relatively intense, large, round inclusions adjacent to nuclei, which have been described in human brain pathology (Heiko Braak et al. 2003; Kuusisto, Parkkinen, and Alafuzoff 2003).

Kuusisto et al. (2003) compiled a comprehensive catalogue of LP in the SNPC, and many of the structures described therein are also observed here. Notably, they described LBs as being spherical inclusions, minimum 4 mm in diameter, and adjacent to the nucleus, which are defining qualities of LB pathology assessed by the analysis pipeline here. Similarly, a range of short and thin, to voluminous and bifurcating LNs, sometimes ending in teardrop shaped enlargements were also observed here, matching descriptions from Braak et al. (2003). One notable difference between cell models and brain pathology is that typically only one LB is reported per neuron in cell models, as observed here, though several bodies can develop and cluster in neurons in the brain (Kuusisto, Parkkinen, and Alafuzoff 2003; Mahul-Mellier et al. 2020).

Many studies in cell models note an initial formation of neuritic pathology, with aggregates being retrogradely transported before somatic inclusions develop (Mahul-Mellier et al. 2020; Volpicelli-Daley, Luk, and Lee 2014; Volpicelli-Daley et al. 2011). I did not observe a substantial change in the balance of axonal and somatodendritic pathology changes with time, by comparing pathological load in different ROIs. While it is difficult to compare time courses between hiPSC and rodent primary culture models, this may be because the earliest timepoint used here is 10 days, at which point there was already substantial somatodendritic pathology, in addition to axonal. Rodent culture models typically observe neuritic pathology within 3-4 days, and somatic inclusions not long afterwards, with hiPSC overexpression models having a similarly rapid time course. It may also be that grouping pathology in axons and dendrites collectively, and comparing this to somatic pathology, would be a more apt distinction than axonal and somatodendritic.

While the shifting ratio of MAP2:β3T axonal pathology over time and genotype roughly reflected observations of LN and LB pathology at different timepoints, a technical limitation of this analysis was the quality of the β3T labelling, which was more variable compared to MAP2. Relatively weak β3T labelling of thin processes, possibly made weaker by neuritic inclusions themselves, either by disrupting the axonal process or by physically blocking antibody access, meant that the analysis pipeline searching for LNs within the β3T ROI often missed the full extent of LN pathology, or cut LNs short. This may also be why LNs do not substantially increase in length or area over time. The criss-crossing of neurites in mature, highly networked cultures, also confounds the definition of axonal compartment vs somatodendritic compartment, despite seeding neurons at a relatively low density to maximise the ability to clearly observe neuritic pathology. The apparent plateauing of LN formation in the PSEN1-Int4Del lines after 30 days is nevertheless in concordance with other studies showing that neuritic aggregates in rodent primary neurons stop increasing after 14 days, with pathology likely being transported to and becoming more centred on the perinuclear region over time (Mahul-Mellier et al. 2020).

Strictly, the LNs and LBs observed in the hiPSC CN cell model here are only LN and LB-like, since ultrastructural characterisation has not been performed, nor labelling for other characteristic markers such as p62, ubiquitin, and organelle inclusion (Kuusisto, Parkkinen, and Alafuzoff 2003; Lam et al. 2024; Lashuel 2020; Mahul-Mellier et al. 2020; Shahmoradian et al. 2019). pASyn-S129 labelling, while a key feature, is not typically considered sufficient to properly define LBs, which have distinct structures, are membrane rich, and appear to undergo several stages of development. Nevertheless, the morphological similarity, localisation to being nuclear adjacent within the cell, and time course over which they appear, make it reasonable to assume that the LBs described here are similar to those deeply characterised in other cell models, shown to reflect brain pathology (Lam et al. 2024; Mahul-Mellier et al. 2020).

Furthermore, the presence of a broad range of structures along the spectrum of ASyn aggregation observed in the brain suggests that the pathological process occurring in the cell model seeded by the PFF insult mirrors that which occurs in disease (Kuusisto, Parkkinen, and Alafuzoff 2003). However, while it is important for the validity of the model to see that the aggregation process is disease relevant, the aim of this study is not to characterise LP itself, but to understand differential susceptibility of these cells to this process.

3.4.4. Selective Vulnerability of PSEN1-Int4Del CNs

Despite the lengths taken to be able to observe ASyn pathology in an hiPSC CN model, severe inclusion formation was largely only observed in the PSEN1-Int4Del clonal pair. This presents the CN model as, indeed, relatively resilient to ASyn pathology, as might be expected from brain pathology and previous studies. However, it also presents the question of what makes the PSEN1-Int4Del CNs so uniquely vulnerable, even compared to other PSEN1 and APP mutations, let alone PD LRRK2 mutations.

The Int4Del mutation is a relatively severe form of fAD, with an earlier age of onset than most other mutations at 37-38 (Janssen et al. 2000; N. S. Ryan et al. 2016), though from the limited autopsies performed, it does not appear to have any more LP than other fAD cases (Janssen et al. 2000). Arber et al. (2020) do note that PSEN1-Int4Del mutations reduce gamma-secretase activity more than other mutations, which might make it more severe. Like many fAD cell lines, PSEN1-Int4Del hiPSC CNs also release A β 42:40 in a higher ratio than healthy controls, though not to a degree that distinguishes it from other fAD lines (Arber et al. 2020; 2019; Hung and Livesey 2018).

The relative resilience of the isogenic corrected control here identifies the vulnerability of the PSEN1-Int4Del lines as a result of the mutation, which produces three PSEN1 splice

transcripts, two of which are truncations (De Jonghe et al. 1999). It may be that this results in a loss of PSEN1 function, in addition to the threonine insertion in the third transcript responsible for elevated A β 42 (De Jonghe et al. 1999). PSEN1 has been implicated in autophagy function in hiPSC neurons (Chong et al. 2018; Hung and Livesey 2018; Kwart et al. 2019; Mustaly-Kalimi et al. 2022). Chong et al. (2018) notably shows that PSEN1-KO leads to suppressed autophagy. Here, the PSEN1-Int4Del cell lines appear to be relatively insensitive to chloroquine, an autophagy blocker, compared to their corrected control and the PSEN1-M146I cell line, suggesting that impaired autophagy may underpin vulnerability here. This hypothesis will be explored in depth in Chapter 5.

3.4.5. Aggregation Independent Effects on Nuclear Morphology and Neuronal Area

Another striking observation here is the reduction in nuclear area, apparently driven by PFF insult. Since this was observed in most cell lines, irrespective of aggregation levels observed, and even in the SNCA-KO line, it would seem this is a direct effect of the PFF, and aggregation independent. Changes in nuclear morphology in the context of protein aggregates have scarcely been examined or reported elsewhere. In a study employing machine learning to identify PD subtypes from imaging hiPSC-derived neurons, D'Sa et al. (2023) note that nuclear morphological features were important discriminating factors in determining subtypes by their response to ASyn oligomers, though it is not clear what these features are. Interestingly, one pathology report indicates that in the AD and PSP brain, tau pathology is associated with larger nuclei and condensed chromatin, whereas in the DLB brain, reduction in nuclei area and relaxed chromatin was observed, compared to healthy controls in frontal cortical tissue (Montalbano et al. 2023). This corresponds with observations here. Though the functional

significance of this as it might relate to nuclear toxicity or DNA regulation is unclear, there is an increasing appreciation for the role ASyn plays as a mRNA binding protein (Hallacli et al. 2022).

I observed little other toxicity of the PFF insult in terms of nuclei number, though some effect on neuronal morphology was seen. Other studies are highly mixed in reporting neurotoxicity and neurodegeneration. Here, neurons receive support that is likely both physical and trophic from rAstros, so might be expected to be more resilient. Nevertheless, there is some time dependent attrition of neuronal area associated with PFF insult, which, since there is no reduction in nuclei number, would appear to reflect a reduction in neuronal size and/or neurite thinning and/or retraction. This also appears aggregation independent. While the toxicity of ASyn PFFs is thought to depend on recruitment of endogenous ASyn, i.e. prion-like aggregation dynamics, Denning et al. (2022) show a toxic effect of ASyn oligomers on mitochondrial membrane potential in *SNCA*-KO mouse primary neurons, suggesting that both aggregation dependent and independent mechanisms might be at play in driving neuronal vulnerability to aggregate insult.

3.4.6. No Effect on Astrocytes

Since effects of nuclear and cell morphology appear to be independent of aggregation, it is reasonable to question whether these are an indirect effect of PFF-driven changes in the rAstros. This is also important to address in considering whether the aggregation effects observed here are the results of cell autonomous factors driving neuronal vulnerability, or an interaction effect with the rAstros. One study on hiPSC-derived mDANs noted that PFF-seeded ASyn inclusions were only observed with concomitant IFN γ immune insult (Bayati et al. 2023), and hiPSC-derived astrocytes and microglia readily take up and break down ASyn PFFs (Rostami et al. 2021). While an impact of the PFF insult on the rAstros and resultant

interactions such as these cannot be definitively ruled out here, I observed no indications of toxicity, inflammation, or aggregation in the rAstros.

Rat astrocytes grown in monoculture showed no pASyn-S129+ labelling in response to PFF insult and had no changes to nuclear or cell morphology that might indicate aggregation independent toxicity, as observed in the neurons. Similarly, there was no upregulation of GFAP or S100 β that might indicate an inflammatory response. ICC data from Chapter 2, as well as western blotting here showed that rAstros do not appear to express ASyn, and therefore might function analogously to *SNCA*-KO cells, with regards to aggregation. Furthermore, the species barrier between rat and human may also prevent seeding of pathology by human PFFs (Luk et al. 2016). In cocultures, similarly, no toxic or inflammatory effect of PFF on rAstros was observed, and pASyn-S129+ labelling which did not match MAP2+ processes also did not correspond to rAstros. This neuritic-type labelling which does not associate with MAP2 has also been observed in other studies (MacIsaac et al. 2020), and here is confirmed to be in β 3T+ axons.

3.4.7. Strengths, Controls, and Limitations

As such, one of the strengths of the experiments presented here is the extent of the controls employed. In addition to confirming all pathology is most likely the result of neuronal-specific effects, I also perform IgG negative control labelling in both PFF untreated and treated cultures, to confirm that antibody immunoreactivity is specific to seeded, phosphorylated aggregates, and not the result of non-specific labelling or interaction with the PFFs themselves. The employment of the *SNCA*-KO control and isogenic corrected cell lines, in addition to multiple timepoints and doses, stitch together a comprehensive picture of a time, dose, endogenous ASyn, and for PSEN1-Int4Del, mutation-dependent effect of PFF insult on seeded aggregation. I also use a much larger array of cell lines and genotypes here, compared to most other hiPSC studies, which have typically examined 2-3 cell lines.

Concerningly, once again, the KOLF2.1J cell line was noted to have aberrant behaviour relative to other healthy controls, especially with regards to neuronal and nuclear morphology, highlighting the importance of employing a larger number of cell lines. While monomer controls are often employed in the context of PFF experiments to examine whether the aggregates formed are specifically seeded by fibrils, and not a generic protein load, here, these were not used. This is because, rather than being interested in the question of what kind of insult drives pathology, this study was more concerned with differences in vulnerability to a disease relevant insults and processes. Since the PFFs here clearly seed pASyn-S219+ pathology which resembles that formed in the brain and other models, this is deemed sufficient to qualify the PFF model as a disease relevant insult for the question of selective vulnerability. Thus, controls are focused on differences between cell types and genotypes, rather than the type of insult.

3.4.8. Conclusions

These experiments have shown that the ASyn PFF insult model in the hiPSC-derived CN paradigm established here leads to substantial, disease-relevant pathology, even without *SNCA* overexpression or mutation. I report LP in AD hiPSC CNs for the first time, including the notable profound vulnerability of the PSEN1-Int4Del, as well as aggregation independent effects on nuclei morphology, which are underexamined in the literature but present an easy opportunity for further research using existing data. Importantly, experiments in this chapter also show that vulnerability to this PFF-seeded pathology is most likely a neuronal cell-autonomous process, rather than driven by interactions with rAstros. Next, is to consider how the vulnerability of these neurons compares to that of mDANs, which will be examined in the subsequent chapter. Effects of the PFF insult beyond simply pASyn-S129+ pathology will also be examined in Chapter 6.

Chapter 4.

Alpha Synuclein PFF-Seeded Pathology in Midbrain Dopaminergic Neurons

4.1. Introduction

In this chapter, I examine the vulnerability of mDANs to ASyn PFF-seeded pathology. mDANs in the brain, especially in the SNPC, are highly vulnerable to LP. Given this, and the functional importance of these cells to PD, there have been remarkably few prior examinations using ASyn PFF insults in hiSPC mDANs. These studies found increased pASyn-S129+ labelling and Lewy-like pathology in cell lines with PD-associated *GBA* mutations (S. Kim et al. 2018), PD-associated *SNCA* mutations and *SNCA* triplication (Tanudjojo et al. 2021; Iannielli et al. 2022), and HCs, though in this case, only with co-application of an immune insult (Bayati et al. 2023).

The effects of ASyn PFFs in mDAN cultures beyond 28-30 days, or in AD PSEN1, or PD LRRK2 neurons have not been examined before in hiPSC models, though rodent primary neurons with LRRK2-G2019S mutations have enhanced vulnerability to PFF-seeded pathology (MacIsaac et al. 2020).

Furthermore, few studies have examined mDANs in comparison with CNs. Here, for comparison with PFF insults in the relatively resilient CNs, I grew hiPSC mDANs alongside rAstros to D36 of differentiation, when ASyn expression was robust, and monitored cells for up to 40 days after insult. This is 10 days longer than has been reported elsewhere in the literature (Bayati et al. 2023).

The aims of this chapter, which is largely designed to mirror the investigation of CNs in the previous chapter, are thus to:

- 1) Demonstrate an inclusionopathy model in hiPSC-derived mDANs for comparison with CNs.
- 2) Determine the degree to which hiPSC mDANs are vulnerable to ASyn PFF seeded pathology in the various AD and PD genotypes examined.

- 3) Measure other effects of ASyn PFFs and resultant pathology on cellular and nuclear morphology, as indicators of vulnerability.

4.2. Methods

The PFF insult paradigm used in the mDANs is described in Chapter 2, and essentially the same as for CNs. Briefly, cells grown in 96-well plates are insulted at D36, and then half media changes resume on D39. This is one day shorter than the 4-day incubation for CNs before first feed, owing to the high media demand of the dense mDAN cultures, though PFF uptake is thought to be extremely rapid (Bieri, Gitler, and Brahic 2018; Gribaudo et al. 2019). As a positive control, in some cases, I also include chloroquine alongside PFF insult. Similarly, this is also not washed out but diluted over time. Consistent with CN culture, mDANs were grown in coculture with rAstros.

As per CN experiments, I use immunocytochemical labelling of pASyn-S129 in PFF-insulted cells as a primary outcome. Immunolabelling and imaging methods are described in Chapter 2, and image analysis pipelines are described alongside results.

4.3. Results

4.3.1. Qualitative Observations of pASyn-S129+ Pathology in mDANs

mDANs cocultured with rAstros were insulted with ASyn PFFs at D36, and subsequently fixed 10, 20, 30, or 40 days later. Some cell lines, including APP-V717I, healthy controls 156 and 856, *SNCA-KO*, and iNDI lines, were only examined at 20 and 30 days. However, for graph consistency, all genotypes have been included at all time points on the X-axes. Fixed cells were immunolabelled for pASyn-S129, as well as dopaminergic cell marker TH, and neuronal cell marker MAP2 or β 3T, and nuclei were stained with DAPI.

Striking pASyn-S129+ pathology with a range of morphologies was evident 30-40 days after PFF insult (Figure 4.1). As with CNs, these structures included small punctate labelling, and thin, thread-like neuritic labelling, typically ~10 μ m but sometimes extending throughout neurites and reaching 50-100 μ m in length. Perikaryal inclusions were also highly abundant, including smaller crescent or triangular shaped inclusions forming around nuclei, and especially larger, oblong accumulations that seemed to fill a portion of the cell body adjacent to the nucleus, with more granular labelling texture, reminiscent of pale bodies. LB-like structures were also observed in addition to these.

These labelling patterns were compared across cell lines at 30 days after a 10 μ g/mL insult in Figure 4.2, and appeared to be typical across all genotypes except *SNCA-KO*, which exhibited no pASyn-S129+ labelling. All other cell lines, including healthy controls, PSEN1, APP, and LRRK2 mutant lines, exhibited similarly heavy burdens of pathology, with extensive neuritic and perikaryal inclusions. Of note, there was no obvious difference in pathological burden between the PSEN1-Int4Del clonal pair and their isogenic corrected control, despite the substantial difference in vulnerability in CNs.

To examine whether, at a 10 µg/mL dose, the apparently highly vulnerable mDANs were at the upper limit of their dose-response curve, thus making the assay insensitive to genotype differences, PFFs were seeded at lower doses of 2.5 and 5 µg/mL in the PSEN1-Int4Del and corrected control pair, and PSEN1-M146I (Figure 4.3). This showed that pASyn-S129+ labelling was dose dependent, and pathological burden was similar across all three genotypes, including control, even at lower dosages. No pASyn-S129+ labelling could be observed in the untreated or vehicle treated condition for any cell line, beyond levels of non-specific labelling in the IgG isotype negative control, which only displayed some light non-specific punctate labelling (Figure 4.3i,ii,vii).

The development of pathology was also clearly time dependent, with only minimal punctate and some short neuritic pathology evident after 10 days, even at higher 10 µg/mL doses, longer neuritic aggregates forming mostly after 20 days, and larger perikaryal aggregations mostly presenting by 30 and 40 days (Figure 4.4).

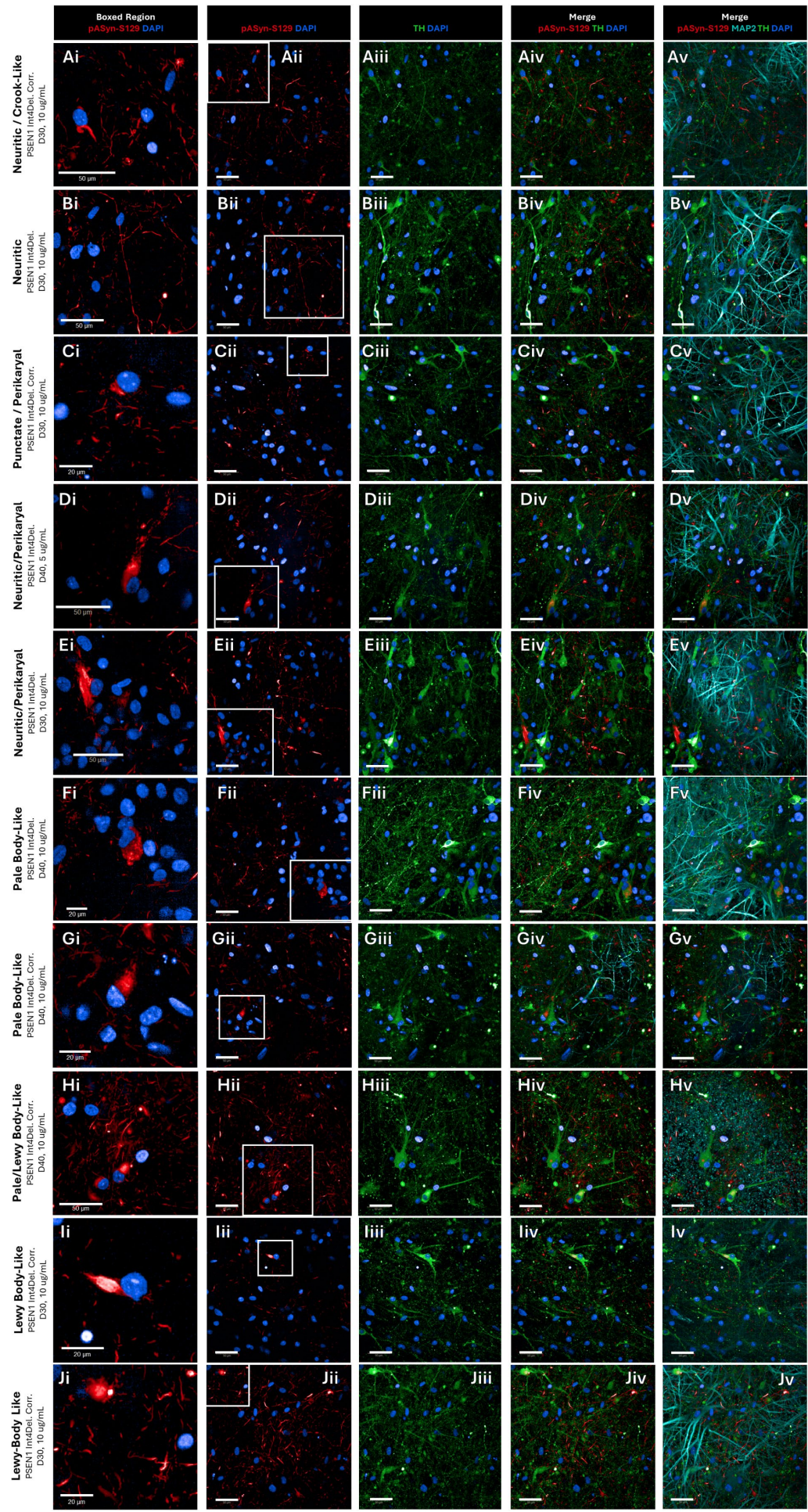


Figure 4.1. Examples of pASyn-S129+ Labelling in MAP2+ TH+ Midbrain Dopaminergic Neurons. Inclusions with a variety of morphologies were observed in PFF-insulted cells after 30-40 days, including **(A)** neuritic labelling, including short, thin structures, as well as larger, thicker structures, with crook-like morphologies **(B)** extremely long, thin neuritic labelling, **(C)** small and punctate labelling adjacent to nuclei in the perikaryon, **(D-E)** larger, perikaryal inclusions that appeared to fill the soma and extend with punctate labelling into neurites, **(F-G)** large, slightly less intense, soma-filling inclusions with a granular texture adjacent to nuclei, resembling pale bodies, **(H)** large, rounder, somatic inclusions, of higher intensity, more resembling Lewy body-like inclusions, **(I)** highly intense Lewy-body like somatic inclusions with spindle tendrils adjacent to nuclei, and **(J)** large, round, dense-appearing Lewy body-like structures. These images are from PSEN1-Int4Del and its corrected control, representative of a range of pathology. Scale bars are 50 μ m, except where otherwise indicated as 20 μ m.

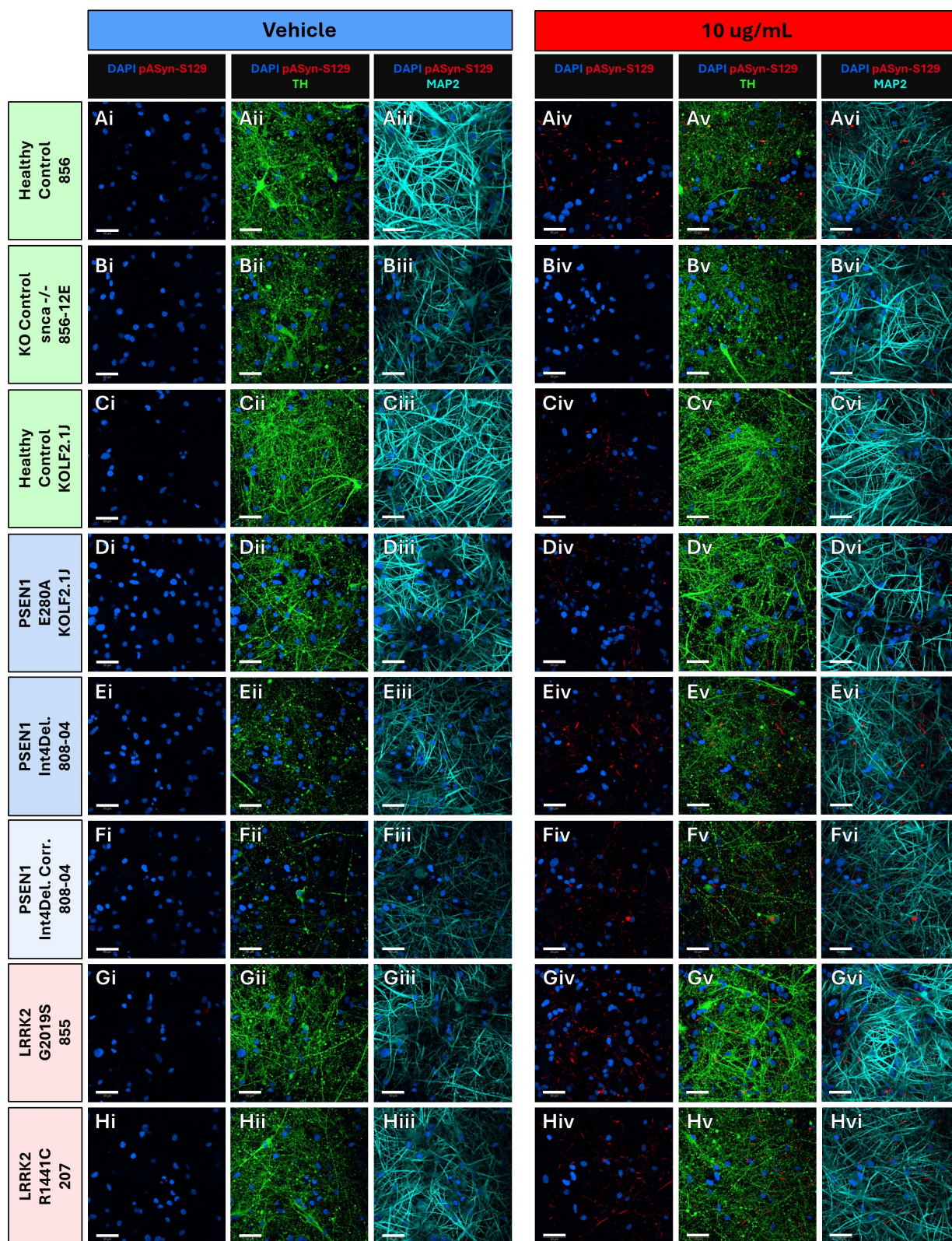


Figure 4.2. High levels of pASyn-S129+ Labelling Evident in All Cell Lines Except SNCA-KO.

Representative images from cells 30 days after 10 μ g/mL PFF insult from a representative range of different genotypes are shown, with vehicle treated cells on the left (i-iii), and PFF-insulted cells on the right (iv-vi). With the exception of SNCA-KO, all genotypes, including healthy and corrected controls, exhibited high degrees of neuronal pASyn-S129+ labelling, without obvious differences. All lines exhibited a variety of inclusion morphologies including neuritic, pale body-like, and Lewy Body-like, as depicted in Figure 4.1. Scale bars are 50 μ m.

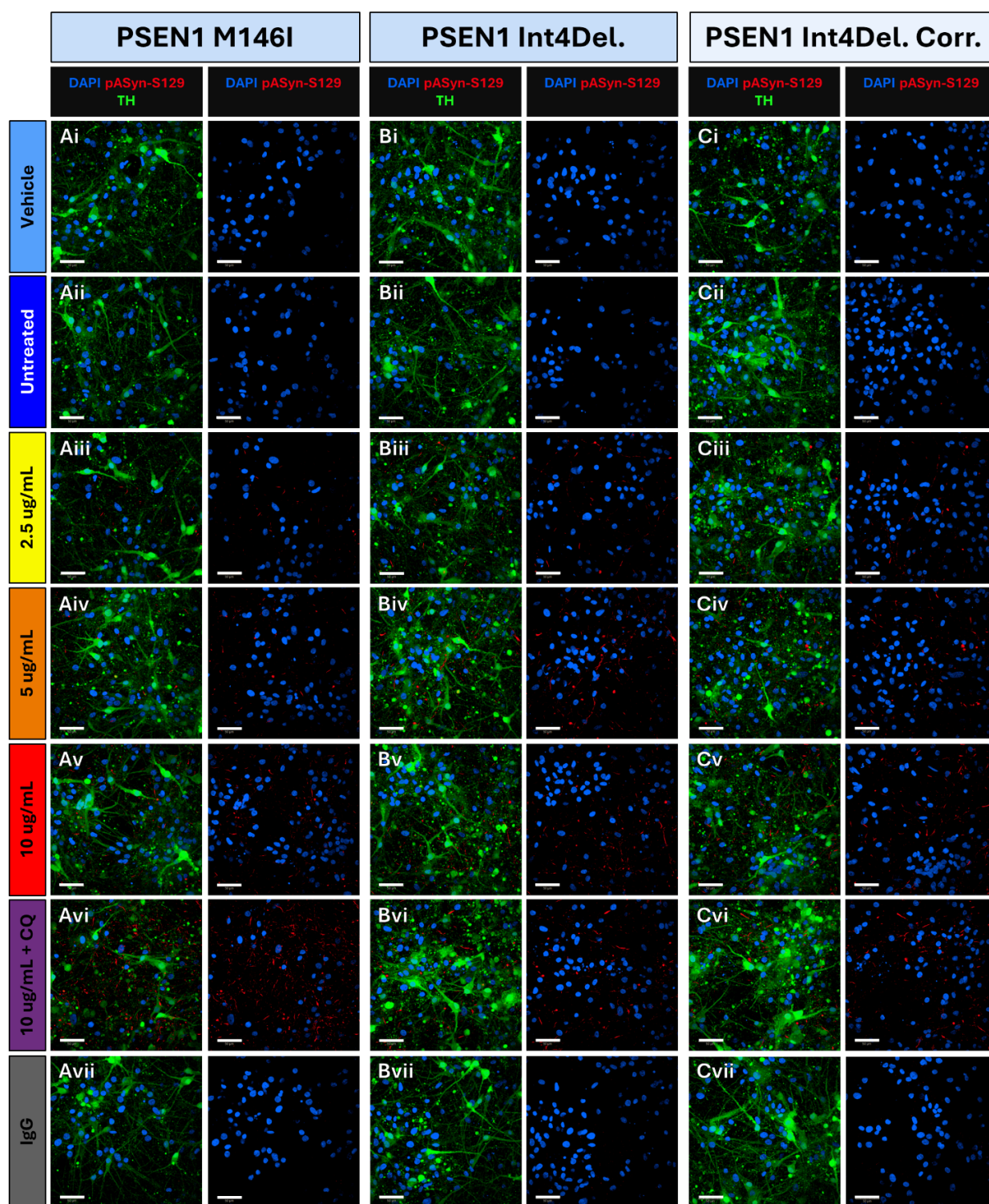


Figure 4.3. pASyn-S129+ Labelling is Dose Dependent. A range of dosages, including co-treatment with chloroquine, was examined in (A) PSEN1-M146I, (B) PSEN1-Int4Del and (C) PSEN1-Int4Del corrected control at 20 days after PFF insult. (i-ii) Only slight punctate labelling was observed in vehicle and untreated cells, not distinct from IgG labelled cells, shown in (vii). (iii-v) Insult with 2.5, 5, and 10 $\mu\text{g/mL}$ PFF led to a dose dependent increase in pathological severity, though no obvious differences between genotypes. Labelling at 2.5 $\mu\text{g/mL}$ was largely restricted to short neuritic pathology, while larger and more somatic structures were present at higher dosages. (vi) 10 $\mu\text{g/mL}$ PFF insult with 20 μM chloroquine, an autophagy blocker, led to substantially more seeded aggregates, especially in the PSEN1-M146I line. (vii) minimal labelling was apparent in the PFF-untreated, IgG negative control labelled cells. Scale bars are 50 μm .

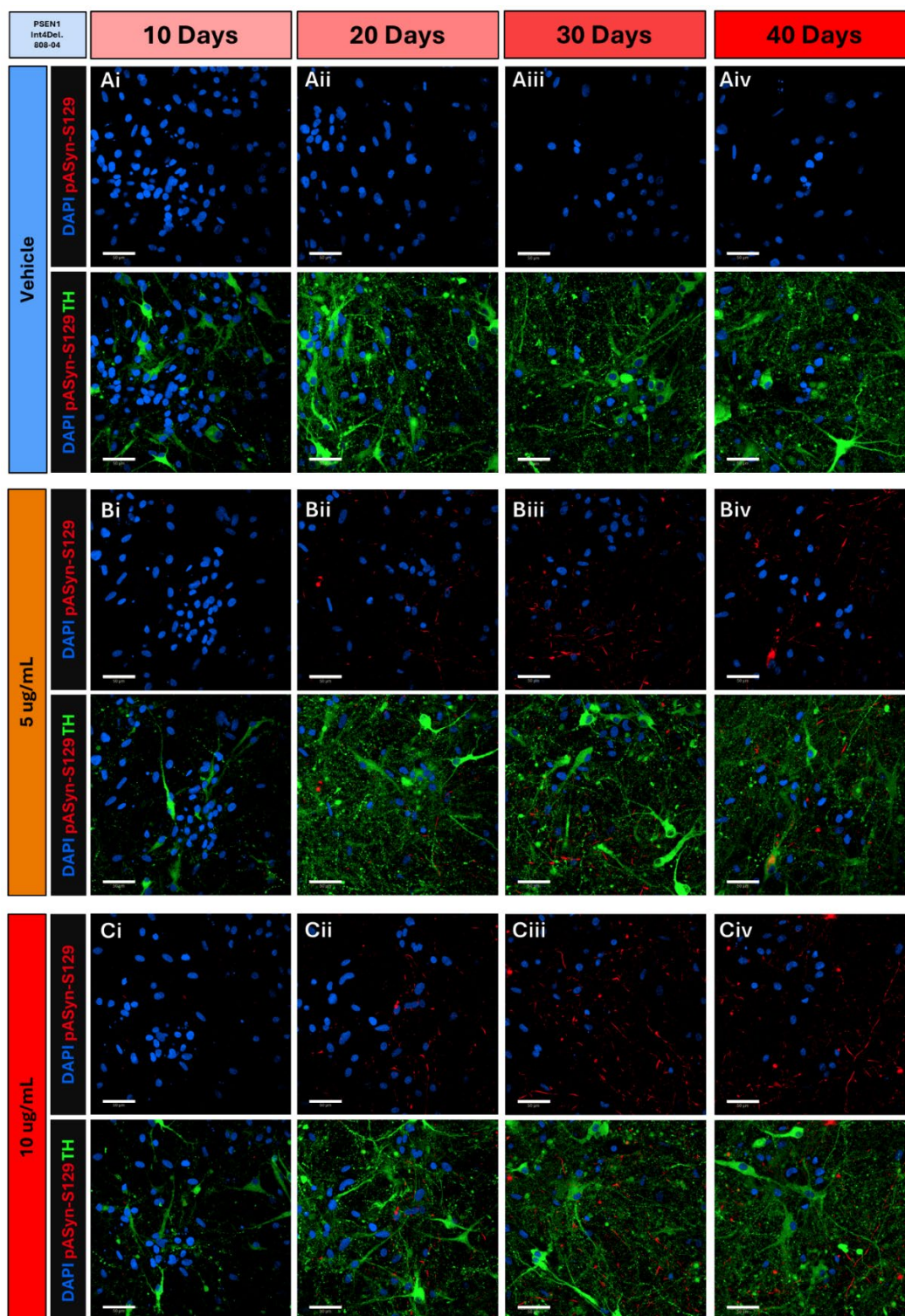


Figure 4.4. pASyn-S129+ Labelling is Time Dependent. Images of PSEN1-Int4Del mDANs, representative of all cell lines other than SNCA-KO, are presented from sequential timepoints after PFF insult at three concentrations. **(i)** At 10 days, labelling is largely punctate, with some small, short, neuritic inclusions. **(ii)** At 20 days, numerous, short, neuritic inclusions are evident, extending discontinuously throughout neuronal processes, with some larger, thicker, and somatic inclusions present at higher concentrations. **(iii)** At 30 days, numerous neuritic inclusions are longer and thicker, some with rounded or club-like endings, and more, small, rounded, somatic inclusions are evident. **(iv)** At 40 days, long, serpentine, and thicker neuritic inclusions are present, often extending throughout neuronal processes, with large, round, pale body or Lewy Body-like structures apparent too. Inclusions thus appear to become more numerous and more severe with time. Scale bars are 50 μ m.

4.3.2. Quantitative Assessment of pASyn-S129+ LP in mDANs

To quantify the pathology burden in these images, a custom pipeline was designed in Harmony 5.2 software, analogous to that for CNs (Figures 4.5-7). First, to generate cellular ROIs, the images were split into pASyn-S129, TH, and MAP2 channels (Figure 4.5). In one differentiation, β 3T was used as a neuronal cell marker, instead of MAP2, and was treated equivalently. Then, four ROIs were created, whose area could be calculated, and could be used to localise pathology: MAP2/ β 3T area (both TH+ and TH- cells), TH cell area, TH+MAP2 "total" cell area, and TH- MAP2 "non-dopaminergic" cells. This thus allowed comparison of dopaminergic and adjacent non-dopaminergic cells. An analogous pipeline to that for CNs, outlined in Figure 4.6, was then used to identify and distinguish TH+, and TH- neuronal nuclei, for downstream analysis and counting.

To quantify the pASyn-S129+ pathological burden in the cells, I categorised labelling patterns into three main categories, as per CNs: puncta of all morphologies (LP), longer "Lewy neurite"-like structures (LNs), and "Lewy Body"-like structures (LBs), as outlined in Figure 4.7.

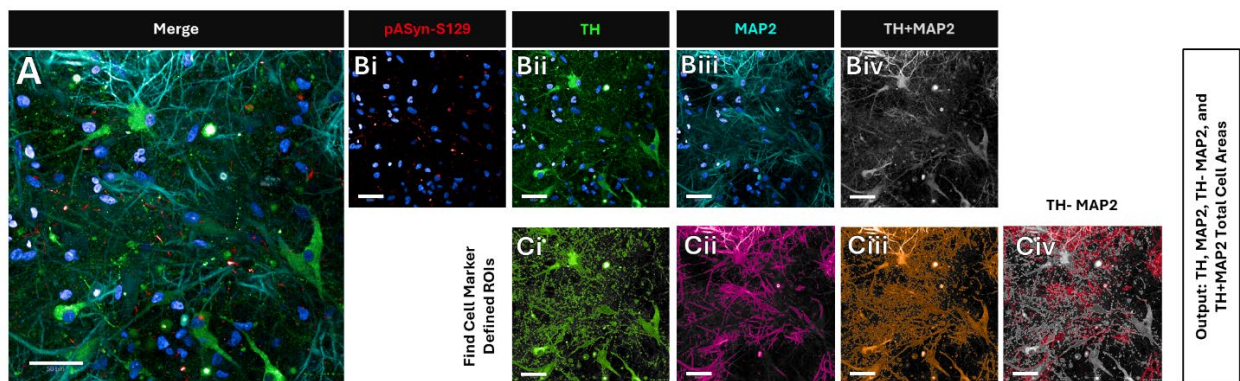


Figure 4.5. Analysis Pipeline for Neuronal Morphology Labelling and ROI Construction in Midbrain Dopaminergic Cultures. To analyse pASyn-S129+ labelling in MAP2 and TH-labelled neurons, neuronal ROIs were defined. **(A)** A merge representative image with pASyn-S129+ labelling of various punctate, neuritic and somatic inclusions in TH+ and MAP2+ neurons. **(Bi-iii)** These pASyn-S129, TH, and MAP2 channels are shown separately. **(Biv)** a new channel, merging the MAP2 and TH channel was created. **(C)** From the MAP2 and TH channels, four ROIs were created, using the Find Image Region function with common threshold: **(Ci)** TH Area, **(Cii)** MAP2 Area, **(Ciii)** TH+MAP2 "Total" Cell Area, and **(Civ)** "TH-MAP2", created by subtracting TH+ image area from the total cell area, thus leaving only non-TH MAP2+ cells. These ROIs were used to spatially compartmentalise pASyn-S129+ labelling, and their area was calculated. Scale bars are 50 μ m.

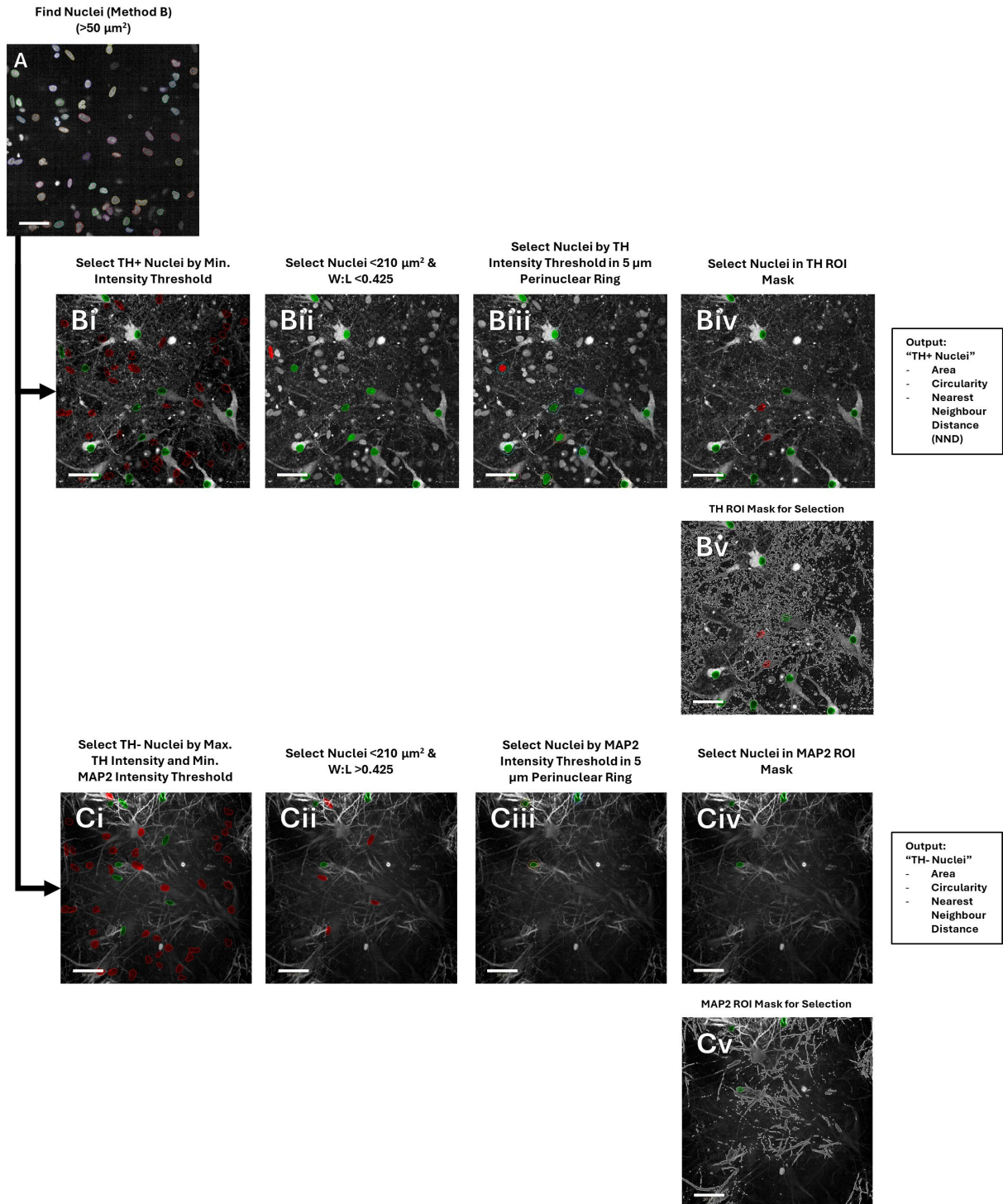


Figure 4.6. Analysis Pipeline for Neuronal Nuclei Labelling in Midbrain Dopaminergic Cultures. To distinguish human neuronal nuclei from rat astrocyte nuclei, since the HuNu antibody was not used here, and to distinguish nuclei in TH+ cells from TH- MAP2+ cells, a series of filters were used to progressively screen out non-neuronal (not clearly in a TH+ or MAP2+ cell body) nuclei and select for nuclei in TH+ or TH- cells. This pipeline was analogous to that for cortical neurons. **(A)** All DAPI+ nuclei were identified using the Find Nuclei Function (Method B). Nuclei were defined as $> 50 \mu\text{m}^2$ to exclude most pyknotic nuclei. **(B)** For TH+ nuclei, **(Bi)** a nuclear ROI was created, and nuclei with intensity below a set threshold in the TH channel were excluded. **(Bii)** Nuclei larger than $210 \mu\text{m}^2$ or with a width:length ratio of <0.425 were excluded. **(Biii)** A perinuclear ring ROI was then created by expanding the nuclear ROI by $5 \mu\text{m}$ and subtracting the DAPI+ nucleus itself. This captured more intense TH+ labelling around the nucleus, since labelling colocalised with DAPI was much weaker. Nuclei with TH+ labelling in the

perinuclear ring below a set intensity threshold were excluded. **(Biv)** Nuclei with <50% overlap with the TH ROI mask were excluded. **(Bv)** shows the same image as **(Biv)** but includes the boundaries of the TH mask. The remaining nuclei were termed TH+ nuclei, and their number, area, circularity, and distance to nearest other neuronal nucleus (nearest neighbour distance, NND) measured. **(C)** An analogous process was used to select for TH- MAP2 nuclei. Here, **(Ci)** nuclei below a minimum MAP2 intensity threshold and above a maximum TH+ intensity threshold were excluded. Following this, in **(Cii-v)**, an identical process was used, but MAP2 intensity used for thresholding. These nuclei were termed TH- Nuclei. In images presented in these figures, DAPI, TH, and MAP2 labelling are shown in greyscale, with green highlights for selected objects and red highlights for excluded objects. Scale bars are 50 μm .

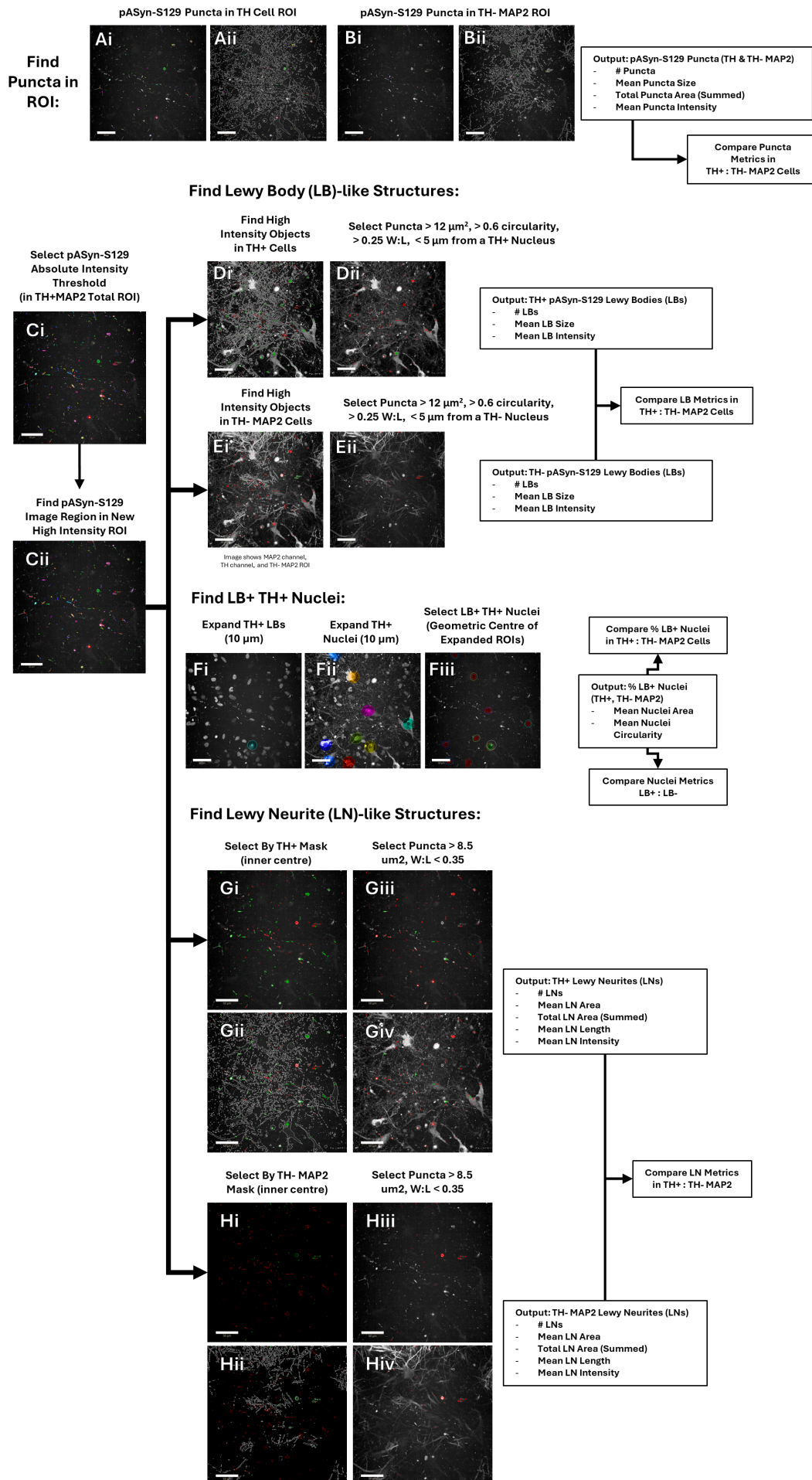


Figure 4.7. Analysis Pipeline for pASyn-S129+ Labelling in Midbrain Dopaminergic Cultures. pASyn-S129 labelling in midbrain dopaminergic cultures was classed into three broad categories: overall labelling, Lewy bodies (LBs), and Lewy neurites (LNs). **(A)** pASyn-S129+ puncta identified by the Find Spots function (Method A) in the input image (same as for Figures 4.5-4.6), within the TH Cell ROI. ROI boundaries are shown in (Aii). **(B)** pASyn-S129+ puncta in the same image identified by the same function within the TH- MAP2 ROI. ROI boundaries are shown in (Bii). For LBs and LNs, from the same input image, **(Ci)** high intensity pASyn-S129+ labelling was first selected using the Find Image Region function and an absolute intensity threshold, within the TH+MAP2 total cell area ROI. **(Cii)** Within this new minimum threshold ROI, the Find Image Region function was used again with common threshold to identify high intensity pASyn-S129+ objects. From these **(D-E)** Lewy Bodies were selected in **(Di)** TH+ cells only, by selecting objects only within the TH ROI, or **(Ei)** TH-MAP2 cells only, by selecting objects only within the TH- MAP2 ROI, and then **(D-Eii)** selecting objects that were $>12 \mu\text{m}^2$, >0.6 circularity, >0.25 width:length ratio, and $<5 \mu\text{m}$ from a TH+ or TH- neuronal nucleus, as appropriate. **(F)** Nuclei in LB laden cells were identified by **(Fi)** expanding the Lewy body mask by $10 \mu\text{m}$, and **(Fii)** expanding the neuronal nuclei mask by $10 \mu\text{m}$, and **(Fiii)** selecting nuclei whose geometric centre overlapped with the expanded Lewy body mask. An analogous process was used to find LB+ TH- nuclei. **(G-H)** To find Lewy neurites, high intensity pASyn-S129+ objects from (Cii) within the MAP2+TH “total” cell area were selected by either **(Gi-ii)** TH Cell ROI, or **(Hi-ii)** TH- MAP2 ROI (ROI boundaries shown in Gii, Hii). **(G-Hiii,iv)** Then, objects $>8.5 \mu\text{m}^2$ and <0.35 W:L ratio were selected as LNs. TH channel is shown in (Giv), and MAP2 channel is shown in (Hiv). In images in this figure, selected objects are highlighted in green, excluded objects are highlighted in red. All scale bars are $50 \mu\text{m}$.

Quantifying puncta of all sizes and morphologies across the TH cell ROI revealed a dose and time dependent accumulation of pASyn-S129+ pathology from as early as 10 days in all cell lines except *SNCA*-KO (Figure 4.8). At each time point, while there was an overall increase in the pathological burden, no effect of disease genotype was found. Puncta size was similar to that in CNs, increasing from $\sim 0.6\text{-}0.8 \mu\text{m}^2$ at 10 days to as much as $1.5 \mu\text{m}^2$ at 40 days (Figure 4.8B).

Puncta density, mean area, area fraction, and intensity were also compared between TH+ and TH- MAP2+ cells (Figure 4.9). This analysis was performed across all genotypes, where available, and revealed a slight preferential vulnerability of the TH+ cells to accumulation of pASyn-S129+ pathology, which did not change with time or dose.

In contrast to punctate labelling, LN pathology was mostly not present at 10 days, with some occasional exceptions, remained low at 20 days for most genotypes, but substantially increased with time and was most pronounced by 30-40 days, without appearing to plateau (Figure 4.10). This time dependent formation of LNs occurred across all cell lines, and there

did not appear to be genotype differences in the number of structures seeded or their size or length. In Figure 4.11, the mean area and length of these LNs are plotted for the same select genotypes as the previous chapter, to clearly illustrate the growth in these structures over time.

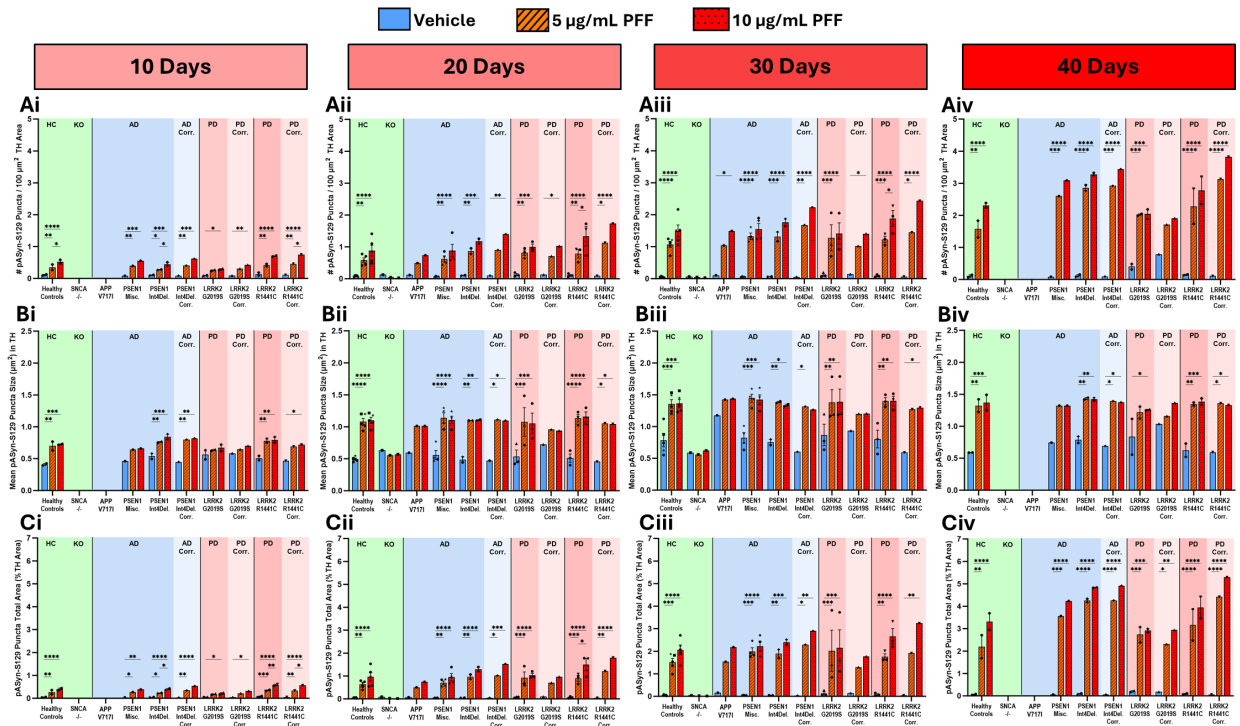


Figure 4.8. pASyn-S129+ Labelling in TH+ Midbrain Dopaminergic Neurons is Elevated in All Cell Lines and Increases with Time. Overall pASyn-S129+ LP labelling within the TH Cell ROI was quantified in three ways. **(A)** the puncta density, calculated as the number of puncta per 100 μm^2 TH area, **(B)** the mean area of each individual puncta, and **(C)** the summed area of all puncta, represented as a percentage of the total TH ROI area (area fraction). All cell lines had significantly elevated pASyn-S129+ labelling compared to their vehicle condition as early as **(i)** 10 Days after PFF insult, and the severity of accumulation appeared to increase at **(ii)** 20 days, **(iii)** 30 days, and **(iv)** 40 days after PFF insult. Minimal labelling was detected in SNCA-KO cell lines, even after 30 days. No effect of genotype was found. Data points represent individual cell lines, averaged over 2-4 independent differentiations, bars represent mean $\pm$ SEM. In each differentiation, 100s-1000s of puncta were measured per well, or 1000-10,000s by D40, or 10s in SNCA-KO, across 3-4 wells per cell line. Isogenic pairs are noted with shared point shape. Healthy Control 856 and its SNCA-KO are square points. iNDI KOLF lines are represented by stars. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and iNDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as “PSEN1 Misc.” APP, SNCA-KO, HC 856 and 156, and iNDI lines were only examined at 20 and 30 days. Statistical comparisons are 2-Way ANOVA with Bonferroni correction for multiple comparisons.

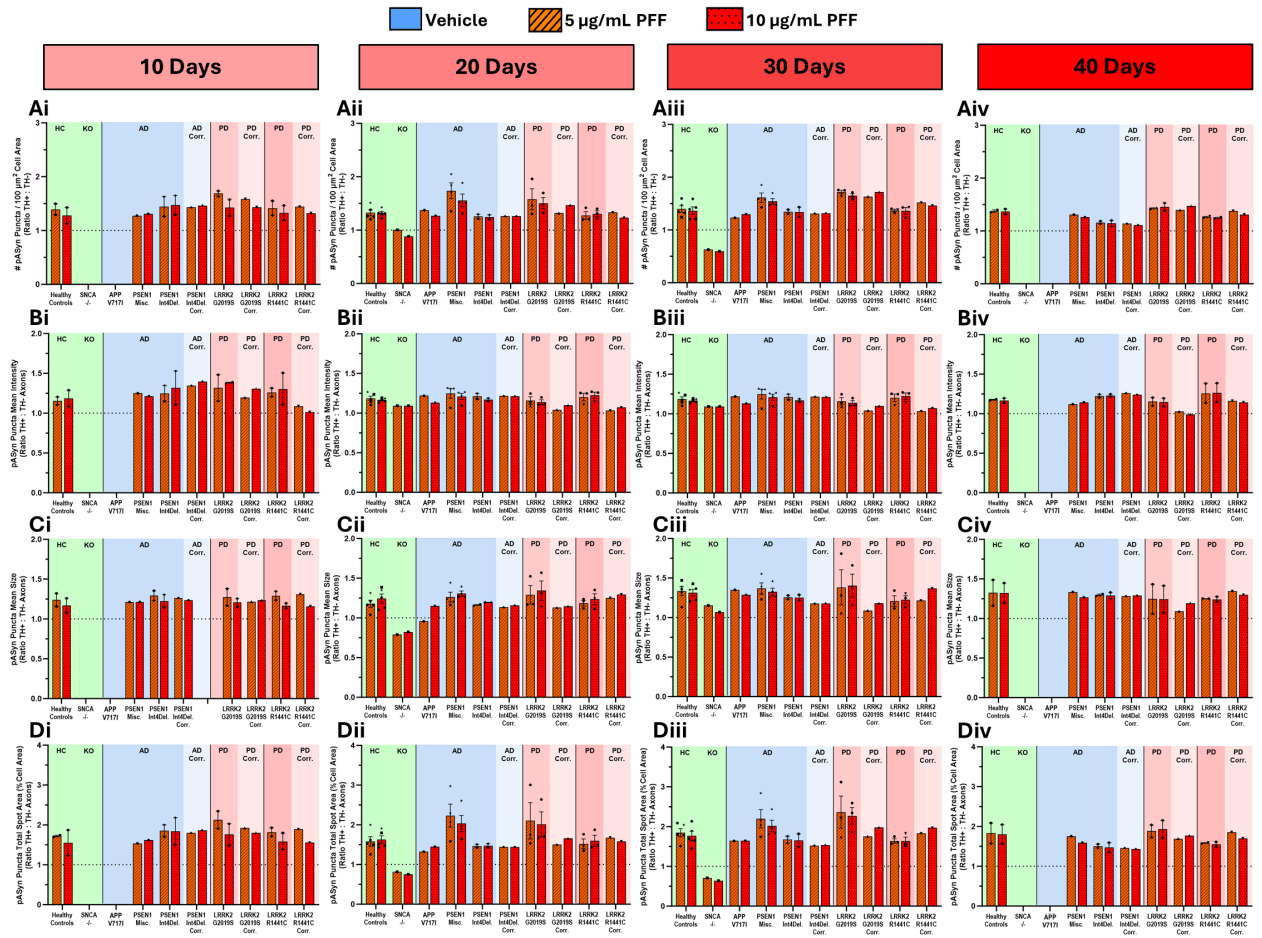


Figure 4.9. pASyn-S129+ Pathology Preferentially Affects TH+ Cells. To examine whether TH+ cells were differentially vulnerable compared to TH-MAP2+ cells, and how this changed with duration post insult, the ratio of various measures of pathology in the TH+ ROI to TH-MAP2+ ROI was calculated. Values above 1 indicate more pathology in TH+ cells. Pathology was measured within each ROI as **(A)** Puncta density within the relevant ROI, where there was ~1.5-fold more pathology in TH+ cells, which did not change with time; **(B)** mean puncta intensity, which was typically ~1.25-fold higher in TH+ cells across all time points; **(C)** mean puncta size, which was typically ~1.25-fold larger in TH+ cells across all time points; and **(D)** total summed puncta area as a proportion of the relevant ROI (area fraction), which was ~1.5-2-fold higher in TH+ cells. The system of denoting particular cell lines with different shaped points was retained from earlier figures.

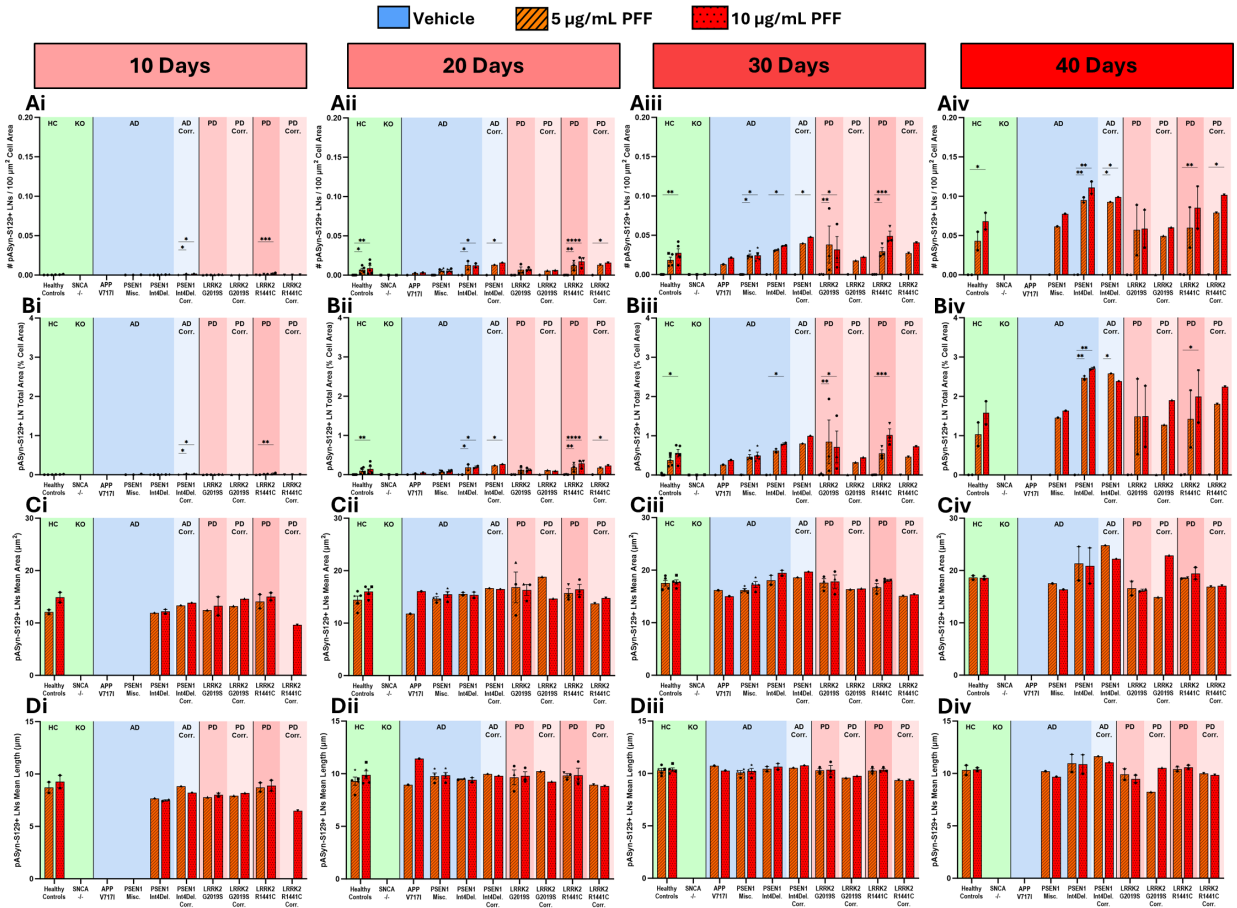


Figure 4.10. The Number of pASyn-S129+ Lewy Neurite Structures in TH+ Midbrain Dopaminergic Neurons is Elevated in All Cell Lines and Increases with Time. pASyn-S129+ Lewy Neurite (LN) load was quantified in two ways. **(A)** LN density, calculated as the number of LNs per 100 µm² TH cell area. **(B)** The area fraction, calculated by the summed area of all LNs, represented as a percentage of the total cell ROI area. Where there were LNs to measure, the **(C)** mean area of individual LNs, and **(D)** the mean length of individual LNs was also measured at each timepoint. Few cell lines had significantly elevated pASyn-S129+ LN structures compared to their vehicle condition as early as **(i)** 10 Days after PFF insult, but the degree of accumulation appeared to increase at **(ii)** 20 days, **(iii)** 30 days, and **(iv)** 40 days after PFF insult. No labelling was detected in SNCA-KO cell lines, even after 30 days. No effect of genotype was found. Data points represent individual cell lines, averaged over 2-4 independent differentiations, bars represent mean $\pm$ SEM. In each differentiation, 1s-10s of LNs were measured at 10 days, 10s at 20 days, and 100s beyond 30 days, per 3-4 wells. Isogenic pairs are noted with shared point shape. Healthy Control 856 and its SNCA-KO are square points. iNDI KOLF lines are represented by stars. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and iNDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as “PSEN1 Misc.”. APP, SNCA-KO, HC 856 and 156, and iNDI lines were only examined at 20 and 30 days. Statistical comparisons are 2-Way ANOVA with Bonferroni correction for multiple comparisons.

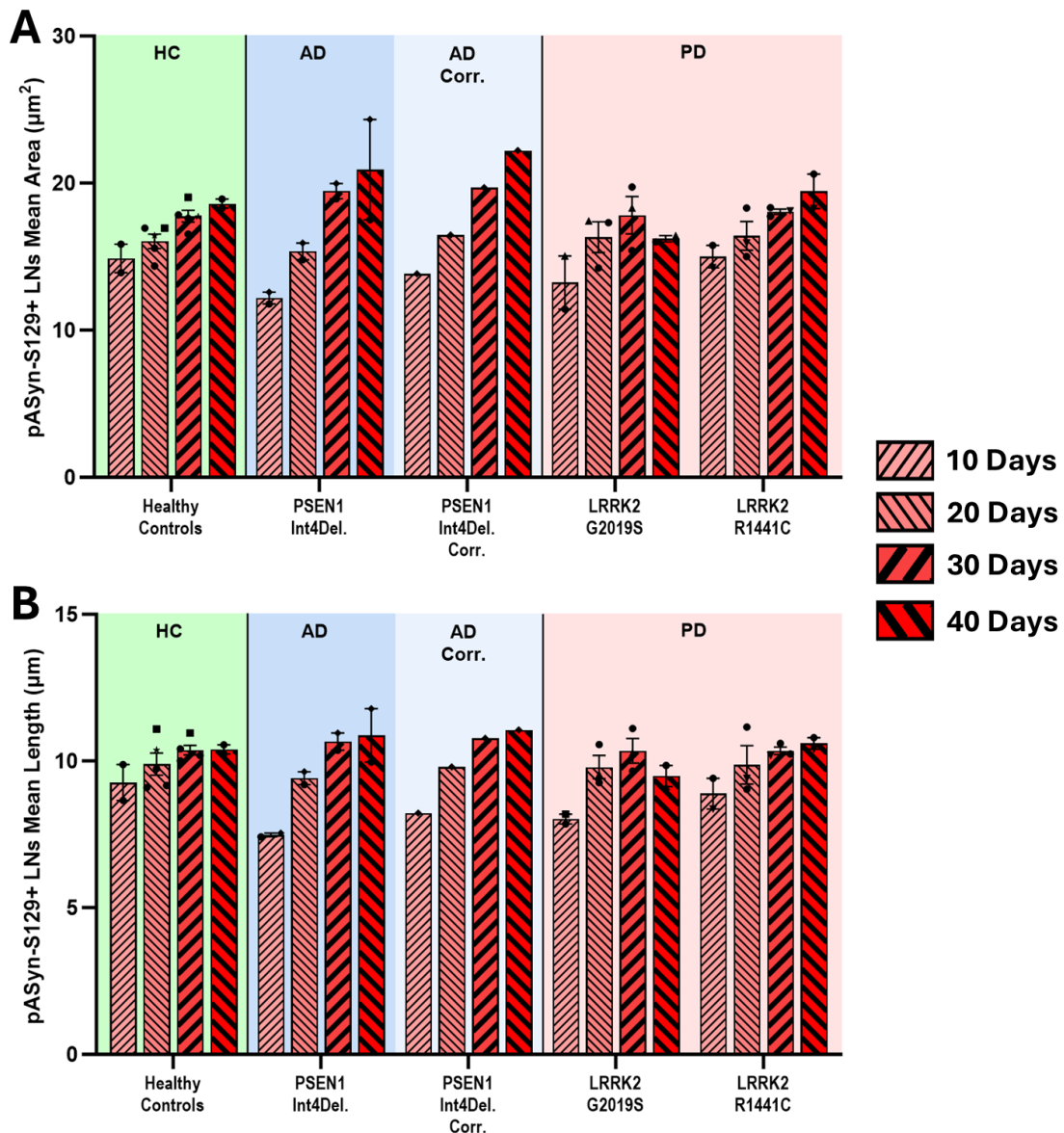


Figure 4.11. Lewy Neurite Length and Area Increase Over Time in TH+ Midbrain Dopaminergic Neurons. The (A) Mean Area, and (B) Mean Length of each Lewy Neurite (LN) was measured and is represented for the genotypes that developed the most pathology as cortical neurons. No effect of genotype but a significant effect of time was found for area ($p=0.0032$) and length ($p=0.0027$) (repeated measures two-way ANOVA). Data points represent individual cell lines averaged across 2-4 differentiations; bars represent mean $\pm$ SEM. In each differentiation, 1s-10s of LNs were measured at 10 days, 10s at 20 days, and 100s beyond 30 days, per 3-4 wells. The system of denoting particular cell lines with different shaped points was retained from earlier figures.

Similarly to LNs, LB pathology was scarcely evident at 10 days but continued to increase in number over time through to 40 days, though there was no genotype difference in LB frequency or size in TH cells (Figure 4.12). LBs also increased in size over time, with the mean size at D40 being almost 2-4-fold larger than at D10 (Figure 4.13).

LB formation was also compared between TH+ and TH- cells (Figure 4.14). TH+ cells appeared to develop LB pathology at a much higher rate than TH- cells. LBs in TH+ cells at 30 and 40 days could also be as much as 2-3-fold larger, and were typically higher intensity. I then examined nuclear area and circularity in TH+ and TH- cells, with and without LB pathology (Figure 4.15). There was a slight trend towards a 'LB dose' dependent reduction in nuclear area, though this was inconsistent and not significant, and there was no clear effect on nuclei roundness in either TH+ or TH- cells.

Given the pronounced vulnerability of the PSEN1-Int4Del CNs compared to the resilience of their corrected control, I quantified labelling of overall, LN, and LB pathology in these cell lines and PSEN1-M146I, at a lower dose of 2.5 µg/mL in one differentiation and found no differences (Figure 4.16).

Additionally, since chloroquine had such a marked effect in CNs in these lines, and since it was included as a positive control in pilot differentiations, I quantified the effect of cotreatment with this autophagy blocker on PFF-seeded aggregation (Figure 4.17). Interestingly, while CQ appeared to lead to only a mild increase in overall punctate pathology, LB and LN frequency was highly sensitive to autophagy blockade, but only in the PSEN1-M146I cells. There were no apparent differences in response to CQ between the PSEN1-Int4Del and corrected control cells, and no effect on LB or LN size.

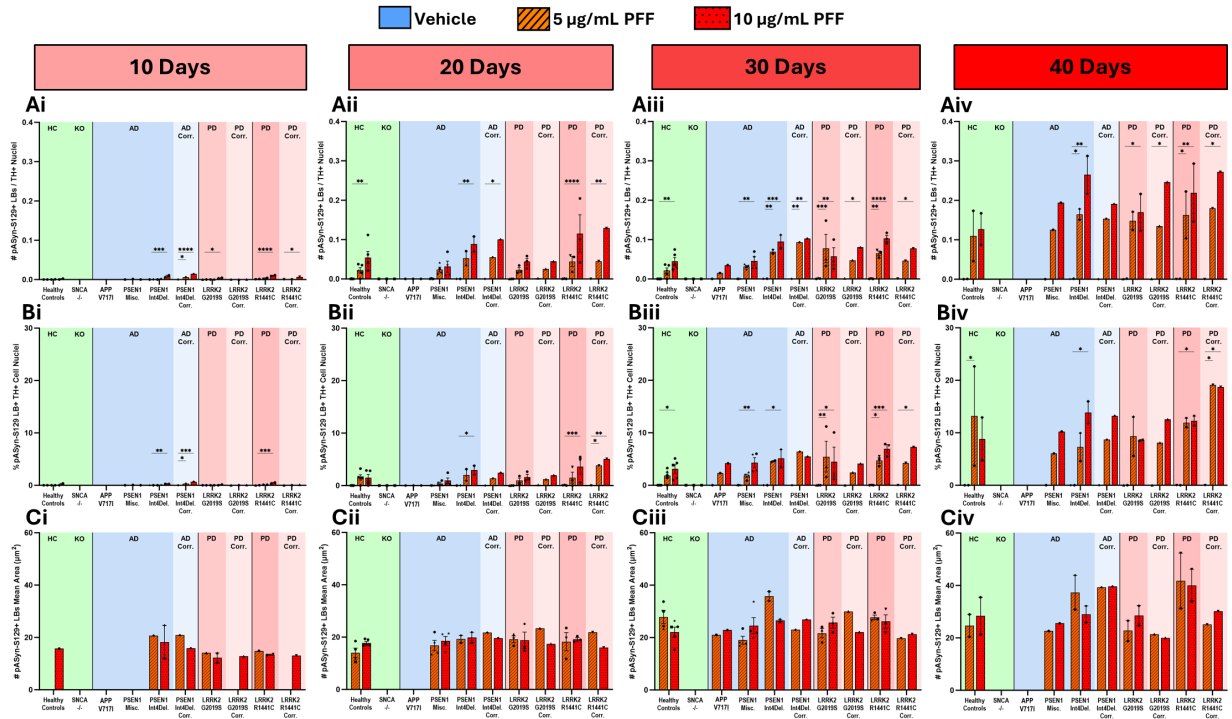


Figure 4.12. The Number of pASyn-S129+ Lewy Body Structures in TH+ Midbrain Dopaminergic Neurons Increases with Time in All Cell Lines. pASyn-S129+ Lewy bodies (LBs) were quantified in two ways. **(A)** The number of LBs per TH+ nuclei. **(B)** The number of TH+ nuclei with adjacent LBs, calculated semi-independently from (A). Several cell lines had significantly elevated pASyn-S129+ LBs compared to their vehicle condition as early as **(i)** 10 Days after PFF insult, and the severity of accumulation appeared to increase at **(ii)** 20 days, **(iii)**, 30 days, and **(iv)** 40 days after PFF insult. No pathology was detected in the SNCA-KO, even after 30 days. No effect of genotype was found. Data points represent individual cell lines, averaged over 2-4 independent differentiations, bars represent mean $\pm$ SEM. In each differentiation, 1s of LBs were measured at 10 days, and 10-70 LBs from 20 days per 3-4 wells. Isogenic pairs are noted with shared point shape. Healthy Control 856 and its SNCA-KO are square points. iNDI KOLF lines are represented by stars. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and iNDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as “PSEN1 Misc.”. APP, SNCA-KO, HC 856 and 156, and iNDI lines were only examined at 20 and 30 days. Statistical comparisons are 2-Way ANOVA with Bonferroni correction for multiple comparisons.

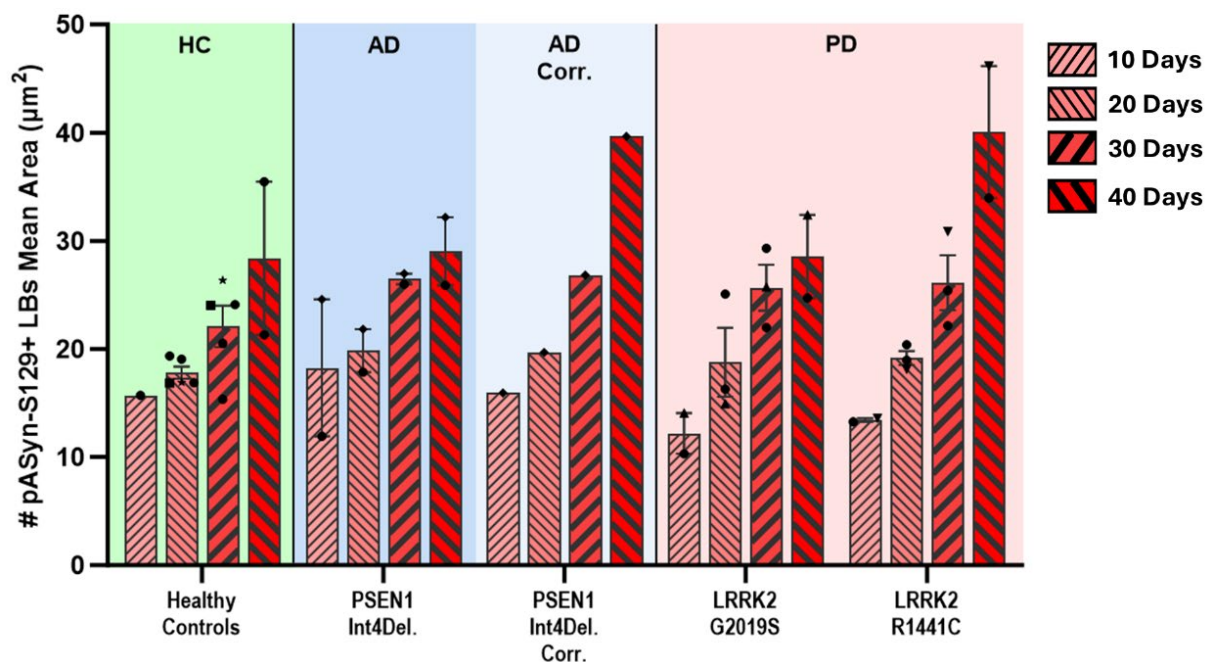


Figure 4.13. Lewy Body Size Increases Over Time in TH+ Midbrain Dopaminergic Neurons. The mean area of each Lewy Body (LB) was measured and is represented for select genotypes that developed the most pathology as cortical neurons. No effect of genotype but a significant effect of time was found ($p=0.0044$) (repeated measures two-way ANOVA). Data points represent individual cell lines averaged across 2-4 differentiations, bars represent mean $\pm$ SEM. In each differentiation, 1s of LBs were measured at 10 days, and 10-70 LBs from 20 days per 3-4 wells. The system of denoting particular cell lines with different shaped points was retained from earlier figures.

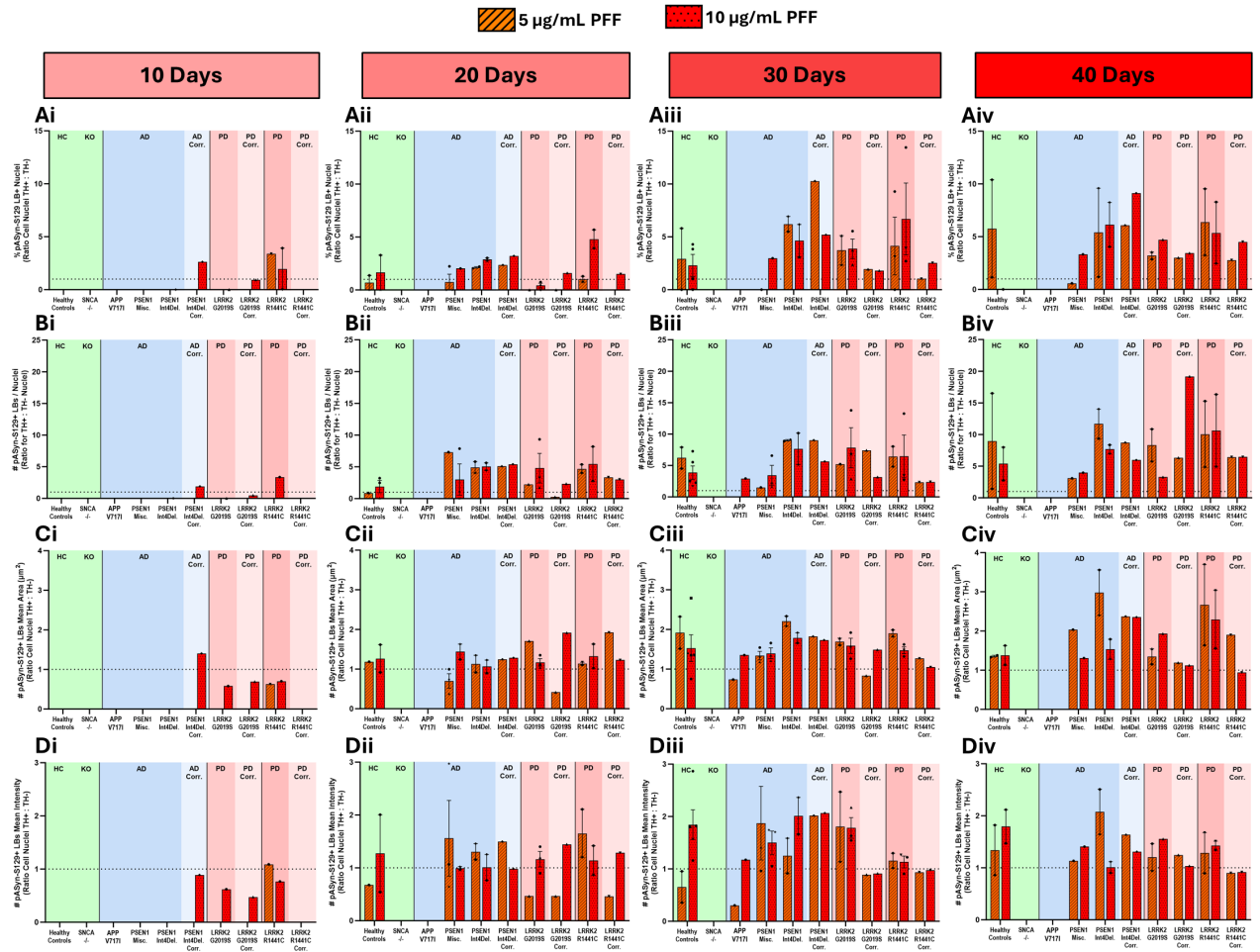


Figure 4.14. pASyn-S129+ Lewy Body Pathology Preferentially Affects TH+ Cells. To examine whether TH+ cells were differentially vulnerable compared to TH- MAP2+ cells, and how this changed with duration post insult, the ratio of various measures of Lewy Body (LB) pathology in the TH+ cells to TH- MAP2+ cells was calculated where LBs were present in both TH+ and TH- cells. Values above 1 indicate more pathology in TH+ cells. LB load was measured in each cell population as **(A)** the percentage of LB+ nuclei, where there was as much as a 10-fold more LB+ TH nuclei in some cell lines by 40 days, and which appeared to increase with time; **(B)** the number of LBs per nuclei, which also appeared to preferentially affect TH+ cells by an increasing degree over time; **(C)** mean LB size, which was typically ~1.5-2-fold larger in TH+ cells by 30 days; and **(D)** mean LB intensity, which was typically similar or higher in TH+ cells from 30 days. Ratios tended to vary per genotype, and no effect of genotype or dosage was found (two-way ANOVA). The system of denoting particular cell lines with different shaped points was retained from earlier figures.

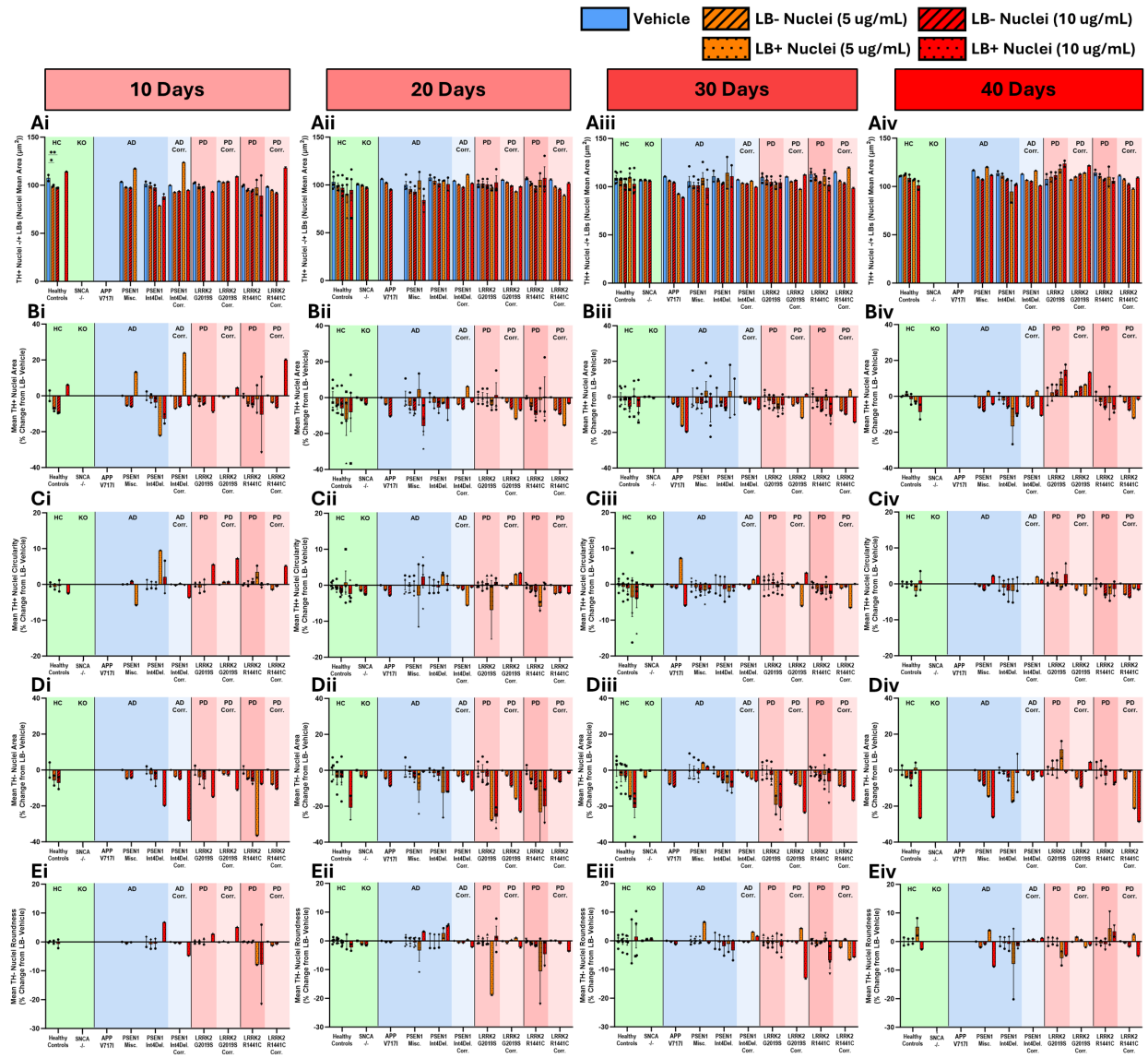


Figure 4.15. Lewy Body Load is Associated with a Trend Towards Reduction in Nuclei Area, not Circularity. The mean area (A-B, D) and mean circularity (C,E) of either TH+ nuclei (A-C) or TH- nuclei (D-E) was measured in vehicle-treated and therefore LB- cells (blue bars), PFF-treated but LB- nuclei (bars with lines), and PFF-treated LB+ nuclei (bars with dots). **(A)** Raw values of mean TH+ nuclei area are shown as an exemplar. **(B-E)** Percentage change in nuclear area or circularity from vehicle treated cells is shown. No significant effects were found, though there was a trend towards reduction in nuclear area with PFF insult and LB load, especially in TH- nuclei. Data points represent individual cell lines, averaged over 2-4 independent differentiations, bars represent mean $\pm$ SEM. In each differentiation, 100s of nuclei were measured in the vehicle condition, 1s of nuclei in the LB-laden conditions at 10 days, and 10s of nuclei from 20 days per 3-4 wells. Isogenic pairs are noted with shared point shape. Healthy Control 856 and its SNCA-KO are square points. iNDI KOLF lines are represented by stars. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and iNDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as “PSEN1 Misc.”. APP, SNCA-KO, HC 856 and 156, and iNDI lines were only examined at 20 and 30 days. Statistical comparisons are 2-Way ANOVA with Bonferroni correction for multiple comparisons.

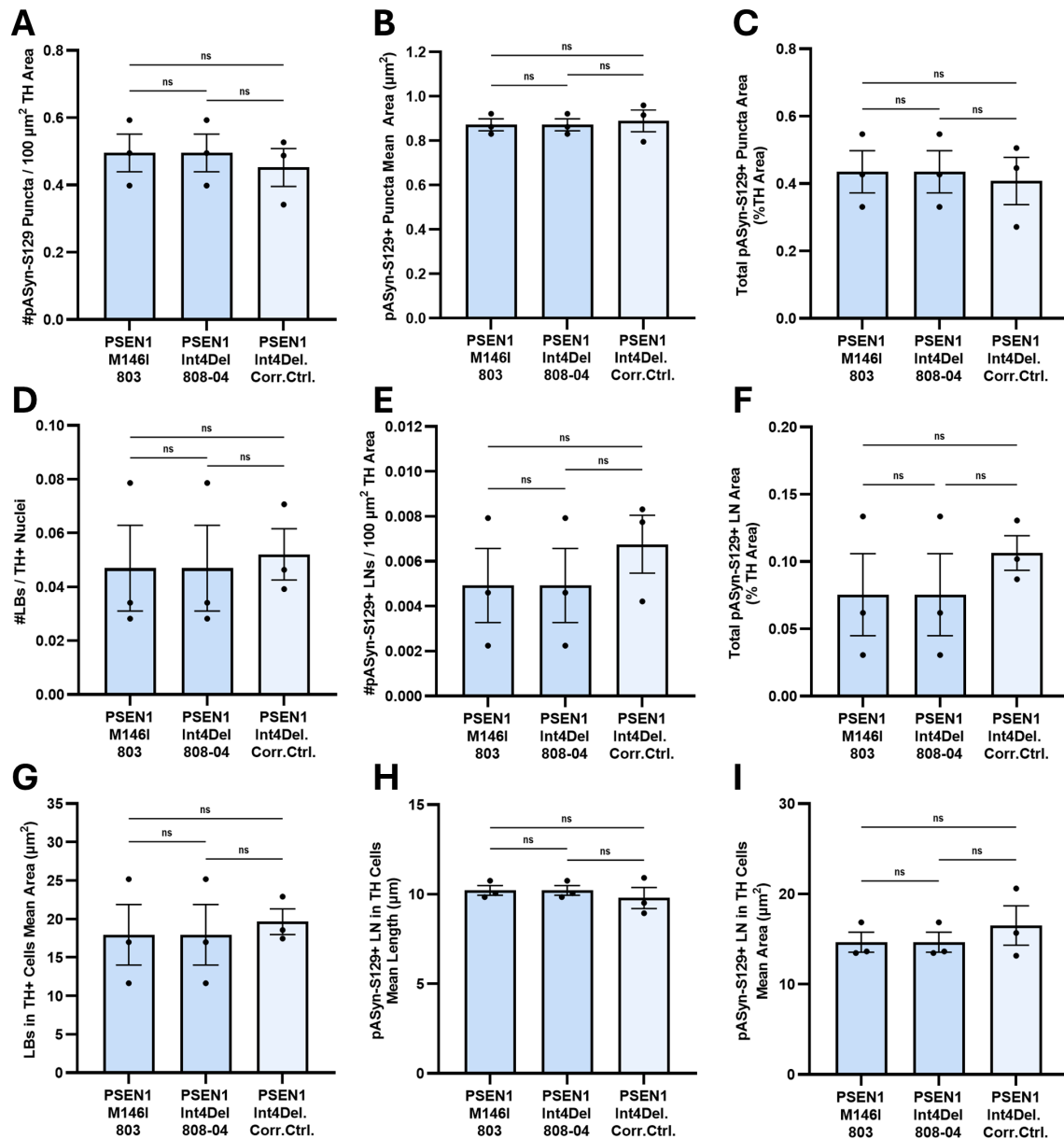


Figure 4.16. No Difference in Pathology Load in PSEN1-Int4Del and M146I Lines at Low PFF Insult Dosage in Midbrain Dopaminergic Neurons. In a single differentiation, PSEN1-M146I, PSEN1-Int4Del 808-04 and its isogenic corrected control were insulted with 2.5 $\mu\text{g}/\text{mL}$ ASyn PFF, alongside other dosages (shown previously), and fixed after 20 days, to examine whether a lower dose would reveal any of the striking differences in vulnerability in the PSEN1-Int4Del lines observed in cortical neurons. No significant difference was observed in **(A)** puncta density in TH cells, **(B)** mean puncta area in TH cells, **(C)** pASyn-S129+ total puncta area as a proportion of TH cell area, **(D)** #LBs in TH cells, **(E)** LN density in TH cells, **(F)** total LN area as a proportion of TH cell area, **(G)** mean LB area, **(H)** mean LN length, and **(I)** mean LN area. Data points represent individual well means, each comprising 31 fields of view, in a single differentiation, bars represent mean $\pm$ SEM. Statistical comparisons are 2-way ANOVA with Bonferroni correction for multiple comparisons.

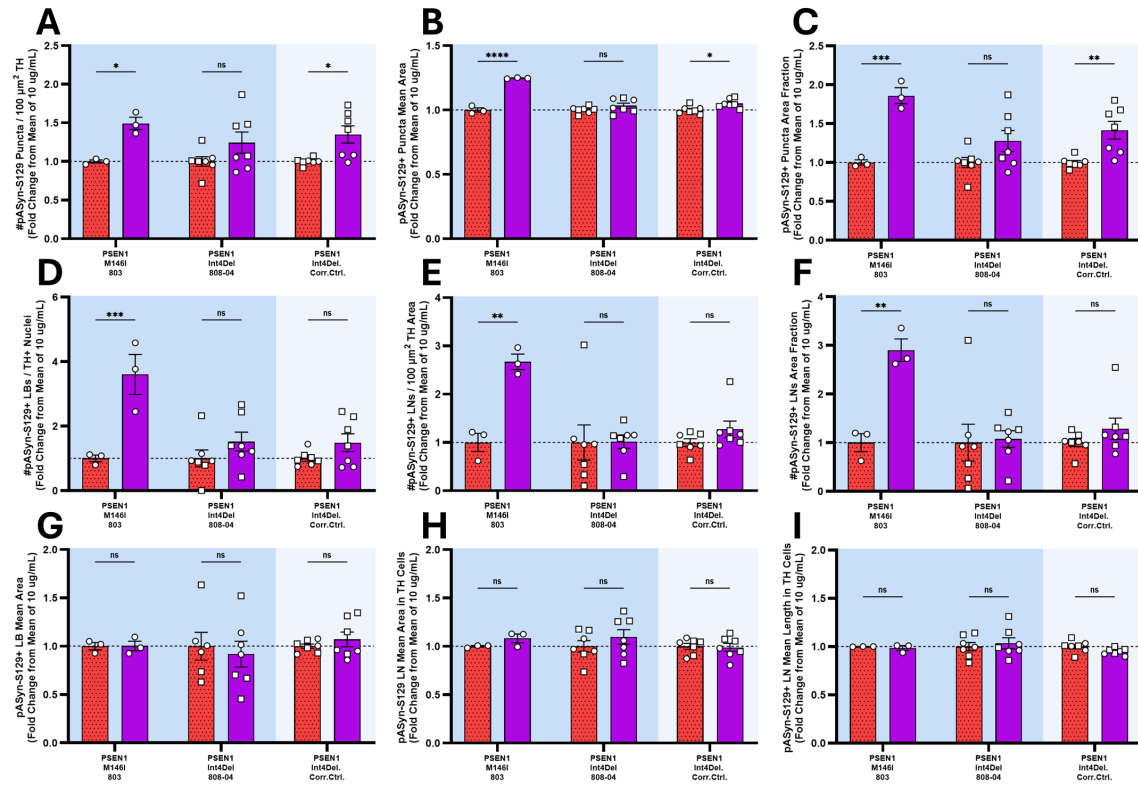


Figure 4.17. Chloroquine Co-treatment with PFF Insult Leads to Greater Pathological Burden in PSEN1-M146I but not in PSEN1-Int4Del Midbrain Dopaminergic Neurons. PSEN1-M146I, PSEN1-Int4Del 808-04 and its isogenic corrected control were co-treated with chloroquine (CQ), an autophagy blocker, and a 10 µg/mL ASyn PFF insult, alongside other dosages (shown previously), and fixed after 20 days, as a positive control to PFF-seeded aggregation. The effect of CQ co-treatment on seeded pathology is shown here as a fold change from 10 µg/mL PFF insult without CQ, for **(A)** puncta density in TH cells, which trended towards being elevated with CQ treatment, **(B)** pASyn-S129+ total puncta area as a proportion of TH cell area (area fraction), which trended towards being elevated with CQ treatment, **(C)** mean puncta area in TH cells, which was significantly elevated with CQ treatment in PSEN1-M146I, **(D)** #LBs in TH cells, which was significantly elevated with CQ treatment in PSEN1-M146I, **(E)** %LB+ TH cell nuclei, which was significantly elevated with CQ treatment in PSEN1-M146I, **(F)** mean LB area, which was unaffected by CQ treatment, **(G)** LN density in TH cells, which was significantly elevated with CQ treatment in PSEN1-M146I, **(H)** total LN area as a proportion of TH cell area (area fraction), which was significantly elevated with CQ treatment in PSEN1-M146I, and **(I)** mean LN area, which was unaffected by CQ treatment. Data points represent mean values of 3-4 wells from independent differentiations, bars represent mean $\pm$ SEM. Points with the same shape are from the same differentiation. PSEN1-M146I was only treated with CQ in one differentiation, PSEN1-Int4Del in two differentiations. Statistical comparisons are 2-way ANOVA with Bonferroni correction for multiple comparisons.

4.3.3. Effects of PFF Insult on mDAN Nuclear and Cellular Morphology

I also measured changes to nuclear and cellular morphology, as a result of PFF insult.

Counting TH+ nuclei, there was substantial attenuation of cultures by 40 days, accompanied by a trend towards increasing nearest neighbour distance (Figure 4.18). However, there was no effect of PFF on nuclei number, though nuclear area was again reduced 5-10%, in a seemingly aggregation independent manner. This change in size was not accompanied by a change in nuclei circularity, except for a small change in some genotypes at later timepoints.

A similar array of findings was also observed for TH- nuclei (Figure 4.19), with the notable exception of some nuclei loss associated with PFF insult in cell lines from 20 days. TH- nuclear size was also depressed to a similar degree by the PFF insult. Total neuronal nuclei numbers, as a measure of overall culture density, were also counted, with expected intermediate neurodegenerative effects of PFF insult observed, and no genotype differences in culture density (Figure 4.20).

Finally, cell area was also examined under the different ROIs, and their response to PFF insult (Figure 4.21). There were no genotype differences in ROI areas, and despite attrition in nuclei number, cell area appeared to increase up to 30 days, before diminishing by 40 days. Interestingly, there was little effect of PFF on TH cell area, even at later time points (Figure 4.21A), but a more consistent neurodegenerative effect of PFF insult on MAP2 (or β 3T) cell area from as early as 10 days that resulted in as much as a ~30% attenuation in cell area by 20-40 days (Figure 4.21B). A similarly pronounced effect on TH- cell area was observed, and these occurred across all genotypes, including SNCA-KO.

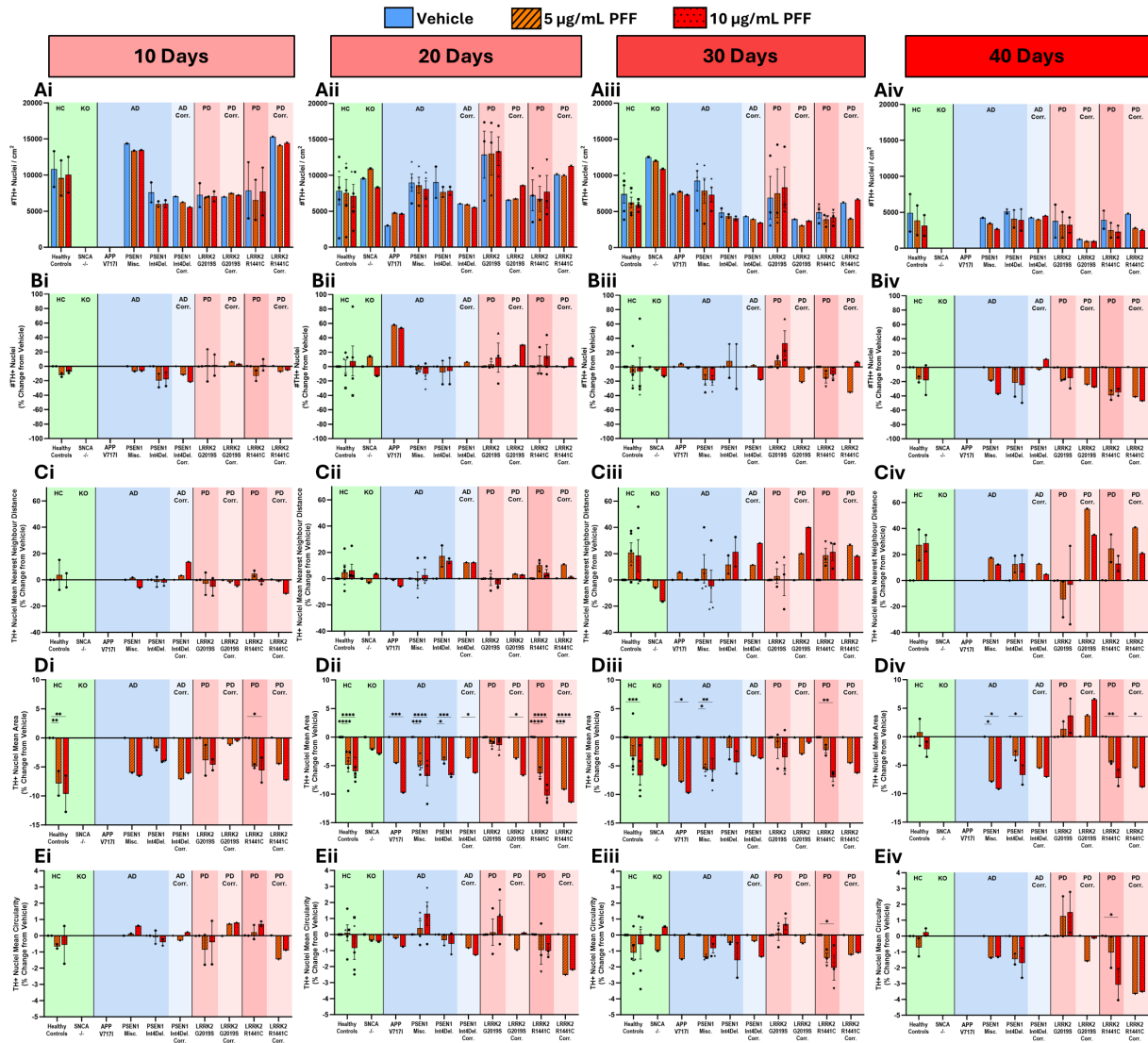


Figure 4.18. ASyn PFF Insult Is Associated with a Reduction in TH+ Nuclei Area, but not Circularity or Number. Various metrics associated with TH+ nuclei were measured and presented as raw values or percentage change from vehicle. **(A-B)** The number of TH+ nuclei declined over time, but was not affected by PFF insult, though there was a trend towards reduction in nuclei number in some genotypes by 40 days. **(C)** Correspondingly, while there were no significant changes in the mean nearest neighbour distance (NND) of each nucleus, genotypes with a trend towards nuclei loss with PFF insult trended towards having increased NND. **(D)** TH+ nuclei area was significantly reduced in most genotypes in an apparently dose dependent fashion by 5-10% by 20 days, and as early as 10 days in some lines. **(E)** No change in nuclei circularity was observed, except for a small effect at later timepoints in cells with higher nuclei loss. Data points represent individual cell lines, averaged over 2-4 independent differentiations, bars represent mean $\pm$ SEM. In each differentiation, 100s of nuclei (typically ~200-400 at 10 days, ~100 at 40 days) were measured per 3-4 wells. Isogenic pairs are noted with shared point shape. Healthy Control 856 and its SNCA-KO are square points. iNDI KOLF lines are represented by stars. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and iNDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as “PSEN1 Misc.”. APP, SNCA-KO, HC 856 and 156, and iNDI lines were only examined at 20 and 30 days. Statistical comparisons are 2-Way ANOVA with Bonferroni correction for multiple comparisons.

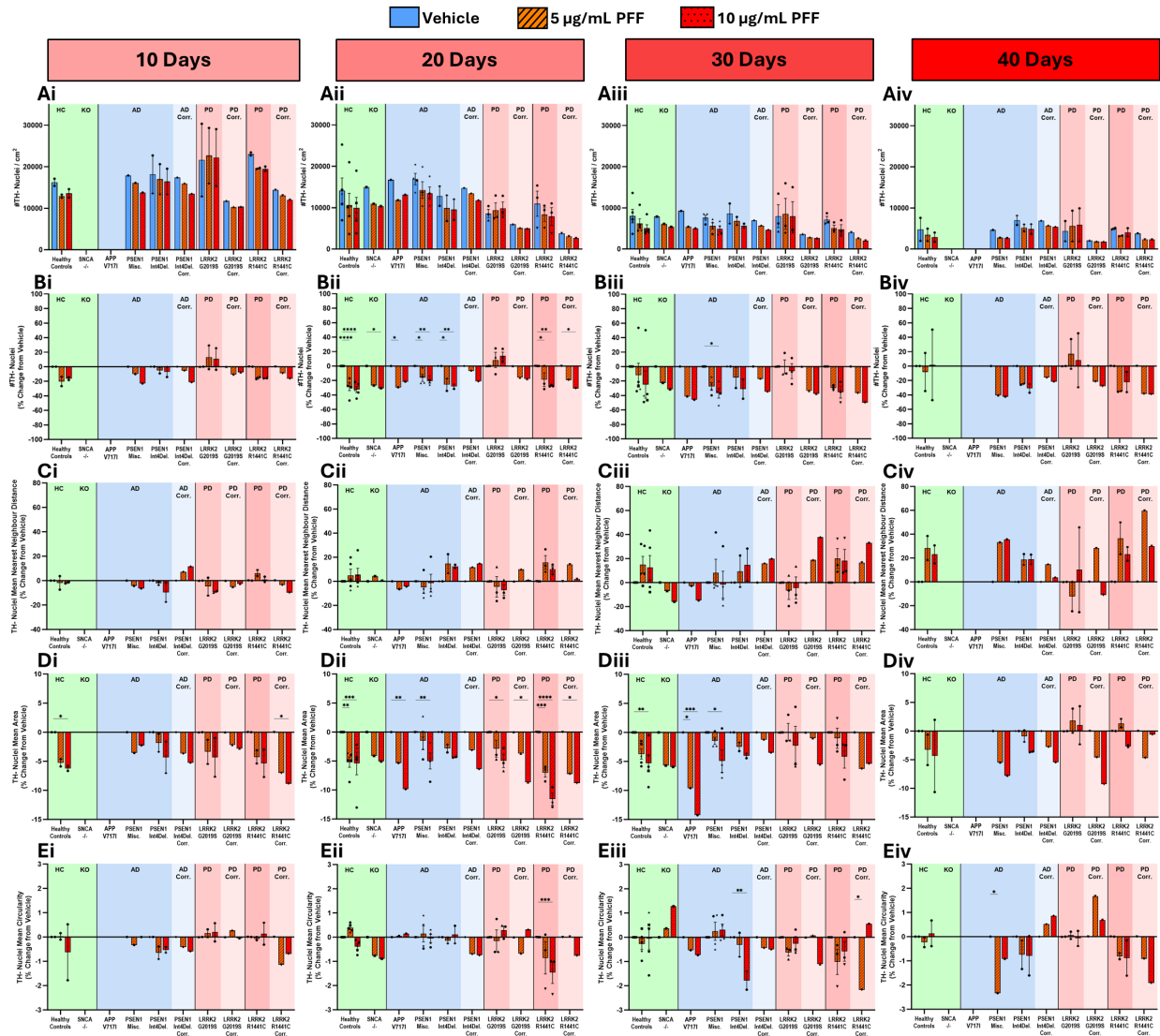


Figure 4.19. ASyn PFF Insult Is Associated with a Reduction in TH- Nuclei Area and Number, but not Circularity. The same metrics measured in TH+ nuclei were also measured in TH- nuclei and presented as raw values or percentage change from vehicle. **(A-B)** The number of TH- nuclei declined over time and was impacted by PFF insult in most genotypes by 20 days. **(C)** Correspondingly, while there were no significant changes in the mean nearest neighbour distance (NND) of each nucleus, genotypes trended towards having increased NND with PFF insult associated with loss of nuclei. **(D)** TH+ nuclei area was significantly reduced in most genotypes in an apparently dose dependent fashion by 5-10% by 20 days, and as early as 10 days in some lines. **(E)** No change in nuclei circularity was observed, except for some small effects typically at later timepoints. Data points represent individual cell lines, averaged over 2-4 independent differentiations, bars represent mean $\pm$ SEM. In each differentiation, 100s of nuclei (typically ~400-600 at 10 days, ~100 at 40 days) were measured per 3-4 wells. Isogenic pairs are noted with shared point shape. Healthy Control 856 and its SNCA-KO are square points. iNDI KOLF lines are represented by stars. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and iNDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as “PSEN1 Misc.”. APP, SNCA-KO, HC 856 and 156, and iNDI lines were only examined at 20 and 30 days. Statistical comparisons are 2-Way ANOVA with Bonferroni correction for multiple comparisons.

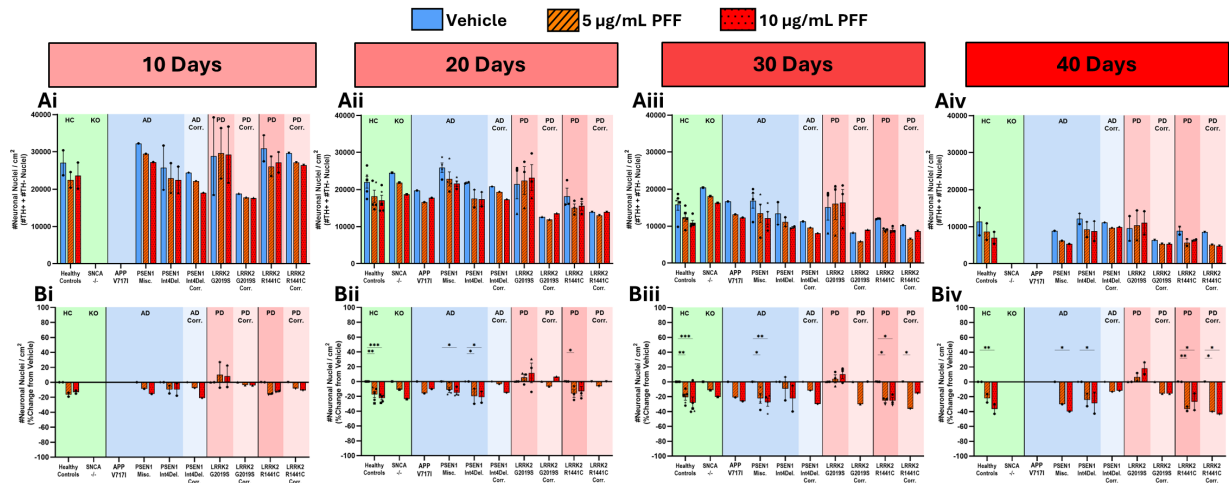


Figure 4.20. Midbrain Dopaminergic Neuron Cultures Undergo Attrition Over Time and ASyn PFF Insult Is Associated with a Reduction in Total Neuronal Nuclei Number. The total neuronal cell number per cm² (i.e. TH+ + TH- nuclei) is presented as **(A)** raw values, and **(B)** percentage change from vehicle. There was no significant difference between genotypes in neuronal density, though a substantial decline across cultures was observed by 40 days. PFF insult resulted in a significant loss of cells in some genotypes by 20 days. Data points represent individual cell lines, averaged over 2-4 independent differentiations, bars represent mean $\pm$ SEM. In each differentiation, 100s of nuclei (typically ~600-800 at 10 days, ~200 at 40 days) were measured per 3-4 wells. Isogenic pairs are noted with shared point shape. Healthy Control 856 and its SNCA-KO are square points. iNDI KOLF lines are represented by stars. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and iNDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as “PSEN1 Misc.”. APP, SNCA-KO, HC 856 and 156, and iNDI lines were only examined at 20 and 30 days. Statistical comparisons are 2-Way ANOVA with Bonferroni correction for multiple comparisons.

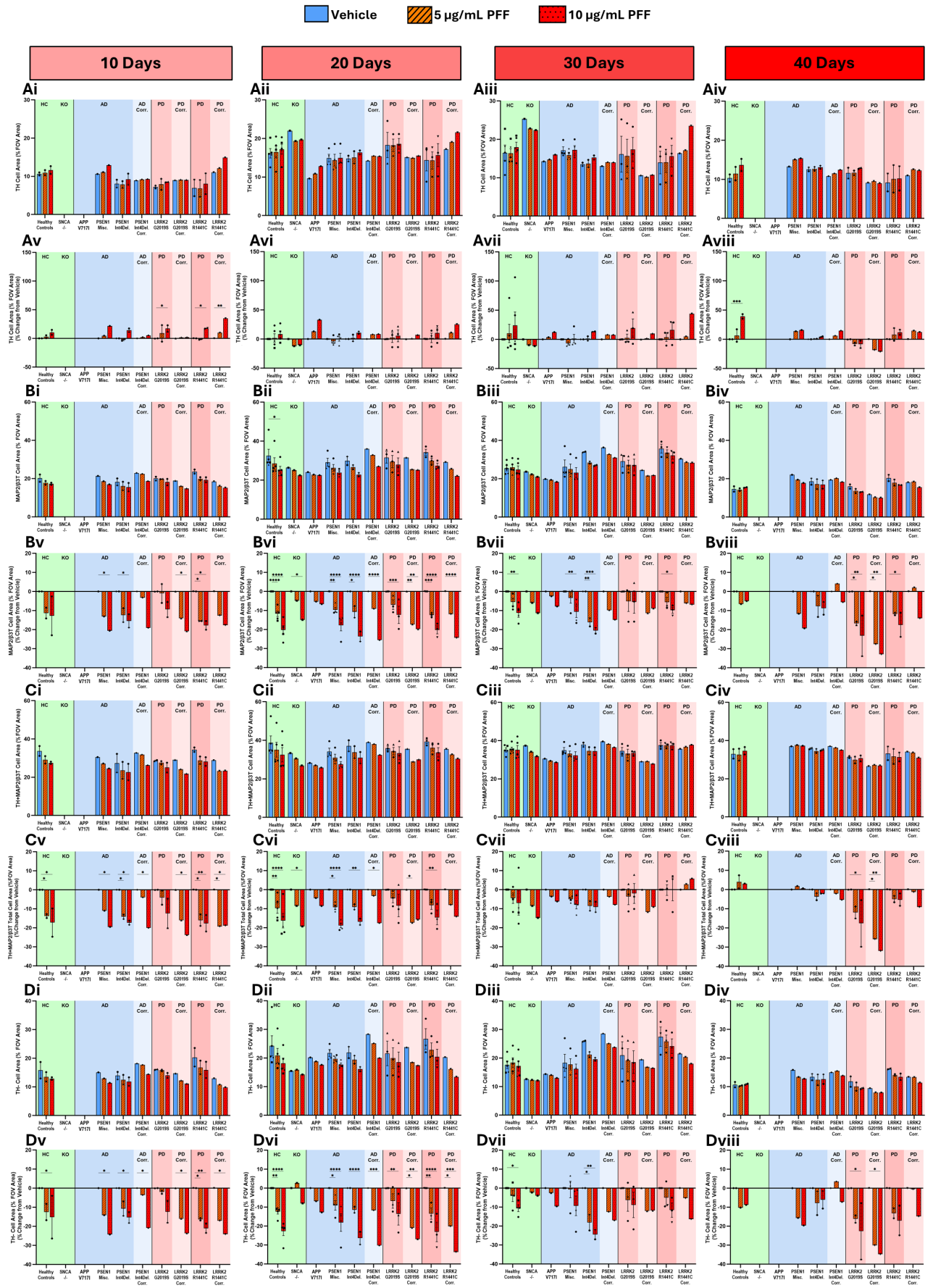


Figure 4.21. ASyn PFF Insult is Associated with Reductions in MAP2+ Cell Area, especially in TH-Cells, in an Aggregation Independent Manner. The mean area of different cellular ROIs was calculated and presented as **(i-iv)** a percentage of the total field of view (FOV) area, and **(v-viii)** as a percentage change from the vehicle treated condition. **(A)** TH+ cell area was typically not substantially affected by PFF insult, though was elevated in some genotypes. **(B)** MAP2 (or in one differentiation, β 3T) cell area was significantly reduced by PFF insult in an apparently dose dependent fashion, from as early as 10 days, but in most genotypes by 20 days, though there was not a clear trend with time beyond 20 days. **(C)** Total neuronal cell area, including TH+ and MAP2+/ β 3T+ cell areas, was similarly significantly reduced by PFF insult in most genotypes, though this attenuated in most cell lines over time. **(D)** TH-MAP2+ cell area was similarly significantly reduced by PFF insult in most genotypes, from as early as 10 days, though there was no clear trend with time. **(A-D)** Of note, there were no significant differences in cell area or neuronal nuclei density between genotypes, and where reductions associated with PFF insult occurred, this typically also occurred in the SNCA-KO line. Data points represent individual cell lines, averaged over 2-4 independent differentiations, bars represent mean $\pm$ SEM. iNDI KOLF lines are represented by stars. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and iNDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as "PSEN1 Misc.". APP, SNCA-KO, HC 856 and 156, and iNDI lines were only examined at 20 and 30 days. Statistical comparisons are 2-Way ANOVA with Bonferroni correction for multiple comparisons.

4.4. Discussion

4.4.1. Results Summary

In this chapter, I sought to examine the vulnerability of hiPSC mDANs to ASyn PFF-seeded aggregation. mDANs were highly susceptible to development of pASyn-S129+ inclusions, with high levels of dose and time dependent labelling across all genotypes, except the *SNCA*-KO, and a diversity of inclusion morphologies, including punctate, neuritic LN-like, and somatic LB-like structures, the latter two of which emerged later in the time course. These are the first observations of ASyn PFF-seeded inclusionopathy in AD and PD LRRK2 hiPSC mDAN models.

In comparing TH+ to adjacent TH- neurons in the same culture, mDANs were preferentially vulnerable to pASyn-S129+ pathology. LB formation had no effect on nuclei morphology alone, but PFF insult broadly across the population of cells did lead to an aggregation independent reduction in nuclear area. However, PFF insult had little effect on TH+ nuclei number or TH cell area, though a neurodegenerative effect on MAP2 area was observed. There were no significant differences in the pathology burden between genotypes. This included the PSEN1-Int4Del lines and corrected control, even at lower concentrations of PFF, or with concomitant CQ treatment. CQ treatment exacerbated pathology, especially LNs and LBs, in PSEN1-M146I neurons.

4.4.2. pASyn-S129+ Labelling Reflects other PFF Studies

Pathology observed here is largely in line with reports of PFF-seeded pASyn-S129+ inclusions in other rodent and hiPSC cell models. Tanudjojo et al. (2021) observe a similar range of speckled, neuritic, and LB-like aggregates in hiPSC mDAN healthy controls, and significantly more in *SNCA*-triplication lines at a similar dosage to here, after 3-4 weeks. Another study saw increases in pASyn-S129+ intensity after 2 weeks with a 5 µg/mL insult on hiPSC mDANs with

GBA mutations (Kim et al. 2018). Pathology I observed, as with CNs and elsewhere, was dependent on endogenous expression of protein (Tapias et al. 2017; Kim et al. 2018; Polinski et al. 2018; Iannielli et al. 2022), and both dose and time dependent (Tapias et al. 2017; Polinski et al. 2018; Tanudjojo et al. 2021).

In rat primary ventral midbrain cultures, Tapias et al. (2017) see more vulnerability of dopaminergic cells to synapse loss and axonal transport deficits, compared to non-dopaminergic cells, as well as observing LB-like assemblies and mitochondrial impairment. Here, I similarly observe a pronounced susceptibility of TH⁺ cells to PFF-seeded aggregates, suggesting that not just midbrain identity, but the dopaminergic machinery itself, or other aspects of these cells' unique physiology drive their vulnerability.

In some studies, more severe aggregates, such as the LB-like inclusions, appear more elusive. Iannielli et al. (2022) found LBs typically in PFF-insulted cells with four copies of the *SNCA* gene, rather than two copies, where LBs were rarer. Their study uses an end point of 21 days however, at which point LBs are still relatively scarce compared to 30 and 40 days in my experiments. Bayati et al. (2023) also see patterns of pathology similar to what is observed here, but only with a concomitant immune insult, whereas pathology readily forms in my culture model. It is possible that the PFF insult leads to an immunogenic stimulation of the rAstros, thus engendering a similar immune co-insult. Bayati et al. (2023) show that the immune insult can consist of direct application of IFN γ , or coculture with activated microglia. However, in the previous chapter, I observed no indication of an inflammatory reaction or any other changes to rAstros with PFF insult. Bayati et al. (2023) also use a much lower dose of 1 μ g/mL, and typically a 14-day endpoint, 30 days at most, to observe LB-like assemblies. The combination of these factors may account for the differences in the model.

As previously discussed, robust expression of endogenous ASyn and electrophysiological activity are likely also important for the development of PFF-seeded pathology. Other studies

showing pASyn-S129+ pathology use a similar growth factor guided Kriks-based protocol to what is employed here, however, typically perform the PFF insult somewhat later, between D45 and D60, once cells have been shown to be electrically active (Kim et al. 2018; Tanudjojo et al. 2021). Using an earlier intervention point of D36, I nevertheless observe robust pathology, likely because of the use of rAstros, which enhance maturation (Hedegaard et al. 2020; Das et al. 2024).

4.4.3. pASyn-S129+ Inclusions Reflect Brain Pathology

In addition to mirroring observations from other *in vitro* models, the inclusionopathy in the hiPSC mDANs here also morphologically reflects pathology observed in the brain, with characteristic speckled, neuritic, and perikaryal inclusions typical of PD (Braak et al. 2003; Kuusisto, Parkkinen, and Alafuzoff 2003; Beach et al. 2009; Lashuel 2020). While ultrastructural characterisation of these structures was beyond the scope of this thesis, and thus the inclusions observed here cannot be claimed to be ‘true’ LBs, electron microscopy performed by Bayati et al. (2023) show LB-like structures seeded in hiPSC mDANs by ASyn PFF insult are filamentous, organelle filled, and structurally analogous to LBs in the brain, while Tanudjojo et al. (2021) also show that such structures are p62+ at 3-4 weeks, another LB marker (Kuusisto, Parkkinen, and Alafuzoff 2003). Since the cells in these experiments are taken to these timepoints and further, at a similar or higher PFF concentration, it might be assumed that the resultant inclusions have similar structural characteristics.

Nevertheless, as with the CN model, the diversity of morphologies of the pathology developed by the mDANs here is also strikingly characteristic of PD neuropathology. Many of the forms of LN-like structures observed, including long, thin, thread-like inclusions, and larger, thicker, crook-shaped or club-shaped structures, with enlarged endings, bear remarkable similarity to those described by Braak et al. (2003). As with the LBs in the CNs in the previous chapter,

neuropathological observations of round inclusions adjacent to nuclei of at least 4 μm diameter (Kuusisto, Parkkinen, and Alafuzoff 2003) were also used to inform the definition of LBs in the analysis pipeline here, and many such structures emerge in a time-dependent manner.

Interestingly, one structure seen much more abundantly in the mDANs than in CNs were large, round inclusions that resembled pale bodies. These are somewhat distinct from LBs in their larger size, disorganised filamentous structure, and more amorphous, oblong shape, tending to fill a large portion of the cell body. They are also described as weakly immunopositive, with a sometimes granular appearance, though generally are not well defined (Takahashi et al. 1994; Braak et al. 2003; Kuusisto, Parkkinen, and Alafuzoff 2003). Neuropathological evidence suggests that pale bodies may be a precursor to LB formation, which subsequently form through a compaction process, though they may also form through a separate parallel process (Kuusisto, Parkkinen, and Alafuzoff 2003). They are also typically observed in the LC and SNPC in the brainstem, rather than in the cortex (Dickson 2012). As such, the frequent observation of pale body-like structures in the mDANs here, but not in the CNs, may be indicative of the model reflecting cell-type specific aggregation processes. This is especially interesting given the same PFF insult is applied to the different cell types, implicating cell-autonomous processes in driving the distinct aggregation outcomes. Cortical and brainstem LBs are also thought to be different structures formed by distinct processes (Fares, Jagannath, and Lashuel 2021), which will be discussed further in Chapter 7.

Interestingly, in the data presented here, both LB and LN-like structures increase in size, as well as frequency, over time. This might suggest that, following nucleation, these structures are formed through a process of gradual accumulation and aggregation. However, rather than growing larger, LBs are thought by some to compact into their highly dense, structured, intensely immunoreactive form, from larger, more disorganised structures (Kuusisto,

Parkkinen, and Alafuzoff 2003; Goedert et al. 2013; McCormack et al. 2019; Fares, Jagannath, and Lashuel 2021; Trinkaus et al. 2021; Lewis et al. 2024). The pipeline for counting and measuring LBs picks up the larger pale-body like structures as well, provided they have sufficient circularity (>0.6). As such, the apparent increase in mean LB area reported here may be a result of LBs growing in size over time or may be a reflection of a growing proportion of the larger pale body-like structures shifting the mean area. Further analysis might try to distinguish these structures, though without additional labelling or ultrastructural imaging, they remain elusive to truly define and distinguish.

Impaired organelle trafficking and distorted, dysfunctional organelles are also highly implicated in the formation of LBs (Shahmoradian et al. 2019; Mahul-Mellier et al. 2020). Autophagic markers such as p62, LC3, and LAMP1, are closely associated with LBs, and some point to a failure of autophagic flux as a point of initiation for LB formation (Kuusisto, Parkkinen, and Alafuzoff 2003; MacIsaac et al. 2020; Fares, Jagannath, and Lashuel 2021; Bayati et al. 2023; Lam et al. 2024; Lewis et al. 2024). Bayati et al. (2023) note that in hiPSC mDANs, ultrastructurally characterised LBs were LC3b+ membrane enclosed, suggesting autophagosomes as a potential initiating structure. Here, in support of this, blockade of autophagy alongside PFF insult with chloroquine treatment led to only a mild increase in overall pathological burden but had a pronounced effect on LN and LB formation in PSEN1-M146I neurons. CQ did not lead to larger LNs or LBs, but rather a higher frequency of inclusion formation.

It is presently unclear why only PSEN1-M146I responded to CQ, while the PSEN1-Int4Del and corrected control lines did not. The relative insensitivity of these lines suggests that they have intrinsically poor functioning autophagy, compared to the PSEN1-M146I line, though they do not exhibit substantially more pathology. This nevertheless posits an important role for

autophagic function in determining vulnerability to this pathology, which will be examined in more depth in Chapter 5.

While the process of LB formation is of great interest and debate in the field, more importantly for the present examination of differential vulnerability, the apparent mirroring of the process of LP formation through the emergence of a diversity of brain relevant structures in a TH+ cell type-specific manner serves to validate the model. As such, the ASyn-PFF insult model in hiPSC neurons, especially at the durations employed in this study, may represent disease relevant processes that enable the examination of differential vulnerability with translational potential.

4.4.4. Lack of genotype dependent differences

The vulnerability of the mDANs to pASyn-S129+ pathology was profound, but given brain pathology, expected. What was more surprising was the lack of any genotype-dependent differences in pathology load. Other studies have observed LP in healthy controls, and since I used a relatively high PFF dose, pathology across genotypes might be expected (Tanudjojo et al. 2021; Jones-Tabah et al. 2024). However, the lack of any difference between disease genotypes and either healthy or corrected controls is striking.

In theory, the lack of disease-related phenotype for PSEN1 cell lines might be expected, given that SNPC mDANs are relatively resilient in AD. In this case, the high levels of pathology, not different from healthy or corrected controls, might simply reflect an intrinsically high vulnerability of mDANs, independent of disease-related genotypes. Since these are heterogenous cultures, it may also be the case that VTA-type neurons, thought to be relatively abundant in Kriks-based protocols (Oosterveen et al. 2021), might exhibit more vulnerability in AD models, while the SNPC-type mDANs might exhibit more vulnerability in PD lines. Since

these subtypes are not distinguished in the culture, the cultures in aggregate might appear equally vulnerable. The differential vulnerability of the VTA and SNPC in AD is less clear than for PD, though VTA-mDAN loss has been shown in an AD APP-mutant model (Nobili et al. 2017). Nevertheless, the lack of phenotype in LRRK2 lines remains surprising. Studies in hiPSC-derived CNs, rodents, rodent primary CNs, and primary ventral midbrain cultures have all shown enhanced vulnerability to ASyn PFF-seeded pathology by LRRK2-G2019S, ameliorated by LRRK2 reduction or KO. (Lin et al. 2009; Volpicelli-Daley et al. 2016; Bieri et al. 2019; Henderson et al. 2019; MacIsaac et al. 2020). While ASyn PFF studies have not been conducted in hiPSC mDANs with LRRK2 mutations, other studies observe enhanced susceptibility to oxidative stress, autophagic dysfunction, and mitochondrial impairments associated with LRRK2-G2019S and LRRK2-R1441C (Nguyen et al. 2011; Sánchez-Danés et al. 2012; Williamson et al. 2023), showing these mutations do have disease-relevant effects in these cell types, generated by similar protocols and assessed at similar timepoints. It may be that LRRK2 is relatively lowly expressed or lowly active in the mDANs, in a manner sufficient to drive mild dysfunction in cellular processes, but insubstantial compared to intrinsic phenotypes of mDANs driving their profound vulnerability to ASyn PFF-seeded pathology, overpowering any LRRK2-related mechanisms. As such, the assay might be insensitive to differences driven by LRRK2 mutations. LRRK2 may also drive pathology in the brain through cell non-autonomous mechanisms instead of acting within the neuron, since LRRK2 is highly expressed in microglia and other macrophages, and may contribute to impaired ASyn degradation by these cells (Lee et al. 2020).

4.4.5. Neurodegenerative Effects of ASyn PFF Insult

As with CNs, ASyn PFF insult led not only to accumulation of pASyn-S129+ pathology, but also to changes in nuclear and cell morphology, that were seemingly aggregation independent. This

was inferred from changes in nuclear and cell ROI area in the *SNCA*-KO cell line, and that nuclei exposed to PFF insult versus a vehicle treatment were reduced in nuclear area, rather than nuclei with versus without adjacent LBs. The effects on nuclei area were not notably different in magnitude, direction, or timing to those observed in CNs, and the functional significance of this remains unclear, and worthy of future investigation.

Further to the effects on cellular morphology being aggregation independent, while TH⁺ cells had a higher burden of pASyn-S129⁺ pathology, the TH⁺ ROI was not significantly changed by PFF insult, whereas MAP2⁺ area was. The lack of a time dependent reduction in cell area beyond 20 days also suggests this may be a transient effect of PFF insult, overcome by the growing cultures, rather than ongoing result of progressive aggregation. The resilience of the TH⁺ cells to morphological changes may also be because these relatively young cultures are still developing and becoming more TH⁺ over the time course, thus counteracting any potential neurodegenerative force. The slight increase in the TH ROI over time may also be related to swelling of the cell body as it accumulates pathology. Some large perikaryal aggregates were noted to be associated with an apparent distention of the soma. The reduction in MAP2⁺ ROI may be because the MAP2 labelling better captured neurites and finer processes, whereas the TH antibody, while still labelling some larger neurites, was most strongly immunoreactive in cell bodies. It may be inferred, especially given the relatively profound effect on β 3T⁺ axons in CNs, that the PFF insult leads to a more severe effect on the distal parts of the cell, i.e. on fine processes such as axons and dendrites, leading either to a thinning or shortening or both of these, and a less pronounced effect on the cell body. This is thus more clearly observed in MAP2⁺ ROIs rather than the TH ROI. The effect of PFF insult on neurites will be examined further in Chapter 6.

In addition to these morphological changes that may be indicative of neurotoxicity, I also observed a diminished nuclei number associated with PFF insult. While debated, there is

substantial evidence for the neurotoxicity of ASyn aggregation (Parkkinen et al. 2011; Mahul-Mellier et al. 2020), and other studies do report neuronal loss in response to PFF insult in a time dependent manner (Tapias et al. 2017; S. Kim et al. 2018), though others, at a lower concentration in rodent primary cultures, do not (MacIsaac et al. 2020). Interestingly, loss of nuclei was particularly seen for TH- rather than TH+ nuclei. It is not clear whether this reflects a distinct vulnerability of the TH- cells in particular, or if the growing proportion of TH+ cells in the growing cultures affords some resilience to this population. Interestingly, the nuclei loss is in contrast with observations in CNs in the previous chapter, and perhaps reflective of the heightened vulnerability and more profound level of aggregation in the cells.

4.4.6. Strengths and Limitations for Examining Cell Type Dependent Vulnerability

As discussed and characterised in this thesis and elsewhere, the Kriks-based mDAN differentiation protocol produces a mixed population of dopaminergic and non-dopaminergic neurons (Kriks et al. 2011; Oosterveen et al. 2021). While this might be a limitation of the model, especially for bulk assays, here, for the purpose of studying cell type-dependent vulnerability, this heterogeneity is an asset of the model, as it allows for the comparison of the relative vulnerability of mDANs to other adjacent ventral midbrain-type neurons, in the same culture, for which all other conditions are thus held equal. Pathology was here observed across both types of cells, but with often many folds more burden in TH+ cells, as compared to TH- cells. This was especially true for the rate of LB formation. Cellular phenotypes intrinsic to the mDANs, therefore, seem to particularly drive vulnerability to ASyn PFF seeded pathology. Future analyses might consider the level of TH expression in each cell in such a heterogeneous culture, and how much this increases susceptibility to PFF-seeded pathology.

One limitation of the model in this regard, however, was the inability to clearly distinguish LNs in TH+ vs TH- neurites, owing in part to the stronger TH labelling in the cell body than neurites relative to MAP2, and in part to the thick cultures with many overlapping processes proving intractable to disentangle. Another challenge of the mDAN cultures was the severe attrition of cells reached by 40 days. This relative instability of the cultures compared to CNs might also explain their being more prone to nuclei loss with PFF insult. Since there was limited pathology evident at 10 days, and cultures could not be reliably maintained through to 40 days, I thus focused on 20- and 30-day endpoints in the mDAN experiments, which still provided substantial overlap with CN experiments and sufficient time to develop LB pathology. As such, while all cell lines are represented at these time points, only a subset of lines were examined at 10 and 40 days. These are nevertheless included for completeness and to demonstrate the full extent of the time dependency of pathology.

4.4.7. Conclusions

As such, these ASyn PFF insult experiments in mDANs, mirroring those performed in CNs, have shown a profound vulnerability not just of the midbrain culture model, but preferentially for TH+ mDANs. The similar array of seeded pathology here, including the presence of pale body-like structures, suggests that the pathological process being modelled reflects cell type-specific processes that occur in the brain in synucleinopathies. This raises the question of why the mDAN cell model is so selectively vulnerable, while in CNs, only the PSEN1-Int4Del cell line exhibited high levels of pathology. The experiments presented here also raise the question of why the PSEN1-Int4Del mutation had such a profound effect in CNs, but no such effect of the mutation was evident in mDANs. These questions arising from the direct comparison of the CN and mDAN models, and an investigation into the pathways that might underly these differences, will be investigated in the next chapter.

Chapter 5.

Cellular Pathways Involved in Differential Vulnerability

5.1. Introduction

In the previous two chapters, I demonstrated an ASyn PFF insult paradigm in CNs and mDANs which produced a range of pASyn-S129+ pathological inclusions resembling LP in the brain. While individually interesting, the overarching aim of this thesis is to examine the differential vulnerability of these two cell types in comparison, and to understand any differences. Therefore, in this chapter, I directly compare the seeded aggregation response in the CNs and mDANs, and then seek to investigate mechanisms underpinning the observed differences in vulnerability.

From the past two chapters, two key differences have emerged. First is the pronounced vulnerability of the mDANs to developing pASyn-S129+ aggregates across all genotypes, while the CNs are relatively resilient. Second, is the striking selective vulnerability of the PSEN1-Int4Del CNs, compared to the corrected control and other genotypes, despite having no difference in vulnerability in mDANs. Many factors may drive these vulnerability differences, including differences in PFF uptake rate, endogenous ASyn expression, or degradative capacity.

Experiments in the previous chapters examining the effect of chloroquine on pathology formation have already suggested a role for autophagic impairment in enhancing vulnerability. Autophagy is thought to play a key role in ASyn fibril degradation, and autophagic malfunction is thought to be important to the formation of LP (Cuervo et al. 2004; Desplats et al. 2009; Gao et al. 2019; Bayati et al. 2023; Jones-Tabah et al. 2024). Furthermore, PSEN1 mutations are also associated with autophagy deficits (Lee et al. 2010; 2015; Nixon 2013; Hung and Livesey 2018; Kwart et al. 2019). However, it is not known how these pathways and deficits might manifest in different cell types to drive differential vulnerability.

PFF experiments in the previous chapters were conducted on neurons in coculture with rAstros. This was essential to the long-term culture and maintenance of CNs and provided

numerous technical and maturational advantages. However, while rAstros were used with both cell types, so divergent pathological outcomes should be driven by neuronal phenotypes, and while no effect of PFFs was observed on rAstros, it remains difficult to disentangle any cell non-autonomous effects that may occur. Therefore, some experiments here are also performed in monocultured neurons, to definitively examine cell autonomous drivers of vulnerability. Other assays of cellular phenotype, such as measurement of endogenous ASyn expression, are conducted with rAstros in coculture to mimic conditions at PFF insult.

As such, the aims of this chapter are to

- 1) Describe the relative burden of pathology in ASyn PFF insulted CNs and mDANs, to establish their differential vulnerability;
- 2) Identify and investigate cell type-dependent and genotype-dependent differences in cellular phenotype or culture conditions that might lead to these differential responses to PFF insult. These include
 - i. Differences in PFF uptake,
 - ii. Differences in culture density,
 - iii. Differences in endogenous ASyn expression, and
 - iv. Differences in autophagic and lysosomal function;
- 3) Identify pathology-relevant differences in cellular phenotype that are cell autonomous, i.e. Independent of any influence of rat astrocytes.

5.2. Methods

5.2.1. Overview and Neuronal Monoculture

Immunocytochemical labelling and western blotting methods have been detailed in Chapter 2. PFF insult paradigms have also been described in previous chapters and are used identically here for labelled ASyn-Alexa 647 PFFs.

Culture conditions for neurons grown in monoculture are also described in Chapter 2. Briefly, CNs are replated at D33 onto PO/L coated plates, and cultured as normal until D50, when assays are performed. mDANs are not treated any differently, rAstros are simply not added on D24, and assays are performed on D36, as normal.

5.2.2. LysoTracker™ Labelling

LysoTracker™ assays were performed on monocultures. To visualise lysosomes in live cells, cell media was fully changed to fresh media with LysoTracker™ Deep Red (Invitrogen) dye made up to a final concentration of 50 nM and incubated for 2 hours. NeuroFluor™ NeuO neuronal dye (Stemcell Technologies), diluted 1:500, and NucBlue™ Live Cell Stain (Invitrogen), added 2 drops/mL, were subsequently added on top 1 hour into the LysoTracker™ incubation. At the end of the incubation, cells were washed with PBS, and imaged on the Opera Phenix at 37 °C and 5% CO₂. For chloroquine treatment, cells were treated with chloroquine dissolved in ddH₂O 24 hours before LysoTracker™ incubation. Harmony analysis pipelines are outlined in the results section.

5.2.3. DQ™ BSA Lysosomal Degradation Assay

As per LysoTracker™, monocultured cells underwent a full media change to fresh media with 45 µg/mL DQ™ Red BSA dextrans. Dextrans were incubated for 4 hours before imaging. NeuroFluor NeuO and NucBlue live cell dyes were added to cells on top, 30 minutes before imaging. Cells were not washed before imaging. As a positive control, cells were treated with 50 µM chloroquine 24 hours in advance.

5.2.4. Fura-2 Calcium Assays:

Calcium assays were performed on monocultures. Fura-2 dye was made up in HBSS buffer with Ca^{2+} and Mg^{2+} (HBSS +/+), 15 mM HEPES, and 10 mM glucose at pH 7.4, and incubated with cells for 1.5 hours in a 1:1 ratio with NGN2 media or mDAN maturation media, as appropriate. Cells were then washed once with HBSS without Ca^{2+} or Mg^{2+} (HBSS -/-), and then left in fresh HBSS -/- with HEPES and glucose.

Evoked Fura-2 signal was measured immediately using the FlexStation 3 (Molecular Devices), at 37 °C, with readings every 4 s for 210 s, and Ca^{2+} eliciting drug delivery at 30 s. To evoke calcium release, two different compounds were used: ionomycin, a calcium-binding ionophore, which evokes Ca^{2+} release from the ER and other cellular stores (Morgan and Jacob 1994), and glycyl-L-phenylalanine 2-naphthylamide (GPN), a lysosomotropic compound that elicits Ca^{2+} release through lysosomal lysis (Hung and Livesey 2018; Yuan et al. 2021). Ionomycin-induced Ca^{2+} release was therefore used as a measure of ER Ca^{2+} , while GPN-induced release was used as measure of lysosomal Ca^{2+} . Compounds were resuspended in DMSO and diluted in HBSS -/- for final concentrations of 5 µM and 200 µM respectively, with DMSO as a negative control.

5.2.5. Autophagic Flux Assay:

Autophagic flux assay was performed based on Rubinsztein et al. (2009). Monocultured CNs were treated with vehicle or 50 μ M chloroquine at D49, and then lysed 24 hours later. Protein was extracted from lysates and blotted for LC3b, as per western blotting protocol.

5.3. Results

5.3.1. Comparison of Pathology in Cortical and Midbrain Dopaminergic Neurons

In the last two chapters I examined ASyn-PFF seeded pASyn-S129+ pathology in CNs and mDANs 10, 20, 30, and 40 days after insult, and their resultant overall levels of punctate pathology, as well as LN and LB formation were analysed separately. Here I present these data side by side on matched Y-axes and consider whether and why mDANs and CNs may have different responses.

Figure 5.1. presents the area fraction of overall pASyn-S129+ pathology in MAP2+ CNs and TH+ mDANs. This measure captures both the rate of seeding and size of aggregates formed. From 10 days, pathological load in mDANs appears higher than in CNs, with as much as 10-fold more pathology in most lines by 40 days. Labelling in PSEN1-Int4Del alone is equivalent to mDANs at 10 days, though by 20-30 days, the 10 µg/mL dose in CNS is more equivalent to a 5 µg/mL dose in mDANs, and at 40 days, PSEN1-Int4Del mDANs have as much as 2-fold more pathology than their CNs. There is no pathology in the SNCA KO line in either CNs or mDANs.

Similarly, in examining the area fraction of specifically LN pathology, PSEN1-Int4Del CNs developed an equivalent pathological load to mDANs at early timepoints, but appeared to plateau after 30 days, while mDANs continued to increase in LN burden (Figure 5.2). As such, at 40 days, the PSEN1-Int4Del mDANs have ~4-fold more LN pathology than their CNs. There also remains negligible LN development in other CN genotypes, despite similarly high levels across mDANs.

Interestingly, the few early LN structures at 10 days in CNs may be slightly larger than in mDANs, although both cell types exhibit similarly sized LNs by 40 days (Figure 5.3). This suggests that the disparity in LN load between CNs and mDANs is due to the rate at which

these structures are seeded, rather than their eventual size. In contrast to LNs, LB pathology developed relatively rapidly in the PSEN1-Int4Del CNs, with approximately 2-fold more at 10 days than in mDANs, where LB pathology relatively lagged (Figure 5.4). However, mDANs quickly caught up, with equivalent numbers of LBs formed at 20 and 30 days to PSEN1-Int4Del CNs, and a slightly higher burden at 40 days. Across all other genotypes, while CNs had much fewer LBs than mDANs, the CNs seemed to be proportionally more prone to LB than LN pathology.

Like LNs, however, LB mean area was relatively similar between cell types at most early time points, though in some cases in mDANs, LBs reached substantially larger average sizes by 40 days (Figure 5.5).

These comparisons raise two key questions. Firstly, why is the mDAN model so much more prone to developing aggregation related pathology in response to the PFF insult than the CN model? Secondly, why does the PSEN1-Int4Del mutation cause such a profound vulnerability to CNs compared to any other genotype tested, including its isogenic control, while there is no difference between the mutant and its corrected control mDANs, where they are equally vulnerable.

To address these questions, I addressed several possible mechanisms. Firstly, uptake of PFFs, then levels of endogenous ASyn, then breakdown of aggregates by the autophagy-lysosomal pathway.

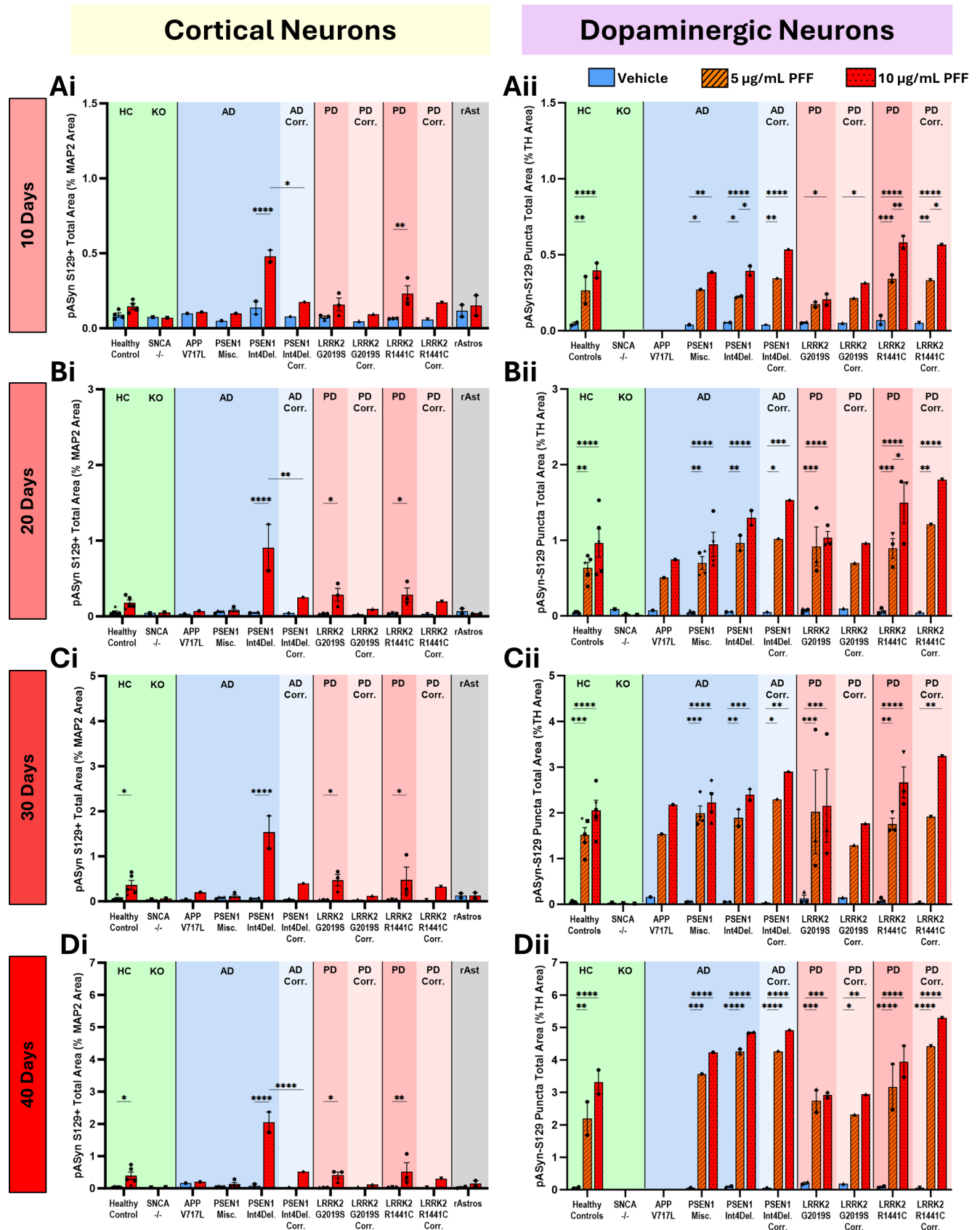


Figure 5.1. Overall ASyn PFF-Seeded pASyn-S129+ Pathology Burden is High in Midbrain Dopaminergic Neurons Relative to Cortical Neurons, Except for PSEN1-Int4Del. The area fraction of pASyn-S129+ pathology in (i) MAP2+ cortical neurons, presented in Chapter 3, and (ii) TH+ midbrain dopaminergic neurons, presented in Chapter 4, is here presented on matched pairs of axes for each of four time points. **(A)** At 10 days, all genotypes assessed in mDANs have significantly elevated pathological burden, but only PSEN1-Int4Del cell lines have an equivalent load at 0.5% in CNs. **(B)** At 20 days, pathology load has approximately doubled in mDANs and PSEN1-Int4Del CNs to ~1%. LRRK2 cell lines are significantly elevated in CNs, but not to the same degree as PSEN1-Int4Del or mDANs. **(C)** At 30 days, pathology burden in PSEN1-Int4Del CNs, seeded by 10 μ g/mL PFF insult, appears more equivalent to that seeded by 5 μ g/mL in mDANs. **(D)** Pathology burden reached in PSEN1-Int4Del CNs, at ~2%, is substantially lower than the 3-5% reached across genotypes in mDANs. PSEN1-Int4Del CNs are also significantly elevated compared to corrected controls, but PSEN1-Int4Del mDANs are equally vulnerable as their corrected controls. Data points represent individual cell lines, averaged over 3-6 independent differentiations in CNs, and 2-4 independent differentiations in mDANs, bars represent mean $\pm$ SEM. Isogenic pairs are noted with shared point shape. Healthy Control 856 and its SNCA-KO are square points. iNDI KOLF lines are represented by stars. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and iNDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as “PSEN1 Misc.”. APP, SNCA-KO, HC 856 and 156, and iNDI mDANs were only examined at 20 and 30 days. Statistical comparisons are 2-Way ANOVA with Bonferroni correction for multiple comparisons.

Cortical Neurons

Dopaminergic Neurons

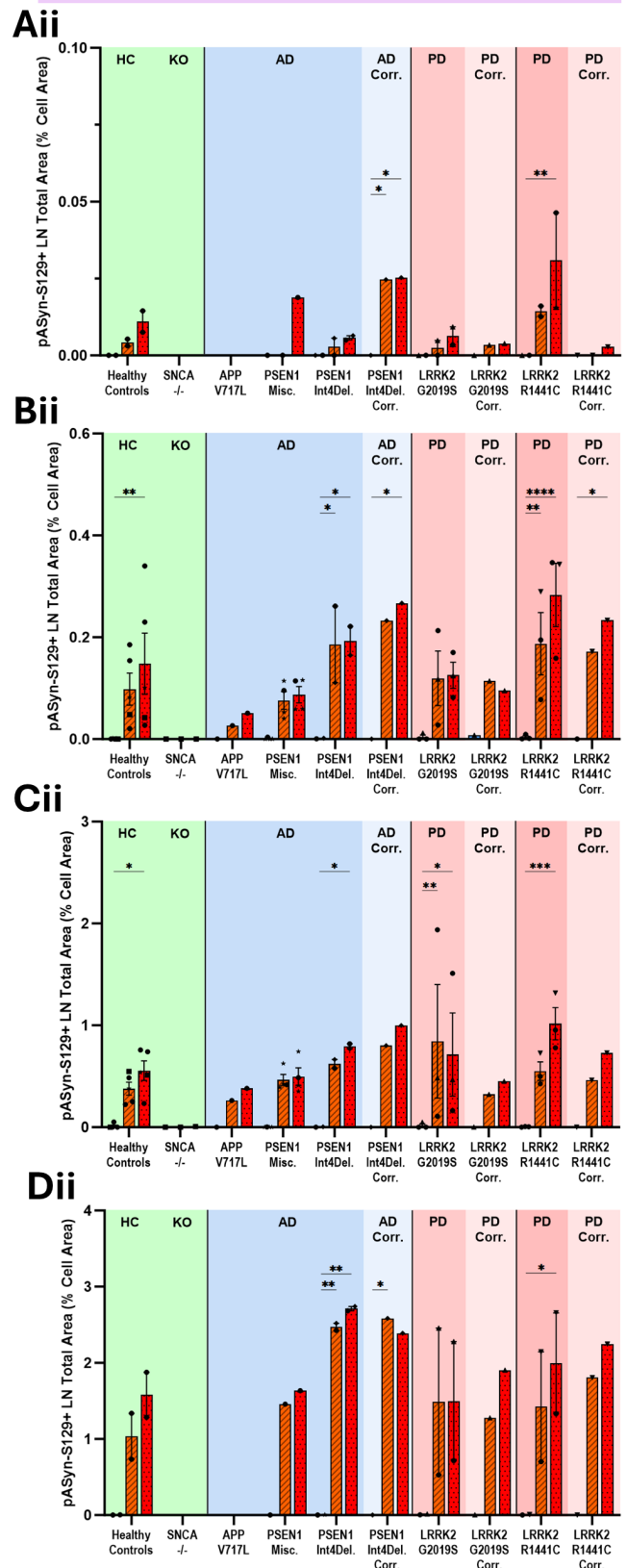
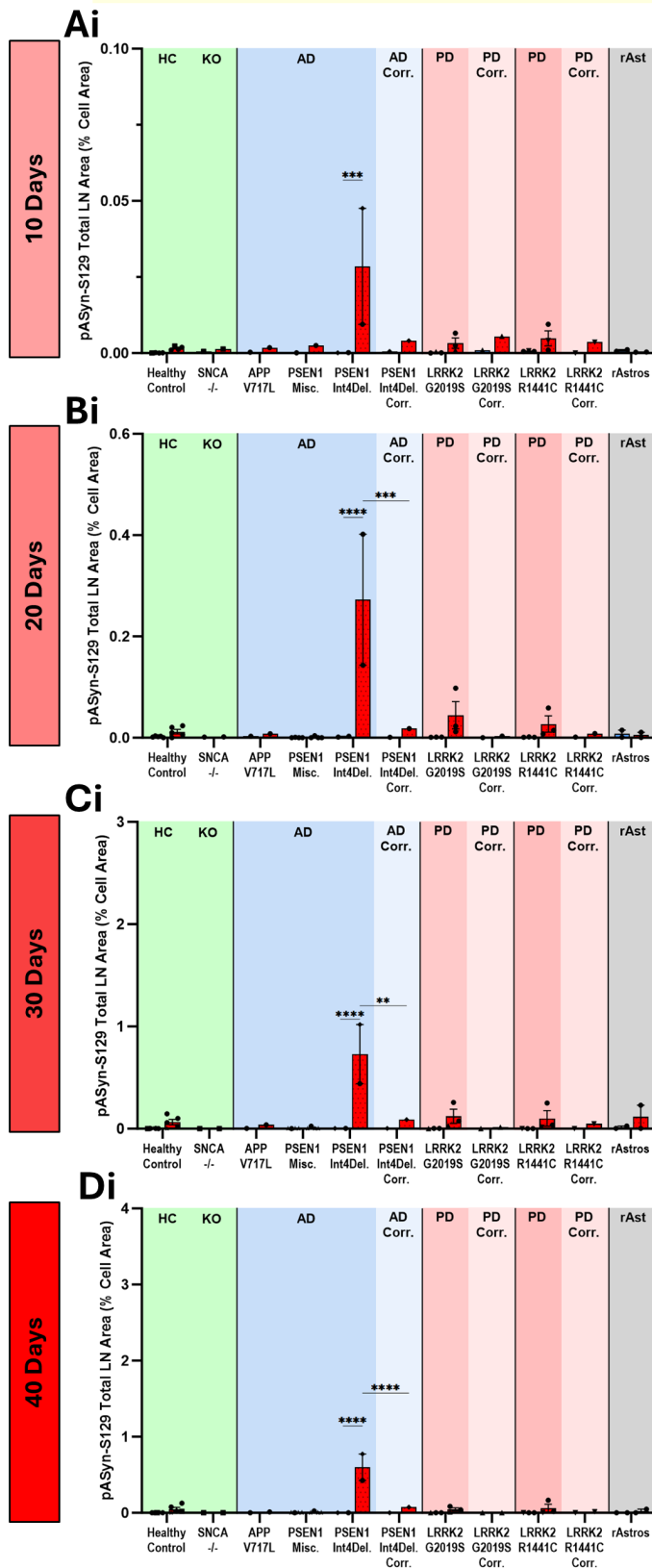


Figure 5.2. ASyn PFF-Seeded pASyn-S129+ Lewy Neurite Pathology is High in Midbrain Dopaminergic Neurons Relative to Cortical Neurons, Except for PSEN1-Int4Del Until 40 Days. The area fraction of Lewy neurite (LN) labelling in (i) MAP2+ CNs, presented in Chapter 3, and (ii) TH+ mDANs, presented in Chapter 4, is here presented on matched pairs of axes for each of four time points, (A) 10 days, (B) 20 days, (C) 30 days, and (D) 40 days. Very few LNs covering little area were present at 10 days. PSEN1-Int4Del CNs developed LNs relatively quickly compared to mDANs, but by 20 days, most mDANs exhibited elevated LN pathology whereas only PSEN1-Int4Del had significantly elevated LNs. LN burden remained approximately equivalent between PSEN1-Int4Del. CNs and mDANs at 30 days, but beyond this, CNs plateau at <1%, while mDANs reach as much as 4-fold greater LN burden at 40 days. There continues to be negligible LN burden in other CN genotypes. Data points represent individual cell lines, averaged over 3-6 independent differentiations in CNs, and 2-4 independent differentiations in mDANs, bars represent mean +/- SEM. Isogenic pairs are noted with shared point shape. Healthy Control 856 and its SNCA-KO are square points. iNDI KOLF lines are represented by stars. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and iNDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as "PSEN1 Misc.". APP, SNCA-KO, HC 856 and 156, and iNDI mDANs were only examined at 20 and 30 days. Statistical comparisons are 2-Way ANOVA with Bonferroni correction for multiple comparisons.

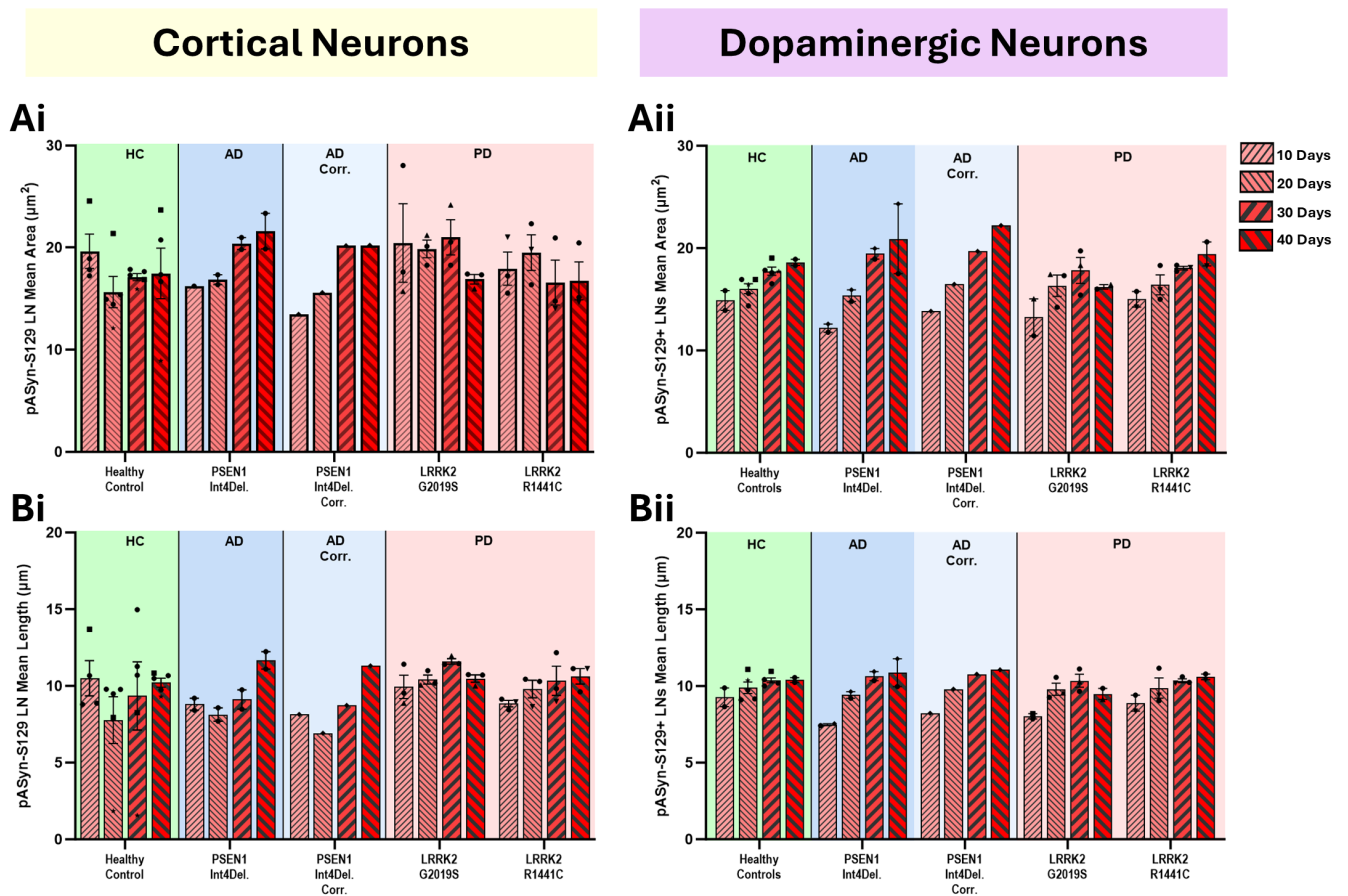
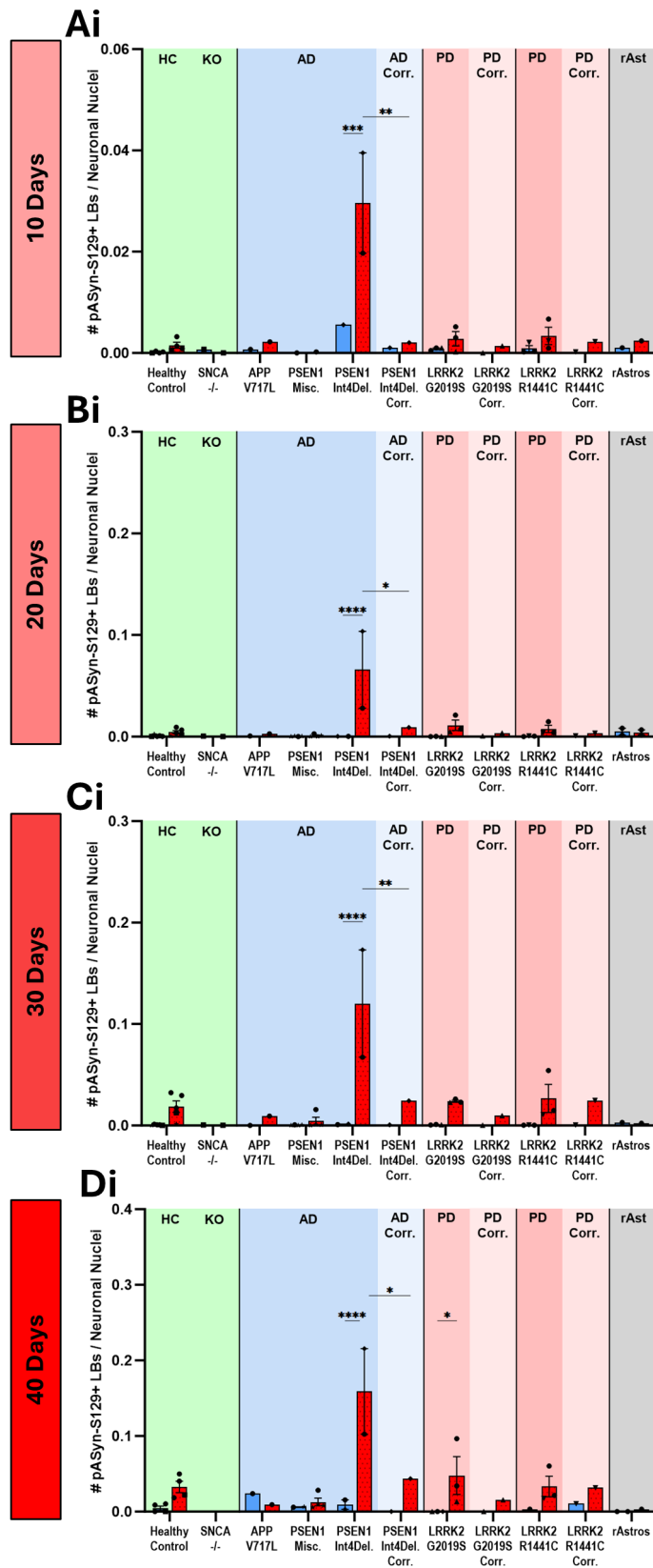


Figure 5.3. LN Length and Area Does Not Differ Between Cortical and Midbrain Dopaminergic Neurons. The (A) mean area, and (B) mean length of pASyn-S129+ LNs seeded by a 10 $\mu\text{g/mL}$ ASyn PFF insult is presented for (i) CNs and (ii) mDANs, for a subset of genotypes that experienced significantly elevated pathology in CNs, on matched axes, with one bar for each time point. LNs in CNs appear slightly larger at earlier timepoints, though there are few LNs being measured here. At later time points, CNs and mDANs reach similar mean lengths. Data points represent individual cell lines, averaged over 3-6 independent differentiations in CNs, and 2-4 independent differentiations in mDANs, bars represent mean $\pm$ SEM. Isogenic pairs are noted with shared point shape, retained from previous figures.

Cortical Neurons



Dopaminergic Neurons

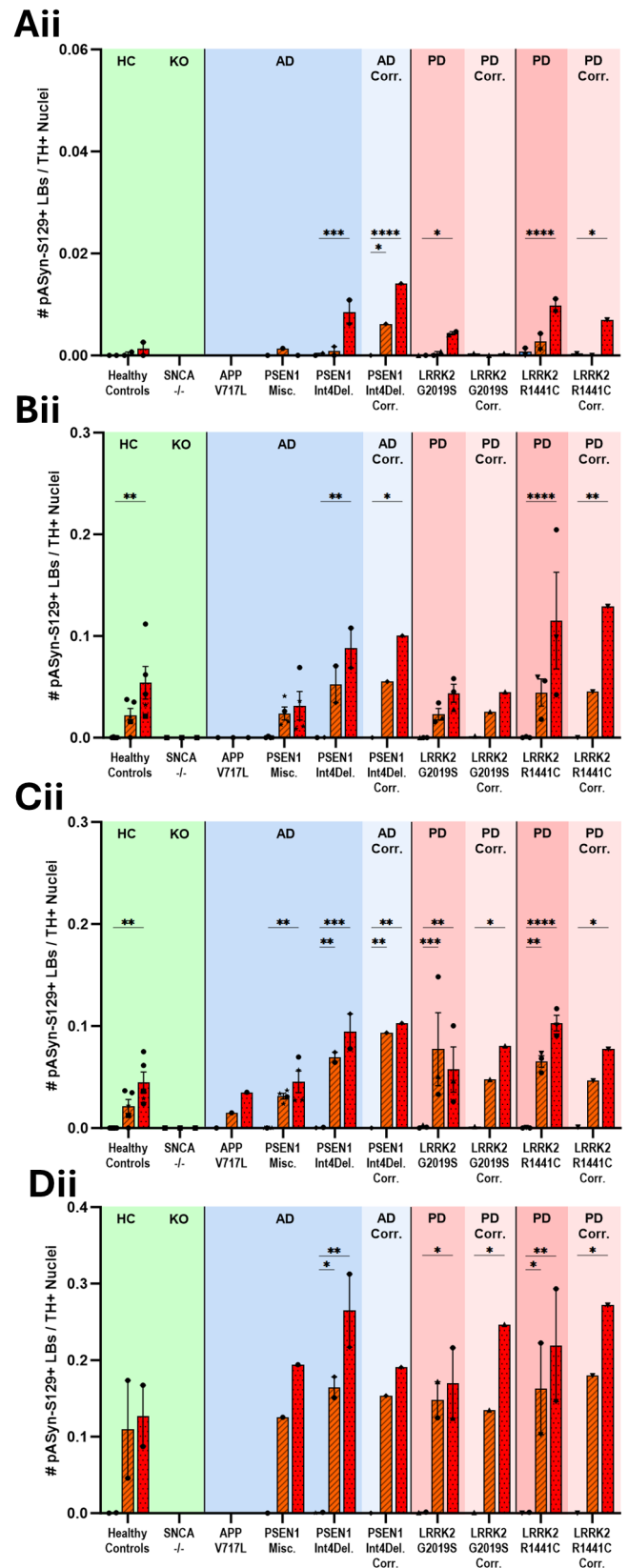


Figure 5.4. ASyn PFF-Seeded pASyn-S129+ Lewy Body Pathology is High in Midbrain Dopaminergic Neurons Relative to Cortical Neurons, Except for PSEN1-Int4Del. The number of Lewy body-like structures (LBs) observed per nucleus in (i) MAP2+ CNs, presented in Chapter 3, and (ii) TH+ mDANs, presented in Chapter 4, is here presented on matched pairs of axes for each of four time points, (A) 10 days, (B) 20 days, (C) 30 days, and (D) 40 days. Very few LBs were present at 10 days in either CNs or mDANs, though they appeared to form relatively rapidly in PSEN1-Int4Del CNs. By 20 days, most mDANs exhibited elevated LN pathology whereas only PSEN1-Int4Del CNs had significantly elevated LBs. LB number increased with time in mDANs and CNs but remained substantially higher in mDANs than all CNs except PSEN1-Int4Del, which remained equivalent at 30 days, though at 40 days, mDANs appeared to have more LBs. Data points represent individual cell lines, averaged over 3-6 independent differentiations in CNs, and 2-4 independent differentiations in mDANs, bars represent mean $\pm$ SEM. Isogenic pairs are noted with shared point shape. Healthy Control 856 and its SNCA-KO are square points. iNDI KOLF lines are represented by stars. PSEN1-Int4Del 808-04 and corrected control are represented by diamonds. The LRRK2-G2019S isogenic pair are represented by upwards triangles, and LRRK2-R1441C by downwards triangles. PSEN1-M146I (dot), and iNDI PSEN1-A79V, M139V, and E280A (stars) are grouped together as “PSEN1 Misc.” APP, SNCA-KO, HC 856 and 156, and iNDI mDANs were only examined at 20 and 30 days. Statistical comparisons are 2-Way ANOVA with Bonferroni correction for multiple comparisons.

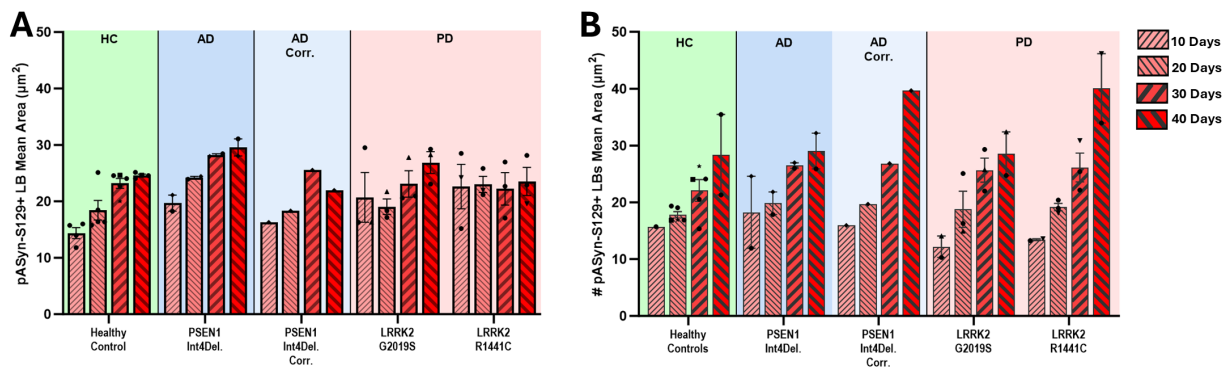


Figure 5.5. LB Area Typically Does Not Differ Between Cortical and Midbrain Dopaminergic Neurons. The mean area of pASyn-S129+ LBs seeded by a 10 μ g/mL ASyn PFF insult is presented for (A) CNs and (B) mDANs, for a subset of genotypes that experienced significantly elevated pathology in CNs, on matched axes, with one bar for each time point. Mean area at each timepoint was usually approximately equivalent in CNs and mDANs, though at later time points, some mDAN genotypes developed LBs with substantially higher mean areas. Data points represent individual cell lines, averaged over 3-6 independent differentiations in CNs, and 2-4 independent differentiations in mDANs, bars represent mean $\pm$ SEM. Isogenic pairs are noted with shared point shape, retained from previous figures.

5.3.2. ASyn PFF Uptake

To first examine uptake, I considered expression of LRP1, a cell surface receptor thought to mediate ASyn PFF entry into neurons (Chen et al. 2022). Western blotting in both mDANs and CNs at D36 and D70 respectively, the point of PFF insult, showed no significant differences between cell types in matched pairs of cell lines, nor any differences between genotypes within CNs that might explain the vulnerability of mDANs or PSEN1-Int4Del CNs (Figure 5.6). Immunocytochemical labelling of LRP1 in CNs similarly bore no discernible differences in LRP1 expression in the cell lines of interest (Figure 5.7).

To further examine uptake in CNs, I also used Alexa Fluor-647-labelled ASyn PFFs, to visualise the degree of PFF uptake into cells. Cells were insulted with 10 µg/mL PFF at D70, and representative images of live cells labelled with NeuO live cell dye are shown in Figure 5.8A-B. Image analysis using a custom Harmony pipeline found no differences in ASyn-647 puncta uptake into NeuO+ neurons (Figure 5.8C-D). To confirm the Alexa-647 tag was not detached from PFFs, cells were fixed after 20 days and labelled for ASyn using the 4D6 antibody, which typically matched the pattern of ASyn-647 PFF signal (Figure 5.9A-C). To confirm the ASyn-647 labelled PFFs were still seed competent, I also labelled cultures for pASyn-S129, and observed pASyn-S129+ pathology in the expected pattern, with punctate, LN, and even early LB-like structures (Figure 5.9D). Interestingly, no ASyn-647 signal was often detected in aggregates (e.g. arrowhead), suggesting a process of secondary nucleation seeded by PFFs.

As such, while these experiments do not definitively rule out a role for fibril uptake, I observed no differences here that might explain mDAN or PSEN1-Int4Del CN vulnerability.

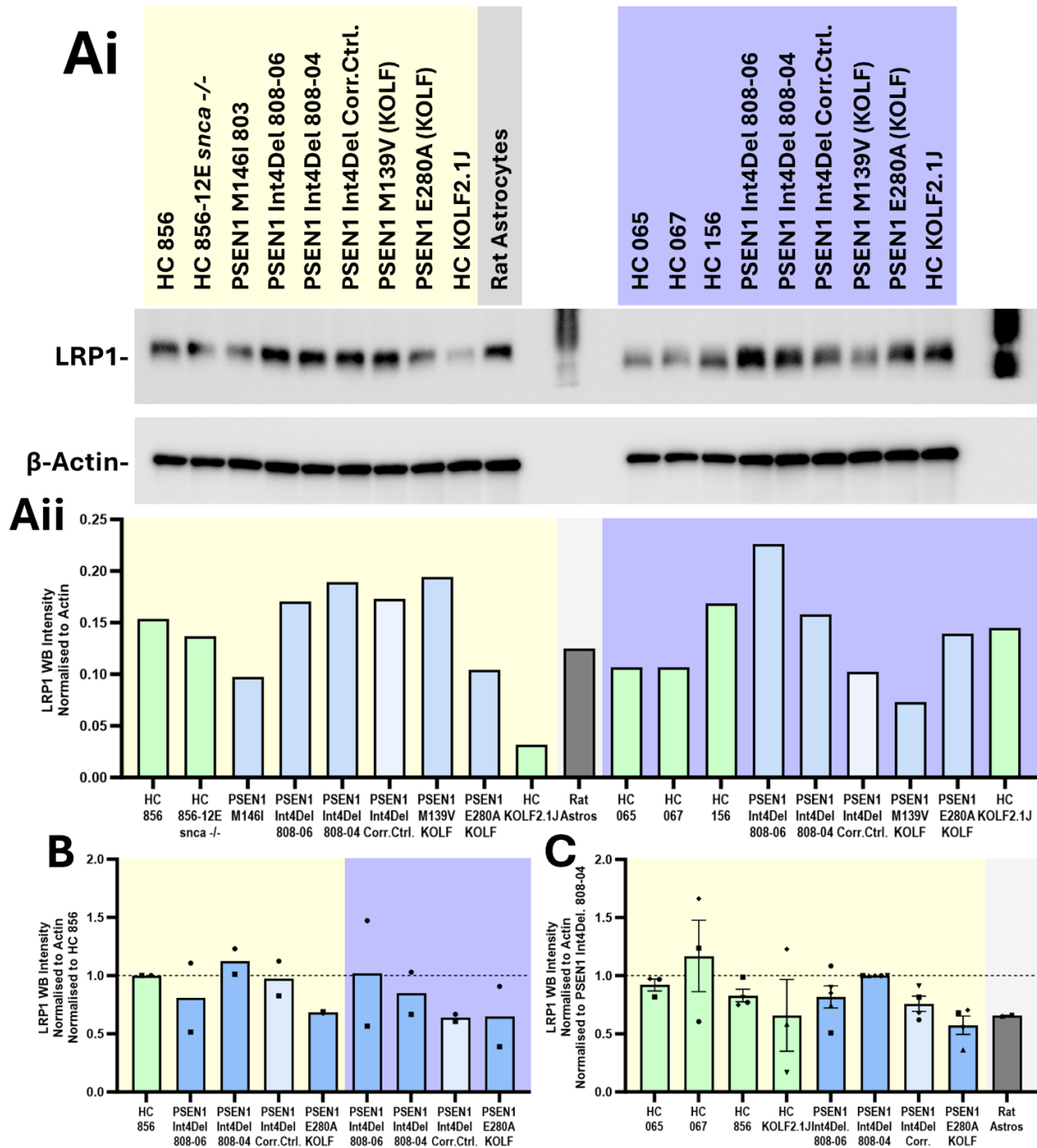


Figure 5.6. Expression of LRP1, a Putative ASyn Fibril Uptake Mediator, Does Not Differ Between CNs and mDANs, or Between CN Genotypes. LRP1 expression was examined by Western blot in CNs and mDANs. **(Ai)** Representative image of 10 μ g protein from various healthy control and PSEN1 cell lines grown as CNs and mDANs, loaded and run on the same gel, and labelled for LRP1, with densitometric quantification of bands in **(Aii)**. **(B)** Lysates from four PSEN1 lines and HC 856 were collected from two independent differentiations of CNs (yellow) and mDANs (purple) and run on the same gel. Quantification of these two blots is shown, normalised to β -actin and HC 856. No significant differences between lines were observed (Kruskal-Wallis test with Dunn's correction for multiple comparisons). Data points represent independent differentiations, bars represent mean $\pm$ SEM. Points from the same differentiation have the same shape (squares and dots). **(C)** Quantification of Western blots from three independent differentiations of CNs, normalised to β -actin and PSEN1-Int4Del 808-04, which was included in each gel. No significant differences between lines were observed (Kruskal-Wallis test with Dunn's correction for multiple comparisons). Data points represent independent differentiations, bars represent mean $\pm$ SEM. Points from the same differentiation have the same shape (squares, dots, and diamonds). LRP1 antibody was kindly provided by Maria Karabova and Sally Cowley.

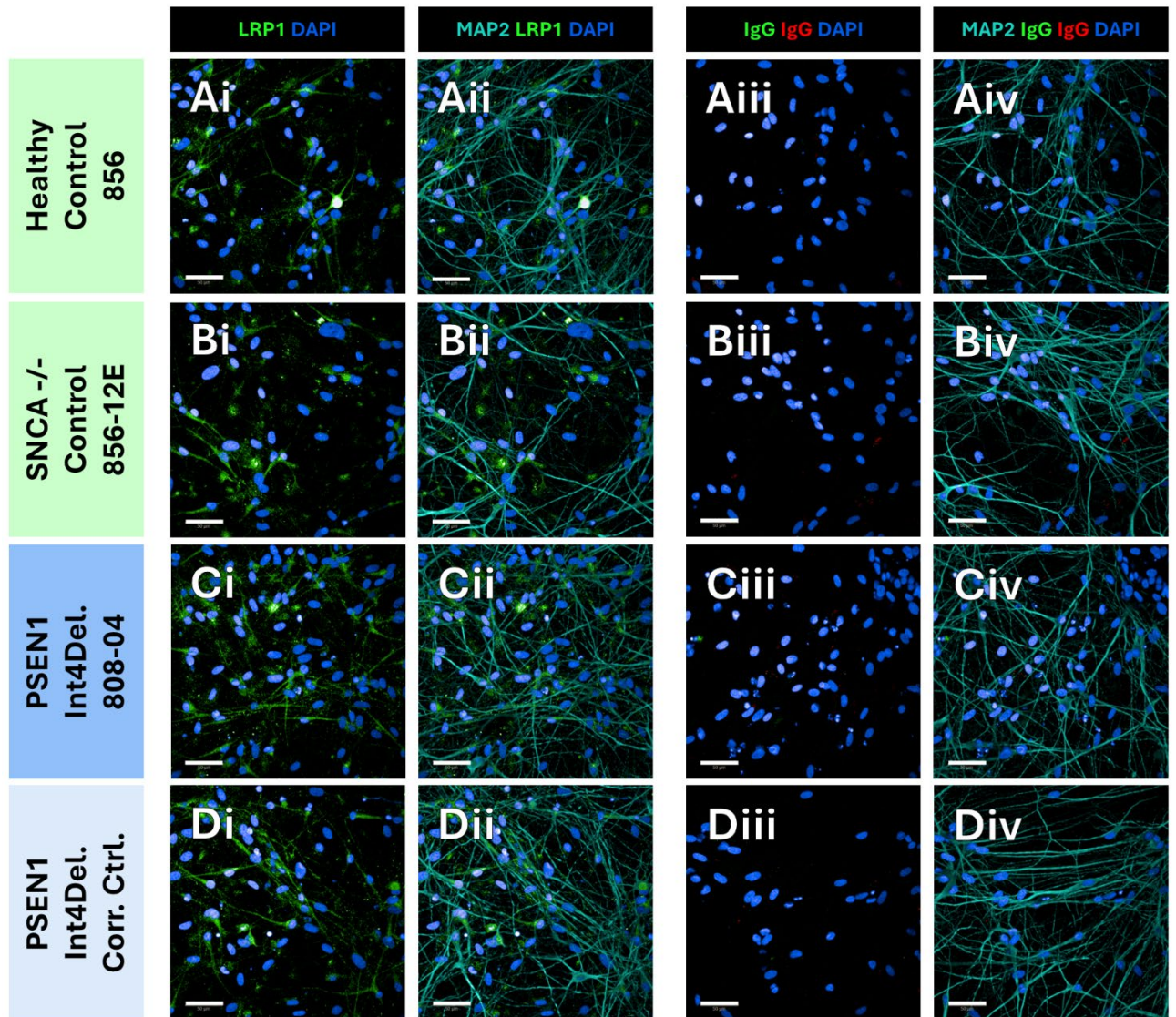


Figure 5.7. Immunolabelling of LRP1 in MAP2+ Cortical Neurons Shows No Differences in Labelling Pattern. ICC for (i) LRP1, alongside neuronal marker (ii) MAP2 was performed in CNs from (A) healthy control 856, (B) SNCA-KO, (C) PSEN1-Int4Del 808-04, and (D) PSEN1-Int4Del corrected control cell lines. No obvious differences in labelling were observed. (iii-iv) IgG negative control labelling showed no immunoreactivity. LRP1 antibody was kindly provided by Maria Karabova and Sally Cowley. Scale bars represent 50 μ m.

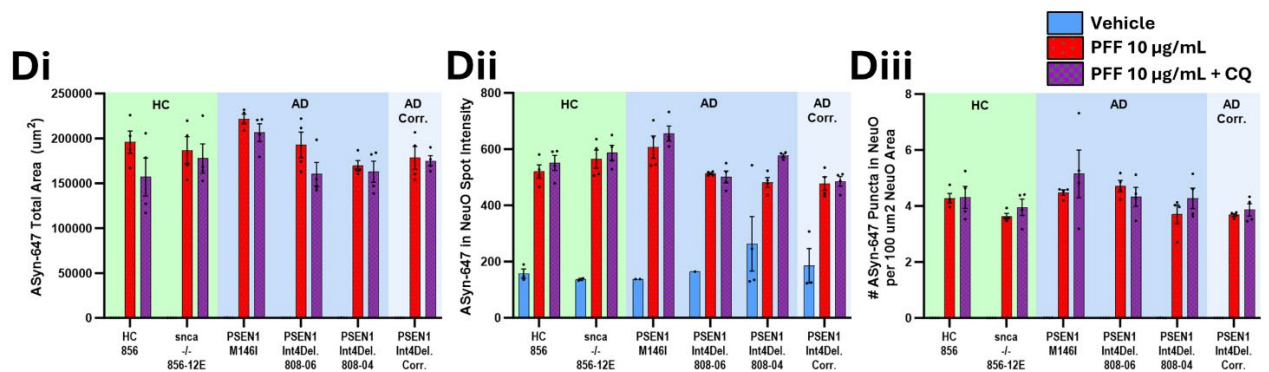
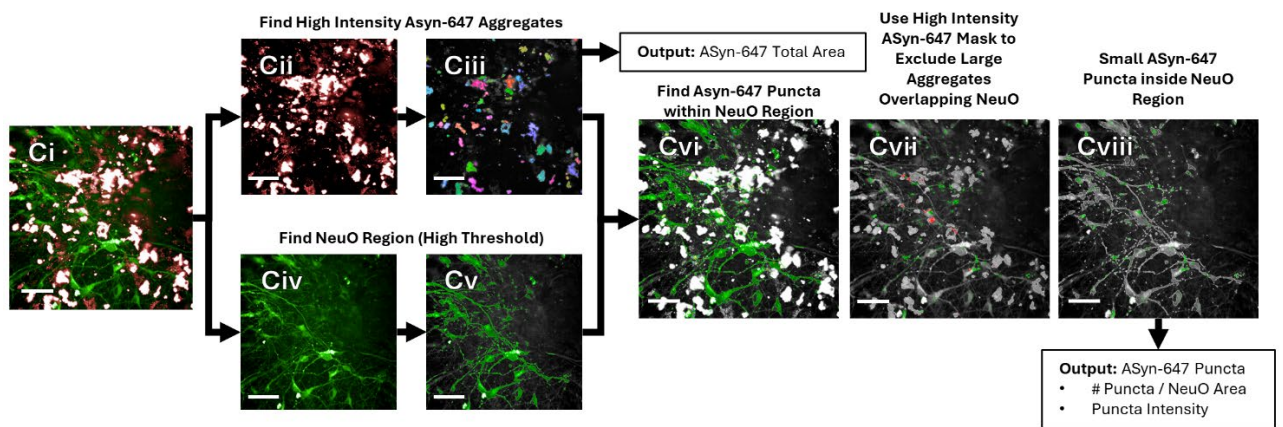
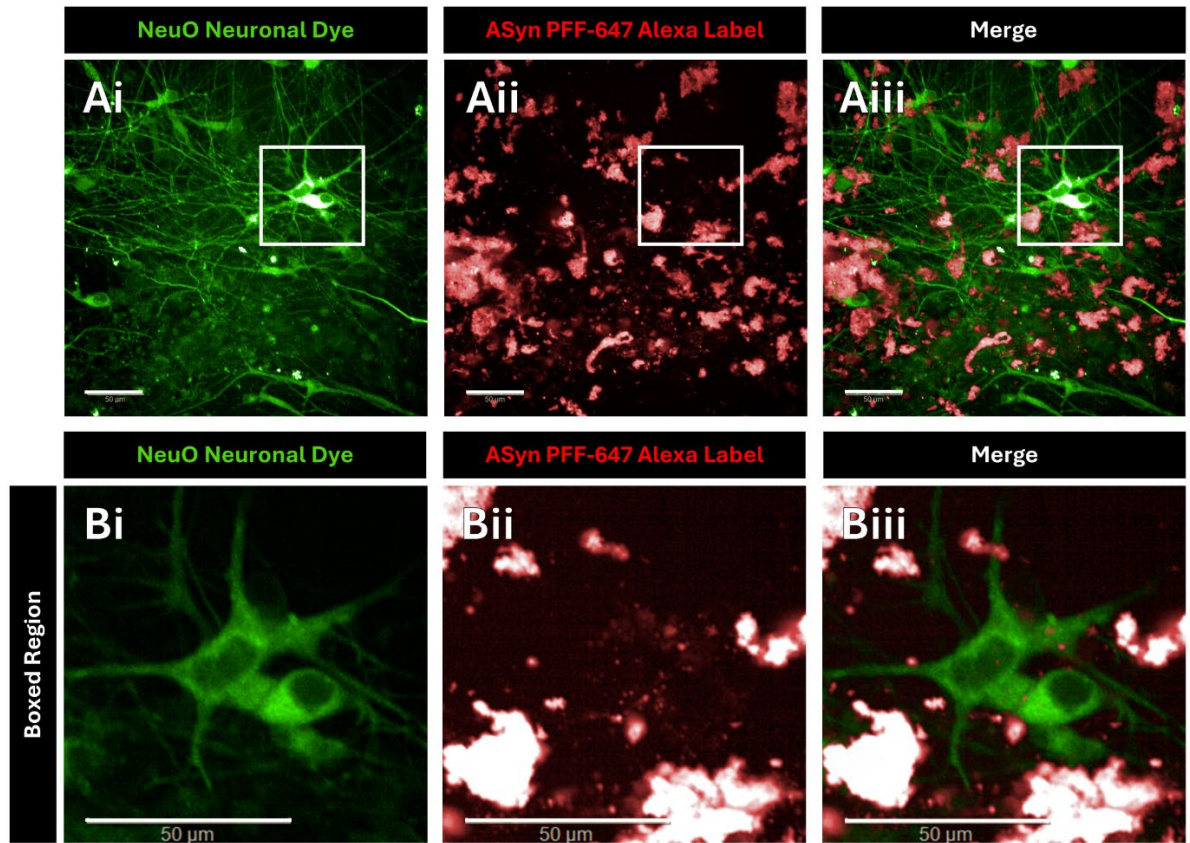


Figure 5.8. Labelled ASyn PFF-Alexa-647 Insult Shows No Differences in PFF Uptake in HC and PSEN1-Int4Del Cortical Neurons. Cortical neurons grown to D70 were treated with Alexa-647-labelled ASyn PFFs at 10 $\mu\text{g/mL}$. **(A-B)** Live imaging of cells (i) stained with NeuO live neuronal cell dye (green) and (ii) ASyn-PFFs (red), 16 days after insult. **(A)** Large-scale image of labelled PFFs among neurons. **(B)** Boxed region enlarged with contrast adjusted to overexpose large extracellular PFF aggregates, and visualise less intense puncta taken up into NeuO+ cells. **(C)** Analysis pipeline for live images. **(Cii-iii)** Find Image Region function was used to find high intensity extracellular PFF aggregates using an intensity threshold. The total area of this high intensity labelling was measured to confirm even PFF dosage. **(Civ-v)** Find Image Region function was also used to define a NeuO ROI with a relatively high threshold, largely capturing cell bodies and large, high intensity neurites. **(Cvi)** Find Spots function was used to identify ASyn-647 puncta within the NeuO ROI. **(Cvii)** Puncta overlapping with the large, high intensity, extracellular aggregates were excluded. **(Cviii)** Remaining puncta in NeuO ROI were counted as taken up fibrils. **(D)** Quantification of live imaging of ASyn-647 uptake into NeuO+ CNs. **(Di)** Total ASyn-647+ area, i.e. high intensity aggregates, **(Dii)** mean spot intensity of puncta in NeuO, and **(Dii)** the number of puncta per NeuO area not significantly changed across all cell lines or by 20 μM CQ co-treatment (Two-way ANOVA with Bonferroni correction for multiple comparison). Scale bars represent 50 μm .

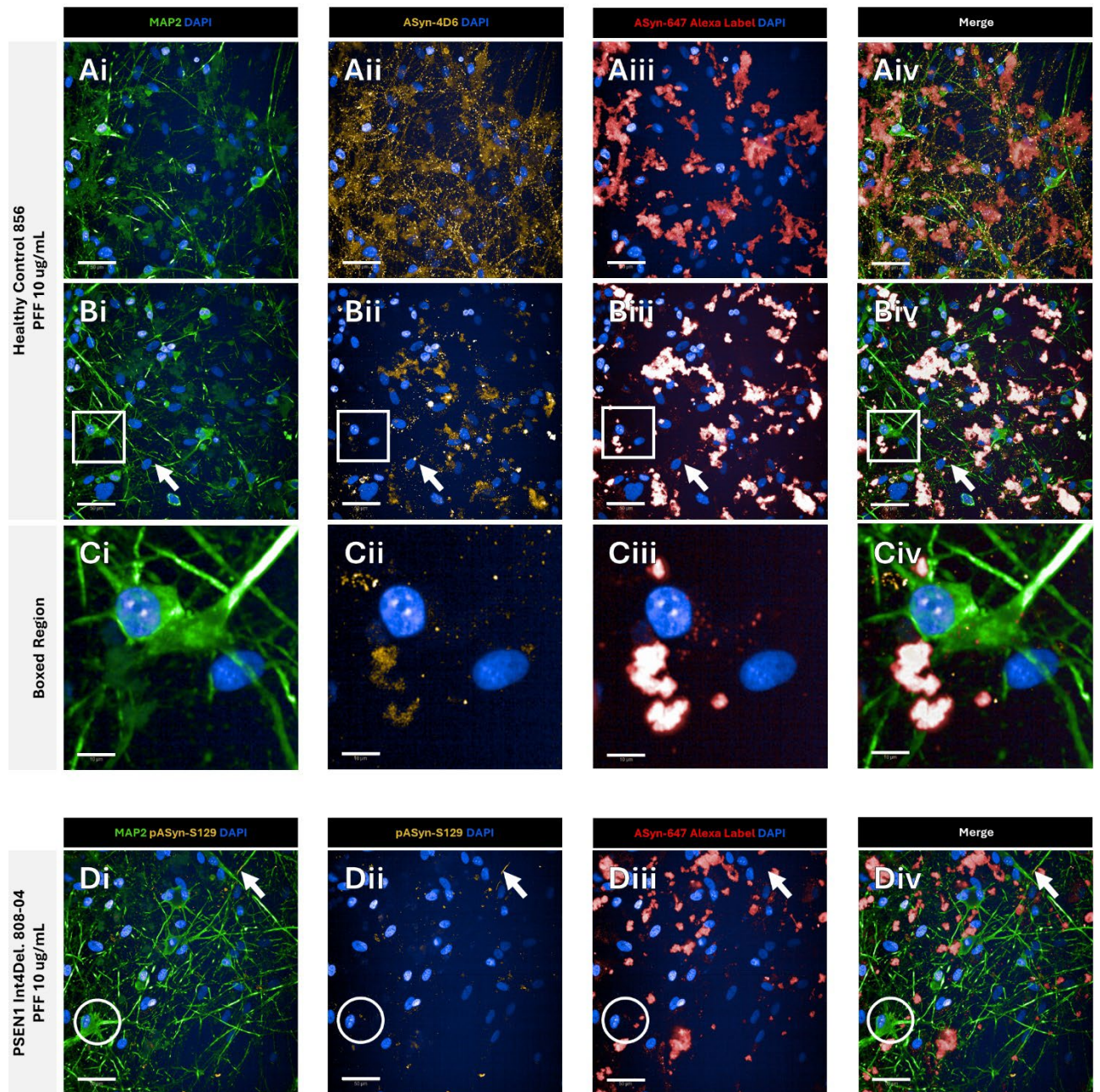


Figure 5.9. Immunolabelling of ASyn-647 Labelled PFFs Show they Colocalise with Alpha Synuclein and Seed pASyn-S129+ Pathology. CNs insulted with ASyn-647 labelled PFFs were fixed after 20 days and labelled with (A-C) ASyn-4D6 and (D) pASyn-S129. **(A-C)** ASyn-4D6 labelling for non-phosphorylated ASyn showed both high levels of endogenous ASyn expression and overlapped with ASyn-647 signal, both in high intensity aggregates seen in (A), and in lower intensity puncta inside cells, visualised with adjusted contrast in (B-C). Cells that were not MAP2+, interpreted as rAstros (arrowhead) also appeared to take up ASyn-647. **(D)** Cells insulted with ASyn-647 labelled PFFs also exhibited pASyn-S129+ pathology, confirming that, despite the Alexa tag, the PFFs were still seed competent, and led to pathology as expected, including LN structures (arrow) and small, perikaryal inclusions (circle). Scale bars represent 50 μm .

5.3.3. Culture Density and Endogenous ASyn Levels Are Associated with Vulnerability

A major difference between the CN and mDAN culture models is the density at which the cells are seeded. CNs were seeded at 25,000 cells/cm², declining to 10,000 cells/cm² 10 days after PFF insult, whereas mDANs are seeded at 200,000 cells/cm², declining to 30,000 cells/cm² 10 days after PFF insult. To examine what effect cell density might play on vulnerability to aggregation, I grew CNs in a series of increasingly high seeding densities, insulted cultures with the same 10 µg/mL dose of ASyn PFF at D70, and fixed cells after 20 days (Figure 5.10). Measuring HuNu+ DAPI nuclei and MAP2 area showed a clear stepwise 'seeding dose'-dependent increase in the number of cells and the amount of neuronal cell area present in the wells (Figure 5.10Bi-ii). Interestingly, even when normalising to the number of nuclei or the MAP2 area, higher density cultures exhibited disproportionately higher levels of pASyn-S129+ pathology in PSEN1-Int4Del CNs (but not the isogenic control), though individual puncta were not any larger with increasing cell density (Figure 5.10Biii-vi). While very few LB and LN structures were observed in this experiment, the vast majority of the LBs were seen in the 8000 cells/well condition (50,000 cells/cm²).

It is not clear what make a denser culture more prone to PFF-seeded aggregation. The effect on puncta number and not size suggests an effect specifically on seeding propensity, implicating higher levels of endogenous ASyn.

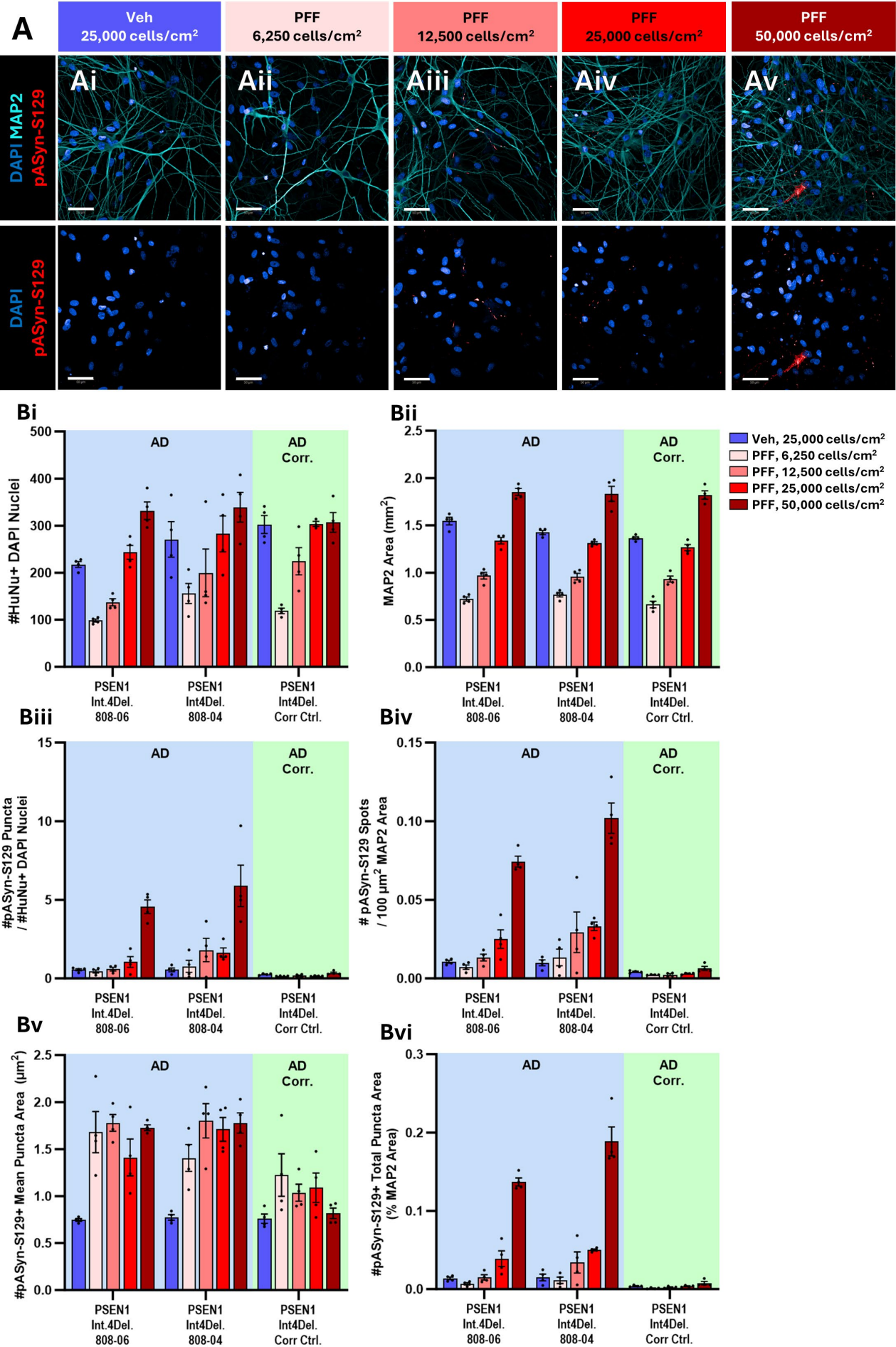


Figure 5.10. Neuronal Density Is Associated with Greater pASyn-S129+ Pathology Seeding in Cortical Neurons. CNs seeded at a range of different densities were insulted with ASyn-PFFs and labelled for pASyn-S129+ pathology after 20 days. **(A)** Representative images of MAP2+ CNs seeded at **(Ai)** 25,000 cells/cm², treated with vehicle (same density used in other experiments), **(Aii-v)** 6250, 12,500, 25000, and 50000 cells/cm² (1000, 2000, 4000, and 8000 cells/well), insulted with 10 ug/mL ASyn PFFs. **(B)** Labelling was quantified using a previously described pipeline. **(Bi)** the number of human (HuNu+) nuclei was counted, and a 'seeded dose' dependent increase in cell number was observed. The number of cells in the vehicle condition matched that in the PFF treated condition with 25,000 cells/cm² seeded. **(Bii)** MAP2 area similarly increased in a seeding dose dependent fashion. **(Biii-iv)** The number of pASyn-S129+ puncta seeded by PFF insult, even once normalised to either **(iii)** HuNu+ nuclei, or to **(iv)** MAP2 area, showed a dose dependent increase in pathology in the PSEN1-Int4Del cell lines, and not in the corrected control. **(Bv)** Mean puncta area was elevated in PFF treated conditions but did not increase with neuronal density. **(Bvi)** the pASyn-S129+ area fraction in MAP2 cells also increased with neuronal density. Data points represent four individual wells per condition from a single differentiation, bars represent mean $\pm$ SEM. Two-way ANOVA found a significant effect of seeding density and genotype for each measure ($p < 0.0001$), except for an effect of seeding density on mean puncta area ($p = 0.7102$).

To examine differences in basal ASyn expression levels, I performed western blotting on CNs and mDANs from cell lines of interest, with proteins run on the same gel and blotted on the same membrane. Cells cocultured with rAstros were harvested at D70 for CNs, and D36 for mDANs, to mimic conditions at PFF insult (Figure 5.11). Here, while ASyn protein levels trended higher in mDANs, it was not significantly or consistently higher than in CNs. In Figure 5.12, ASyn levels are compared across a wider range of PSEN1 CNs. While expression trended higher in the vulnerable PSEN1-Int4Del 808-04 cell line compared to its clonal 808-06 pair and to its isogenic corrected control, this was not significant, nor was it substantially different from other PSEN1 and healthy control lines which were far more resilient to PFF-seeded pathology. The lack of ASyn signal in rAstros confirmed that all ASyn signal in these coculture blots was from neurons, and specific labelling was confirmed through use of the SNCA-KO as a negative control.

Thus, while these experiments may go some way to explaining the differences in response to PFF insult by the mDANs and CNs and confirm the lack of ASyn protein expression in the SNCA-KO and rAstros, they do not seem sufficient to explain the particular vulnerability of the PSEN1-Int4Del cell line relative to its corrected control or other lines in CNs.

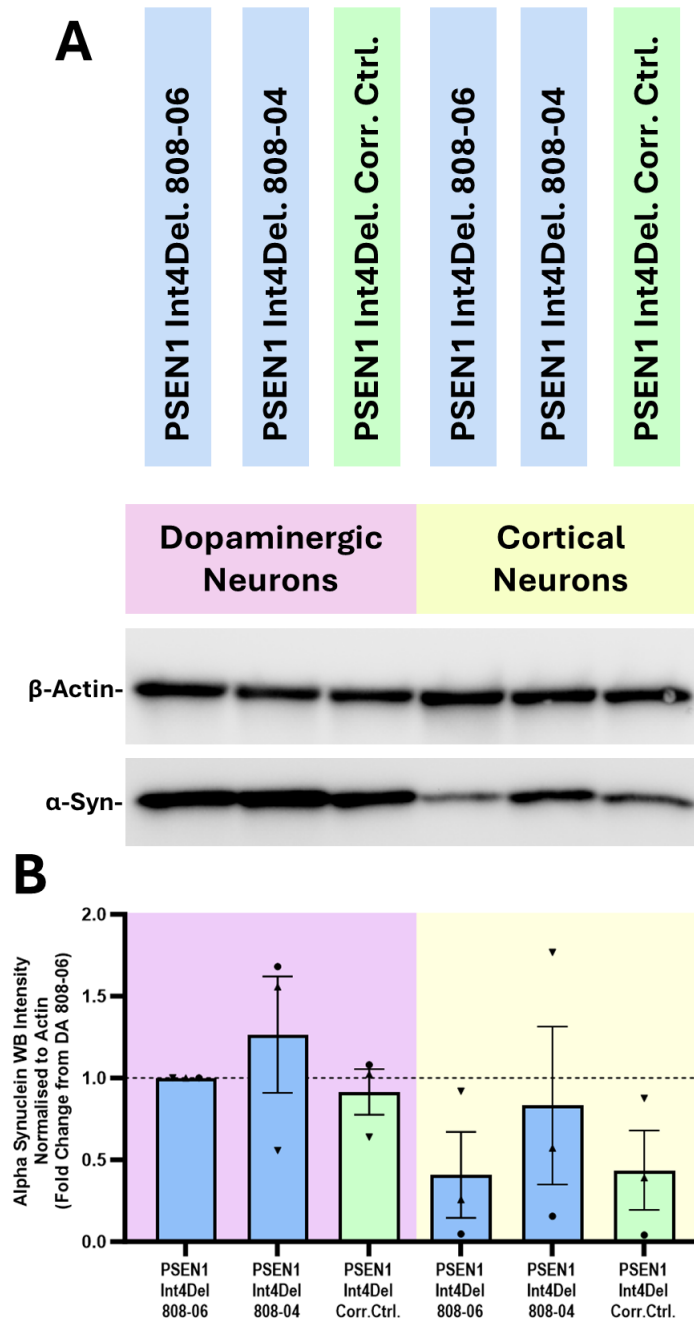


Figure 5.11. Endogenous Alpha Synuclein Expression Trends Higher in PSEN1-Int4Del Midbrain Dopaminergic Neurons than Cortical Neurons But Is Not Significantly Different Between Cell Types or PSEN1-Int4Del Genotypes. (A) A representative Western blot for ASyn performed on 10 μ g protein from lysates of CNs at D70 and mDANs and D36, run simultaneously on the same gel. **(B)** Quantification of Western blots from three pairs of independent differentiations, with each pair run on the same gel, with intensity normalised to β -actin and PSEN1-Int4Del 808-06 mDANs. Protein levels trended higher in mDANs compared to CNs and trended slightly higher in PSEN1-Int4Del, though this was inconsistent differentiation to differentiation, and no significant effect was found for genotype or cell type (two-way ANOVA). Data points represent intensity from a single blot from a single differentiation, bars represent mean $\pm$ SEM. Points from the same blot have the same shape (upright triangles, downward triangles, dots).

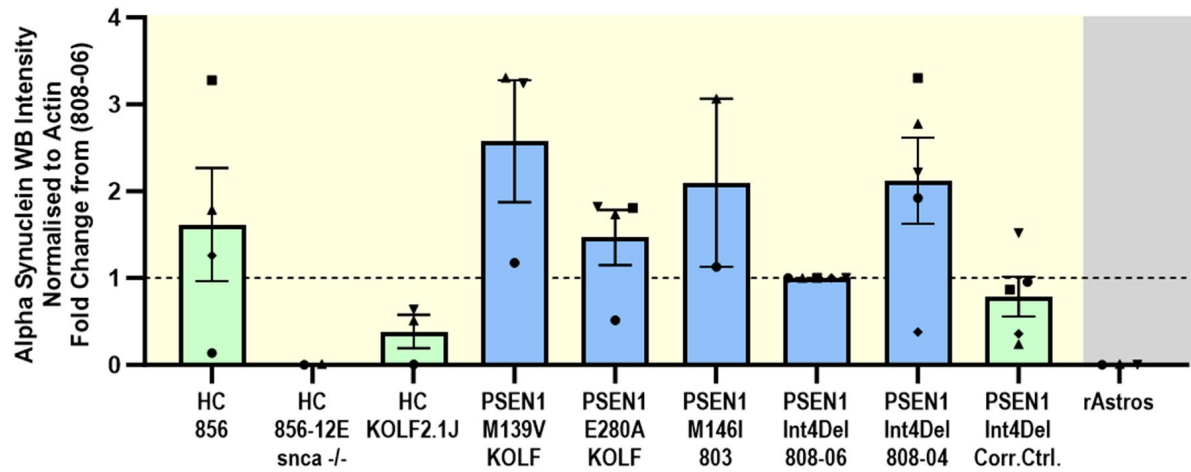


Figure 5.12. Endogenous Alpha Synuclein Expression Varies Between PSEN1 Cortical Neurons and Is Not Significantly Different Between PSEN1-Int4Del Isogenic Pairs. Quantification of western blots from up to 5 independent differentiations in healthy control and PSEN1 CNs show variable levels of ASyn protein levels between cell lines, with a significant effect of genotype ($p=0.0061$) but no significant differences between genotypes (Kruskal-Wallis test with Dunn's Correction for multiple comparisons). No expression was seen in the SNCA-KO or rAstros, from three separate cultures. A separate statistical test comparing the PSEN1-Int4Del 808-04 to its corrected control alone found a trend towards higher ASyn expression in the mutant line (Wilcoxon matched-pairs signed rank test, $p=0.0625$). Data points represent blots from independent differentiations, bars represent mean $\pm$ SEM. Blots from the same gel share the same point shape (squares, upright triangles, downward triangles, dots, and diamonds).

5.3.4. Cell Type-Dependent Lysosomal Dysfunction in PSEN1-Int4Del CNs

To further examine pathways that may be responsible for underlying the cellular response to PFF insult, I examined lysosomes, part of the autophagy pathway responsible for degrading ASyn. To examine lysosomes in neurons alone, and to confirm any effects were cell autonomous, these experiments were performed on live neurons in monoculture.

I first used LysoTracker™, which preferentially labels acidic organelles in live cells. LysoTracker™ signal was chloroquine dependent (Figure 5.13). Analysis of LysoTracker™ signal in this experiment, using a customised Harmony pipeline detailed in Figure 5.13D, showed that puncta number and intensity were significantly elevated in PSEN1-Int4Del CNs, compared to corrected control (Figure 5.13Gi,li). Further differentiations of this isogenic pair found that the effect of CQ treatment on LysoTracker™ intensity, number, and area were not different between genotypes, though the number of LysoTracker™+ puncta trended towards being elevated in PSEN1-Int4Del (Figure 5.14A).

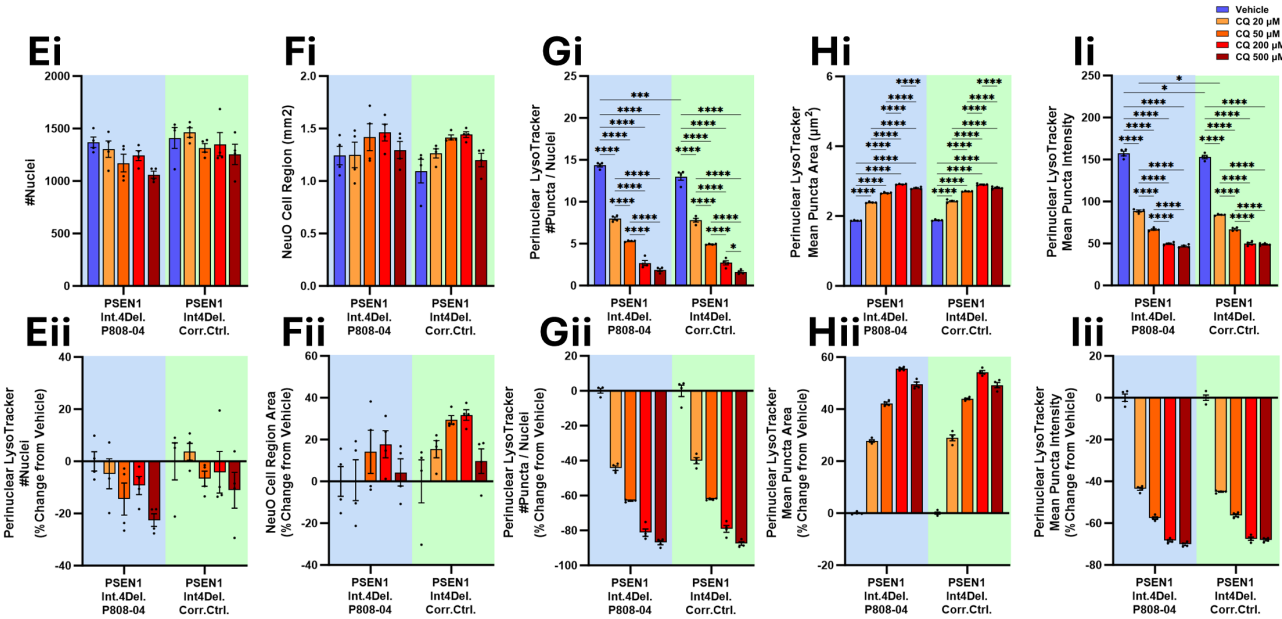
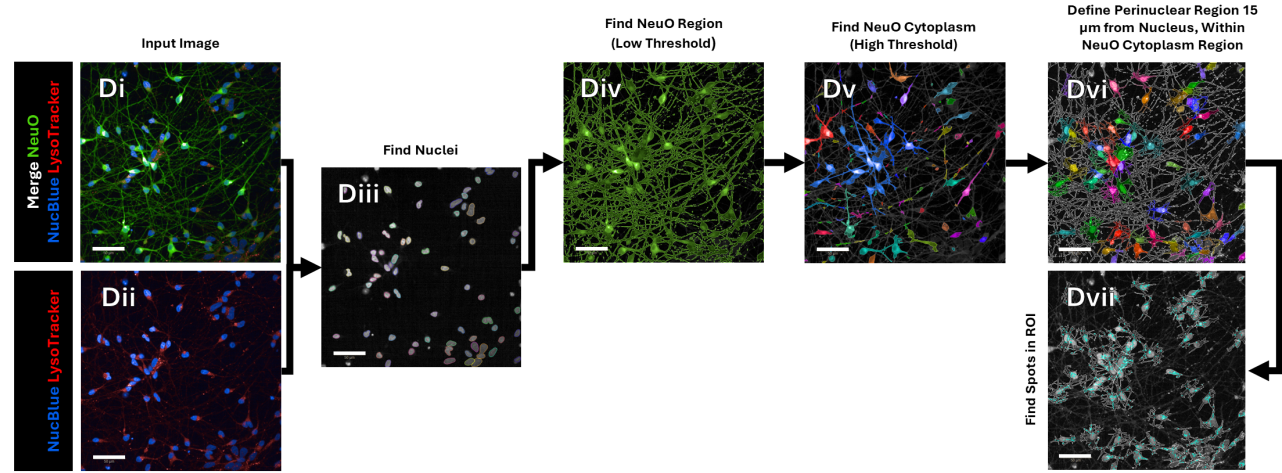
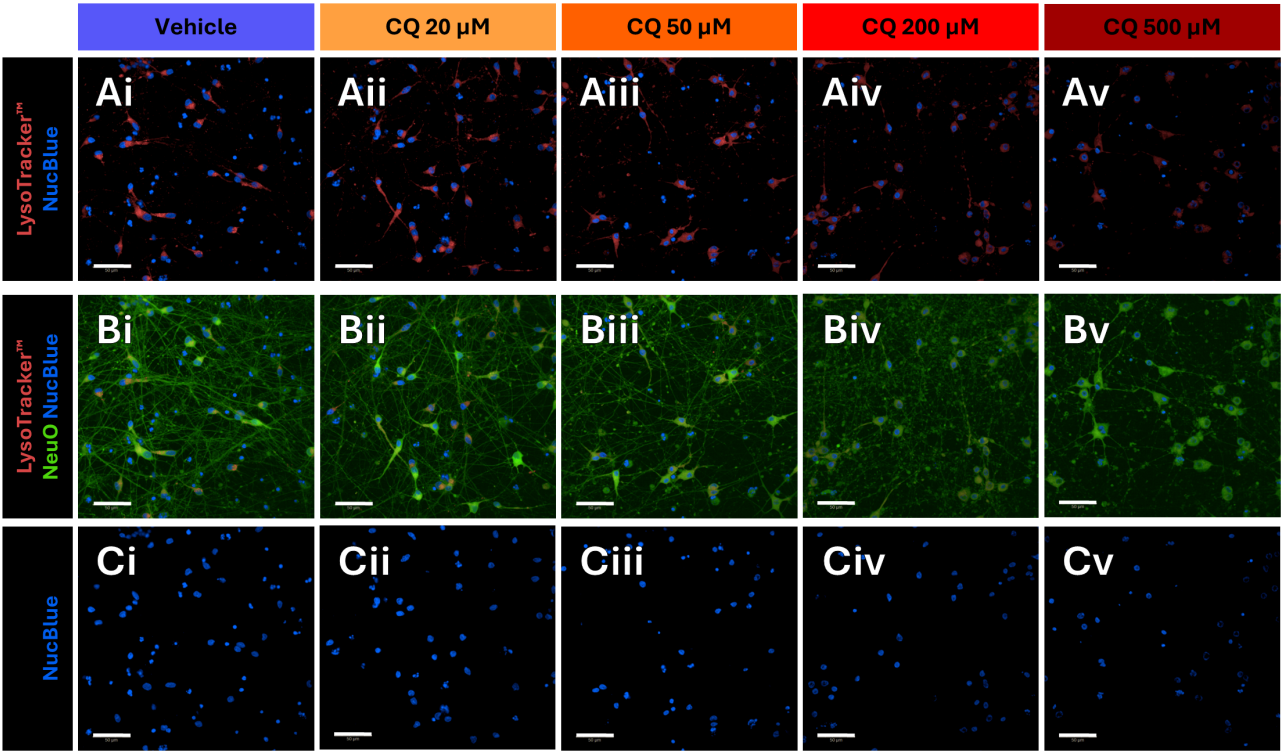


Figure 5.13. LysoTracker™ Deep Red Measures Lysosomes in Live Cells in a CQ-dependent Manner.

To visualise lysosomes in live cells in monoculture, cells were stained with lysosomal dye LysoTracker™ Deep Red, as well as neuronal cytoplasm dye NeuroFluor™ NeuO, and nuclear dye NucBlue™. **(A-C)** Representative live imaging of the three dyes staining cells pretreated with **(i)** vehicle, or **(ii-v)** chloroquine (CQ), at 20 μ M, 50 μ M, 200 μ M, and 500 μ M, for 24 hours. **(D)** Analysis pipeline for LysoTracker labelling. **(Di-ii)** shows an exemplar input image. **(Diii)** Find Nuclei Function method B was used to identify and count NucBlue™ nuclei. **(Div)** The Find Image Region function was used with a low threshold to define a NeuO neuronal ROI, including neurites, to create a 'whole cell' ROI, and the area of this was measured. **(Dv)** The Find Image Region function was used at a higher threshold to define a second NeuO ROI, capturing high intensity NeuO, typically around the cytoplasm and large neurites. **(Dvi)** This high threshold NeuO ROI was restricted to 15 μ m around nuclei, to create a neuronal cytoplasm ROI. **(Dvii)** LysoTracker™ puncta were also counted within this more restrictive, perinuclear ROI (blue spots). **(E-I)** Quantification of **(i)** raw values, and **(ii)** percentage change from vehicle, of **(E)** Number of nuclei, and **(F)** NeuO 'Total' cell area, which did not decline with CQ treatment, showing minimal toxicity; **(G)** the number of perinuclear lysotracker puncta, which declined with CQ treatment, and in vehicle treated cells, was significantly higher in PSEN1-Int4Del than in the corrected control; **(H)** the mean area of each LysoTracker™ punctum, which increased with CQ treatment; and **(I)** the mean intensity of LysoTracker™ puncta, which decreased with CQ treatment, and at vehicle and 20 μ M CQ treated conditions, was significantly higher in PSEN1-Int4Del than in the corrected control. Points represent individual wells from a single differentiation. Statistical comparisons are two-way ANOVA, with Bonferroni correction for multiple comparisons. Scale bars represent 50 μ m.

LysoTracker™ puncta number was therefore examined in both CNs and mDANs, in the PSEN1-Int4Del and corrected control (Figure 5.14B-C). Here, I observed a consistent elevation in LysoTracker™ puncta across multiple differentiations in PSEN1-Int4Del CNs relative to its corrected control, but not such difference in mDAN cultures. To confirm this, I sought to independently measure lysosomal levels by western blotting for LAMP1, a lysosomal protein (Figure 5.15). Here, as with LysoTracker™, LAMP1 levels in PSEN1-Int4Del CNs were consistently higher than the corrected control across many differentiations, though this same difference was not evident in mDAN cultures from the same isogenic pair.

To further examine lysosomal function and content, I assayed intracellular Ca^{2+} in this pair of lines in CNs (Figure 5.16A-B) and mDANs (Figure 5.16C-D), using a Fura-2 assay under basal conditions. The lysosome is an intracellular Ca^{2+} store, and Ca^{2+} release is essential for normal lysosomal functioning. Here, I used GPN to evoke Ca^{2+} release from lysosomes as a measure of lysosomal Ca^{2+} content (Figure 5.16A,C), and ionomycin to evoke Ca^{2+} release from the ER and other organelles, as a measure of cellular Ca^{2+} stores (Figure 5.16B,D). PSEN1-Int4Del CNs trended toward having higher lysosomal calcium release than the corrected control,

consistent with higher lysosomal content, whereas in mDAN cultures, they trended in the opposite direction. Interestingly, when using ionomycin to trigger Ca^{2+} release, PSEN1-Int4Del CNs also trended towards elevated Ca^{2+} release compared to the corrected control, while in mDANs the trend was again in the opposite direction.

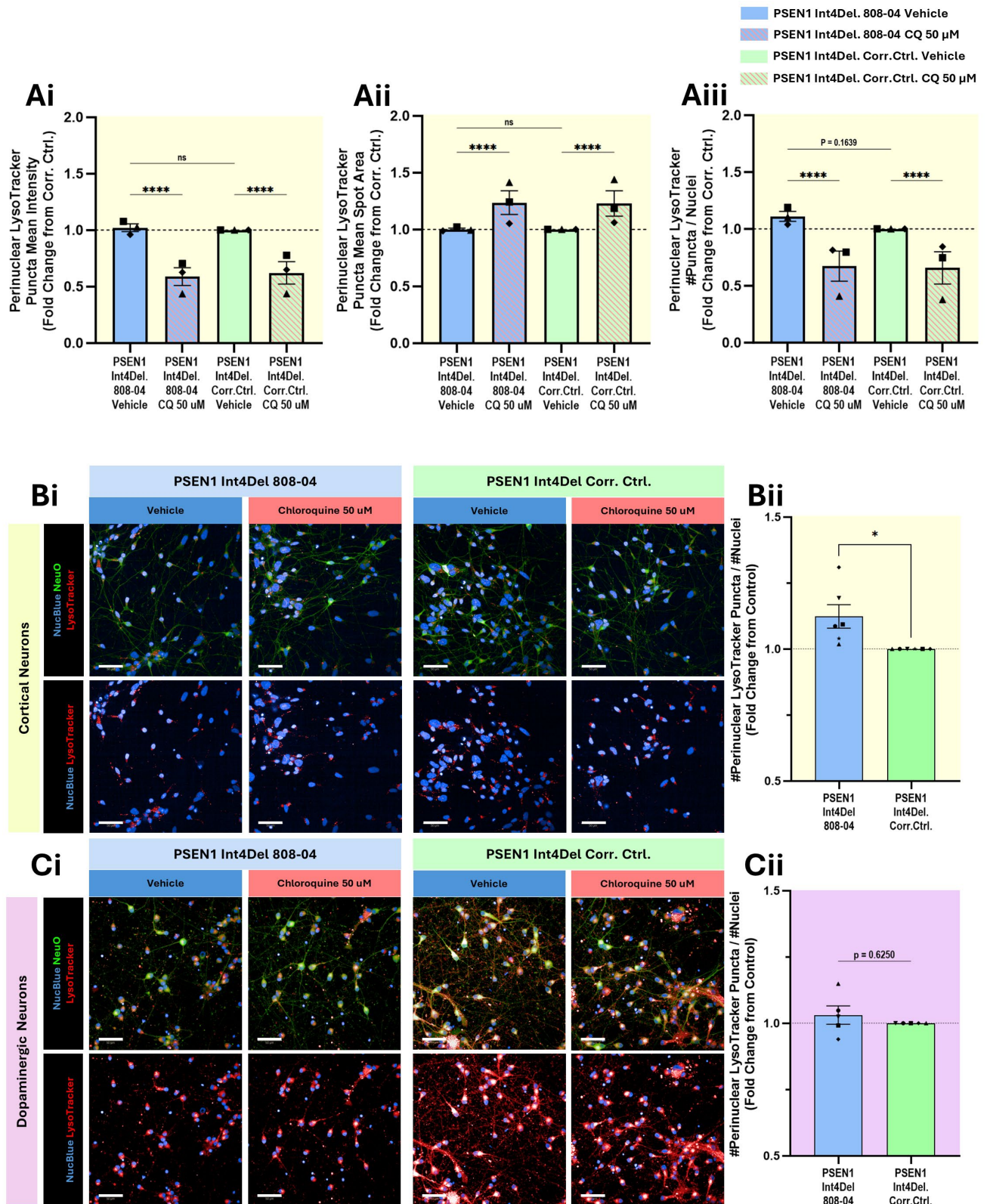


Figure 5.14. Live LysoTracker™ Imaging Shows PSEN1-Int4Del Neurons Have Higher Basal Lysosomal Content in Cortical Neurons, not Midbrain Dopaminergic Neurons. (A) LysoTracker puncta were analysed in D50 CNs grown in monoculture, pretreated with vehicle or 50 μ M chloroquine (CQ). CQ had a significant impact on (Ai) LysoTracker™ mean puncta intensity, (Aii) LysoTracker™ mean puncta area, and (Aiii) LysoTracker™ mean puncta number per cell. There was no significant difference

in the effect of CQ on the PSEN1-Int4Del cell line compared with its corrected control, or at baseline, though there was a trend towards elevated LysoTracker™ number in PSEN1-Int4Del. **(B-Ci)** Representative images of LysoTracker™ dye, alongside NeuO™ and NucBlue™ in **(B)** CNs, and **(C)** mDANs. The number of LysoTracker™ puncta was quantified in both CNs and mDANs. **(Bii)** The number of LysoTracker™ puncta per cell was significantly elevated in PSEN1-Int4Del cortical neurons compared to their corrected control, in basal conditions at D50. **(Cii)** The number of LysoTracker™ puncta per cell was not significantly different in PSEN1-Int4Del mDANs, compared to their corrected control, under basal conditions, at D36. For (A) Data points represent pairs of three independent differentiations, each with at least 4 wells per condition. Statistical comparisons are 2-way ANOVA. For (B-Cii) data points represent 6 or 5 pairs of independent differentiations respectively, each with at least 4 wells per cell line. Bars represent mean $\pm$ SEM. Statistical comparisons are Wilcoxon matched pairs signed rank test. Scale bars represent 50 μ m.

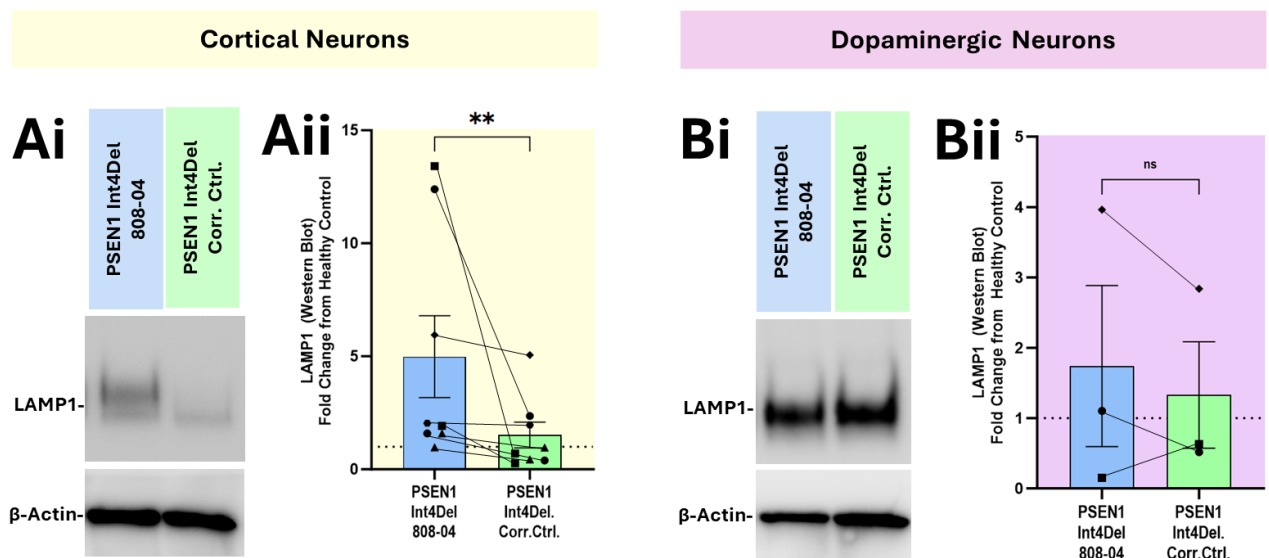


Figure 5.15. Western Blotting for Lysosomal Marker LAMP1 Shows PSEN1-Int4Del Neurons have Higher Basal Lysosomal Content in Cortical Neurons, not Midbrain Dopaminergic Neurons. Western blots were performed in PSEN1-Int4Del and corrected control monocultures of **(A)** CNs and **(B)** mDANs for lysosomal marker LAMP1. **(A-Bi)** show images of representative LAMP1 blots. **(Aii)** Quantification of LAMP1 blot intensity in CNs show significantly elevated levels of LAMP1 under basal conditions at D50. Data points represent 8 blots from 5 pairs independent differentiations. **(Bii)** Quantification of LAMP1 blot intensity in mDANs show no significant difference in LAMP1 levels under basal conditions at D36. Data points represent blots from 3 pairs independent differentiations. Data points in Aii and Bii are values normalised to β -actin and normalised to a healthy control, included in differentiation. Statistical comparisons are Wilcoxon matched pairs signed rank test. Bars represent mean $\pm$ SEM.

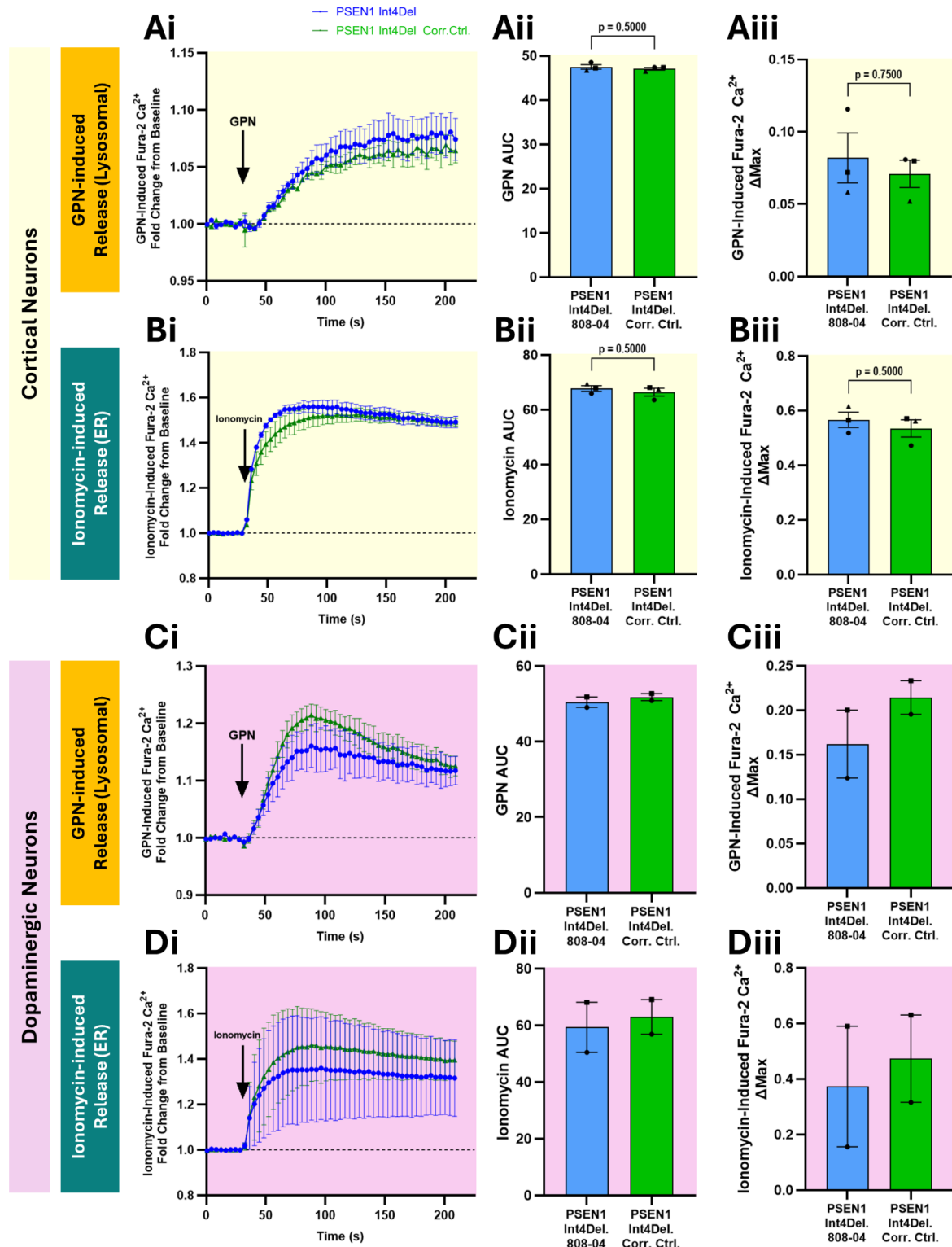


Figure 5.16. Evoked Lysosomal and ER Calcium Release Trends Towards Being Increased in PSEN1-Int4Del Cortical Neurons and Decreased in PSEN1-Int4Del Dopaminergic Neurons. Fura-2 assays of evoked Ca^{2+} release from (A,C) the lysosome, by GPN, or (B,D) the ER, by ionomycin, were conducted in (A-B) cortical neurons in monoculture at D50, and (C-D) mDANs in monoculture at D36. **(i)** Traces of Fura-2 ratio are presented as fold change from baseline, where baseline was the average of the first 8 measures before drug injection at 30 s. **(ii)** Area under the curve for each trace (arbitrary units). **(iii)** Maximum change in Fura-2 ratio from baseline. Data points represent 3 independent differentiations in CNs and 2 independent differentiations in mDANs, each with at least 8 wells per line, bars represent mean $\pm$ SEM. Statistical comparisons are Wilcoxon matched pairs signed rank test.

These data collectively point towards a higher lysosomal content in the PSEN1-Int4Del CNs under basal conditions than their corrected control, an effect of the mutation that does not appear to be present in the mDANs. This may explain why this pair of lines responds differently to the PFF insult as CNs, but are equally as vulnerable as mDANs. I therefore sought to better understand the functional significance to the autophagy-lysosomal pathway of the elevated lysosomal levels in the PSEN1-Int4Del CNs.

First, I used DQ™ BSA to examine lysosomal degradation. DQ™ BSA dextrans are self-quenching, and upon lysosomal degradation, begin to fluoresce. Thus, fluorescence intensity is a measure of lysosomal degradation. DQ™ signal was sensitive to CQ in both the PSEN1-Int4Del and corrected control CNs to a similar degree, however, there was no baseline difference in DQ™ intensity between PSEN1-Int4Del and control (Figure 5.17). Therefore, the PSEN1-Int4Del mutation did not appear to cause a deficit in lysosomal degradation.

I also assayed autophagic flux, to see if elevated lysosomal content represented a blockage of autophagosome-lysosome fusion. Chloroquine normally blocks autophagy through preventing autophagosome-lysosome fusion. However, if this flux is constitutively impaired, chloroquine should have a reduced effect (Rubinsztein et al. 2009; Klionsky et al. 2012; Jiménez-Moreno et al. 2017). Here, chloroquine treatment produced almost 2-fold higher levels of LC3b-II, an autophagosome marker, in the PSEN1-Int4Del corrected control, as compared to the CQ-treated mutant line. This implies that the PSEN1-Int4Del mutation leads to impaired autophagic flux in this cell line (Figure 5.18).

This blockage of autophagic flux may underly the elevated lysosomal content in the PSEN1-Int4Del CNs and may drive the selective vulnerability of the PSEN1-Int4Del cell line to the PFF insult, relative to other CNs.

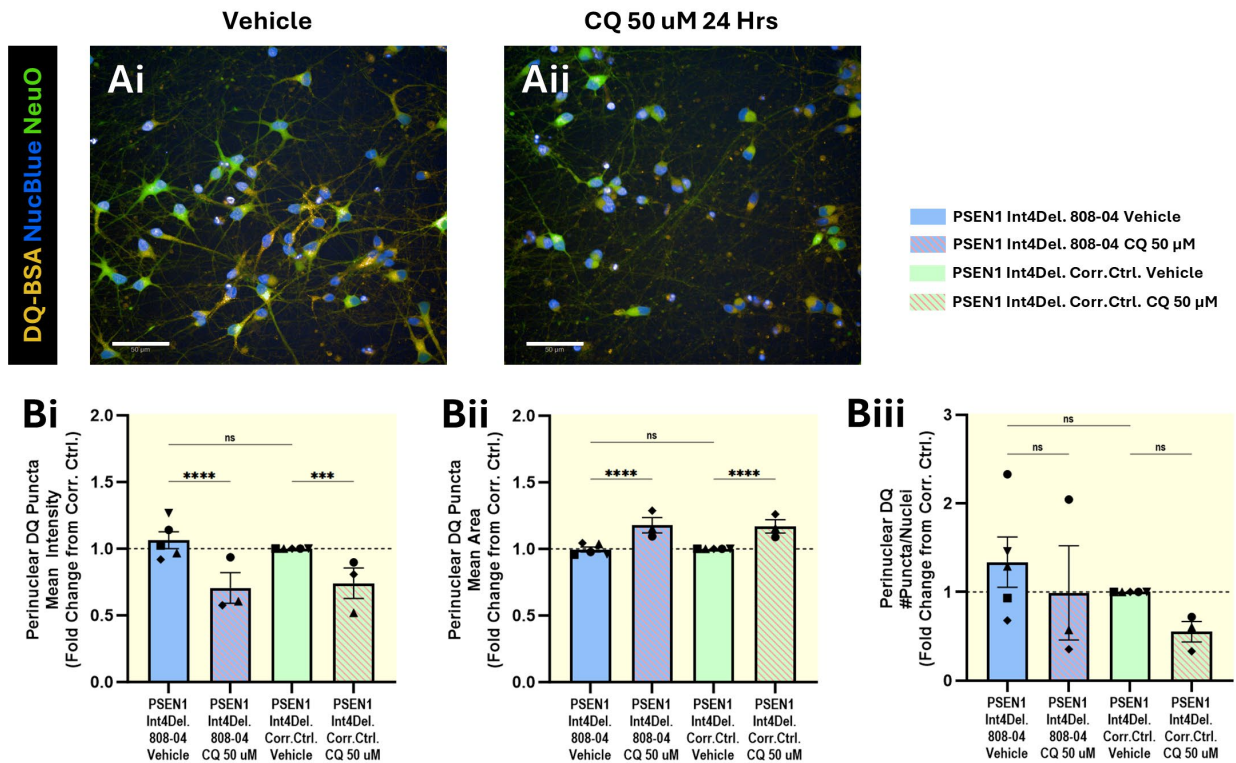


Figure 5.17. DQ™ BSA Assay Shows No Difference in Lysosomal Degradation Capacity in PSEN1-Int4Del Cortical Neurons. (A) Representative images of CNs at D50 grown in monoculture, labelled with NeuO™ and NucBlue™, and incubated with DQ™-BSA. CQ pretreatment for 24 hours reduced DQ™ BSA intensity, a measure of lysosomal degradation. (B) Images were analysed using a pipeline analogous to that for LysoTracker™. There was a significant effect of CQ on (Bi) DQ™ BSA puncta intensity, and (Bii) DQ™ BSA puncta area, but not on (Biii) the number of DQ™ BSA puncta per nuclei. There were no significant differences between genotypes, though there was a trend towards an increased number of DQ™ BSA puncta in PSEN1-Int4Del ($p=0.6922$). Data points represent five independent differentiations, each with at least 4 wells per condition, bars represent mean $\pm$ SEM. Statistical comparisons are two-way ANOVA.

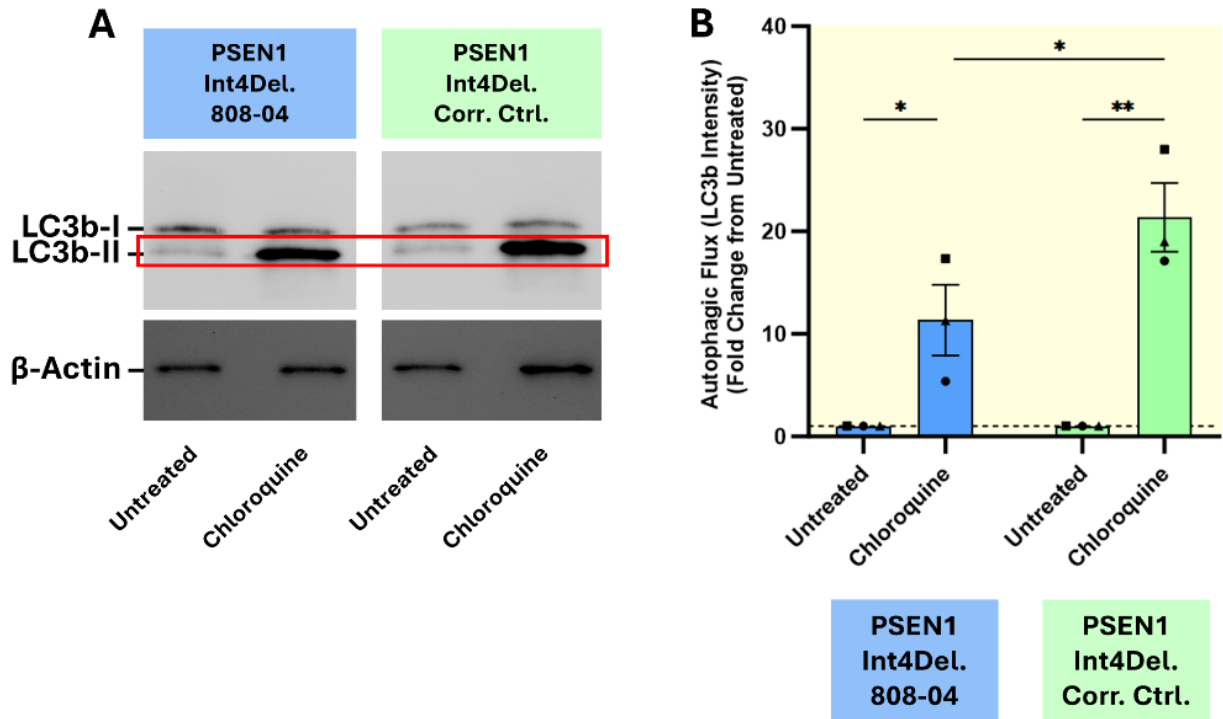


Figure 5.18. Autophagic Flux is Blocked in PSEN1-Int4Del Cortical Neurons. PSEN1-Int4Del CNs grown in monoculture were treated with 50 μ M chloroquine for 24 hours, then lysed for Western blotting at D50. **(A)** Representative blot showing autophagosome markers LC3b-I and LC3b-II, elevated in CQ treated conditions. **(B)** Quantification of LC3b-II Western blots from three independent differentiations shows a significantly greater effect of CQ on the corrected control CNs than the PSEN1-Int4Del CNs. Data points represent blots normalised to β -actin and to the untreated cells, from pairs of three independent differentiations, bars represent mean $\pm$ SEM. Statistical comparisons are two-way ANOVA.

5.4. Discussion

5.4.1. Results Summary

Whereas in the previous two chapters I examined CNs and mDANs individually, in this chapter, I sought to directly compare the responses of cortical and dopaminergic neurons, to gain insight into their differential vulnerability, and then to investigate what might underlie observed differences in cell type-based or genotype-based vulnerability. Side-by-side comparison of pASyn-S129+ labelling in CNs and mDANs clearly demonstrated that mDANs had a substantially faster and more severe aggregation response than CNs. Even the PSEN1-Int4Del neurons, which experienced substantial pathology as CNs, developed many fold more pathology as mDANs. The size of larger aggregates such as LNs and LBs was not substantially different between cell types, but they were much more numerous in mDANs. In addition to the difference in cell type-based vulnerability, it was also evident that the PSEN1-Int4Del mutation had a substantial effect on vulnerability in CNs, but not so in mDANs.

In searching for differences in cellular phenotype that might explain these, no difference in LRP1 expression was observed between cell types or genotypes, nor was there any difference in labelled ASyn-647 PFF uptake into CNs of differing vulnerabilities. Higher culture density led to more seeded aggregation, but endogenous ASyn expression only trended higher in mDANs and PSEN1-Int4Del CNs. However, PSEN1-Int4Del CNs had elevated LysoTracker™ puncta levels and LAMP1 protein levels, compared to corrected controls, while there was no effect of the mutation in mDANs. PSEN1-Int4Del CNs and mDANs also had opposing trends in ER and lysosomal Ca²⁺ release that were consistent with elevated lysosomal content selectively in CNs. PSEN1-Int4Del was not associated with impaired lysosomal degradation in CNs, but reduced autophagic flux was observed.

5.4.2. Cell-Type Based Preferential Vulnerability of Dopaminergic Neurons

A key collective outcome of PFF insult experiments Chapters 3-5 is the differential vulnerability CNs and mDANs derived from hiPSCs from the same individual. The cell type dependent differences in vulnerability to pASyn-S129+ pathology observed here concur with other PFF insult studies that examine both CNs and mDANs (Iannielli et al. 2022; Bayati et al. 2023). Here, however, this is achieved without *SNCA* overexpression, and without additional insults, in a wide range of genotypes, including AD and PD LRRK2 patient cells for the first time, as well as healthy controls. Importantly, this differential vulnerability also reflects that which is observed in the PD brain. mDANs, especially those in the SNPC, are more vulnerable to ASyn pathology in PD in terms of time of onset and degree of involvement than many other brain regions and especially the cortex, even in ‘brain-first’ patterns of LP (Braak et al. 2003; Surmeier, Obeso, and Halliday 2017b; Fu, Hardy, and Duff 2018; Javier Alegre-Abarrategui et al. 2019; Borghammer et al. 2021; Kampmann 2024).

The validity of direct comparisons between the hiPSC-derived CN and mDAN models, and the relationship to selective vulnerability observations in AD and PD neuropathology, will be further discussed in Chapter 7. The focus of the discussion in this chapter will be the hypothesised mechanisms underlying the observed differences in vulnerability.

The mean size of inclusions such as LNs and LBs was similar between mDANs and CNs, while the overall pathological load in mDANs was much higher. This suggests more aggregation seeding events, rather than formation of larger aggregates, was responsible for much of the cell type-dependent differences in pathology load. ASyn fibrillisation is nucleation dependent, and thus, more seeding opportunities should lead to more pathology (Wood et al. 1999). Since dose concentration and volume of insult were held constant between cell types, a higher frequency of seeding events might be due to greater PFF uptake into mDANs or might be due to a larger pool of endogenous ASyn available to be seeded.

LRP1 has recently been shown to be a receptor mediating not only ASyn PFF uptake in neurons, but also tau (Rauch et al. 2020; Chen et al. 2022). Higher LRP1 expression might therefore lead to more PFF uptake, and more seeding of pathology. LRP1 expression, however, did not differ between CNs and mDANs, and did not differ between genotypes. This does not rule out differences in LRP1 function, or the function of other uptake mechanisms though. Lymphocyte activation gene 3 (LAG3) has also been proposed to be a mediator of ASyn uptake, though a recent consensus paper has shown that it is not expressed in human or murine neurons (Emmenegger et al. 2021).

To circumvent the myriad other pathways that might mediate uptake and be differentially functional (Bieri, Gitler, and Brahic 2018; Gribaudo et al. 2019; Bayati et al. 2022), I used ASyn-647 labelled PFFs to examine if rates of PFF uptake were different in live CNs. No genotype dependent differences were observed here that might lead to the enhanced vulnerability of the PSEN1-Int4Del neurons, though this was not performed in mDANs, so cell type dependent differences in uptake remain an avenue of future investigation.

5.4.3. ASyn Levels as a Driver of Vulnerability

There is substantial precedent for the hypothesis that higher levels of endogenous ASyn might predispose cells to vulnerability to pathology. Elevating *SNCA* expression through copy number variation is causative for PD, and leads to greater vulnerability to PFF-seeded pASyn-S129+ pathology in cell models (Singleton et al. 2003; Byers et al. 2011; Zambon et al. 2019; Tanudjojo et al. 2021; Iannielli et al. 2022; Lam et al. 2024). Conversely, *SNCA*-KO, here and in other studies, abolishes any pASyn-S129+ aggregation response (Volpicelli-Daley, Luk, and Lee 2014; Polinski et al. 2018; Mahul-Mellier et al. 2020; Dening et al. 2022; Iannielli et al. 2022; Lam et al. 2024). Furthermore, insults in older neurons with more ASyn expression develop more pathology (Volpicelli-Daley et al. 2011; Tapias et al. 2017), while reducing free ASyn

monomers using the drug misglustat, or reducing expression using antisense oligonucleotide knock-down, leads to a protective effect in hiPSC and rodent models respectively (Kim et al. 2018; Luna et al. 2018). Indeed, as risk of PD and other synucleinopathies increases with age, so too does *SNCA* expression (Chu and Kordower 2007), and *SNCA* expression appears to be enriched in PD vulnerable cells in the brain (Taguchi et al. 2016). It would seem, thus, that greater amounts of ASyn, either by endogenous expression, or even by dose, leads to more pathology, likely on account of more nucleation (Wood et al. 1999; Volpicelli-Daley et al. 2011). However, western blotting for ASyn expression in the mDANs and CNs here, showed no significant differences between the two cell types, though some variation between differentiations. While ASyn levels trend higher in mDANs, this does not seem entirely sufficient to explain the stark difference in vulnerability. Moreover, examining for differences in ASyn expression levels between CN genotypes again found variation but no significant differences or clear pattern that might explain the selective vulnerability of the PSEN1-Int4Del cell lines, compared not just to the corrected control but also to other disease and healthy genotypes.

When comparing between cell types by western blot, the same 10 µg amount of protein was loaded from each cell type. Thus, more properly, the protein concentration of ASyn in mDANs and CNs was not significantly different. However, since mDANs were cultured at much higher density, they likely have much higher absolute levels of protein, and thus more absolute levels of ASyn available for seeding. Indeed, increasing the culture density of CNs led to a 'cell density dose'-dependent rise in pathological load in response to PFF insult.

It is not certain whether increased culture density drives vulnerability through a higher absolute level of free, available, endogenous ASyn. For example, other mechanisms such as increased synaptic activity, different maturation rates, or enhanced ability to spread might be at play instead, or additionally. However, many other such mechanisms would likely also lead to

higher levels of ASyn (e.g. greater synaptic density), and this seems the most parsimonious conclusion.

Differences in culture density here are not entirely a mere artefact of the *in vitro* model. mDANs are seeded in high numbers by necessity, since differentiations collapse at lower densities, likely reflecting the developmental conditions in the ventral midbrain, especially the densely populated substantia nigra *pars compacta* (Cui et al. 2016; Luo et al. 2020). The dense arrangement of cells in the mDAN culture, relative to the CN model, could be considered as reflective of the relative densities of cells growing in different parts of the brain, though this is difficult to compare directly. mDANs, with their highly arborised axons, varicosities, and enormous synaptic load, are also likely to have a high endogenous expression of ASyn, since it plays a crucial role in vesicle dynamics in the presynaptic terminal (Matsuda et al. 2009; Bolam and Pissadaki 2012; Burré, Sharma, and Südhof 2015).

While these experiments thus show a role for high available ASyn levels in enhancing the vulnerability of the mDAN model, this is unlikely to be the only driver of the cell-type dependent differences. Dopamine production, for example, is thought to be relatively neurotoxic, owing to the associated production of free radicals (Alegre-Abarrategui et al. 2019; Evans, Defensor, and Shamloo 2022). Oxidative stress and mitochondrial dysfunction are thus frequently observed in mDAN cell models (Deas et al. 2016; Tapias et al. 2017; Zambon et al. 2019), even specifically in TH⁺ cells (Fernandes et al. 2016). While these mechanisms were not investigated here, I did find that TH⁺ cells were substantially more vulnerable than TH⁻ cells, suggesting that TH expression itself plays a role in vulnerability. Comparisons of the CNs and mDANs in this regard might also therefore be an interesting avenue for further research.

ASyn levels in CNs also cannot be the sole driver of the selective vulnerability of the PSEN1-Int4Del cell lines, since these do not have substantially different ASyn protein concentrations to other more resilient cell lines and did not have significantly different neuronal densities from

other cell lines in the aggregation experiments. Thus, autophagy was investigated as a mechanism driving the selective vulnerability of PSEN1-Int4Del CNs.

5.4.4. Autophagy Deficits Drive Vulnerability of PSEN1-Int4Del Cortical Neurons

There is a similarly strong precedent for examining autophagy deficits here, since they relate to both fAD mutations, and vulnerability to ASyn PFFs. Autophagy and the endolysosomal system are implicated in AD GWAS and has been shown to be disordered in AD (Nixon and Yang 2011; Nixon 2013; Cai and Ganesan 2022; Szabo et al. 2022; Udayar et al. 2022; Yamamoto, Zhang, and Mizushima 2023). Furthermore, autophagy and endosomal deficits, including impaired autophagic flux and suppressed autophagosome formation, have been observed in PSEN1 and other fAD models, including hiPSC CNs (Cataldo et al. 2000; Bordi et al. 2016; Chong et al. 2018; Hung and Livesey 2018; Kwart et al. 2019). This pathway is therefore clearly relevant to the disease and model, though has not been compared between different cell types.

The autophagy and endolysosomal systems have also been shown to be crucial for ASyn degradation. Internalised ASyn is targeted to the lysosome, and autophagy is one of the main ways it is degraded (Cuervo et al. 2004; Lee et al. 2008; Madureira, Connor-Robson, and Wade-Martins 2020; Sanyal et al. 2024). Modulation of autophagy, either through KO or KD of autophagy genes like DJ-1 or lysosomal cathepsins, or through small molecule modulators like chloroquine and rapamycin, all modulate PFF-seeded ASyn pathology (Desplats et al. 2009; Mazzulli, Zunke, Tsunemi, et al. 2016b; Gao et al. 2019; Tanudjojo et al. 2021; Kim et al. 2023; Jones-Tabah et al. 2024). Experiments in earlier chapters in this thesis have also shown that

chloroquine enhances vulnerability to pASyn-S129+ pathology accumulation, though typically not in PSEN1-Int4Del neurons.

At the centre of these autophagy and endosomal degradation systems is the lysosome. I found several lines of evidence converging on lysosomal dysfunction in the PSEN1-Int4Del CNs. Firstly, the number of puncta labelled with LysoTracker™ dye, which marks lysosomes and other acidic organelles (such as late endosomes), was significantly elevated compared to corrected controls. Secondly, levels of LAMP1, a lysosomal marker, were significantly elevated compared to corrected controls. Thirdly, both GPN-induced lysosomal calcium release and DQ™ puncta number, consistent with elevated lysosomal content, trended towards elevation compared to corrected controls. These experiments collectively paint a picture of lysosomal build up in CNs because of the PSEN1-Int4Del mutation.

Interestingly, these findings contrast with observations by Hung and Livesey (2018). There, they find enlarged early endosomes marked by RAB5A-GFP and greater LAMP1 western blot levels in APP, not PSEN1 cell lines, including PSEN1-Int4Del CNs. They also observe enlarged and more numerous LAMP1-RFP puncta in APP and PSEN1 genotypes, but not in PSEN1-Int4Del, which they thus do not include for the remainder of their experiments examining lysosomal transport and autophagic flux. It is unclear how to account for these differences, considering it is likely that the same STEMbancc cell line was employed. It is possible that the use of the Shi et al. (2012)-based protocol alone in their study, in contrast to the blend with NGN2 differentiation here, leads to a slightly different neuronal phenotype. It is also possible that the expression of the LAMP1-RFP probe, in contrast to LysoTracker™ dye, alters the lysosome. However, the alignment of multiple independent measures across many differentiations here, in addition to the comparison to a isogenic corrected control, not used by Hung and Livesey (2018), make the phenotype presented here convincing.

Furthermore, in line with these observations of lysosomal perturbation in PSEN1-Int4Del CNs is the impairment of autophagic flux. A blockage of autophagosome and lysosomal fusion could lead to a build-up of lysosomal cargo. Here, chloroquine is used to block autophagic flux, rather than bafilomycin A1, which is typically used (Rubinsztein et al. 2009; Hung and Livesey 2018; Lam et al. 2024). Bafilomycin A1 blocks autophagic flux through inhibition of v-ATPase mediated acidification of the lysosome, leading to lysosomal alkalinisation and degradative impairment (Mauvezin and Neufeld 2015; Mauthe et al. 2018). By contrast, chloroquine blocks flux by specifically impairing lysosomal-autophagosome fusion, and leading to a build-up of both autophagosomes and lysosomes (Mauthe et al. 2018). The use of chloroquine to measure flux here specifically implicates fusion, rather than pH impediments resulting from the PSEN1-Int4Del mutation.

The impairment in autophagic flux here also corresponds to observations from earlier chapters that chloroquine treatment alongside PFF insult led to increased pathological burden in PSEN1-Int4Del corrected control CNs, and PSEN1-M146I CNs, but not PSEN1-Int4Del CNs. This implies that the constitutive deficit in autophagosome-lysosome fusion resulting from the PSEN1-Int4Del mutation was driving the high pathological burden in these cells, such that additional blockade with chloroquine had minimal additive effect, as it did in the corrected control.

Interestingly, the measures of pathology most sensitive to chloroquine treatment, i.e. impaired autophagic flux, as a mechanism driving vulnerability, were the formation of LNs and LBs. In CNs, overall punctate pathology burden following chloroquine treatment was not substantially different between PSEN1-Int4Del and the corrected control, but there was a two-fold difference in LB seeding and approximately 10-fold difference in LN seeding. It is therefore perhaps unsurprising that here, in comparing the vulnerability of CNs and mDANs to various measures of pathology, the difference between PSEN1-Int4Del CNs and mDANs is smallest,

and stays equivalent for longest, for measures of LN and LB pathology, rather than overall pASyn-S129+ puncta. i.e. the PSEN1-Int4Del CNs, whose selective vulnerability appears to be driven by impaired autophagic flux, is most profoundly vulnerable, relative to mDANs, to those pathologies most sensitive to impairment of autophagic flux. This also further implicates autophagy and defective flux in the process of LP formation, in accord with other studies (Bayati et al. 2023).

5.4.5. PSEN1-Int4Del Autophagy Deficits are Cell Type-Dependent

Crucially for the investigation here, these lysosomal abnormalities in the PSEN1-Int4Del cell line appear to be specific to CNs. Neither LysoTracker™ puncta nor LAMP1 western blotting levels were elevated in PSEN1-Int4Del mDANs, and lysosomal Ca^{2+} release trended in the opposite direction to CNs. This lack of difference in autophagic function between the PSEN1-Int4Del and its corrected control in the mDANs corresponds to their equal vulnerability to ASyn PFF-seeded pASyn-S129+ pathology. Furthermore, while autophagic flux was not directly measured by western blotting in the mDANs, chloroquine treatment alongside PFF insult in the mDANs had an equal effect on both the mutation and corrected control line, with regards to resultant accumulation of pASyn-S129+ pathology. Therefore, the PSEN1-Int4Del mutation drives a cell type specific impairment of autophagic flux in CNs, leading to the selective vulnerability of these lines to PFF-seeded pASyn-S129+ pathology, while there is no difference between the isogenic pair in mDANs, whose vulnerability is likely driven by ASyn expression levels and the expression of dopaminergic machinery. Interestingly, CQ treatment had an equal yet minimal impact on pASyn-S129+ accumulation in the PSEN1-Int4Del and corrected control mDANs, suggesting that while there was no effect of the mutation on autophagic flux in the mDANs, impaired autophagic flux in both lines in the mDANs may also contribute to their vulnerability.

The observations of impaired autophagosome-lysosome fusion do not, however, rule out other mechanisms of autophagic dysfunction that could be at play. While I do not observe impairment of lysosomal degradation by DQ™ BSA assay, it is unclear what lysosomal enzymes are specifically responsible for degradation of DQ™ dextrans, how these map to the enzymes responsible for degrading ASyn fibrils, and what kind of compensatory mechanisms might be at play to maintain degradation in such a way that DQ™ degradation is unimpaired, but ASyn degradation is. For example, some studies have shown that Cathepsin D, a crucial lysosomal hydrolase for degrading ASyn and other protein aggregates, becomes impaired in fAD models, and is competitively inhibited by Aβ42 (Hung and Livesey 2018; Terron et al. 2023). PSEN1-Int4Del cell lines, and other fAD lines, have been shown to have elevated release of Aβ42 (Arber et al. 2019; 2020), and increased Aβ42:40 ratios have been shown to be specific to forebrain neurons, rather than hindbrain neurons, in a different fAD hiPSC model (Muratore et al. 2017). It is thus possible that the PSEN1-Int4Del neurons impair lysosomal degradation of ASyn PFFs through inhibition of cathepsin D by elevated release of Aβ42 selectively in CNs, not mDANs.

PSEN1-Int4Del mutations may also impair autophagy through disruption of lysosomal pH. Several studies have proposed a role for PSEN1 in lysosomal acidification, through targeting of the V0a1 subunit of the vATPase pump from the ER to the lysosome (Lee et al. 2010; Lie et al. 2022; Mustaly-Kalimi et al. 2022). These are based on PSEN1-KO in mice or fAD mutations in hiPSC neurons respectively, leading to loss of PSEN1 function, and are in line with other studies showing PSEN1 loss-of-function related dysregulation of ER, lysosome, and intracellular Ca²⁺ (Nelson et al. 2007; 2010; Bezprozvanny 2013; Lie et al. 2022). These studies collectively suggest that PSEN1 acts as a Ca²⁺ leak channel on the ER, and loss of function leads to elevated ER Ca²⁺ and excessive release.

Here, I show that ER calcium in PSEN1-Int4Del CNs trends towards being significantly elevated, compared to corrected controls. It is possible that the Int4Del mutation, which leads

to two PSEN1 truncation transcripts (De Jonghe et al. 1999), leads to a partial loss of PSEN1 function, subsequent ER Ca^{2+} dysfunction, and further downstream effects on lysosomal pH and Ca^{2+} , both of which are essential to normal lysosomal functioning. Lysosomal pH is not examined directly here. LysoTracker™ is thought to be an indirect measure of pH, since it preferentially targets acidic organelles and loses fluorescence above pH 6.5, though it is not strictly a pH sensor and does not correlate with lysosomal pH (Mauthe et al. 2018). However, the elevated presence of LysoTracker™ puncta and lack of difference in intensity does rule out severe pH deficits in the PSEN1-Int4Del CNs. Lysosomal Ca^{2+} was assessed here, and, as with ER Ca^{2+} , trended towards being elevated in PSEN1-Int4Del CNs, compared to corrected controls. It is not clear whether this may be due to elevated lysosomal content generally, or because Ca^{2+} release from lysosomes is impaired, though either would be consistent with blocked autophagic flux. While these Ca^{2+} assays only showed trends when averaged across differentiations, the large number of wells employed per differentiation (at least 9 per plate), meant that individual differentiations comparing wells as Ns showed significant differences, at least for ionomycin-induced release in CNs.

Importantly, the trends in Ca^{2+} release were also cell-type specific, being reversed in mDANs. This is consistent with all other observations of lysosomal perturbations in the PSEN1-Int4Del neurons. While the association of lysosomal, autophagy, and calcium impairment with AD and PSEN1 mutations is not unexpected, the cell type specific manifestation of these deficits is greatly interesting, and highlights this as a likely mechanism driving the selective vulnerability of these neurons to ASyn pathology.

5.4.6. Limitations and Conclusions

One of the limitations of the live assays (such as LysoTracker™ and DQ™ BSA) and bulk assays (such as western blotting and Fura-2 Ca^{2+} imaging) performed here is that individual cell types

within mixed cultures cannot be distinguished. This less of an issue for CN cultures, which are relatively homogenous and grown in monoculture for these assays. However, for the mDANs, these assays examine both TH⁺ and TH⁻ neurons indiscriminately. These cells have already been observed here to be differentially vulnerable to pASyn-S129⁺ pathology, and elsewhere, to autophagic dysfunction (Fernandes et al. 2016; Lang et al. 2019). Thus, inclusion of the relatively resilient TH⁻ cells here may mask some of the vulnerability of the TH⁺ cells.

Nevertheless, the use of monoculture assays, alongside experiments showing that rAstros do not express ASyn, strengthen the case that cell autonomous phenotypes intrinsic to neurons are driving the differential vulnerability observed here.

As such, the pathology seeded by the ASyn PFF model in the previous two chapters is here observed to occur in a cell type-specific manner, matching patterns of vulnerability as might be predicted from brain pathology. This is likely due to increased levels of ASyn in the culture, and other intrinsic properties of TH⁺ neurons driven by their dopaminergic machinery. The selective vulnerability of the PSEN1-Int4Del CNs, meanwhile, may be explained by mutation specific and cell type-dependent impairment of autophagic flux, associated with elevated lysosomal content and dysregulated calcium. These deficits in basal conditions are consistent with observations of differential levels of pathology and the effect of chloroquine on pathology. Importantly, these effects of the PSEN1-Int4Del mutation were cell type-specific, highlighting how differential vulnerability may arise in different cell types, and illustrating the utility of the selective vulnerability framework.

While these three chapters have thus examined cell type dependent differences in vulnerability to pathology, the differential effect on cellular function of the PFF insult on different genotypes, and the impact of the different disease genotypes themselves on cellular function, will be investigated further in the next chapter, before a final discussion.

Chapter 6.

Cortical Synapses & Neurite

Outgrowth

6.1. Introduction

In the previous chapters, I examined the vulnerability of CNs and mDANs to the accumulation of pASyn-S129+ inclusions seeded by ASyn PFFs and sought to understand the cellular phenotypes that predispose different cell types and genotypes to this pathology. However, the development of protein aggregates is not the only form of pathological change or dysfunction that occurs in the degenerating brain. Furthermore, while the development of proteinaceous inclusions is indicative of proteostatic dysfunction in the cell, the functional effects of this have not yet been examined.

Synapse loss is a feature of AD and PD and regarded as the best correlate of cognitive decline (DeKosky and Scheff 1990; Terry et al. 1991; Bellucci et al. 2016). A meta-analysis of postmortem studies reporting synapse loss in AD found that patterns of loss across the hippocampus, cingulate, entorhinal, temporal, and frontal cortices matched patterns of vulnerability to cell loss and protein aggregation (de Wilde et al. 2016). Presynaptic dysfunction also appears to be a feature of dopaminergic neuron terminals in the PD striatum and DLB cortex, both related to and in advance of ASyn aggregation (Schulz-Schaeffer 2010; Janezic et al. 2013; Chou et al. 2014; Beccano-Kelly et al. 2015; Cramb et al. 2023).

In cell models, A β , tau, and ASyn aggregates have been shown to cause synaptic dysfunction and loss in rodent models (Volpicelli-Daley et al. 2011; Tapias et al. 2017; Roux et al. 2020; Stoyka et al. 2021) and hiPSC CNs (Usenovic et al. 2015; Prots et al. 2018; Gribaudo et al. 2019; Ng et al. 2022; Stephens et al. 2023; Kapucu et al. 2024). ApoE4 status also led to worse ASyn accumulation and synapse loss in a *Snca* overexpression rodent model (Zhao et al. 2020). Therefore, I sought to also examine the effect of the ASyn PFF insult on synapses here, focusing on CNs, since synapses within mDAN cultures are not as disease relevant.

A key advantage of the hiPSC neuronal model, however, is the ability to investigate the effects of disease related insults on live cell functions. In previous chapters, PFFs were noted to have

a neurodegenerative effect on neuronal area, with a potentially pronounced effect on dendrites and axons. Here, I therefore sought to further examine neurite outgrowth and whether this neurodegenerative effect was a cell autonomous effect in the neurons.

Neurite outgrowth has rarely been measured in cell models with ASyn PFF insult, though Zhao et al. (2020) find reduced MAP2+ neurites in hiPSC-derived neurons treated with ASyn aggregates amplified from brain homogenates. PSEN1 may play an important role in neurogenesis and neurite outgrowth through Notch1 signalling (Arber et al. 2021; Hernández-Sapiéns et al. 2022; Hurley et al. 2023), though the effect of PSEN1 mutations on neurite outgrowth has only rarely been characterised (Pigino et al. 2001).

By contrast, LRRK2 plays a clear role in cytoskeletal regulation (Cookson 2012; Civiero et al. 2018; Mancini et al. 2020). LRRK2 overexpression and G2019S mutations have frequently been associated with neurite outgrowth impairments, while LRRK2-KO is associated with neurite elongation in SH-SY5Y cells (Plowey et al. 2008), PC12 cells (Heo, Kim, and Seol 2010) rodent cell models (Parisiadou et al. 2009; Winner et al. 2011; Sloan, Alegre-Abarategui, and Wade-Martins 2012; Sepulveda et al. 2013), dopaminergic neurons derived from mice brains (Ramonet et al. 2011) and hiPSC-derived dopamine neurons (Sánchez-Danés et al. 2012; Reinhardt et al. 2013; Korecka et al. 2019), whereas hiPSC-derived sensory neurons displayed normal neurite length (Schwab and Ebert 2015). Neurite outgrowth has not been as extensively assessed in LRRK2-R1441C models, though a scratch assay showed neurite regrowth was impaired in both LRRK2-G2019S and R1441C hiPSC-derived mDANs (Connor-Robson et al. 2019), and other LRRK2 mutations have additionally been associated with impaired outgrowth in hiPSC-derived mDANs (Luo et al. 2020).

While this thoroughly implicates LRRK2 and fPD related mutations in neurite outgrowth deficits in vulnerable cell types in PD, hiPSC-derived CNs, which might be more resilient, have not been examined for neurite outgrowth deficits in PD cell lines.

Therefore, in this chapter, to examine functional effects of mutations and PFF insult, and confirm cell autonomous effects, I sought to

- 1) Characterise the effects of ASyn PFF insult on synaptic density in CNs,
- 2) Characterise the effects of AD PSEN1 and PD LRRK2 mutations on neurite outgrowth in monocultured CNs,
- 3) Characterise the effects of ASyn PFF insult on neurite outgrowth in monocultured CNs with AD PSEN1 and PD LRRK2 mutations, and
- 4) Examine differential vulnerability between AD and PD genotypes in the CNs

6.2. Methods

6.2.1. Synaptic Density Assay

The approach for examining synaptic density in the hiPSC CN cultures was adapted from Ng et al. (2022) for ASyn PFF insults here. CNs were insulted with ASyn PFFs at D70 and fixed up to 20 days later, as described in previous chapters, although fixation was conducted for 5 minutes rather than the full 20 minutes for other markers. ICC for pre and post synaptic markers was then conducted as described in Chapter 2, with permeabilisation conducted with 0.05% saponin in TBS for 20 minutes, to help preserve delicate structures. Analysis methods for measuring synaptic density are described in the results section in Figure 6.1.

6.2.2. Neurite Outgrowth Assay

6.2.2.1. GFP Transduction and Cell Culture

The approach for examining neurite outgrowth in the hiPSC CN cultures was adapted from Ng et al. (2024), with some modifications. The principle of the assay is to express GFP in a subset of cells to clearly visualise their outgrowth in live culture imaging, with subsequent automated analysis using custom pipelines in Harmony software. To achieve this, NPCs thawed at D25 were transfected with a low titre of lentivirus which expressed NGN2 and GFP, alongside NGN2 and rtTA lentiviruses for 8 hrs, as per the usual protocol. 0.25 μ L of the NGN2-GFP virus was added per well (i.e. per 500K NPCs thawed), such that ~1% of neurons expressed GFP, at the time of replating on D33. The remaining differentiations steps remain identical to the protocol described in Chapter 2, and cells were replated on to PO/L-coated 96-well imaging plates, to enable analysis of phenotypes absent influence from rAstros.

ASyn PFF insult was conducted the day after replating on D34, and cells were imaged immediately after insult. This was D0 of neurite tracing. Cells were subsequently imaged every 4 days, immediately before half media changes, for 16 days (D50).

6.2.2.2. Live Neurite Imaging and Analysis

Live imaging was conducted on the Opera Phenix™ at 37 °C and 5% CO₂. Using a 20x water objective, the whole well was imaged, though excluding the well edge to avoid spurious signal. An overlap of 1% between fields of view was used to ensure continuity across the image for neurite tracing. Image analysis was conducted on Harmony software with a custom pipeline. GFP signal was used to identify nuclei (Method B), alongside additional morphological filters (circularity >0.25, length > 10 µm, width:length ratio >0.02) to remove growth cones and other false positives. The Find Neurites function was then used to identify neurites associated with the nuclei. Since imaging here was conducted for substantially longer than in Ng et al. (2024), such that cells eventually grew substantially overlapping neurites and individual arbours could no longer be distinguished, I focused on a measure of total neurite length normalised to the number of nuclei, as a measure of total mean neurite length per cell measured. This is also the most commonly reported metric in other studies (Civiero et al. 2018). Typically, 15-30 GFP+ cells were measured per well. Wells with less than 5 cells were excluded from analysis.

6.3. Results

6.3.1. Synaptic Density Assays

I quantified synaptic density in CN cultures using immunolabelling of synapsin I/II, a presynaptic marker, and Homer1, a postsynaptic marker, as per Figure 6.1. These puncta were counted within an expanded MAP2 neuronal ROI, and juxtaposed puncta were counted as synapses. Synaptic density was assessed under basal conditions in a selection of cell lines from different AD and PD genotypes at multiple timepoints (Figure 6.2), and appeared to increase at a similar rate over time.

A 5 µg/mL PFF insult at D70 resulted in ~50% loss of synaptic density in all genotypes examined by 10 days after insult (Figure 6.3Ai), which was similarly depressed, if not slightly recovered by 15-20 days after insult (Figure 6.3Bi). Interestingly, most of the synaptic density loss was substantially driven by a loss of synapsin I/II puncta, rather than Homer1, which trended towards being more depressed at the later timepoints (Figure 6.3).

While there was thus an interestingly acute (pre)synaptic toxicity of ASyn PFFs in the CNs, there did not appear to be any differential vulnerability between genotypes, with all cell lines similarly affected.

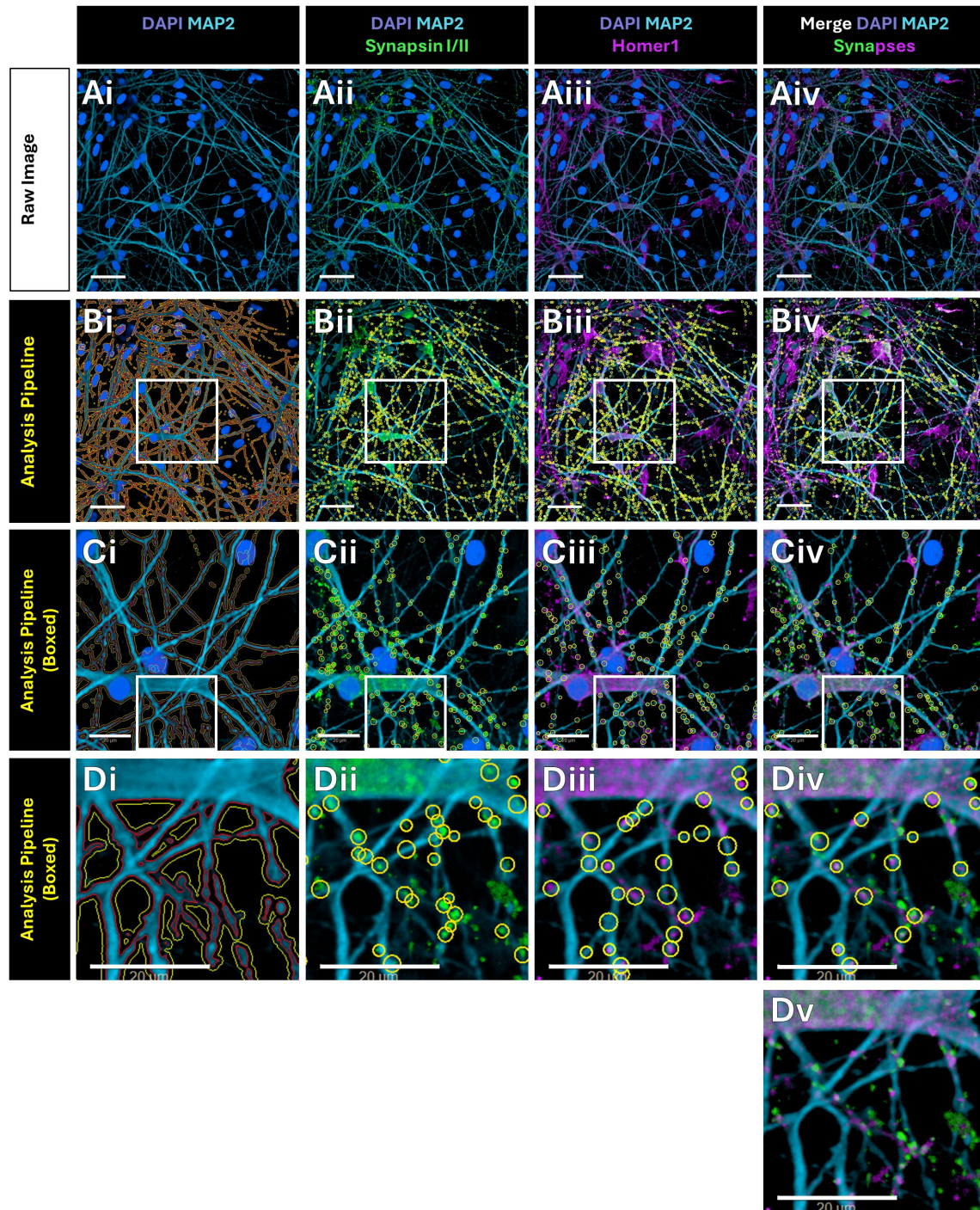


Figure 6.1. Synaptic Density Analysis in Cortical Neurons. (A) Cortical neurons were labelled with (i) MAP2, (ii) presynaptic marker synapsin I/II, and (iii) postsynaptic marker homer1. (iv) Merge image of MAP2, and apposed pre and postsynaptic markers. (B–D) Harmony analysis pipeline for counting synapses. (i) MAP2 area ROI was defined using the Find Image Region function with common threshold (red lines) and expanded 1.5 μm (yellow lines) to be able to capture presynaptic markers on the ‘surface’ of MAP2 labelling. (ii) Presynaptic synapsin I/II puncta were identified (yellow circles) and counted in the expanded MAP2 ROI using the Find Spots function with common threshold. (iii) Postsynaptic homer1 puncta were identified (yellow circles) and counted in the expanded MAP2 ROI using the Find Spots function with common threshold. (iv) Synapsin I/II puncta boundaries were expanded by 1.5 μm , and homer1 puncta that had >50% overlap with expanded synapsin I/II puncta were counted as synapses (yellow circles). This captured synapsin I/II and homer1 puncta in apposition. Synaptic density was calculated as the number of apposed puncta per MAP2 area. (Dv) Boxed boxed region shown without analysis pipeline markers. Scale bars are 50 μm for (A–B), and 20 μm for (C–D).

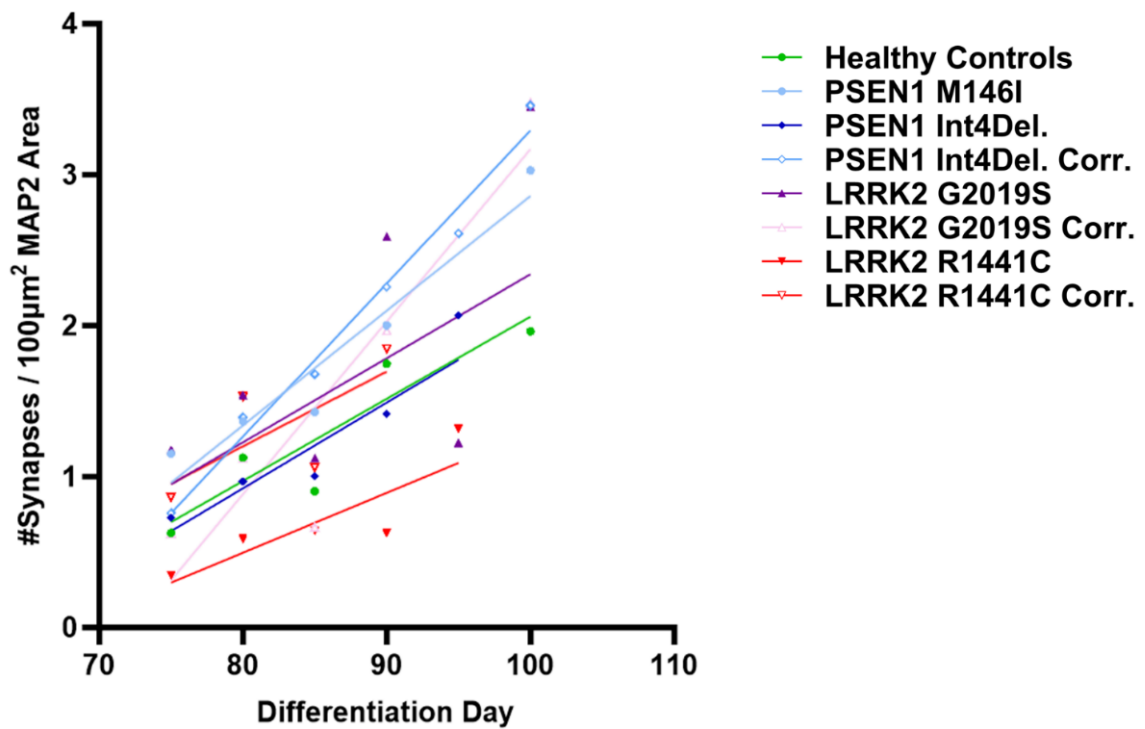


Figure 6.2. Synaptic Density Increases Over Time in PSEN1 and LRRK2 Genotypes. Synaptic density was quantified across various untreated cell lines from D75 to D100. Simple linear regression showed that synaptic density significantly increased with time in healthy controls (N=3, $p=0.0073$), PSEN1-M146I ($p=0.008$), PSEN1-Int4Del clones (N=2, $p=0.0241$) and corrected control ($p=0.001$), LRRK2-G2019S corrected control ($p=0.0269$), and LRRK2-R1441C (N=2, $p=0.447$), and trended towards significant increase in LRRK2-G2019S (N=2, $p=0.1801$) and LRRK2-R1441C corrected control ($p=0.2823$). Data points represent individual genotypes averaged across the stated number of cell lines (N) in 2-4 differentiations.

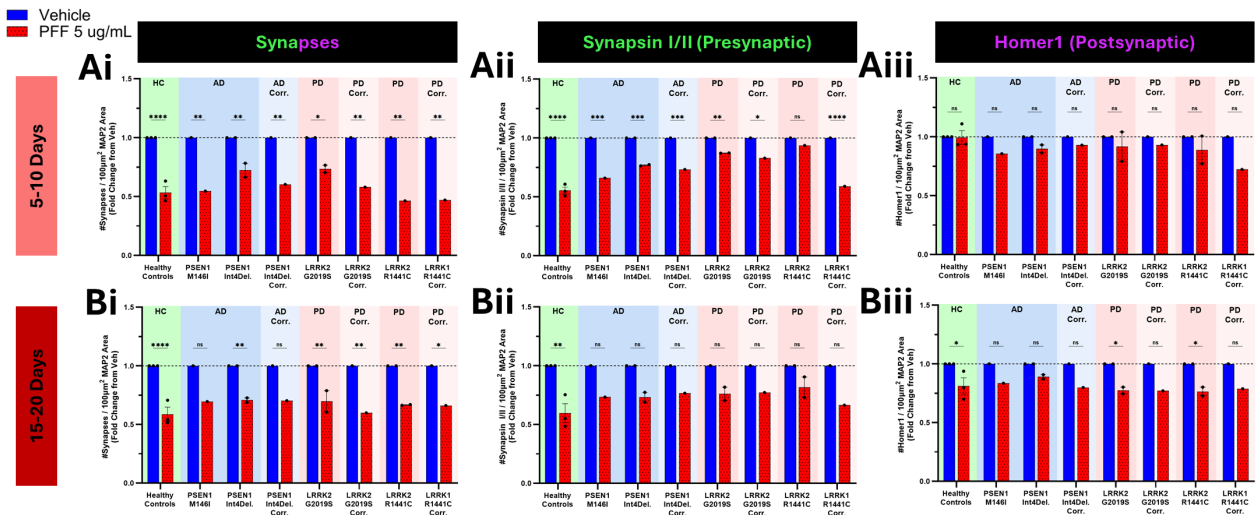


Figure 6.3. ASyn PFF Insult Reduces Synaptic Density in Healthy, AD, and PD Cortical Neurons, with Preferential Loss of Presynaptic Markers. Cortical neurons were insulted with 5 µg/mL ASyn PFF and fixed, immunolabelled, and analysed for synaptic density (A) 5-10, or (B) 15-20 days after PFF insult. Puncta density is presented as a fold change from vehicle treated cells. **(A-Bi)** Synaptic density was significantly reduced by as much as 50% by 5-10 days. No significant effect of genotype was found at either timepoint. **(ii-iii)** Synaptic density was also separated out to its constituent presynaptic and postsynaptic markers. **(Aii)** Synapsin I/II density was significantly reduced in most genotypes at 5-10 days, whereas **(Aiii)** homer1 density only had a mild trend towards reduction at 5-10 days. **(Bii-iii)** Both synapsin I/II and homer1 density trended towards reduction by PFF insult to a similar degree at D15-20. Statistical comparisons are 2-way ANOVA with Bonferroni correction for multiple comparisons. Data points represent individual cell lines averaged across 2-4 differentiations, bars represent mean $\pm$ SEM.

6.3.2. Neurite Outgrowth Assays

Neurite outgrowth was examined in live CN monocultures using low titre GFP paradigm, such that ~1% of cells in the culture expressed GFP (Figure 6.4A). Cells were longitudinally imaged over time and neurites traced using automated image analysis (Figure 6.4B).

Mean neurite length increased over time across all 20 cell lines examined under basal conditions (Figure 6.5). There were no significant differences between genotypes, though PSEN1-M146I and PSEN1-E280A trended towards a much more rapid arborisation relative to controls, and LRRK2-R1441C cell lines trended towards impaired outgrowth relative to healthy and corrected controls. A transient difference between healthy controls and LRRK2-G2019S and LRRK2-R1441C cell lines was observed only at 8 days, between unnormalised values, without correcting for multiple comparisons.

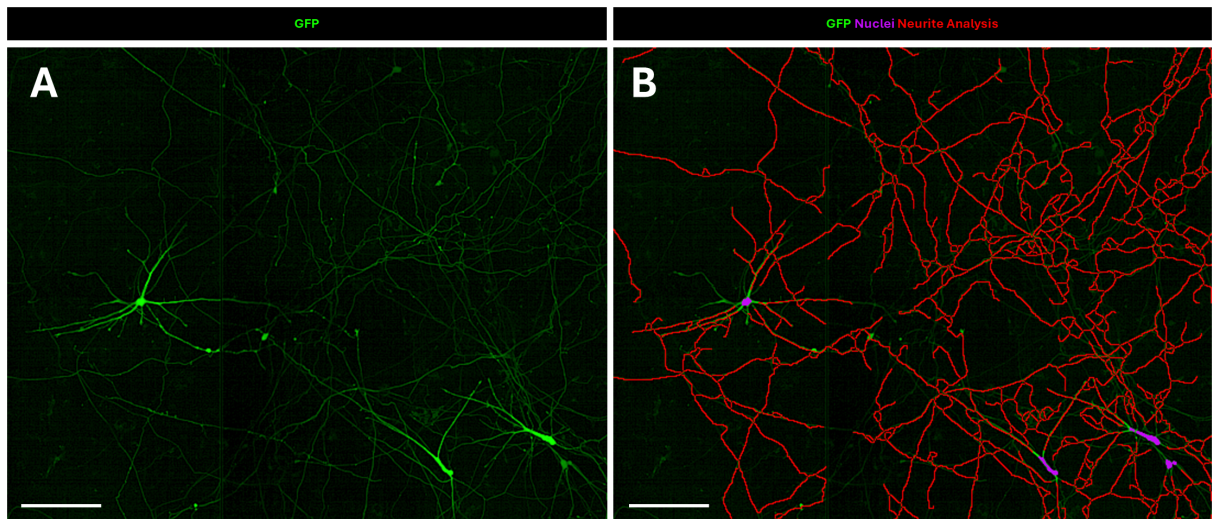


Figure 6.4. GFP Neurite Outgrowth Assay Analysis in Cortical Neurons. (A) Representative image of cortical neurons, where a subset of cells (~1%) express GFP, for neurite tracing. (B) Harmony image analysis neurite tracing, with nuclei identified in purple, and associated neurites in red. Scale bars represent 50 μm .

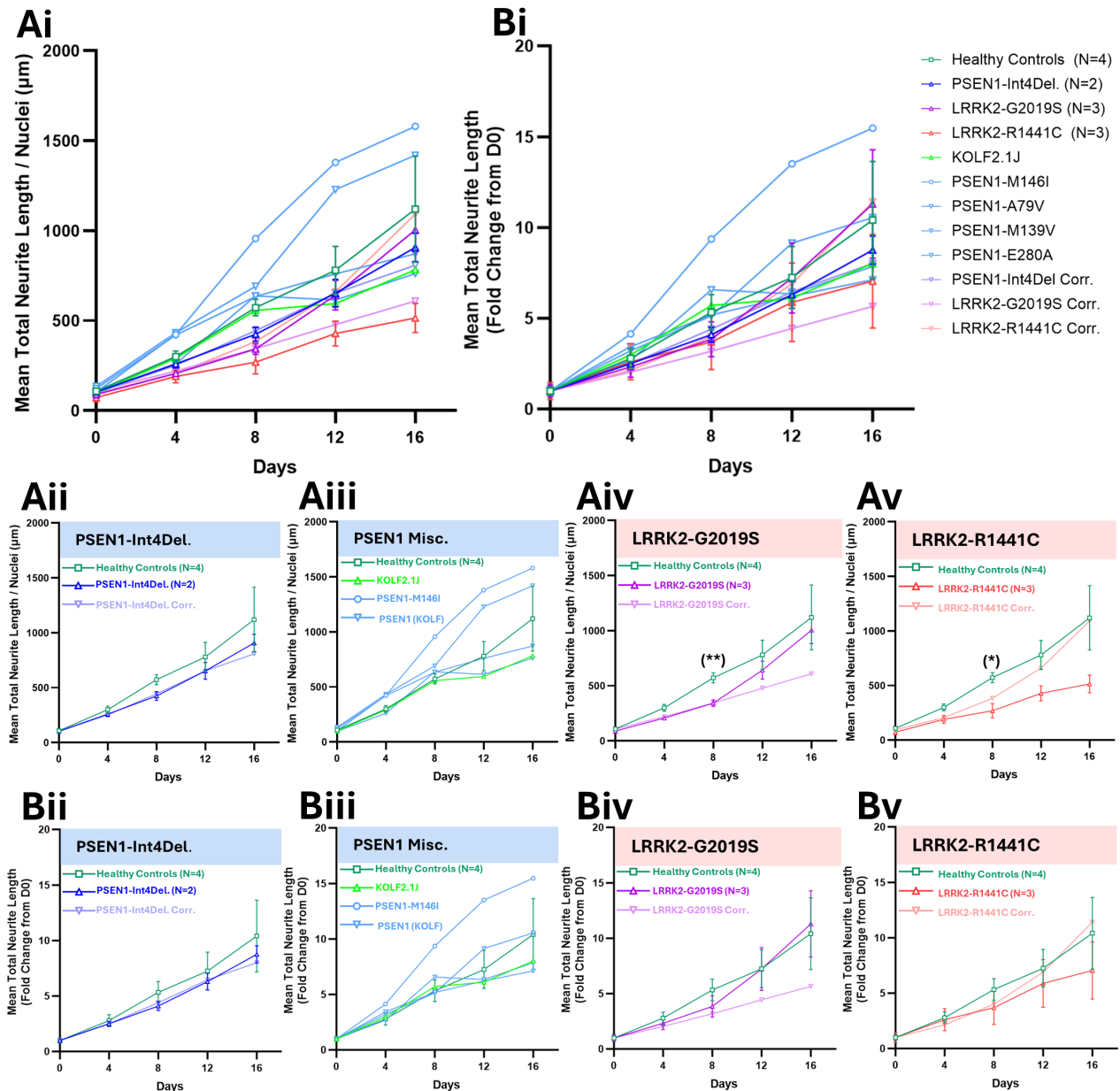


Figure 6.5. Neurite Outgrowth Increases Over Time in Untreated Cortical Neuron Monocultures.

The mean total length of all neurites per neuron was measured for healthy control, AD, and PD genotypes, and presented as **(A)** raw values, or **(B)** fold change from D0 (D34 of differentiation). Repeated measures two-way ANOVA found that for **(Ai)** raw values, there was a significant effect of time ($p < 0.0001$) but not of genotype ($p = 0.1261$) across genotypes. Genotypes were separated out into individual graphs comparing healthy controls with **(Aii)** PSEN1-Int4Del and corrected control; **(Aiii)** other PSEN1 mutant lines, and KOLF2.1J separated out from healthy controls; **(Aiv)** LRRK2-G2019S and corrected control, where a significant difference was found between healthy control and LRRK2-G2019S at 8 days, only when multiple comparisons correction was not applied ($p = 0.0089$); and **(Av)** LRRK2-R1441C and corrected control, where a significant difference was found between healthy control and LRRK2-R1441C at 8 days ($p = 0.0205$), only when multiple comparisons correction was not applied. **(B)** Repeated measures two-way ANOVA found that there was a significant effect of time ($p = 0.0002$) but not genotype, and no significant differences between genotypes, even without correcting for multiple comparisons. Data points represent genotypes averaged across multiple cell lines where available, from 2-6 differentiations per line, bars represent mean $\pm$ SEM.

I then sought to examine the effect of a 10 µg/mL PFF insult at D34 ("D0" in neurite time course) on this neurite outgrowth. Example images of neurite outgrowth are shown in Figure 6.6 and quantification in individual cell lines is shown in Figure 6.7. Most healthy controls, including *SNCA*-KO, were unaffected by PFF insult. There was also no effect on KOLF2.1J, though a small effect and trend towards impairment in the isogenic PSEN1-M139V and PSEN1-A79V lines respectively. PSEN1-M146I and both PSEN1-Int4Del CNs were significantly impaired, with no impairment in the corrected control. Two of three LRRK2-G2019S lines were affected by PFF insult, though a third line and its corrected control were unaffected. None of the LRRK2-R1441C cell lines were impaired by the PFF insult. Interestingly, PFF insult did not necessarily result in a loss of neurites, merely an apparent slowing or delay of outgrowth, in impaired cell lines.

Pooling cell lines per genotype showed that there was, overall, a significant impairment in PSEN1-Int4Del cell lines, and trend towards impairment in the other PSEN1 and LRRK2-G2019S cell lines, but not in corrected controls, healthy controls, or LRRK2-R1441C (Figure 6.7).

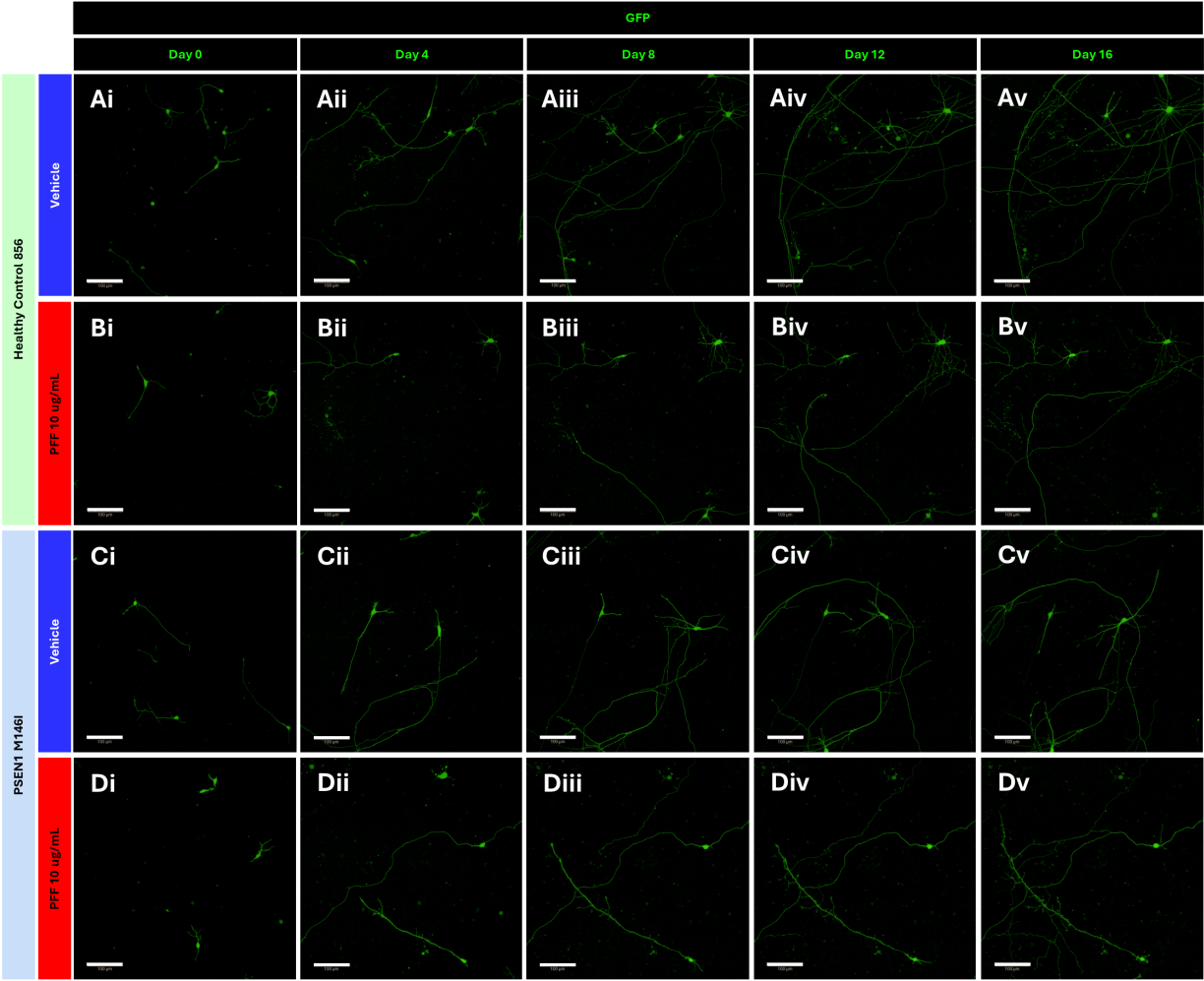


Figure 6.6. Representative Images of Neurite Outgrowth with ASyn PFF Insult. Live images of GFP-expressing monocultured cortical neurons, insulted at D34 (D0) with 10 µg/mL ASyn PFF, and imaged every 4 days subsequently. Example images are shown of (A-B) healthy control neurons, and (C-D) PSEN1-M146I neurons, treated with (A-C) vehicle, or (B-D) PFF. Scale bars represent 100 µm.

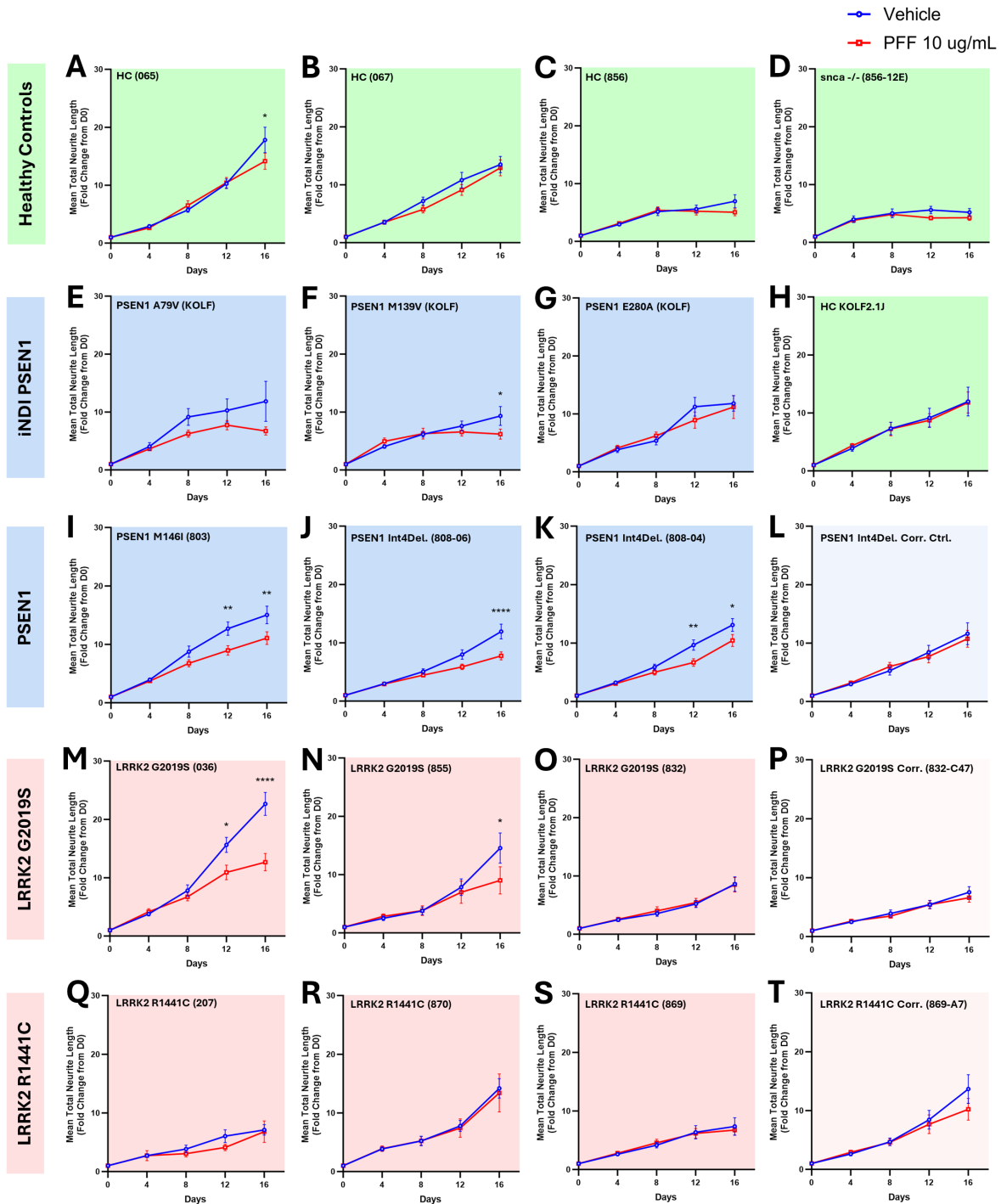


Figure 6.7. Neurite Outgrowth Is Significantly Impaired by ASyn PFF Insult in some PSEN1 and LRRK2-G2019S Cell Lines. Neurite outgrowth is presented as fold change from the time of PFF insult for 20 lines from different genotypes, presented on individual graphs. Statistical comparisons are two-way ANOVA with Bonferroni correction for multiple comparisons. **(A-D)** No effect of PFF insult was observed on healthy control lines or the SNCA-KO, except for at 16 days in 065. **(E-H)** No consistent effect of PFF insult was observed in the iNDI PSEN1 cell lines or healthy control KOLF2.1J, though a trend towards impaired outgrowth was observed in some PSEN1 genotypes, becoming significant by 16 days in PSEN1-M139V. **(I-L)** Neurite outgrowth was impaired by the PFF insult in PSEN1-M146I and both PSEN1-Int4Del CN clones, but not in the Int4Del corrected control. **(M-P)** Neurite outgrowth was impaired in two LRRK2-G2019S cell lines but was unimpaired in the isogenic pair. **(Q-T)** Neurite outgrowth was unimpaired in the LRRK2-R1441C cell lines and corrected control. Data points represent the mean of 2-6 differentiations per line, averaged across at least 4 wells per differentiation, bars represent mean $\pm$ SEM.

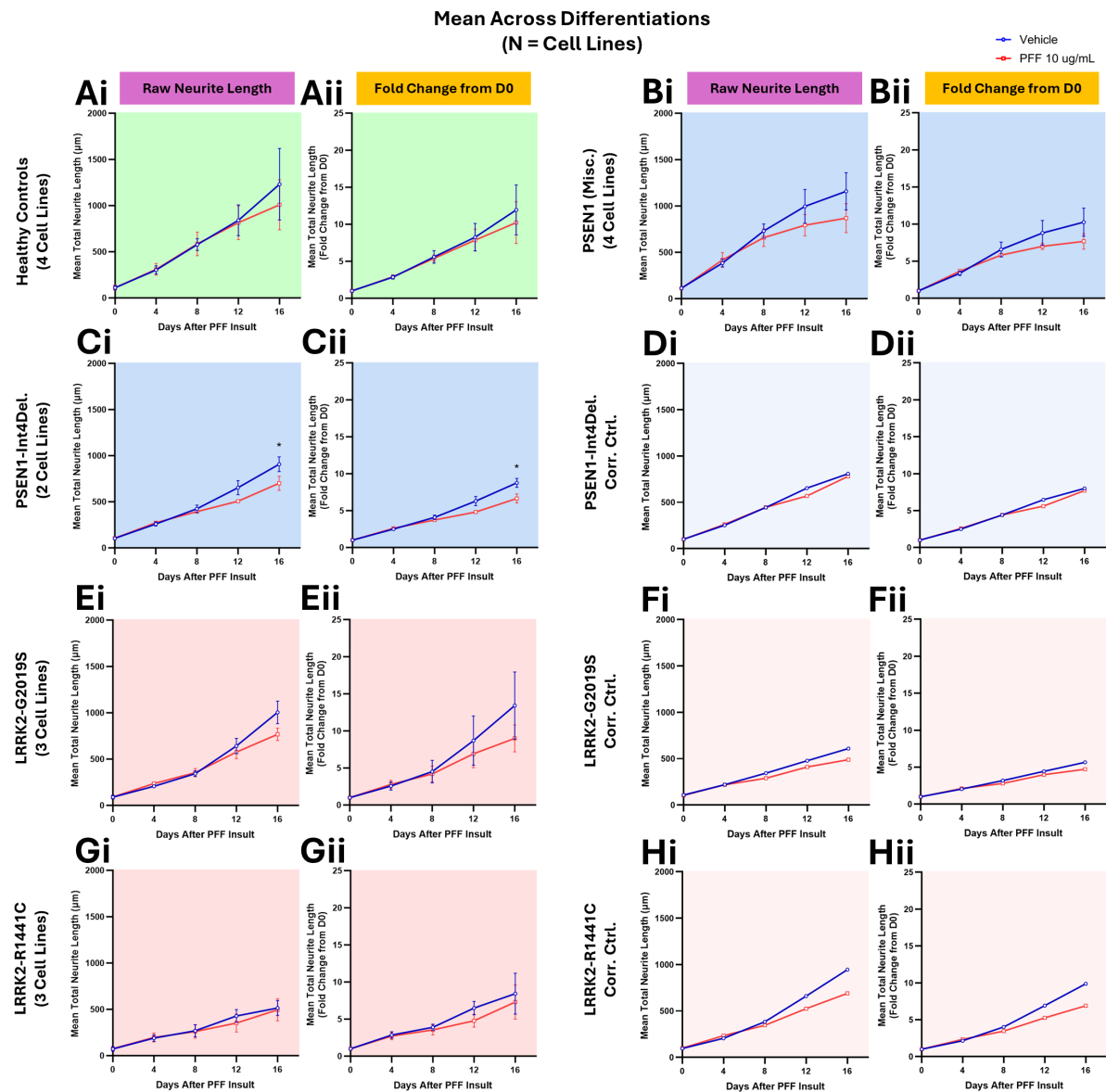


Figure 6.8. Neurite Outgrowth is Significantly Impaired in PSEN1-Int4Del Cortical Neurons by ASyn PFF Insult. Cell lines from Figure 6.7 were pooled per genotype, and presented as (i) raw values, and (ii) values normalised to the time of PFF insult (D0). (B) PSEN1 lines include PSEN1-M146I and iNDI lines (PSEN1-A79V, PSEN1-M139V, PSEN1-E280A), and collectively trended towards impairment. (C) PSEN1-Int4Del cell lines were significantly impaired by the ASyn PFF insult. (E) LRRK2-G2019S trended towards impairment. (G) LRRK2-R1441C was not impaired by PFF insult. Statistical comparisons were two-way ANOVA with Bonferroni correction for multiple comparisons. Data points represent cell line means from 2-6 differentiations per line, with at least 4 well replicates per differentiation, bars represent mean $\pm$ SEM.

6.4. Discussion

6.4.1. Results Summary

In this chapter, I sought to examine functional consequences of PFF insult in CNs, beyond seeded aggregation, and examine any genotype-based differences in vulnerability, independent of PFF insult. I first examined synaptic density, and found that in D70 cocultured CNs, synaptogenesis is an ongoing process, and ASyn PFF insult led to a severe but possibly transient loss of synaptic density, preferentially affecting presynaptic puncta. Synaptic density was reduced similarly in healthy controls, PSEN1, and LRRK2 genotypes. I also examined neurite outgrowth in monocultured CNs. In untreated cultures, there was no little significant effect of AD or PD genotypes on neurite outgrowth, highlighting the resilience of CNs in PD. However, several AD PSEN1 and PD LRRK2-G2019S cell lines were significantly impaired in their neurite outgrowth by PFF insult, whereas LRRK2-R1441C and control lines were not.

6.4.2. Cortical Neuron (Pre)Synaptic Density is Reduced by ASyn PFF Insult

The reduction in synaptic density here is in line with other studies of PFF insults in rodent primary cell models which find loss of synaptic proteins in response to ASyn PFF insult (Volpicelli-Daley et al. 2011; Tapias et al. 2017; Mahul-Mellier et al. 2020). While most of the studies have that have examined ASyn PFFs in hiPSC CNs have not examined synaptic density, Prots et al. (2018), using lentiviral overexpression of oligomer forming ASyn in hiPSC CNs, also find synaptic loss. However, a strength of the analysis here is the examination of apposed pre and postsynaptic puncta, which might be a better reflection of functional synapses, as opposed to simply examining the expression of synaptic proteins, as is done in most other studies.

Surprisingly, despite having observed stark differences in the vulnerability of these cell lines to pASyn-S129+ pathology, I observed no difference in sensitivity of healthy controls, AD PSEN1, and PD LRRK2 genotypes to synapse loss. This might suggest that the PFFs exert synaptic toxicity in an aggregation independent manner. Indeed, the synapse loss occurs very rapidly, within 5 days, before substantial aggregation has occurred, as in other PFF studies (Volpicelli-Daley et al. 2011; Tapias et al. 2017; Mahul-Mellier et al. 2020), and in the AD and PD brain (Pereira et al. 2021). However, the synaptic toxicity of the PFFs also appeared to be preferentially presynaptic. This is consistent with the presynaptic terminal representing the main site of endogenous ASyn in the cell. These sites of high local ASyn concentration may thus represent points of vulnerability to PFF-seeded aggregation. Several other *in vitro* studies have shown a similarly selective effect on presynaptic proteins (Volpicelli-Daley et al. 2011; Tapias et al. 2017; Gcwensa et al. 2024; Kapucu et al. 2024).

While monomer and SNCA-KO controls were not employed here, this suggests that the synaptic toxicity is not a physical effect of the PFFs on synapses, since then post synaptic puncta would be expected to be equally affected, and is not simply an effect of protein load, since it is likely that the PFFs are seeding pathology at these sites of local ASyn concentration. Another study has also shown a selective toxicity of ASyn fibrils, not monomers, on presynaptic vesicles (Stephens et al. 2023). It may be that synaptic toxicity of ASyn PFFs is a result of early stage seeding of endogenous ASyn at the presynapse, causing a loss of function. Simultaneously, ASyn PFFs may also have aggregation independent effects on mitochondria, which are also clustered and highly vulnerable at the presynapse (Völgyi et al. 2015; Prots et al. 2018; Denning et al. 2022).

The rapidity of synaptic alterations and selectivity of presynaptic vulnerability help to validate the ASyn PFF and hiPSC CN models. Synaptic dysfunction is thought to be one of the earliest pathological events in AD and PD, and accumulations of ASyn have been found localised at

the presynapse, before substantial LP (Kramer and Schulz-Schaeffer 2007; Schulz-Schaeffer 2010; Pereira et al. 2021). A metaanalysis of 417 AD postmortem studies reporting synapse loss in AD also noted a selective vulnerability of presynaptic proteins (de Wilde et al. 2016). Interestingly, higher presynaptic A β concentrations are associated with vGLUT1+ corticofugal projections into the basolateral amygdala, which are preferentially vulnerable to A β aggregation, compared to vGLUT2+ thalamofugal projections, in rodents with PFFs injected into the striatum (Chen et al. 2022; Gcwensa et al. 2024). Projection neurons in deeper cortical layers which represent subcortical output, and the amygdala itself, are vulnerable loci in synucleinopathies (Heiko Braak et al. 2003; Borghammer et al. 2021). The hiPSC NGN2 CNs used here also represent vGLUT1+ excitatory CNs, thus modelling the synaptically-vulnerable cells.

However, the hiPSC CNs are nevertheless a developing cell model, and synaptogenesis appears to be ongoing despite PFF insult, leading to the seemingly transient effect of PFF loss. Bieri et al. (2019), for example, do not observe synapse loss in LRRK2-G2019S NGN2 hiPSC-derived neurons, in response to a 6.5 μ g/mL A β PFF insult. This may be because at a 3-week endpoint, the transient synapse loss has already been ameliorated by the growing cultures, and earlier timepoints need to be examined.

Interestingly, this suggests that, despite their persistent presence in the culture, the PFFs provide an acute, rather than an ongoing source of toxicity to the neurons (at least not a chronic stressor that is unable to be overcome by the synaptogenesis of developing neurons). There may then still be downstream, time-delayed toxicity resulting from seeded aggregation, rather than the PFFs themselves. In a mouse slice culture model, PFF-seeded accumulation of pA β -S129+ pathology was inversely associated with synaptophysin labelling (Roux et al. 2020). It is therefore possible that beyond 20 days, with more profound Lewy-like pathology in the PSEN1-Int4Del CNs, a second diminishment of synaptic density might occur.

Nevertheless, owing to the lack of differential vulnerability observed between genotypes here, and since the hiPSC mDAN cultures do not provide a model of the nigrostriatal synapses relevant to PD, synaptic pathology was not further investigated in this thesis, and there is substantial scope for further experimentation. For example, it is unclear why PSEN1-Int4Del CNs are no more vulnerable to synapse loss (if anything, they trend towards being comparatively resilient) than other genotypes, given their vulnerability to seeded pASyn-S129+ aggregation, relatively high expression of vGLUT1, and a basal synaptic density not substantially different from other genotypes. It is also unclear what the functional effects on neuronal activity of this synaptic pathology is. ASyn PFF insult has led to mixed effects observed in MEA (Valderhaug et al. 2021; Kapucu et al. 2024). Given the profound effect on synaptic density observed here, and the relevance of the hiPSC CN model to synuclein synaptopathy, this presents an interesting route for further investigation, alongside a better understanding of the mechanisms that lead to this synaptic toxicity.

6.4.3. Genotype-Dependent Vulnerability to ASyn PFFs in Neurite Outgrowth

Despite the lack of differential vulnerability to synapse loss between genotypes, neurite outgrowth proved to be more selectively impaired in vulnerable genotypes. Specifically, the PSEN1-Int4Del CNs, which were shown in Chapter 3 to selectively develop profound pASyn-S129+ pathology, also show deficits in neurite outgrowth in response to ASyn PFF insult. This was mutation dependent, given the resilience of the corrected control to this insult. Some LRRK2-G2019S and other PSEN1 CNs appeared to have impaired outgrowth as well. These observations are the first of this kind of functional impairment as a result of ASyn PFF insult, and add to the novel observations of PSEN1-related vulnerability to ASyn pathology, and add to the series of cellular changes including protein aggregation, neuronal and nuclear

morphological abnormalities, and synaptic loss, resulting from ASyn PFF insult in the CN neurons shown here. The mechanism for this deficit, however, remains unclear.

Overexpression of wildtype, A53T, and A30P ASyn has been shown to impair neurite outgrowth in rat primary midbrain neurons (Koch et al. 2015). Seebauer et al. (2022) and Oliveira et al. (2015) found that *SNCA*-duplication and triplication, respectively, led to impaired neurite outgrowth in hiPSC mDANs. These would suggest that an excessive ASyn protein load, not necessarily fibrillar in nature, is sufficient to impair neurite outgrowth. Unlike many studies which typically analyse neurite outgrowth via immunolabelling of processes at a fixed timepoint, an advantage of the neurite outgrowth paradigm here is the ability to longitudinally track outgrowth over time. This allowed me to see that the neurite outgrowth deficit observed here is time dependent, with no difference in outgrowth between PFF and vehicle treated neurites typically until 12-16 days. This is the timeframe in which I have previously observed accumulation of seeded pASyn-S129+ pathology in these cells, especially in the vulnerable PSEN1-Int4Del CNs.

However, given that the CNs at D34-D50 are not as mature as the neurons insulted at D70, and since there are no cocultured rAstros to enhance maturation, and since the duration from the point of insult is only 16 days, it is unlikely that the CNs under this paradigm express high levels of endogenous ASyn, and it is unlikely that they develop substantial Lewy-like pathology. Furthermore, while the PSEN1-Int4Del cell lines develop profound pASyn-S129+ pathology and show neurite outgrowth deficits, PSEN1-M146I and LRRK2-G2019S cell lines also show similar neurite outgrowth impairment, but significantly less seeded aggregate pathology. As such, more factors must be at play than just the development of intracellular inclusions in driving the neurite outgrowth impairment, which, like synaptic loss, appears to be an early deficit, before substantial aggregation.

Aggregation independent effects of ASyn PFFs have been observed on mitochondrial membrane potential in *SNCA*-KO rodent primary neurons (Denning et al. 2022), and mitochondrial impairments in PD models have also been shown to lead to impaired outgrowth (Su and Qi 2013; Neely et al. 2017). However, since I do not observe neurite outgrowth deficits in the *SNCA*-KO cell line, or other healthy or corrected controls, it is likely a result of the mutation. Autophagy deficits were identified as a phenotype of *PSEN1*-Int4Del CNs in the previous chapter. Efficient autophagic functioning is crucial for neurite outgrowth and remodelling, and various in vitro studies have linked neurite outgrowth impairment with autophagic dysfunction (Heo, Kim, and Seol 2010; Ramonet et al. 2011; Sánchez-Danés et al. 2012; Reinhardt et al. 2013). Since ASyn PFFs have been shown to lead to lysosomal impairment and aggregate pathology is associated with overwhelmed autophagic machinery, this provides a likely mediator for PFF-related neurite outgrowth impairment (Mazzulli, Zunke, Isacson, et al. 2016a; Zambon et al. 2019; MacIsaac et al. 2020; Stojkovska et al. 2021; Bayati et al. 2023).

However, despite basal autophagy deficits in the *PSEN1*-Int4Del CNs, these cell lines display only a slight trend but no significant impairment in neurite outgrowth in untreated conditions. It may thus be the case that a confluence of ASyn protein load and aggregation-independent mitochondrial impairment overwhelm the autophagic system, already under reduced capacity in these disease cell lines. This, coupled with the additional time dependent burden of the development of early stage inclusionopathy, may lead to the neurite outgrowth deficit observed in select genotypes.

If this is the case, it is particularly striking that *LRRK2*-R1441C cells display no neurite outgrowth impairment, despite having similar levels of pASyn-S129 accumulation as *LRRK2*-G2019S and *PSEN1*-M146I here, and despite impaired scar healing observed in iPSC mDANs (Connor-Robson et al., 2019), and being thought of as having more severe autophagic and

mitochondrial deficits than LRRK2-G2019S (Max Sloan et al. 2016; Wallings, Connor-Robson, and Wade-Martins 2019; Wallings et al. 2019; Williamson et al. 2023). This could be a reflection of cell type-dependent vulnerability and differences between models, as LRRK2-R1441C lines have not been examined in an hiPSC CN model before, which may be more resilient than rodent primary neurons and hiPSC mDANs. Wallings et al. (2019) note that elevated levels of LC3 puncta were observed in mDANs of BAC transgenic rats with LRRK2-G2019S or LRRK2-R1441C, but only in the cortex of LRRK2-R1441C. While this is the inverse pattern for my observations of LRRK2-G2019S vulnerability and LRRK2-R1441C resilience in CNs here, it does speak to the potential for cell-type dependent vulnerability to mutation related autophagic deficits. Thus, future research might prioritise examining differences in autophagic function between CNs and mDANs.

Crucially, these experiments point to mutation and PFF-related deficits in CNs, in line with other observations in this thesis, that are on account of cell autonomous factors. This is also of interest since it represents an early functional deficit in live cells, before substantial aggregate pathology has probably formed. Early-stage dysfunction before protein pathology and cell death, such as neuronal signalling, synaptic deficits, and axonopathy, are becoming increasingly appreciated as a key mechanism in AD and PD, as models such as these make them more tractable for study (Janezic et al. 2013; Hunn et al. 2015; Essayan-Perez et al. 2019; Mancini et al. 2020). While the paradigm employed here is more a model of outgrowth rather than neurodegeneration, it may equally be applied to more mature cultures to examine neurite loss in response to later stage insults. The outgrowth model also has implications for impaired neurogenesis in the context of LP, which could be an important mechanism in both AD and PD (Winner et al. 2011; Arber et al. 2021). This might be especially relevant in the hippocampus and dentate gyrus, which provides a locus for the intersection of a neurogenic niche in the adult brain, a site of high plasticity and remodelling, and a region relatively prone to ASyn accumulation in AD and PD.

6.4.4. Neurite Abnormalities in PSEN1 Cortical Neurons

The low titre GFP assay also enabled the visualisation of longitudinal neurite outgrowth in cells under basal conditions, to examine the effect of AD and PD genotypes in hiPSC CNs.

PSEN1 mutations did not lead to significant abnormalities in neurite outgrowth, though PSEN1-M146I and PSEN1-E280A lines trended towards having substantially elevated neurite outgrowth. The role PSEN1 and AD associated mutations might play in neurite outgrowth, if any, is much more unclear than for LRRK2. Neurite outgrowth dysfunction has rarely been examined before in a PSEN1 fAD context. There is, however, a potential link between PSEN1 and neurite regulation through Notch signalling.

In addition to cleaving APP as part of the gamma-secretase complex, PSEN1 is also required for cleaving Notch1, a transmembrane receptor important for neuronal development and cell fate regulation (Song et al. 1999; Lee and Ch'ng 2020). Notch1 cleavage releases the Notch intracellular domain (NICD) that acts as a transcription factor to activate genes which, among other functions, inhibits neurite outgrowth (Berezovska et al. 1999; Lathia, Mattson, and Cheng 2008). fAD PSEN1 mutations have been shown to impair Notch1 signalling (Song et al. 1999; Pigino et al. 2001), which might lead to dysregulation of neurite outgrowth through a disinhibition effect. Indeed, Pigino et al. (2001) show three fAD PSEN1 mutations which lead to impaired Notch1 signalling, reduced nuclear NICD, and increased neurite length. One of these was the PSEN1-M146V, a mutation at the same site as the PSEN1-M146I mutation employed here, which displayed a trend towards aberrantly elevated neurite outgrowth. Pigino et al. (2001) also suggest that PSEN1 associates with microtubules and other cytoskeletal elements itself and that these interactions are impaired by fAD mutations.

Other and more recent studies have also pointed to PSEN1 mutant related alterations in Notch1 signalling leading to premature differentiation, and potentially to depletion of neurogenic niches (Wang et al. 2004; Yang et al. 2016; Arber et al. 2021; Hernández-Sapiéns et

al. 2022; Hurley et al. 2023). In Arber et al. (2021), PSEN1-M146I, PSEN1-M139V, and especially PSEN1-Int4Del hiPSC CNs are all shown to exhibit premature differentiation through impaired Notch1 signalling, and increased TUJ1+ neurites were also observed in a PSEN1-M139V patient's hippocampal DGGC layer. This aligns with the trend towards neurite deregulation in the PSEN1-M146I line observed here, though outgrowth in PSEN1-Int4Del and PSEN1-M139V was not notably aberrant. In the case of PSEN1-Int4Del, it is possible that defective autophagy impairing neurite outgrowth and impaired Notch1 signalling disinhibiting neurite outgrowth act as opposing and relatively balanced forces. In the case of PSEN1-M139V, Arber et al. (2021) note that the effect of the mutation on NICD is less severe than other mutations, concurring with other studies showing minimal effect of this particular mutation on Notch1 cleavage (Chávez-Gutiérrez et al. 2012). Confusingly, however, another study found reduced neurite outgrowth in a PSEN1-M146V hiPSC CN model, amid other indicators of premature differentiation (Ghatak et al. 2019).

Thus, while the findings of my experiments are in alignment with some other studies, more apparent is the uncertain relationship between the many and varied PSEN1 mutations and the amyloid processing-independent functions of the protein, which are not as well characterised as A β catabolism. The results here regarding PSEN1-related neurite deregulation are uncertain, and whether the PSEN1 cell lines here exhibit a premature differentiation phenotype will be discussed further in the subsequent chapter. However, in the context of the literature, these data add to a growing picture of a role for PSEN1 in neurogenesis, for which the hiPSC neurite outgrowth paradigm could prove useful for future study.

6.4.5. Resilience to LRRK2-related Neurite Outgrowth Deficits in Cortical Neurons

Given the clearer role of LRRK2 in regulating neurite outgrowth, the lack of neurite outgrowth deficit in the LRRK2-G2019S cell lines here is perhaps more striking. LRRK2 regulates microtubules through its kinase function (Cookson 2012; Civiero et al. 2018), and is even known to phosphorylate tau (Henderson et al. 2019; Outeiro et al. 2019; Castro-Sánchez et al. 2020). Accordingly, overexpression of LRRK2, and/or the G2019S mutation, which causes a gain of function, have been associated with neurite shortening, while LRRK2 KO or KD is associated with neurite elongation (Cookson 2012; Sánchez-Danés et al. 2012; Sepulveda et al. 2013). This has been observed in a variety of models, including drosophila, SH-SY5Y, PC12, and other cell models, as well as rodent primary midbrain, hippocampal, and cortical cultures (MacLeod et al. 2006; Heo, Kim, and Seol 2010; Cookson 2012; Sepulveda et al. 2013; Martin et al. 2014; Civiero et al. 2018). While many of these models utilise overexpression of LRRK2, and the neurite outgrowth deficit may depend on this (Dächsel et al. 2010), there are now also numerous studies in hiPSC mDANs from patients bearing LRRK2 mutations that recapitulate this, typically without transgenic overexpression (Sánchez-Danés et al. 2012; Reinhardt et al. 2013; Su and Qi 2013; Borgs et al. 2016; Qing et al. 2017; Korecka et al. 2019).

In contrast to this, here, I show for the first time that hiPSC CNs are apparently resilient to LRRK2-G2019S related neurite outgrowth deficits. Only a slight trend towards impairment compared to healthy controls was seen transiently at 8 days, which became significant only without applying any correction for multiple comparisons testing. However, this was not seen in normalised data, and there was no such trend between the G2019S lines and the corrected control. This suggests that neurite outgrowth impairment is a cell-type specific phenotype in LRRK2-G2019S hiPSC models and reflects the differential vulnerability of CNs and mDANs in

the PD brain. While the mechanism of this resilience in CNs is unclear, relative LRRK2 expression levels and kinase activity would seem logical places to begin further investigation.

Interestingly, LRRK2-R1441C cell lines showed a clearer trend towards impairment in the hiPSC CNs here, compared both to healthy and corrected controls, though was similarly not significant. Neurite outgrowth has not been as thoroughly characterised for LRRK2-R1441C models. Overexpression in SH-SY5Y and rodent primary neurons led to neurite shortening (Sheng et al. 2012; Verma et al. 2017), and hiPSC mDANs with LRRK2-G2019S and LRRK2-R1441C have similarly impaired neurite regeneration in a scratch insult assay (Connor-Robson et al. 2019). However, in a drosophila model, dopaminergic neurons expressing LRRK2-G2019S, but not LRRK2-R1441C, showed arborisation deficits compared to wild-type LRRK2 (Lin et al. 2010).

These deficits (or lack thereof, here) are of interest as they are the result of the LRRK2 mutation, absent alpha synuclein pathology. Not only do LRRK2 PD patients frequently present without ASyn pathology (Henderson et al. 2019), but having a deeper understanding of what cellular dysfunction lies upstream or independent of protein pathology in neurodegenerative disease is of great interest, and presents a wide opportunity set for innovative therapeutic targets. The cell type dependency here also reflects the value of the selective vulnerability framework, and examination of how LRRK2 differentially regulates neurite process morphology in different cell types could elicit such therapeutic targets.

6.4.6. Conclusions

The findings in this chapter present a picture of developing CNs undergoing synaptogenesis and neurite outgrowth, both of which are susceptible to ASyn PFF insult in a disease-relevant manner. The neurite and synaptic density assays here also build on previous chapters

examining ASyn PFF seeded pASyn-S129+ pathology by showing early-stage deficits with functional significance to build up a wider picture of the cellular effects of the ASyn PFF insult. They also allow for observations of disease related abnormalities independent of protein aggregation. These experiments not only confirm cell autonomous effects of ASyn PFF insults and disease related mutations in the hiPSC CN model, but also demonstrate cell type-dependent differences in vulnerability that reflect differences in brain pathology. Thus, these results collectively strengthen the validity of the model for examining neurodegenerative disease, and point especially to the opportunity to examine neurogenesis, since this converges with both AD and PD pathology in the vulnerable hippocampal region and takes advantage of the developing hiPSC-derived neuronal model.

Chapter 7.

Discussion

7.1. Introduction

The phenomenon of selective vulnerability is, simply put, quite strange. It is unexpected that some proteins, which are expressed widely across the brain, become pathological only in small populations of cells. It is striking that, amid the vast dysfunction and destruction of neurons in late-stage disease, some cells remain perfectly healthy. It is surprising how stereotyped and spatiotemporally defined the pattern of pathological developments is in AD and PD. It is perplexing, given the convergent pathways implicated in these diseases, that sometimes the cells which are vulnerable to one disease are resilient in another. It is also remarkable how common these pathways and phenomena are across the broad landscape of neurodegenerative disease.

In this thesis, I argue that this quirk of neurodegenerative diseases is both interesting and useful; that in the garb of a natural experiment, it allows insight into the fundamental biochemical and cellular driving factors that lead to neurodegenerative disease; that by studying differentially vulnerable cells from different diseases, we might come to better understand common and unique elements of each disease, and thence, strategies for their amelioration or prevention.

Therefore, in Chapter 1, I outlined vulnerable and resilient cells in AD and PD, the two most common neurodegenerative diseases, whose patterns of vulnerability are overlapping but opposed. I then examined the differences between these populations, to identify cell non-autonomous and autonomous pathways that might predispose their vulnerability, but noted the limitations of examining this through postmortem pathology. I therefore set out to investigate selective neuronal vulnerability in AD and PD using hiPSC-derived neurons. The approach of comparing multiple cell types from the same patient line has rarely been examined before, and never across multiple diseases.

In Chapter 2, I detailed an approach for generating both CNs, which are vulnerable in AD but relatively resilient to PD, and mDANs, which are vulnerable in PD and relatively resilient in AD. These protocols were designed to be as comparable as possible for a PFF insult paradigm, and cells were electrophysiologically active, with distinct neuronal identities.

In Chapter 3, I demonstrated an ASyn PFF-seeded inclusionopathy model in the CNs, showing time and dose dependent aggregation of ASyn for the first time in an hiPSC CN model without *SNCA* overexpression or mutation. This was also the first time AD PSEN1 patient cell lines had been examined, and PSEN1-Int4Del was noted to develop profound levels of pathology. The pathology observed was morphologically akin to LP observed in the brain. Diminished nuclear area, independent of aggregation levels, was also a PFF-related phenotype, noted in cell models here for the first time.

In Chapter 4, I demonstrated the ASyn PFF insult also seeded extensive pathology in the mDANs, demonstrating equally high levels of accumulation in healthy and corrected controls, and for the first time, PD LRRK2 and AD PSEN1 cell lines. In these mixed cultures, TH⁺ neurons were noted to develop more pathology than TH⁻ neurons, and pathology was again morphologically characteristic of LP observed in the human midbrain. Diminished neuronal and nuclear area were also associated with PFF insult.

In Chapter 5, these models were directly compared, and I noted that while the mDANs developed pASyn-S129⁺ pathology more rapidly and to a greater degree than the CNs, the PSEN1-Int4Del mutation had a striking effect on the vulnerability of the CNs, but not the mDANs. While no differences in PFF uptake were observed, culture density, levels of ASyn, and the dopaminergic machinery itself were all identified as likely contributors to the vulnerability of mDANs. Meanwhile, cell type-dependent lysosomal build up was identified in PSEN1-Int4Del CNs, as a result of a blockage of autophagic flux, likely the cause of the specific vulnerability of these CNs.

Finally, in Chapter 6, I showed that the effects of the PFF insult extended beyond pASyn-S129+ aggregation to both synapse loss across all genotypes, and neurite outgrowth impairments in select PSEN1 and LRRK2-G2019S lines. Independent of PFF insult, I also noted that while some PSEN1 cell lines trended towards aberrantly high neurite outgrowth, and LRRK2-R1441C CNs trended towards impaired neurite outgrowth, LRRK2-G2019S CNs were remarkably resilient to neurite outgrowth deficits, highlighting the cell type dependency of this well-characterised phenotype.

In these experiments, collectively, I sought to show that hiPSC-derived neurons could sufficiently model the phenomenon of differential vulnerability observed in neurodegenerative disease. To do this, I aimed to demonstrate that different neuronal subtypes exhibited differential vulnerability to PFF insult, that differences would be cell type-based and genotype-based in a manner that matched differences observed in the brain, and that the model could be used to examine early-stage dysfunction and underlying pathways relevant to disease.

In order to assess whether these aims have been fulfilled, the following has to be discussed:

- 1) Is there a cell type-dependent and genotype-dependent difference in pathology that reflects the brain?
- 2) Does the pathology observed in each cell type reflect pathology in the brain?
- 3) Is the PFF model a good operationalisation of vulnerability in neurodegenerative disease?
- 4) Are the hiPSC-derived neurons a good model for cells in the brain?
- 5) Is it legitimate to compare the two different cell models?
- 6) If so, what do these models demonstrate regarding selective neuronal vulnerability and the pathways driving it?

These questions, in addition to the limitations, strengths, novelty, and significance of the models and findings outlined in this thesis, will be discussed here.

7.1. Discussion Part I: Evaluation of the Model

7.1.1. Do Differences in hiPSC-Derived Neurons Match Differences in the Brain?

The first and most important question to address is whether the hiPSC neurons do in fact model differences in vulnerability seen in the brain. In the experiments conducted here, I observed several instances of differential vulnerability.

First is the cell type-dependent vulnerability between CNs and mDANs to PFF-seeded pASyn-S129+ pathology where mDANs developed many-fold times more pathology than their CNs generated from the same cell line, even in the case of PSEN1-Int4Del, where CNs developed substantial pathology. This difference in susceptibility matches patterns of pathological development in the brain, where mDANs are highly susceptible, and the cortex, especially upper layer neurons modelled here, are relatively spared of ASyn inclusions (Alegre-Abarategui et al. 2019; Borghammer et al. 2021; Braak et al. 2003; Surmeier, Obeso, and Halliday 2017b). This also corresponds with other hiPSC studies showing differences in disease-relevant phenotypes between cortical/forebrain neurons and midbrain dopaminergic/caudal neurons (Brazdis et al. 2020; Muratore et al. 2017), and studies showing differential response to ASyn PFFs in *SNCA*-overexpressing or dual-insult hiPSC models (Bayati et al. 2023; Iannielli et al. 2022).

Second, I also observed that TH+ cells were more prone to developing pASyn-S129+ inclusions than TH- cells in the mDAN cultures, mirroring the selective vulnerability of dopaminergic neurons in the ventral midbrain (Damier et al. 1999; McRitchie, Cartwright, and Halliday 1997; Surmeier, Obeso, and Halliday 2017b). This also corresponds with other studies of hiPSC-derived mDANs, where autophagy deficits were observed more prominently in TH+ cells than TH- cells (Fernandes et al. 2016). In primary cultures of rat ventral midbrain neurons,

mitochondrial impairment and oxidative stress were also seen more in TH+ than TH- neurons (Tapias et al. 2017).

Third, I also observed genotype-dependent differences in neurite outgrowth deficits that, in part, correspond to patterns in disease. Specifically, in the CNs, vulnerable in AD and more resilient to PD, neurite outgrowth was impaired or trended in that direction in several PSEN1 genotypes, but unhindered by the PFF insult in LRRK2-R1441C neurons. This is more difficult to interpret since not all PSEN1 lines were impaired, and some LRRK2-G2019S lines were also impaired. There is some mixed evidence for involvement of the cortex in LRRK2-G2019S PD in particular (Gomez and Ferrer 2010; Pouloupoulos et al. 2012). Nevertheless, the resilience of the LRRK2-R1441C CNs to either pASyn-S129+ inclusions or neurite outgrowth deficits, relative to AD cell lines, is quite striking, especially for an ASyn insult.

Similarly, the lack of neurite outgrowth impairment in LRRK2-G2019S CNs, absent PFF insult, is also noteworthy. While I do not directly compare different cell models here, the neurite outgrowth deficit phenotype in LRRK2-G2019S mDAN models is extremely well characterised (Civiero et al. 2018; Cookson 2012; Sloan, Alegre-Abarrategui, and Wade-Martins 2012). Given this, it should be highly surprising that LRRK2-G2019S CNs have unimpaired outgrowth, unless the hiPSC CN model is interpreted as being an inherently resilient cell type to LRRK2-G2019S PD phenotypes.

The evidence presented in this thesis thus adds to the emerging understanding that cell models and especially hiPSC-derived neurons exhibit cell type and genotype-dependent differences in vulnerability. Crucially, these reflect disease-relevant differences in the brain, highlighting the potential of this approach.

7.1.2. Does the Pathology Observed Model Brain Pathology?

While it is crucial that hiPSC-derived neurons do reflect brain regional differences in vulnerability, it is also important that the vulnerability exhibited also reflects disease. In this thesis, I primarily use an ASyn PFF model, and observe the formation of pASyn-S129+ pathology as a main outcome, in addition to observing changes in nuclear and cellular morphology, synapse loss, and neurite outgrowth. The relevance of these phenotypes and the PFF insult itself to disease is discussed here.

7.1.2.1. *Lewy Pathology and Cortical vs Brainstem Lewy Bodies*

In both the CNs and mDANs, PFFs seeded dose, time, and endogenous ASyn-dependent pathology, that adopted morphological forms highly reminiscent of LP in the brain. These were not limited to LN-like structures and LB-like structures, the main hallmarks of synucleinopathy in AD and PD. They in fact included a wide range of pathological inclusions mirroring those described in the human brain (Kuusisto, Parkkinen, and Alafuzoff 2003). The importance of this should not be understated. Many cell models including HEK293 and SH-SY5Y cells typically only develop diffuse, sometimes punctate pASyn-S129+ labelling, and human and rodent neuronal models typically have required *SNCA* overexpression and/or mutations to generate inclusions, even seeded by PFFs (Baena-Montes, Avazzadeh, and Quinlan 2021; Fares, Jagannath, and Lashuel 2021). The observation of a range of brain LB-resembling aggregates in the human neuronal models here, without these accelerants, is a meaningful advance and suggests a mirroring of disease processes.

However, LBs in the brain have distinct structural characteristics. They are lipid rich, organelle containing, and have their own diverse proteome of 100s of proteins (den Jager 1969; Leverenz et al. 2007; Goedert et al. 2013; McCormack et al. 2019; Shahmoradian et al. 2019; Trinkaus et al. 2021). Of these, ASyn, phosphorylated at S129, as well as p62 and ubiquitin have been established as the most common identifiers of LP (Fares, Jagannath, and Lashuel 2021). In my

experiments, I opted for performing large scale studies across multiple cell lines, timepoints, and cell types, rather than performing deeper characterisation of the LP developed. It is therefore uncertain whether the pASyn-S129+ LB-like structures I observe are ‘proper’ LBs. However, other studies using PFF insults have observed inclusions bearing ultrastructural and biochemical hallmarks of LBs in rodent primary cells (Mahul-Mellier et al. 2020; Trinkaus et al. 2021) and hiPSC-derived neurons (Bayati et al. 2023; Bieri et al. 2019; I. Lam et al. 2024; Tanudjojo et al. 2021). Since these occur on a shorter timescale and in less mature neurons, it might be inferred that the array of pASyn-S129+ structures I observe here likely reflect a process relevant to LB formation in the brain.

It is unclear from evidence in the human brain whether this heterogeneity of inclusions represents different stages of LB formation or independently forming structures. LBs seem to take on distinct structures in different parts of the brain (Estaun-Panzano, Arotcarena, and Bezard 2023; Fares, Jagannath, and Lashuel 2021). Specifically, cortical LBs show a more diffuse and disorganised filamentous structure, while brainstem LBs are more eosinophilic, with a dense core and halo (Fares, Jagannath, and Lashuel 2021). It would be interesting, in future, to examine whether the LB-like structures formed in the cell types here reflect these differences. Few previous studies have reported LB-like pathology in both hiPSC-derived cortical and dopaminergic neurons from the same patient lines, so this has not been examined before.

Notably, while these were not quantified distinct from LB-like structures, I observed that mDANs appeared to exhibit inclusions that were strongly reminiscent of pale bodies in much higher numbers compared to CNs. Pale bodies are typically found in the LC and SNPC, and may represent an early stage in the formation of brainstem LBs (Fares, Jagannath, and Lashuel 2021; Kuusisto, Parkkinen, and Alafuzoff 2003). While only qualitative evidence, this observation supports the idea that the Lewy-like pathology observed in the PFF-induced cell

models here does in fact reflect cell type-specific aggregation processes that occur in the brain. If confirmed, this would make the model highly relevant for the examination of selective vulnerability.

7.1.3. Is PFF-induced Protein Aggregation a Good Operationalisation of Vulnerability?

7.1.3.1. *Supraphysiological Concentrations and Timescales*

A related question regarding the relevance of the pathology modelled here to the brain is the validity of the PFF model itself. One criticism of this model is that supraphysiological levels of protein are needed to achieve a rapid appearance of aggregates, not reflective of disease (Baena-Montes, Avazzadeh, and Quinlan 2021; Estaun-Panzano, Arotcarena, and Bezard 2023). The high concentrations of protein are needed to model a disease process in a practical timeframe that otherwise occurs over years. However, the observations here and elsewhere that PFF-seeded pathology appears to reflect the same cell-type dependent distribution and aggregation process as in the brain act as a counter to this criticism, and help to validate the model.

It is in fact particularly striking that despite the supraphysiological levels of protein, some cells remain remarkably resilient to aggregation. This calibration of insult allows not only the examination of what makes cells vulnerable to disease, but also what makes them able to cope with such a severe impact. Incidentally, the time course in which individual inclusions form in humans is also largely unknown (Meisl et al. 2021; Meisl, Knowles, and Klenerman 2020). While the kinetics of aggregation are slower *in vivo* and in human cells rather than *in tubo* or in other cell models, severe AD and Lewy-type aggregates in infants with lysosomal storage disorders and other genetic disorders like Krabbe disease suggest that formation of aggregates

can also be relatively rapid (Hatton et al. 2022; Lloyd-Evans and Haslett 2016; Plotegher and Duchon 2017).

7.1.3.2. PFF Conformations and Batch Effects

Another limitation of the PFF model is the structure of the aggregates themselves. While the PFFs used here represent a mixture of fibril lengths and structures, brain-derived ASyn aggregates have distinct and specific structures not reflected in the *in vitro* model (Lee, Civitelli, and Parkkinen 2024; Yang et al. 2022). Moreover, distinct conformational structures or strains of ASyn aggregates have differing toxicities (Bousset et al. 2013; Gribaudo et al. 2019; Guo et al. 2013; Polinski et al. 2018; Tanudjojo et al. 2021; Tarutani et al. 2016) and lead to differing levels of pathology in different cell types (Peelaerts et al. 2015; Gao et al. 2019; Rey et al. 2019; Lau et al. 2020; Holec and Woerman 2021; Holec, Liu, and Woerman 2022). Different conformational strains, delightfully analogised to pasta shapes in a review by Ronald Melki (2024), likely alter the interactome of the synuclein spaghetti in the cellular sauce, leading to different pathological outcomes. It is thus unclear to what extent the differential vulnerability and toxicity observed here is dependent on the strain of ASyn PFF, as opposed to cell type dependent differences.

The fact that the cell type-dependent differences in pathology here do match the brain, however, does support the relevance of the PFF model employed here. While future studies might consider the effects of brain-derived ASyn aggregates, these are not as scalable, and may contain other active elements that could constitute a co-insult, such as other proteins or immune factors. While PFFs also tend to have batch-to-batch variability problems (Polinski et al. 2018), I consistently observed the same differences in pASyn-S129+ pathology across multiple PFF batches. As such, the PFF model appears to remain the most appropriate and disease relevant for the questions examined here.

7.1.3.3. Is Protein Aggregation a Good Measure of Vulnerability?

Given that the LP observed in the cell models here reflects that in disease, an important question is whether this protein aggregation is a good measure of vulnerability at all. Vulnerability in the context of neurodegenerative disease typically refers to vulnerability to protein aggregation, though could also refer to neuronal dysfunction and death, which might be independent of inclusion formation (Fu, Hardy, and Duff 2018). While protein aggregation is thus not the sole indicator of vulnerability, it is nevertheless a key outcome in virtually all neurodegenerative diseases. Moreover, while aggregates themselves may not be toxic, they nevertheless report on a dysregulated cellular environment and reflect underlying disease-related processes (Freer et al. 2016). As such, the propensity of cells to develop protein aggregates remains an important measure of their vulnerability to disease.

7.1.3.4. Beyond Lewy Pathology: Further Evidence of Disease Relevant Processes

Nevertheless, in addition to examining vulnerability to protein aggregation, I also examine other pathologies here which are shown to be disease relevant. One such key phenotype I observed is synapse loss in response to the PFF insult. Synapse loss an important, disease-relevant process in both AD and PD (Bellucci et al. 2016; DeKosky and Scheff 1990; Javier Rodriguez-Jimenez et al. 2023; Mancini et al. 2020). Here, I additionally showed synaptic toxicity was preferentially observed in the presynaptic terminal. Since synaptic pathology is associated especially with presynaptic dysfunction in both the AD and PD brain, this also validates the model as reflecting disease processes (Kramer and Schulz-Schaeffer 2007; Schulz-Schaeffer 2010; W. Wang et al. 2023).

Another PFF-related change I observed reflecting brain pathology was the depression of neuronal nuclei area in both CNs and mDANs. Some studies report altered nuclear morphology in AD (Bundgaard et al. 2001; S. Das and Ramanan 2023; Rinne, Paljärvi, and Rinne 1987; Riudavets et al. 2007), but none in PD. However, reduced nuclear size in the context of LP has been observed in the DLB brain, while enlarged nuclei are associated with tau pathology

in AD and PSP (Montalbano et al. 2023). Though this has thus only been rarely reported, it concurs with my observations here and presents an interesting and novel line of enquiry into the effects of these disease processes beyond aggregation itself.

In addition to these alterations, I also observed neurodegenerative phenotypes in the form of reduced neuronal area, reduced neurite area, and reduced neurite outgrowth. Since the reduced neurite outgrowth in the monoculture CN assays was genotype specific, but neurite and cell area reduction in the coculture PFF experiments was aggregation independent across all lines, these findings should be interpreted cautiously. Nuclei loss and neuronal area reduction might be an artefact of culture conditions or interactions through rAstros, rather than a direct effect on neurons. Nevertheless, neurodegeneration remains, of course, a disease-relevant phenotype. While neurite outgrowth cannot be measured in the postmortem patient brain, many models implicate this as a disease-relevant phenotype, and a feature of the live cell model is the ability to examine these early-stage disease manifestations in functioning cells.

Therefore, the pathological changes observed in these cell models span pASyn-S129+ aggregates that resemble LP, (pre)synaptic dysfunction, neurite outgrowth deficits, and aberrations in nuclei morphology. These together present a wider picture of various facets of neuronal vulnerability relevant to important and novel aspects of neurodegenerative disease in the brain, that are present in the cell model.

7.1.4. Are the Cell Types Generated a Good Model?

Given that the PFF-induced pathology reflects relevant disease processes in a cell type-dependent manner, it is important to also consider whether the cell models themselves are appropriate for investigating differential vulnerability. This was largely discussed in chapter 2, though here I also consider the limitations of the hiPSC model itself.

7.1.4.1. Opposing and Distinct Identities

In these experiments, I decided to compare cortical and midbrain dopaminergic neurons. A key advantage of these in the context of AD and PD is their inverse pattern of vulnerability. The CUX2⁺ upper layer CNs represent cells vulnerable in AD but relatively resilient to LP in PD, while TH⁺ mDANs are highly vulnerable in PD, but not typically affected in AD. As such, the pair of cell types here represent a balanced and opposing internally controlled comparison. Future studies might try and improve on these models by generating a purer SNPC-type dopaminergic identity or generating other cell types of interest. Nevertheless, the cells generated here display distinct neuronal identities of interest. Importantly, they are also electrophysiologically active with apparently functional synapses and even distinct firing patterns, reflective of differentially vulnerable neurons in the brain. As such, they seem to be reasonable models of cells in the brain.

7.1.4.2. Premature Differentiation

One phenotype that has been observed in hiPSCs with PSEN1 mutations is premature differentiation, related to impaired Notch1 signalling. Indeed, in some PSEN1 mutant lines here, I observed a trend towards rapid and dysregulated neurite growth, potentially reflective of such a phenotype. However, this was not consistent across PSEN1 genotypes, including in lines such as PSEN1-Int4Del, which have been shown elsewhere to exhibit impaired Notch1 signalling (Arber et al. 2021).

While I did not specifically examine differentiation through proliferation markers such as PAX6, I did in fact note that some cell lines, including PSEN1-Int4Del, as well as some LRRK2 patient lines in fact remained relatively proliferative during pilot CN differentiations. This necessitated the increased puromycin and AraC treatments of the CNs relative to other protocols, which produced a more even neuronal differentiation phenotype across the different cell lines. It is not clear to what extent premature differentiation is observed in the other assays presented

here. PSEN1-Int4Del CN synapses exhibited a slight relative resilience to PFF insult compared to other cell lines, and had relatively high vGLUT1 expression, which might reflect more robust and mature synapses. However, synaptic density was no higher in these cell lines, nor was ASyn expression significantly different. Moreover, while it might be expected that rapidly differentiating neurons are more resilient to neurite outgrowth deficits, these cell lines were in fact particularly vulnerable.

Possibly, the rapid, forced NGN2 differentiation method enables a more consistent neuronal phenotype and overcomes issues with premature differentiation (Das et al. 2024; Sheta et al. 2022; Yingsha Zhang et al. 2013). However, the PSEN1 mutant lines were not notably distinct on any markers of mDAN identity either. As such, while Notch1 signalling and cell fate regulation may be an important aspect of PSEN1 function, the methods employed here appear to circumvent any issues of varying rates of differentiation.

7.1.4.3. Further Limitations of the hiPSC Model

The hiPSC model more generally, however, is not without fault. There may be unknown or unmeasured effects of the reprogramming process, though most of the cell lines employed here were generated using Sendai virus, which is integration free (Baena-Montes, Avazzadeh, and Quinlan 2021). hiPSC-derived neurons do, however, typically represent developing neurons, and lose any aging and epigenetic phenotypes. This is less of a concern for the study of differential vulnerability here, where the fundamental differences in neuronal phenotypes which predisposes them to disease, rather than their aging phenotype, is under consideration. Nevertheless, an understanding of how aging affects neuronal subtypes differently would be valuable. While direct conversion protocols do reproduce aging phenotypes and transcriptomic signatures of disease, these are not sufficiently scalable and lack isogenic controls, which proved important for experiments here (Albert et al. 2022; Aversano, Caiazza, and Caiazza 2022; Balusu et al. 2023).

The inherent variability of hiPSCs is a further intrinsic limitation of the model. While I employed many lines and differentiations in experiments to try and overcome this, this variation likely impaired the ability to find significant differences, for example, in calcium measurements. In the future, mixed-model statistical approaches which are better able to account for variance in different levels of comparison might be employed. Despite this variability, however, I nevertheless observed consistent, differential effects of the PFF insult in the CNs and mDANs, which may reflect the strength of the cell-type dependency of their vulnerability.

7.1.5. Are the hiPSC-Derived Neurons Appropriate for Direct Comparison?

Having shown in these experiments that patient hiPSCs can be differentiated into distinct and differentially vulnerable cell types which are relevant to disease, and that they develop an array of pathological changes that are similarly disease-relevant and cell type-dependent, one key question remains: is it valid to directly compare the two different cell models?

On the one hand, many critical aspects of the paradigm designed here are held even in each model. Assays in monoculture cells occur at similar DIV. The concentration and duration of PFF insult is the same in both cells, and both models employ the same rAstrocytes, applied equally, such that any difference should be neuronal. Both also express similar A β levels, and while the CN model is more electrophysiologically active at the time of insult, the mDAN model rapidly matures during the first 10-20 days of the PFF time course to match this.

Nevertheless, important differences remain. It is not clear, for example, what effects the different media compositions have on aggregation dynamics. The culture models are also grown at very different densities, which is shown to influence aggregation. While this difference is arguably relevant to the differences between cell populations in the brain, it does raise the question of how much the results observed here are dependent on the differentiation method

used and the differentiation day assayed. Chung et al. (2016) show that mDANs generated using a floor-plate-based protocol, as I employed here, but not a neural-rosette-based method, exhibited PD-related phenotypes. Relatedly, transcriptional profiling of NGN2 neurons has shown that these best reflect foetal or early CNs (Das et al., 2024), while mDANs generated by similar protocols show substantial overlap with mature, adult neurons (Sandor et al. 2017). Possibly, a difference in developmental stage may account for some difference in vulnerability, though CNs were insulted at a much later timepoint, accounting for this.

This is why the characterisation of the cells produced by the model is crucially important. Dependency on differentiation method and insult timepoint only matters insofar as they change the neuronal phenotype. A different cortical differentiation protocol, for example, might produce neurons that more reflect deeper cortical layers, which might be more vulnerable. Alternatively, it might produce a more heterogeneous culture of excitatory and inhibitory neurons, and thus be more resilient. This is not a flaw in the model but an opportunity to understand what characteristics of cells and cultures predispose them to pathology. The very advantage of the comparison paradigm is not that the cell types are perfectly matched, but that there are natural discrepancies that are of interest.

The downside of the comparison here, and why it might seem inappropriate, is because the differences between the cell models are multifactorial, with many independent variables. This does make interpretation of differences between the models more challenging than a perfectly controlled experiment with a single variable. However, this system allows for exploratory investigation in a living, brain-relevant model, that can later be experimentally tested, with modulation of individual, identified pathways.

It remains possible, though unlikely, that different culture conditions, such as media constituents or plate coating, may artefactually impact pathology. For example, it is unclear whether the difference in NGN2 differentiation for CNs, in contrast to growth factor-mediated

differentiation for mDANs might have an influence. Future studies might take advantage of recent NGN2-induced mDAN protocols, for maximum comparability (Sheta et al. 2022). Another way around this is by comparing to controls in each cell type. For example, differences in plate coating may lead to faster neurite outgrowth in one cell type, and slower in another (Sepulveda et al. 2013). However, since outgrowth is slower than controls in LRRK2 mDANs elsewhere, but not different to controls in CNs here, this points to cell autonomous factors driving the difference, rather than substrate differences.

Thus, while I avoid making direct statistical comparisons between the two different models, it should be legitimate to consider how the same insult, equally administered and identically measured in each cell model, might produce different outcomes. Provided that differences in vulnerability result from differences in neuronal phenotype rather than artefacts of culture method, with the appropriate cell models and insult, as employed here, these differences should be relevant to disease. Investigating the differences in neuronal phenotypes should, therefore, yield insight into what factors might drive vulnerability.

7.2. Discussion Part II: Outcomes from the Examination of the Model

7.2.1. What Do Differentially Vulnerable hiPSC-Derived Neurons Demonstrate?

A major overarching aim of this thesis is thus fulfilled: hiPSCs from patients can be grown into distinct cell types that reflect differential vulnerability to disease-relevant pathology in a pattern that matches the brain. These neuronal models can thus be used to examine selective neuronal vulnerability. As such, the final aim remains: what does the model actually tell us about the pathways underlying selective neuronal vulnerability?

Key mechanisms observed in this thesis are discussed, as well as other notable findings and opportunities for further investigation, before a final assessment of the limitations and significance of these.

7.2.2. Cell Autonomous Mechanisms Partially Drive Selective Vulnerability

Since the cell model isolates neurons from non-autonomous mechanisms, this implies that cell autonomous mechanisms must play a role driving the differential vulnerability observed. While this does not rule out non-autonomous mechanisms, it does suggest that factors like glial cells, vascular-related vulnerability, and prion-like spreading cannot be the only drivers of pathology. The balance of autonomous and non-autonomous factors driving vulnerability has long been debated from postmortem tissue (Morrison and Hof 2002; Surmeier, Obeso, and Halliday 2017b; Wang et al. 2020). The hiPSC model here and in similar studies shows more definitively how cell intrinsic factors can predispose cells to disease (Bayati et al. 2023; Brazdis et al. 2020; Iannielli et al. 2022; Muratore et al. 2017; Oosterveen et al. 2021). Prashberger et

al. (2023) also make a convincing argument for cell intrinsic factors driving protein aggregate-related toxicity.

In some cases, differences between cell models or genotypes that were predicted based on brain tissue were not observed. A hypothesis arising from this might be that differences here in the brain therefore result from non-autonomous factors. For example, no difference between AD, PD, and healthy control genotypes was observed in mDANs. Possibly, non-autonomous mechanisms mitigate the vulnerability of these cells in the AD and healthy brain but are lost in PD. These are also removed in the cell model, isolating the otherwise highly intrinsically vulnerable cells. For example, many PD risk genes also converge on glial function, presenting a role for dysfunction in these cells driving vulnerability (Cragolini et al. 2019; Olsen and Feany 2021). While much research has focused on what makes mDANs vulnerable, this suggests that a renewed focus on what makes them so resilient, despite their intrinsic vulnerability, in other circumstances might be fruitful.

7.2.3. Endogenous Seeding Potential

Several lines of evidence point to endogenous ASyn levels contributing to vulnerability. Firstly, ASyn expression trended towards being higher in the vulnerable mDANs and trended towards being higher in the vulnerable PSEN1-Int4Del line, and when knocked out, abolished aggregation. Secondly, pathology burden between differentially vulnerable cell types differed primarily in the frequency of inclusion formation, not on the inclusion size. Thirdly, presynaptic terminals, likely enriched in ASyn, were selectively vulnerable relative to postsynaptic puncta. Fourth, denser cell cultures, likely with higher raw levels of ASyn, had a density-dose dependent impact on aggregation. These collectively implicate protein concentration and nucleation opportunities in predisposing cells to vulnerability (Fu, Hardy, and Duff 2018; Wood et al. 1999).

The contribution of endogenous ASyn to PFF-seeded pathology in cell models is well understood, and presents approaches that slow seeding dynamics as a potent therapeutic strategy (Fountaine et al. 2008; Longhena et al. 2020; Luna et al. 2018; Menon et al. 2022; Polinski et al. 2018). While *SNCA* knock down strategies risk loss of function deficits, future examination might consider the specific mechanisms by which higher density cultures exhibit more vulnerability, and whether other means of preventing seeding of aggregation might be effective.

7.2.4. Neuronal Activity

While the most vulnerable CNs were the PSEN1-Int4Del lines, CNs from the LRRK2-R1441C 207 line were also relatively vulnerable. Interestingly, one phenotype that tied these lines together was relatively high expression of vGLUT1 puncta. It is unclear whether this is due to a developmental or maturational phenotype, or another cause. Regardless, this does point to a role of glutamatergic transmission in driving pathology. This has been postulated from postmortem pathology and observed in rodent models (Dudek, Alexander, and Farris 2016; Wu et al. 2016; Wu et al. 2020; Yamada and Iwatsubo 2018). The specific vulnerability of vGLUT1+ terminals, and their coinciding with higher levels of synaptic ASyn has also been noted (Gcwensa et al. 2024). If higher expression of vGLUT1 additionally represents more mature synapses and/or higher electrophysiological activity, this could further inform the hypothesis that higher levels of neuronal activity also drive vulnerability.

In AD, hyperexcitability is an early disease-related phenotype, that likely drives excitotoxic damage and contributes to cellular dysfunction (Deng et al. 2022; Ghatak et al. 2021; 2019; Tweedy et al. 2021). Vulnerable brain regions tend to contain less interneurons, and hyperexcitability is possibly related to loss of input from inhibitory cells (Alkadhi 2019; Deng et al. 2022; Dudek, Alexander, and Farris 2016; Leng et al. 2021; Marinaro et al. 2020; Mathys et

al. 2023; Ruden, Dugan, and Konradi 2021). The NGN2 CN protocol generates a relatively pure glutamatergic phenotype, and I observed little to no GAD65/67 labelling, suggesting a lack of inhibitory cells. Muratore et al. (2017) note that vulnerable forebrain cultures in their study had a lower proportion of inhibitory neurons than the more resilient hindbrain cultures. A lack of inhibitory cells, therefore, might explain the relative vulnerability of my CN model compared to other studies attempting to seed pASyn-S129+ pathology in CNs.

Interestingly, activity from the likely inhibitory TH- cells in the mDAN cultures was not sufficient to confer resilience to these cultures, nor were the higher levels of neuronal activity in the D70 CNs sufficient to match the intrinsic vulnerability of the mDANs. It is possible that the TH- cells which are lost in the mDAN cultures are inhibitory neurons, contributing to TH+ mDAN vulnerability. A more thorough characterisation of the cell populations in the mDAN cultures might yield more insights here. Additionally, coculture of different ratios of inhibitory and glutamatergic neurons, deeper electrophysiological characterisation, and modulation of neuronal activity through small molecule inhibition or stimulation might also grant insight into these mechanisms.

7.2.5. Cell Type-Dependent Autophagy Deficits

A particular focus of the experiments conducted here was examining cell-type dependent deficits in autophagy. Autophagy has been well understood to be an important factor in mitigating ASyn aggregation (Cuervo et al. 2004; Desplats et al. 2009; Jones-Tabah et al. 2024; Karpowicz et al. 2017), especially in the context of PFF insults, and autophagy failure as a result of ASyn aggregation has also been observed (Bayati et al. 2023; Tanik et al. 2013). However, it is not well understood how autophagy regulation differs cell-to-cell. Here, I show that mutation-related autophagy deficits in PSEN1-Int4Del occur specifically in CNs, not mDANs, suggesting that differential autophagy regulation predisposes them to selective vulnerability.

This is furthered by the observation that in mDANs, both PSEN1-Int4Del and corrected control were insensitive to chloroquine, suggesting a cell type-related reduction in autophagic flux, rather than the genotype-dependent reduction observed in CNs.

Differential expression of some autophagy regulators and lysosomal proteins has been observed that may correspond to selective vulnerability (Aman et al. 2021; Chen, Acosta, et al. 2022; Chen et al. 2021; Fernandes et al. 2016; Freer et al. 2016; Fu et al. 2019; He et al. 2020; Wallings, Connor-Robson, and Wade-Martins 2019). Autophagy is an enormously complex and heavily regulated process, with many points of potential failure. It is likely that differences in autophagy regulation and function across the brain overlap with disease-related deficits or mutations that are cell type specific, and thus converge on consequently vulnerable cells (Nixon 2013; Nixon and Yang 2011). It is important to understand how this occurs in a cell-specific manner, for therapeutic design. For example, if autophagic flux is blocked, as I show here, drugs such as rapamycin, which induce further autophagy, are likely to exacerbate the problem, rather than rescue it. This points to the need for a better understanding of how specific cell types regulate proteostasis, which could lead to more targeted therapeutics.

7.2.6. Phenotypes Driving PSEN1-Int4Del Vulnerability

Importantly, none of the above discussed pathways are alone sufficient to explain the patterns of vulnerability observed in these cell models and observed in the brain. A complex confluence of interacting pathways converges to make cells in certain diseases vulnerable. This is exemplified in the differential cell type- and genotype-dependent vulnerability observed for PSEN1-Int4Del. Vulnerability here was likely driven by a combination of observed phenotypes, including slightly higher density, slightly higher A β expression, higher vGLUT1 expression, and cell type-dependent autophagic flux and calcium handling deficits. More still may be at play, such as cell type-dependent elevated A β 42 release and/or reduction in gamma secretase

activity (Arber et al. 2020; 2019; Muratore et al. 2017). Future studies might consider specific modulation of these contributing factors, to determine their relative contribution to vulnerability.

7.2.7. Limitations and Future Directions

Many of the limitations in this study, such as those associated with the PFF and hiPSC model, have already been discussed. One unaddressed thus far is the use of pASyn-S129+ labelling as the main endpoint compared between the two cell models. While this is regarded as the best sole marker of ASyn pathology, not all pASyn is pathological, not all pathological ASyn is phosphorylated, and other modifications to ASyn may interfere with antibody immunoreactivity (Bezard 2022; Estaun-Panzano, Arotcarena, and Bezard 2023; Fares, Jagannath, and Lashuel 2021; Lashuel et al. 2022; Ramalingam et al. 2023; Ramalingam and Dettmer 2023). The scale of the number of genotypes employed was traded off against ultrastructural and biochemical characterisation of the pathology here, limiting a fuller understanding of the pathology developed by these cells. Nevertheless, I do also examine other PFF-associated phenotypes, including morphological changes, synapse loss, and neurite outgrowth, which paint a broader picture of pathological changes in the cells. In the future, object specific analyses of cells and pathological markers, rather than analysis of means across many aggregates in each plate well, may also yield more insights with more power.

A further shortcoming of the project is the reliance on familial models for AD and PD. While these appear to model relevant disease processes, and LRRK2 in particular may bear a strong connection to sporadic PD, the applicability of findings in relatively rare and not necessarily representative mutations is unclear. Nevertheless, for an initial and exploratory study such as this, these models suffice for proof of concept and future studies might examine a broader

range of cell lines derived from sporadic patients, which have recently been shown to mirror patient vulnerability (Ng et al. 2022; Lee et al. 2024).

Another limitation is the examination of only one kind of insult. Tau aggregate insults present an interesting opportunity for further research (Xu et al. 2021), and might be expected to have the opposite effect with regards to differential vulnerability of the cells examined here. If AD CNs were vulnerable to tau pathology while AD mDANs and PD cell lines were not, it would substantially improve our understanding of cell type-specific, disease-specific, and protein-specific drivers of vulnerability, and further validate the model. Immune related insults would also prove an interesting paradigm to examine selective vulnerability under. While the impact of disease genotype was examined as an insult *per se* in neurite outgrowth, this raises another limitation of the study, which is the reliance on monogenic familial forms of the disease. These replicate many features of the sporadic condition, but are ultimately extremely rare, and of unknown translational validity. Similarly, while aggregate formation is of great interest to vulnerability, other mechanisms of PFF toxicity such as generation of ROS and mitochondrial impairment, and how and why these might differ between cell types, should be of substantial future interest (Deas et al. 2016; Prots et al. 2018; Brazdis et al. 2020; Cascella et al. 2021; Oosterveen et al. 2021).

While the mechanisms such as autophagy and cell density examined in this study were largely chosen by observation and expected disease-related phenotypes, future investigations would be better off taking an unbiased approach. Single cell characterisation of the two different cultures would give a much more comprehensive understanding of the compositions and phenotypes of the cells present, and would present a clear list of differentially expressed pathways (Balusu et al. 2023). These could then be individually modulated, to understand their contribution to the differential vulnerability of the models.

7.2.8. Novelty, Strengths, and Significance

The main outcome of this thesis is the examination of differentially vulnerable cell types, and the cell type-dependent phenotypes that predispose their susceptibility to disease. The demonstration of differential pathology across two differentially vulnerable cell types here is significant and has been rarely reported before. An important strength here and one of the more novel approaches of this study is the number of cell lines of different disease genotypes employed. Many more cell lines than are typical for this kind of study were employed, including both healthy and isogenic corrected controls for most genotypes. Crucially, genotypes from both AD and PD were also used at the same time, for comparison between diseases, which has rarely been examined before in hiPSC models. Indeed, this is the first examination of hiPSC-derived mDANs from AD patients. Research in AD and PD tends to be relatively narrow and focused, but the overlapping and parallel pathways, pathologies, and cell types involved in these two diseases present many opportunities for comparison and contrast, especially in the question of selective vulnerability.

The first observations of synucleinopathy in an AD hiPSC model, presented here, are particularly important in this regard. Lewy co-pathology in AD is underappreciated and its role in disease is poorly understood, despite AD being the most common synucleinopathy, making this worthy of future examination (Jin et al. 2022; Leverenz et al. 2006; Robinson et al. 2021; Uchikado et al. 2006; Visanji, Lang, and Kovacs 2019). As noted, these observations of sometimes profound synucleinopathy in the AD patient neurons are also among the first to show ASyn inclusions in models without employing *SNCA* overexpression or mutation, making these methods potentially more widely applicable.

Also notable here is evidence potentially supporting amyloid processing-independent functions of *PSEN1*, which contribute to disease (Kelleher and Shen 2017; Shen and Kelleher

2007). The case for the role of PSEN1 in lysosomal function and at the ER has been controversial and, possibly as a consequence, overlooked (Bezprozvanny 2013; Chou et al. 2023; Mustaly-Kalimi et al. 2022; Nelson et al. 2010; 2007; Zhang et al. 2012). Data I present here supports a role for PSEN1 in autophagy and calcium regulation and shows that this occurs in a cell type-dependent fashion, in line with selective vulnerability in disease.

Another curious and novel observation here is the diminished nuclear area associated with ASyn PFF insult, aligning with recent observations in the brain in the context of LP (Montalbano et al. 2023). These may point to alterations in DNA damage or regulation, worthy of further investigation, and add to growing evidence of aggregation independent toxicity associated with PFF insults (Denning et al. 2022).

Finally, an important strength of the hiPSC neuronal model is the ability to examine live cell processes. It is becoming increasingly clear that early changes in neuronal and synaptic function, before profound aggregation and cell loss, are vitally important in these diseases (Bellucci et al. 2016; Cramb et al. 2023; De Strooper and Karran 2016; Mancini et al. 2020). These can be examined in the hiPSC model, and here, I show both synapse loss and, for the first time, neurite outgrowth deficits in the CNs associated with ASyn PFF insult. Notably, I also examine neurites in hiPSC CNs from LRRK2 patients for the first time, showing a striking resilience to genotype-related outgrowth deficits. Future studies might utilise these cell models for examining the role of neurogenesis and/or axonal degeneration under different disease conditions. The further advantage of the cell model, however, is the opportunity to not just observe differences, but experimentally test what leads to them. This will be an important future direction for the models presented here.

7.3. Conclusion

In neurodegenerative disease research, vulnerable cell types are too often focused on, at the expense of understanding why other cell types remain resilient. These diseases, however, present a remarkable opportunity to examine healthy and vulnerable cell types in comparison, a ready-made control study, to better understand their underlying causes. An understanding of neurodegenerative disease and therapeutic strategies for ameliorating them can be just as much gleaned from the vulnerable cells as from the cells which are exposed to disease but withstand it.

Therefore, I sought to establish and demonstrate a paradigm for examining selective neuronal vulnerability in hiPSC-derived neuronal models. In doing so, I demonstrated novel approaches that successfully recapitulated disease-relevant pathology in disease-relevant cell types in disease-relevant patterns. From these experiments, I showed evidence for the role of several mechanisms in driving cell autonomous vulnerability and made novel observations of various pathologies and deficits in these models which contribute to our understanding of disease and to the broader validity of the hiPSC neuronal model.

More important than these methods and observations, however, is the paradigm of selective neuronal vulnerability itself, that has been explored and demonstrated here. Examination of differentially vulnerable cell types and genotypes is a powerful framework for understanding the underlying cellular conditions that cause neurodegenerative disease. This strange and remarkable phenomenon is not particular to AD and PD, but observed and applicable across many neurodegenerative diseases. Future research should employ this principle to better understand the fundamental drivers of cellular vulnerability and resilience, and so harness them for therapeutic ends. It is, after all, in embracing the strange and unexpected that discovery is made.

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Appendix

Table A1. Vulnerable and Resilient Cell Populations in AD and their Phenotypic Characteristics.

Structure	Notable Characteristics	References
Locus Coeruleus (LC)	• Neuromodulatory nucleus in pontine tegmentum • Modulates attention, mood, blood flow, neuroinflammation • Widespread noradrenergic afferents • Cell loss first reported in 1976 • Tau pathology present here before allocortex • More prominent cell loss in rostral (which projects to cerebral cortex) than ventral portion (which projects to brainstem and spinal cord)	(Matchett et al. 2021); (Davies and Maloney 1976); (Bueichekú et al. 2024); (H. Braak and Braak 1991); (Heiko Braak et al. 2006); (Heiko Braak et al. 2011); (Evans, Defensor, and Shamloo 2022)
(Trans) Entorhinal Cortex (EC)	• Transitional region between temporal neocortex and hippocampal allocortex • Comprise input into the hippocampus through perforant pathway • Profound cell loss underpins memory and orientation deficits in early AD (32% in mild cognitive impairment, more in severe AD) • Cell loss selectively in pre-alpha projection neurons, stellate and large pyramidal neurons in Layer II especially, Layer IV to a lesser extent • First site of Gallyas argyrophilic NFTs • Vulnerable cells are Reelin+, SMI32+, RORB+	(Heiko Braak et al. 2011); (Hyman et al. 1984; H. Braak and Braak 1985; Van Hoesen and Hyman 1990; Gómez-Isla et al. 1996; Stranahan and Mattson 2010; Grubman et al. 2019; Mrdjen et al. 2019; Leng et al. 2021)
Hippocampal Formation (CA1, CA3)	• Profound cell loss related to anterograde memory dysfunction • Subfields have differing vulnerability • CA1 and subiculum show highest susceptibility to tau pathology and neurodegeneration, CA3 also affected • Vulnerable cells receive projections from EC layer II and are most important for corticopetal projections • CA2 is less vulnerable, with more interneurons, less synaptic plasticity, • Small dentate gyrus granule cells (DGGCs) are relatively resilient	(Hyman et al. 1984; Van Hoesen and Hyman 1990; Morrison and Hof 2002; Dudek, Alexander, and Farris 2016; Fu, Hardy, and Duff 2018; Mrdjen et al. 2019)
Cerebral Cortex	• Cerebral neocortex is typically affected by neurofibrillary pathology and cell loss after limbic and subcortical regions in a staged and lamina specific manner • Higher order associational cortices are more vulnerable while primary cortical areas (pre and post central gyri, occipital pole) are remarkably spared • Temporal gyri are affected from inferior to superior, as well as parietal and frontal, and finally occipital • Layers II and III are especially implicated in tau neurofibrillary pathology, then layer V, typically large pyramidal neurons, responsible for inter and intrahemispheric projections • Deeper layers, especially Layer V, are more susceptible to Lewy co-pathology	(Pearson et al. 1985; Heiko Braak et al. 2006; Beach et al. 2010; Mrdjen et al. 2019; S. Chen, Chang, et al. 2022; Leuzy et al. 2022; Mathys et al. 2023; Zwang et al. 2023)
Basal Forebrain Cholinergic Nuclei	• Magnocellular nuclei in the basal forebrain important for attentional deficits in early AD • Pronounced changes associated with tau pathology, especially in the nucleus basalis of Meynert, and upper Raphe nucleus	(Heiko Braak et al. 2011; Kilimann et al. 2014)
Midbrain Dopaminergic Neurons	• Relatively resilient to AD, compared to PD • Cell loss in AD typically more associated with VTA than SNPC, and may relate to affective and motivational changes	(Dietmar R. Thal et al. 2002; Heiko Braak et al. 2006; 2011; Freer et al.

	- Experiences plaque pathology only in later stages of disease - Relatively spared by Lewy co-pathology, when present, which predominantly affects the amygdala and limbic structures	2016; Nobili et al. 2017; Raunio et al. 2019; Borghammer et al. 2021)
Inhibitory Interneurons	- Evidence is mixed regarding different classes of inhibitory interneurons which may be lost or be resilient to neurodegeneration, and are variably associated with tau pathology - Calretinin+ neurons largely spared of tau pathology - Caplain and parvalbumin interneurons exhibit some limited tau pathology - LAMP5+, somatostatin, and parvalbumin interneurons seem to be lost, without neurofibrillary pathology involvement - Interneurons tend to be smaller, fast spiking, and highly express calcium binding proteins	(Iwamoto and Emson 1991; P. R. Hof et al. 1993; Marinaro et al. 2020; Ruden, Dugan, and Konradi 2021; Deng et al. 2022; Mathys et al. 2023)
Resilient Cell Types	- Pyramidal motor neurons in the precentral gyrus are notably spared, despite resembling many other large projection neurons that are vulnerable in AD - Large purkinje cells in the cerebellum similarly are resilient to tau pathology, with cerebellar plaques emerging only late in disease - Smaller neurons such as in the DGGC, pyramidal neurons in the primary visual cortex, and smaller cortical neurons in layers II and IV are relatively spared - Brainstem and neuromodulatory nuclei are relatively spared of Lewy pathology	(Dietmar R. Thal et al. 2002; Fu, Hardy, and Duff 2018; Mrdjen et al. 2019; Ossenkoppele et al. 2019)

Table A2. Vulnerable and Resilient Cell Populations in PD and their Phenotypic Characteristics.

Structure	Characteristics	References
DMNV and Olfactory Bulbs	- Often identified as among the earliest affected regions in PD - Functionally unlike, except that both are among the few parts of the brain exposed to the external environment through the Vagus and olfactory nerves respectively	(Heiko Braak et al. 2003; Kalaitzakis et al. 2008; Beach et al. 2009; Ubeda-Bañon et al. 2020; Borghammer et al. 2021; Paß, Wiesner, and Pla-Martín 2021)
Pontine, Medullary, and Basal Forebrain Neuromodulatory Nuclei	- A large number of nuclei throughout the brainstem and basal forebrain are affected, including the serotonergic Raphe nuclei, noradrenergic locus coeruleus, gigantocellular populations in the reticular nucleus, and cholinergic neurons in the basal forebrain and pedunculopontine nucleus - These nuclei are typically neuromodulatory, with extensive and diffuse projections throughout the brain - GABAergic neurons in and around these nuclei are typically spared - Glutamatergic neurons in the PPN are also spared	(G. M. Halliday et al. 1990; Heiko Braak et al. 2003; Surmeier, Obeso, and Halliday 2017b)
Midbrain Dopaminergic Neurons in the VTA and SNPC	- Pigmented cells in the ventral midbrain which project to the ventral and dorsal striatum respectively. VTA neurons form the mesolimbic pathway, with roles in reward, mood, and motivation. SNPC neurons form the nigrostriatal pathway, with roles in movement, decision making, and regulation of basal ganglia pathway activity. - Both are neuromodulatory nuclei with diffuse projections - The SNPC, as with the above neuromodulatory nuclei, is heavily affected by Lewy pathology and cell loss in PD, and its pronounced vulnerability and functional relevance to motor symptoms in PD have afforded it particular attention - The SNPC is not affected equally. Ventral tier neurons are more susceptible than the dorsal tier. Cell loss appears to follow a gradient from caudal to rostral, lateral to medial, ventral to dorsal. - Resilient dorsal cells are PCP4+, and enriched for other calcium ion homeostasis and vesicle trafficking genes, but not PD GWAS genes - Vulnerable neurons have been identified as SOX6+/AGTR1+, enriched for PD GWAS loci - mDANs in the VTA are not unaffected, but remain relatively resilient, providing a valuable control population.	(Fearnley and Lees 1991; Damier et al. 1999; Heiko Braak et al. 2003; Surmeier, Obeso, and Halliday 2017b; Fu, Hardy, and Duff 2018; Monzon-Sandoval et al. 2020; Kamath et al. 2022)
Amygdala and Limbic Structures	- The amygdala is most frequently involved in LP, after the SNPC, and also experiences cell loss - Cortical and basolateral nuclei are most heavily affected. These have major olfactory connections. - Animal models with ASyn injection into other parts of the brain frequently develop amygdala LP - LP often observed in the amygdala in AD too	(Harding et al. 2002; Masuda-Suzukake et al. 2014; Borghammer et al. 2021; Y.-J. Guo et al. 2022; Jin et al. 2022)
Limbic Structures	- Mixed relationship between cell loss and LP in other limbic structures - Tuberomammillary nucleus, lateral hypothalamus, thalamic intralaminar nuclei are all affected	(Masuda-Suzukake et al. 2014; Surmeier, Obeso, and Halliday 2017b; Luna et al. 2018; Borghammer et al.

	• Hippocampal CA2, interestingly inverse of AD, is affected • DGGCs are relatively spared	2021; Y.-J. Guo et al. 2022; Jin et al. 2022)
Cerebral Cortex	• Cortical regions are typically affected in later stage PD, and to a lesser extent than the midbrain and limbic structures • Associational cortices are typically more affected than primary cortices, especially cingulate and frontal cortex • LBs are typically observed in deeper layers, while LNs are typically observed in upper layers. • Excitatory, intratelencephalic layer V neurons are relatively vulnerable	(Heiko Braak et al. 2003; Beach et al. 2009; Surmeier, Obeso, and Halliday 2017b; Borghammer et al. 2021; Goralski et al. 2024)
GABAergic Neurons	• In the cortex, and other brain regions such as limbic structures, and the PPN, GABAergic neurons have typically been reported as resilient to LP • Parvalbumin and somatostatin expressing interneurons were relatively resilient to inclusion formation in a mouse model	(Kingsbury et al. 2010; Double 2012; Goralski et al. 2024)

Table A3. Cell Non-Autonomous and Autonomous Pathways Associated with Selective Neuronal Vulnerability in AD and PD and Key Evidence For Them.

Structure	Characteristics	References
Astrocyte and Microglial Composition and Phenotype Differences	- Single cell analyses show region specific subtypes and proportions of glial cells across the brain, including between the VTA and SNPC - The functional significance of these differences is largely understudied but could underpin the differential susceptibility of different regions in different diseases	(Cragnolini et al. 2019; di Domenico et al. 2019; Kostuk, Cai, and Iacovitti 2019; Agarwal et al. 2020; Balusu et al. 2023)
Myelination	- Oligodendroglial myelination plays a clear role in neuroprotection - Poorly myelinated cells (e.g. in the SNPC and LC) need higher energy expenditure to maintain axonal function and transport - Heavily myelinated neurons in primary cortical areas are resilient in AD and PD - Tau progression is inversely related to myelination - Gaps in myelin may allow for additional exposure to environmental toxins	(H. Braak and Braak 1996; Fu, Hardy, and Duff 2018; Matchett et al. 2021; Rubinski et al. 2022)
Vasculature-related Vulnerability	- Coupled blood flow is crucial for nutrient supply to neurons, and impaired blood brain barrier integrity may result in poorer clearance of waste and a source of toxins - Vascular pathology such as atherosclerosis and CAA are especially associated with AD - Microhaemorrhages have been linked to sites of plaque formation, especially adjacent to points of vessel bifurcation, where walls may be weaker - Blood vessel leakiness associated with pathology initiation is consistent with the theory of Aβ being an antimicrobial response	(Tarasoff-Conway et al. 2015; Boland et al. 2018; Boyle et al. 2018; Javier Alegre-Abarrategui et al. 2019) (Cullen, Kócsi, and Stone 2006; Ando et al. 2008; Gosztyla, Brothers, and Robinson 2018; Frölich 2020; Z.-T. Wang et al. 2020) (Patrick R. Hof and Morrison 2004; Montagne et al. 2015; Grubman et al. 2019; Z.-T. Wang et al. 2020; Rubinski et al. 2022; Balusu et al. 2023) (Matchett et al. 2021)
Viral Susceptibility	- The HC and temporal cortex are selectively vulnerable to herpes simplex virus 1 and microbiome-derived lipopolysaccharides - HC and especially CA1 is implicated in impaired blood flow and BBB integrity - Understanding of selective vulnerability of the HC may be because of selectively focused research	
Vasculature and the LC	- Noradrenergic innervation from the LC regulates cerebral blood flow - Vascular efferents and adjacency to the 4th ventricle may additionally expose the LC to toxins and predispose it to iron accumulation - LC pathology is associated with CAA in terminal fields - Early LC dysfunction could lead to subsequent cerebrovascular dysfunction in AD, predisposing these regions to damage. - Direction of causality is poorly understood	
Heavy Metal Accumulation	- Iron, copper, manganese, and other heavy metals, have neurotoxic effects, and accumulate with age in selectively vulnerable brain regions - In AD, iron accumulates in the LC and frontal cortex - In PD, iron accumulates in the LC, SNPC, and basal ganglia	(Zecca et al. 2004; Double 2012; Hare et al. 2014; Javier Alegre-Abarrategui et al. 2019; 2019;

	• Iron accumulation is higher in SNPC than VTA • pTau in LC is associated with cells with heavy metal accumulation • heavy metals are frequently a component of pathological inclusions, and promote ASyn oligomerisation	Matchett et al. 2021; Y.-J. Guo et al. 2022)
External Exposure	• Exposure to the external environment links the olfactory bulbs and DMNV • Paraquat and rotenone (pesticides), as well as heavy metal exposure, increases PD risk • Appendectomy reduces risk for developing PD, but selectively in rural populations, not urban, suggesting exposure to rural toxins such as pesticides through the nose or gut may predispose PD in these brain regions • This does not account for the resilience of other externally exposed structures, such as peripheral nerves. Type Ia and Ib afferents are highly myelinated, as are peripheral motor neurons, but C-type pain fibres are long and unmyelinated, in addition to being peripherally exposed. Examination of these in PD may be insightful. • The retina contains the only neurons visibly exposed to the external world, and this has recently been recognised to also undergo neuropathological changes in AD and PD. However, rather than preceding brain pathology, retinal involvement seems to correlate with pathology in the brain itself, and any directionality remains unclear.	(Nave and Werner 2014; Killinger et al. 2018; Raunio et al. 2022; Hart de Ruyter et al. 2023)
Prion-like Spreading	• A hypothesis arising from Braak staging of AD and PD is that pathology spreads from sites of initial, primary vulnerability, to sites of secondary vulnerability, through synaptic and anatomical links • Spreading has not been directly shown in humans and is contested • Pathological proteins such as ASyn and tau seem to be able to act in a prion-like manner, in that they can spread and seed pathology elsewhere	(Jucker and Walker 2013; Brettschneider et al. 2015; Walker and Jucker 2015; Carceles-Cordon, Weintraub, and Chen-Plotkin 2023)
Conformational strains	• Different strains of pathological proteins lead to different patterns of vulnerability in the brain • Structural imaging advances have shown that tau and ASyn adopt distinct and specific structures in different diseases with distinct distributions of pathology • Injection of conformationally distinct protein aggregates or aggregates from different diseases leads to different patterns of pathology in rodents, reflective of the original disease • There is strong evidence that variable patterns of regional involvement in disease may be a result of conformational strains, but the mechanisms underlying this are uncertain.	(Clavaguera et al. 2013; Boluda et al. 2015; Falcon et al. 2015; Kaufman et al. 2016; Rey et al. 2019; Lau et al. 2020; Vaquer-Alicea, Diamond, and Joachimiak 2021; Y. Yang et al. 2022)
Evidence for Prion-like Spreading	• AD and PD pathology appears along anatomically defined pathways such as the Papez circuit or olfactory-amygdala pathways in a staged fashion • Mathematical modelling of pathology progression in the brain implicates spreading along connected circuitry • Tau and ASyn have the ability to seed and template pathology in a prion-like manner	(H. Braak and Braak 1991; Heiko Braak et al. 2003, 199; J. L. Guo and Lee 2011; L. Liu et al. 2012; Recasens et al. 2014; Walker and Jucker 2015;

Evidence against Prion-like Spreading	- Cell and animal models have shown that cells can release and uptake pathological forms of tau and ASyn, allowing for a spreading mechanisms - Spreading has not been observed directly in human disease - ASyn pathology has been observed within transplanted tissue in a PD patient, though whether this is evidence of spreading is contested. - Downstream connectivity is an imperfect explanation for selective vulnerability and strongly argued against by some authors - EC layer II cells project through the perforant pathway to both the CA3 and DGGCs, though one is relatively resilient, and the other vulnerable - Tau paired helical filament injection into rat eyes does not lead to pathology in the lateral geniculate, superior colliculus, or pretectal nucleus - ASyn pathology has not been observed in the peripheral nervous system, absent involvement of the brain - Pathology in the DMNV is frequent but not always present - The VTA, globus pallidus, striatal cholinergic interneurons, and cerebellar purkinje cells are all anatomically connected to PD vulnerable regions but relatively resilient to PD - Injection of ASyn PFFs into the PPN resulted in pathology in all connected brain regions, though the kinetics, magnitude, and persistence of pathology was not related to connectivity. - Prion-like spreading remains a possible and even likely disease mechanism, but is alone, insufficient to explain the relative vulnerability of downstream neurons - The alternate hypothesis that all cells possess the capacity to develop pathology, and happen to do spontaneously yet in an anatomically orderly manner also seems unlikely - An interaction vulnerability driven by spreading and potentiated or mitigated by intrinsic characteristics is likely	Surmeier, Obeso, and Halliday 2017a; Okuzumi et al. 2018; Javier Alegre-Abarrategui et al. 2019; Franzmeier et al. 2020; Abey et al. 2021; Carceles-Cordon, Weintraub, and Chen-Plotkin 2023) (Amaral and Witter 1989; Heiko Braak et al. 2003; Kalaitzakis et al. 2008; Kingsbury et al. 2010; Sulzer and Surmeier 2013; Engelender and Isacson 2017; Surmeier, Obeso, and Halliday 2017a; Lionnet et al. 2018; Javier Alegre-Abarrategui et al. 2019; Henrich et al. 2020; K. Yamamoto 2021; de Fisenne et al. 2022)
Alternate Connectivity Mechanisms	- Connectivity may confer vulnerability or resilience through mechanisms beyond transsynaptic spread of pathology - Loss of rostral LC noradrenergic innervation and thus loss of neuroinflammatory regulation may leave cortical, hippocampal, and septal regions vulnerable in AD, but cerebellum and spinal cord resilient - LC innervation of the resilient precentral gyrus is segregated from innervation to the rest of the PFC - Changes in gut microbiota may regulate pathology in the brain through modulation of neuroinflammation - Increased inhibitory GABAergic signalling in the CA2 HC may be protective	(L. A. Schwarz and Luo 2015; Dudek, Alexander, and Farris 2016; Sampson et al. 2016; Evans, Defensor, and Shamloo 2022; Waterhouse et al. 2022)
Neurotransmitters and Receptors	- Synthesis of and signalling through certain neurotransmitters is postulated to drive cell autonomous and non-autonomous vulnerability - Excitotoxicity through excessive glutamatergic signalling and Ca^{2+} entry is thought to play a role in AD, mediated by receptor dysfunction, changes in receptor composition, or loss of inhibitory input - vGLUT2 expression is thought to be important for mDAN vulnerability in the SNPC	(Yan Zhang et al. 2016; Burbulla et al. 2017; Fu, Hardy, and Duff 2018; Steinkellner et al. 2018; Javier Alegre-Abarrategui et al. 2019; Marinaro et al. 2020; Matchett et al.

	• Catecholaminergic neurons are linked to neurodegeneration since their transmitters are relatively reactive • Dopamine is readily oxidised into ROS, leading to impaired lysosomes and mitochondria • ASyn increases reuptake of dopamine through enhanced dopamine transporter function, without increasing repackaging into synaptic vesicles, resulting in higher dopamine levels in the cytosol • Noradrenergic signalling similarly produces metabolites that contribute to tau pathogenesis and accumulate free radicals • Catecholaminergic neurons also accumulate neuromelanin, though it is unclear if this is protective or harmful	2021; Ruden, Dugan, and Konradi 2021; Evans, Defensor, and Shamloo 2022; S. S. Kang et al. 2022)
Neuronal Hyperactivity	• Loss of parvalbumin and GAD1 interneurons is implicated in hyperactivity in fAD • Populations in vivo and in vitro with higher proportion of inhibitory neurons are relatively resilient • Release of tau and ASyn is activity dependent • Hyperactivity enhances ASyn phosphorylation, trafficking, and pathology • Highly active neurons such as mDANs and BFCNs have higher energy demand • Hyperactivity of glutamatergic neurons leads to elevated Ca^{2+} and excitotoxicity • Structures comprising the default mode network have high resting state activity levels and are vulnerable in AD	(Dudek, Alexander, and Farris 2016; J. W. Wu et al. 2016; Q. Wu et al. 2020; Muratore et al. 2017; Yamada and Iwatsubo 2018; Javier Alegre-Abarrategui et al. 2019; Marinaro et al. 2020; Z.-T. Wang et al. 2020; Mahady et al. 2021; Y.-J. Guo et al. 2022; Ramalingam et al. 2023)
Calcium Buffering Capacity	• Expression or lack of calcium buffering proteins is a key marker of resilient and vulnerable cell populations across neurodegenerative diseases • Calbindin is highly expressed in DGGCs, but less in CA1 • Variations in Ca^{2+} buffering proteins correlate with inhibitory neuron susceptibility to Aβ • Neurons in associational cortices are more reliant on calcium signalling • A key difference between the otherwise similar SNPC and VTA is lower calcium buffering capacity and higher calcium influx in SNPC mDANs • In the SNPC, calcium ion homeostasis pathways are overrepresented in the resilient dorsal tier neurons • Calbinin is lowly expressed in the DMNV and neuromodulatory nuclei such as the LC and raphe, and declines with age in the BFCNs, where greater loss is associated with being more prone to degeneration. • Substantial evidence for Ca^{2+} buffering playing a role in Huntington's disease, motor neuron disease, and spinocerebellar ataxia • Interneurons, cerebellar neurons, DGGCs, CA2 pyramidal cells, hilar and mossy fibre neurons are all relatively resilient and have high expression of calcium binding proteins, including PCP4, somatostatin, parvalbumin, and calretinin • Calcium channel expression is varied between vulnerable and resilient cells	(P. R. Hof et al. 1993; Alexianu et al. 1994; Shaw and Eggett 2000; Greene, Dingledine, and Greenamyre 2005; Sulzer and Surmeier 2013; Riascos et al. 2014; Ahmadian et al. 2015; Morigaki and Goto 2017; Recabarren and Alarcón 2017; Fu, Hardy, and Duff 2018; Javier Alegre-Abarrategui et al. 2019; Huang and Verbeek 2019; Mrdjen et al. 2019; Monzon-Sandoval et al. 2020; A. Shi et al. 2020; Z.-T. Wang et al. 2020; Paß, Wiesner, and Pla-Martín 2021; K. Yamamoto 2021)

Calcium Channel Expression	• Cav1 channels allow high calcium flux in SNPC mDANs, combined with low calcium buffering, which may lead to mitochondrial dysfunction • Forebrain neurons have age related increases in Ca²⁺ entry through Cav1 channels in AD • Inhibition of Cav1 channels by dihydropyridines is associated with less cellular vulnerability and lower PD risk • Ca²⁺ entry is also implicated in the LC, DMNV, and PPD in PD • Differential calcium channel expression between CA1, CA3, and DGFCs may account for differential hippocampal vulnerability	(Goldberg et al. 2012; Sanchez-Padilla et al. 2014; Y. Wang and Mattson 2014; Kalia and Lang 2015; Surmeier, Obeso, and Halliday 2017b; Fu, Hardy, and Duff 2018; K. Yamamoto 2021; Colbourne and Harrison 2022)
Oxidative Stress & Mitochondrial Dysfunction	• Unbuffered calcium entry and neurotransmitter synthesis can exert toxicity through impact on mitochondrial function, leading to impaired energy production and oxidative stress • Combinations of mitochondrial impairment with high energy demand in certain neurons likely leads to selective dysfunction • LC neurons have higher levels of mitochondrial oxidative stress under physiological conditions • Oxidative stress and mitochondrial energy homeostasis changes precede neurodegeneration in the LC in aged mice • Calcium oscillations in mDANs in the SNPC drive activation of mitochondrial oxidative phosphorylation, leading to increased ROS generation • The selective vulnerability of SNPC mDAN mitochondria to systemically administered complex I inhibitors such as MPTP, rotenone, and paraquat, points to the role for mitochondrial dysfunction driving vulnerability in PD • In a PFF injection model, SNPC mDANs were also selectively vulnerable to respiration deficits compared to the amygdala and striatum, where pathology developed across all three regions • ROS and reactive nitrogen species can potentiate ASyn aggregation, in addition to damaging cellular machinery including mitochondria and autophagic systems, and lead to oxidative DNA damage and transcriptional errors, compounding the issue in LC and SNPC neurons • Oxidative stress in the SNPC follows a gradient that matches subregional vulnerability • Oxidative damage is also common in AD vulnerable brain regions • SNPC mDANs are selectively vulnerable to mtDNA mutations, compared to the striatum and frontal cortex	(Andreas Bender et al. 2006; A. Bender et al. 2008; Fountaine et al. 2008, 200; Gupta, Dawson, and Dawson 2008; G. Halliday et al. 2009; Wong and Cuervo 2010; Neuhaus et al. 2014; Surmeier, Obeso, and Halliday 2017b; Javier Alegre-Abarrategui et al. 2019; Burtscher et al. 2020; Monzon-Sandoval et al. 2020; Z.-T. Wang et al. 2020; Evans et al. 2021; Paß, Wiesner, and Pla-Martín 2021; Evans, Defensor, and Shamloo 2022; Zuniga and Frost 2023)
Energy Demand and Neuronal Morphology	• Vulnerable neurons typically have higher energy demands relative to resilient ones • CA1 and EC neurons have long projections with 3x more extensive neurite trees than smaller DGFCs, making them more susceptible to decreased oxygen and glucose	(Patrick R. Hof and Morrison 2004; Matsuda et al. 2009; Heiko Braak et al. 2011; Bolam and Pissadaki 2012; Montagne et al.

	• Cortical neurons vulnerable to lewy and amyloid pathology are typically part of the default mode network, with high levels of resting state activity • Some PV interneurons may be vulnerable in AD because of their fast spiking activity • Neuromodulatory neurons such as in the BFCNs and LC and especially mDANs in the VTA and SNPC have extensive projections and arborisation, with additionally energy intensive volume signalling through varicosities • SNPC mDANs in particular are estimated to have a total arborisation of 4.5m and give rise to up to 2.4 million synapses, a unique challenge to mitochondrial bioenergetics • Mitochondrial toxins are selectively toxic to SNPC and not VTA type cells • Reducing the arborisation of these cells in mice using axonal guidance inhibitors reduces their vulnerability • Purkinje cells remain resilient, despite their large dendritic trees and hundreds of thousands of synapses per cell • Striatal cholinergic neurons have similarly highly branched axons and volume neuromodulatory release signalling, but also remain resilient in PD • In addition to higher energy demand, larger neurons require more proteins and have more transcriptional activity, leading to a higher likelihood of DNA damage • Cells with longer axons and more synapses likely express higher raw levels of tau and ASyn, key components of these structures, • Larger neurons have a larger surface area and therefore possibly higher exposure to toxins and oxidants • Larger cells have different membrane surface area to volume ratios, which may also change the kinetics of aggregating proteins, especially for membrane associating proteins such as ASyn	2015; Pacelli et al. 2015; Surmeier, Obeso, and Halliday 2017b; Fu, Hardy, and Duff 2018; Javier Alegre-Abarategui et al. 2019; Alkadhi 2019; Matchett et al. 2021; Oosterveen et al. 2021; Paß, Wiesner, and Pla-Martín 2021; Ruden, Dugan, and Konradi 2021; Denning et al. 2022; Evans, Defensor, and Shamloo 2022; Zuniga and Frost 2023) (Dudek, Alexander, and Farris 2016; Zuniga and Frost 2023; Zwang et al. 2023)
Synaptic Plasticity	• Regions with higher synaptic plasticity might be expected to have a higher energy demand and higher transcriptional demand • This makes the HC and associational cortices especially vulnerable • Notably, the resilient CA2 and DGGCs show lower synaptic plasticity • The neurogenic niche in the hippocampus and frontal cortex may also contribute to this, with axonal remodelling and axonogenesis in these relatively plastic regions leading to tau hyperphosphorylation as part of microtubule binding regulation and greater reliance on proteostatic mechanisms	(Dudek, Alexander, and Farris 2016; Grothe et al. 2018; Alkadhi 2019)
Proteostatic Differences	• Differences in cellular pathways underpinning uptake, expression, clearance, and release of aggregating proteins may logically impart vulnerability to certain cells • Proteins most prone to aggregation make up a 'metastable proteome' whose concentrations are high relative to their solubility	(Singleton et al. 2003; Ciryam et al. 2013; Freer et al. 2016; Fu, Hardy, and Duff 2018; Grothe et al. 2018; Luna et al. 2018; Javier Alegre-Abarategui et al. 2019; Mrdjen et al.
Expression of Disease Proteins		

Protein Uptake	• Converging and compounding age related stressors in vulnerable cells may compromise the maintenance of the solubility of these proteins • Overexpression models and copy number genetic variants causing familial version of AD and PD implicate protein expression levels in driving cellular vulnerability and disease. • Math2+ neurons in the HC CA regions express higher levels of SNCA than DGGCs and are more susceptible to ASyn aggregation, ameliorated by knock down by antisense oligonucleotides • MAPT expression levels correlate with neurodegeneration and vulnerable regions • Mixed evidence from single cell analyses versus brain homogenates and diverse localisation of proteins across large cells makes accurately measuring protein expression challenging	2019; Monzon-Sandoval et al. 2020)
Protein Clearance	• Cells may have higher levels of protein due to uptake, not just expression. • Receptors such as LAMP1 have been shown to mediate both tau and ASyn uptake, so expression of such receptors or mechanisms may differ across the brain, though this has not been closely examined	(Rauch et al. 2020; K. Chen et al. 2022)
	• Clearance mechanisms such as the endolysosomal system, autophagy, and ubiquitin-proteasome system are all crucial for normal cell functioning and clearance of disease related pathology • Impaired clearance mechanisms are highly implicated in neurodegenerative diseases through GWAS • Autophagy and vesicle trafficking genes are differentially expressed between dorsal and ventral neurons in the human SNPC, and between cortical and SNPC neurons in rodent models, and in TH+ compared to TH- neurons in mDAN iPSC cultures • Autophagic vacuoles accumulate in LRRK2 KO mice in an age dependent manner in the LC and SNPC but not cortex or cerebellum • Expression of proteostasis genes changes occur in aggregate laden cells such as in the nucleus basalis, Layer III cortical neurons in the precuneus, or CA1 hippocampal neurons with tau pathology • These studies examining one cell type in disease cannot tell cause from effect • Tissue microarray analysis of gene expression in healthy tissue found expression of tau and amyloid co-aggregating proteins matched regions vulnerable to developing pathology , while genes associated with protection from aggregation, such as BAG3, an autophagy homeostasis regulator, were more expressed in resilient brain regions. The same applied to PD and LP. • BAG3 is highly expressed in inhibitory neurons, resilient to tau pathology • AIMP2, an ASyn co-aggregate, is thought to increase its aggregation propensity, and may be differentially expressed throughout the brain	(Gharesouran et al. 2014; Bordi et al. 2016; Fernandes et al. 2016; Freer et al. 2016; Gonçalves et al. 2016; Tiernan et al. 2016; Giaime et al. 2017; Fu, Hardy, and Duff 2018; Mrdjen et al. 2019; Nalls et al. 2019; R. Wallings, Connor-Robson, and Wade-Martins 2019; Ham et al. 2020; He et al. 2020; Monzon-Sandoval et al. 2020; Andrews et al. 2023)

Expression of Genetic Risk Factors	• Many PD risk genes are expressed in nigral mDANs, consistent with their vulnerability • Vulnerable SOX6/AGTR1+ neurons in the ventral SNPC are enriched for PD GWAS hits • Differential expression of other risk genes in different cell populations as single cell studies emerge is of interest	(Kamath et al. 2022; Balusu et al. 2023)
Aging	• Frequently posited as a driver of selective vulnerability, but without explanation of how this might differentially affect distinctly vulnerable cell populations • Biological aging remains nebulously defined, but hallmarks of aging have been outlined, as well as putative aging clocks, and examining how these differ across vulnerable and resilient regions would be of interest. • Precuneus neurons show reduced telomere length in AD • RNA sequencing in the mouse brain throughout aging identified glia-associated transcriptional signatures of aging which were accelerated in certain brain regions, raising the question of whether the brain ages at different rates. • Some age-related changes, such as calbindin expression and heavy metal accumulation occur in a regionally selective manner	(Fu, Hardy, and Duff 2018; Mahady et al. 2021; Y.-J. Guo et al. 2022; Hahn et al. 2023)